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**Screening of multi-class veterinary drugs in animal-derived
food using hyphenated high-performance thin-layer
chromatography techniques**

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Declaration

I declare: this dissertation submitted is a work of my own, written without any illegitimate help by any third party and only with the materials indicated in the dissertation. All passages in the text that have been taken verbatim or in spirit from published work of others, as well as all information relating to verbal communications, are identified as such. At any time during the investigations carried out by me and described in the dissertation, I followed the principles of good scientific practice as laid down in the “Justus Liebig University Giessen Statute for Ensuring Good Scientific Practice”.

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Scientific contributions

Peer-reviewed original research papers

Publications reflecting the main scope of this thesis

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- III. A. Mehl, L. Hudel, M. Bückner, G.E. Morlock, Validated Screening Method for 81 Multiclass Veterinary Drug Residues in Food via Online-Coupling High-Throughput Planar Solid-Phase Extraction to High-Performance Liquid Chromatography–Orbitrap Tandem Mass Spectrometry, *J. Agric. Food Chem* 70 (2022), 10886–10898. <https://doi.org/10.1021/acs.jafc.2c03925>.

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- VI. G.E. Morlock, A. Ziltener, S. Geyer, A. Mehl, T. Schreiner, T. Kamel, J. Tersteegen, F. Brümmer, Evidence that Indo-Pacific bottlenose dolphins self-medicate with invertebrates in coral reefs, *iScience* 25 (2022), 104271. <https://doi.org/10.1016/j.isci.2022.104271>.

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Poster presentations

Presenter in italics

- I. Curious2022 Future Insight™ conference, Darmstadt, 12-14th July 2022: *G.E. Morlock, A. Ziltener, S. Geyer, A. Mehl, T. Schreiner, T. Kamel, J. Tersteegen, F. Brümmer*, Dolphins' beauty secrets, invited poster presentation, awarded with the poster price.

List of Abbreviations

ADI	acceptable daily intake
AIF	all ion fragmentation
<i>A. fischeri</i>	<i>Aliivibrio fischeri</i>
APCI	atmospheric pressure chemical ionization
API	active pharmaceutical ingredient
APPI	atmospheric pressure photoionization
ASE	accelerated solvent extraction
<i>B. subtilis</i>	<i>Bacillus subtilis</i>
CE	capillary electrophoresis
ddMS ²	data-dependent tandem mass spectrometry
CC β	detection capability for screening
C-trap	curved linear trap
CVMP	Committee for Veterinary Medicinal Products
DB	direct bioautography
(v)DIA	(variable) data-independent acquisition
DLLME	dispersive liquid-liquid microextraction
(d)SPE	(dispersive) solid-phase extraction
EDA	effect-directed analysis
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ESI	electrospray ionization
EU	European Union
FEC	frontally eluted components
FLD	fluorescence light detection
FT	Fourier-transform
FWHM	full width at half maximum
GC	gas chromatography
HCD	higher-energy collision-induced dissociation
HILIC	hydrophilic interaction liquid chromatography
HPLC	high-performance liquid chromatography
(HP)TLC	(high-performance) thin-layer chromatography
HRMS	high-resolution mass spectrometry

HTpSPE	high-throughput planar solid-phase extraction
LFIA	lateral flow immunoassay
LLE	liquid-liquid extraction
LOD	limit of detection
LRMS	low-resolution mass spectrometry
MEKC	micellar electrokinetic capillary electrophoresis
MIP	molecularly imprinted polymer
MRM	multiple reaction monitoring
MRL	maximum residue limit
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MTT	3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyltetrazolium bromide
NADH	nicotinamide adenine dinucleotide + H ⁺
NCE	normalized collision energy
NMR	nuclear magnetic resonance
NSAID	non-steroidal anti-inflammatory drug
PLE	pressurized liquid extraction
PRM	parallel reaction monitoring
QqQ	triple quadrupole
QuEChERS	quick, easy, cheap, effective, rugged, safe
RP	reversed-phase
RPA	reference point for action
SFPE	solvent front position extraction
TOF	time-of-flight
tSIM	targeted selected ion monitoring
UHPLC	ultra-high-performance liquid chromatography
UV	ultraviolet light
VD	veterinary drug
vis	visible light
VMP	veterinary medicinal product

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1. Introduction

1.1 Veterinary drug residues in food – legislative aspects

Veterinary medicinal products (VMPs) are defined as substances or a combination of substances with properties for the treatment or prevention of disease in animals. They are used to restore, correct, or modify physiological functions by exerting pharmacological, immunological, or metabolic action [1]. A VMP consists of the active pharmaceutical ingredient (API) and, depending on the dosage form, various excipients without pharmacological activity. In this work, the APIs of the respective VMPs are referred to as veterinary drugs (VDs).

In factory farming, anti-infective VDs such as antibiotics, antifungals, and antiparasitics are used to avoid the rapid spreading of infections amongst livestock [2]. In addition, non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids are administered for the treatment of painful conditions and inflammatory diseases, whereas β -agonists, hormones, and steroids are used for improving the growth rate [2,3]. Residues of these VDs in food of animal origin, such as meat, milk, eggs, and honey, can harm the health of consumers by altering the human gut microbiota, chronic toxicity, mutagenicity, carcinogenicity, and provoking mild hypersensitivity reactions or even severe anaphylaxis [4,5]. Furthermore, excessive use of antibiotics carries the risk of antibiotic-resistant bacteria developing, leading to infections that are difficult to treat [4]. Despite the risks posed by the use of VDs, their use in animal husbandry is essential not only for animal welfare reasons, but also to prevent infections of humans with zoonoses [1]. Currently, there are over 1,200 approved VMPs on the European market [6]. For different indications, they contain a wide variety of VDs and thus, structural classes. Important VD classes are listed in Annex I of Council Directive 96/23/EC [7]. Accordingly, Group A substances include unauthorized compounds and compounds with anabolic effects, while Group B substances include antibacterial compounds, antiparasitics, anthelmintics, sedatives, and NSAIDs, as well as substances such as plant protection products and mycotoxins or other environmental contaminants.

To protect consumers from adverse effects and to ensure animal welfare, the use of VMPs is regulated in the European Union (EU). Regulation (EU) 2019/6, “lays down rules for the placing on the market, manufacturing, import, export, supply, distribution, pharmacovigilance, control and use of veterinary medicinal products” [1]. One of its main objectives is to fight antimicrobial resistance by prohibiting the preventive and growth-

promoting use of antibiotics either directly or in medicated feed as well as reserving certain antimicrobials for use in humans only [6].

Before a VMP is authorized in the EU, a safety evaluation is carried out by the Committee for Medicinal Products for Veterinary Use (CVMP) of the European Medicines Agency, and maximum residue limits (MRLs) for VDs are recommended on the basis of Regulation (EC) 470/2009 [8,9]. MRLs are the maximum allowed concentrations of residues in food of animal origin and are expressed in mg/kg or µg/kg fresh weight [9]. To recommend MRLs, the CVMP conducts a scientific risk assessment concerning the metabolism and drug excretion rate in the respective animal species [9]. The type and amount of residues that may be ingested by humans consuming contaminated food products throughout their lives without a significant health risk are considered the acceptable daily intake (ADI) [9]. MRLs are set individually for different matrices in Commission Regulation (EU) 37/2010 [10], taking into account the ADIs. For those substances for which no MRLs have been set, reference points for action (RPAs) are established on the basis of Commission Regulation (EU) 2019/1871 [11]. RPAs must be as low as reasonably possible from an analytical point of view with regard to the respective VDs' toxic potential. If a VD is potentially harmful even in very low concentrations, its use is prohibited and a zero-tolerance principle applies. Animal products containing prohibited or non-authorized VDs in concentrations exceeding the RPA levels or residual amounts of authorized VDs exceeding the established MRLs are considered non-compliant and may not be placed on the market. High residual VD concentrations in food can result from insufficient withdrawal periods before slaughter or milking [5,12], inappropriate dosage [12], improper mixing of medicated feed or cross-contamination of feed due to improper cleaning [12], or environmental contamination by the use of feces as fertilizer [4,12,13]. To verify that food of animal origin complies with the legal requirements, official controls are mandatory as laid down in Regulation (EU) 2017/625 [14], which recently replaced Council Directive 96/23/EC [7]. The official control procedures are implemented in each EU Member State through a national residue control plan (Germany: *Nationaler Rückstandskontrollplan*). The samples taken must be analyzed by accredited control laboratories using analytical methods that meet the requirements of Commission Implementing Regulation 2021/808 [15], which recently replaced Commission Decision 2002/657/EC [16]. It divides methods for residue analysis into screening and confirmatory methods and specifies the performance criteria they must meet. Accordingly, screening methods may be qualitative, semi-quantitative, or quantitative

with a false-negative rate of no more than 5% at the level of interest, and a reasonable percentage of false detects. If, after analysis by a screening method, a sample is suspected of being contaminated, it must be subjected to a confirmatory method. The latter must provide “full or complementary information enabling the substance to be unequivocally identified and if necessary quantified [...]” [15]. To ensure the quality of the results obtained, quality control procedures must be implemented to continuously verify the performance of screening and confirmatory methods. In 2020, 0.45% of all planned samples (those that were not taken on suspicion) in Germany were confirmed as non-compliant [17].

1.2 Veterinary drug residue analysis – an overview

The ideal residue analysis method should be simple, low-cost, accurate, highly sensitive and selective, have a high sample throughput, and cover as many analyte classes as possible to avoid repeated analyses of the same sample. However, the development of such multi-class methods is difficult because the various classes often differ significantly in their physicochemical properties. Sufficient sensitivity and selectivity often cannot be guaranteed for all VDs in the analytical focus, as MRLs and RPAs are mostly in the low µg/kg-range. The matrix load of animal-derived food is generally very high, and so residue analysis of VDs can be likened to the proverbial search for a needle in a haystack.

To cope with the large sample volumes to be analyzed, screening methods, in particular, must be fast, simple, and inexpensive. These can be biological methods such as inhibitor tests measuring a cellular response, biochemical methods based on antibodies, enzymes, or receptors, as well as physicochemical methods such as high-performance liquid chromatography (HPLC) with mass spectrometric (MS), photodiode array, or fluorescence detection [18].

Confirmatory methods consist of an often time-consuming and costly sample preparation step, followed by instrumental determination, including separation and detection. They must provide information on the analyte structure and thus, separation and detection are commonly based on HPLC–tandem MS (MS/MS) [2,15].

1.2.1 Sample preparation techniques

Sample preparation usually consists of comminution, homogenization, extraction, clean-up, and sometimes solvent evaporation followed by reconstitution to concentrate the sample solution before instrumental analysis. The sample preparation techniques mentioned in this section are intended to give an overview of the most important methods

for VD residue analysis and are not complete, as there are still many advancements and modifications available.

VDs are usually extracted from food matrices using polar organic solvents such as (acidified) acetonitrile, methanol, ethyl acetate, and sometimes water or ethylenediaminetetraacetic acid (EDTA) solutions [19]. For multi-class methods, acetonitrile is widely preferred because it can extract a broad range of analytes and yields cleaner extracts because it precipitates proteins sufficiently [19]. A “traditional” and simple extraction method is liquid-liquid extraction (LLE) [20]. In LLE, either solid samples are first mixed with an aqueous buffer solution, or liquid samples, *e.g.*, milk, urine, or serum, are extracted directly. For extraction, the solution is shaken after the addition of an immiscible organic solvent, and the analytes pass into the organic phase, which can then be evaporated and reconstituted [21–23]. Without further clean-up, this technique leads to intense matrix effects (see also section 1.4.1) during instrumental determination [2]. Other disadvantages are emulsion formation, high consumption of sample material, and the use of toxic organic solvents, which make it expensive, time-consuming, and environmentally harmful [2]. There are numerous modifications of LLE, for instance, dispersive liquid-liquid microextraction (DLLME) [24,25], which represents a miniaturized, greener version with less solvent consumption. For extraction of solids or semi-solids, accelerated solvent extraction (ASE) [26–29], also known as pressurized liquid extraction (PLE), represents an automated and more environmentally friendly method that is widely used for VD residues [20]. The samples are extracted with organic solvents at high pressures (3.4–20.7 MPa) and temperatures (50–200 °C) [20], resulting in less solvent consumption, higher extraction speed, and efficiency [29].

Solid phase extraction (SPE) [30–33] is one of the most commonly used methods for the extraction of VDs, in which extracts are purified using an adsorbent material packed in an SPE cartridge. Depending on the characteristics of the adsorbent and the physicochemical properties of the analytes, the latter interact with the adsorbent and are retained, while some matrix compounds are very effectively removed in several washing steps [34]. The analytes are then eluted from the cartridge, while some matrix compounds are retained so strongly that they cannot be eluted from the adsorbent, separating analytes and matrix interferences [34]. The eluates are either directly diluted or evaporated and reconstituted in the appropriate solvent and sometimes filtered before injection into the determination system [34]. In multi-class methods, the SPE approach is often problematic because very polar analytes are removed together with the polar matrix compounds in reversed-phase

(RP) SPE, whereas very lipophilic analytes bind so strongly to the sorbent bed that they cannot be recovered [34]. Consequently, some analytes or even analyte classes may be completely lost during clean-up. In addition, “classic” SPE consumes high solvent volumes, is time-consuming, and includes many manual steps [34]. Online SPE [35,36] represents a fully-automated evolution of this technique to overcome the latter issues but has not been widely used for VD residues in food [34]. Another version of SPE is dispersive SPE (dSPE), which is part of the Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) method. This approach was originally developed for pesticide residue analysis in fruits and vegetables [37] and is quicker and easier than traditional SPE [2]. Using this technique, the samples are extracted with acetonitrile and a mixture of salts is added to remove water and induce partitioning of the analytes into the acetonitrile phase. The dSPE step is performed by adding small amounts of bulk adsorbents to the solution, such as primary secondary amine, C18, and/or graphitized carbon black that adsorb matrix compounds [34]. After centrifugation, the upper acetonitrile phase is subjected to instrumental analysis. Compared to SPE, this technique provides a less efficient clean-up, resulting in higher matrix effects [2]. Since 2006, many studies [38–42] were published that diversely optimized the QuEChERS procedure for VD residues [43]. The simplest approach of all is “dilute-and-shoot” [44–46], which means that samples are just diluted and/or extracted to diminish matrix effects and then injected into the determination system. Although this technique has a high sample throughput, peak shapes of polar analytes are often poor [47] and excessive dilution can be problematic in terms of detection limits (LODs) and has not widely been applied in VD residue analysis so far.

1.2.2 Determination systems

Modern multi-residue methods for VDs mostly rely on HPLC–MS detection. HPLC–MS/MS with triple quadrupole or ion trap multi-stage instruments was long considered the gold standard [2,48] for VD residue analysis. Over the last decades, the use of high-resolution mass spectrometry (HRMS) with orbitrap or time-of-flight (TOF) analyzers has increased [49,50]. MS detectors offer the ability to detect a vast number of analytes simultaneously and have a high recovery and selectivity as well as good reproducibility. Other HPLC-compatible detectors such as fluorescence detectors [51], diode array detectors [52], ultraviolet detectors [53], and evaporative light scattering detectors [54] are less commonly used because they can only detect certain analytes with specific structural properties and sometimes require previous analyte derivatization [55]. For thermostable

VDs, also GC–MS [56] or GC–MS/MS is used [2]. Other possible, but less frequently used GC detectors are the “classic” nitrogen-phosphorous detectors and electron capture detectors. Another chromatographic technique for VD residues is capillary electrophoresis (CE), which employs capillaries as separation channels. A high-voltage direct current electrical field is the driving force for the chromatographic separation of analytes in their ionic form. It is compatible with MS detectors [57], but also with multiple other highly sensitive detectors such as diode array detectors [58], ultraviolet [59], laser-induced fluorescence [60], chemiluminescence [61], and electrochemiluminescence detectors [62]. Micellar electrokinetic capillary chromatography (MEKC) is a modification of CE, in which a surfactant is added to the analyte solution to form micelles. The analytes distribute between the solution and the micelles and migrate with the electroosmotic flow [63], enabling the separation of both ionic and neutral analytes [64]. In recent years, MEKC has become a popular technique for the separation of VDs. [20]

The above-mentioned techniques can qualitatively and quantitatively determine the VDs present in the food sample. Therefore, they are suited for confirmatory analysis but are also used for screening, especially in multi-residue analysis [2]. Prior to the 21st century, microbial growth inhibition tests were one of the most common detection methods for antimicrobial residues [65]. They are still widely used for screening purposes and will be discussed in detail in section 1.3. Another screening method is the enzyme-linked immunosorbent assay (ELISA) which is based on a specific antigen-antibody immunological reaction combined with an amplified enzymatic catalytic reaction to display the primary immune response [20]. It is a sensitive and rapid detection method, but due to its operating principle, it is often preferentially specific for a few analytes [66] or a single analyte class [67] with the same structural moiety. Nevertheless, multi-class ELISA methods have also been developed [68,69]. The lateral flow immunoassay (LFIA) is another immunochemical method used for VD residue analysis [70,71] not only for one but also for multiple VD classes [72]. It combines ELISA and thin-layer chromatography (TLC) and is suitable for fast on-site residue detection [73]. Instead of using antibodies as analyte recognition elements, other approaches include aptamers or molecularly imprinted polymers (MIPs) [73]. In recent years, advanced biosensor-based detection technology has emerged as an alternative to conventional methods [20]. It can be expected that they will become a trend in VD residue analysis, as they are rapid, efficient, and cost-effective [20].

1.3 Bioassay detection

Microbial growth inhibition assays were the earliest methods to detect antibacterial residues and they are still a widely used screening tool [74]. They visualize the specific activity of antibacterial agents via their inhibitory effect on bacterial growth and can therefore be used for the detection of antibiotic residues in various matrices [74]. The advantages of these tests are their ease of use, their commercial availability, and the facts that the sample preparation is reduced to a minimum and no expensive equipment or specialized technicians are required [75]. They represent a non-target screening as they can detect antibacterial residues outside the analytical scope, such as metabolites, degradation products, or unknown antibacterial substances. Nevertheless, they only provide sum parameters and lack identification of the compounds [75]. Another problem is that each microorganism is preferentially sensitive to only a few antibiotic classes, so several different bioassays must be performed for comprehensive screening of a single sample.

There are two general types of growth inhibition assays: the tube test and the (multi-) plate assay. The first type commonly consists of a growth medium inoculated with bacterial spores and a pH or redox indicator. Without the presence of antimicrobials, the growth of the test bacteria during incubation will acidify the medium, resulting in a color change of the indicator. If bacterial growth is inhibited by antimicrobial agents, no or delayed color change is observed. These tests are almost exclusively based on *Bacillus stearothermophilus* var. *calidolactis* and are commercially available, e.g., Premi®Test [76,77], or Delvotest® SP-NT [78,79]. They were originally developed for screening in milk [80,81], but are also suitable for other matrices such as muscle tissue fluid or honey [76,77,82]. Plate tests consist of a layer of nutrient agar inoculated with bacteria and are usually used for tissue samples. The samples are applied on top of the layer or in wells inside the agar. After overnight incubation, bacterial growth is visible as an opaque layer. The presence of antibacterial residues is indicated by a clear inhibition zone around the samples. The size of the inhibition zone depends on the residue type and concentration. Sensitivity depends on several factors, such as the test organism, growth medium, layer thickness, pH value, and matrix type. Therefore, the detection of all relevant antibiotics at their MRLs requires a multi-plate system. The European four-plate test [83,84] has long been considered the gold standard and comprises four individual plates, three containing *Bacillus subtilis* BGA (*B. subtilis*) at pH 6, pH 7.2 including trimethoprim, and pH 9. The fourth contains *Kocuria rhizophila*. Other tests [85–87] using additional indicator

organisms were developed with up to seven plates to enable presumptive identification of the antibiotic class but still result in more laborious screening. Despite all efforts, microbial growth inhibition assays are often not sensitive enough to detect all important antibiotic residues in all relevant matrices at their regulated limits. [74,75]

Nowadays, the three-plate method (*B. subtilis* at three different pH values) is still applied for the screening of tissue samples in Germany's official control laboratories. In 2019, an exceptionally high percentage of 87% positive screening results in the three-plate test (0.07% of all tested samples) remained without confirmation by physicochemical methods [88] and ranged 57–66% in other reporting years [17,89,90]. Although false-positive results from bioassay detection have already been described as problematic [91], to the best of our knowledge, this methodological inconsistency has remained unexplained to date.

1.4 Mass spectrometric detection

1.4.1 Matrix effects

Animal-derived foods represent challenging matrices for residue analysis. Muscle tissue [92], milk [93,94], and eggs [95] contain high amounts of proteins, amino acids, lipids, and fatty acids, whereas honey [92] mostly consists of saccharides. All of these substances can cause intense matrix effects. These can lead to poor and unreliable data in quantitative analysis, as they affect the reproducibility, linearity, and accuracy of the method [96] and can also cause poor chromatography, poor analyte recovery, and false-positive or false-negative results [46,97]. The extent of matrix effects is often unpredictable, depending on the nature of matrix compounds and analytes [96]. High matrix amounts may also contaminate the ion source and ion optics of the MS, increasing maintenance expenditures and unwanted instrument downtime [46].

In LC-MS, matrix effects alter the ionization efficiency by the presence of compounds co-eluting with the analytes, leading to ion suppression or enhancement, and were referred to as the “Achilles heel” of quantitative HPLC-electrospray ionization (ESI)-MS [98]. Atmospheric pressure ionization sources, such as ESI and atmospheric pressure chemical ionization (APCI) are commonly used interfaces for HPLC-MS coupling in many application fields [99]. In an ESI source, the analyte solution is guided through a spray capillary to the tip of which a voltage is applied so that the analytes in the liquid phase are charged. Ions with the same charge collect at the tip of the capillary and repel each other, leaving the capillary as a fine aerosol through Taylor cone formation, often supported by

a nebulizer gas, *e.g.*, nitrogen. The droplet size decreases due to solvent evaporation and Coulombic fission and the ions are emitted into the gas phase. There are two proposed models for this process, charge residue [100] and ion evaporation [101]. The ions are then introduced into the first stage of the MS. There are different theories about ion suppression mechanisms in ESI: In the liquid phase, analytes and matrix compounds may compete for the available charges and access to the droplet surface and in addition, the linearity of the ESI response is often lost at high concentrations of dissolved compounds [102]. Matrix compounds increase the viscosity of the solution, and thus the surface tension of the droplets, impeding droplet formation, and reducing the amount of gas-phase ions [103]. Furthermore, non-volatile interfering compounds can lead to the formation of solid analyte-inclusion particles [103]. Mobile phase additives must also be considered, as they can form ion pairs with the pre-formed analyte ions [104]. In the gas phase, the charge of the analytes can be lost via neutralization reactions or charge transfer [103]. ESI is suited for ionization of polar to middle-polar analytes [105] such as VD_s, however, it is sometimes more affected by matrix effects than APCI [106]. In APCI, analytes are first transferred into the gas phase as neutral molecules using a nitrogen stream and a heater. The analytes are then ionized in the gas phase by a corona-discharge needle [103,107], which is why APCI is better suited for non-polar compounds [105,108,109]. Consequently, matrix effects in APCI can only occur in the gas phase, while in ESI they can occur in both the liquid phase and the gas phase. Two hypotheses were formulated for ion suppression effects in APCI: formation of solid precipitates of analytes with non-volatile matrix compounds [103] and modification of the charge transfer efficiency due to electron affinity differences between compounds in the gas phase [110]. Polar analytes [111,112] with low molecular weight [113] are often most affected by matrix effects in both ESI and APCI. [96]

Depending on the analytical problem, matrix effects can be simply compensated or must be minimized. If sufficient sensitivity is achieved for the analytes, compensation of matrix effects by standard addition or matrix-matched calibration via post-extraction spiking of a representative blank matrix is possible [114]. If the quantification accuracy is still not sufficient, it can be improved by the use of isotope-labeled internal standards [115,116], but this approach is not suitable for multi-residue analysis due to the high purchase costs of these standards [96]. However, in residue analysis of trace VD amounts, sufficient sensitivity is a critical parameter, and therefore, matrix effects should be minimized. One option to minimize matrix effects is the dilution of the samples [117], which is, however,

detrimental for the LODs. Another approach is the modification of the MS conditions. For example, negative ionization is less susceptible to matrix effects than positive ionization [97,118], but this can only be applied if the analytes ionize sufficiently under these conditions. Changing the chromatographic conditions in such a way that as few matrix compounds as possible co-elute with the analytes improves their ionization efficiency [96]. However, this is difficult to achieve, especially in multi-residue analysis. The most effective way to remove matrix interferences is the application of sample preparation methods (section 1.2.1) before instrumental determination although these are often time-consuming. [99]

1.4.2 HPLC–MS/MS in veterinary drug residue analysis

HPLC–MS/MS for VD residue analysis in food has become widely used due to its ability to provide identification, confirmation, and quantification of the substances. Before the 21st century, low-resolution MS (LRMS) such as triple quadrupole (QqQ) instruments were used that required relatively long dwell times (>100 ms). Combined with the low resolution of the HPLC system, the number of compounds that could be detected in one run was limited. The use of HPLC systems with higher resolution and sensitive fast-scanning QqQ instruments (<10 ms) emerged in the last decades, allowing the detection of a wider range of target compounds. The current standard for qualitative and quantitative residue analysis is the use of QqQs operated in the multiple reaction monitoring (MRM) acquisition mode: the first quadrupole is set to a single precursor mass, the second quadrupole serves as a collision cell that fragments the precursor ion, and the third quadrupole is set to a selected fragment ion. The second most commonly used LRMS detectors are quadrupole linear ion trap instruments also operated in MRM mode [19]. Monitoring of two transitions with ion ratios and retention times of all monitored masses corresponding to those of a standard compound is suitable for confirmation [15].

Ultra-high performance LC (UHPLC) offers a higher peak capacity than HPLC through the introduction of sub-2 μm particle columns and higher linear velocities delivered by UHPLC pumps at pressures >40 MPa [19]. In the last decade, this technique has been frequently applied to residue analysis of VDs [119–122]. To get the most out of UHPLC, it should be combined with fast scanning MS instruments with the ability of fast polarity switching, allowing a suitable number of data points (12–15) to be simultaneously acquired for several UHPLC peaks that are only a few seconds wide. The high costs of

hardware and the reduced lifetime of UHPLC columns are the main drawbacks of this technique. To avoid clogging of UHPLC columns and the resulting high backpressure, thorough sample preparation is required. Commonly used (U)HPLC separation modes are RP [123,124] and also hydrophilic interaction liquid chromatography (HILIC) [21,125] for hydrophilic compounds that are not retained under RP conditions [19]. [2]

LRMS instruments only provide unit mass resolution, and although MRM is very selective and usually yields reliable results, false negatives are still possible if the responses for the transitions are weak at low concentrations [19] or if matrix compounds shift the ion ratios of the confirming MRM transitions and therefore the product ion spectra differ from those of a solvent standard [126]. The occurrence of such an event is nowadays more likely than a few decades ago because QqQ instruments are now one or two orders of magnitude more sensitive without a significant increase in selectivity [126]. Isobaric interferences can occur if matrix compounds with the same nominal mass as the analytes reach the detector at the same time and mimic the supposed analyte-specific MRM transition [126], leading to false detects [15].

Another problem is that the MRM detection approach is targeted and therefore blind to any unknown contaminants, active metabolization, or degradation products outside of the analytical focus. In other words, you will only find what you are looking for, which is very problematic in terms of food safety. In particular, the detection of metabolites can be considered proof that the drug has been administered to the animal and that the samples have not been contaminated with drug residues in the laboratory. However, no reference substances are available for metabolites or unknown substances, so that target analysis is not possible for these compounds. Therefore, non-target MS data acquisition methods such as full scan should be used to obtain comprehensive information on the samples. Unfortunately, QqQ instruments lack sufficient sensitivity and selectivity in full scan acquisition mode and are therefore not suited for non-target screening analysis. [127]

1.4.3 HRMS in veterinary drug residue analysis

HRMS instruments provide higher mass resolution than LRMS and mass accuracies below 5 ppm [128]. According to Commission Implementing Regulation 2021/808 [15], HRMS must provide resolutions of at least 10,000 at 10% valley or 20,000 at full width at half-maximum (FWHM). During the past two decades, the use of HRMS for residue analysis has been increasingly accepted in fields where an ever-increasing number of new analytes

has had to be covered [127], for example in multi-class pesticide [129,130] and VD residue analysis [31,131–134] or for combinations of multiple contaminants [135–138].

Today, the user base of HRMS instruments in residue analysis is still smaller than those of QqQ instruments despite their advantages: HRMS instruments allow the measurement of accurate masses and isotopic fine structures, enabling the determination of molecular formulas. A key advantage of the resulting higher selectivity is the ability to perform non-target or retrospective analysis of full scan data [48]. Thus, data can be acquired without having to define compound-specific settings, easing straightforward method setup. This makes HRMS instruments ideal for screening of compounds for which no physical reference standards are available. Even if fragmentation is required for confirmation, an additional full scan can be incorporated into the acquisition method, enabling data quality monitoring and detection of critical issues. [127]

Compared to QqQ instruments, LODs achieved with the current HRMS instruments are generally higher due to their limited dynamic range, but this sensitivity gap has narrowed over the last decade. It can be expected that HRMS instruments will take the lead in the coming years [126]. Regarding selectivity, multiple studies [139–142] concluded that a mass resolution of 50,000 FWHM in a full scan acquisition combined with corresponding narrow mass windows provides a selectivity comparable to or even exceeding that of a QqQ-based MRM. Selectivity can also be improved using other acquisition modes (see section 1.4.4). While the scanning speed of QqQs has dramatically increased in the last decade, HRMS instruments often require much longer cycle times for good data quality. [127]

The HRMS instruments that are widely used for residue analysis can be divided into orbitrap and TOF detectors. Although both techniques provide data of similarly good quality when used correctly, they offer different advantages and disadvantages due to their different operating principles. In general, TOFs are less robust than orbitraps, as their mass axis stability can be affected by many factors, even with modern TOF instruments, so they must be constantly recalibrated. They are also more susceptible to material wear than orbitraps. However, the purchase costs of orbitrap instruments are significantly higher than those of TOF instruments. [127]

In this work, a hybrid quadrupole-orbitrap instrument was used, so the orbitrap technology is described in detail in the following section.

1.4.4 Orbitrap instruments

Orbitrap mass analyzers are ion traps that consist of a spindle-like central electrode and two symmetrical electrically isolated outer barrel-like electrodes. Ion packets are injected into the space between the inner and outer electrodes through a slot in one of the outer electrodes. When a voltage is applied between the inner and outer electrodes, a radial electric field bends the ion trajectory toward the inner electrode, while the tangential velocity causes an opposing centrifugal force. The ions stay on a circular spiral around the central electrode while they are pushed toward the widest part of the trap by the axial electric field, which initiates harmonic axial oscillations. The oscillation frequency in the electrostatic field depends on the mass-over-charge ratio (m/z ratio) of the ions. Hence, ions of different m/z ratios spread into rings that oscillate along the central electrode. The image current of the axial oscillations is detected as a time-domain signal by the outer electrodes that act as receiver plates. The signal is transformed into the frequency domain via Fourier Transform and is then converted into a mass spectrum. The more oscillations recorded, the higher the resolution, which can reach up to 1,000,000 FWHM [143]. Thus, the resolution in orbitrap analyzers is inversely proportional to the scanning speed. [144] A curved linear trap (C-trap) serves as an external storage device to control the number of ions injected into the discontinuously operated orbitrap analyzer and enables coupling to continuous ion sources such as ESI interfaces [145]. Incoming ions are first stored in the C-trap until the maximum number of charges or the maximum inject time is reached and the ion packet is then injected into the orbitrap, minimizing space charge effects [143]. Hence, sensitivity is directly proportional to the number of ions collected in the C-trap and injected into the analyzer [48]. In trace analysis, the operating principle of these systems can be detrimental because minor ions may not be detected if interfering ions fill the C-trap before a sufficient number of analyte ions have accumulated. Therefore, at low concentrations, the analyte signals do not hide within the noise as commonly observed using QqQ or TOF instruments, but disappear completely from a virtual noise-free background [146], limiting the linear dynamic range of the instrument [127]. To improve selectivity, hybrid quadrupole-orbitrap instruments have an additional quadrupole in front of the C-trap for mass filtering, which allows the isolation of precursor ions, and a higher-energy collision-induced dissociation (HCD) cell consisting of a gas-filled multipole after the C-trap for fragmentation at normalized collision energy (NCE) or stepped NCE in multi-class analysis [145]. This setup enables a variety of different acquisition modes. In full scan mode, ions of a broad mass range are allowed to pass the

quadrupole, are collected in the C-trap, and subsequently detected in the orbitrap to gain comprehensive information about the sample and possible precursor ions. In targeted selected ion monitoring (tSIM), the quadrupole is set on a narrow mass window or multiple narrow mass windows at once to isolate the analytes from the matrix ions, which increases sensitivity. The risk of false assignments increases if the detection is based solely on the exact masses of precursors and their adducts. Therefore, quadrupole-orbitrap instruments offer fragmentation in different modes. Parallel reaction monitoring (PRM) can be used to quantify only a few analytes. In PRM, the quadrupole is used as a narrow-band mass filter set on the target precursors and the ions present are fragmented in the HCD cell. All fragments generated from the precursors are then simultaneously monitored with full scan characteristics. In data-dependent MS/MS (ddMS²), an initial survey scan detects potentially present precursors, and fragmentation is triggered if certain predefined criteria are met. This acquisition mode can be problematic if a large number of low-abundant analytes are to be confirmed because in this case the instruments often fail the data-dependent trigger due to long cycle times or they falsely trigger fragmentation due to interfering ions. ddMS² can also be combined with tSIM to enhance sensitivity and selectivity. The non-target all ion fragmentation (AIF) and data-independent acquisition (DIA) mode have shorter cycle times and are therefore more suitable to confirm a large number of analytes. They represent a generic approach that requires no modifications for specific analytes and is often combined with additional full scans, facilitating retrospective data analysis. In AIF, all ions of a broad mass range are fragmented in the HCD cell at the same time and detected in the orbitrap, which makes it difficult to associate fragments with the correct precursors and may also result in decreased sensitivity. To overcome these limitations, the DIA mode divides the full m/z range of interest into several smaller sections, which are consecutively filtered by the quadrupole, undergo HCD fragmentation and the resulting product ions are then detected in the orbitrap before the next section. Alternating with a full scan, these successive fragmentation events are constantly repeated. In this way, DIA represents a compromise between the high selectivity of targeted ddMS² or PRM and the fast-scanning speed of AIF while maintaining full scan information. These characteristics make DIA the preferred acquisition mode for screening, whereas full scan, tSIM, and PRM are mostly used for quantitative analysis. However, data generated by DIA are commonly compared to compound databases comprising the target analytes for confirmation. Even if the data are additionally compared to larger accurate-mass databases available online, this detection

approach is still not a true non-target workflow because this would require the identification of all mass signals present in the spectrum. [143]

1.5 High-performance thin-layer chromatography

1.5.1 General application area and benefits

High-performance thin-layer chromatography (HPTLC) is a more sophisticated development of the long-known TLC. The stationary phase in (HP)TLC is a planar adsorbent (silica gel, chemically modified silica gel, cellulose, alumina, *etc.*) fixed on a carrier material (usually glass or aluminum) [147]. HPTLC adsorbents are more powerful than TLC adsorbents because of their smaller layer thickness, a smaller average particle size of 5–7 μm , and a narrower particle size distribution [148], enabling faster developments. Another advancement of HPTLC is the use of modular automated instrumentation for sample application, development, and derivatization [149]. An advantage of planar chromatography over other chromatographic techniques is that any combination of solvents can be used as the mobile phase, since it is evaporated after chromatography, ensuring compatibility with various detection systems. Other advantages are a high sample throughput, enabling the separation of up to 46 samples on the same plate [150] under the same analytical conditions, resulting in minimal separation times and costs per sample. Furthermore, HPTLC plates have high matrix robustness, and thus, high sample volumes of up to 1 mL can be applied as areas [151], concentrating the sample on the adsorbent and reducing sample preparation to a minimum [152]. In addition, all components of the sample remain on the plate and, if stable, are accessible for post-chromatographic chemical derivatizations or effect-directed analysis (EDA). EDA is the *in situ* detection of biochemically (enzymatic induction/inhibition, immunostaining) or biologically (bioautography) active compounds [152]. Detection is performed via multi-imaging with visible light (vis), ultraviolet (UV) light absorption, fluorescence light detection (FLD), or luminescence [152]. Quantitative results can be obtained by densitometry [153,154], digital image evaluation [155,156], or (HR)MS [157,158]. For further characterization and structure elucidation, hyphenations to HRMS [159,160] or nuclear magnetic resonance (NMR) spectroscopy [159,161,162] are possible.

1.5.2 High-throughput planar solid-phase extraction

In 2011, high-throughput planar solid-phase extraction (HTpSPE) was introduced as a promising new clean-up concept for pesticide residues in fruits and vegetables utilizing HPTLC techniques [163]. Up to 20 sample extracts were applied on a single HPTLC plate as areas. Two developments were carried out: The first almost to the upper plate edge served the purpose of front-eluting the target analytes, whereas matrix compounds with higher adsorption energies to the plate remained closer to the application zone, thus extracting the pesticides from their matrices. The second development was carried out to a shorter migration distance in the reverse direction, focusing the slightly separated analytes and matrix compounds with lower adsorption energy in a sharp target zone (Fig. 1A). The target zones were then eluted into autosampler vials using a TLC–MS Interface 2 and were subsequently injected into an HPLC–MS system for detection of the analytes. This resulted in minimum clean-up times per sample and very low matrix effects. Since then, HTpSPE was proven to be applicable for other matrices [164,165] and analytes [164–169], and also for bioassay instead of MS detection [170]. A similar approach termed frontally eluted components (FEC) procedure was presented in 2016 [171]. It was based on two-fold development in the same direction. The first development was carried out with a mobile phase low in elution strength to front-elute such matrix compounds with lower adsorption energies than the target analytes. With the second development, using a mobile phase of higher elution strength, the analytes were front-eluted to a shorter migration distance while the residual matrix remained close to the application area. Thus, the analytes were separated from matrix compounds both with higher and lower adsorption energies. Further research in the field of multi-residue drug analysis demonstrated that multifold developments were necessary to focus all analytes in a sharp zone and has led the authors to rename this concept to solvent front position extraction (SFPE; Fig. 1B) [172–174].

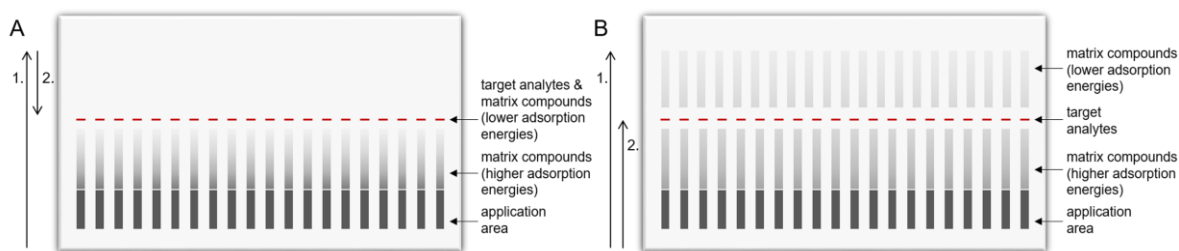


Fig. 1. Comparison of two different planar clean-up approaches: HTpSPE with the second front-elution carried out in the reverse direction (A) versus SFPE with two front-elutions in the same direction (B).

1.5.3 Planar antibacterial bioassays

In (HP)TLC-EDA, the open planar system enables rapid sample screening with low effort and data handling. Due to its matrix tolerance, sample preparation can be reduced which offers a comprehensive view on the samples. Compared to conventional sum parameter assays, (HP)TLC-EDA includes a chromatographic separation and therefore the observed effect can be directly linked to the respective zone, which avoids false results due to interferences or antagonistic/synergistic effects. All compounds applied on the plate remain on the adsorbent (except for volatile compounds), and even those that do not migrate with the mobile phase and remain at the start zone are accessible to the bioassay and visible thanks to image-based detection. Only the zones that show biological effects are then further investigated using MS or NMR hyphenations, reducing the number of analyses to a reasonable level. Organic solvents used during extraction and chromatography are evaporated and therefore do not interfere with biological detection. Memory effects from a previous run are avoided since a new plate is used for each analysis [175]. Detectabilities are high with LODs typically in the sub-ng to pg-range. [152]

Bioautography is a type of EDA that describes the detection of biological effects using microorganisms. It is differentiated into agar diffusion bioautography, for which the (HP)TLC plate is placed onto an agar medium containing the respective microorganism [176], and agar overlay bioautography, for which the plate is immersed in or covered with an inoculated agar medium [177], and direct bioautography (DB). For the latter, the plate is immersed into a microorganism suspension or the plate is piezoelectrically sprayed with the suspension, followed by incubation in a moist atmosphere. [175]

For antibacterial compounds, DB methods were reported based on, for example, *B. subtilis* [178–183], *Escherichia coli* [184,185], *Pseudomonas savastanoi* pv. *phaseolicola* [186], *Pseudomonas syringae* pv. *maculicola* [187], and *Staphylococcus aureus* [178] bacteria [188]. An (HP)TLC-direct bioautography kit using *B. subtilis* is commercially available [188], which might be the reason why applications with *B. subtilis* are most frequently reported [175]. After dipping or spraying the bacteria suspension and incubation in a moist chamber, usually, the yellow 3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) is sprayed onto the plate and during subsequent incubation, the MTT dye is reduced to a rose formazan dye in living bacteria only (Fig. 2) [189]. Hence, antibacterial zones are visible as white/yellowish zones on a rose background (Fig. 2) [175]. (HP)TLC-DB with the Gram-negative *Aliivibrio fischeri* (*A. fischeri*) bacterium [178,181–183] is based on a different detection principle. If *A. fischeri* bacteria are

exposed to antibacterial compounds or compounds affecting the bacterial metabolism, their natural bioluminescence is quickly reduced or inhibited altogether [175]. The inhibition of the bioluminescence can be detected by placing the (HP)TLC plate covered with the bacteria suspension in a dark chamber with a sensitive charge-coupled device camera, generating a grey-scale picture [175]. Thus, metabolism-affecting/antibacterial compounds are visible as dark zones on a grey background, whereas metabolism-enhancing compounds are visible as bright zones.

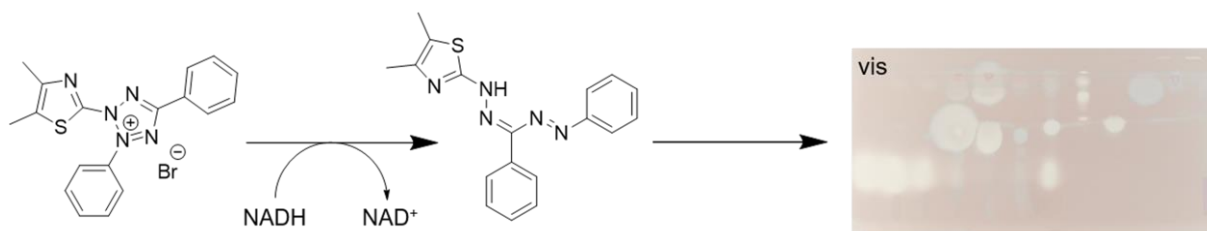


Fig. 2. Conversion of the yellow dye MTT into its rose formazan reaction product mostly by nicotinamide adenine dinucleotide + H⁺ (NADH) in living cells, and corresponding HPTLC-*B. subtilis*-vis image.

1.5.4 TLC-MS hyphenations

Hyphenation of (HP)TLC with MS is an essential option to obtain more information about the analytes contained in the zones of interest for confirmation of suspects or identification of unknowns. The storage of the chromatogram on the open planar stationary phase allows the direct link of mass signals to the substance zones that were previously evaluated [152]. There are two general principles for the direct coupling of planar chromatography to MS: desorption- and elution-based techniques. Desorption-based techniques rely on atom bombardment, ion bombardment, laser light beams, spray beams, or excited gas beams [190]. These techniques hit only a portion of the zone size on the adsorbent surface and are therefore more suited for the detection of the main compounds, but not for trace analysis. They require internal standards to compensate for fluctuations of desorption, ionization, sputtering, and conduction into the MS. [190]

There were different approaches for elution-based techniques, such as micro capillary arrow [191], surface sampling [192–194], overrun chromatography on a TLC strip unit [195], and forced-flow chromatography [196–198]. Today, elution head-based interfaces are the most commonly used devices. They can extract the whole substance zone including its depth profile and thus, offer better detectability than desorption-based techniques [199,200]. They are flexible plug-and-play devices compatible with any ion source used in column chromatography, *e.g.*, ESI, APCI, and atmospheric pressure photoionization (APPI) [190]. The first commercially available elution head-based TLC-

MS interface [201] was launched by CAMAG in 2009. An upgraded TLC–MS Interface 2 followed in 2015. It operates with a pneumatic elution head movement, activated by a toggle switch. After manual plate positioning, the cutting edge of the elution head is pressed tightly onto the silica gel layer and valve elution is activated via a six-port valve (Fig. 3A/B). The solvent flowing through the elution head then dissolves the retained compounds and elutes them into the ion source of the MS. Elution head cleaning is carried out by pushing a button, employing a parallel gas flow through the six-port valve. Dried particles are captured by a drawer underneath, moving simultaneously during the head cleaning cycle. All of the above steps must be performed manually, and if a step was forgotten or the order was reversed, a pressure increase may occur, resulting in zone leakage, requiring the repetition of analyses. Hence, automation was needed for a high number of elutions from the same plate in particular. It had also been demonstrated that manual plate positioning might lead to up to 6% of the total random error [202], compromising the quantitative precision of the measurement. In 2017, the first low-cost, fully automated interface was presented [203], termed autoTLC–MS (Fig. 3C) [204]. It is an open-source upgraded CAMAG TLC–MS Interface 2, facilitating target zone selection by simple clicks-on-the-image, automated plate positioning, and advanced automation of the elution and cleaning process. Stepper motors move a plate holder along the x- and y-axes directly on the metal base plate. The elution head movement is still pneumatically driven and head cleaning is adjustable in interval and intensity. An adjustable gas beam directed on the plate ensures a particle-free surface for the next zone elution, provided by an electric six-port valve. Thus, it enables a fully automated sequence analysis of all zones of interest.

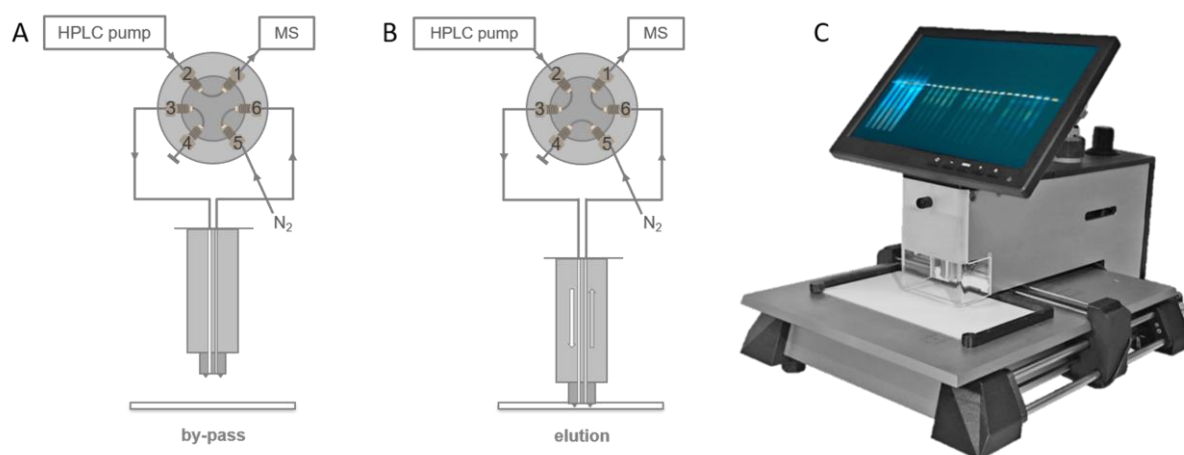


Fig. 3. Scheme of the elution head and six-port valve of the CAMAG TLC–MS Interface 2 in by-pass (A) or elution position (B), and image of the open-source upgraded autoTLC–MS interface with a 20 cm × 10-cm plate installed (C).

1.5.5 TLC–HPLC–MS hyphenations

Coupling planar chromatography with HPLC–MS is a promising tool to elucidate the nature of compounds co-eluting in a single zone, combining the advantages of both techniques. It maximizes the information obtained from a single sample run. An orthogonal dimension can be added if normal-phase HPTLC is combined with RP-HPLC. The simplest way of a TLC–HPLC–MS hyphenation is the elution of the target zones with a TLC–MS interface and collection of the eluates in autosampler vials followed by injection of a few microliters of the sample solutions into an HPLC–MS system [163,164]. The disadvantage of this workflow is obvious: Only a small portion of the sample or target analyte on the HPTLC zone is introduced into the MS. When the analyte amount on the plate is small as in trace analysis, the performance of the method is compromised with regard to LODs and quantification limits. Using online techniques, the complete sample amount is directly transferred to the HPLC column, improving detectability. The simplest online technique for hyphenation of HPTLC and HPLC is the installation of an HPLC column in between the TLC–MS interface and the MS, as first used for the analysis of Sudan dyes in food [205,206]. The TLC–MS interface was manually operated and all elutions from the plate were directly guided into the column. Other authors also demonstrated the usefulness of such an online-coupling technique for plant alkaloids [207,208]. A monolithic column was used because this column type generates lower backpressure than particulate columns [209], which is essential because a high backpressure over approximately 6.5 MPa can cause the elution head to leak in this setup [210]. Another study that focused on EDA of lemon balm isomers used a particulate column, but only at a flow rate of 0.2 mL/min to avoid backpressure problems, resulting in long run times [210]. The mentioned studies employed isocratic HPLC conditions probably due to a lack of automation of the elution process and missing communication between the interface and the HPLC pump. Nevertheless, HPTLC–HPLC–MS has been online-coupled to EDA, eluting the active zones directly from the bioautogram using gradient separation [211]. An improved eight-dimensional workflow including an additional valve switch compatible with any HPLC column, flow rate, and gradient was recently published [212]. Thereby, both the elution process and the chromatographic run were started manually. Although online coupling of HPTLC–HPLC–MS is very advantageous, the automation of all steps is still missing, but essential for time- and cost-efficient measurements. Despite the recent progress described, it remains unattractive for routine analysis if human intervention would be required after every HPLC run.

2. Scope

Analysis of VD residues in food of animal origin is of utmost importance to detect non-compliant food and protect consumers from adverse effects. However, the trace analysis of multi-class VD residues in heavy food matrices still poses a major challenge for state-of-the-art analytical methods. Effective, high-throughput, and inexpensive clean-up methods are needed to handle the large sample volumes in routine analysis. Therefore, the main scope of this work is to investigate the potential of different hyphenated HPTLC techniques for trace-level analysis of VDs in food of animal origin.

HTpSPE, a new clean-up concept in pesticide residue analysis, represents an interesting option that could also be suitable for VD residues in animal-derived food. However, the application of HTpSPE has not yet been shown for either multi-class VDs or food of animal origin. Hence, the first objective of this study is to investigate for the first time whether HTpSPE is suited for residue analysis of VDs in the most commonly consumed animal-derived foods, namely muscle meat, cow milk, and chicken eggs. Therefore, an HTpSPE method for screening of 66 multi-class antibiotics in water-based food extracts should be developed. Detection should be based on fully automated direct elution to orbitrap-HRMS via the autoTLC–MS interface to ensure high-throughput analyses without compromising the LODs.

The HPLC separation of compounds co-eluting in a single HPTLC zone or, in particular, in an HTpSPE target zone, is advantageous. Therefore, the next aim is the modification of the hardware of the autoTLC–MS interface to enable a fully automated hyphenation of HPTLC or HTpSPE with HPLC–MS by the transmission of contact closure signals to the HPLC pump. Further modifications of hardware and software should ensure convenient handling of the then renamed autoTLC–LC–MS interface in routine analysis.

The next goal is to extend the scope of the HTpSPE method to 81 multiclass VDs, which include five drug classes other than antibiotics. In addition to muscle, milk, and eggs, the applicability of HTpSPE should also be investigated for the first time for the matrix honey. Therefore, various optimizations of the first HTpSPE approach should be carried out. The modified autoTLC–LC–MS interface should be used for detection via automated HTpSPE–HPLC–HRMS/MS.

In addition to physicochemical methods, microbiological detection is another screening tool for antibiotics. However, positive bioassay screening results often remain unconfirmed by routine HPLC–MS/MS methods. As a first approach to explain this

inconsistency, the potential of non-target planar bioassay screening should be investigated using the example of fluoroquinolone residues in milk.

Next, the application of planar bioassay screening to HTPSPE chromatograms of pig muscle, cow milk, chicken eggs, and honey should be examined. Unexpectedly strong antibacterial effects caused by the food matrices themselves should be further investigated by direct coupling to HRMS using the autoTLC–LC–MS interface to elucidate the above-mentioned methodological inconsistency and to characterize the antibacterial zones.

Furthermore, HPTLC–HRMS is a promising tool to obtain structural information on bioactive compounds in a wide variety of samples. To demonstrate this, it should also be used for the identification of bioactive compounds in coral samples and honey, and for the characterization of lipid composition in the liver of *Schistosoma mansoni*-infected hamsters, which, however, is not the main subject of this thesis.

3. Progress achieved

The results and achievements of this work are presented in condensed form in the following sections.

3.1 Development of an HTPSPE–HRMS/MS screening method for antibiotics in muscle meat, cow milk, and chicken eggs (Publication I)

HTpSPE was introduced as an efficient clean-up procedure for pesticide residue analysis in fruits, vegetables [163,165], and tea [164], and its scope has been expanded to other matrices and analytes [166–169], and also to bioassay [170] detection. However, its applicability for multi-residue analysis of VDs in animal-derived food has not yet been demonstrated. Hence, in this study, an HTPSPE–HRMS/MS method has been developed for 66 antibiotic residues in muscle meat, cow milk, and chicken eggs for the first time. The antibiotics were of nine different structural classes, *i.e.*, sulfonamides, diaminopyrimidines, lincosamides, pleuromutilins, macrolides, cephalosporins, penicillins, amphenicols, and nitroimidazoles.

First, the raw extraction method [213] based on an aqueous McIlvaine-EDTA buffer was adapted to be suited for planar analysis, followed by the development of a mobile phase system for the HTPSPE clean-up. This was a major challenge due to the vast number of analytes, the structural differences between them, and the resulting different chemical properties as well as the heavy matrix load of the aqueous raw extracts. Various mobile phase combinations were tested and different stationary phases were assessed. After HTPSPE optimization, the planar clean-up of 16 samples in parallel including application took only 7 min per sample, which was about 5-fold faster than routine methods used in Germany's official control laboratories [30,213,214], and consumed only 0.6 mL organic solvent per sample, representing a more ecological alternative. The sharply focused target zones were automatically eluted into a Q Exactive Plus hybrid Quadrupole Orbitrap mass spectrometer using the autoTLC–MS interface with a short monolithic column installed in between to slightly retard the analytes. This direct elution into the HRMS instrument was preferred over the usual HTPSPE workflow of manually eluting the analyte zones into autosampler vials and then injecting them into an HPLC system with regard to the LODs, and to minimize the manual interventions required. A non-target variable DIA (vDIA) acquisition method was optimized for screening of the different VDs and a compound database was built for MS detection of one representative of each antibiotic class.

The method was validated as a screening method according to Commission Decision 2002/657/EC [16] for each representative, proving its applicability for residue analysis of sulfonamides, diaminopyrimidines, lincosamides, pleuromutilins, macrolides, and nitroimidazoles at 25 and 50 µg/kg in muscle and milk, and at 50 and 100 µg/kg in eggs, depending on the particular antibiotic. Cephalosporins, penicillins, and amphenicols could not be recovered at all because they co-eluted with matrix compounds far below the target zone. Nevertheless, the developed HTpSPE–HRMS/MS method showed great potential for VD residue analysis and represents a promising time- and cost-efficient alternative to routine methods. However, further studies are needed to evaluate its applicability for other antibiotics not yet covered by the proposed method, and for further improvement of performance and detection limits.

3.2 Modification of the autoTLC–MS interface (Publication II)

The autoTLC–MS interface [204], an open-source automated CAMAG TLC–MS Interface 2, has been an essential part of the HTpSPE–HRMS/MS workflow described above. The fully automated image-based target zone positioning and elution into the HRMS proved to be a reliable tool for high-throughput analyses. However, during routine use, some weaknesses of the system became obvious: The clamp-like plate holder design provided only a limited extractable plate surface area so that plates smaller than 20 cm × 10 cm could not be extracted, and occasionally, plates came completely detached from the plate holder during measurement. In addition, the system lacked full automation for sequential HPLC analysis, and the software design was confusing with several features remaining unclear in the initial publication [204].

Hence, a newly designed plate holder with a thin support base was 3D printed. It is suitable for all common plate sizes in (HP)TLC and allows zone elution with only a 1-cm distance to the plate edges. An additional 5-V relay was installed to send contact closure signals to an HPLC pump, enabling communication between the instruments and thus allowing sequential zone extraction followed by HPLC separation. The software was trimmed for fast loading and stable operation, and additional information about G-code commands required for versatile operation was given.

As proof-of-principle, the HPLC separation of a mixture of multi-class antibiotics applied on an HPTLC plate three times as a five-level calibration pattern was demonstrated. Two identical plates were prepared and only one was subjected to the *B. subtilis* bioassay, whereas the other was kept clean and fixed on the plate holder. In this way, the tedious

manual marking step for transfer of the elution positions from the bioautogram to the clean plate was avoided. The mixture was separated on a monolithic HPLC column installed directly in between the interface and the HRMS. In between each zone elution, a blank space on the plate was eluted to clean the elution head from residual analyte amounts. A disadvantage is that each blank elution required an additional HPLC run in this setup. The vDIA mode used in the previous work was modified for a faster acquisition rate to obtain a sufficient number of data points per peak. For five calibration levels of seven antibiotics, intra-day precisions ($n = 3$) ranged 2.1–14.1%, whereas inter-day precisions ($n = 3$) ranged 2.5–16.1% with calibration curves of $R^2 > 0.993$. This proved the renamed autoTLC–LC–MS interface to be a reliable and convenient on-surface autosampler applicable for routinely extracting compounds from planar materials followed by automated online transfer to HPLC–MS.

3.3 Development of an HTPSPE–UV/vis/FLD–HPLC–HRMS/MS screening method for VDs in muscle meat, cow milk, chicken eggs, and honey (Publication III)

The previously developed HTPSPE–HRMS/MS (Publication I) procedure was adapted for screening of 81 widely used VDs in muscle meat, cow milk, chicken eggs, and also honey. Thereby its scope was expanded to other VDs than antibiotics, namely glucocorticoids, anthelmintics, antiparasitics, coccidiostats, and NSAIDs. Instead of water-based extracts, higher concentrated acetonitrile-based extracts for sufficient protein precipitation and wider polarity range [34,50] were produced in a two-step extraction, followed by salting-out induced partitioning to adjust the water content [215], and thus the volume of each extract. Since the food matrices contain a different percentage of water and the dosing on the TLC plate is volumetric, this was considered necessary to obtain quantitative results. The acetonitrile extracts showed a completely different behavior on the TLC plate than the water-based extracts and made adaptations necessary. Hence, the mobile phase composition for the HTPSPE clean-up was optimized using UV/vis/FLD imaging.

The redesigned autoTLC–LC–MS interface automatically eluted the VD target zones (the eluent was provided by a standalone pump) into an electric six-port, two-position switching valve with a 100- μ L sample loop installed. During an optimized period of 40 s, the loop was filled with the sample solution extracted from the plate. Then the valve was switched and the eluent provided by the HPLC pump flushed the loop, transferring the analytes to the HPLC column. After the valve was switched back into the load position, the low-pressure autoTLC–LC–MS system was decoupled from the high-pressure HPLC–

HRMS/MS system so that the analytes could be gradient separated and the elution head could be simultaneously cleaned during the HPLC run, allowing short analysis times. The analytes were detected using the vDIA mode optimized in the previous publication and the mass signals were compared to an in-house compound database that was built for all 81 analytes for confirmation.

For appropriate quantification, different extraction conditions and salt combinations were studied and optimized, but the quantitative performance of the method was poor and could not be further improved. Hence, it was decided to validate the method as a screening method according to the latest Commission Implementing Regulation 2021/808 [15]. Therefore, the so-called “detection capability for screening (CC β)” was determined for each VD. CC β is the analyte concentration in the sample, at which the false negative rate is below 5%. It was determined by the analysis of 20 previously analyzed blank samples spiked at the 5- and 25- $\mu\text{g}/\text{kg}$ levels (Fig. 4). Thus, if a particular analyte was identified in at least 19 out of 20 samples, validation was considered successful. Most analytes were successfully validated at the 5- $\mu\text{g}/\text{kg}$ level in meat, milk, and eggs, which is far below the MRLs of most compounds except for glucocorticoids. Honey was more challenging, as the analytes could only be validated at the five-fold higher 25- $\mu\text{g}/\text{kg}$ level. Validation was not successful for penicillins and cephalosporins and only partly for amphenicols and a few other analytes, confirming the results of the first HTpSPE–HRMS/MS study (Publication I). The method was applied for screening of 36 real samples (nine samples per matrix) and no residues of the target analytes exceeding 5 $\mu\text{g}/\text{kg}$ were found. Thereby, 24 tracks in parallel were applied on one TLC plate, including 21 samples and three quality control samples. For this, the HTpSPE clean-up including application took 6.5 min (about five times faster than routine methods) with 1.9 mL solvent consumption and a total cost for consumables of 3.60 € per sample. A sample can be prepared, extracted, purified, column separated, detected, and confirmed in approximately 32 min with 11 min of manual steps mostly spent for primary preparation. The developed HTpSPE–UV/vis/FLD–HPLC–HRMS/MS method is the first HTpSPE approach to cover such a wide target analyte range and represents the first generic screening across the different classes and matrices with regard to sample preparation and analysis and will increase the currently limited screening capacities.

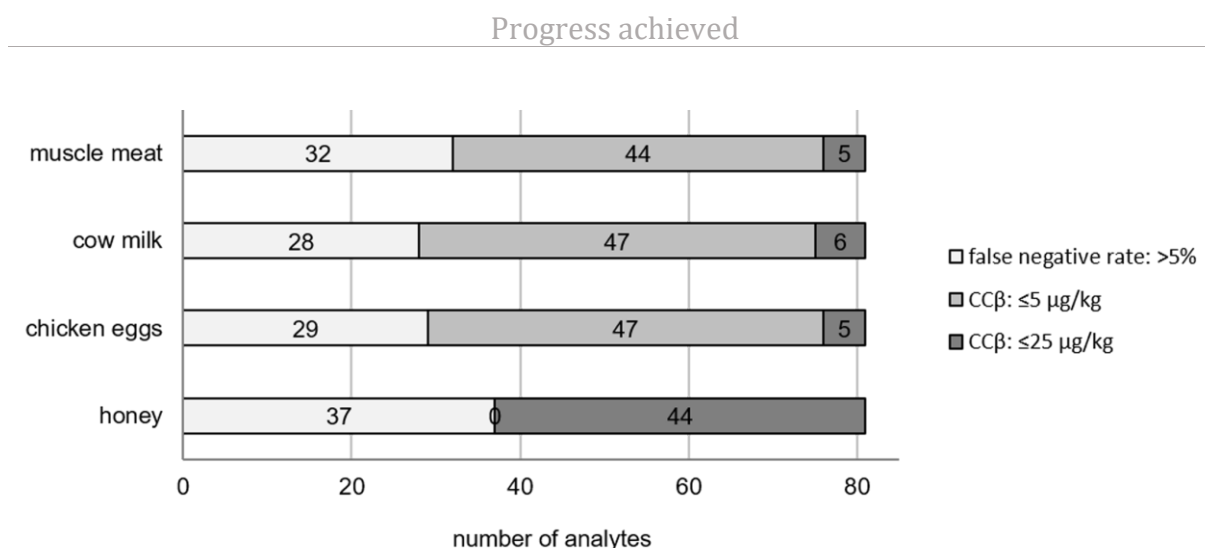


Fig. 4. Results of the validation study for 81 VDs in muscle meat, cow milk, chicken eggs, and honey presented as the number of analytes that had a false negative rate >5% compared to the number of analytes that were successfully validated with a detection capability for screening (CCβ) at the 5- or 25-μg/kg level.

3.4 Potential of non-target HPTLC–UV/vis/FLD–bioassay screening for antibacterial compounds in milk (Publication IV)

Screening for antibacterial residues in food is largely performed by sum-parameter bioassays followed by targeted HPLC–MS/MS analysis for confirmation of potential residues. However, in 2019, an exceptionally high percentage of 87% positive results in the three-plate test remained without confirmation by HPLC–MS/MS in Germany’s official control laboratories [88]. This could be because the targeted HPLC–MS/MS approach is blind to compounds outside the analytical focus. Non-target planar bioassay screening could be an option to clarify this inconsistency. It can detect antibacterial degradation or metabolization products as well as unknowns and directly link the effect to the respective zone due to the precedent chromatographic separation and the nature of these compounds can then be elucidated by coupling to HRMS.

As a first approach to evaluate the potential of HPTLC–UV/vis/FLD–bioassay screening for the field of VD residue analysis, its applicability was investigated exemplarily for two fluoroquinolone antibiotics in milk. For comprehensive effect-directed screening, the samples were only diluted with acetonitrile and centrifuged to keep them as native as possible. After mobile phase optimization for the separation of the fluoroquinolones from the milk matrix, several commercially available milk samples were screened for antibacterial activity via the planar Gram-positive *B. subtilis* bioassay. The standards marbofloxacin and ciprofloxacin were barely visible at 100 μg/kg (12 ng/zone), but three very prominent antibacterial zones in the milk samples were detected. One was at the application area and two others were close to the solvent front, indicating the presence of

other unknown antibacterial compounds. The strongest response was observed for raw milk. It was assumed that fatty acids or acylglycerols from the milk matrix could have been responsible for this effect, as these have already been discussed as alternative antimicrobials [216]. This could be an explanation for the methodological inconsistencies between biological and MS detection, but still must be confirmed via coupling to HRMS. Nevertheless, the results highlighted the high levels of active compounds in the samples if compared to the low response to the fluoroquinolones. Further optimizations were carried out to lower the biological detection limits to 200 µg/kg for ciprofloxacin and 800 µg/kg for marbofloxacin and to increase the sample throughput to 18 samples per plate. Using the final method, 54 samples (three plates) could be prepared in one day with a mean of 10 min analysis time and 4 min manual operating time for about 0.5 € per sample. In addition, the samples were screened using the *A. fischeri* bioassay. As expected, the fluoroquinolones did not show an effect against the Gram-negative bacteria, whereas five antibacterial zones were evident in the upper bioautogram, which will most likely influence the gut microbiome when consumed. These findings challenge the current prevailing understanding of food safety based on target analysis.

3.5 Proof that endogenous fatty acids and lipids cause strong antibacterial effects in bioassay screening methods for VD residues (Publication V)

The results of the previous study demonstrated the potential of planar bioassay screening for the detection of VDs and unknown antibacterial compounds in milk. To further investigate this, HTpSPE plates with extracts of blank muscle meat, milk, eggs, and honey were subjected to the planar *B. subtilis* bioassay. All blank samples investigated were either organically produced or known to be free of VD residues and previously analyzed using the HTpSPE–UV/vis/FLD–HPLC–HRMS/MS method (Publication III), which showed no positive screening results. Still, prominent antibacterial responses were observed in the blank extracts of all matrices, which were much stronger than those to a mixture of 81 VDs. No difference in the response to blank extracts and blank extracts that were oversprayed with the 81-standard mixture was evident as the response to the extracts superimposed the response to the standards. This demonstrated that this detection approach is not suitable for HTpSPE. It also showed the high level of antibacterial activity present in the samples, which was not caused by target compounds but by unknowns outside the analytical focus. For further investigation, blank extracts, reagent blanks, and the 81-standard mixture were applied side-by-side and the plates were only developed

once to improve the separation of the antibacterial zones instead of focusing them by performing a full HTPSPE procedure. The plates were then subjected to different chemical derivatizations as well as the *B. subtilis* and *A. fischeri* bioassays. Comparison to reagent blanks revealed that antibacterial zones in the lower part of the plate and very close to the elution front were mainly caused by chemicals used during extraction. Primuline derivatization indicated the presence of lipophilic compounds such as fatty acids or lipids at the same position as the antibacterial zones in the upper plate part for muscle meat and milk in particular and less intense for eggs and honey. The active zones were further characterized by direct HPTLC–HRMS, which revealed very abundant mass signals, and assigned the respective molecular formulas mostly matching fatty acids and various lipid classes. The results were confirmed using standard solutions of fatty acids as well as mono-, di-, and triacylglycerols. Their antibacterial effect has already been discussed in the literature [216] and has also been observed in other HPTLC–EDA–HRMS studies [182,183,217]. However, to the best of our knowledge, they have not yet been recognized to be the cause of false-positive bioassay results. Depending on the animal species, sex, and type of feed, animal-derived foods may vary in lipid amount and composition. This results in different degrees of antibacterial activity and causes false-positive results in sum-parameter bioassays.

The presented planar non-target screening strategy coupled to HRMS thus explained for the first time the high number of positive results obtained from biological screening for VD residues that remain unconfirmed by physicochemical methods. Thanks to chromatographic separation and effect detection on the same surface, it has bridged the gap between non-target sum parameter bioassays and targeted chromatographic methods blind to effective off-target compounds. It has to be clarified if the antibacterial activity of fatty acids and lipids is problematic, as the effect of the various foods on the human gut microbiota still needs to be investigated. However, the present understanding of food safety based on targeted HPLC–MS/MS analysis often combined with previous positive bioassay screening is to be questioned. Which compounds count for food safety, only exogenous target-residues or all compounds exerting a specific effect?

3.6 Analysis of unknown bioactive compounds in extracts of coral samples and honey (Publication VI & VII)

Because the previous study demonstrated the potential of HPTLC–EDA–HRMS hyphenations to identify compounds exerting biological effects, two further studies were conducted using the same approach for completely different samples.

In the first study, Indo-pacific bottle-nose dolphins in the Egyptian Northern Red Sea have been observed queueing up in natural environments to rub particular body parts against selected corals (*Rumphella aggregata* and *Sarcophyton* sp.) and sponges (*Ircinia* sp.). The presence of bioactive compounds with beneficial effects on skin health was hypothesized to be the reason for this selective rubbing behavior. Hence, via HPTLC–EDA, multi-potent bioactive zones were identified, which were further investigated by direct coupling with HRMS, assigned to the respective molecular formulas, and tentatively identified by comparison to the literature, while some remained unknown and need to be further elucidated. Nevertheless, the observed effects and their assignments provided for the first time an explanation for the astounding rubbing behavior of the dolphins.

In the second study, honey as a complex, healthy, and nutrient-dense food has been investigated. Honey mainly consists of different saccharides, but also of bioactive phytochemicals derived from the plant nectar taken up by the bees and has therefore been used in traditional medicine [218,219]. For characterization of these bioactive constituents, an HPTLC–EDA–HRMS workflow was applied, whereby some multiple bioactive compounds in different Ethiopian honey sorts were tentatively identified, whereas others remained unknown. Although further structure elucidation is required, the study provided new insights helpful for the valorization of Ethiopian honey.

3.7 Quantification and HRMS profiling of lipid composition in the liver of *Schistosoma mansoni*-infected hamsters (Publication VIII)

Schistosomiasis is a neglected parasitic infection caused by *Schistosoma mansoni* that results in liver morbidity. However, the pathomechanisms underlying this disease are not yet fully understood. Hence, schistosomiasis-associated metabolic reprogramming was investigated using various analytical methods. Quantification and profiling of lipid composition in the liver of hamsters infected with *Schistosoma mansoni* via HPTLC–FLD–HRMS helped to understand that, among other effects, it is mainly the eggs that deplete the lipid reservoirs of the host, as they completely reprogram lipid and carbohydrate metabolism by soluble factors.

4. References

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5. Publication I

High-throughput planar solid-phase extraction coupled to orbitrap high-resolution mass spectrometry via the autoTLC-MS interface for screening of 66 multi-class antibiotic residues in food of animal origin

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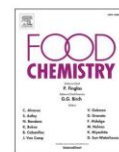
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ABSTRACT

Antibiotic residues in food pose a major threat to the health of humans and animals worldwide. Their trace-level analysis is still too time- and cost-intensive to be adequately covered in routine analysis. Thus, a new high-throughput planar solid-phase extraction method has been developed for rapid screening of 66 antibiotics. Via simple clicks on the image, the autoTLC-MS interface automatically eluted the target analyte zones directly into an orbitrap high-resolution mass spectrometer operated in the variable data-independent acquisition mode. Muscle tissue, cow milk and chicken eggs were analyzed regarding nine different antibiotic classes, including sulfonamides, diaminopyrimidines, lincosamides, pleuromutilins, macrolides, cephalosporins, penicillins, amphenicols and nitroimidazoles. The planar clean-up took 7 min per sample, which is 5-fold faster than the routine state-of-the-art. The screening method has been validated for one representative of each class according to the European Commission Decision 2002/657/EC. Most analytes were successfully detected at half of their required maximum residue limit.

1. Introduction

Veterinary drugs and their residues in meat or animal-derived products are a major concern in ensuring food safety since they are widely used in mass livestock farming. The use of anti-infectives is indispensable to avoid the rapid spreading of infections amongst livestock. In particular, antibiotics are often used not only for therapeutic or prophylactic treatments, but also to promote growth and to increase feed efficiency (Bacanli & Başaran, 2019; Baynes et al., 2016). This may lead to residues of antibiotics in animal products and to the contamination of feedstuffs, crops and vegetables through the recycling of faeces as fertilizer (Bacanli & Başaran, 2019). In the European Union, the use of veterinary medicinal products for food-producing animals is regulated by setting maximum residue limits (MRLs) for a large variety of different drug substances in foodstuffs of animal origin (European Commission, 2010). Antibiotic residues above the MRLs, resulting from inadequate withdrawal periods before slaughter or milking, inappropriate dosage or environmental contamination, can have adverse effects on the health of consumers, such as changes in the human gut microbiota, chronic toxic effects, mutagenicity, carcinogenicity or hypersensitivity reactions

including anaphylaxis (Bacanli & Başaran, 2019; Baynes et al., 2016). There is also a high risk of transmitting antibiotic-resistant bacteria to humans, leading to infections in humans and animals that are difficult to treat (Bacanli & Başaran, 2019). To reduce the health risks to consumers to a minimum, low MRLs in the µg/kg-range have been established for most antibiotics. For veterinary medicinal products without MRLs, a zero-tolerance principle applies, which is a major challenge for trace analysis with regard to the minimum performance criteria required. Qualitative or semi-quantitative screening methods as in Commission Decision 2002/657/EC (European Commission, 2002) should be designed in such a way that they do not lead to false-compliant results with a reasonable percentage of false-detects. Screening methods can be biological methods, measuring a cellular response (sum parameter), biochemical methods based on antibodies or receptors, as well as physicochemical methods. Samples that are found to be non-compliant should be subjected to confirmatory methods, which must provide information about the analyte structure and are therefore based on chromatographic separation with spectrometric detection.

High-performance liquid chromatography-electrospray ionization-tandem mass spectrometry, utilizing triple-quadrupole or ion trap multi-

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stage instruments, has been the method of choice for confirmatory analysis and is still considered to be the gold standard in targeted pesticide and veterinary drug multi-residue analysis (Mainero Rocca et al., 2017). The use of high-resolution instruments such as time-of-flight and orbitrap instruments for food analysis has increased over the last decade (Senyuva et al., 2015). Due to their high mass resolution and accuracy, these instruments offer the possibility to use full scan spectra in retrospective data analysis for non-target workflows. In addition, they enable structure elucidation for suspects or unknowns, often allowing identification and quantification in a single analytical run. Several multi-residue methods covering a very large number of analytes in different food matrices have been recently published in the field of pesticide and veterinary drug analysis using quadrupole orbitrap instruments (Jia et al., 2017, 2018a, 2018b; Wang et al., 2019, 2018) and variable data-independent acquisition (vDIA) mode (Wang et al., 2019; Raetz et al., 2020). Nevertheless, one of the main drawbacks of electrospray ionization in high-resolution mass spectrometry (ESI-HRMS) is the occurrence of matrix effects during ionization. Non-volatile organic compounds, co-extracted from heavy matrices, hinder the evaporation of analytes by increasing the surface tension of the droplets and competing with the analytes for access to their surface (Taylor, 2005). This compromises the maximum evaporation efficiency, leading to a reduction of sensitivity, thus influencing the reliability of the results in terms of accuracy. To a lesser extent, also signal enhancement is possible (Taylor, 2005).

The removal of co-extracted matrix compounds is the key to a reliable residue analysis. The simplest method to diminish matrix effects is the "dilute-and-shoot" strategy, in which raw extracts are diluted and injected into the LC-MS system subsequently (Granelli et al., 2009). However, excessive dilution is problematic in trace analysis with regard to limits of detection and quantification. Most commonly, multi-class methods are based on extraction with organic solvents such as acetonitrile (ACN) or methanol with subsequent solid-phase extraction (SPE), dispersive solid-phase extraction (dSPE) and gel permeation chromatography (Masiá et al., 2016; Mainero Rocca et al., 2017). Also diversely modified versions of the quick, easy, cheap, effective, rugged and safe (QuEChERS) extraction (Anastassiades et al., 2003), which includes a dSPE step, are frequently used. Despite continuous improvements, such as online-SPE or SPE with molecularly imprinted polymers, the clean-up efficiency of these methods is limited since very extensive purification would lead to the total loss of some analytes or substance classes (Mainero Rocca et al., 2017). Based on discussions on sample preparation and chromatography at one go with good accuracy and sensitivity despite high matrix load (Alpmann & Morlock, 2008; Morlock & Vega-Herrera, 2007; Morlock et al., 2011), HTpSPE was introduced as a clean-up concept for residue analysis of pesticides in fruits and vegetables (Oellig & Schwack, 2011). This approach represents a rapid and effective clean-up on planar adsorbents. After application of raw extracts, the target analytes were front-eluted on a TLC plate, isolating them from most matrices. A second front-elution in the reverse direction focused the analytes to sharp target zones. It was possible to purify up to 20 samples simultaneously under the same analytical conditions on a single TLC plate, resulting in minimum clean-up times per sample and very low matrix effects. HTpSPE was extended to further matrices (Oellig & Schwack, 2012; 2014) and analytes (Oellig & Hammel, 2019; Oellig & Melde, 2016; Schick & Oellig, 2019; Schrack, Hohl, & Schwack, 2016; Wagner & Oellig, 2019). A modified strategy was based on a two-step front-elution in the same direction, termed front eluting components (Klimek-Turek et al., 2016). In this strategy, the first front-elution served as front-elution of matrix compounds that had lower adsorption energies than the target analytes. With the second front-elution utilizing a higher solvent strength, the analytes were front-eluted to a shorter developing distance, while highly adsorbing matrix compounds remained at the lower part of the plate. Although the analytes were thus separated from interfering matrix compounds, later research required multifold front-elutions to focus all analytes into a sharp zone, and this concept was

renamed to solvent front position extraction (SFPE) (Klimek-Turek et al., 2017, 2019).

The good results shown so far led to the challenging hypothesis that HTpSPE can also be used as a purification concept for residue analysis of antibiotics in food of animal origin. The screening method to be developed should also explore for the first time, the use of a fully automated elution of the analytes from the HPTLC plate and its direct transfer to a HRMS system operating in the vDIA mode. The applicability of the developed trace-level screening should be proven by validation.

2. Materials and methods

2.1. Chemicals and materials

Double-distilled water was produced by a Heraeus Destamat Bi-18e (Fisher Scientific, Schwerte, Germany); from the latter also water, formic acid (both Optima LC-MS grade) and ethanol (HPLC grade, $\geq 99.8\%$) were obtained. Methanol (Chromasolv LC-MS grade, $\geq 99.9\%$) was from Honeywell Riedel-de Haën (Seelze, Germany). Acetonitrile (Chemsolute LC-MS grade, $\geq 99.95\%$) and o-phosphoric acid (85% puriss.) were from Th. Geyer (Renningen, Germany). 4-Anisaldehyde ($\geq 97.5\%$), glacial acetic acid (p. a., 100%), sulfuric acid (p. a., 96%), disodium hydrogen phosphate ($\geq 99\%$), sodium hydroxide solution ($\geq 99\%$) and magnesium chloride hexahydrate ($\geq 97.5\%$) were purchased from Carl Roth (Karlsruhe, Germany). Ethylenediaminetetraacetic acid disodium salt dihydrate (99–101%), ammonium formate (LC-MS grade, $\geq 99.9\%$), rhodamine B (HPLC grade, $\geq 95\%$), diphenylamine ($\geq 99\%$) and aniline ($\geq 99.5\%$) were obtained from Sigma-Aldrich (Steinheim, Germany). Ammonia solution (Normatom, for trace analysis, 25–27%) and acetone (HiPerSolv, HPLC grade) were from VWR (Darmstadt, Germany). Citric acid monohydrate (p. a.) and TLC silica gel 60 F₂₅₄ MS grade plates 20 × 20 cm with a layer thickness of 0.18 mm, TLC plates silica gel 60 F₂₅₄, LiChrospher HPTLC plates silica gel 60 F₂₅₄ and HPTLC plates silica gel 60 F₂₅₄ were provided by Merck (Darmstadt, Germany). Prior to analysis, the plates were cut into 20 × 10 cm pieces. Porcine, bovine, chicken and turkey muscle meat was organically produced and obtained from local grocery stores, while game meat was obtained from local hunters. Raw milk samples were self-collected at a local organic farm. Chicken eggs were either organically produced and purchased at local supermarkets or were laid and provided by our own chickens. The 66 different antibiotics of the nine different structural classes are listed in Table S-1.

2.2. Stock solutions and standard mixtures

Stock solutions of each antibiotic of 0.1 or 1 mg/mL in different solvents (Table S-1) were proven to be storable at $-20\text{ }^{\circ}\text{C}$ in the dark for up to one year, except for penicillins, which were prepared freshly in double-distilled water every three months. Standard mixtures were prepared by combining the single stock solutions of each structural class in ACN, resulting in a concentration of 100 $\mu\text{g/mL}$ each for sulfonamides, diaminopyrimidines, lincosamides, pleuromutins, penicillins, macrolides and amphenicols, and 10 $\mu\text{g/mL}$ each for cephalosporins and nitroimidazoles. For spiking experiments, a working standard mixture comprising all standard compounds was prepared in ACN (1 $\mu\text{g/mL}$ each). All mixtures were stored at $-20\text{ }^{\circ}\text{C}$ in amber glass vials and prepared freshly every four weeks. A rhodamine B stock solution was prepared in ACN (1 mg/mL) and diluted with ACN to a concentration of 1 $\mu\text{g/mL}$.

2.3. Sample extraction and spiking

Meat samples were minced with a multi moulinette (Moulinex, Alençon, France), whereas milk and whole eggs were homogenized with an Ultra-turrax T25 (IKA, Staufen, Germany) at 24000 rpm for 2 min. All samples were stored at $-20\text{ }^{\circ}\text{C}$ until usage. Each homogenized meat, milk

(2 g each) and egg sample (1 g) was weighed into a 50-mL polypropylene tube, 10 mL McIlvaine-EDTA buffer (37.2 g ethylenediaminetetraacetic acid disodium salt, 21 g citric acid and 17.8 g disodium hydrogen phosphate in 1 L double-distilled water adjusted to pH 4 with 10 N sodium hydroxide solution) were added and vortexed at the highest speed for 1 min (Vortex Genie 2 with multi-tube holder, Scientific Industries, New York, USA), and then shaken manually end-over-end for 15 min. After centrifugation at $3000 \times g$ for 15 min, each supernatant was filtered through a MN 704 $\frac{1}{4}$ filter paper (Macherey-Nagel, Düren, Germany). Slightly turbid raw extracts were stored at 4 °C until further usage.

Before application, 6 μ L rhodamine B solution was added to 600 μ L of each raw extract, and for spiking, in addition 3 or 6 μ L of the working standard mixture (1 μ g/mL) were added and filled up to 1 mL with ACN. Thus, muscle and milk raw extracts (in 40% ACN) were spiked at 25 μ g/kg or 50 μ g/kg and egg raw extracts at 50 μ g/kg or 100 μ g/kg. The samples were vortexed for 1 min and centrifuged at $16200 \times g$ for 15 min, and the supernatants were transferred into autosampler vials.

2.4. HTPSPE clean-up and derivatization

An Automatic TLC Sampler (ATS 4) was used to apply 166 μ L of each sample or standard solution as 6×30 -mm area. Application parameters were set to dosage speed 1200 nL/s, predosage volume 200 nL, filling speed 10 μ L/s, filling vacuum time 2 s, retraction volume 200 nL, rinsing vacuum time 4 s, rinsing cycles 1, empty syringe before filling, and filling cycles 1. Water was used as rinsing solvent. The plate layout was defined as application position Y 10.0 mm, first track position X 15.2 mm and track distance 11.3 mm, resulting in 16 tracks per 20×10 cm plate. Plates were front-eluted in the Automatic Developing Chamber (ADC 2) after 5 min humidity control with a saturated MgCl_2 solution of 33% relative humidity. The first front-elution up to 85 mm was carried out using ACN/methanol/ammonia, 5:3:2 (V/V/V), while the second front-elution in the reverse direction up to 25 mm used ACN/water/ammonia 9:7:4 (V/V/V). After each front-elution, the plates were automatically dried in a stream of cold air for 3 min. The plate image was documented at UV 254 nm and FLD 366 nm (automatic exposure, TLC Visualizer 2).

For method development, the plates were derivatized by piezoelectric spraying (Derivatizer), followed by heating (110 °C, 3 min, TLC Plate Heater III). *p*-Anisaldehyde sulfuric acid reagent (methanol, glacial acetic acid, sulfuric acid and *p*-anisaldehyde 420:50:26:3, V/V/V; 4 mL, blue nozzle, level 3) was used for visualization of macrolides, ninhydrin reagent (500 mg ninhydrin in ethanol and glacial acetic acid 23:2, V/V; 4 mL, blue nozzle, level 4) for penicillins and aniline diphenylamine *o*-phosphoric acid reagent (2 g diphenylamine in methanol, *o*-phosphoric acid and aniline 90:10:1, V/V/V; 2 mL, yellow nozzle, level 5) for lincosamides. Instrumentation was controlled by visionCATS 2.5 (all CAMAG, Muttenz, Switzerland).

2.5. HRMS/MS analysis

The fully automated autoTLC-MS interface (Häbe & Morlock, 2020), equipped with a round elution head of 4 mm diameter, was connected to a Q Exactive Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific) with an Ion-Max HESI-II probe (sheath gas 20 AU, aux gas 10 AU, spray voltage 3.5 kV, capillary temperature 270 °C, probe heater temperature 200 °C and S-lens RF level 50 AU). In between the interface and the mass spectrometer, a filter frit (Upchurch Scientific A-356 and PEEK-Frit Blue UPA-703, Techlab, Erkerode, Germany) and a Chromolith Flash RP-18e 25–2 mm monolithic column were installed. The analytes were eluted from the TLC plates with a 0.1% solution of formic acid and 4 mM ammonium formate in water/methanol 55:45 (V/V) for 30 s at a flow rate of 0.2 mL/min (1100 HPLC system controlled by the handheld control module, Agilent Technologies, Waldbronn, Germany), which was subsequently held for 6.5 min before the next zone

elution. Spectra were recorded with a modified version of the non-targeted vDIA (Wang et al., 2019). In short, a full scan (m/z 100–1100) in the positive ionization mode with a resolution of 70,000 (FWHM at m/z 200), automatic gain control (AGC) target 1e6, maximum inject time 250 ms, lock masses m/z 301.14103 (dibutyl phthalate, $[\text{M} + \text{H}]^+$) and m/z 413.26623 (diisooctyl phthalate, $[\text{M} + \text{H}]^+$), was followed by sequential vDIA scans. DIA 1 covered a mass range of m/z 100–500 with an isolation window of 52 Da (loop count 8), while DIA 2 covered m/z 500–1100 with an isolation window of 104 Da (loop count 6). The general settings for the DIA scans were a resolution of 35000, AGC target 1e6, with stepped normalized collision energy (NCE) of 20, 40 and 60 eV. The inclusion list for the DIA scans consisted of m/z 125.00000, 175.00000, 225.00000, 275.00000, 325.00000, 375.00000, 425.00000, 475.00000, 550.00000, 650.00000, 750.00000, 850.00000, 950.00000 and 1050.00000. For build-up of a compound database, each antibiotic (20 ng) was applied on a TLC plate as 6-mm band and directly eluted with the autoTLC-MS interface into the mass spectrometer using a filter frit without a column in full scan acquisition mode (m/z 100–1100). The most abundant adduct was selected as precursor and fragmented by parallel reaction monitoring with an isolation window of 1.0 Da and stepped NCE of 20, 40 and 60 eV. External mass calibration was performed daily with PierceTM LTQ Velos ESI positive ion calibration solution (Thermo Scientific). The instrument was controlled by Xcalibur 3.0.63.3 with Foundation 3.0.152 and the data were processed with the target screening function of TraceFinder 3.2.512.0 (all Thermo Scientific). Mass Frontier 7.0.5.9 SR3 (HighChem, Thermo Scientific) and Chemdraw Ultra 12.0.2.1076 (Cambridgesoft, PerkinElmer Informatics, Rodgau, Germany) were used for fragmentation pathway simulations.

3. Results and discussion

3.1. Study design

A new HTPSPE-autoTLC-HRMS/MS screening tool has been developed suited for trace analysis. The analytes were extracted from complex matrices using the HTPSPE concept. For the first time, via simple clicks on the image, the autoTLC-MS interface automatically eluted the target zones at the trace level from the TLC plate into a Q Exactive Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer. This fully automated interface was self-upgraded by open-source technologies based on a commercially available interface (Häbe & Morlock, 2020). A fully automated transfer of all zones from the HPTLC plate to the HRMS system was found to be crucial for an efficient screening. The design and setup of the newly developed screening is presented in Fig. 1. The proof-of-principle was intended to be performed for trace level screening of antibiotics in complex food matrices. Antibiotics and their residues in food pose a major threat to the health of humans and animals worldwide. Their analysis at the trace level is still too time- and cost-intensive to be adequately covered in routine analysis. Hence, the newly developed screening tool was explored in this field and intended to support the currently limited screening capacities. Muscle tissue, cow milk and chicken eggs were selected as three quite different examples with a highly complex matrix. All in all, 66 antibiotics were chosen as analytes (Table S-1). These representatives were selected of nine different structural classes, including sulfonamides, diaminopyrimidines, lincosamides, pleuromutilins, macrolides, cephalosporins, penicillins, amphenicols and nitroimidazoles. For one representative analyte from each antibiotic class, the developed screening method was validated according to the requirements of Commission Decision 2002/657/EC (European Commission, 2002).

3.2. Extraction procedure

A previously reported raw extract preparation (2 g sample extracted with 10 mL McIlvaine EDTA buffer and 0.1 mL 20% trichloroacetic acid

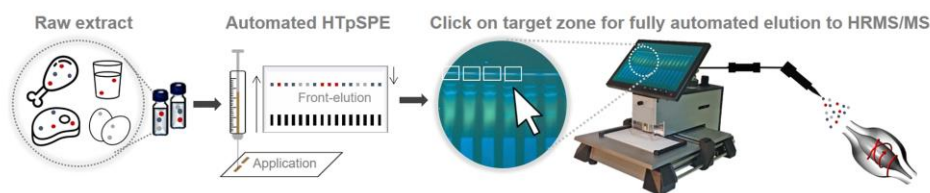


Fig. 1. Scheme of the new HTPSPE-autoTLC-HRMS/MS screening tool exploited for the new purification concept of antibiotic residues at the trace level in food: via simple clicks on the image, autoTLC-MS automatically eluted target zones of HTPSPE directly to orbitrap HRMS/MS. The HTPSPE steps, i.e. application and front-elution, were automated for up to 16 samples in parallel, consumed 0.6 mL solvent/sample and took 7 min/sample, followed by automated target zone elution taking 7 min/sample.

solution; Bohm et al., 2009) required modifications. The egg extract was too viscous to be filtered and could only be made filterable after halving the sample concentration to 1 g/10 mL. Application on the TLC plate using nozzle heating at 60 °C caused blockage of the application syringe due to protein precipitation, and thus, 40% ACN were added to the raw extracts. The trichloroacetic acid (used in the original preparation) front-eluted, interfered with the visual detection of antibiotics and caused cluster formation as well as ion suppression during HRMS measurements (Fig. S-1). Hence, the addition of trichloroacetic acid was skipped, as the addition of ACN proved to be sufficient for protein precipitation. All these modifications were essential to proceed.

3.3. Planar solid-phase extraction

The development of a solvent system for front-elution of antibiotics out of animal-derived food matrices posed a major challenge, caused by the very complex matrices and the diversity in chemical structures of the different antibiotic classes. Various pure solvents with high solvent strength were tested on normal-phase silica gel HPTLC plates. None of them was able to front-elute every antibiotic class at one go, as reported in multi-component drug analysis (Klimek-Turek et al., 2017). The latter authors also concluded that only multifold front-elutions in the same direction led to satisfactory results. To avoid additional time and material consuming steps, it was aimed to further investigate more complex solvent systems with the ability to front-elute all multi-class antibiotics in a single front-elution at one go.

A mixture of acetone/water/ammonia, 7.5:2:0.5 (V/V/V), on HPTLC silica gel 60 F₂₅₄s was able to focus almost all of the investigated antibiotics in the elution front. The solvent strength of the solvent system was increased by using the same plate type in the second trough as counter plate to adsorb gas phase vapors. Performing a single front-elution in the ADC 2 system after a 5-min humidity control (0–5% relative humidity with 0.3 nm-mesh molecular sieve) led to the formation of focused analyte zones, independent of the ambient conditions. Unfortunately, this solvent mixture was not suited for extraction of the antibiotics out of the food extracts, as it was too polar to sufficiently flow through the applied start zones of the extracts. Attempts to pre-treat the extract matrix by developing or immersing it in isopropanol or acetone, followed by the solvent mixture itself, did not bring much progress but increased the total clean-up time by at least one hour. Moreover, other tested stationary phases with different modifications, such as cyano, amino and water-wettable reversed phases did not bring progress. On HPTLC plates with spherical particles (LiChrospher silica gel 60 F₂₅₄s), the antibiotics were focused into sharp bands and the solvent penetrated the extracts more easily, if compared to the HPTLC plate with irregular particles (HPTLC silica gel 60 F₂₅₄s). However, the experiments had to

be discontinued due to a world-wide shortage of LiChrospher HPTLC plates. Instead, the usage of TLC plates silica gel 60 F₂₅₄ with a wider particle size distribution and ca. 25% higher layer thickness than HPTLC plates enabled the successful front-elution of all antibiotic classes from the three different matrices with acetone/water/ammonia, 7:2:1 (V/V/V). As expected, the focusing of the antibiotics in the solvent front was worse on TLC plates, which showed a lower separation efficiency than LiChrospher plates. A second front-elution with the same solvent system in the reverse direction resulted in sharply focused analytes (Fig. S-2). A prior front-elution with a weaker solvent strength (as shown in the SFPE procedure) resulted in total loss of nitroimidazole antibiotics, and thus was not possible for the whole spectrum of 66 antibiotics. Nitroimidazole and partly also sulfonamide antibiotics showed a very low adsorption energy towards the silica gel surface and were front-eluted even with toluene, but not with *n*-hexane. However, *n*-hexane with the lowest solvent strength on normal phase silica gel (Snyder, 1978) was not able to elute the matrix interferences. Therefore, the SFPE concept was not appropriate for the given very broad spectrum of nine different structural classes of antibiotics. Although the developed HTPSPE method was successful and reproducible, it unfortunately impaired the subsequent HRMS measurement due to the high background signals produced by the solvents as interferences (Fig. S-3). These interferences derived from the reaction of ammonia with acetone and were identified by HRMS/MS experiments to be acetoin, diacetoneamine, triacetoneamine and triacetonediamine. Two further abundant ions at *m/z* 138.0887 and *m/z* 138.1274 were not identified. It was not possible to separate the background signals from the analytes and the resulting ion suppression effect still made the detection of trace amounts of antibiotics impossible.

To avoid the formation of such reaction products, acetone was replaced by ACN of the same selectivity group (Snyder, 1978). However, this solvent mixture did not evenly flow through the extracts. An improvement was achieved by replacing water with methanol and by adjusting the proportions of the individual solvents. Finally, a mixture of ACN/methanol/ammonia, 5:3:2 (V/V/V), was able to separate all antibiotic classes from the three complex matrices. To focus the analytes in the elution front, the plate was front-eluted in the reverse direction with a mixture of ACN/water/ammonia, 9:7:4 (V/V/V), which had a higher solvent strength than the first. By doing so, the extraction of all antibiotic classes was successfully achieved from spiked muscle, milk and egg matrices in comparison to blank matrices (Fig. 2). However, not all targeted antibiotics of the cephalosporin (Fig. 2E) and penicillin classes (Fig. 2F/G) were separated from interfering matrix components. Furthermore, the high matrix load of the muscle extract compared to the milk and egg extracts was obvious and still challenging. Hence, both front-eluting solvent systems were compared using blank matrices. The

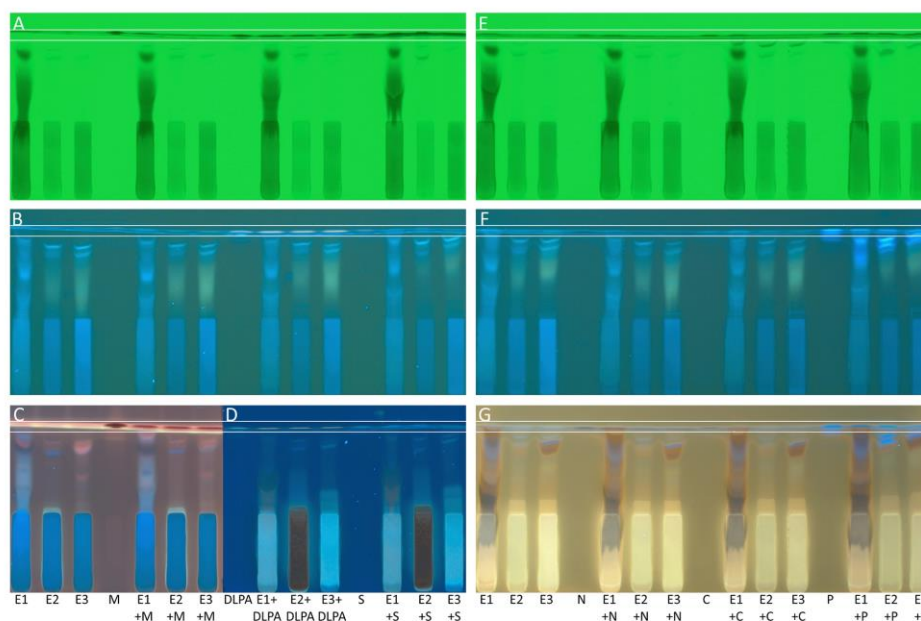


Fig. 2. Visualization of the antibiotics target zone (framed white) in the different complex matrices: HTPSPE images of McIlvaine-EDTA extracts from muscle tissue (E1), raw cow milk (E2) and whole chicken eggs (E3) spiked with visible amounts of macrolides (+M, 1 $\mu\text{g}/\text{area}$); diaminopyrimidines, lincosamides, pleuromutilins and amphenicols (+DLPA, 3 $\mu\text{g}/\text{area}$); sulfonamides (+S, 0.3 $\mu\text{g}/\text{area}$); nitroimidazoles (+N, 0.1 $\mu\text{g}/\text{area}$); cephalosporins (+C, 0.3 $\mu\text{g}/\text{area}$); penicillins (+P, 3 $\mu\text{g}/\text{area}$), 2-fold front-eluted on TLC silica gel 60 F₂₅₄ MS grade plates, first with ACN/methanol/ammonia, 5:3:2 (V/V/V) up to 85 mm, then in the reverse direction with ACN/water/ammonia, 9:7:4 (V/V/V) up to 25 mm and detected at (A/E) UV 254 nm, (B/F) FLD 366 nm, and the latter after the (C) *p*-anisaldehyde sulfuric acid, (D) aniline diphenylamine *o*-phosphoric acid and (G) ninhydrin reagents. For comparison, standard mixtures were applied in equal amounts per area as well as non-spiked extracts. Results of evaluation are depicted in Fig. 4.

firstly developed front-elution system (acetone-based, Fig. 3A) carried green fluorescent matrix compounds into the elution front and thus, into the target zone. In contrast, a better removal of matrix compounds and HRMS-compatibility was obtained with the secondly developed front-eluting solvent system (ACN-based, Fig. 3B). The front-elution was performed in the ADC 2 system including control of the plate activity to ensure reproducible results. In the HRMS spectrum, solvent reaction products or other abundant interfering background signals were not observed (Fig. S-3). MS grade TLC plates generated less background signals than the TLC plates silica gel 60 F₂₅₄ and did not show differences in the chromatographic behavior; thus, these were used for further investigations. Since the antibiotics were not visible in the quantities to be expected in the samples, the fluorescent dye rhodamine B, which showed a similar behavior on the plate as the analytes, was added as a marker compound for visualization. The method allowed the clean-up of 16 samples in parallel with a low solvent consumption of 0.6 mL per sample. With the application as the most time-consuming step, clean-up times of 7 min per sample were possible, which was 5-fold faster than the routine state-of-the-art. Given the current limited capacity, faster screening will provide broader screening and improve food safety. To the best of our knowledge, this is the first time that an HTPSPE multi-method covering a number of 66 different antibiotics has been developed.

3.4. Compound database

An in-house compound database was built using one representative

antibiotic of each structural class (Table 1). Each antibiotic was applied on a TLC plate and directly eluted into the mass spectrometer. The most abundant adduct, mostly the protonated molecule $[M + H]^+$, was selected as precursor ion for MS2 experiments, which was in accordance with other databases resulting from LC-MS (Wang et al., 2015; 2018; Jia et al., 2017). The precursors were fragmented and the most abundant ions (precursor excluded) were chosen for further investigation. It was aimed to include four fragments in the database, but not all of the precursors resulted in multiple fragments of suitable abundance. Florfenicol even produced only one product ion. Commission Decision 2002/657/EC (European Commission, 2002) does not specify on MS criteria for screening methods, but for confirmatory methods utilizing HRMS, only one exact precursor and one exact product ion is required for unambiguous confirmation, thus no further optimization of the collision energy was carried out. Fragments lower than m/z 90, which could result from multiple precursors, were excluded from the database. The fragments were identified by elucidating detailed fragmentation pathways based on simulations with Mass Frontier and Chemdraw Ultra. The proposed structure candidates are depicted in Figure S-2. The identified molecular formulas were entered into our in-house TraceFinder compound database, which calculated the theoretical exact masses of precursors and fragments.

3.5. Acquisition method

As a non-target acquisition method, vDIA was chosen to provide the possibility of retrospective data analysis. Compared to data-dependent

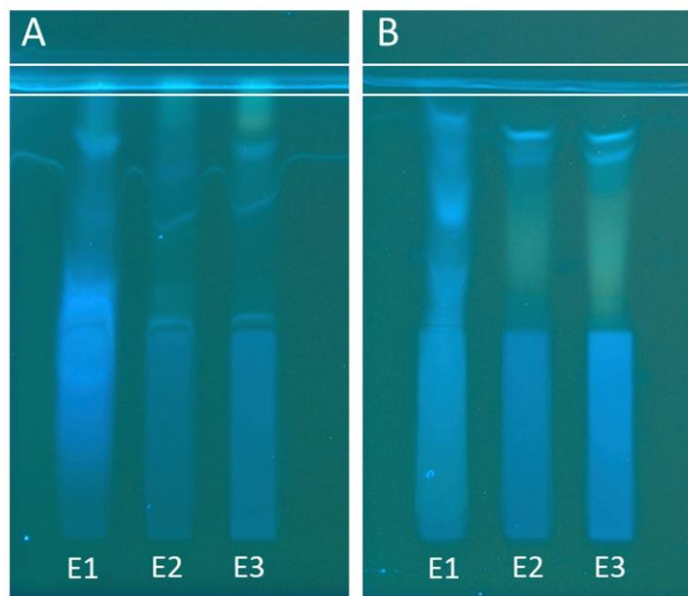


Fig. 3. Comparison of two different HTPSPE methods: FLD 366-nm images (target analyte zone framed white) of non-spiked McIlvaine-EDTA extracts on TLC silica gel 60 F₂₅₄ MS grade plates after 2-fold front-elution with (A) acetone/water/ammonia, 7:2:1 (V/V/V; $\leq 5\%$ relative humidity on plate) first up to 80 mm, then in the reverse direction up to 25 mm versus (B) ACN/methanol/ammonia, 5:3:2 (V/V/V; 33% relative humidity on plate) up to 85 mm, then in the reverse direction with ACN/water/ammonia, 9:7:4 (V/V/V) up to 25 mm.

MS2 (ddMS2) (Wang et al., 2015; Jia et al., 2014) and all-ion fragmentation (AIF) (Turnipseed et al., 2017), medium mass selectivity is provided by vDIA. ddMS² acquisition methods can be problematic, because orbitrap instruments tend to fail the data-dependent trigger if they cover a large number of low abundant analytes due to their long duty cycle, thus making ddMS² only reliable for a limited number of compounds. AIF on the other hand tends to produce noisy spectra, since all present ions are fragmented in the higher-energy collisional dissociation cell and sent to the orbitrap analyzer in a single injection (Wu et al., 2020; Rajski et al., 2017; Kaufmann, 2018). During method development, a multi-method in the field of pesticide residue analysis (Wang et al., 2019) was modified by extending the mass range of the full scan to m/z 1100 and adding one additional DIA scan from m/z 1000 – 1100 to cover the full mass range required for all antibiotics on the analyte list, resulting in a cycle time of approximately 2.4 s. An illustration of the acquisition method is depicted in the [supplementary material](#) (Fig. S-4).

3.6. Automated zone elution and chromatographic conditions

The targeted antibiotics zones were automatically eluted and transferred by the autoTLC-MS interface (Häbe & Morlock, 2020) into the mass spectrometer. This eliminated the time-consuming manual interventions for eluting each analyte zone (mostly into autosampler vials, then injected into an HPLC system), which has commonly been used for hyphenation of HTPSPE and MS. Briefly, after sample purification by HTPSPE, the plate was marked with a soft pencil cross at a position not interfering with any target zone. An image of the plate was taken at FLD 366 nm, which was then uploaded to the open-source-based autoTLC-MS software. Each targeted elution position was indicated via the orange fluorescent rhodamine B marker. These target zones were easily selectable by clicking on the respective band on the image. The manually marked cross was used to check, and if necessary, to adjust the autoTLC-MS interface to each individual plate. By clicking on the cross

on the image, the laser crosshair of the interface (indicating the stamp position) was positioned over it and a potential bias was adjusted in the software. A following test stamp confirmed the correct adjustment, and the previously clicked target zones were directly measured automatically.

In between the interface and the HRMS system, a filter frit and a short RP-18e monolithic column were installed to protect from particles and to slightly retard the analytes. A separation was not pursued, since the developed method was intended for screening. Still to achieve the highest signal intensities of the elution of the antibiotics zones, the isocratic eluent composition was optimized to a 0.1% solution of formic acid and 4 mM ammonium formate in water/methanol 55:45, V/V. In between each 30-s elution, a blank position above the elution front was eluted to clean the elution head from residues, which doubled the measurement time. Shorter zone elution times of 15 s resulted in equal signal intensities (indicating a sharp front-elution of each analyte), but were not sufficient for the elution head cleaning. The current software version does not provide an option for choosing analyte elution times separately from those of blanks (same period fixed). Hence, 30-s elutions were chosen as a compromise. After elution, the flow was held for 6.5 min, as nafcillin as the most retained substance of the representatives eluted at a retention time of 4.0 – 4.5 min. The remaining time was used for column equilibration, but could have been shortened by 1 min, because no late eluting contaminants were observed after 5.5 min.

3.7. Data processing

The data were processed using the target screening function of TraceFinder with the developed in-house compound database. Contrary to the findings of another study (Wang et al., 2019), it was not necessary to set threshold values in the compound database for each individual compound as no false-positive results were observed with a threshold value of zero, due to the fact that the database consisted of theoretical exact masses derived from the previously determined molecular

Table 1
Built-up compound database for one representative per antibiotic class.

Antibiotics	Class	Molecular formula	Adduct	Precursor Exact mass [m/z]	Fragment 1 Exact mass [m/z]	Elemental composition	Fragment 2 Exact mass [m/z]	Elemental composition	Fragment 3 Exact mass [m/z]	Elemental composition	Fragment 4 Exact mass [m/z]	Elemental composition
Sulfaquinoxaline	Sulfonamides	$C_{14}H_{12}N_4O_5S$	$[M]^+$	301.07537	156.01138	$C_8H_6NO_2S^+$	108.04439	$C_8H_6NO^+$	92.04948	$C_8H_6N^+$	123.06652	$C_8H_6N_4$
Trimethoprim	Diaminopyrimidines	$C_{14}H_{18}N_4O_3$	$[M]^+$	291.14517	261.09822	$C_{12}H_{13}N_4O_3^+$	245.10330	$C_{12}H_{13}N_4O_2^+$	230.11621	$C_{12}H_{14}N_4O^+$		
Clindamycin	Lincosamides	$C_{18}H_{33}ClN_2O_5S$	$[H]^+$	425.18715	377.18378	$C_{17}H_{30}ClN_2O_5^+$	126.12768	$C_8H_{10}N^+$				
Tiamulin	Pleuromutilins	$C_{28}H_{47}NO_4S$	$[M]^+$	494.32986	192.10528	$C_8H_{16}NO_2S^+$	119.01613	$C_8H_7O_2S^+$				
Erythromycin A	Macrolides	$C_{27}H_{45}NO_{13}$	$[M]^+$	734.46852	158.11756	$C_8H_{16}NO_2^+$	116.10699	$C_8H_{14}NO^+$	98.09643	$C_8H_{12}N^+$		
Cefoperazone	Cephalosporins	$C_{23}H_{27}N_5O_6S_2$	$[M]^+$	668.13162	526.05739	$C_{18}H_{17}N_5NaO_6S_2^+$	410.04173	$C_{17}H_{13}NaO_6S^+$	177.99332	$C_8H_{18}NNaO_2S^+$	165.06345	$C_8H_{10}N_2NaO_2^+$
Nafcillin	Penicillins	$C_{21}H_{28}N_2O_5S$	$[Na]^+$	415.13222	256.09682	$C_{18}H_{14}NO_5^+$	199.07536	$C_{13}H_{11}O_5^+$	181.06479	$C_{13}H_9O^+$	171.04406	$C_{11}H_9O_2^+$
Florfenicol	Amphenicols	$C_{12}H_{14}Cl_2FNO_2S$	$[M]^+$	379.98968	359.98346	$C_{12}H_{13}Cl_2NNaO_4S^+$						
Iprnidazole	Nitroimidazoles	$C_7H_{11}N_3O_2$	$[Na]^+$	170.09240	140.09441	$C_7H_{12}N_2O^+$	123.09167	$C_7H_{11}N_2^+$	109.07602	$C_6H_8N_2^+$		

formulas and not of experimentally determined masses. An analyte was considered as identified, if the precursor (full scan) and one fragment ion (vDIA) were present at the same time in the spectrum within a mass extraction window of 5 ppm. Identification was solely based on the fragment ion criterion and not on additional retention time or ion ratio criteria, which is only demanded for confirmatory methods (European Commission, 2002). The full scan data provided 7–10 points per peak for all analytes except nafcillin (4–5 points per peak), which might already be sufficient for quantitation with orbitrap instruments as suggested in the literature (Rajski et al., 2017). Nevertheless, this would require reproducible peak shapes via a complete (gradient) separation. So far, no fully automated online hyphenation of TLC and HPLC with gradient elution has been demonstrated, which will be part of future investigations.

3.8. Method validation

To prove the method's applicability for residue analysis of antibiotics in food, it was validated according to the requirements of Commission Decision 2002/657/EC (European Commission, 2002) and the recommendations of the Community Reference Laboratories (CRLs) for Residues (Community Reference Laboratories Residues, 2010) for one representative analyte per group. For qualitative screening methods, the European Commission demands the determination of specificity/selectivity, ruggedness and detection capability (CCB), which corresponds to "the smallest content of the substance that may be detected, identified and/or quantified with an error probability of 8".

According to the CRLs guidelines, threshold values for each analyte were determined by the analysis of 20 blank samples per matrix (muscle, cow milk and chicken eggs), which were either organically produced or known to be free from antibiotic residues. For muscle tissue, four samples each were taken from chicken, turkey, pig, beef and two each from wild boar and deer. The 20 individual samples from different suppliers were stored over varying periods to test the method's ruggedness. No false-positive results were obtained in the analysis of the blank samples, proving the method's selectivity. The calculated threshold values were zero in all cases except for ipronidazole and tiamulin, which showed a low response in some blank samples due to isobaric interferences (Fig. 4).

For the determination of CCB, the same 20 blank samples were spiked with a complete standard mixture with the whole set of 66 antibiotics to test the method's specificity. According to the CRLs guidelines, the determination of CCB requires only 20 replicates if the tested concentrations are ≤ 0.5 MRL; for spiking levels of 0.5–0.9 MRL, 40 replicates are necessary, while for 0.9–1.0 MRL, 60 replicates are needed. The chosen spiking levels were 25 $\mu\text{g}/\text{kg}$ and 50 $\mu\text{g}/\text{kg}$ (muscle and milk) or 50 $\mu\text{g}/\text{kg}$ and 100 $\mu\text{g}/\text{kg}$ (eggs), which corresponded to ≤ 0.5 MRL of the respective analytes. The samples were spiked after the extraction because it was the aim of this work to assess the performance of the HTpSPE procedure, and not the initial extraction. The samples were analyzed on two consecutive days by two different analysts to determine the CCB values at a false negative rate $\leq 5\%$, which corresponds to a maximum of one false-negative sample from the 20 spiked samples. The achieved CCB values proved the method's applicability for residue analysis of trimethoprim, clindamycin, tiamulin, erythromycin A and ipronidazole in all tested matrices at the required MRL (Table 2, Fig. 4). Sulfaquinoxaline showed a false negative rate of 15% at 50 $\mu\text{g}/\text{kg}$ in the egg extract. In the other matrices, this analyte was successfully validated at half of the required MRL of 100 $\mu\text{g}/\text{kg}$. Nafcillin showed a false negative rate of 10% in milk and 45% in muscle and eggs, whereas cefoperazone and florfenicol were not detectable at the required concentration level, which might have been caused by ion suppression by other co-eluting analytes. A full chromatographic separation might be more suitable for these analyte groups, which should be investigated in future work as well as the application of the method to further analytes.

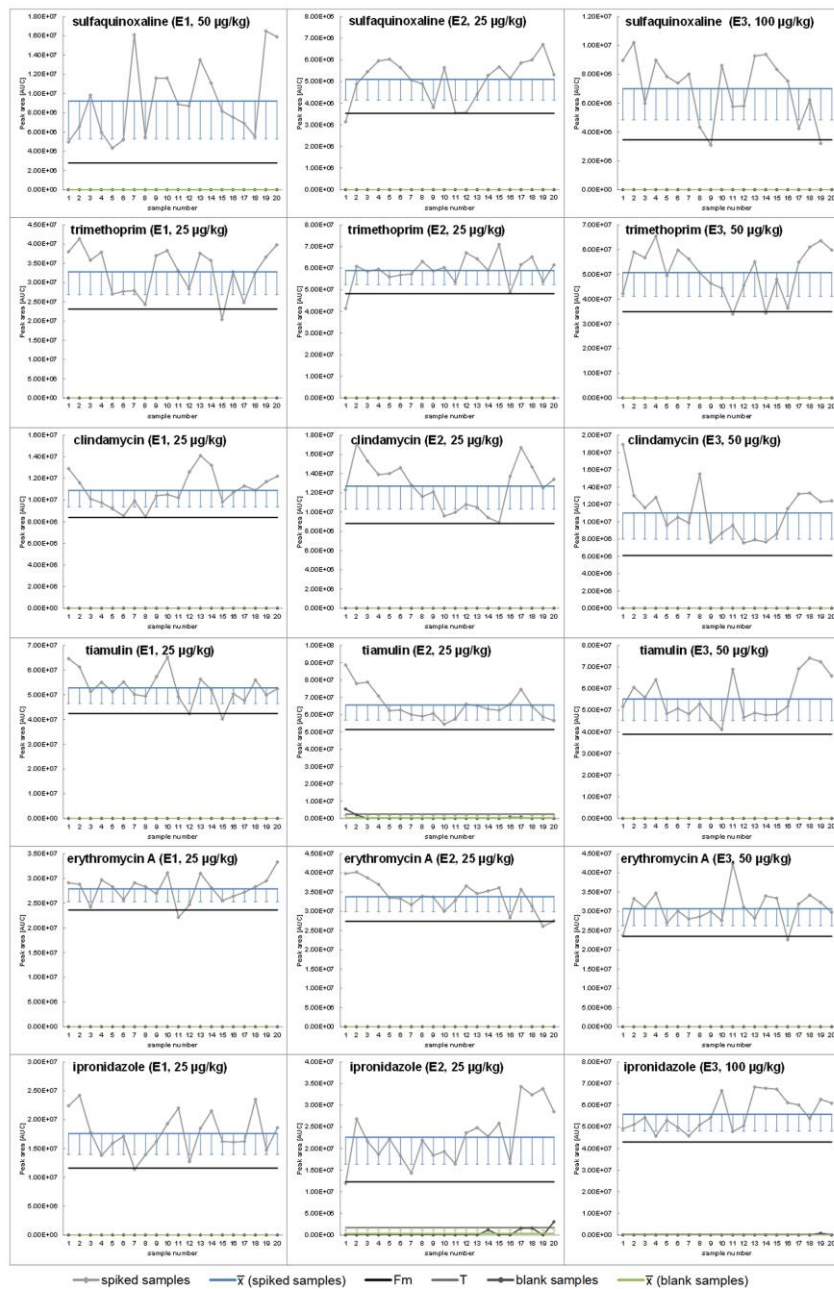


Fig. 4. Graphical evaluation of the validation data according to Commission Decision 2002/657/EC for six representatives of different antibiotic classes in three different matrices (muscle tissue: E1, raw cow milk: E2, and whole chicken eggs: E3) at concentrations ranged 25 – 100 µg/kg showing each the individual results of the blank and spiked samples as well as their respective means (green and blue horizontal lines, respectively), cut-off factor (Fm, black horizontal line) and threshold value (T, grey horizontal line). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2

MRLs and determined CC_β values for one representative per antibiotic class; in case of MRLs varying between animal species, the lowest and thus strictest value was applied.

Antibiotics	MRL muscle [μg/kg]	CC _β E1 [μg/kg]	No. positives/No. samples E1	MRL milk [μg/kg]	CC _β E2 [μg/kg]	No. positives/No. samples E2	MRL egg [μg/ kg]	CC _β E3 [μg/kg]	No. positives/No. samples E3
Sulfaquinoxaline	100	≤50	20/20	100	≤25	20/20	prohibited	≤100	19/20
Trimethoprim	50	≤25	20/20	50	≤25	20/20	prohibited	≤50	20/20
Clindamycin	n. a.	≤25	20/20	n. a.	≤25	20/20	n. a.	≤50	20/20
Tiamulin	100	≤25	20/20	n. a.	≤25	20/20	1000	≤50	20/20
Erythromycin A	200	≤25	20/20	40	≤25	20/20	150	≤50	20/20
Cefoperazone	n. a.	–	–	50	–	–	n. a.	–	–
Nafcillin	300	–	–	30	–	–	n. a.	–	–
Florfenicol	100	–	–	prohibited	–	–	prohibited	–	–
Iprnidazole	n. a.	≤25	20/20	n. a.	≤25	20/20	n. a.	≤100	20/20

MRL: maximum residue limit, CC_β: detection capability, E1: muscle tissue, E2: cow milk, E3: chicken eggs, n. a.: not applicable.

4. Conclusions

HTpSPE was successfully coupled to orbitrap HRMS via the autoTLC-MS interface for the first time. The newly developed HTpSPE-autoTLC-HRMS/MS screening proved to be a time- and cost-efficient tool for trace level analysis. After planar extraction of up to 16 samples in parallel, by simply clicking on the targeted zones in the image, these were automatically eluted and transferred to HRMS/MS. Exploiting the new tool, a rapid screening of muscle tissue, cow milk and chicken eggs for 66 antibiotics from nine different classes has successfully been developed and thus, HTpSPE proved to be suitable for the intended purpose. The planar clean-up took only 7 min per sample, which was 5-fold faster than the routine state-of-the-art technology. Given the current limited capacity, this faster screening will provide a more comprehensive investigation of samples, and thus improve food safety. Solvent consumption was only 0.6 mL per sample. Owing to the built-up compound database and non-target vDIA method, the antibiotics were successfully identified at the trace level, based on detection of the exactly determined masses of precursor and fragment ions with a mass error ≤ 5 ppm. The validation according to Commission Decision 2002/657/EC (European Commission, 2002) proved the method's applicability for residue analysis of representatives of sulfonamides, diaminopyrimidines, lincosamides, pleuromutilins, macrolides and nitroimidazoles. The method is not applicable for residue analysis of penicillins, cephalosporins and amphenicols in its current state. In future investigations, a compound database for all analytes will be built and a complete, fully automated online hyphenation of TLC and HPLC with gradient elution will be explored to enable rapid clean-up, confirmation and quantitation of the residues in a single analytical run.

CRedit authorship contribution statement

Annabel Mehl: Methodology, Investigation, Validation, Formal analysis, Writing - original draft. **Lena J. Schmidt:** Investigation, Validation, Formal analysis. **Laszlo Schmidt:** Investigation, Formal analysis. **Gertrud E. Morlock:** Conceptualization, Methodology, Supervision, Resources, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2021.129211>.

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Supplementary material

**High-throughput planar solid-phase extraction coupled to orbitrap
high-resolution mass spectrometry via the autoTLC-MS interface for
screening of 66 multi-class antibiotic residues in food of animal origin**

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Table S-1 Complete list of antibiotic standards and solutions prepared.

Structural class	Antibiotic standard	Manufacturer	Purity [%]	Concentration [mg mL ⁻¹]	Solvent* (V/V)
Sulfonamide (S)	Dapsone (DA)	Sigma-Aldrich	97.2	1	ACN
	Sulfacetamide (SA)	Sigma-Aldrich	99.9		
	Sulfachloropyridazine (SCP)	Sigma-Aldrich	99.7		
	Sulfaclozine (SC)	Chemos	99.8		
	Sulfadiazine (SD)	Sigma-Aldrich	99.8		
	Sulfadimethoxine (SDM)	Sigma-Aldrich	99.9		
	Sulfadimidine (SDI)	Sigma-Aldrich	99.8		
	Sulfadoxine (SDX)	Sigma-Aldrich	98.9		
	Sulfamerazine (SM)	Sigma-Aldrich	99.7		
	Sulfamethoxazole (SMX)	Sigma-Aldrich	99.9		
	Sulfamethoxypyridazine (SMP)	Sigma-Aldrich	99.8		
	Sulfanilamide (SN)	Sigma-Aldrich	99.9		
	Sulfaquinoxaline (SQ)	Sigma-Aldrich	97.8		DMF
	Sulfathiazole (ST)	Sigma-Aldrich	99.9		ACN
Diaminopyrimidine (D)	Baileloprim (BAQ)	Witega	99.89	1	MeOH
	Trimethoprim (TRI)	Sigma-Aldrich	99.7		
Lincosamide (L)	Clindamycin (CLI)	Sigma-Aldrich	99	1	MeOH
	Lincomycin (LIN)	Sigma-Aldrich	n.a.		
Pleuromutilin (P)	Tiamulin (TIA)	Sigma-Aldrich	99.9	1	MeOH
	Valnemulin (VAL)	Sigma-Aldrich	96.2		
Macrolide (M)	3-ortho-Acetyltylosin (3-o-A)	TRC	95	1	MeOH
	Clarithromycin (CLA)	Sigma-Aldrich	98.4		
	Erythromycin A (ERY)	Sigma-Aldrich	93		
	Gamithromycin (GAM)	Sigma-Aldrich	93.2		
	Josamycin (JOS)	Sigma-Aldrich	n.a.		
	Neospiramycin (NEO)	TRC	96		
	Oleandomycin (OLE)	SantaCruz	98		
	Spiramycin (SPI)	Sigma-Aldrich	87.7		
	Tilmicosin (TIL)	Sigma-Aldrich	94		
	Tulathromycin 2 (TUL)	SantaCruz	99		
	Tylosin A (TYA)	Sigma-Aldrich	89.7		
	Tylosin B (TYB)	TRC	95		
	Tylvalosin (TYL)	ClearSynth	81.5		
Cephalosporin (C)	Cefalexin (CLX)	Dr. Ehrenstorfer	99	0.1	ACN/H ₂ O 3:1
	Cefalonium (CL)	Sigma-Aldrich	99.4		
	Cefapirin (CP)	Sigma-Aldrich	98		
	Cefazolin (CZ)	Sigma-Aldrich	97.4		
	Cefoperazon (COP)	Sigma-Aldrich	98.2		
	Cefquinom (CQ)	Sigma-Aldrich	98.3		
	Ceftiofur (CF)	Sigma-Aldrich	97.4		
	Desacetyl-Cefapirin (CPD)	TRC	95		

Table S-1 continued

Structural class	Antibiotics	Manufacturer	Purity [%]	Concentration [mg mL ⁻¹]	Solvent* (V/V)
Penicillin (P)	Amoxicillin (AMO)	Sigma-Aldrich	98.7	1	H ₂ O
	Ampicillin (AMP)	Sigma-Aldrich	98.8		
	Cloxacillin (CLO)	Sigma-Aldrich	96		
	Dicloxacillin (DIC)	Sigma-Aldrich	99		
	Methicillin (MET)	Sigma-Aldrich	97.1		
	Nafcillin (NAF)	Sigma-Aldrich	98.7		
	Oxacillin (OXA)	Sigma-Aldrich	99.2		
	Penicillin G (PENG)	Dr. Ehrenstorfer	99.8		
	Penicillin V (PENV)	Sigma-Aldrich	98.7		
Amphenicol (A)	Chloramphenicol (CAP)	Sigma-Aldrich	100	1	MeOH
	Florfenicol (FF)	Sigma-Aldrich	98		
	Florfenicolamine (FFA)	Sigma-Aldrich	99.3		
	Thiamphenicol (TP)	Sigma-Aldrich	>99.9		
Nitroimidazole (N)	Carnidazole (CNZ)	Witega	99.4	0.1	MeOH
	Dimetridazole (DMZ)	Sigma-Aldrich	n.a.		
	Hydroxydimetridazole (DMZOH)	Witega	99.9		
	Hydroxyipronidazole (IPZOH)	Witega	99.7		
	Hydroxymetronidazole (MZNOH)	Witega	99.37		
	Iprnidazole (IPZ)	Witega	99.4		
	Metronidazole (MNZ)	Sigma-Aldrich	100		
	Ornidazole (ONZ)	Sigma-Aldrich	>99		
	Ronidazole (RNZ)	Sigma-Aldrich	>99		
	Secnidazole (SNZ)	Witega	99.8		
	Ternidazole (TENZ)	Witega	99.8		
	Tinidazole (TINZ)	Sigma-Aldrich	99.7		

purity not available: n.a.

*Acetonitrile: ACN, dimethylformamide: DMF, methanol: MeOH

Table S-2. Structures of the representative antibiotics and proposed fragment candidates.

Antibiotics	Structure neutral molecule	Structure fragment 1	Structure fragment 2	Structure fragment 3	Structure fragment 4
Sulfaquinoxaline					
Trimethoprim					
Clindamycin					
Tiamulin					
Erythromycin A					
Cefoperazon					
Nafcillin				unknown	
Florfenicol					
Iprnidazole					

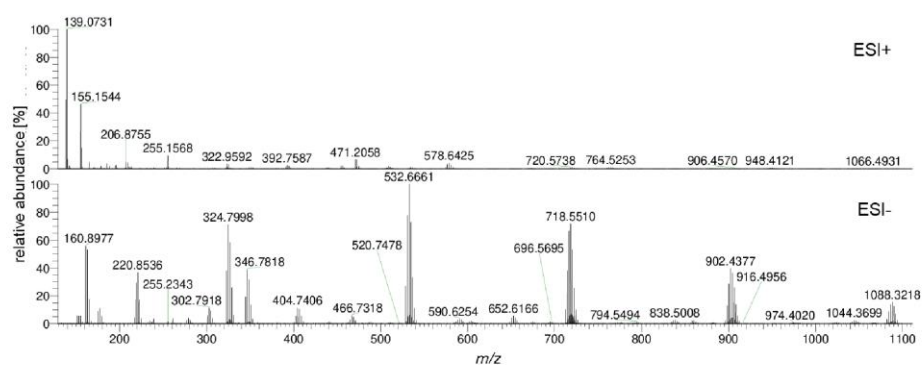


Figure S-1. Cluster formation caused by TCA sodium adducts (Δ 185.8845 Da) in raw cow milk (E2) after HTPsPE-HRMS in the positive and negative ionization mode.

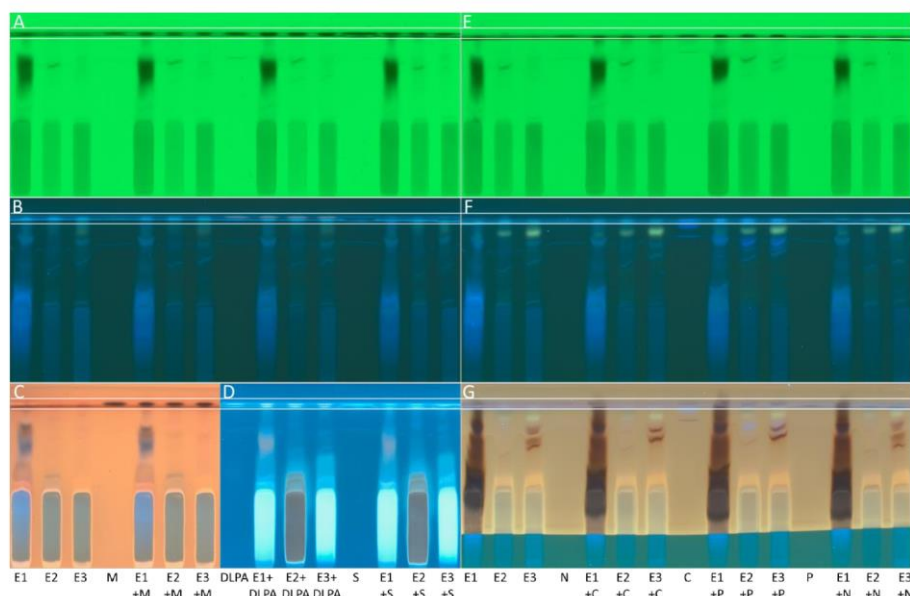


Figure S-2. HTpSPE of Mcllvaine-EDTA extracts from muscle (E1), raw cow milk (E2) and whole chicken eggs (E3) on two TLC silica gel 60 F₂₅₄ plates. A twofold development was carried out with acetone/water/ammonia 7:2:1 (V/V/V), each after 5 min humidity control (0-5% relative humidity) in the ADC 2 system, first up to 80 mm, then in the reverse direction up to 25 mm. Extracts were spiked with visible amounts of macrolides (+M, 1 µg area⁻¹); diaminopyrimidines, lincosamides, pleuromutilines and amphenicols (+DLPA, 3 µg area⁻¹); sulfonamides (+S, 0.2 µg area⁻¹); nitroimidazoles (+N, 0.1 µg area⁻¹); cephalosporins (+C, 0.3 µg area⁻¹); penicillins (+P, 3 µg area⁻¹). Standard mixtures were applied in equal amounts per area for comparison. The target analyte zone is framed white. Documentation at UV 254 nm (A, E), FLD 366 nm (B, F), and the latter via the *p*-anisaldehyde sulfuric acid (C), aniline diphenylamine phosphoric acid (D) and ninhydrin (G) reagents.

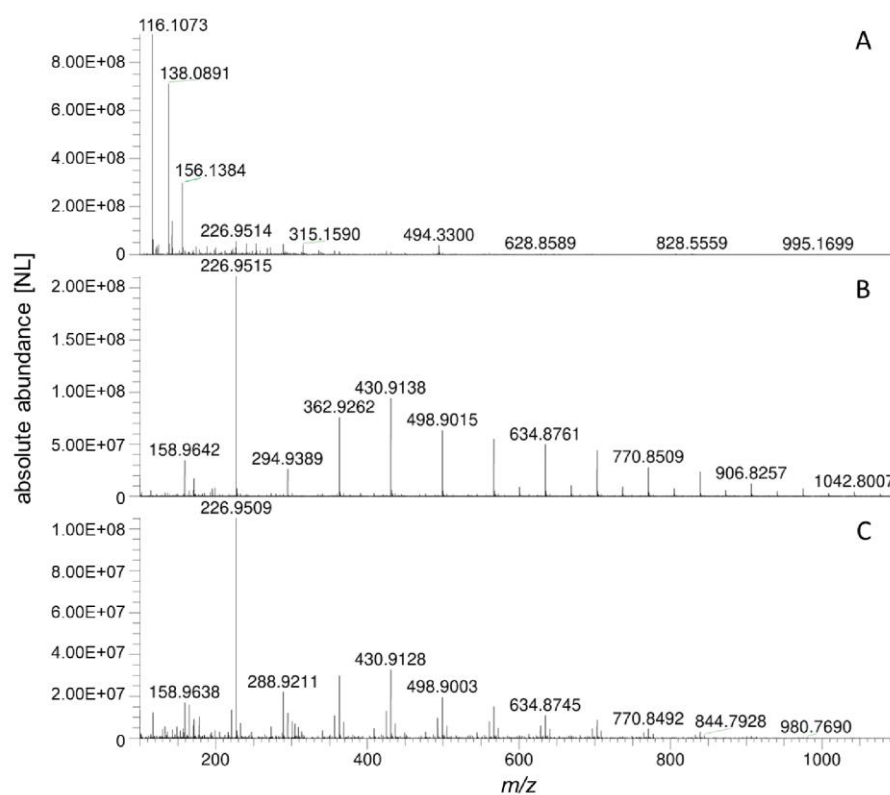


Figure S-3. Comparison of background mass spectra in the positive ionization mode: target zone elution after HTPSPE with acetone/water/ammonia 7:2:1 (V/V/V) in both directions on TLC plate silica gel 60 F₂₅₄ (A), first with ACN/methanol/ammonia 5:3:2 (V/V/V) and in the reverse direction with ACN/water/ammonia 9:7:4 (V/V/V) on TLC plate silica gel 60 F₂₅₄ (B) and on TLC plate silica gel 60 F₂₅₄ MS grade (C).

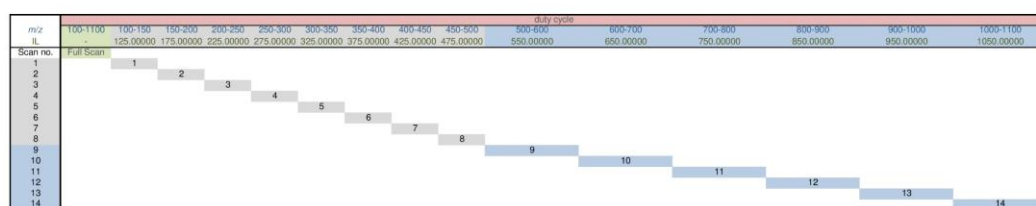


Figure S-4. Acquisition method with full scan and consecutive vDIA scans including m/z windows (inclusion list entry; IL).

6. Publication II

On-surface autosampling for liquid chromatography– mass spectrometry

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On-surface autosampling for liquid chromatography–mass spectrometry[☆]

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ABSTRACT

An on-surface multi-purpose autosampler was built for liquid chromatography–mass spectrometry (LC–MS) based on the autoTLC–MS interface, taking advantage of open-source hard- and software developments as well as 3D printing. Termed autoTLC–LC–MS system, it is introduced for orthogonal hyphenation of normal phase high-performance thin-layer chromatography with reversed phase high-performance LC (HPLC) and high-resolution MS (HRMS). For verification of its functionality, a multi-class antibiotic mixture was applied as a calibration band pattern on an adsorbent layer and detected by the *Bacillus subtilis* bioassay. This effect-image was uploaded as a template in the updated TLC–MS_manager software. The clicked-on antibiotic zones were sequentially eluted without intervention from the planar counterpart (without bioassay) via a monolithic HPLC column into the HRMS system. For elution of antibiotics of 7 structural classes at 5 different calibration levels, the new on-surface autosampler achieved intra-day precisions of 2.1–14.1%, while inter-day precisions ranged 2.5–16.1% (all $n = 3$). The new hyphenation offers potential for planar sample clean-up prior to HPLC, concentration of liquid samples, increase of peak capacity and proof of peak purity or isomers. The integrated autoTLC–LC–MS system enabled high sample throughput, efficiency and reproducibility for the first time through fully automated TLC–LC–MS sequence operation. Its contact-closure signal functionality, versatile 3D printed planar sample holder and open-source software made it readily adjustable for new analytical tasks. Undoubtedly, any planar material can be investigated for leachables, such as textiles, foils, papers and other packagings, as well as planar biological samples for ingredients.

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1. Introduction

Technical progress paves the way for new coupling techniques but it also inspires new ideas or potential for improvement. An elution head-based TLC–MS interface, usually used to elute zones from a planar adsorbent [1,2], could also be used as on-surface sampler to extract any planar material. Extractable compounds of textile fabrics, foils, food packagings, papers, commodities, leaves, etc. could conveniently be sampled and transferred to liquid chromatography–mass spectrometry (LC–MS). The extraction of plastic foils of food packagings for leachables was already shown [3]. In contrast to desorption-based techniques [4–6], only elution head-based techniques are suited for coupling with

LC–MS and also offer a better detectability [1,7,8]. However, they lack in full automation for elution and sequential LC analysis. In all reported studies, each elution process needed several manual interventions, which hindered high-throughput, time- and cost-efficient measurements. To circumvent this lack, the eluted zones were manually collected in autosampler vials and then injected as sequence [9,10]. As a disadvantage, only an aliquot of the eluted zone was introduced into the LC–MS. Hence, trace analysis and high-throughput planar solid-phase extraction methods are currently compromised with regard to limits of detection and quantification.

Apart from extraction of materials [3], on-surface sampling is used in hyphenated methods for heart-cut zone elutions. The hyphenation of separation and detection techniques is a powerful tool to maximize the information obtained by a single sample run. A promising orthogonal hyphenation combining different stationary phase selectivities and detection principles is normal phase high-performance thin-layer chromatography–reversed

[☆] Dedicated to the 70th birthday of Prof. Dr. Colin Poole, Wayne State University, Detroit, MI, USA.

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phase high-performance liquid chromatography–high-resolution mass spectrometry (NP-HPTLC–RP-HPLC–HRMS). It combines the advantages of TLC with HPLC. In terms of matrix robustness, sample throughput and efficiency, this coupling technique is even superior to 2D HPLC, as it can readily be combined with bioassays. The bioactive compound zones are directly detectable in the (bio)autogram. In 2008, such a manual coupling was first used for Sudan dye analysis of foods to remove the intense background signals originating from a caffeine-impregnated HPTLC plate [11,12]. Therefore, a short monolithic column consisting of a single porous silica rod was installed in between the elution head-based TLC–MS interface and MS system. The eluted Sudan dye was separated from coeluting caffeine in the orthogonal HPLC dimension, and the resulting Sudan dye mass spectra were free from plate background signals. Discussed at symposia (www.uni-giessen.de/food), other studies followed such a heart-cut coupling for plant alkaloids [13,14] and lemon balm isomers [15]. Thereby, all HPLC separations were performed under isocratic conditions due to the lack of automation and communication between the instruments. Hence, latest research showed the use of gradients and even the implementation of the zone elution directly from the bioautogram [16,17], when a purification or desalting step was integrated due to the high load of the coeluting polar bioassay medium ingredients. Unless monolithic columns were used, flow rates common for HPLC separations were not possible. The elution head sealing of the TLC–MS interface could not withstand the high backpressure of particle-packed HPLC columns, which led to leakage of the eluent onto the HPTLC plate. To overcome the high backpressure, an additional valve switch was integrated for analyte trapping. This trapping/desalting step allowed the usage of any HPLC column and gradient [17].

Concerning TLC–MS interfaces, two different elution head-based interfaces [18,19] were introduced in 2004 for coupling of planar chromatography with any liquid ionization technique. In 2006, the ChromeXtractor [19] interface was modified for its functioning not only on aluminium foils but also on HPTLC glass plates [20]. In 2007, an automated interface provided a convenient zone selection by click-on-the-image, an automated zone positioning and an online elution into the MS system [21]. Unfortunately, the mechanical construction was expensive which compromised wider use. Based on the ChromeXtractor, the “TLC–MS Interface” was launched by CAMAG on the market in 2009 [22]. An upgraded but still not automated “TLC–MS Interface 2” followed in 2015. After manual positioning of a zone under the elution head, the cutting edge is pressed onto the silica gel layer sealing the zone for elution. Thereby, the pneumatic elution head movement must be manually activated with a toggle switch. Then, the zone elution is manually activated by a deflection lever, by which the six-port valve is switched and solvent flow is directed onto the silica gel. The elution head is cleaned by manually pressing a button, initiating a parallel gas flow through the six-port valve. During this head cleaning, a drawer quickly moves forward under the cutting edge to catch the dried falling adsorbent particles. “Plate Express” (Advion), another commercially available interface, was launched in 2015. It exploited the same elution head principle, but the elution head is two-parted and thus exchangeable without removing the capillaries to the six-port valve. After manual zone positioning, the elution head is lowered by a stepper motor via a contact closure signal. Elution time and contact pressure are software controlled. The head cleaning cycle is performed sequentially after each zone elution. Contrary to both commercial interfaces, a simple low-cost device was introduced in 2015 [23]. It consisted of a capillary system and 6-port valve switch, and was operated manually. The “Plate Express” on the other hand represents the state-of-the-art available for elution-head based interfaces until 2017, when a first low-cost, open-source modified and automated interface was

presented [24], termed autoTLC–MS [25]. It enabled click-on-image zone selection, software-controlled plate positioning, and an advanced elution and cleaning process. The positioning system relies on stepper motors moving a plate holder along the x- and y-axis directly on the base plate of the “TLC–MS Interface 2”. The elution head movement was pneumatically driven and head cleaning adjustable in interval and intensity. An adjustable gas beam directed on the plate ensured a particle-free surface for the next zone elution, provided by an electric six-port valve.

In this study, the autoTLC–MS interface [25] was upgraded to serve for the first time as a fully automated open-source multi-purpose on-surface autosampler for LC–MS. It enables the automated analysis of sample sequences for HPTLC–HPLC–HRMS for the first time. Its proper functioning was exemplarily investigated and proven for multi-class antibiotics.

2. Materials and methods

2.1. Chemicals and materials

Sulfaquinoxaline ($\geq 97.8\%$), trimethoprim ($\geq 99.7\%$), clindamycin ($\geq 99\%$), tiamulin ($\geq 99.9\%$), erythromycin A ($\geq 98.4\%$), cefoperazone ($\geq 98.2\%$), nafcillin ($\geq 98.7\%$), ammonium formate ($\geq 99.9\%$), and Müller-Hinton-Broth were obtained from Sigma-Aldrich, Steinheim, Germany. Iprnidazole ($\geq 99.4\%$) was obtained from Witega, Berlin, Germany. Double-distilled water was produced by a Heraeus Destamat Bi-18e, Fisher Scientific, Schwerte, Germany. For HPLC–HRMS, formic acid ($\geq 99.9\%$) was obtained from Fisher Scientific, whereas LC–MS grade water (HiPerSolv CHROMANORM) was obtained from VWR, Darmstadt, Germany. Methanol ($\geq 99.9\%$) was purchased from Honeywell Riedel-de Haën, Seelze, Germany. Thiazolyl blue (MTT, $\geq 98\%$) was purchased from Carl Roth, Karlsruhe, Germany. Dulbecco's phosphate buffered saline (PBS), *Bacillus subtilis* spore suspension (BGA), and HPTLC plates silica gel 60 (20 cm $\times$ 10 cm) were delivered by Merck, Darmstadt, Germany.

2.2. Standard solutions

A stock solution of each antibiotic was prepared (1 mg/mL), whereby sulfaquinoxaline was dissolved in acetonitrile, cefoperazone in acetonitrile/water 3:1 (V/V), nafcillin in water and trimethoprim, clindamycin, tiamulin, erythromycin A and ipronidazole in methanol. Each stock solution was diluted 1:100 with acetonitrile, resulting in a 10- μ g/mL standard solution. Aliquots (100 μ L) of each standard solution were combined in an amber glass vial and 200 μ L of acetonitrile was added, resulting in a standard mixture of 1 μ g/mL each.

2.3. Planar *Bacillus subtilis* bioassay

The standard mixture was applied as a 6-mm calibration band pattern at five equidistant volumes (0.5–2.5 μ L or ng/band) in triplicate on a 5 cm $\times$ 10 cm plate piece (Automatic TLC Sampler ATS 4, controlled by FreeMode option of winCATS 1.4.7 software, CAMAG, Muttens, Switzerland). The layout of the band pattern was spaced 1.0 cm to the left, right, and lower plate edge and 1.5 cm in the x- and y-direction. The application of the triplicate band pattern was carried out in duplicate (one plate part for the bioassay and the other for HPLC–HRMS). The bioassay was performed as reported [26–28]. Briefly, 23 g Müller-Hinton broth were weighed into a 1 L volumetric flask, made up to volume with double-distilled water and autoclaved. The bacterial suspension was prepared by diluting 200 μ L *B. subtilis* spore suspension in 40 mL Müller-Hinton broth in a 125 mL chicane conical flask and incubated in an incubator shaker at 37 °C overnight. The cell number was checked measuring the optical density (OD) at 600 nm

against the medium using a spectrophotometer (M501, Spectronic Camspec, Leeds, UK). Directly before the assay, the $OD_{600\text{ nm}}$ was in a range of 0.7–1.0. The cell suspension (4 mL) was piezoelectrically sprayed onto the plate (red nozzle, level 6, Derivatizer, CAMAG). The plate was horizontally incubated for 2 h at 37 °C in a moistened plastic box (22 cm × 14 cm × 8 cm, KIS, ABM, Wolframs-Eschenbach, Germany, with water-wetted filter paper at the bottom and sides). Then, the plate was piezoelectrically sprayed with 500 µL of a 0.2% PBS-buffered MTT solution (blue nozzle, level 6), incubated for 30 min (37 °C), dried at 50 °C for 10 min (TLC Plate Heater III, CAMAG), and documented under white light illumination in the reflectance mode (Digistore 2, CAMAG).

2.4. 3D-printed plate holder

The 3D model of the two-piece plate holder was designed with OpenSCAD (<http://openscad.org>). To improve practicability, it consisted of a new x-carrier and a new plate holder (Figure S2). The open-source files for 3D printing are available online (<https://github.com/OfficeChromatography/autoTLC-LC-MS>), as well as for the other functional parts of the autoTLC-MS [25], freely allowing to reproduce the interface including the electronic parts. Both plate holder pieces were 3D printed (Prusa i3 MK3 3D printer; Prusa Research, Praha, Czech Republic) with polylactic acid (PLA; Filament-world, Neu-Ulm, Germany). Threads were cut into the holes placed on the right side of the plate holder and the front side of the x-carrier using an M3 tap. The x-carrier was slid over the rods of the x-moving, and the rods were pushed into the left y-carrier. The timing belt was tightened in such a way that smooth movement in both directions was possible and was fixed with two screws from the top. The new plate holder was attached to the x-carrier with two screws (M3 × 15 mm) from the front side and can easily be replaced. Two screws (M3 × 25 mm) were inserted into the threads on the right side of the plate holder to securely fix the plate with a 3D-printed pusher.

2.5. Board electronics with contact-closure signal

The control voltage contacts of a 5-V reed relay (SIL05-1A72-71 L, Meder, Standex Electronics, Fairfield, OH, USA) were connected to ground and pin D57 on the RAMPS 1.4 of the autoTLC-MS [25]. The working contacts were connected with pins 3 (ground) and 4 (digital input) of a signal cable (P/N: 6000.1004; Thermo Fisher Scientific, Dreieich, Germany), leading to the digital input of an Ultimate HPG-3200SD standard binary pump (Dionex Softron, Germering, Germany). The Dionex Chromatography MS (DCMS) link 2.14 software (Dionex Softron) was programmed to wait for a contact closure signal before starting a chromatographic run. The start signal for the HPLC pump was provided by the autoTLC-LC-MS interface, while the start signal for the HRMS was provided by the HPLC pump by implementing relay commands in the DCMS link software (Figure S1).

2.6. autoTLC-LC-MS installation

The installation protocol (Instruction S1), start protocol (Instruction S2) and a manual of the open-source TLC-MS_manager software (Instruction S3) are freely available online (https://github.com/OfficeChromatography/TLC-MS_manager). Its operation is available as video at <https://youtu.be/nVx9VL45JSY>. The autoTLC-LC-MS interface was equipped with a round elution head of 4 mm diameter and connected to a Chromolith Performance RP-18e 100 mm × 3 mm monolithic column including the corresponding guard column (Merck). The column was connected to a Q Exactive Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific) equipped with an Ion-Max HESI-II probe operated

in positive ionization mode (sheath gas 20 AU, aux gas 10 AU, spray voltage 3.5 kV, capillary temperature 320 °C, probe heater temperature 350 °C and S-lens RF level 50 AU).

Mobile phase A was 0.1% formic acid and 4 mM ammonium formate in water, while mobile phase B was 0.1% formic acid and 4 mM ammonium formate in methanol. The analytes were directly eluted from the HPTLC plate into the monolithic column via the autoTLC-LC-MS using an Ultimate HPG-3200SD standard binary pump (Dionex Softron) in the first 30 s of the gradient with the initial mobile phase composition of 10% B and a flow rate of 0.2 mL/min. The following 10-min gradient was used for elution (0–0.5 min), separation (0.5–8.0 min) and column equilibration (8.0–10 min): 0–1 min: 10% B at 0.2 mL/min flow rate; 1–2 min: composition and flow rate were linearly increased to 40% B and 0.5 mL/min; 2–4 min: 40% B was held; 4–6 min: the composition was linearly increased to 90% B; 6–8 min: 90% B was held; 8–8.1 min: the composition and flow rate were decreased to 10% B and 0.2 mL/min and these conditions were held until 10 min for column equilibration. Between each zone elution, a blank space on the HPTLC plate was eluted to clean the elution head from residual analyte traces. The settings in the TLC-MS_manager software were 30 s elution time and 580 s pausing time.

2.7. Acquisition of HRMS spectra

HRMS spectra were recorded with a modified version of a non-targeted variable data-independent acquisition (vDIA) method as recently published [29]. In short, a full scan (m/z 100–1100) was performed in the positive ionization mode with a resolution of 70,000 (FWHM at m/z 200), automatic gain control (AGC) target $1e6$, maximum inject time 200 ms, lock masses m/z 301.14103 (dibutyl phthalate, $[M+H]^+$) and m/z 413.26623 (diisooctyl phthalate, $[M+H]^+$). It was followed by sequential vDIA scans. DIA 1 covered a mass range of m/z 100–500 with an isolation window of 52 Da (loop count 8), while DIA 2 covered m/z 500–1100 with an isolation window of 600 Da (loop count 1). The general settings for the DIA scans were a resolution of 35,000, AGC target $1e6$, with stepped normalized collision energy (NCE) of 20, 40, and 60 eV. The inclusion list for the DIA scans consisted of m/z 125.00000, 175.00000, 225.00000, 275.00000, 325.00000, 375.00000, 425.00000, 475.00000 and 800.00000. External mass calibration was performed daily with Pierce™ LTQ Velos ESI positive ion calibration solution (Thermo Scientific). The instrument was controlled by Xcalibur 3.0.63.3 with Foundation 3.0.152 under DCMS link 2.14, and the data were processed with TraceFinder 3.2.512.0 (all Thermo Scientific) using the latest compound database [29]. Statistical analysis was done with Excel 2010 (Microsoft Corporation, Redmond, WA, USA).

3. Results and discussion

The following manual interventions have to be performed for each zone elution via the status quo “TLC-MS Interface 2”, i.e. (1) zone positioning (laser cross switch on/off), (2) press toggle switch, (3) turn deflection lever, and (4) press cleaning button. A typical scenario is the elution of 25 zones from an HPTLC plate. This means that 100 manual operations have to be performed in the correct order within an hour. If one operation of the 100 was forgotten or the order was reversed, pressure increase and zone leakage (distorting adjacent bands and tracks) occurred, which meant that the analyses (including plate preparation) had to be repeated. Thus, the need for full automation and technical improvement has been obvious for routine use. For over a decade, a commercial system for automated sequential sample analysis has not been available. Hence, using open-source technology, the relevant manual operations to perform TLC-MS experiments were fully automated,



Fig. 1. autoTLC-LC-MS: Open-source add-on hardware (A) and software (B) of the multi-purpose autosampler for LC-MS.

allowing the sequential elution of dozens of zones into a single MS run [25]. However, for coupling with LC-MS, it was not suitable and further decisive optimizations were necessary. In the following sections, these improvements are discussed and resulted in the first multi-purpose on-surface autosampler for LC-MS (Fig. 1).

For LC-MS analysis, any planar material generally can be extracted by the autoTLC-MS (Fig. 2). In particular, the HPTLC ad-

sorbent can be used for planar sample clean-up [30–32] or sample concentration [33,34]. The layer adsorbent is used only once, and thus it can be used for clean-up and highly loaded with sample matrix. Thereby, organic solvents are readily evaporated, and thus, the analyte is enriched on the adsorbent, e.g., by a factor of up to 1000 when 500 μ L were applied instead of 0.5 μ L [2]. The orthogonal hyphenation of NP-HPTLC and RP-HPLC increases peak

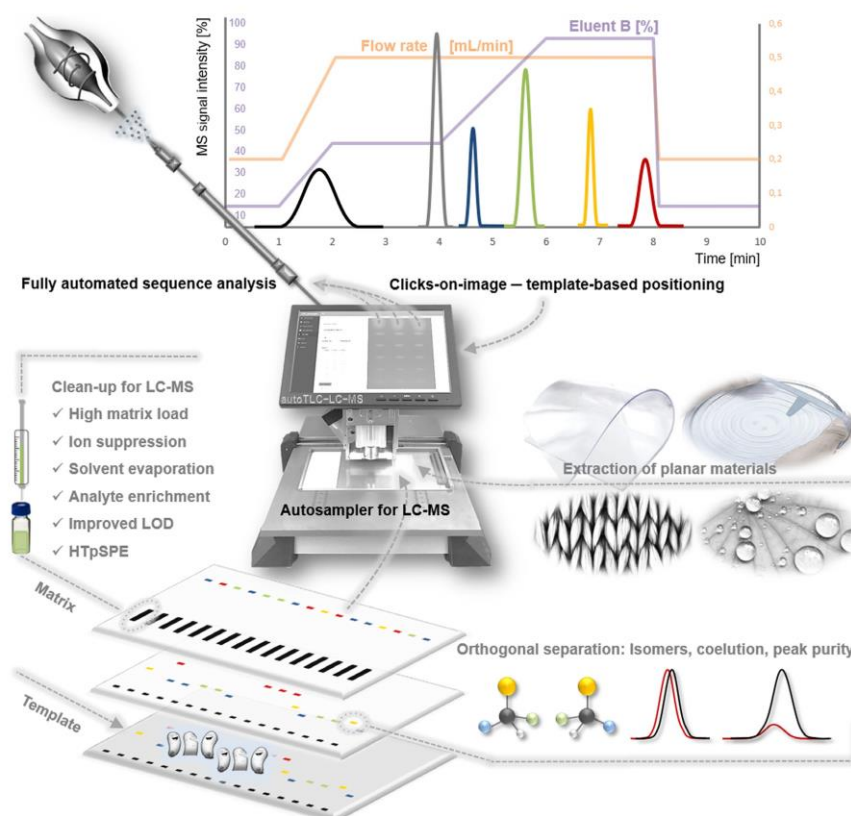


Fig. 2. Scheme of autoTLC-LC-MS offering the new options and versatile potential as multi-purpose autosampler for LC-MS.

capacity and provides comfortable options for the separation of isomers and other coelutions as well as for peak purity proof [15]. But above all, it would allow the fully automated sequential analysis of samples via online HPTLC-HPLC-HRMS. This would substantially ease the coupling of high-throughput planar-solid phase extraction to MS [9,10]. A further advantageous new option would be a template-based positioning. In particular, this is highly attractive for the template-assisted selection of bioactive zones by clicks on the effect-image, instead of compound marking by a soft pencil. The versatile advantages for its multi-purpose application are evident, especially in combination with the HPTLC adsorbent. Hence, the interface was termed autoTLC-LC-MS. A video demonstrating its operation is available at <https://youtu.be/nVx9VL45JSY>.

3.1. Hardware development

To enable a fully automated online TLC-LC-MS hyphenation, an additional 5-V relay was installed in the RAMPS 1.4 shield (Fig. 3A) to send contact closure signals to the HPLC pump after the software-controlled start of the zone elution process. For synchronization, the DCMS link software was programmed to wait for a contact closure signal before starting a chromatographic run,

which can also be programmed in Chromeleon SII (Figure S1). Consecutively, the start signal for the HPLC pump was provided by the autoTLC-LC-MS interface, while the start signal for the HRMS was provided by the HPLC pump.

The plate holder is intended to move the HPTLC glass plates directly over the metal base plate of the interface. In the former design [25], the plate was placed on the base plate of the "TLC-MS Interface" and clamped by the flanks of the plate holder. However, the practical experience showed obstacles for routine use. Firstly, the former design allowed zone elution only up to 2 cm distance to the left, right and upper plate edge. Secondly, when the plate was not clamped properly, it shifted during the plate holder movements. When larger plate formats were not exactly cut into 20 cm × 10 cm pieces, shifting in the y-direction occurred, and the plate was even completely lost from the holder. The same hurdles occurred for smaller cut plate pieces, which often shifted during the movements in the former adaptor for 10 cm × 10 cm plates (3D printable also for other dimensions). Having no own carrier base, the clamp adaptor had to keep the smaller glass plates completely on the metal base plate to prevent it from falling off the edge. As it was centred, its 0/0 mm coordinates did not correspond to the image documentation software (the plate-in-the-adaptor im-

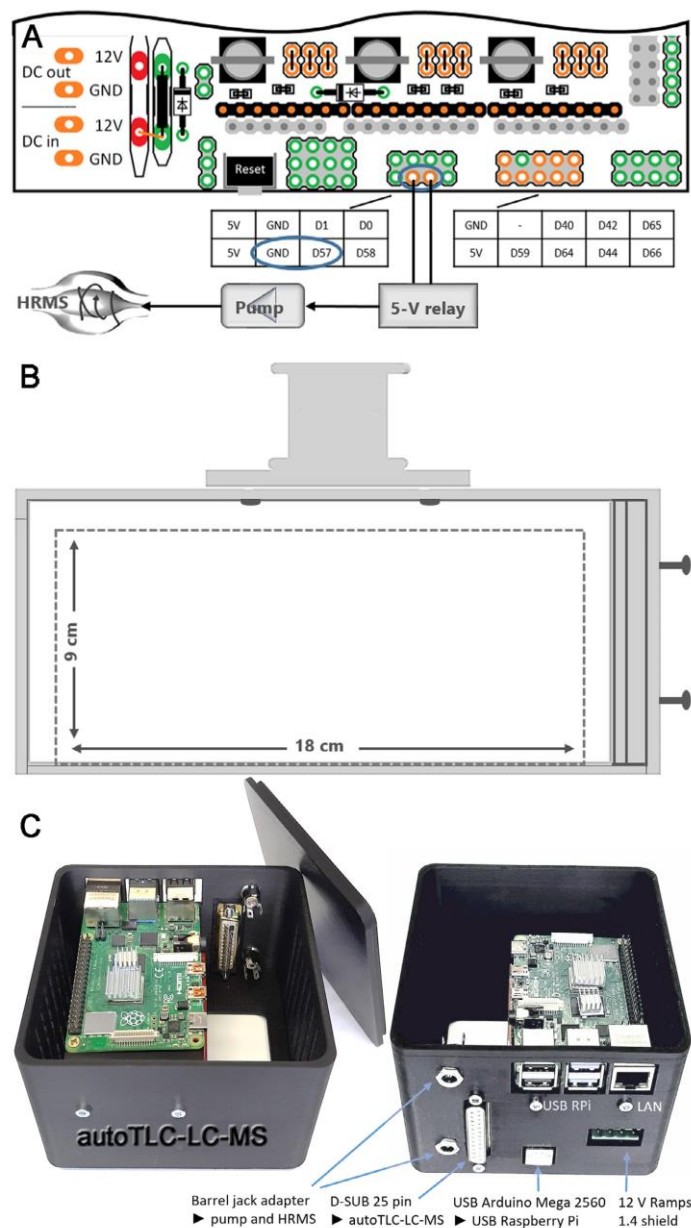


Fig. 3. View on three crucial modifications: Scheme of the RAMPs 1.4 shield and cable connections with pins (circled blue) for installation of the 5-V relay (A). Two-piece 3D printed plate holder with enlarged elution zone area (B, comparison to former in Figure S2). Front and rear side (depicted opened, not wired) of the Raspberry Pi 4B housing including Arduino Mega 2560 and Ramps 1.4 shield (C).

age had to be captured or positions had to be calculated by 5-cm addition to the x-direction in a comma-separated values file).

The above mentioned impairments were eliminated by a new design of the plate holder (Fig. 3B). By reducing the height of the plate holder, zone elution is now possible up to 1 cm distance to all edges, which supports the optimum usage of the entire plate size for high-throughput methods. A new design now ensured accurate plate positioning and was found to be the key for reliable zone elution and reproducible results. A 0.2-mm thick PLA layer was 3D printed to carry the glass plates over the base plate. Thus, small plate sizes could not fall off the base plate edge. The material was so elastic that the force of the elution head did not break the glass carrier of the HPTLC plate. Additionally, the plate was securely fixed with a pusher and two screws on the right side of the plate holder. With no hardware or software adjustments, the new design fits all common plate sizes in HPTLC when simply a counter plate is used to fill the gap. The two-piece design allowed easy replacement of the plate holder from the x-carrier. In case of damage or fatigue of material, the plate holder can now be exchanged within a minute. Advantages in terms of practicality and applicability have been associated with the new design of the plate holder, which now allows reliable, convenient and accurate handling of the plates at high throughput.

3.2. G-code commands and software development

For reproducible functioning, G-code commands are key for batch-wise zone elution but have not been provided so far. Including functional translations, the G-code commands used in this study (Table S1) help to understand the operating principle. The data were stored in individual files for each chromatographic run. Start signals for the HPLC pump and optionally for the HRMS were given in between each zone elution. G-code command files were tailored for different setups and saved as profiles (Instruction S3) for usage in different autoTLC–LC–MS setups. Prior to zone elution, the suitable profile has to be loaded. These can easily be modified further. For example, if all zone elutions should be stored in a single data file, the start signals have to be implemented in the code “before batch”.

The autoTLC–LC–MS is operated by the open-source TLC–MS_manager software, derived from the former OC_manager software [25]. It was trimmed to ensure fast loading and stable operation. Redundant files and folders were deleted, and scripts were updated. The use of the software is described as well as distinct instructions as ‘Read me’ tab, installation protocol (Instruction S1), start protocol (Instruction S2), software manual (Instruction S3) and tailored G-code command file (Table S1). All instructions are available at https://github.com/OfficeChromatography/TLC-MS_manager.

The recently introduced Raspberry Pi 4B with 4 GB or 8 GB RAM is a rather powerful PC. The TLC–MS_manager software perfectly runs on this system, which is 40% cheaper than the formerly used NUC-Mini-PC [25] (150 Euro versus 60 Euro, Fig. 3C). Therefore, a respective SD card image file and an installation script are also available for the Raspberry Pi along with other setup options (Instruction S1). The cost savings by use of the Raspberry Pi 4B (90 Euro) make up for the additional costs of the 5-V relay (4 Euro) installed in the RAMPS 1.4 shield. Hence, the costs for the open-source extension [25] are still ca. 2000 Euro for the new autoTLC–LC–MS.

3.3. Online elution into HPLC column

The analysis of multi-class antibiotics was chosen as an example to verify the applicability of the autoTLC–LC–MS for the fully

automated online hyphenation of planar materials (here HPTLC adsorbent layer) with HPLC and HRMS. Therefore, a mixture of eight antibiotics was applied onto a 5 cm × 10 cm HPTLC plate as a five-point calibration pattern in three replicates per plate and on three different days. The application was carried out twice, one plate was subjected to the *B. subtilis* bioassay and the other analogous plate was used for zone elution via the autoTLC–LC–MS (to avoid contamination of the HRMS system with bioassay medium). The bioassay image was uploaded in the TLC-MS_manager software, and for the first time, it was used as a template for automated zone elution from the analogous plate. By manually clicking on each bioactive zone of the image, the respective zone was simply selected for elution on the analogous plate (Fig. 4A). This eliminated the previous tedious, time-consuming, error- and contamination-prone manual marking (to transfer coordinates from the bioassay plate to the other plate). Any positioning bias was adjusted as reported [25].

A monolithic column was chosen for separation of the multi-class antibiotic mixture zone applied onto the HPTLC plate (without chromatography). Monolithic columns create lower backpressure than particle-packed columns [35], and thus, do not cause leakage of the elution head during zone elution. Leakage can occur if the pressure exceeds ca. 65 bar [16]. This was considered in the used elution flow rate. For zone elution, a low flow rate of 0.2 mL/min was applied, which led to a system pressure of only 20 bar. After switching the valve to the by-pass position to decouple the flow from the elution head, the flow rate was increased to 0.5 mL/min for the HPLC separation, which resulted in a system pressure of 80 bar. It was decided to initially use a mobile phase composition of 10% B for zone elution, which had a very high elution strength on the NP-HPTLC plate, but a low elution strength on the RP-HPLC column. This enabled rapid transfer of the analytes from a zone on the HPTLC plate to the column and suitable retention on the monolithic RP-18 silica gel. A higher organic content during zone elution was detrimental to retention on the column and worsened the peak shapes. Lower organic content was avoided to ensure the solubility of the analytes in the mobile phase. The following mobile phase gradient was optimized to rapidly separate the multi-class antibiotics within 10 min (including column equilibration). Note that for elution head cleaning, a blank elution was automatically performed between the zone elutions.

3.4. Acquisition of HRMS data

Baseline separation was not required because the non-targeted vDIA method with an already established compound database [29] was very selective for each antibiotic, and reproducible peak shapes were achieved (Fig. 4B). For data evaluation, the precursor ion was used as quantifier, while the fragment ion with the highest abundance was chosen as qualifier (Table 1). Peak integration of the extracted ion chromatograms of the quantifier was performed with the TraceFinder software within a 5-ppm extraction window, while the qualifier confirmed the presence of the respective analyte. The acquisition method [29] had to be modified because the peaks obtained for sulfaquinoxaline, clindamycin and nafcillin were surprisingly narrow and consisted only of 4–5 data points, which is not necessarily sufficient to obtain quantitative results. Consequently, the cycle time of the orbitrap instrument had to be decreased. This was achieved by replacing the original six DIA windows of 100 Da in the range of m/z 500–1100 with a single DIA window with 600 Da. The selectivity of the acquisition method was not impaired because the molecular weight of the chosen antibiotics was mostly below 500 Da. By doing so, the cycle time was decreased from 2.4 s to 1.7 s, which resulted in 8–10 data points per peak.

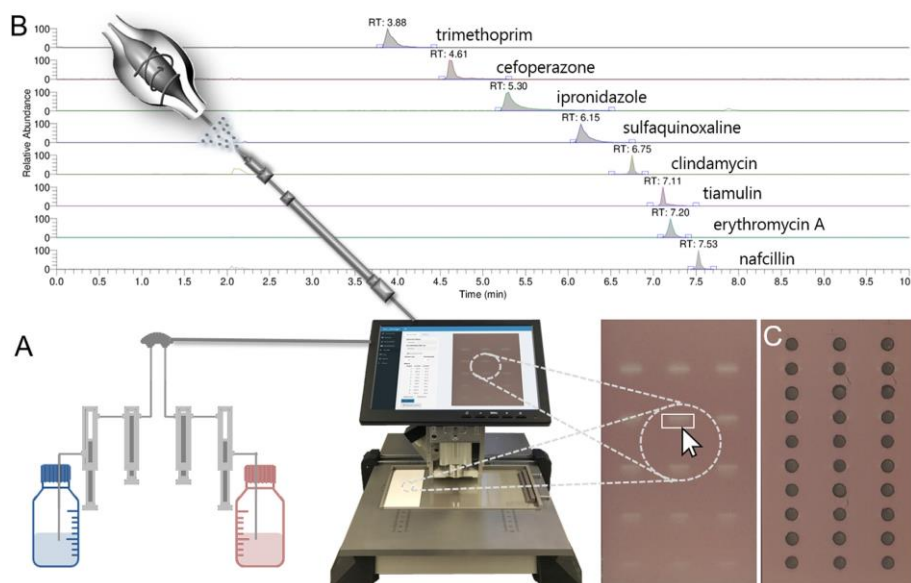


Fig. 4. Verification of the fully automated online coupling via the autoTLC–LC–MS: After zone selection by clicks-on-the-image, the elution gradient of the HPLC pump automatically transferred sequentially each antibiotic multi-class mixture zone to the orthogonal monolithic HPLC column and HESI-MS system (A). Resulting HPTLC–HPLC–HRMS extracted ion chromatograms of each antibiotic are depicted (B). Correct zone positioning verified by a post-elution *B. subtilis* bioassay, showing the eluted zone imprints (C).

Table 1

Precision and repeatability obtained from the calibration experiments for each respective antibiotic standard.

Drug	Retention time [min]	Adduct	Exact mass precursor (quantifier) [Da]	Exact mass fragment (qualifier) [Da]	R ²	Precision [%RSD]									
						Intra-day (n = 3)					Inter-day (n = 3)				
						Calibration level [ng]									
						0.5	1.0	1.5	2.0	2.5	0.5	1.0	1.5	2.0	2.5
Trimethoprim	3.88	[M+H] ⁺	291.14517	123.06652	0.999	6.0	7.6	2.8	10.6	5.4	8.3	6.3	5.2	12.2	4.9
Cefoperazone	4.61	[M+Na] ⁺	668.13162	165.06345	0.986	17.5	30.4	16.2	36.0	12.2	28.9	34.6	37.4	49.3	42.2
Ipronidazole	5.30	[M+H] ⁺	170.09240	109.07602	0.999	5.7	8.5	2.1	7.6	3.3	7.7	7.3	6.1	12.0	9.3
Sulfaquinoxaline	6.15	[M+H] ⁺	301.07537	156.01138	0.993	4.0	13.6	6.4	10.4	2.9	13.8	13.7	7.7	12.6	2.5
Clindamycin	6.75	[M+H] ⁺	425.18715	126.12768	0.999	5.5	9.8	4.3	8.1	7.1	8.1	12.9	7.7	6.9	15.0
Tiamulin	7.11	[M+H] ⁺	494.32986	192.10528	0.996	10.6	12.4	8.8	14.1	4.8	12.9	12.1	7.6	13.8	10.8
Erythromycin A	7.20	[M+H] ⁺	734.46852	158.11756	0.999	7.6	7.2	3.9	10.3	7.7	7.8	9.4	3.3	9.1	8.9
Nafcillin	7.53	[M+H] ⁺	415.13222	199.07536	0.998	10.3	8.8	5.1	12.7	4.5	16.1	15.7	7.4	12.4	6.8

3.5. Verification and performance data

Most antibiotics, especially trimethoprim, sulfaquinoxaline, and ipronidazole showed intense peak tailing (Fig. 4B), which could not be avoided with any of the tested mobile phase gradients. Nevertheless, calibration curves with $R^2 > 0.993$ and reproducible results were achieved when the experiment was repeated on three different days. Intra-day and inter-day precision were calculated with Microsoft Excel 2010 according to ISO 5725-2 [36] for each respective analyte and calibration level (Table 1). The results for intra-day precision for 5 different calibration levels of 7 antibiotics of quite different structural classes ranged 2.1–14.1%, while inter-day precisions ranged 2.5–16.1%. Cefoperazone was excluded due to its poor ionization efficiency compared to the other antibiotics. No reproducible peak shapes were obtained (%RSD up to 49.3%, $R^2 = 0.986$).

This needs further studies, as it could also be explained by the 30-s zone elution, which might have been too short for this antibiotic. Note that all analytes were detected at low levels (down to 0.5 ng/band) and the signals and elution head positioning were not corrected by an internal standard to improve precision. Post-elution, the imprinted plate was also subjected to the *B. subtilis* bioassay. Comparison of the two bioautograms before and after the elution (Fig. 4B/C) verified the correct positioning of the elution head. Although the batch-wise zone elution process was completely automated, the elution zones had to be chosen by manual clicks-on-the-image. This led to random deviations from the ideal elution position. Hence, automated band-picking by artificial intelligence or machine learning algorithms can improve future precision data. During the measurements, the entrance of air bubbles through the elution head into the chromatographic system can-

not be avoided, which, however, did not disturb the MS measurement as reported [15]. No severe variations in total ion current or system pressure were observed. Nevertheless, the usage of a switching valve as presented recently [17] could be a good option and additionally offers the possibility to clean the elution head from residual analytes during the actual chromatographic run. In the present study, we had to perform a full chromatographic run for each blank zone elution too, which doubled the measurement time. These mentioned aspects will therefore be part of future improvements.

4. Conclusions

The newly developed autoTLC–LC–MS provided as an on-surface autosampler for LC–MS all the basic functions of a conventional autosampler in HPLC or gas chromatography. For the first time, the hyphenation of on-surface elution from planar materials (e.g., NP-HPTLC plate) with RP-HPLC and HRMS was fully automated. It can now be applied for online analysis of sample sequences in routine, and it is suited for high-throughput analysis. It is a powerful orthogonal tool to maximize information about a complex sample. Fully automated sequences of multi-class antibiotic zones were online eluted into a monolithic HPLC column for rapid separation and direct transfer to HRMS. The proper functioning of the new on-surface autosampler for LC–MS was verified by the good performance data.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Annel Mehl: Conceptualization, Investigation, Formal analysis, Writing – original draft. **Wolfgang Schwack:** Software, 3D design, Writing – original draft. **Gertrud E. Morlock:** Supervision, Conceptualization, Resources, Writing – review & editing.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2021.462334.

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Supplementary material

**On-surface autosampling for
liquid chromatography–mass spectrometry**

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Table S1. Exemplary G-code commands for automated online-HPTLC-HPLC-HRMS coupling.

Before batch		Between head				After batch	
G-code	Translation	G-code	Translation	G-code	Translation	G-code	Translation
G90	Start	M42 P42 S255	Plate gas on	M42 P66 S0	Rotate valve into bypass position	G4 P2000	Waiting time
G28 X	Home x	G4 P5000	Waiting time	G4 P1000	Waiting time	G1 X100 Y150 Z150 M84	Go to position (100 150) End
G1 X100 F2500	Home x	M42 P57 S255	Contact closure to pump	M42 P64 S0	Elution head up		
G92 Y150 Z150	Home y/z	G4 P250	Waiting time	G4 P1000	Waiting security		
G1 Y0 Z0	Home y/z	M42 P57 S0	Open contact to pump	M42 P42 S0	Plate gas off		
G28 Y Z	Home y/z	G4 P250	Waiting time	M42 P65 S255	Drawer out		
G1 Y10 Z10 F2000	Home y/z	M42 P40 S255	Contact closure to MS (if not provided by pump)	G4 P500	Waiting time		
G4 P500	Waiting time	G4 P250	Waiting time (if necessary)	M42 P44 S255	Purge start		
M42 P59 S0	LED off	M42 P40 S0	Open contact to MS (if not provided by pump)	G4 P5000	Waiting time		
		G4 P250	Waiting time (if necessary)	M42 P44 S0	Purge stop		
		M42 P64 S255	Elution head down	G4 P500	Waiting time		
		G4 P1000	Waiting time	M42 P65 S0	Drawer in		
		M42 P66 S255 G4 P2000	Rotate valve into elution position Insert numeric elution time	G4 P2000	Insert numeric rinsing time		

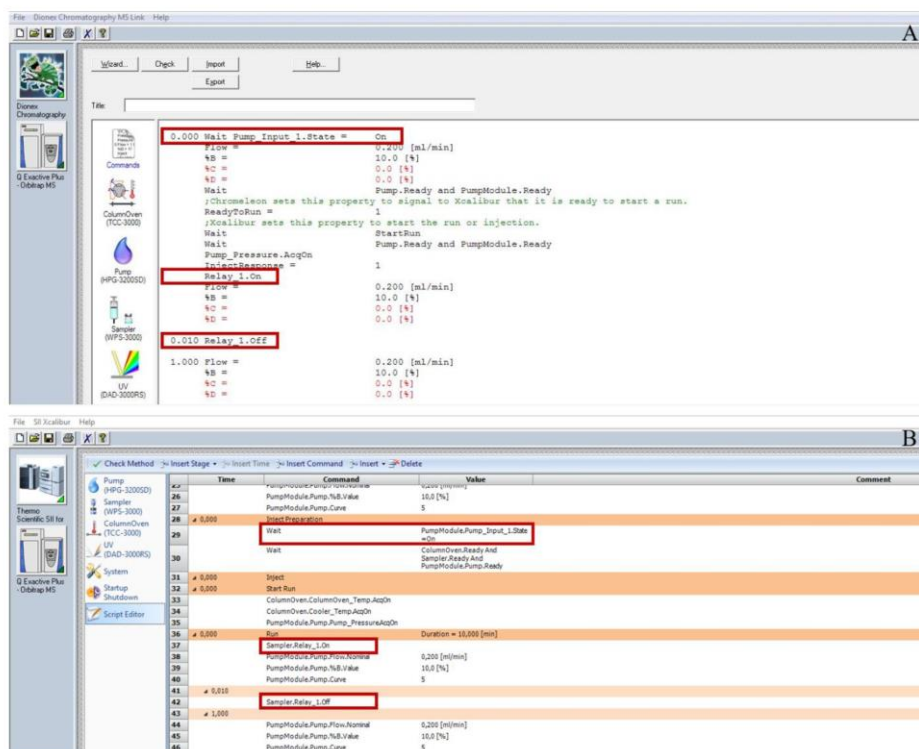


Figure S1. Instrument methods used for TLC-HPLC-HRMS, written either in Dionex Chromatography MS link (A) or Chromeleon SII (B). The implemented relay commands are outlined in red.

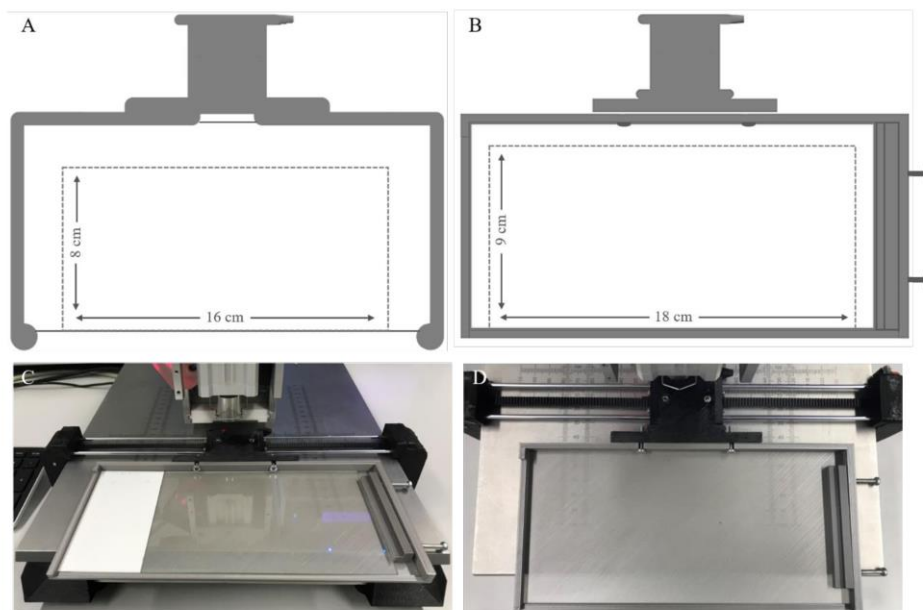


Figure S2. Comparison of 3D printed plate holders: Former (**A**) versus new two-piece design with enlarged elution zone area (**B**). Front view photograph of the new 3D plate holder with 5 cm × 10 cm plate mounted (**C**) and top view of the new plain plate holder (**D**).

Instruction S1. Installation protocol of the TLC-MS_manager software

The installation can be performed via 4 options as follows.

Ubuntu 18.04.5 LTS image

Follow [this link](#) to download an image of Ubuntu 18.04.5 with the installed TLC-

MS_manager, last updated: 2021-02-10 (3.7 GB), and copy it onto a USB stick.

Higher versions of Ubuntu cannot be used, because some needed libraries are not available for Ubuntu >18.

To flash the image, boot the NUC from DVD (Ubuntu 18.04.5 LTS) (Live-CD).

- Therefore, download Ubuntu 18 (<https://releases.ubuntu.com/18.04/>) and flash the ISO onto a DVD.
- Attach the DVD to the NUC and start the PC. On booting select "Try Ubuntu without installation" and wait for the booting process.
- Attach the USB stick with the downloaded image file (TLCMS_Ubuntu18.gz).
- Open a terminal and type:
- `sudo fdisk -l` to identify the NUC-SSD (typically `/dev/sda`) and the USB stick (typically `/dev/sdb`).
- Mount the USB stick to the folder `/mnt`: `sudo mount /dev/sdb /mnt`
- Unmount the SSD: `sudo umount /dev/sda` followed by `sudo su`, to get root privileges
- Start flashing with: `cat /mnt/TLCMS_Ubuntu18.gz | gunzip -d -c | dd of =/dev/sda bs=32k`.
- After finishing, check the values of 'in' and 'out', which should be fully identical, if nothing went wrong.
- Reboot the NUC while detaching the DVD.
- User name: pi, password: tlcms
- Configure Ubuntu for your purposes (language, keyboard).
- Configure a proper time server:
`sudo nano /etc/systemd/timesyncd.conf`
"edit the line `fallbackNTP=` ... followed by"

```
sudo timedatectl set-ntp true
```

```
sudo reboot
```

- After date and time is correctly synchronized, perform update und upgrade:

```
sudo apt update
```

```
sudo apt upgrade
```

Manual installation of TLC-MS_manager on NUC with Ubuntu 18.04.5 LTS already installed, using the installation script

Firstly, install the git library:

```
sudo apt install git
```

Secondly, clone this github repository:

```
git clone https://github.com/OfficeChromatography/TLC-MS_manager.git
```

Finally, run the install script:

In the downloaded folder home/TLC-MS_manager, there is an installation script (TLC-MS_manager_Ubuntu-18-04_install.sh) to be executed (last updated and tested 2021-02-10). Therefore, open the file sudo_bash.txt, and copy the repective command line into the terminal.

In case of installation problems, the log file TLC-MS_manager-Ubuntu_install.txt (in home/) can be checked for error messages concerning, for example, missing dependencies.

Raspberry Pi 4B image

Follow [this link](#) to download an image of a Raspberry Pi OS with the installed TLC-MS_manager, last updated 2021-02-10 (2.1 GB).

- Flash the image onto an SD card (>= 16 GB), best using the Raspberry Pi Imager (<https://www.raspberrypi.org/software/>).
- Insert the SD card into the Raspberry Pi 4B and boot the system.
- Configure the Raspberry Pi for your purposes (language, keyboard).
- User name: pi, password: tlcms
- Configure a proper time server:

```
sudo nano /etc/systemd/timesyncd.conf
```

"edit the line fallbackNTP= ... followed by"

```
sudo timedatectl set-ntp true
```

```
sudo reboot
```

- After date and time is correctly synchronized, perform update und upgrade:

```
sudo apt-get update
```

```
sudo apt-get upgrade
```

Manual installation of TLC-MS_manager on a Raspberry Pi 4B with an OS already installed, using the installation script

There is a different installation script for the Raspberry Pi in the downloaded folder `home/pi/TLC-MS_manager`. Use the respective sudo bash command to be found in the file `sudo_bash.txt`.

Unfortunately, the new Ubuntu 20.10 OS for the Raspberry Pi 4B does not work, because some needed libraries are not available for this OS.

Instruction S2. Start protocol of the TLC-MS_manager software

The open-source software can be started via 2 options as follows.

Start at reboot with crontab

Crontab is a job scheduler for UNIX-like system. The file can be accessed with the following command and the lines inside will be executed at the appropriate moment.

```
sudo crontab -e
```

Once in the editor, add this line for both the NUC and the Raspberry Pi:

```
@reboot Rscript /home/pi/TLC-MS_manager/app_exec.R
```

After reboot, the TLC-MS_manager will be available in the browser at <http://127.0.0.1> (localhost).

Therefore, best configure the browser to start with the custom URL <http://127.0.0.1/>.

If a static IP had been set, the application will also be available at the IP (in the browser) from a remote computer in the network.

Start directly from R (in case of problems to catch the errors)

Being in a terminal:

```
cd TLC-MS_manager
```

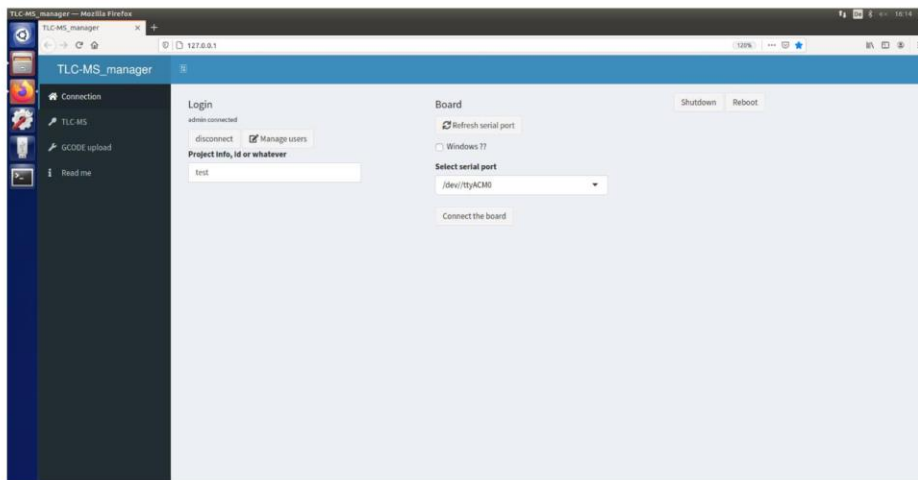
```
R
```

```
shiny::runApp()
```

Instruction S3. Manual of the TLC-MS_manager software

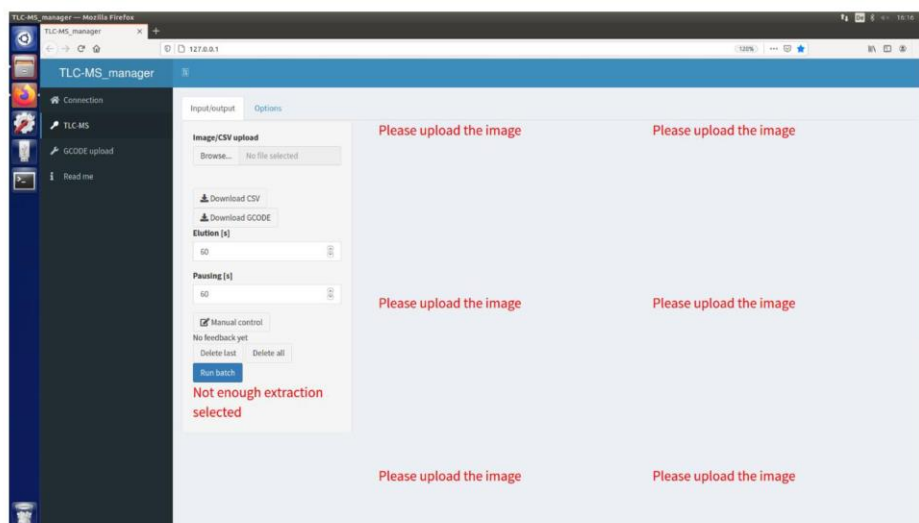
The TLC-MS_manager is an open-source software to control the autoTLC-MS interface. A manual (pdf) is available in the downloaded folder TLC-MS_manager/Instruction. The same instruction is also available in the "Read me" tab of the TLC-MS_manager software as follows. The TLC-MS_manager is divided into three main functions, which can be accessed via the main menu on the left side.

Connection tab



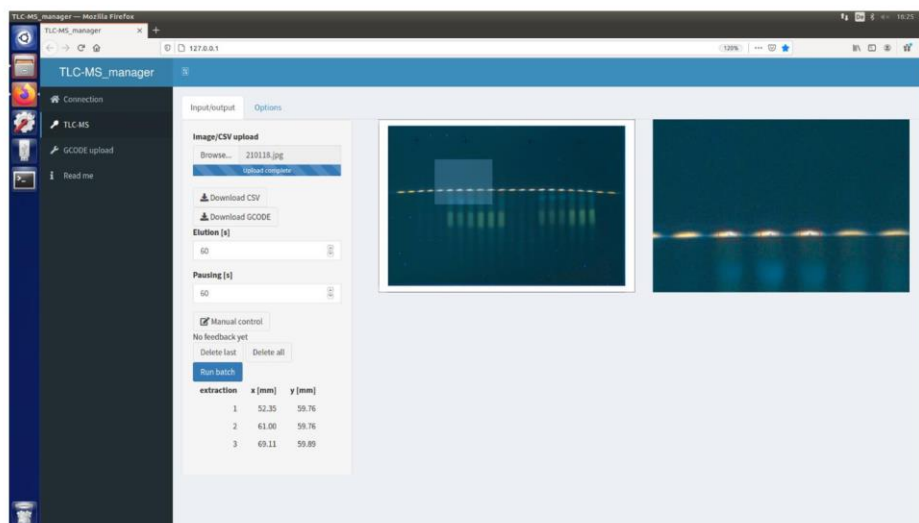
Before you can use the autoTLC-MS interface, you have to establish a connection between the interface and the TLC-MS_manager software. Click on the **Connection** tab. Choose the appropriate serial port in the drop-down menu on the right and click on the "Connect the board" button. After the connection is established, the button displays "Disconnect the board". In case of communication problems, click the "Refresh serial port" button and click on "Connect the board" again. With the help of the buttons "disconnect", "Manage users", "Project info, id or whatever", a user management system can be established if desired.

TLC-MS tab



Click on the **TLC-MS** tab. Under this tab you can find all functions necessary for automated TLC-MS measurements.

Image upload and zone selection

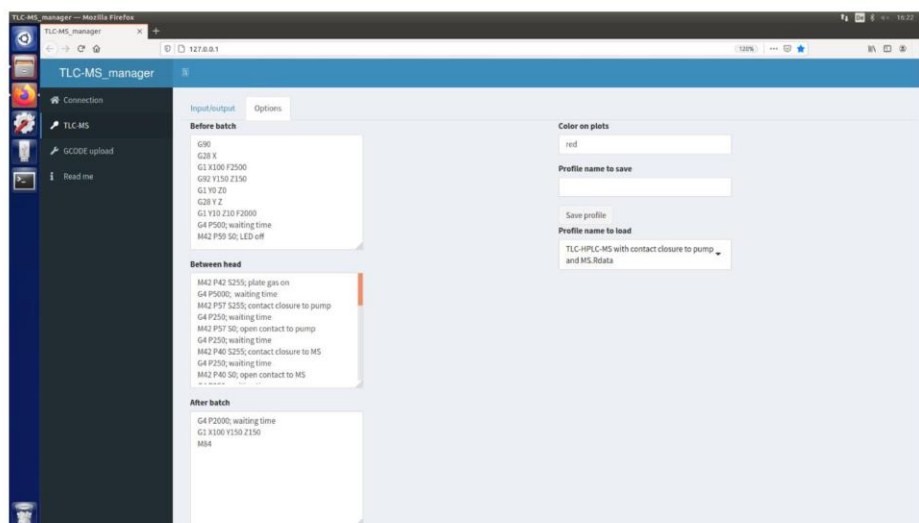


Click on the "Input/output" tab in the top left corner. Images (file formats .jpg, .png, .bmp, .tif) of chromatograms can be uploaded by clicking on the "Browse" button under "Image/CSV upload". You can upload up to four images at once by marking all desired image files before clicking "okay". The same image is displayed two times. Click on the image on the left side and drag the cursor over the image to create a rectangle selection to be zoomed out. Double-click on the selection. The selected area is displayed enlarged on the right side. On the enhanced image on the right side, click on the band or spot to be selected for elution. The selected spot is marked with a red square. The chosen position is translated into a batch, displayed on the left side below the buttons "Delete last", "Delete all" and "Run batch". After you selected all positions in the enhanced area, double-click on the image on the left side outside the selection to undo the selection. The image is displayed in full frame again on the right side. Continue with other areas on your image until you have chosen all zones for elution. Be careful to only click once on a spot for elution. If you double-click, the same spot is chosen twice. You can undo the last selection by clicking on the "Delete last" button. You can delete all selected positions by clicking the "Delete all" button. Alternatively, by clicking on "Image/CSV upload", you can upload a comma-separated values (CSV) file written in the following format:

	extraction	x [mm]	y [mm]
1	1	x1	y1
2	2	x2	y2
3	3	x3	y3

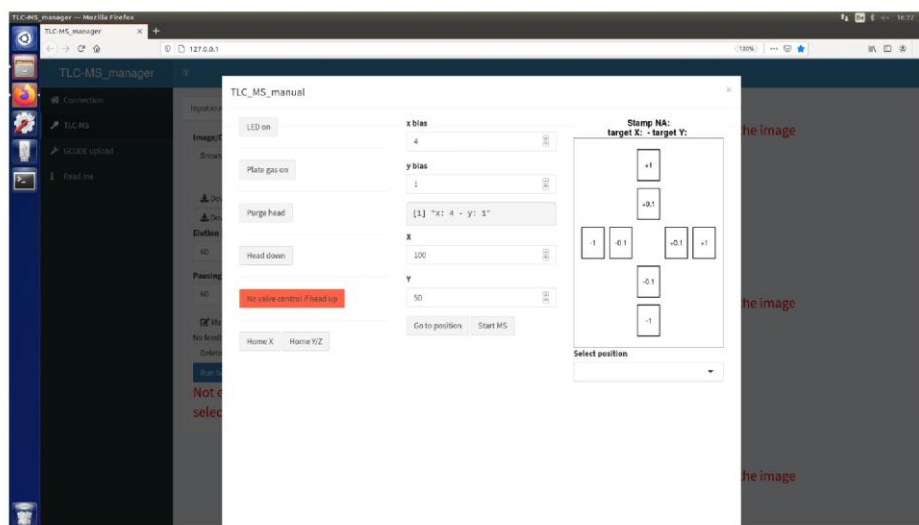
The CSV file is translated into a batch and the elution positions are marked with red squares. If you would like to control the marked positions with the help of a plate image, you can upload images after you have uploaded the CSV file. To delete the batch, click on "Delete all". For documentation of manually chosen elution zones, download the batch in a CSV format by clicking on the "Download CSV" button before running a batch. To download commands as G-code, click on the "Download GCODE" button.

Profile selection



Before you can run a batch, it is important to choose the right elution profile. Click on the "Options" tab to enter the profile selection menu. In the "Profile name to load" drop-down menu, choose the appropriate G-code command file. Different profiles for diverse setups have been provided by default. After loading a profile, you can adjust it according to your needs. All commands under "Before batch" are only performed once before the batch. If you want to record one single file for all zone elutions, you must set the start signal for MS or LC here. All commands under "Between head" are repeated in between each zone elution. If you want to record individual files for each zone elution, you must set the start signal for MS or LC here. Do not change the order of commands except for start signals. If necessary, you can adjust the waiting times between each command by adjusting the time [ms] written directly behind "G4 P". All commands under "After batch" are only performed once after the batch. The new file can be saved by inserting a name into the empty field under "Profile name to save" and clicking on "Save profile". You can also modify profiles in R and save them as .R data files in the folder TLC-MS_manager\tlcms_profile. Enter a color under "Color on plots" to change the color of the squares on the image that was uploaded under the "Input/output" tab.

Manual control



The manual control menu provides all functions necessary for manual TLC-MS measurements. Click on the "LED on" button to switch on the LED. If the LED is on, the same button displays "LED off". Use this button to switch off the LED. Click on the "Plate gas on" button to switch on the plate gas for removal of particles on the plate surface. If the plate gas is on, the same button displays "Plate gas off". Use this button to switch off the plate gas. Click on the "Purge head" button to clean the elution head with a gas stream. The drawer is pushed out and catches the particles blown out of the elution head. The cleaning cycle is finished after 5 seconds. Click on the "Head down" button to lower the elution head onto the plate. The button now displays "Head up", while the button below now displays "Valve elution". Click on the "Valve elution" button to rotate the electric six-port valve into elution position. The "Head up" button cannot be activated during valve elution. After the desired elution time, click on "Valve bypass" to rotate the valve into bypass position. Click on the "Head up" button to move the elution head up. After each elution, purge the elution head. To start an MS acquisition, click on the "Start MS" button. These are the only functions necessary for manual zone elution. Before every automated measurement, subsequently click on the "Home X" and "Home Y/Z" buttons.

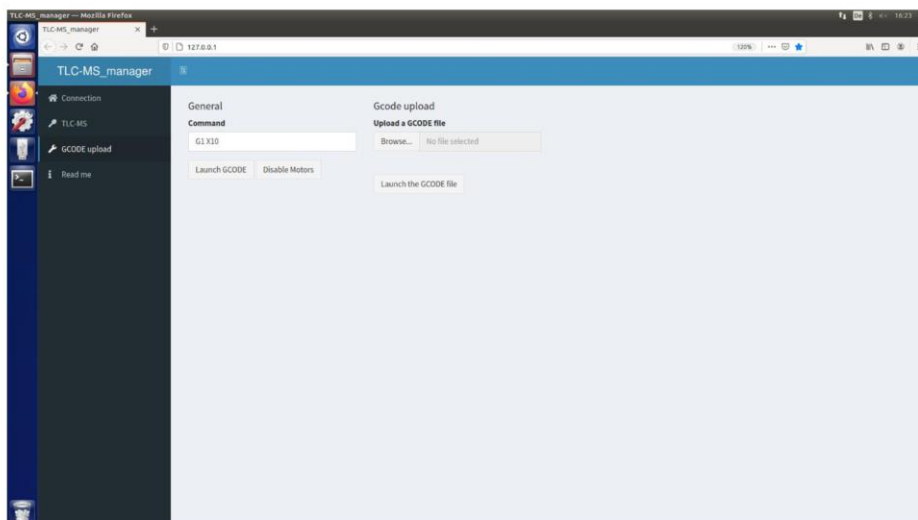
"Home X" induces movement along the x-axis, while "Home Y/Z" induces movement along the y-axis. For the latter, two motors are necessary, which is why the button displays "Y/Z", one for each motor. The plate holder then moves to the end stop of the respective axis. After that, you have to adjust the positioning bias. Move the plate holder towards you by entering the values "100" in the "X" field and "150" in the "Y" field. Click on "Go to position" to move the plate holder to the selected position. Mount the plate you would like to work with, upload an image of the plate and choose a prominent zone or a manually made mark for elution as described in the previous section of this manual. Go back to "Manual control" and in the "Select position" drop-down menu, choose the spot you have just marked as an elution zone.

The coordinates of the selected position are now displayed in the "X" and "Y" fields. Alternatively, enter a value in the "X" and "Y" fields instead of choosing it on the image. Click on "Go to position" to move the plate holder to the selected position. Switch on the LED and check the position. If the LED cross is not exactly in the middle of the selected spot, adjust the bias by clicking on the +/- 1/0.1 mm buttons on the right until the LED cross is centered over the selected spot. Alternatively, enter an appropriate value in the "x bias" or "y bias" fields. Now the positioning error is compensated. For this way of adjustment, it is recommended to verify that the LED is correctly aligned with the elution head position beforehand.

Running a batch

After you have chosen all positions for elution or uploaded a CSV file, chosen the right profile, have adjusted the positioning bias and downloaded manually chosen elution positions as a CSV file, everything is ready for your zones to be eluted. In the "Input/output" tab, enter the desired elution time and rinsing time (waiting time in between elutions) into the fields. Click on the "Run batch" button to start the run. Note that after you have started a batch, there is no option to stop the run with the help of the software. In case of any problems, you need to disconnect the NUC PC or Raspberry Pi from the power supply. To rapidly restore manually chosen elution zones, upload the CSV file you have downloaded before running the batch.

GCODE upload tab



Click on the **GCODE upload tab**. It can be used to try out G-code commands, for example after modification of the autoTLC-MS interface or if you would like to test which pin is responsible for which function. Enter the G-code you would like to execute and click on the "Launch G-code" button.

7. Publication III

Validated Screening Method for 81 Multi-Class Veterinary Drug Residues in Food via Online-Coupling High-Throughput Planar Solid-Phase Extraction to High-Performance Liquid Chromatography–Orbitrap Tandem Mass Spectrometry

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Validated Screening Method for 81 Multiclass Veterinary Drug Residues in Food via Online-Coupling High-Throughput Planar Solid-Phase Extraction to High-Performance Liquid Chromatography–Orbitrap Tandem Mass Spectrometry

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ABSTRACT: Current screening capabilities for veterinary drugs (VDs) in foods are limited, requiring time-consuming and expensive trace-level analyses. For the first time, a high-throughput planar solid-phase extraction (HTpSPE) cleanup, controlled by UV/vis/FLD imaging, was developed for screening 81 VDs from 6 different groups (glucocorticoids, anthelmintics, antiparasitics, coccidiostats, nonsteroidal anti-inflammatory drugs, and antibiotics) in 4 different matrices (honey, pig muscle, cow milk, and chicken eggs). It consumed 13 times less solvent and was more eco-friendly and 5 times faster than routine methods. The VDs were automatically eluted using the autoTLC–LC–MS interface, separated online on a high-performance liquid chromatography column via a 10-min gradient, and detected by Orbitrap high-resolution tandem mass spectrometry. The screening method was validated according to the latest European Commission Implementing Regulation 2021/808. Most VDs except penicillins and cephalosporins were detected at the 5- $\mu\text{g}/\text{kg}$ level in pig muscle, cow milk, and chicken eggs and 25- $\mu\text{g}/\text{kg}$ level in honey.

KEYWORDS: automated HTpSPE–UV/vis/FLD–HPLC–HRMS/MS, autoTLC–LC–MS interface, cleanup, multiclass veterinary medicines, food safety, Commission Implementing Regulation 2021/808

1. INTRODUCTION

Animal-derived products such as meat, milk, cheese, and eggs are consumed all over the world¹ and account for about one-third of the global protein consumption.² Honey is considered a healthy, nutritious sweetener containing natural sugars, enzymes, amino acids, antioxidants, and minerals.³ To meet the demand for animal products, most industrialized countries practice mass livestock farming, which necessitates the use of anti-infective drugs. This, however, leads to residues in food products that can be harmful to humans.^{4,5} Also, antibiotics are administered to treat bacterial brood diseases in apiculture.^{6,7} In addition, anthelmintics and coccidiostats are used to protect livestock from parasitic infections.^{8,9} Hormones, steroids, and β -agonists are administered to improve the growth rate, whereas corticosteroids and nonsteroidal anti-inflammatory drugs (NSAIDs) are used to treat inflammatory diseases and painful conditions.^{8,9} For consumer protection, the European Union has set maximum residue limits (MRLs) for drug substances in foodstuffs of animal origin.¹⁰ The use of some veterinary drugs (VDs) is prohibited for certain matrices, resulting in a zero-tolerance for these drugs.¹⁰ Official controls are mandatory¹¹ to verify the compliance of food products with the legal requirements, ensuring food safety and consumer health.

Performance criteria and validation of analytical methods are regulated in the Commission Implementing Regulation 2021/808.¹² Accordingly, screening methods may, and confirmatory methods must be based on chromatographic separation with spectrometric detection, providing information about the analyte structure. High-performance liquid chromatography–

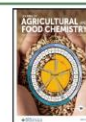
electrospray ionization–tandem mass spectrometry (HPLC–ESI–MS/MS) utilizing ion trap multistage or triple quadrupole instruments has been considered the gold standard for confirmatory analysis^{9,13,14} for a long time. Over the last decade, the use of high-resolution (HR) instruments has increased.^{15,16} In the field of VDs and pesticide residue analysis of food, several non-target multi-residue methods using quadrupole Orbitrap instruments operated in the data-independent acquisition (DIA) mode have been reported.^{17–19} The occurrence of matrix effects is a major limitation of ESI–MS. Cleanup procedures are required to avoid false positives, false negatives, or inaccurate quantification.^{9,14,20} State-of-the-art multiclass methods are most commonly based on extraction with acidified acetonitrile or methanol, followed by solid-phase extraction (SPE), dispersive SPE (dSPE), or gel permeation chromatography.^{9,14} In particular, quick, easy, cheap, effective, rugged, and safe (QuEChERS)²¹ procedures, including a dSPE step, have been applied in recent years.^{9,14} While these sometimes provide insufficiently purified extracts, the more efficient and partly automated SPE cleanup can result in the total loss of some analytes or substance classes if the purification is too extensive.⁹ To the best of our knowledge, dilute-and-shoot methods are

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very rarely applied for VDs in animal-derived food because coextracted matrices are very complex and result in unpredictable matrix effects and severe contamination of the HRMS system.

Despite all efforts and analytical progress, the trace-level analysis of VDs in food is still too time- and cost-intensive to be adequately covered in routine analysis. Addressing this issue and based on our first approaches to sample preparation and chromatography at one go with good accuracy and sensitivity despite high matrix load,^{22–24} high-throughput planar SPE (HTpSPE) was coupled to Orbitrap HRMS as rapid screening for antibiotics in water-based extracts of meat, milk, and eggs.²⁵ The cleanup was performed on a planar adsorbent utilizing high-performance thin-layer chromatography (HPTLC) instrumentation. Up to 16 raw extracts were applied on the adsorbent, and their different matrices were simultaneously 2D-separated from front-eluting VDs, present as one zone per sample. Automated zone elution is crucial for high-throughput online hyphenations of TLC/HPTLC with HPLC–MS,^{26,27} however, lacked full automation for a long time. For HTpSPE applied in the pesticide field so far, target zones have been manually eluted via an elution head-based TLC–MS interface from the adsorbent into autosampler vials and subsequently injected into a separate HPLC system with MS detection.^{28,29} Unfortunately, only a portion of the total analyte amount in the TLC/HPTLC zone is thus transferred to HPLC–MS, which can compromise the method performance regarding the detection limit. Online HPTLC–HPLC–MS was first presented at conferences in 2008, when a short monolithic column was installed between the interface and the MS, separating Sudan dyes from coeluting caffeine under isocratic conditions.^{30,31} Such online coupling has also been shown for plant alkaloids^{32,33} and coeluting lemon balm isomers.³⁴ Recently, the online elution of bioautogram zones directly from the bioassay plate to HPLC–MS was shown in an eight-dimensional hyphenation using gradient separation.^{35,36} Desalting cartridges and an additional valve switch for analyte trapping were integrated, uncoupling the low-pressure TLC–MS interface from the high-pressure HPLC–MS system.³⁶ This allowed for any HPLC column, flow rate, and gradient to be used without risking leakage of the elution head tightening during zone elution due to high backpressure. Serving the need for automation, an open-source modified, fully automated version of the CAMAG TLC–MS interface 2³⁷ was recently built termed autoTLC–MS.³⁸ After click-on-the-image selection of zones, it operates fully automated (plate positioning, zone elution, head cleaning, etc.). An upgraded version, termed autoTLC–LC–MS, has recently been introduced as a multipurpose on-surface autosampler for LC–MS.³⁹

This study aimed to develop an automated HTpSPE–UV/vis/FLD–HPLC–HRMS/MS method for the analysis of 81 VDs from 6 different groups in 4 matrices (pig muscle, cow milk, chicken eggs, and honey) for the first time. It was hypothesized that after the development of a generic HTpSPE–UV/vis/FLD cleanup, a fully automated sequence analysis of the VD zones can be performed, eluting the VD zones one-by-one via the autoTLC–LC–MS interface onto an HPLC column with subsequent gradient separation and HRMS/MS detection. An additional valve switch will be used to clean the elution head during the HPLC run, halving the analysis time. Such a hyphenation would be faster and less expensive than current analytical workflows. To find out the limitations of the developed method, it should be validated for each analyte

according to the latest EU requirements¹² and successfully applied to real samples.

2. MATERIALS AND METHODS

2.1. Chemicals and Materials. Acetonitrile (ACN, LC–MS grade, ≥99.95%) was obtained from Th. Geyer, Renningen, Germany. Formic acid (LC–MS grade, ≥99.0%) was purchased from Carlo Erba Reagents, Emmendingen, Germany. Methanol (LC–MS grade, ≥99.9%, Riedel-de Haën), sodium acetate (NaOAc, >99%), and anhydrous sodium sulfate (Na₂SO₄, ≥99%) were from Honeywell, Seelze, Germany. Water (LC–MS grade, HiPerSolv CHROMA-NORM), sodium chloride (NaCl, ≥99.5%), and anhydrous magnesium sulfate (MgSO₄, 99%) were purchased from VWR, Darmstadt, Germany. Ethanol (≥99.8%) and ACN (≥99.9%, used for extraction), ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA, 99–101%), ammonium formate (LC–MS grade, ≥99.9%), and rhodamine B (≥95%) were purchased from Sigma-Aldrich, Steinheim, Germany. A molecular sieve (4 Å) was purchased from Carl Roth, Karlsruhe, Germany. Double-distilled water (used for extraction) was produced using a Heraeus Destamat Bi-18e (Fisher Scientific, Schwerte, Germany). Polypropylene (PP) falcon tubes (15 and 50 mL) were obtained from Sarstedt, Nümbrecht, Germany. Ammonia solution (Suprapur, 25%), glacial acetic acid (HOAc, 100%), HPTLC silica gel 60 F₂₅₄ plates 10 cm × 20 cm with a layer thickness of 0.17 mm, and TLC silica gel 60 F₂₅₄ MS grade plates 20 cm × 20 cm with a layer thickness of 0.21 mm (cut to 20 cm × 10 cm) were provided by Merck, Darmstadt, Germany. The 81 VD standards were obtained as listed (Table S1). Validation samples (Table S2) were obtained either organically produced or known to be free from VD residues. Not organically produced samples used for routine testing were obtained from local supermarkets (Table S3).

2.2. Stock Solutions and Standard Mixtures. VD stock solutions at 0.1 or 1 mg/mL in different solvents (Table S1) were stored at –20 °C in the dark for up to 1 year, except for penicillins, which were prepared freshly in double-distilled water every 4 weeks. For the extraction study, a representative 26-standard mixture (10 µg/mL each) was prepared by combining 100 µL of each 1 mg/mL stock solution of the glucocorticoid, anthelmintic, avermectin, coccidiostat, and NSAID groups (Table S1) and one representative of each antibiotic group, namely sulfaquinolone, trimethoprim, clindamycin, tiamulin, erythromycin A, nafcillin, florfenicol, and 1000 µL of the 0.1 mg/mL stock solutions of cefoperazone and ipronidazole in a 10 mL volumetric flask. The solution was made up to volume with ACN. For the validation experiments, an 81-standard mixture comprising all standard compounds (1 µg/mL each) was prepared analogously. A rhodamine B stock solution was prepared in (1 mg/mL) and diluted with ACN (1 µg/mL).

2.3. Sample Extraction and Spiking. Muscle meat samples were minced for 30 s (Multi Moulinette, Moulinex, Alençon, France), whereas raw cow milk was vortexed for 30 s at speed 8 (Vortex Genie 2 with a multitube holder, Scientific Industries, New York, NY, USA). Whole chicken eggs were homogenized at 24 000 rpm for 30 s (ULTRA-TURRAX T25, IKA, Staufen, Germany). The samples were stored at –20 °C until use. Honey samples were stored at 4 °C and gently warmed to 40 °C and stirred before weighing. For pooled samples, 10 g each of 5 individual samples of the same matrix were homogenized altogether. Each sample (2.5 g) was weighed into a 50 mL PP tube. Depending on the experiment, the samples were spiked with respective volumes of the standard solutions. For the extraction study, the samples were spiked with 12.5 µL of the 26-standard mixture (10 µg/mL each) and made up to 200 µL with ACN (50 µg/kg). For the validation experiments, the samples were spiked with 12.5 or 62.5 µL of the 81-standard mixture (1 µg/mL each) and made up to 200 µL with ACN (5 or 25 µg/kg). To produce blank extracts, 200 µL of pure ACN was added to keep the same volume as in the spiked sample extracts. Each PP tube was vortexed for 30 s at speed 8 and allowed to stand for 15 min. Then, 2.5 mL of an aqueous 0.1 M EDTA solution was added to the samples and vortexed at speed 8 for 30 s. Subsequently, 2.5 mL of a 1%-HOAc solution in ACN was added to the pig muscle meat, raw cow

Table 1. Built-Up Compound Database; The Highest Abundant Fragment (Bold) Was Chosen as the Confirming Fragment

veterinary drug	retention time [min]	molecular formula	adduct	precursor	fragment 1		fragment 2		fragment 3		fragment 4	
					exact mass [m/z]	elemental composition	exact mass [m/z]	elemental composition	exact mass [m/z]	elemental composition	exact mass [m/z]	elemental composition
dapsone (DA)	4.26	C ₁₂ H ₈ N ₂ O ₅ S	[M+H] ⁺	249.06922	156.01138	C ₈ H ₆ NO ₃ S ⁺	108.04439	C ₆ H ₄ NO ⁺	92.04948	C ₄ H ₂ N ⁺		
sulfacetamide (SA)	3.33	C ₈ H ₉ N ₃ O ₃ S	[M+Na] ⁺	237.03043	no fragmentation							
sulfachloropyridazine (SCP)	4.58	C ₁₀ H ₈ ClN ₂ O ₃ S	[M+H] ⁺	285.02075	156.01138	C ₈ H ₆ NO ₃ S ⁺	108.04439	C ₆ H ₄ NO ⁺	92.04948	C ₄ H ₂ N ⁺		
sulfafloxacin (SC)	5.52	C ₁₆ H ₁₂ ClN ₄ O ₃ S	[M+H] ⁺	285.02075	156.01138	C ₈ H ₆ NO ₃ S ⁺	130.01665	C ₆ H ₄ ClN ₂ ⁺	108.04439	C ₄ H ₂ NO ⁺	94.06513	C ₂ H ₂ N ⁺
sulfadiazine (SD)	3.86	C ₈ H ₈ N ₄ O ₂ S	[M+H] ⁺	251.05972	156.01138	C ₈ H ₆ NO ₃ S ⁺	108.04439	C ₆ H ₄ NO ⁺	96.05562	C ₄ H ₂ N ₂ ⁺	92.04948	C ₂ H ₂ N ⁺
sulfadimethoxine (SDM)	5.80	C ₁₂ H ₁₀ N ₄ O ₅ S	[M+H] ⁺	311.08085	170.05602	C ₈ H ₆ N ₂ O ₃ S ⁺	156.07684	C ₆ H ₄ N ₂ O ₃ ⁺	108.04439	C ₄ H ₂ NO ⁺	92.04948	C ₂ H ₂ N ⁺
sulfadimidine (SDI)	4.30	C ₁₂ H ₁₀ N ₄ O ₅ S	[M+H] ⁺	279.09102	204.04374	C ₁₀ H ₈ N ₂ O ₃ S ⁺	156.01138	C ₈ H ₆ NO ₃ S ⁺	124.08692	C ₆ H ₄ N ₂ ⁺	108.04439	C ₄ H ₂ NO ⁺
sulfadoxine (SDX)	4.74	C ₁₂ H ₁₀ N ₄ O ₅ S	[M+H] ⁺	311.08085	156.01138	C ₈ H ₆ NO ₃ S ⁺	140.04536	C ₆ H ₄ N ₂ O ₃ ⁺	108.04439	C ₄ H ₂ NO ⁺	92.04948	C ₂ H ₂ N ⁺
sulfamerazine (SM)	4.10	C ₁₁ H ₉ N ₃ O ₃ S	[M+H] ⁺	265.07537	190.02809	C ₈ H ₆ O ₂ N ₃ S ⁺	156.01138	C ₆ H ₄ NO ₃ S ⁺	110.07127	C ₄ H ₂ N ₂ ⁺	108.04439	C ₂ H ₂ NO ⁺
sulfamethoxazole (SMX)	4.60	C ₁₀ H ₉ N ₃ O ₃ S	[M+H] ⁺	254.05939	156.01138	C ₈ H ₆ NO ₃ S ⁺	108.04439	C ₆ H ₄ NO ⁺	99.05529	C ₄ H ₂ NO ⁺	92.04948	C ₂ H ₂ N ⁺
sulfamethoxypyridazine (SMP)	4.40	C ₁₁ H ₉ N ₃ O ₃ S	[M+H] ⁺	281.07029	156.01138	C ₈ H ₆ NO ₃ S ⁺	126.06650	C ₆ H ₄ N ₂ O ⁺	108.04439	C ₄ H ₂ NO ⁺	92.04948	C ₂ H ₂ N ⁺
sulfanilamide (SN)	2.55	C ₈ H ₈ N ₂ O ₂ S	[M+H] ⁺	195.01987	no fragmentation							
sulfaguinoxaline (SQ)	6.12	C ₁₂ H ₁₀ N ₄ O ₅ S	[M+H] ⁺	301.07537	156.01138	C ₈ H ₆ NO ₃ S ⁺	108.04439	C ₆ H ₄ NO ⁺	92.04948	C ₄ H ₂ N ⁺		
sulfathiazole (ST)	3.91	C ₈ H ₈ N ₂ O ₂ S ₂	[M+H] ⁺	256.02089	156.01138	C ₈ H ₆ NO ₃ S ⁺	108.04439	C ₆ H ₄ NO ⁺	92.04948	C ₄ H ₂ N ⁺		
baquloprim (BAQ)	3.41	C ₁₀ H ₁₀ N ₄	[M+H] ⁺	309.18222	294.15875	C ₈ H ₈ N ₄ ⁺	279.13527	C ₆ H ₆ N ₄ ⁺	266.14002	C ₄ H ₄ N ₄ ⁺	171.09167	C ₂ H ₂ N ₂ ⁺
trimethoprim (TRI)	4.11	C ₁₀ H ₁₂ N ₄ O	[M+H] ⁺	291.14517	261.09822	C ₈ H ₁₀ N ₄ O ⁺	245.10330	C ₆ H ₈ N ₄ O ⁺	230.11621	C ₄ H ₆ N ₄ O ⁺	123.06652	C ₂ H ₄ N ₂ ⁺
clindamycin (CLI)	6.65	C ₁₈ H ₂₈ ClN ₂ O ₅ S	[M+H] ⁺	425.18715	377.18378	C ₁₆ H ₂₆ ClN ₂ O ₅ ⁺	126.12768	C ₁₄ H ₂₄ N ⁺				
lincomycin (LIN)	4.09	C ₁₈ H ₂₈ N ₂ O ₅ S	[M+H] ⁺	407.22103	389.21047	C ₁₆ H ₂₆ N ₂ O ₅ S ⁺	359.21766	C ₁₄ H ₂₄ N ₂ O ⁺	126.12773	C ₁₂ H ₂₂ N ⁺		
tiamulin (TIA)	7.02	C ₁₈ H ₂₈ NO ₅ S	[M+H] ⁺	494.32986	192.10528	C ₁₆ H ₂₆ NO ₃ S ⁺	119.01613	C ₁₄ H ₂₄ O ₃ ⁺				
valnemulin (VAL)	7.42	C ₁₈ H ₂₈ N ₂ O ₅ S	[M+H] ⁺	565.36697	156.01138	C ₈ H ₆ NO ₃ S ⁺	108.04439	C ₆ H ₄ NO ⁺	92.04948	C ₄ H ₂ N ⁺		
clarithromycin (CLA)	7.35	C ₂₁ H ₃₃ NO ₅	[M+H] ⁺	748.48417	158.11756	C ₁₀ H ₁₄ NO ₃ ⁺	116.07060	C ₈ H ₁₀ NO ₃ ⁺	98.09643	C ₆ H ₈ N ⁺		
erythromycin A (ERY)	7.09	C ₂₁ H ₃₃ NO ₅	[M+H] ⁺	734.46852	158.11756	C ₁₀ H ₁₄ NO ₃ ⁺	116.10699	C ₈ H ₁₀ NO ⁺	98.09643	C ₆ H ₈ N ⁺		
gamithromycin (GAM)	6.35	C ₂₀ H ₃₁ NO ₅	[M+2H] ²⁺	389.27719	619.45281	C ₁₀ H ₁₄ N ₂ O ₄ ⁺	158.11756	C ₈ H ₁₀ NO ₃ ⁺	116.10699	C ₆ H ₈ NO ⁺		
josamycin (JOS)	7.29	C ₂₀ H ₃₁ NO ₅	[M+H] ⁺	828.47400	174.11247	C ₁₀ H ₁₄ NO ₃ ⁺	109.06479	C ₈ H ₁₀ O ⁺				
neopiramycin (NEO)	4.99	C ₁₈ H ₃₄ NO ₅	[M+2H] ²⁺	350.22496	174.11247	C ₁₀ H ₁₄ NO ₃ ⁺	160.13321	C ₈ H ₁₀ NO ₃ ⁺	98.09643	C ₆ H ₈ N ⁺		
spiramycin (SPI)	7.13	C ₁₈ H ₃₄ NO ₅	[M+H] ⁺	772.44778	158.11756	C ₁₀ H ₁₄ NO ₃ ⁺	116.07060	C ₈ H ₁₀ NO ₃ ⁺	95.04914	C ₆ H ₈ O ⁺		
oleandomycin (OLE)	5.50	C ₂₀ H ₃₄ NO ₅	[M+2H] ²⁺	422.26428	174.11247	C ₁₀ H ₁₄ NO ₃ ⁺	101.05971	C ₈ H ₁₀ O ⁺				
tilmicosin (TIL)	6.60	C ₂₀ H ₃₄ NO ₅	[M+2H] ²⁺	435.29030	695.48411	C ₁₀ H ₁₄ N ₂ O ₄ ⁺	174.11247	C ₈ H ₁₀ NO ₃ ⁺	99.04406	C ₆ H ₈ O ⁺		
tulathromycin 2 (TUL)	4.30	C ₂₀ H ₃₄ NO ₅	[M+2H] ²⁺	403.79046	577.40557	C ₁₀ H ₁₄ N ₂ O ₄ ⁺	158.11756	C ₈ H ₁₀ NO ₃ ⁺	98.09643	C ₆ H ₈ N ⁺		
tylosin A (TYA)	7.05	C ₂₀ H ₃₄ NO ₅	[M+H] ⁺	916.52643	174.11247	C ₁₀ H ₁₄ NO ₃ ⁺	101.05971	C ₈ H ₁₀ O ⁺				
tylosin B (TYB)	6.90	C ₂₀ H ₃₄ NO ₅	[M+H] ⁺	772.44778	174.11247	C ₁₀ H ₁₄ NO ₃ ⁺	116.07060	C ₈ H ₁₀ NO ₃ ⁺	98.09643			
cefalexin (CLX)	4.33	C ₁₅ H ₁₄ N ₂ O ₃ S	[M+H] ⁺	348.10125	160.04268	C ₈ H ₆ NO ₃ S ⁺	158.02703	C ₆ H ₄ NO ₃ S ⁺				
cefalonium (CL)	4.18	C ₁₅ H ₁₄ N ₂ O ₃ S	[M+H] ⁺	459.07914	158.02703	C ₈ H ₆ NO ₃ S ⁺	152.01646	C ₆ H ₄ NO ₃ S ⁺	123.05529	C ₄ H ₂ N ₂ O ⁺	97.01065	C ₂ H ₂ S ⁺
cefazolin (CZ)	3.90	C ₁₅ H ₁₄ N ₂ O ₃ S	[M+H] ⁺	424.06315	292.05728	C ₈ H ₆ N ₂ O ₃ S ⁺	152.01646	C ₆ H ₄ NO ₃ S ⁺	124.02155	C ₄ H ₂ N ₂ ⁺	112.02155	C ₂ H ₂ N ₂ S ⁺
cefepazone (COP)	4.55	C ₁₈ H ₁₆ N ₂ O ₃ S	[M+Na] ⁺	477.01923	317.04273	C ₁₀ H ₈ N ₂ NaO ⁺	177.99332	C ₈ H ₆ NNaO ₃ S ⁺				
cefquinome (CQ)	4.73	C ₁₈ H ₁₆ N ₂ O ₃ S	[M+Na] ⁺	668.13162	526.05739	C ₁₀ H ₈ N ₂ NaO ₃ S ⁺	410.04173	C ₈ H ₆ NNaO ₃ S ⁺	177.99332	C ₆ H ₄ NNaO ₃ S ⁺	165.06345	C ₄ H ₂ NNaO ₃ S ⁺
ceftriaxone (CT)	4.03	C ₁₈ H ₁₆ N ₂ O ₃ S	[M+H] ⁺	529.13224	167.02736	C ₈ H ₆ N ₂ O ₃ S ⁺	134.09643	C ₆ H ₄ N ₂ ⁺	125.00422	C ₄ H ₂ N ₂ ⁺	112.02155	C ₂ H ₂ N ₂ ⁺
cefepirin (CF)	6.39	C ₁₈ H ₁₆ N ₂ O ₃ S	[M+H] ⁺	524.03629	241.03899	C ₈ H ₆ N ₂ O ₃ S ⁺	126.01204	C ₆ H ₄ N ₂ ⁺	125.00422	C ₄ H ₂ N ₂ ⁺	95.01276	C ₂ H ₂ O ⁺
desacetil-cefepirin (CPO)	3.35	C ₁₈ H ₁₆ N ₂ O ₃ S	[M+H] ⁺	382.05259	193.04301	C ₈ H ₆ N ₂ O ₃ S ⁺	152.01646	C ₆ H ₄ NO ₃ S ⁺	124.02155	C ₄ H ₂ N ₂ ⁺	112.02155	C ₂ H ₂ N ₂ S ⁺
amoxicillin (AMO)	3.37	C ₁₆ H ₁₈ N ₂ O ₅ S	[M+H] ⁺	366.11182	208.04268	C ₈ H ₆ NO ₃ S ⁺	160.04268	C ₆ H ₄ NO ₃ S ⁺	134.06004	C ₄ H ₂ NO ⁺	114.00081	C ₂ H ₂ NO ⁺
ampicillin (AMP)	4.52	C ₁₆ H ₁₈ N ₂ O ₅ S	[M+H] ⁺	350.11690	192.04776	C ₈ H ₆ NO ₃ S ⁺	174.05495	C ₆ H ₄ NO ₃ S ⁺	160.04268	C ₄ H ₂ NO ⁺	106.05513	C ₂ H ₂ N ⁺
cloxacillin (CLO)	7.30	C ₁₈ H ₁₆ ClN ₂ O ₃ S	[M+H] ⁺	436.07285	277.03745	C ₁₀ H ₈ ClN ₂ O ₃ S ⁺	178.05542	C ₈ H ₆ ClNO ⁺	160.04268	C ₆ H ₄ NO ₃ S ⁺	114.03720	C ₄ H ₂ N ⁺
dicloxacillin (DIC)	7.41	C ₁₈ H ₁₆ Cl ₂ N ₂ O ₃ S	[M+H] ⁺	470.03387	310.99847	C ₁₀ H ₈ Cl ₂ N ₂ O ₃ S ⁺	211.96645	C ₈ H ₆ Cl ₂ NO ⁺	160.04268	C ₆ H ₄ NO ₃ S ⁺	114.03720	C ₄ H ₂ N ⁺
methicillin (MET)	6.70	C ₁₉ H ₁₈ ClN ₂ O ₃ S	[M+H] ⁺	381.11148	222.07068	C ₁₀ H ₈ ClN ₂ O ₃ S ⁺	165.05462	C ₈ H ₆ O ⁺				
oxacillin (OXA)	7.41	C ₁₉ H ₁₈ NO ₃ S	[M+H] ⁺	415.13222	256.09682	C ₁₀ H ₈ NO ₃ S ⁺	199.07536	C ₈ H ₁₀ O ₃ ⁺	181.06479	C ₆ H ₈ O ⁺	171.04406	C ₄ H ₂ O ⁺
oxacillin (OXA)	7.25	C ₁₉ H ₁₈ NO ₃ S	[M+H] ⁺	402.11182	243.07642	C ₁₀ H ₈ NO ₃ S ⁺	160.04268	C ₈ H ₁₀ NO ₃ ⁺	144.04439	C ₆ H ₄ NO ⁺	114.03720	C ₄ H ₂ N ⁺
penicillin G (PENG)	6.98	C ₁₆ H ₁₈ N ₂ O ₅ S	[M+H] ⁺	335.10600	217.06414	C ₈ H ₆ N ₂ O ₃ S ⁺	176.07060	C ₆ H ₄ NO ₃ S ⁺	160.04268	C ₄ H ₂ NO ₃ S ⁺	91.05423	C ₂ H ₂ ⁺
penicillin V (PENV)	7.											

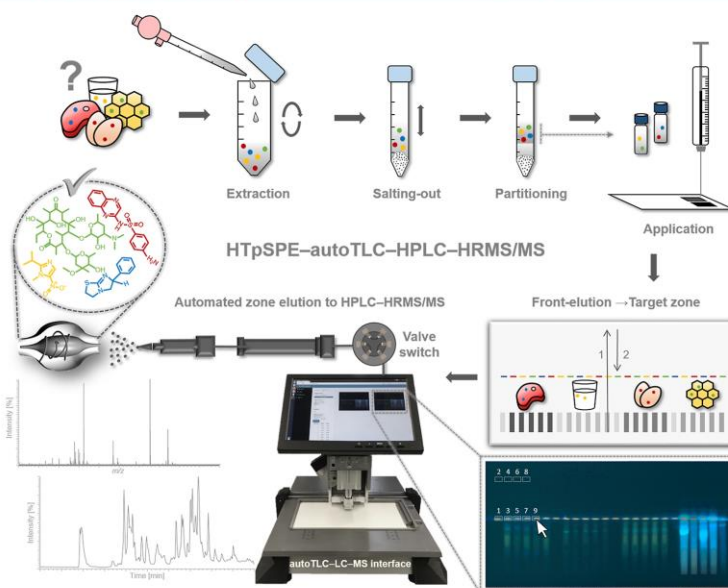


Figure 1. Workflow of the developed HTPSPE–UV/vis/FLD–autoTLC–LC–HRMS/MS method.

milk, and chicken egg samples, whereas 5% HOAc in ACN was used for the honey samples. The tubes were then shaken end-over-end for 15 min at 70 rpm using a Reax 2 shaker (Heidolph Instruments, Schwabach, Germany). Centrifugation followed at $3000 \times g$ for 5 min (ROTANTA 460 R, Hettich, Tuttingen, Germany). The supernatants were decanted into 15 mL PP tubes containing the QuEChERS salt mixtures (1 g MgSO_4 and 0.25 g NaOAc for muscle meat and honey, 1.5 g Na_2SO_4 and 0.25 g NaCl for raw cow milk and chicken eggs) and immediately shaken vigorously for 15 s. The 15 mL tubes were centrifuged at $3000 \times g$ for 10 min, and supernatants were carefully transferred into autosampler vials.

2.4. HTPSPE Cleanup. Each extract (100 μL) was applied on TLC silica gel 60 F_{254} MS grade plates as 4 mm $\times$ 20 mm area using the following settings: dosage speed 1200 nL/s, predosage volume 200 nL, filling speed 15 $\mu\text{L}/\text{s}$, filling vacuum time 4 s, retraction volume 200 nL, rinsing solvent ACN, rinsing vacuum time 4 s, rinsing cycles 2, filling cycles 1, and nozzle heating to 60 $^\circ\text{C}$ (Automatic TLC Sampler ATS 4, CAMAG, Muttensz, Switzerland). The layout was as follows: application position Y 10.0 mm, first track position X 10.3 mm, and track distance 7.8 mm, allowing 24 tracks to be applied on a 20 cm $\times$ 10 cm plate. A rhodamine B solution (1 $\mu\text{g}/\text{mL}$) was oversprayed on the samples (5 μL). After a 5 min humidity control of the layer (0–5% relative humidity using a dry molecular sieve), the VDs were first front-eluted with ACN/ammonia/ethanol 13:4:3 (V/V/V) up to 80 mm (from the lower plate edge) and then in the reverse direction with methanol/ACN/ethanol/ammonia 6:2:1:1 (V/V/V/V) up to 45 mm (Automatic Developing Chamber ADC 2, CAMAG). After each step, the plates were automatically dried in a cold stream of air for 3 min. Images were recorded at UV 254 nm and fluorescence light detection (FLD) 366 nm (automatic exposure, TLC Visualizer 2, CAMAG). Instrumentation was controlled by visionCATS 3.1.21109.3 (Ultimate software package, CAMAG) using File S1.

2.5. autoTLC–LC–HRMS/MS Analysis. The VD zones were eluted from the plates for 60 s using the fully automated autoTLC–LC–MS interface (Files S2 and S3),³⁹ equipped with a round elution head of 4 mm diameter. The elution solvent (0.1% formic acid and 4 mM ammonium formate solution in water/methanol 9:1, V/V) was

provided at a flow rate of 0.2 mL/min by a standalone HPLC pump (MX010PFT, Teledyne SSI, State College, PA, USA). Using a PEEK capillary of 230 mm length and 0.13 mm inner diameter, the outlet of the autoTLC–LC–MS interface was connected to a six-port, two-position switching valve (MXT series, PD715-000, Rheodyne, IDEX Health & Science, Rohnert Park, CA, USA). In the latter, a 100- μL sample loop was installed and filled with the eluted VDs coming from the autoTLC–LC–MS interface. After 40 s of filling time, the valve was automatically switched by contact closure signals configured in Xcalibur MS software (File S4). The HPLC eluent (provided by an Ultimate HPG-3200SD standard binary pump, Dionex Softron, Germering, Germany) flushed the sample loop, and the VDs were guided to a Chromolith Performance RP-18e 100 mm $\times$ 3 mm monolithic HPLC column thermostated at 25 $^\circ\text{C}$ in a column oven. The valve was switched back into bypass position at 60 s. Mobile phase A was 0.1% formic acid and 4 mM ammonium formate in water, while mobile phase B was 0.1% formic acid and 4 mM ammonium formate in methanol. The following gradient was used at a 0.5 mL/min flow rate: 0–1 min: 5% B; 1–2 min: the composition was linearly increased to 40% B; 2–4 min: 40% B was held; 4–6 min: increase to 95% B; 6–8 min: 95% B was held; 8–8.1 min: decrease to 5% B and held until 10 min for column equilibration (Files S4 and S5). The start of each chromatographic run was controlled by the autoTLC–LC–MS interface (File S2) as described recently³⁹ and thus synchronized with the start of each zone elution. A blank spot above the elution front on the plate was eluted to clean the elution head valve and tubings, as well as the sample loop, from potentially remaining traces after each VD zone elution. This cleaning was performed in the middle of each 10-min gradient when the switching valve was still in the bypass position, and thus, the solvent flow was directed to the waste without disturbing the chromatographic run. The corresponding settings in the autoTLC–LC–MS software TLC–MS_manager were 60 s elution time and 250 s pausing time before the next elution (including 10 s delay time).

The analytes were detected with a Q Exactive Plus Hybrid Quadrupole–Orbitrap mass spectrometer (Thermo Scientific, Dreieich, Germany) with an Ion-Max HESI-II probe (sheath gas 20 AU, aux gas 10 AU, spray voltage 3.5 kV, capillary temperature 320 $^\circ\text{C}$, probe heater

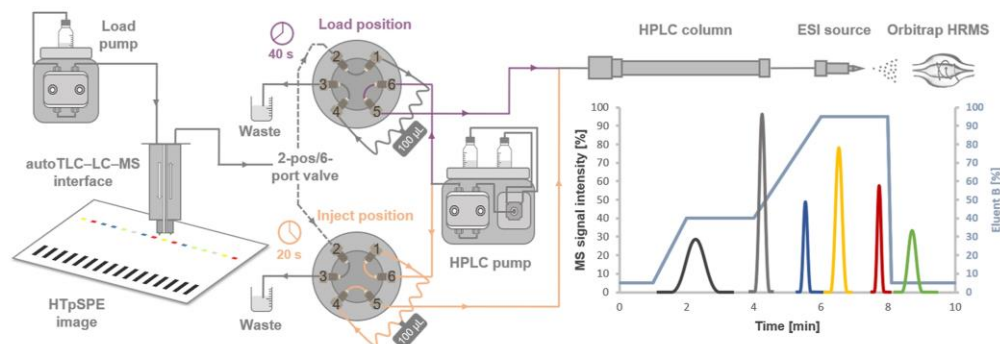


Figure 2. Flow scheme of the autoTLC-LC-MS-HRMS/MS instrumentation.

temperature 350 °C, and S-lens RF level 50 AU). Spectra were recorded using the variable (v) DIA mode as recently published.³⁹ Briefly, a full scan (m/z 100–1100) in the positive ionization mode, with a resolution of 70,000 full width at half-maximum (FWHM) at m/z 200, automatic gain control (AGC) target of 1×10^6 , maximum inject time of 200 ms, without lock mass injection, was followed by sequential vDIA scans. DIA 1 covered a mass range of m/z 100–500 with an isolation window of 52 Da (loop count 8), whereas DIA 2 covered m/z 500–1100 with an isolation window of 600 Da (loop count 1). The general settings for the DIA scans were a resolution of 35,000, AGC target 1×10^6 , with stepped normalized collision energy of 20, 40, and 60 eV. The inclusion list for the DIA scans consisted of m/z 125.00000, 175.00000, 225.00000, 275.00000, 325.00000, 375.00000, 425.00000, 475.00000, and 800.00000 (File S4). External mass calibration was performed daily with Pierce LTQ Velos ESI positive ion calibration solution (Thermo Scientific). The instruments were controlled by Xcalibur 4.2.47 with Foundation 3.1.261.0 and SII for Xcalibur 1.5.0.10747. The data were processed with TraceFinder 3.2.512.0 General Quan using File S6 (all Thermo Scientific). Mass Frontier 7.0.5.9 SR3 (HighChem, Thermo Scientific) and ChemDraw Ultra 12.0.2.1076 (CambridgeSoft, PerkinElmer Informatics, Rodgau, Germany) were used for fragmentation pathway simulations. Statistical analyses were performed with Excel 2016 (Microsoft, Redmond, WA, USA).

2.6. Method Validation. The method was validated as a screening method according to the latest Commission Implementing Regulation 2021/808¹² and the recommendations of the Community Reference Laboratories (CRLs) for Residues.⁴⁰ 20 individual blank samples per matrix were analyzed. These originated from different suppliers and were either organically produced or known to be free from VD residues. The analyses were divided into four series of five analyses for each of the four matrices, whereby each series was performed on one day. This led to a total of 20 analyses per day on one TLC plate, carried out on four consecutive days by two different analysts. The same 20 blank samples were spiked at the two validation levels of 5 and 25 $\mu\text{g}/\text{kg}$ and analyzed analogously. Additionally, a reagent blank and three standard solutions corresponding to the respective validation level were applied on each TLC plate, resulting in 24 tracks per plate.

3. RESULTS AND DISCUSSION

3.1. Study Design. Food extracts were prepared on an aqueous basis mixed with ACN for protein precipitation in our first HTPSPE approach.²⁵ However, apart from combinations with buffers or EDTA solutions,^{41–45} VD multiresidue methods rely on the extraction with acidified ACN for sufficient protein precipitation and a wider polarity range.^{44,45} Combined with salting out, the remaining water content in the ACN extract is standardized to obtain quantitative results.⁴⁶ Hence, the HTPSPE was newly developed for acidified ACN-based extracts.

Salt combinations and extraction conditions were investigated. In addition to the previous meat, milk, and eggs matrices, the matrix honey was newly included. Besides 64 multiclass antibiotics (not available were 3-*o*-acetyltylosin and tylvalosin if compared to ref 25), 17 VDs from 5 further groups (Table S1), that is, glucocorticoids, anthelmintics, antiparasitics, coccidiostats, and NSAIDs were investigated for the first time via HTPSPE. All of the selected VDs are widely used in veterinary medicine, and MRLs have been established for most of them in pig muscle and milk, while some are banned altogether.¹⁰

It is the first generic screening across the different classes and matrices concerning sample preparation and analysis. After HTPSPE, the VD zones were automatically eluted from the planar adsorbent using the latest autoTLC-LC-MS interface³⁹ and separated online on an HPLC column. An additional valve switch was integrated to shorten the analysis times. Unambiguous confirmation of each VD was realized using an in-house-built compound database (Table 1, File S6). The quantitative performance of the final method (Figure 1) was evaluated, and it was validated as a screening method according to the latest Commission Implementing Regulation 2021/808.¹² All files concerning the method, sequence, database, and commands relevant to operating the instrument and to run the final method are made available (Files S1–S6). Finally, to assess the method performance and applicability for routine testing, 36 samples bought in local discounters (Table S3) were analyzed.

3.2. autoTLC-LC-HRMS/MS Analysis. The autoTLC-LC-MS interface³⁹ was chosen for rapid online transfer of the VD zones to the HPLC column and then the HRMS. As an on-surface autosampler, it can automatically position the adsorbent plate, elute the VD zones, and clean the elution head and tubing from residual analytes, thus replacing any laborious manual interventions.⁴⁷ The plate image can be uploaded in the TLC-MS_manager software, and the elution positions can be selected by simply clicking on the respective zones on the image. These zones are then automatically eluted one by one as a sequence without the need for further manual intervention.^{25,38,39} In the former study,³⁹ a monolithic HPLC column was installed directly in between the interface and the mass spectrometer, enabling a fully automated online elution into the column. Consequently, also elutions intended for cleaning (between sample runs) were transferred to the column, doubling the analysis time. During the elutions, the flow rate had to be reduced to avoid leakage of the elution head due to high backpressure. To overcome these limitations, an additional

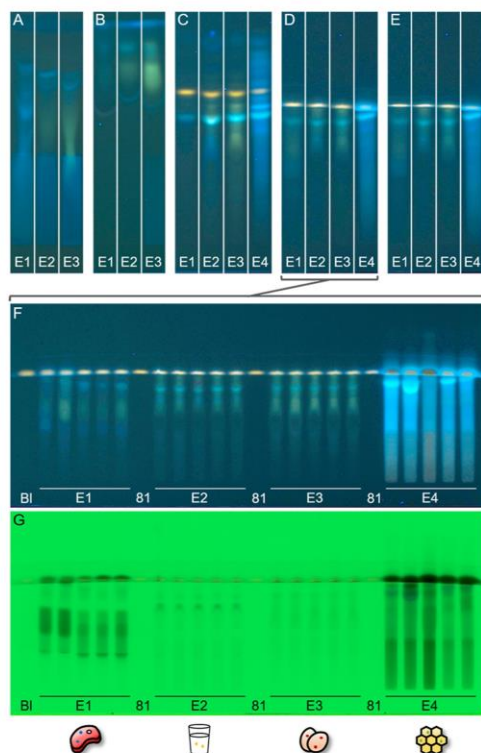


Figure 3. HTpSPE images at FLD 366 nm of McIlvaine-EDTA extracts (A) and ACN-based extracts (B), after a single front elution with ACN/methanol/ammonia 5:3:2 (V/V/V) until 85 mm and of the final QuEChERS extracts after a single front elution with ACN/ammonia/ethanol 13:4:3 (V/V/V) until 80 mm (C), after a second front elution in the reverse direction with methanol/ACN/ethanol/ammonia 6:2:1:1 (V/V/V/V) until 45 mm (D), and after a third front elution with the latter conditions (E) on MS-grade TLC silica gel 60 F₂₅₄. Typical HTpSPE image of the validation studies after twofold front elution at FLD 366 nm (F) and UV 254 nm (G). E1: pig muscle, E2: raw cow milk, E3: chicken eggs, E4: honey, Bl: reagent blank, 81: 81-standard mixture (5 µg/kg), orange band: marker rhodamine B, indicating the analyte position.

switching valve and standalone pump were installed for the first time in between the autoTLC–LC–MS interface and the Chromolith Performance RP-18e 100 mm × 3 mm monolithic HPLC column (Figure 2), as performed manually in an eight-dimensional hyphenation.³⁶ Thus, the low-pressure interface and the high-pressure HPLC–MS system were decoupled, which allowed using any HPLC column, gradient, and flow rate. As the solvent for zone elution, 10% aqueous methanol containing 0.1% formic acid and 4 mM ammonium formate was found to be optimal for a 60 s elution at a 0.2 mL/min flow rate whereby the eluted VDs were still properly retained on the reversed-phase HPLC column. Just before starting the elution of each zone, the chromatographic run and the MS data acquisition were started by a contact closure signal to the HPLC pump.³⁹ For 40 s, the switching valve was held in the load position during

the zone elution by the autoTLC–LC–MS interface (Figure 2) to fill the 100 µL sample loop with the eluted VDs. During this time, at a 0.2 mL/min flow rate, theoretically, 133 µL of VD solution should have been transferred from the plate. Nevertheless, the interface took 1.5 s (5 µL) after the gradient start to lower the elution head and start the elution process, so the actual volume of the transferred solution was 128 µL. The combined dead volumes of the outlet stainless-steel capillary of the elution head (10 µL; 300 mm length and 0.205 mm inner diameter), the connecting PEEK capillary (3 µL; 230 mm length and 0.13 mm inner diameter), and the sample loop (100 µL) was calculated to be 113 µL. Hence, the first 15 µL of the eluent was directed into the waste. Still, 40 s until the valve switch from load to inject proved to be optimal for transfer (shorter or longer times led to a loss of the signal intensity). A possible reason for this could be that the VDs did not immediately front-elute in the elution solvent. Instead, matrix compounds with lower adsorption energy to the plate front-eluted first, and thus, these were discarded. After the 40 s transfer, the valve was automatically switched to the inject position for 20 s (Figure 2) by contact closure signals configured in Xcalibur MS software. The eluent provided by the HPLC pump flushed the 100 µL sample loop at a 0.5 mL/min flow rate and guided the VDs into the column, and thus, a total of 166 µL, consisting of the VD eluate stored in the sample loop and the initial mobile phase, was transferred to the column as flow injection. The previous mobile phase gradient³⁹ was optimized for a rapid 10 min separation (including column equilibration) of the multiclass VDs, starting with 5% methanol content and ending with 95%. After 20 s, the valve was switched back into the load position and was kept in this position during the remaining 9 min of the chromatographic run. Simultaneously, the elution process of the autoTLC–LC–MS interface was completed (60 s total run time). In the middle of the running gradient separation, the autoTLC–LC–MS interface tubings were cleaned from potential remaining traces by a 60 s elution of blank space (above the elution front of the plate). The flow was directed to the waste without disturbing the chromatographic run since the switching valve was still in the load position (Figure 2), enabling fast analysis times.

Since the autoTLC–LC–MS interface is a plug-and-play device, it can be coupled to any HPLC pump and any mass spectrometer available. In this study, a quadrupole Orbitrap instrument operated in the vDIA mode³⁹ (Figure S1) was chosen for MS data acquisition, which allows simultaneous detection of precursors and fragments of a large number of analytes and also allows retrospective data analysis for the detection of metabolites, analogs, homologs, or unknowns. An in-house compound database (Table 1, File S1) containing the accurate masses of precursors and fragments and the retention time of each analyte was built analogously to our first study.²⁵ Sulfacetamide, sulfanilamide, chloramphenicol, and thiamphenicol did not produce fragments under the chosen fragmentation conditions of 20, 40, and 60 eV. Oxytetracycline and rifaximin did only ionize in the negative mode, whereas carprofen and ibuprofen did not ionize at all. For the latter, the ionization settings were not further optimized due to the optimal settings for all other VDs, and thus, both were excluded.

Confirmation according to the new Commission Implementing Regulation 2021/808¹² relies on a system of identification points. This regulation recently replaced the old Commission Decision 2002/657/EC,⁴⁸ whereby the identification point system was revised. For banned compounds, now a minimum of five identification points is required, whereas, for regulated VDs,

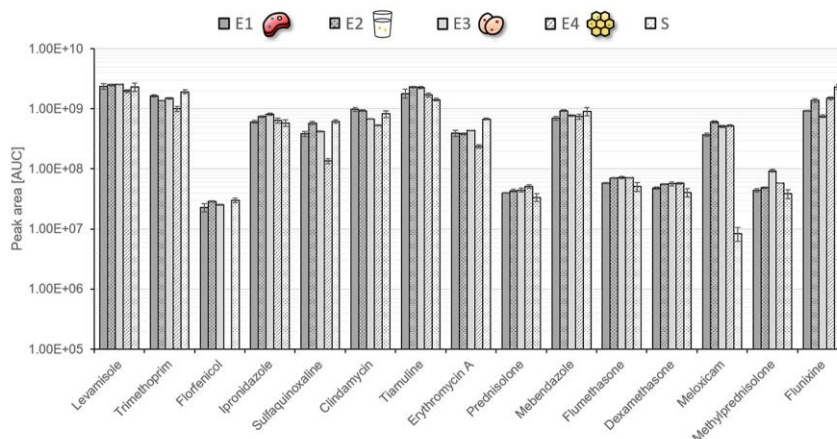


Figure 4. Comparison of the peak area signals ($n = 3$, error bars = standard deviation) of VD standards.

only four points are necessary. This was fulfilled by measuring two diagnostic ions per analyte, one exact precursor ion at a resolution of 70,000 (FWHM) and one exact fragment ion at a resolution of 35,000 (FWHM) with a maximum mass deviation of 5 ppm, resulting in four identification points. Ion ratios had to correspond to those of a standard solution in a range of $\pm 40\%$. An additional point was achieved by the retention time criterion, which was newly added to the identification point system of the regulation. The retention time of the analyte in the extract had to correspond to that of a calibration standard within a tolerance of ± 0.1 min. In short, an analyte was considered confirmed if the respective masses of the diagnostic ions (precursor and confirming fragment) were observed at a retention time of ± 0.1 min tolerance within a 5 ppm mass extraction window using TraceFinder 3.2 software. Other fragments included in the database and isotopic ratios of the precursor gave further confidence but were not used as confirmation criteria.

3.3. Optimization of the Extraction Procedure and HTPSPE. The calculated maximal sample matrix amount applied as 6 mm $\times$ 30 mm area on the plate was 20 mg for muscle meat and milk and 10 mg for eggs in the previous study.²⁵ Higher amounts were not possible because the raw extracts still needed to be filterable before application, and the applied volume of the final solution (166 μ L) was already near to overloading the adsorbent. Thus, a more selective extraction solvent and then feasible higher extract application volume were needed to lower the detection limits. This was realized with ACN, for which 100 μ L of the final extract corresponded to 93 mg of muscle, milk, eggs, and honey matrix. In addition, the size of the areas was reduced to 4 mm $\times$ 20 mm, which allowed the application of 24 areas per plate (Figure 3F,G) and thus increased sample throughput and efficiency.

For the ACN-based extracts, the ACN/methanol/ammonia 5:3:2 (V/V/V) mixture used for front elution of the McIlvaine-EDTA extracts in the previous study²⁵ was too high in the elution strength, as most matrix compounds were at $hR_F > 60$ (Figure 3A vs 3B). Consequently, the mixture was adjusted, and elution strength was reduced. Using ACN/ammonia/ethanol 13:4:3 (V/V/V), the matrix compounds were partially front-eluted (Figure 3C), which could not be avoided with any of the

tested mobile phases. This made the development of a second solvent system for front elution in the reverse direction very challenging. The second had to be more polar compared to the first to focus all VDs in a sharp zone. However, if it was too polar, it was not able to pass through the matrix compounds in the elution front evenly, thus leading to distorted bands or no focusing at all (Figure S2). After the addition of methanol, the mixture methanol/ACN/ethanol/ammonia 6:2:1:1 (V/V/V/V) was able to penetrate the matrix compounds and focus the analytes on the elution front (Figure 3D). A repetition of the latter front elution sharpened the VD zones even more (Figure 3E) and improved the precision of the results (Section 3.4). All front elutions were carried out in the ADC 2 system after a 5-min plate humidity control to 0–5% relative humidity to ensure reproducible results. Since the VD standards were not visible at the trace level, the orange-fluorescent marker rhodamine B, which showed a similar elution behavior as the VDs,²⁵ was applied as overspray on the extracts to indicate their position (Figure 3C–F). Their presence in the VD zone was verified via autoTLC–LC–HRMS/MS (Section 3.2), and if applied at higher amounts (0.1–5 μ g/area), on the HTPSPE image using derivatization reagents.²⁵ As reported,²⁵ not only some cephalosporins and penicillins but also the NSAID carprofen were retained at a low hR_F by food matrix compounds (Figure S3C,D). For all the other VDs, the planar extraction and focusing on the elution front was successful (Figure S3A,B).²⁵

In preliminary experiments, different extraction solvents were tested. For that purpose, three 2.5-g aliquots of a pooled blank sample were spiked at 50 μ g/kg using the 26-standard mixture consisting of representative standards of each group instead of the 81-standard mixture to reduce the standard consumption and facilitate data evaluation. Then, two different parameters were compared per plate as described in the following: extracts that were produced using a 0.1-M EDTA solution in the first and 1% HOAc in ACN⁴⁹ in the second step resulted in higher peak areas than extracts produced using acidified ACN only. EDTA was added to avoid the chelation of macrolides with polyvalent cations in the aqueous phase.⁵⁰ Additionally, 1% HOAc in acetone was also tested; however, the extracts were very turbid due to stronger coextraction of lipids, proteins, and pigments.⁴⁹

Table 2. MRLs and Validation Results, in the Case of MRLs Varying between Species, the Lowest and Thus Strictest Value Was Applied

veterinary drug	pig muscle			cow milk			chicken egg			honey		
	MRL [$\mu\text{g/kg}$]	CC β [$\mu\text{g/kg}$]	no. positives/no. samples	MRL [$\mu\text{g/kg}$]	CC β [$\mu\text{g/kg}$]	no. positives/no. samples	MRL [$\mu\text{g/kg}$]	CC β [$\mu\text{g/kg}$]	no. positives/no. samples	MRL [$\mu\text{g/kg}$]	CC β [$\mu\text{g/kg}$]	no. positives/no. samples
dapsone (DA)	prohibited	≤ 5	20/20	prohibited	≤ 5	20/20	prohibited	≤ 5	20/20	prohibited		
sulfacetamide (SA)	100 ^b	≤ 5	20/20	100 ^b	≤ 5	20/20	n.a. ^a	≤ 5	20/20	n.a.	≤ 25	20/20
sulfachloropyridazine (SCP)		≤ 5	20/20		≤ 5	20/20	n.a.	≤ 5	20/20	n.a.	≤ 25	20/20
sulfadiazine (SD)		≤ 5	19/20		≤ 5	20/20	n.a.	≤ 25	20/20	n.a.	≤ 25	20/20
sulfadimethoxine (SDM)		≤ 5	20/20		≤ 5	20/20	n.a.	≤ 5	20/20	n.a.	≤ 25	20/20
sulfadimidine (SDI)		≤ 5	20/20		≤ 5	20/20	n.a.	≤ 5	20/20	n.a.	≤ 25	20/20
sulfadoxine (SDX)		≤ 5	20/20		≤ 5	20/20	n.a.	≤ 5	20/20	n.a.	≤ 25	20/20
sulfamerazine (SM)		≤ 5	20/20		≤ 25	20/20	n.a.	≤ 5	20/20	n.a.	≤ 25	20/20
sulfamethoxazole (SMX)		≤ 5	20/20		≤ 5	20/20	n.a.	≤ 5	20/20	n.a.	≤ 25	20/20
sulfamethoxyipyridazine (SMP)		≤ 5	20/20		≤ 5	20/20	n.a.	≤ 5	20/20	n.a.	≤ 25	20/20
sulfanilamide (SN)		≤ 5	20/20		≤ 5	20/20	n.a.			n.a.		
sulfquinolone (SQ)		≤ 5	20/20		≤ 5	20/20	n.a.	≤ 5	20/20	n.a.	≤ 25	20/20
sulfathiazole (ST)		≤ 5	20/20		≤ 5	20/20	n.a.	≤ 5	20/20	n.a.	≤ 25	19/20
baquoloprim (BAQ)	n.a.	≤ 5	20/20	30	≤ 5	20/20	n.a.	≤ 5	20/20	n.a.	≤ 25	20/20
trimethoprim (TRI)	50	≤ 5	20/20	50	≤ 5	20/20	n.a.	≤ 5	20/20	n.a.	≤ 25	20/20
clindamycin (CLI)	n.a.	≤ 5	20/20	n.a.	≤ 5	20/20	n.a.	≤ 5	20/20	n.a.	≤ 25	20/20
lincomycin (LIN)	100	≤ 5	20/20	150	≤ 5	20/20	50	≤ 5	20/20	n.a.	≤ 25	20/20
tiamulin (TIA)	100	≤ 5	20/20	n.a.	≤ 5	20/20	1000	≤ 5	20/20	n.a.	≤ 25	20/20
valnemuline (VAL)	50	≤ 5	20/20	n.a.	≤ 5	20/20	n.a.	≤ 5	20/20	n.a.	≤ 25	20/20
clarithromycin (CLA)	n.a.	≤ 5	20/20	n.a.	≤ 5	20/20	n.a.	≤ 5	20/20	n.a.	≤ 25	20/20
erythromycin A (ERY)	200	≤ 5	20/20	40	≤ 5	20/20	150	≤ 5	20/20	n.a.	≤ 25	20/20
ganithromycin (GAM)	50	≤ 5	20/20	n.a.	≤ 5	20/20	n.a.	≤ 5	20/20	n.a.	≤ 25	20/20
josamycin (JOS)	n.a.	≤ 5	20/20	n.a.	≤ 5	20/20	n.a.	≤ 5	20/20	n.a.	≤ 25	20/20
neospiramycin (NEO)	200 ^c	≤ 5	20/20	200 ^c	≤ 5	20/20	n.a.	≤ 5	20/20	n.a.	≤ 25	20/20
spiramycin (SPI)		≤ 25	20/20		≤ 5	20/20	n.a.	≤ 5	20/20	n.a.	≤ 25	20/20
oleandomycin (OLE)	n.a.	≤ 5	20/20	n.a.	≤ 5	20/20	n.a.	≤ 5	20/20	n.a.	≤ 25	20/20
tilmicosin (TIL)	50	≤ 5	20/20	50	≤ 5	20/20	n.a.	≤ 5	20/20	n.a.	≤ 25	20/20
tulathromycin 2 (TUL)	300	≤ 5	20/20	n.a.	≤ 25	20/20	n.a.	≤ 25	20/20	n.a.	≤ 25	20/20
tylosin A (TYA)	100	≤ 5	19/20	50	≤ 5	20/20	200	≤ 5	20/20	n.a.	≤ 25	20/20
tylosin B (TYB)	n.a.	≤ 5	19/20	n.a.	≤ 5	20/20	n.a.	≤ 5	20/20	n.a.	≤ 25	20/20
cefalestin (CLX)	200	≤ 5	19/20	100	≤ 5	20/20	n.a.	≤ 5	20/20	n.a.	≤ 25	20/20
cefalonium (CL)	n.a.	≤ 5	20/20	100	≤ 5	20/20	n.a.	≤ 5	20/20	n.a.	≤ 25	20/20
cefazolin (CZ)	n.a.	≤ 5	20/20	50	≤ 5	20/20	n.a.	≤ 5	20/20	n.a.	≤ 25	20/20
cefoperazone (COP)	n.a.	≤ 5	20/20	50	≤ 5	20/20	n.a.	≤ 5	20/20	n.a.	≤ 25	20/20
cefquinome (CQ)	50	≤ 5	20/20	n.a.	≤ 25	20/20	n.a.	≤ 25	20/20	n.a.	≤ 25	20/20
ceftiofur (CF)	1000 ^d	≤ 5	19/20	100 ^d	≤ 5	20/20	n.a.	≤ 5	20/20	n.a.	≤ 25	20/20
cefapirin (CP)	50 ^c	≤ 5	19/20	60 ^c	≤ 5	20/20	n.a.	≤ 5	20/20	n.a.	≤ 25	20/20
desacetyl cefapirin (CPD)		≤ 5	19/20		≤ 5	20/20	n.a.	≤ 5	20/20	n.a.	≤ 25	20/20
amoxicillin (AMO)	50	≤ 5	19/20	4	≤ 5	20/20	n.a.	≤ 5	20/20	n.a.	≤ 25	20/20

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Table 2. continued

veterinary drug	pig muscle			cow milk			chicken egg			honey		
	MRL [μg/kg]	CCβ [μg/kg]	no. positives/no. samples	MRL [μg/kg]	CCβ [μg/kg]	no. positives/no. samples	MRL [μg/kg]	CCβ [μg/kg]	no. positives/no. samples	MRL [μg/kg]	CCβ [μg/kg]	no. positives/no. samples
ampicillin (AMP)	50		4				prohibited			n.a.		
cloxacillin (CLO)	300		30				prohibited			n.a.		
dicloxacillin (DIC)	300		30				prohibited			n.a.		
methicillin (MET)	n.a.		n.a.				n.a.			n.a.		
nafcillin (NAF)	300		30				n.a.			n.a.		
oxacillin (OXA)	300		30				prohibited			n.a.		
penicillin G (PENG)	n.a.		n.a.				n.a.			n.a.		
penicillin V (PENV)	n.a.		n.a.				n.a.			n.a.		
chloramphenicol (CAP)	prohibited			prohibited	≤25	20/20	prohibited	≤25	19/20	prohibited		
florfenicol (FF)	100 ^c	≤25		prohibited	≤5	20/20	prohibited	≤5	20/20	n.a.		
florfenicol amine (FFA)	50	≤25	20/20	50	≤5	20/20	prohibited	≤5	20/20	n.a.		
thiamphenicol (TP)	n.a.			n.a.	≤25	19/20	n.a.			n.a.		
carindazole (CNZ)	prohibited	≤5	20/20	prohibited	≤5	20/20	prohibited	≤5	20/20	n.a.		
dimetridazole (DMZ)	n.a.	≤5	20/20	prohibited	≤5	20/20	prohibited	≤5	20/20	n.a.		
hydroxydimetridazole (DMZOH)	n.a.	≤5	20/20	n.a.	≤5	20/20	n.a.			n.a.		
hydroxyipronidazole (IPZOH)	n.a.	≤5	20/20	n.a.	≤5	20/20	n.a.			n.a.		
hydroxymetronidazole (MZNOH)	n.a.	≤5	20/20	n.a.	≤5	20/20	n.a.			n.a.		
ipronidazole (IPZ)	n.a.	≤5	20/20	n.a.	≤5	20/20	n.a.			n.a.		
metronidazole (MNZ)	prohibited	≤5	20/20	prohibited	≤5	20/20	prohibited	≤5	20/20	prohibited		
ornidazole (ONZ)	n.a.	≤5	20/20	n.a.	≤5	20/20	n.a.			n.a.		
ronidazole (RNZ)	n.a.	≤5	19/20	n.a.	≤5	20/20	n.a.			n.a.		
secnidazole (SNZ)	n.a.	≤5	20/20	n.a.	≤5	20/20	n.a.			n.a.		
ternidazole (TENZ)	n.a.	≤5	20/20	n.a.	≤5	20/20	n.a.			n.a.		
tinidazole (TINZ)	n.a.	≤5	20/20	n.a.	≤5	20/20	n.a.			n.a.		
dexamethasone (DEXA)	0.75	≤5	19/20	0.3	≤5	20/20	n.a.			n.a.		
flumethasone (FLU)	n.a.	≤5	19/20	n.a.	≤5	20/20	n.a.			n.a.		
methylprednisolone (MPR)	10	≤25	20/20	2	≤5	20/20	n.a.			n.a.		
prednisolone (PRED)	4	≤5	20/20	6	≤5	20/20	n.a.			n.a.		
levamisole (LEV)	10	≤5	20/20	n.a.	≤5	20/20	n.a.			n.a.		
mebendazole (MEB)	60	≤5	20/20	prohibited	≤5	20/20	n.a.			n.a.		
clorsulone (CLS)	35			16			n.a.			n.a.		
avermectin B _{1a} (AB)	20	≤25	20/20	prohibited	≤5	20/20	n.a.			n.a.		
ivermectin B _{1a} (IV)	30			prohibited	≤25	20/20	n.a.			n.a.		
decoquinat (DECO)	n.a.			prohibited			n.a.			n.a.		
flunixin (FLUX)	10	≤5	20/20	40 ^e	≤5	20/20	n.a.			n.a.		
meloxicam (MEL)	20	≤5	20/20	15	≤5	20/20	n.a.			n.a.		
phenylbutazone (PHEN)	n.a.			n.a.			n.a.			n.a.		

^aSum of all sulfonamides. ^bSum of spiramycin and neospiramycin. ^cSum of all residues retaining the beta-lactam structure expressed as desfurylecefotiofur. ^dSum of cefapirin and desacyl cefapirin. ^eSum of florfenicol and its metabolites measured as florfenicol amine. ^f5-Hydroxyflunixin.

As the matrix load on the plate was very high, this approach was rejected. Attempts to reduce the matrix load by a defatting step with *n*-hexane or by freezing out overnight were not successful with any of the extracts. Without the addition of salts in a partitioning step, most VD_s could not be found. The cephalosporin cefoperazone and the penicillin nafcillin could not be recovered in any of the experiments. Decoquinat, clorsulone, phenylbutazone, avermectin B_{1a}, and ivermectin B_{1a} had such low ionization efficiencies and such high relative standard deviations (% RSD)⁵¹ that they were excluded from data evaluation. Based on these findings, the extraction parameters were further optimized to obtain the highest peak areas and lowest % RSDs. Initially, the mass ratio of the salt mixture was 4:1 (W/W), as in the original QuEChERS method,²¹ but on a smaller scale to reduce the consumption of solvents and standard solutions. It was observed that phase separation with only 1.0 g of Na₂SO₄ was not sufficient, especially for the raw cow milk extracts, which was then increased to 1.5 g. Four different salt mixtures for the partitioning step were investigated, that is, (I) 1.0 g MgSO₄ + 0.25 g NaOAc, (II) 1.5 g Na₂SO₄ + 0.25 g NaOAc, (III) 1.0 g MgSO₄ + 0.25 g NaCl, and (IV) 1.5 g Na₂SO₄ + 0.25 g NaCl. Note that the combination of HOAc for extraction and NaOAc for partitioning results in a buffer system with a pH of about 4.8.

For the chicken egg extracts, no phase separation (or first after a prolonged 10-min centrifugation) was observed using mixtures I and III, whereas phase separation was always sufficient using mixtures II and IV. Nevertheless, no significant differences in the peak area signals were observed for the different salt mixtures (Figure S4). It was decided to use mixture I for pig muscle and honey and mixture IV for raw cow milk and chicken eggs, which showed slightly higher peak area signals and precisions for the respective matrices. The peak area signals obtained from extraction with an end-over-end shaker, an ultrasonic bath, and a vortex mixer were compared. For all matrices, the highest peak areas were obtained using the end-over-end shaker and the lowest with the ultrasonic bath (Figure S5). Hence, the end-over-end shaker was chosen. For the extraction time, 15 min was chosen, as it led to the highest peak area signals for most VD_s in all matrices (Figure S6), except for muscle meat, for which no clear trend was evident (Figure S6A). Since VD_s are known to form complexes or covalent bonds with the sugars of the honey matrix, an acid hydrolysis step is often required.⁵² Hence, the effect of increasing the HOAc content from 1 to 5% in ACN was investigated. However, the peak area signals obtained from spiked pig muscle, raw cow milk, and chicken eggs were clearly reduced (Figure S7A–C). In contrast, the peak area signals were slightly increased for honey (Figure S7D), and it was decided to use 5% HOAc only for honey. Contrary to adding the salt mixture to the raw extracts,²¹ the raw extracts were centrifuged before partitioning, and the supernatants were decanted into 15 mL PP tubes containing the salt mixture. This enabled the use of low extraction volumes because the smaller tube diameter eased pipetting the final solution into autosampler vials. The first centrifugation of the raw extracts was carried out in the 50 mL PP tubes to remove solid sample parts and during decanting, to avoid blockage of the 15 mL PP tubes. This workflow was also crucial for recovery (Figure S8), as, without the first centrifugation, peak area signals were significantly smaller for all matrices, except for honey (Figure S8D), where sufficient phase separation was observed even without centrifugation. For the centrifugation time, 5 min at 3000 × *g* was found to be optimal, whereas 10 min led to a decrease in peak area signals for

all matrices, and even more so without the first centrifugation (Figure S8). After adding the salt mixture and vigorous shaking, the extracts were centrifuged again to induce phase separation. A second 10 min centrifugation (instead of 5 min) at 3000 × *g* led to higher peak area signals and precisions for most VD_s in pig muscle, raw cow milk, and honey (Figure S9). Only for chicken eggs, a 5 min centrifugation resulted in slightly higher peak area signals (Figure S9C). Nevertheless, to standardize the procedure for all matrices, the 10 min centrifugation was chosen.

The pig muscle and honey extracts proved to be more challenging using the final extraction method, yielding lower peak area signals for most VD_s than raw cow milk and chicken egg extracts. The peak area signals of florfenicol and the glucocorticoid group were about 1 to 2 orders of magnitude smaller than the peak areas of the other analytes. This was explained by their low ionization efficiencies, which were verified using VD standards. Thus, florfenicol could not be found in honey. For meloxicam, a positive matrix effect was observed, as peak area signals of standards were significantly smaller than those of spiked samples (Figure 4).

3.4. Quantitative Performance. In pesticide residue analysis of fruits and vegetables, good recoveries and precision were achieved after HTpSPE cleanup,²⁸ which led to the hypothesis that the developed method could be suitable for quantification despite the complex animal-derived food matrix. However, during the optimization of the extraction procedure, the poor precision of the method became obvious. The % RSDs were often above 50% for most VD_s. In preliminary tests, a two-fold front elution in the backward direction sharpened the VD zones (Figure 3E) and slightly improved all precisions. Thus, to assess the quantitative performance of the method in detail, pooled blank samples of each matrix were spiked with the 81-standard mixture at the calibration levels 0, 25, 50, 75, 100, and 200 µg/kg, extracted, and subjected to the cleanup by HTpSPE, but still, % RSDs mostly exceeded 30–40%. Instead of less expensive TLC silica gel 60 F₂₅₄ MS grade plates used so far, HPTLC silica gel 60 F₂₅₄ plates were assessed because it was assumed that HPTLC plates with smaller silica gel particles and narrower particle size distribution could improve the cleanup and thus precision. This was proven to be true to a small extent for pure standard solutions. However, the comparatively thinner HPTLC plate was overloaded with the extract solutions, hindering the separation of the VD_s from the matrix compounds; therefore, this approach was not suitable. Rhodamine B, which was sprayed on the extracts to serve as a fluorescent marker for the analyte position was considered to be an internal standard but did not improve the precision. Isotopically labeled standards were also considered possible internal standards and were added to the samples before extraction but did not bring improvement since only a few standards of each analyte group were available.

Dixon's *Q* test ($\alpha = 5\%$) revealed a high percentage of outliers. In most cases, one out of four replicates was identified as an outlier. Possible reasons for this could be errors occurring during extraction, especially when decanting the raw extract supernatants into the 15 mL tubes. In the HTpSPE study for pesticide residues,²⁸ the extracts were spiked postextraction, avoiding such errors. However, fluctuating ambient conditions could also affect front elution during HTpSPE even though the plate activity was previously adjusted in the ADC 2 because the humidity cannot be adjusted in the Twin-Trough Chamber itself. In addition, the matrix load originating from the animal-derived foods was higher and more challenging than that from

fruits and vegetables, comparing respective UV/FLD images.²⁸ Pure ACN and acetone were sufficient as the mobile phases for the separation of the pesticides from the plant-based matrices, whereas, for the VDs, two different complex solvent systems were necessary. A possible reason for this could be that VDs differ less in polarity and physicochemical properties from animal-derived matrix compounds than the more lipophilic pesticides that differ from polar plant matrices. Hence, after the cleanup, a high amount of matrix compounds coeluting with the VDs (indicated by the orange fluorescent rhodamine B) was evident, especially for muscle and honey (Figure 3F,G), whereas in pesticide residue analysis,²⁸ the “target analyte zone” was almost free from matrix interferences. The coeluting matrix might have influenced the front-elution behavior of the VDs in a nonreproducible way, which could have led to precision problems. Another HTpSPE study investigated pesticide residues in tea with good recoveries and precision.²⁹ Since tea is a much more challenging matrix than vegetables, prior dSPE cleanup was required to reduce the matrix load and successfully apply HTpSPE. The application of a dSPE step could also be a precision-improving option for animal-derived foods but carries the risk of loss of certain analytes or analyte groups. Furthermore, the positioning of the elution head of the autoTLC–LC–MS interface over each VD zone is automated after manually clicking on the respective zones in the image. This can lead to random deviations from the ideal elution position (Figure S10).³⁹

After removal of the outliers from the measurement series, precisions mostly below 10% were reached, but the method was considered not to be suited for quantification for the above-mentioned reasons. In addition, proper quantification would require the application and planar extraction of at least five standard solutions, matrix-matched or matrix-fortified standards, to establish a calibration curve on the same plate as the test samples. Together with three quality control (QC) samples, namely the system suitability QC sample, a compliant (blank) QC sample, and a noncompliant (spiked) QC sample, this would require 8 of the 24 tracks on the TLC plate for QC only, reducing the sample throughput to 16 samples per plate. Including the analysis of the 3 QC samples on each plate, the method was recommended for screening 21 samples in parallel.

3.5. Validation Results and Real Samples. For validation as a screening method according to Commission Implementing Regulation 2021/808¹² and the recommendations of the CRLs,⁴⁰ 20 blank samples per matrix (Table S2) were analyzed for the determination of threshold values on 4 different days by 2 different analysts. For the muscle tissue, eight samples were taken from pigs, and four samples each were taken from beef, chicken, and turkey. Honey was from various floral and geographical origins. Organic raw milk (natural fat content) and organic chicken egg samples were of different sorts and origins. All samples obtained from different suppliers were known to be free from VD residues or organically produced and stored over varying periods for testing robustness. Since quantification was no longer intended, only two front elutions were performed, discarding the second front elution in the reverse direction, reducing analysis time. No interfering matrix compounds or false positives were observed during blank sample analyses. The calculated threshold values were mostly zero, with few exceptions. The same 20 blank samples were spiked with the 81-standard mixture at 5 and 25 $\mu\text{g}/\text{kg}$ to determine selectivity and detection capability for screening ($CC\beta$), which means the smallest content of each VD that may be detected or quantified

in each sample with an error probability of β .¹² The validation level, at which the error probability is $\leq 5\%$, corresponding to 1 false negative sample out of 20 spiked samples, equals the $CC\beta$. The validation was successful, as for most analytes, $CC\beta$ was $\leq 5 \mu\text{g}/\text{kg}$ in muscle, milk, and eggs (Table 2), which is mostly far below the MRLs of the regulated compounds, except for the glucocorticoids, whose MRLs were not met and should therefore be further investigated. In contrast to the previous study,²⁵ $CC\beta$ was five times lower.

No mutual interferences between VDs were observed, proving selectivity. Sulfacetamide and sulfanilamide could not be confirmed in the samples at the chosen validation levels because they were not strongly retained by the RP-18e column and eluted at a retention time close to the column void volume; thus, their peak shapes were distorted (the same holds true for florfenicol amine); furthermore, they did not form fragments at the chosen conditions. For the β -lactam antibiotics, no $CC\beta$ values could be determined because they were not recovered as mentioned (Section 3.3). Clorsulone, ivermectin B_{1a}, decoquinat, and phenylbutazone were also problematic (Section 3.3). Honey proved to be a comparatively more challenging matrix: the achieved $CC\beta$ values were at the 5-fold higher 25 $\mu\text{g}/\text{kg}$ level for all VDs (Table 2) due to ion suppression effects. Also, amphenicols could not be recovered in the honey matrix but partly in the other three matrices. This might be because the method was originally developed for meat, milk, and eggs and not for matrices with very high sugar content. The strong blue fluorescence of the coeluting honey matrix compounds was obvious in the HTpSPE images at FLD 366 nm (Figure 3C–E). No MRLs are established for VD residues in honey, and thus, $CC\beta$ should be in the low $\mu\text{g}/\text{kg}$ range. With the current method, this could not be reached, and the front elution of the VDs from the polar honey matrix needs still to be investigated in more detail.

Nine samples per matrix (36 samples, Table S3) bought in local discounters were analyzed to evaluate the feasibility for routine testing. A standard solution at the 5 $\mu\text{g}/\text{kg}$ level was applied as the system suitability QC sample, followed by a blank QC sample as a negative control, the nine real samples, and a blank QC sample spiked at 5 $\mu\text{g}/\text{kg}$ as a positive control. With this plate layout including three QC samples, a total of 21 test samples of 1 matrix can be applied and purified on a single plate. The HTpSPE cleanup, including the application step, took 6.5 min per sample, which is five times faster (Table S4) than the standard SPE–LC–MS/MS methods for six samples in parallel used in Germany’s official control laboratories.^{41–43} The solvent consumption for the cleanup was 1.9 mL per sample, which is 13 times less than for SPE (Table S4). The total costs for all consumables were about 3.60 € per sample. This more ecofriendly and more sustainable method will increase the current screening capacities, thus improving food safety. In total, one sample can be prepared, extracted, purified, separated on the RP18e column, detected, and confirmed from start to finish in roughly 32 min with 11 min of manual interventions, mostly spent for the primary preparation of the sample. No VD residues exceeding 5 $\mu\text{g}/\text{kg}$ were found in the 36 real samples.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jafc.2c03925>.

Complete lists of standards and samples, additional HTPSPE images, and peak area comparisons of the extraction optimization experiments; for method operation, all relevant device files zipped as follows (PDF) HTPSPE visionCATS method file, command file and sequence file for operation of the autoTLC–LC–MS interface, Xcalibur method file and sequence file for the HRMS measurement as well as in-house built Trace-Finder compound database file (ZIP)

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Author Contributions

A.M. developed the methodology, designed experiments, carried out validation studies, analyzed data, and wrote the manuscript. L.H. performed experiments and analyzed data. M.B. performed experiments and validation studies. G.E.M. conceptualized the project, developed the methodology, supervised work, obtained resources, and reviewed and edited the manuscript.

Notes

The authors declare no competing financial interest.

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DEDICATION

Dedicated to the 70th birthday of Prof. Dr. Wolfgang Schwack, University of Hohenheim, Germany.

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Supporting Information

**Validated Screening Method for 81 Multi-Class Veterinary Drug Residues
in Food via Online-Coupling High-Throughput Planar Solid-Phase Extraction to
High-Performance Liquid Chromatography–Orbitrap Tandem Mass Spectrometry**

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Dedicated to the 70th birthday of Prof. Dr. Wolfgang Schwack, University of Hohenheim, Germany

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File S6. TraceFinder compound database.

Table S1 Complete list of VD standards and solutions prepared.

therapeutic class	structural class	VD standard	manufacturer	purity [%]	concentration [mg/mL]	solvent* (V/V)
antibiotic	sulfonamide (S)	dapsone (DA)	Sigma-Aldrich	97.2	1	acetonitrile
		sulfacetamide (SA)	Sigma-Aldrich	99.9		
		sulfachloropyridazine (SCP)	Sigma-Aldrich	99.7		
		sulfaclozine (SC)	Chemos ^a	99.8		
		sulfadiazine (SD)	Sigma-Aldrich	99.8		
		sulfadimethoxine (SDM)	Sigma-Aldrich	99.9		
		sulfadimidine (SDI)	Sigma-Aldrich	99.8		
		sulfadoxine (SDX)	Sigma-Aldrich	98.9		
		sulfamerazine (SM)	Sigma-Aldrich	99.7		
		sulfamethoxazole (SMX)	Sigma-Aldrich	99.9		
		sulfamethoxypyridazine (SMP)	Sigma-Aldrich	99.8		
		sulfanilamide (SN)	Sigma-Aldrich	99.9		
		sulfaquinoxaline (SQ)	Sigma-Aldrich	97.8		dimethylformamide
		sulfathiazole (ST)	Sigma-Aldrich	99.9		acetonitrile
	diaminopyrimidine (D)	baquiloprim (BAQ)	Witega ^b	99.89	1	methanol
		trimethoprim (TRI)	Sigma-Aldrich	99.7		
	lincosamide (L)	clindamycin (CLI)	Sigma-Aldrich	99	1	methanol
		lincomycin (LIN)	Sigma-Aldrich	n. a.		
	pleuromutilin (P)	tiamulin (TIA)	Sigma-Aldrich	99.9	1	methanol
		valnemulin (VAL)	Sigma-Aldrich	96.2		
	macrolide (M)	clarithromycin (CLA)	Sigma-Aldrich	98.4	1	methanol
		erythromycin A (ERY)	Sigma-Aldrich	93		
		gamithromycin (GAM)	Sigma-Aldrich	93.2		
		josamycin (JOS)	Sigma-Aldrich	n. a.		
		neospiramycin (NEO)	TRC ^c	96		
		oleandomycin (OLE)	SantaCruz ^d	98		
		spiramycin (SPI)	Sigma-Aldrich	87.7		
		tilmicosin (TIL)	Sigma-Aldrich	94		
		tulathromycin 2 (TUL)	SantaCruz ^d	99		
		tylosin A (TYA)	Sigma-Aldrich	89.7		
		tylosin B (TYB)	TRC ^c	95		
	cephalosporin (C)	cefalexin (CLX)	Dr. Ehrenstorfer ^e	99	0.1	acetonitrile/water 3:1 (V/V)
		cefalonium (CL)	Sigma-Aldrich	99.4		
		cefapirin (CP)	Sigma-Aldrich	98		
		cefazolin (CZ)	Sigma-Aldrich	97.4		
		cefoperazone (COP)	Sigma-Aldrich	98.2		
		cefquinome (CQ)	Sigma-Aldrich	98.3		
		ceftiofur (CF)	Sigma-Aldrich	97.4		
		desacetyl-cefapirin (CPD)	TRC ^c	95		

Table S1 continued.

therapeutic class	structural class	VD standard	manufacturer	purity [%]	concentration [mg/mL]	solvent (V/V)
antibiotic	penicillin (P)	amoxicillin (AMO)	Sigma-Aldrich	98.7	1	water
		ampicillin (AMP)	Sigma-Aldrich	98.8		
		cloxacillin (CLO)	Sigma-Aldrich	96		
		dicloxacillin (DIC)	Sigma-Aldrich	99		
		methicillin (MET)	Sigma-Aldrich	97.1		
		nafticillin (NAF)	Sigma-Aldrich	98.7		
		oxacillin (OXA)	Sigma-Aldrich	99.2		
		penicillin G (PENG)	Dr. Ehrenstorfer ^c	99.8		
		penicillin V (PENV)	Sigma-Aldrich	98.7		
	amphenicol (A)	chloramphenicol (CAP)	Sigma-Aldrich	100	1	methanol
		florfenicol (FF)	Sigma-Aldrich	98		
		florfenicolamine (FFA)	Sigma-Aldrich	99.3		
		thiamphenicol (TP)	Sigma-Aldrich	>99.9		
	nitroimidazole (N)	carnidazole (CNZ)	Witega ^b	99.4	0.1	methanol
		dimetridazole (DMZ)	Sigma-Aldrich	n. a. ³		
		hydroxydimetridazole (DMZOH)	Witega ^b	99.9		
		hydroxyipronidazole (IPZOH)	Witega ^b	99.7		
		hydroxymetronidazole (MZNOH)	Witega ^b	99.37		
		ipronidazole (IPZ)	Witega ^b	99.4		
		metronidazole (MNZ)	Sigma-Aldrich	100		
		ornidazole (ONZ)	Sigma-Aldrich	>99		
		ronidazole (RNZ)	Sigma-Aldrich	>99		
		secnidazole (SNZ)	Witega ^b	99.8		
		ternidazole (TENZ)	Witega ^b	99.8		
		tinidazole (TINZ)	Sigma-Aldrich	99.7		
anti-inflammatory	glucocorticoid (G)	dexamethasone (DEX)	Sigma-Aldrich	99.6	1	methanol
		flumethasone (FLU)	Sigma-Aldrich	99.6		
		methylprednisolone (MPR)	Sigma-Aldrich	98.3		
		prednisolone (PRED)	Sigma-Aldrich	99		
anthelmintic	salicylate (SA)	oxyclozanide (OXY)	Sigma-Aldrich	99.6	1	methanol
		rafoxanide (RAF)	Sigma-Aldrich	99.53		
	imidazothiazole (I)	levamisole (LEV)	Sigma-Aldrich	99.9	1	dimethylformamide
	benzimidazole (B)	mebendazole (MEB)	Sigma-Aldrich	99.7	1	dimethylformamide
	benzolsulfonamide (BS)	clorsulon (CLS)	Sigma-Aldrich	99.5	1	methanol
antiparasitic	avermectin (A)	avermectin B _{1a} (AB)	Sigma-Aldrich	98.7	1	methanol
		ivermectin B _{1a} (IV)	Sigma-Aldrich	96		
coccidiostat	quinolone (Q)	decoquinat (DECO)	Sigma-Aldrich	99.2	1	acetonitrile/formic acid 9:1 (V/V)
non-steroidal anti-inflammatory drug	arylpropionic acid (AA)	carprofen (CAR)	Sigma-Aldrich	99.7	1	acetonitrile/methanol 9:1 (V/V)
		ibuprofen (IBU)	Sigma-Aldrich	100		
	fenamate (F)	flunixin (FLUX)	Sigma-Aldrich	99.8	1	acetonitrile/methanol 9:1 (V/V)
	oxicam (O)	meloxicam (MEL)	Sigma-Aldrich	99.9	1	acetonitrile/methanol 9:1 (V/V)
	pyrazolidindione (P)	phenylbutazone (PHEN)	Sigma-Aldrich	99.9	1	acetonitrile/methanol 9:1 (V/V)

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^eDr. Ehrenstorfer, Augsburg, Germany

Table S2. Complete list of validation samples.

no.	muscle meat				raw cow milk			chicken egg			honey			
	type	label	German supplier	date of purchase	label	German producer	Date of sampling	label	German producer	date of laying	type	label	German producer	best before
1	chicken breast	organic (Bioland)	Tegut I, Giessen	05-27-20	organic (Naturland)	Burkhard Czyscholl, Driedorf-Waldaubach	05-07-21	-	private chicken farmer	04-16-20	honey creamy (mixture)	Organic (EU)	Honigland, Stuttgart	07-22
2	chicken breast	organic (Bioland)	Tegut I, Giessen	05-27-20	organic (Naturland)	Burkhard Czyscholl, Driedorf-Waldaubach	05-07-21	-	private chicken farmer	04-12-20	honey fluid (mixture)	Organic (EU)	Honigland, Stuttgart	08-22
3	chicken breast	organic (Bioland)	Tegut I, Giessen	05-27-20	organic (Naturland)	Burkhard Czyscholl, Driedorf-Waldaubach	05-07-21	-	private chicken farmer	04-15-20	rape honey	Organic (EU)	Imkerei Rüdiger Feldt, Eichede	03-23
4	chicken breast	organic (Bioland)	Tegut I, Giessen	05-27-20	organic (EU)	Burkhard Neuß, Grünberg	16-05-20	-	private chicken farmer	04-26-20	summer honeysuckle (turbid)	Organic (EU)	Imkerei Caroline Quednau, Frankenthal	04-22
5	turkey cutlet	organic (EU)	Tegut I, Giessen	06-03-20	organic (EU)	Burkhard Neuß, Grünberg	16-05-20	-	private chicken farmer	04-26-20	acacia honey	Organic (EU)	Dreyer-Bienenhonig, Uelzen	03-23
6	turkey breast	organic (Naturland)	Tegut I, Giessen	06-03-20	organic (EU)	Burkhard Neuß, Grünberg	16-05-20	-	private chicken farmer	04-27-20	avocado honey, Mexico	-	Imkerei Rüdiger Feldt, Eichede	unknown
7	turkey breast	organic (Naturland)	Tegut I, Giessen	06-03-20	organic (EU)	Burkhard Neuß, Grünberg	16-05-20	-	private chicken farmer	11-20-20	ulmo honey, Chile	-	Imkerei Rüdiger Feldt, Eichede	unknown
8	turkey breast	organic (EU)	Klatschmohn Naturkost GmbH, Giessen	06-03-20	organic (EU)	Burkhard Neuß, Grünberg	16-05-20	-	private chicken farmer	11-20-20	acacia honey, Hungary	-	Imkerei Rüdiger Feldt, Eichede	unknown
9	beef leg slice	organic (EU)	Tegut I, Giessen	05-27-20	organic (EU)	Burkhard Neuß, Grünberg	16-05-20	organic (Demeter)	Ernst Geißler, Lollar	06-24-21	chestnut honey, Italy	-	Imkerei Rüdiger Feldt, Eichede	unknown
10	roast beef	organic (EU)	Tegut I, Giessen	05-27-20	organic (EU)	Burkhard Neuß, Grünberg	16-05-20	organic (Demeter)	Ernst Geißler, Lollar	06-24-21	clover honey, New Zealand	-	Imkerei Rüdiger Feldt, Eichede	unknown
11	beef tenderloin	organic (EU)	Tegut I, Giessen	06-03-20	organic (EU)	Burkhard Neuß, Grünberg	16-05-20	organic (EU)	Denree, Töpen	07-10-21	pine honey, Turkey	-	Imkerei Rüdiger Feldt, Eichede	unknown
12	ground beef	organic (EU)	Klatschmohn Naturkost GmbH, Giessen	06-03-20	organic (EU)	Burkhard Neuß, Grünberg	16-05-20	organic (EU)	Denree, Töpen	07-10-21	heath honey, Norway	-	Imkerei Rüdiger Feldt, Eichede	unknown
13	pork loin	organic (EU)	Tegut II, Giessen	06-22-21	organic (EU)	Burkhard Neuß, Grünberg	16-05-20	organic (EU)	Eierhof Hennes, Töpen	07-06-21	buckwheat, Europe	-	Imkerei Rüdiger Feldt, Eichede	unknown
14	pork loin	organic (EU)	Metzgerei Reuther, Brettscheid	06-23-21	organic (EU)	Burkhard Neuß, Grünberg	16-05-20	organic (EU)	Eierhof Hennes, Töpen	07-06-21	pampas honey, Argentina	-	Imkerei Rüdiger Feldt, Eichede	unknown
15	pork tenderloin	organic (EU)	EDEKA, Herborn	06-22-21	organic (EU)	Burkhard Neuß, Grünberg	16-05-20	organic (EU)	Eierhof Hennes, Töpen	07-06-21	quillaya honey, Chile	-	Imkerei Rüdiger Feldt, Eichede	unknown
16	pork neck steak	organic (EU)	EDEKA, Herborn	06-22-21	organic (EU)	Burkhard Neuß, Grünberg	16-05-20	organic (EU)	Eierhof Hennes, Töpen	07-06-21	calluna heath honey, France	-	Imkerei Rüdiger Feldt, Eichede	unknown
17	pork steak	organic (EU)	REWE, Giessen	07-07-21	organic (EU)	Burkhard Neuß, Grünberg	16-05-20	organic (EU)	Eierhof Hennes, Töpen	07-13-21	lavender honey, France	-	Imkerei Rüdiger Feldt, Eichede	unknown
18	ground pork	organic (EU)	REWE, Giessen	07-07-21	organic (EU)	Burkhard Neuß, Grünberg	16-05-20	organic (EU)	Eierhof Hennes, Töpen	07-13-21	oak forest honey, Spain	-	Imkerei Rüdiger Feldt, Eichede	unknown
19	pork ham cutlets	organic (EU)	Dennis BioMarkt, Giessen	07-07-21	organic (EU)	Burkhard Neuß, Grünberg	16-05-20	organic (EU)	Eierhof Hennes, Töpen	07-13-21	eucalyptus honey, Argentina	-	Imkerei Rüdiger Feldt, Eichede	unknown
20	pork rump steak	organic (EU)	Tegut II, Gießen	07-07-21	Organic (EU)	Burkhard Neuß, Grünberg	16-05-20	organic (EU)	Eierhof Hennes, Töpen	07-13-21	honey creamy (mixture)	Organic (EU)	Dr. W. Krieger's Bienenhonig, Magdeburg	07-21

Table S3. Complete list of test samples.

no.	type	muscle meat producer	German vendor	type	cow milk producer	German vendor	type	chicken egg producer	German vendor	type	honey producer	German vendor
1	ground pork	Real Meistermetzger	Real, Wetzlar	Weihenstephan fresh milk 1.5% fat	Molkerei Weihenstephan, Freising	Real, Wetzlar	Beste Wahl free-range egg	Columbus Frischei, Schöppingen	REWE, Giesen	Bihophar acacia honey with spring honeysuckle	Fürsten-Reform, Braunschweig	Real, Wetzlar
2	ground beef	Real Meistermetzger	Real, Wetzlar	Schwarzwaldmilch fresh meadow-grazed milk 3.6% fat	Schwarzwaldmilch, Freiburg	Real, Wetzlar	Landfreude barn egg	Moos-Butzen, Viersen	Aldi Süd I, Wetzlar	Bihophar mountain flower honey	Fürsten-Reform, Braunschweig	Real, Wetzlar
3	beef goulash	Real Meistermetzger	Real, Wetzlar	TIP fresh whole milk, 3.5% fat	Zentrale Handelsgesellschaft, Offenburg	Real, Wetzlar	Landfreude free-range egg	Moos-Butzen, Viersen	Aldi Süd II, Wetzlar	Bihophar forest honey	Fürsten-Reform, Braunschweig	Real, Wetzlar
4	pork goulash	Real Meistermetzger	Real, Wetzlar	Milbona fresh low-fat milk, 1.5% fat	Privatmolkerei Bechtel, Schwarzenfeld	Lidl, Wetzlar	Landfreude free-range egg	Moos-Butzen, Viersen	Aldi Süd I, Wetzlar	Breitsamer forest honey	Breitsamer Honig, Munich	Real, Wetzlar
5	gourmet naturel young bull rump steak	Fa. Huber Tirol, St. Johann, Austria	Real, Wetzlar	Milbona durable whole milk, 3.5% fat	Privatmolkerei Bechtel, Schwarzenfeld	Lidl, Wetzlar	Henne & Hahn barn egg	Ovopack, Grammetal	Aldi Süd I, Wetzlar	Breitsamer sunflower honey	Breitsamer Honig, Munich	Real, Wetzlar
6	Real pork roulades	Real, Mönchengladbach	Real, Wetzlar	Milksani fresh low-fat milk 1.5% fat	Hochwald Foods, Hünfeld	Aldi Süd I, Wetzlar	Ein Herz für Küken barn egg	Eierhof Hennes, Töpen	Lidl, Wetzlar	Breitsamer summer blossom honey	Breitsamer Honig, Munich	Real, Wetzlar
7	Meine Metzgerei chicken cutlet	Pluken GmbH, Gudensberg	Aldi Süd I, Wetzlar	Fair & Gut meadow-grazed milk, 3.5% fat	NordseeMilch, Witzwort	Aldi Süd I, Wetzlar	Ein Herz für Küken free-range egg	Kwetters Eierhof, Hötensleben	Lidl, Wetzlar	Summer blossom honey	Imkerei Uhlmann, Callenberg	Imkerei Uhlmann, Callenberg
8	Landjunker chicken cutlet	Landgeflügel FG Vertriebsgesellschaft, Haren	Lidl, Wetzlar	Milksani durable whole milk, 3.5% fat	Gmundner Molkerei, Gmunden, Austria	Aldi Süd I, Wetzlar	KAT barn egg	Fa. Goertz, Niederkrüchten	Real, Wetzlar	Spring blossom honey with rape	Imkerei Uhlmann, Callenberg	Imkerei Uhlmann, Callenberg
9	Landjunker turkey cutlet	Frischland Premium Spezialitäten, Bogen	Lidl, Wetzlar	Milksani lactose-free durable low-fat milk, 1.5% fat	AF Deutschland, Düsseldorf	Aldi Süd I, Wetzlar	Landfreude free-range egg	Moos-Butzen, Viersen	Aldi Süd I, Wetzlar	Goldland flower honey	Honigland, Stuttgart	Aldi Süd I, Wetzlar

Table S4. Comparison of time and solvent consumption: HTPSPE–UV–Vis/FLD–LC–HRMS/MS method (new clean-up consumed **13 times less solvent** and was **5 times faster**) versus routine SPE–LC–MS/MS methods.

HTPSPE–UV/Vis/FLD–LC–HRMS/MS for 21 samples in parallel				SPE–LC–MS/MS [45–47] estimated for 6 samples in parallel			
analysis step	time [min] 21 samples	time [min] per sample	solvent [mL] per sample	analysis step	time [min] 6 samples	time [min] per sample	solvent [mL] per sample
extraction							
primary preparation	120	5.7	-	primary preparation	34	5.7	-
weighing	26	1.2	-	weighing	7	1.2	-
extraction	30	1.4	5	extraction	8–32	1.3–5.3	10.1–17.1
1 st centrifugation	5	0.2	-	centrifugation	10–30	1.6–5.0	-
salting-out	20	1.0	-	filtration of supernatant	15	2.5	-
2 nd centrifugation	10	0.5	-				
clean up							
transfer to vials	15	0.5	-	SPE and solvent evaporation	180	30.0	24.0
application and HTPSPE	135	6.5	1.9	reconstitution and transfer to vials	12	2.0	1.0–2.0
data recording							
autoTLC–LC–HRMS/MS	285	13.6	8.4	LC–MS/MS	162	27.0	7.8
data evaluation	25	1.2	-	data evaluation	7	1.2	-
total	671	32	15	total	435–479	73–80	43–51



Fig. S1. Acquisition method with full scan and consecutive vDIA scans including *m/z* windows (inclusion list entry: IL).

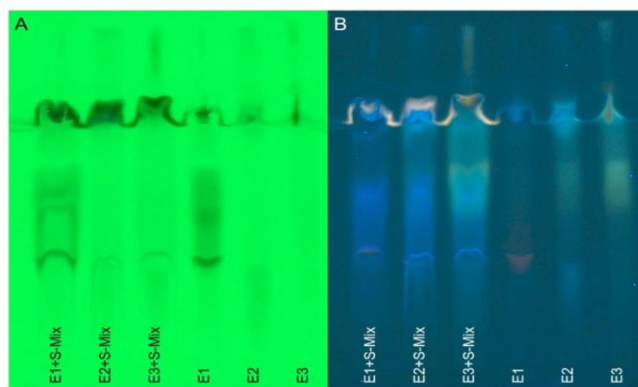


Fig. S2. HTPSPE images at UV 254 nm (A) and FLD 366 nm (B) of the blank extracts E1 (pig muscle), E2 (cow milk), E3 (chicken egg), and overspray of a sulfonamide standard mixture (S-Mix) after a first front-elution with ACN/ethanol/ammonia 6:2:2 (V/V/V) until 80 mm, followed by a second front-elution in the reverse direction with ACN/methanol/ammonia/water 5:2:2:1 (V/V/V/V) until 38 mm on MS grade TLC silica gel 60 F₂₅₄. The high polarity of the second mobile phase led to distorted bands.

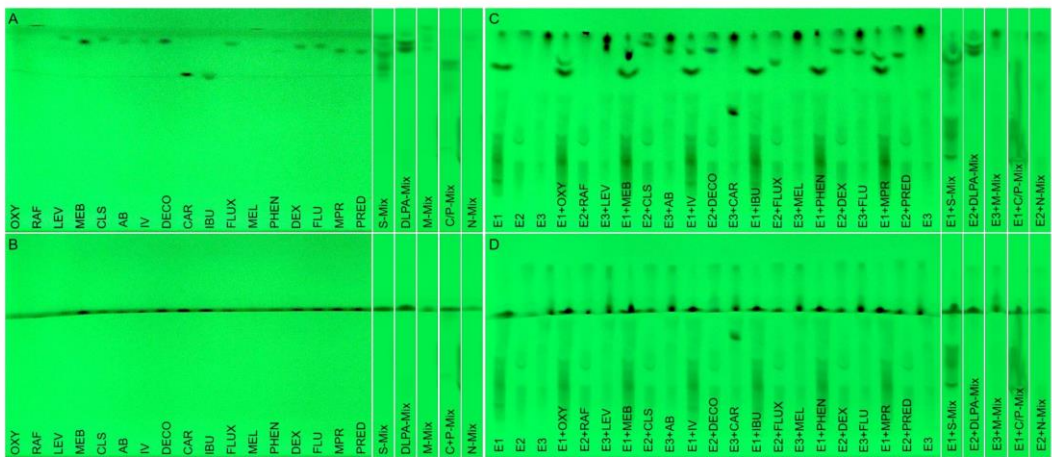


Fig. S3. HTpSPE images at UV 254 nm of VD standards (abbreviated as in Table S1) without (A/B) and with overspray on the extracts E1 (pig muscle), E2 (raw cow milk), E3 (chicken egg) (C/D) on MS grade TLC silica gel 60 F₂₅₄; after a single front-elution (A/C) with ACN/ammonia/ethanol 6:2:2 (V/V/V) until 85 mm, after a second front-elution (B/D) in the reverse direction until 45 mm with methanol/ACN/ammonia/ethanol 14:3:2:1 (V/V/V/V).

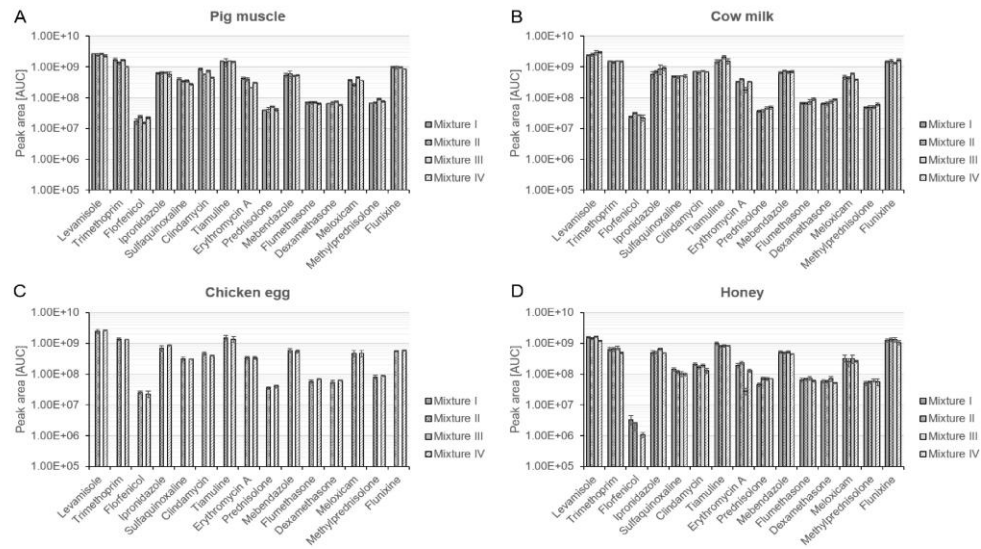


Fig. S4. Comparison of the peak areas ($n = 3$, error bars = standard deviation) of representative standards obtained from the analysis of fortified extracts (50 $\mu\text{g/kg}$) of pig muscle (A), cow milk (B), chicken egg (C), and honey (D). Different salt mixtures were used for the partitioning step after extraction: 1 g $\text{MgSO}_4 + 0.25$ g NaOAc (mixture I), 1.5 g $\text{Na}_2\text{SO}_4 + 0.25$ g NaOAc (mixture II), 1 g $\text{MgSO}_4 + 0.25$ g NaCl (mixture III), and 1.5 g $\text{Na}_2\text{SO}_4 + 0.25$ g NaCl (mixture IV).

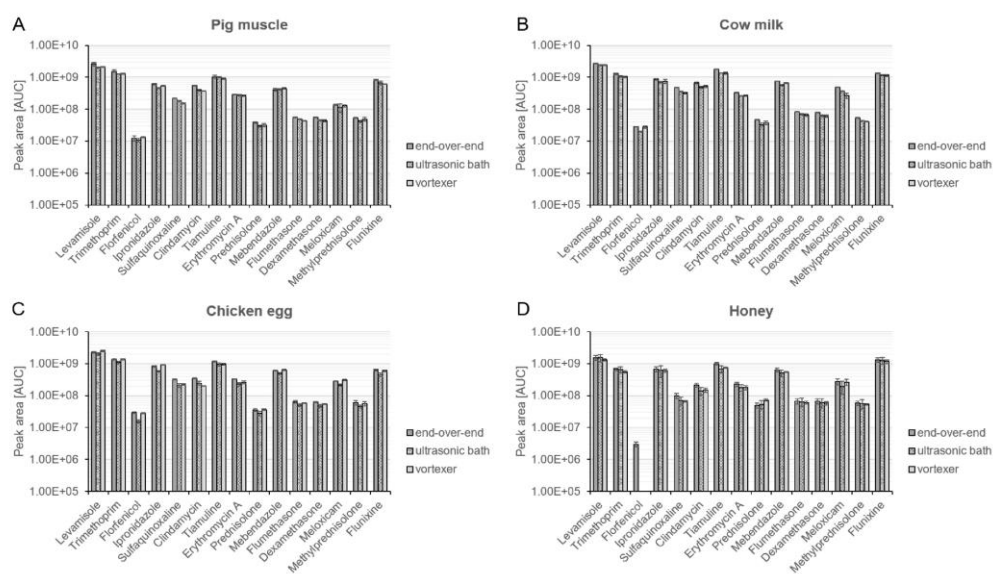


Fig. S5. Comparison of the peak areas ($n = 3$, error bars = standard deviation) of representative standards obtained from the analysis of fortified extracts (50 $\mu\text{g/kg}$) of pig muscle (A), cow milk (B), chicken egg (C), and honey (D), extracted using either an end-over-end shaker or an ultrasonic bath or a vortex mixer.

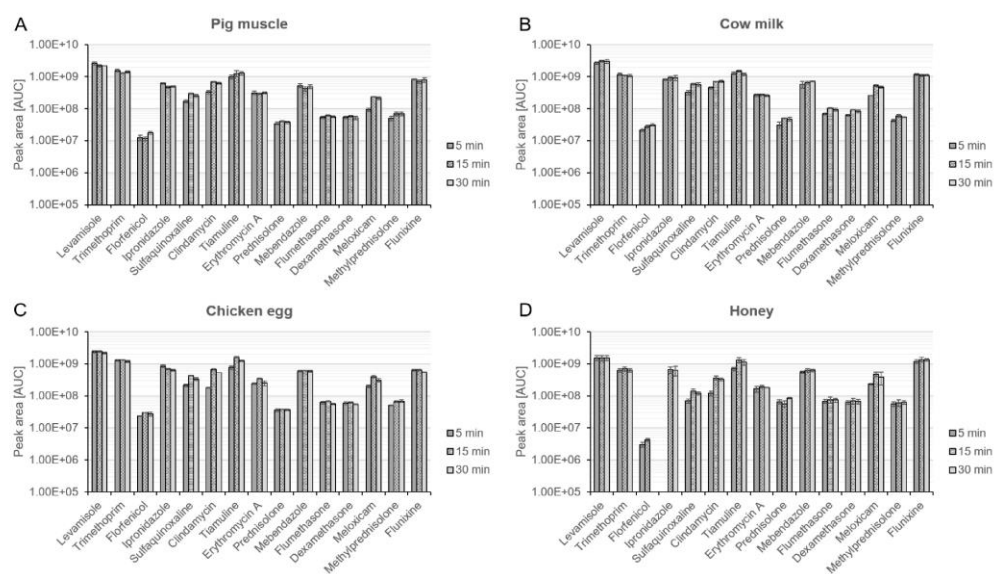


Fig. S6. Comparison of the peak areas ($n = 3$, error bars = standard deviation) of representative standards obtained from the analysis of fortified extracts (50 $\mu\text{g/kg}$) of pig muscle (A), cow milk (B), chicken egg (C), and honey (D), extracted for 5, 15 and 30 min using an end-over-end shaker.

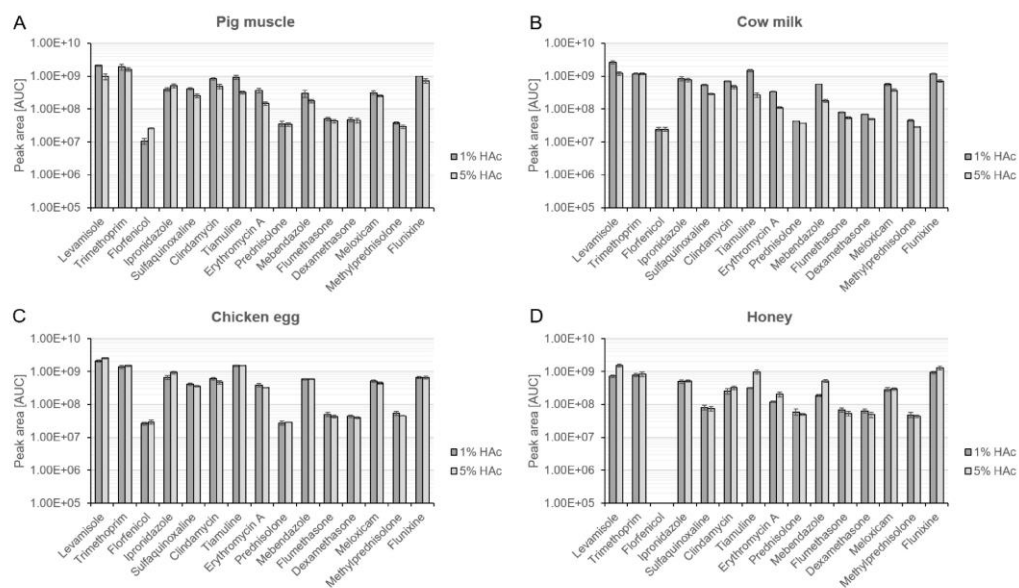


Fig. S7. Comparison of the peak areas ($n = 3$, error bars = standard deviation) of representative standards obtained from the analysis of fortified extracts (50 $\mu\text{g/kg}$) of pig muscle (A), cow milk (B), chicken egg (C), and honey (D), extracted with 1% and 5% HOAc in ACN.

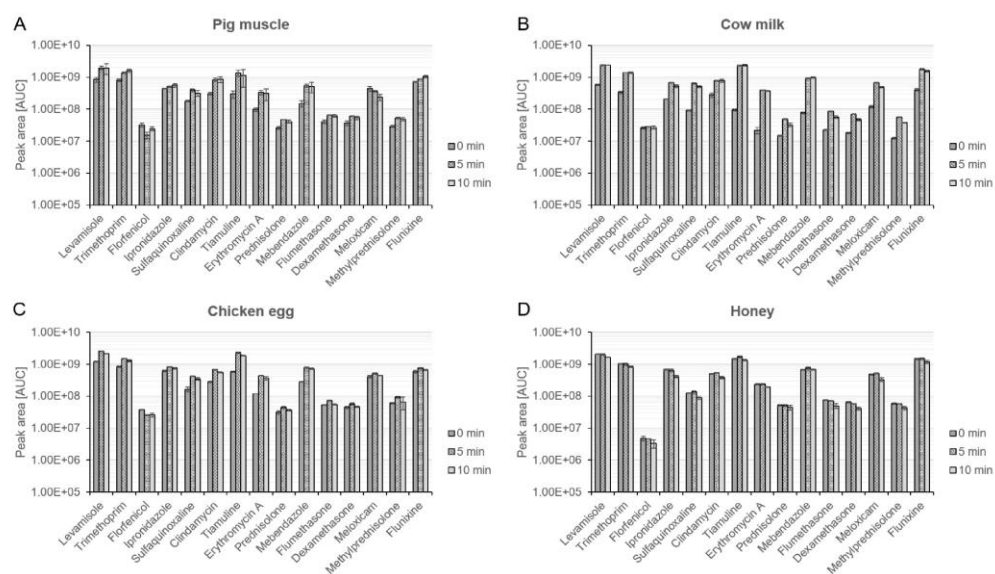


Fig. S8. Comparison of the peak areas ($n = 3$, error bars = standard deviation) of representative standards obtained from the analysis of fortified extracts (50 $\mu\text{g/kg}$) of pig muscle (**A**), cow milk (**B**), chicken egg (**C**), and honey (**D**), extracted using a first centrifugation time of 0, 5 and 10 min.

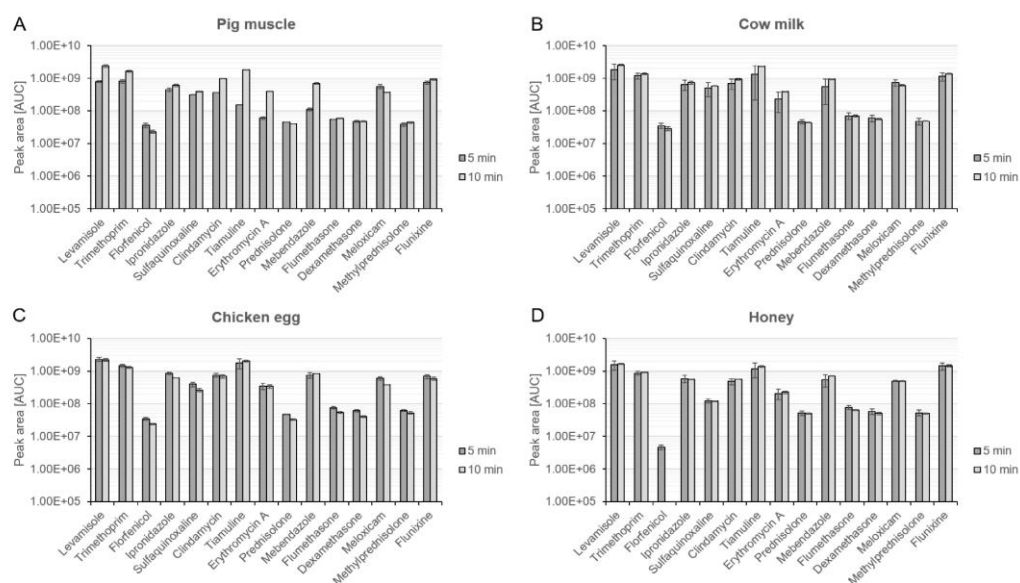


Fig. 59. Comparison of the peak areas ($n = 3$, error bars = standard deviation) of representative standards obtained from the analysis of fortified extracts ($50 \mu\text{g/kg}$) of pig muscle (A), cow milk (B), chicken egg (C), and honey (D), extracted using a second centrifugation time of 5 and 10 min.

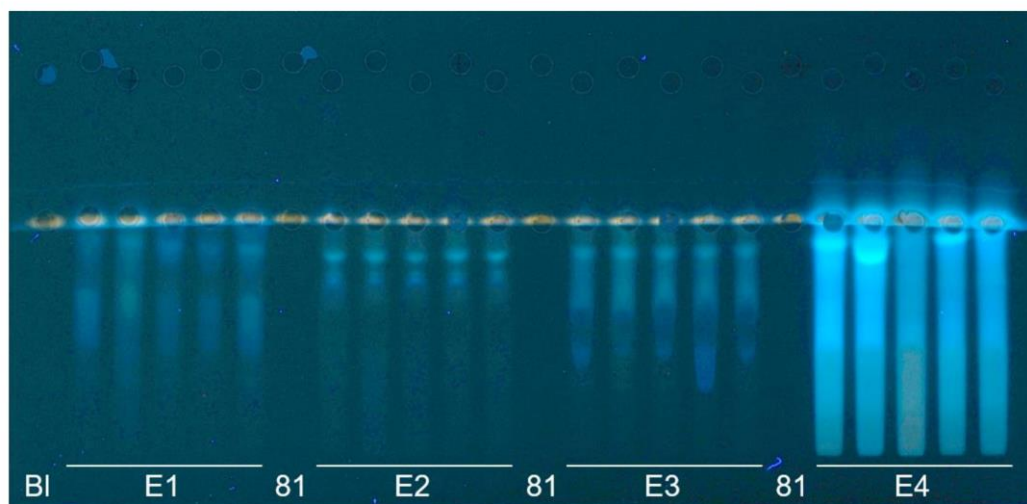


Fig. S10. Typical HTpSPE image of the validation studies at FLD 366 nm after two-fold front-elution and subsequent autoTLC–LC–HRMS/MS measurement on MS grade TLC silica gel F₂₅₄. Random deviations from the ideal elution position are evident. E1: pig muscle, E2: raw cow milk, E3: chicken eggs, E4: honey, BI: reagent blank, 81: 81-standard mixture (5 µg/kg), orange band: marker rhodamine B.

File S1. visionCATS analysis file for HTPSPE of three QC samples and 21 test samples.

File S2. G-code command file for the autoTLC–LC–MS interface [39].

File S3. Typical autoTLC–LC–MS sequence file created by clicking on VD zones in the HTPSPE image [39].

File S4. Xcalibur instrument method file for autoTLC–LC–HRMS/MS measurement.

File S5. Xcalibur sequence file for the autoTLC–LC–HRMS/MS measurement for three QC samples and 21 test samples (analogously to File S3).

File S6. TraceFinder compound database.

8. Publication IV

Potential of simple, rapid, and non-target planar bioassay screening of veterinary drug residues

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ABSTRACT

Veterinary drug residues in food samples of animal origin are currently analyzed by target analysis using high-performance liquid chromatography combined with sophisticated mass spectrometers. Since the results are only partially consistent with the microbiological results and positive findings occur rarely (in the per mil range in Germany), the potential of a simple planar bioassay screening was studied in the field of veterinary drug residue analysis. Using only a simple dilution of the milk for sample preparation, it was challenging to meet the maximum residue limits for antibiotic drug residues, exemplarily shown for the screening of two fluoroquinolones. However, the potential was evident for a simple, rapid, eco-friendly, and non-target screening without expensive instrumentation. Regardless of whether it is an active metabolite, contaminant, degradation product, or veterinary drug residue, the effect indicated on the planar surface due to bioassay detection will most likely also affect the human microbiome when consumed. The non-target screening of the milk samples revealed compounds with substantial antibacterial effects, which were not in the previous focus of interest. These antibacterial compounds will most likely also affect the human microbiome. Is it only the regulated antibiotic residues or generally all antibiotic compounds in a sample that count for consumer protection? The current prevailing understanding of food safety and antimicrobial resistance, based on the results of target (rather than effect) analyses, is being challenged. Non-target planar bioassay screening has been shown to fill a current gap by providing an understanding of inconsistencies and complementing routine target analysis of veterinary drug residues. As a highlight, it provides the full picture of the real levels of active compounds, regardless of the permitted limits of antibiotics.

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1. Introduction

Approximately 160,000 tons of antibiotics were fed to livestock in 2020, with the global market valued at 40.7 billion USD [1,2]. Among the various antibiotic groups, fluoroquinolone antibiotics are among the last-choice agents for humans [3], and their market value is expected to increase in this decade [2]. As long as antibiotics are used extensively for therapeutic purposes in human medicine and especially in veterinary medicine, antibiotic resistance infections can be expected to increase due to misuse or overuse of antibiotics in the global food chain [4]. Concerning dairy animals, maximum residue limits (MRL) for antibiotic drug residues in milk have been established in many countries to mitigate risks [5–7]. Reducing antibiotic residues is particularly impor-

tant for milk (as a basic food source including its versatile products) not only in terms of potential risks to human health and the human microbiome but also to the environment [8]. In addition, the antibiotic residues present can affect milk processing and interfere with fermentation [9].

Biological screening tests that provide a sum parameter for antibiotic activity present (e.g., in Petri dish or microtiter plate) have been increasingly replaced by analyses using high-performance liquid chromatography (HPLC) combined with powerful mass spectrometers (MS) with triple-quadrupole (MS/MS), time-of-flight, or orbitrap analyzers [10–13]. However, a non-negligible number (30%, private communication with EU reference laboratory Anses Laboratoire de Fougères, Javené, France, www.anses.fr) of positive screening results in the bioassay remained with no confirmation by HPLC–MS. This inconsistency is supported by a proficiency test which demonstrated that 73% of the microbial, 50% of the biochemical, and 22% of the instrumental methods led to false negative results [11]. The combination of planar chromatography [14,15]

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with bioassay detection could be useful to understand such inconsistencies [16] and to complement food safety currently based on target analysis results [17].

The benefit is that high-performance thin-layer chromatography hyphenated with multi-imaging and non-targeted effect-directed detection (HPTLC–UV/Vis/FLD–EDA) can also detect active metabolites, breakdown products, and contaminants that were not in the previous focus of interest [18]. Screening of veterinary drug residues by planar chromatography has not played a major role despite several advantages [19]. Early methods were not quantitatively performed at the required MRL (trace analysis) due to diffuse antibacterial zones [20,21]. Without any bioassay detection, quantitative HPTLC–FLD studies followed [22,23], however, required elaborate plate treatment and sample preparation [23] or used non-environmentally friendly halogenated mobile phases [20–23].

The current routine analysis of samples via HPLC–MS/MS is expensive for society since positive findings of veterinary drug residues in food of animal origin occur rarely (found in the per mil range in Germany). Still, simple, rapid, eco-friendly, low-cost, and sensitive screening methods are of great interest. Especially the inclusion of the non-target bioassay detection would detect also compounds not in the previous focus of interest. Analytes can simply be enriched on the planar layer during spray-on application through evaporation of the solvent, and sample preparation can be performed during separation and affords no extra steps if the matrix remains in the start zone or elutes to the front [24]. Hence, the potential of a simple planar bioassay screening (HPTLC–UV/Vis/FLD–bioassay) at the trace level was studied on the example of two fluoroquinolone antibiotics in milk. It was studied how far one can go under these circumstances with a simple non-target planar bioassay screening to face the limits of the method.

2. Materials and methods

2.1. Chemicals

HPTLC plates silica gel 60 (20 cm × 10 cm, layer thickness 150 µm), HPTLC plates silica gel 60 F₂₅₄ (20 cm × 10 cm, layer thickness 170 µm), *Bacillus subtilis* spore suspension (BGA, DSM 618 strain), isopropyl acetate (gradient grade), acetonitrile, ethanol, methanol (MS grade), and ammonia solution (25%, Suprapur) were obtained from Merck, Darmstadt, Germany. *Aliivibrio fischeri* bacteria strain NRRL B-11,177 (DSM-7151) was obtained from the German Collection of Microorganisms and Cell Cultures, Berlin, Germany. Ammonium formate (reagent grade), caffeine (99%), and ciprofloxacin (98%) were obtained from Sigma-Aldrich, Steinheim, Germany. Marbofloxacin (98%) was purchased from Tokyo Chemical Industry, Tokyo, Japan. Tetracycline hydrochloride (reagent grade) was obtained from Serva Electrophoresis, Heidelberg, Germany. The medium and substrate for the *Bacillus subtilis* bioassay [18] as well as the medium for the *Aliivibrio fischeri* bioassay are listed elsewhere [25]. Various sorts of milk samples were bought at the local market (Table 1).

2.2. Stock solutions, standard mixture, sample preparation, and spiked samples

Ciprofloxacin and marbofloxacin were dissolved each in methanol containing 0.5% ammonia solution (0.1 mg/mL stock solutions). Working standards (5 ng/µL) were prepared by 1:20 dilution of 50 µL stock solution in 950 µL acetonitrile. A standard mixture was prepared by diluting 50 µL of each stock solution in 900 µL acetonitrile (5 ng/µL each). Working standards and the standard mixture were stored at 4 °C in the dark and were prepared

freshly every four weeks. Milk samples (750 µL each) were 1:2 diluted with 750 µL acetonitrile, vortexed for 5 s, and centrifuged at 14,000 × g for 10 min. The clear supernatant was transferred to sampler vials. Depending on the experiment, 750 µL of the milk samples were spiked at 200 µg/kg with 1.5 µL of the ciprofloxacin stock solution and at 800 µg/kg with 6 µL of the marbofloxacin stock solution, diluted with 743 µL acetonitrile and prepared accordingly.

2.3. Planar antibacterial bioassay screening

The operation of all HPTLC system devices (CAMAG, Muttentz, Switzerland) and evaluation were performed via winCATS software, version 1.4.6. For 19 tracks per plate, 18 1:2-diluted milk sample solutions (100 µL each) and an antibiotic reference mixture (2 µL ciprofloxacin working solution oversprayed with 8 µL marbofloxacin working solution to be 10 and 40 ng/band, respectively) were applied as 7-mm × 18-mm area each on the HPTLC plate silica gel 60 with or without F₂₅₄ using an application speed of 1200 nL/s and nozzle heating of 60 °C. The distance from the lower plate edge was 8 mm and from the left side edge 5 mm with a distance between tracks of 9.7 mm (Automatic TLC Sampler 4). The blank silica gel on the left and right side edge was scraped off (HPTLC Scoring Unit). The areas were focused four times with 5 mL acetonitrile – methanol – ammonia 5.5:2.5:2 (V/V/V) up to 30 mm (ca. 3.5 min) with intermediate 1-min drying (hair dryer). After each focusing step, the remaining solvent in the chamber was replaced with a fresh mobile phase. After the second focusing, 15 mm of the lower plate edge was cut off (Smart-Cut), which shortened the focusing time to 45 s. Chromatography was performed with 7 mL isopropyl acetate – methanol – ammonia 5:3.5:1.4 (V/V/V) up to 1 cm below the upper plate edge (ca. 38 min) and dried (5 min, hair dryer, followed by 30 min at 50 °C, TLC Plate Heater). The chromatogram was documented at FLD 366 nm (TLC Visualizer). Optional fluorescence measurement was performed at 313/–400 nm (mercury lamp, measurement slit 8 mm × 0.4 mm, scanning speed 20 mm/s; TLC Scanner 4).

For bioassay detection, the chromatogram was further dried (15 min; Automated Developing Chamber 2), and as a positive control, tetracycline (10 µg/mL in ethanol, 0.4 – 1.2 µL/band) or caffeine (1 mg/mL in methanol, 0.5–3 µL/band) was applied above the solvent front for the *Bacillus subtilis* [18] or *Aliivibrio fischeri* bioassay [25], respectively. The plate was piezoelectrically sprayed (red nozzle, level 6; Derivatizer) with 3.5 mL of the *Bacillus subtilis* cell suspension prepared in Müller-Hinton broth (5.5 × 10⁶ cells per mL; 0.8–1.1 optical density at 600 nm) and horizontally incubated at 37 °C for 2 h in a moistened plastic box (KIS 26.5 cm × 16 cm × 10 cm; ABM, Wolframs-Eschenbach, Germany). After incubation, the plate was sprayed with 500 µL of a 0.2% PBS-buffered tetrazolium salt solution (blue nozzle, level 5), incubated at 37 °C for 30 min (or 1.5 h for plates with fluorescence indicator), and dried at 50 °C for 10 min (TLC Plate Heater). The bioautogram was documented under white light illumination (reflectance mode; TLC Visualizer). For the *Aliivibrio fischeri* bioassay, the dried plate was sprayed (red nozzle, level 6) with 3.5 mL of the *Aliivibrio fischeri* cell suspension prepared according to DIN EN ISO 11,348–1 [25]. The bioluminescent image was directly recorded (50 s exposure time; BioLuminizer 2).

3. Results and discussion

3.1. Development of the dilute & screen strategy

For a comprehensive effect-directed screening, the sample should be used as originally as possible, and the workflow should be as simple as possible. Hence, dedicated sample clean-up, e.g.,

Table 1
Milk samples obtained from the local market or directly from the farmer.

ID Track	Milk type	Fat (%)	Brand	Manufacturer in Germany
1	Fresh	1.5	Weihenstephan	Molkerei Weihenstephan, Freising
2	Fresh	1.5	Milbona	Privatmolkerei Bechtel, Schwarzenfeld
3	Fresh	1.5	Milsani	Hochwald Foods, Thalfang
4	Long-life lactose-free	1.5	Milsani	AF Deutschland, Düsseldorf
5	Fresh	3.5	TiP	Zentrale Handelsgesellschaft, Offenburg
6	Fresh Pasture	3.5	Fair & Gut	NordseeMilch, Witzwort
7	Long-life	3.5	Milbona	Privatmolkerei Bechtel, Schwarzenfeld
8	Long-life	3.5	Milsani	Gmundner Molkerei, Gmunden*
9	Fresh Pasture	3.8	Schwarzwaldmilch	Schwarzwaldmilch, Freiburg
10	Raw	n.a.	Cow 347	Biohof Burkhard, Czyscholl
11	Raw	n.a.	Cow 475	Biohof Burkhard, Czyscholl
12	Raw	n.a.	Cow 128	Biohof Neuss, Weitersshain

n.a. not available, *in Austria.

Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) strategy or solid-phase extraction (SPE) followed by solvent evaporation, and HPTLC plate pre-treatment (with disodium ethylenediaminetetraacetate dihydrate, needed to meet required MRLs) [23] should be avoided. Instead, the milk samples were only 1:2 (750 μ L milk + 750 μ L acetonitrile) diluted and centrifuged to avoid any change or loss of compounds. Also, lower dilutions of milk (750 μ L milk) with only 500 μ L and 625 μ L acetonitrile were tested, but the mixture was turbid and remained so. Solvent systems reported for separation of fluoroquinolones on the HPTLC plate silica gel 60 [20–23] contained halogenated solvents, required a modification of the layer or sample preparation steps, or resulted in tailing or diffused zones for the intended high milk matrix load (without clean-up). Among the tested solvent systems, isopropyl acetate – methanol – ammonia 5:5:2 (V/V/V) based on equal ratios of isopropyl acetate and methanol plus a substantial portion (17%) of ammonia, resulted in a good separation from the milk matrix. It took about 34 min for a 65-mm migration distance. The separation performance depended on the vapor space of the chamber, and for chambers with a reduced vapor space (10 cm x 10 cm twin trough chamber; horizontal developing chambers), the ratio was adjusted to 7:7:2 (13% ammonia). The elution power was adjusted to avoid the coelution of marbofloxacin (Fig. 1) with a yellow fluorescent compound zone (possibly a riboflavin-like compound) in the milk matrix (Fig. S-1).

Spectra were recorded (200–700 nm) to obtain the optimal measurement wavelength for densitometric evaluation (Fig. S-2). In accordance with the literature [23], ciprofloxacin showed a maximum at 279 nm, whereas the maximum of marbofloxacin was located at 300 nm. However, measurement at these maximal wavelengths of absorbance showed that the milk matrix interfered, especially evident for marbofloxacin (Fig. S-3A). A multi-wavelength scan comprising absorbance measurement at 280 nm, 300 nm, 320 nm, and 340 nm and fluorescence measurement at 313/>400 nm and 366/>400 nm followed to study this matrix influence. Fluorescence measurement at 313/>400 nm was the best selection for measurement with regard to a good signal-to-noise ratio for both antibiotics without milk matrix interference (Fig. S-3B). In contrast, fluorescence measurement at 366/>400 nm and absorbance measurement at 320 nm and 340 nm (Fig. S-3A/C) were not as good concerning signal intensities and reduction of matrix interference (no clean-up of the milk).

For an impression on the physicochemical HPTLC–FLD method performance, ciprofloxacin and marbofloxacin were applied as 6-mm bands, i.e. halved in the band length and same for the measurement slit, allowing along with applied milk samples more tracks per plate to be studied. The working range started at the limit of quantification of 2.5 ng/zone (S/N > 10 for each via peak

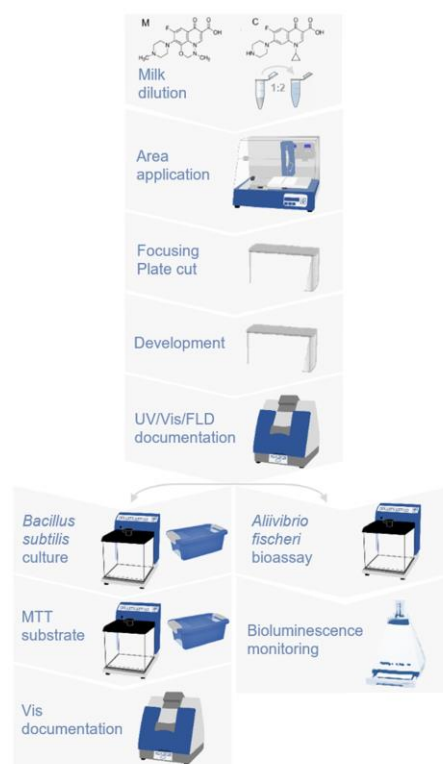


Fig. 1. Developed non-target planar bioassay screening of milk for Gram-negative and Gram-positive antibacterial compounds: scheme of the workflow steps as well as the structures of the two fluoroquinolones marbofloxacin **M** and ciprofloxacin **C**.

area) and covered a range of 1:30 via a 4-point calibration (S1–S4; 2.5–75 ng/zone; 0.5, 5, 10 and 15 μ L, Fig. S-4). The regression for fluorescence measurement at 313/>400 nm showed a linear response and good correlation coefficients of 0.9999 (peak area evaluation). In contrast, absorbance measurement at 340 nm showed a noisy baseline (Fig. S-5) and polynomial fit (Fig. S-6). Recovery rates of ciprofloxacin and marbofloxacin in different milk samples

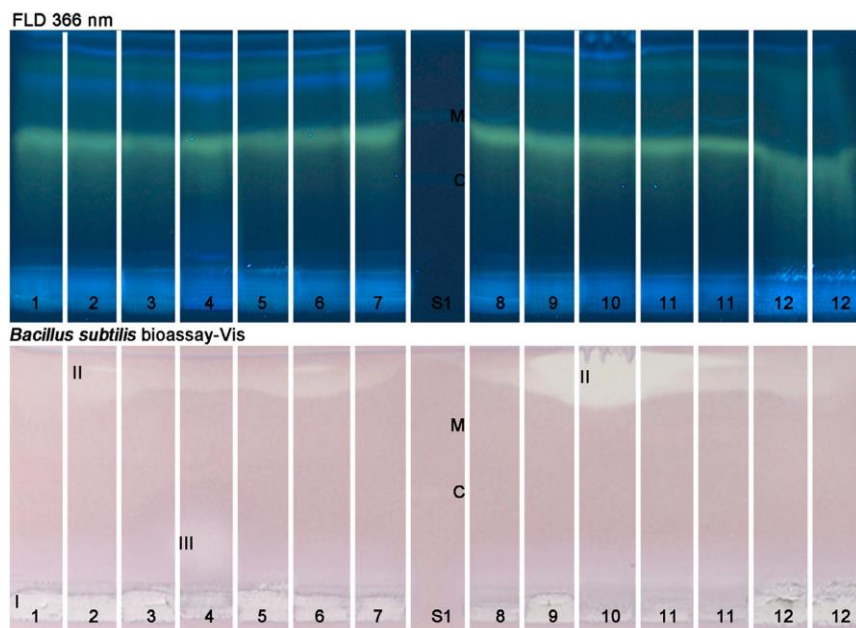


Fig. 2. Initial planar bioassay screening: separation of 12 different milk samples (IDs 1 – 12, assigned in Table 1, 1:2 diluted, 120 μ L, 12 mm $\times$ 7 mm) and standard mixture S1 with ciprofloxacin C and marbofloxacin M corresponding to 100 μ g/kg (each 12 ng, both hardly visible) on the HPTLC plate silica gel 60 with isopropyl acetate – methanol – ammonia 5:5:2 (V/V/V), after focusing with the same solvents in the ratio 3:8:2:2 (V/V/V), detected at FLD 366 nm and white light illumination after the *Bacillus subtilis* bioassay, revealing antibiotic responses as colorless zones I–III on a rose background.

were studied (two-fold determination). Each milk sample (741 μ L) was spiked with 4.5 μ L of each 0.1-mg/mL stock solution (each 450 ng/750 μ L; 600 μ g/kg). The spiked sample was allowed to stand overnight in the refrigerator and then diluted as described. There was no loss in analytes expected because sample preparation only included a dilution and centrifugation step. The mean recovery rate for both fluoroquinolones was 115% (Table S-1). The excess finding was explained by the slight narrowing of the bands due to the matrix during the development. The whole procedure repeated two times (2 plates per day) and four times (4 plates per day) achieved intra-day repeatabilities of $\leq 3\%$ and reproducibilities in milk matrix of $\leq 1.4\%$ ($n = 2$, Table S-1). This proved HPTLC to be a reliable tool per se, suited as the basis for biological on-surface detection.

3.2. Lowering the detection limit

In the European Union, the MRL of marbofloxacin in milk is 75 μ g/kg. Ciprofloxacin is the main metabolism product of enrofloxacin, for which the MRL is 100 μ g/kg. [5] Such trace level concentrations in milk can only be reached in HPTLC using area application (for both, biological and fluorescence detection), as recently demonstrated for lactose-free milk and milk products [24] or high-throughput planar solid-phase extraction of multi-class antibiotic residues [17]. Application volumes and application geometries were studied to find out the maximal milk matrix load on the adsorbent. The application of different volumes of a milk sample 1:3 diluted with acetonitrile showed that up to 160 μ L could be applied without major distortion of the chromatogram (Fig. S-7). It was decided to equivalently apply only 120 μ L of a 1:2 diluted milk to be faster in sample application (Fig. S-8). Dif-

ferent geometries were studied, whereby the 12 mm $\times$ 7 mm area was found to be most robust concerning the heavy matrix load and a fast dosage speed. The dosage speed for methanolic samples was increased up to 1200 nL/s and the spray nozzle heating was set at 60 $^{\circ}$ C. Using these settings (120 μ L, 12 mm $\times$ 7 mm, 1200 nL/s, heated nozzle), the application of one sample including system rinsing took less than 4 min, which was found to be acceptable, since milk samples were only diluted for sample preparation.

Next, the focusing of the compounds was studied for the area-applied milk samples. The solvent mixture had to be stronger in elution power for zone focusing than separation to move the lower onto the upper application part, thus generating a sharp start band for subsequent separation. Using isopropyl acetate – methanol – ammonia 1:1:1 (V/V/V), the focusing mixture could not migrate evenly through the start area and migration was hampered, whereas the ratio 3:2:1 could penetrate but did not front-elute the analytes anymore. Eventually, ciprofloxacin and marbofloxacin were focused to a band using isopropyl acetate – methanol – ammonia 3.5:2:1.5 (V/V/V) up to the upper edge of the applied area, followed by drying for 2 min (Fig. S-9). The investigation of the maximum matrix load, optimal area application, and efficient focusing led to the maximal possible dilute & screen strategy, whereby the loading of the HPTLC system reached its limits.

3.3. Initial planar bioassay screening of milk samples

The developed method (Fig. 1) was applied to the planar *Bacillus subtilis* bioassay screening of 12 commercially available milk samples. The standard mixture was applied at 100 μ g/kg (12 ng/area) as a reference. The samples included all kinds of pos-

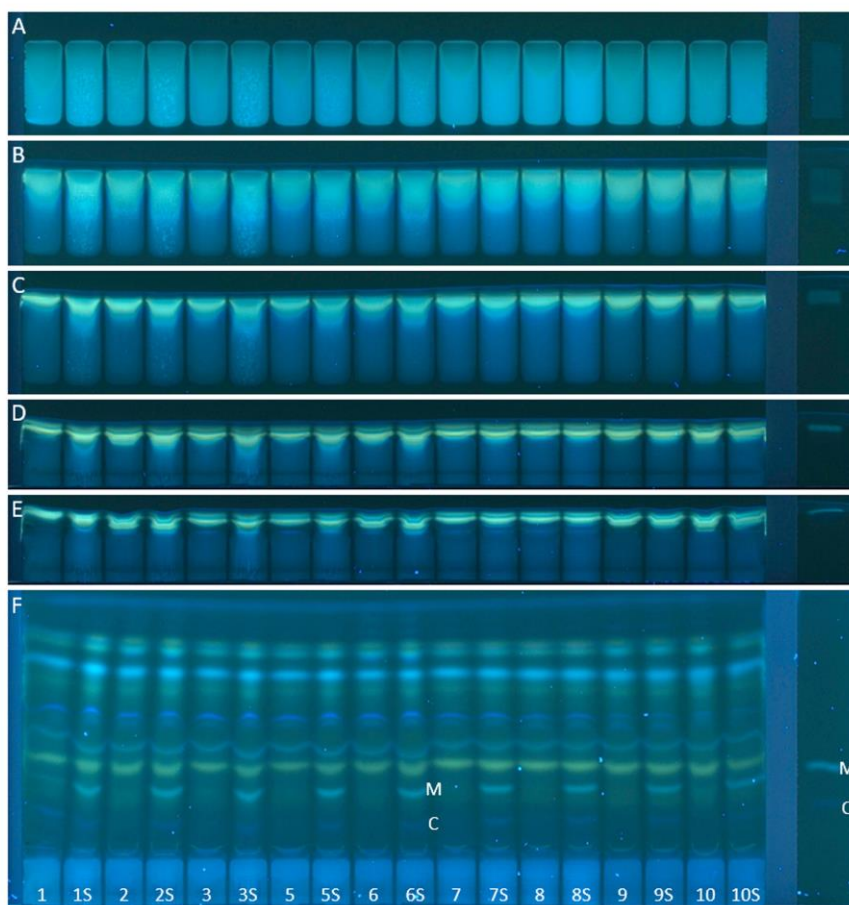


Fig. 3. HPTLC–FLD chromatograms at 366 nm after application (A) of different milk samples (tracks **1** – **10**, Table 1, 1:2 diluted, 100 μ L, 7 mm $\times$ 12 mm), each also spiked (S) with ciprofloxacin **C** at 200 μ g/kg and marbofloxacin **M** at 800 μ g/kg (last reference track), on HPTLC plate silica gel 60 F₂₅₄ (adsorbent scraped off on left and right plate sides to avoid zone distortion), first (B), second (C, thereafter plate cut at 1.5 cm), third (D) and fourth (E) focusing using acetonitrile – methanol – ammonia 5.5:2.5:2 (V/V/V) up to 3 cm as well as separation with isopropyl acetate – methanol – ammonia 5:3.5:1.4 (V/V/V) up to 1 cm below the upper plate edge.

sible milk types, i.e. fresh or long-life milk with fat contents of 1.5% and 3.5% fat, and even higher fat contents for raw milk, as well as lactose-free milk and organic milk (Table 1). Antibacterial zones active against Gram-positive bacteria were observed as colorless zones on a rose background. The advantage of biological detection is that antibiotics and further active compounds are detected, including metabolites, degradation products, and contaminants that were not previously in focus. Ciprofloxacin was hardly visible and marbofloxacin could not be detected at the chosen concentration, but three colorless antibacterial zones were evident in the samples (Fig. 2). On the start zone, a strong antibacterial response (zone I) was evident which needs further clarification as it could be caused by other antibiotic residues [17] remaining at the start zone. The compound zone II near the front was observed with the highest antibiotic response in raw milk (track/ID 10) which influenced neighboring tracks through its spreading. At a lower antibacterial response, this compound was detected also in fresh milk with 1.5% fat (track/ID 2) and fresh pasture milk with 3.5%

fat (track/ID 6). Most likely this antibacterial response was caused by triacylglycerides, which should be proven in future studies by coupling to high-resolution MS. Recently, the properties of fatty acids and monoacylglycerides have been discussed as alternative antimicrobials [26], and the antibiotic effect of some fatty acids in this planar bioassay has already been reported [27–29]. The compound zone III showing a diffuse antibiotic response (which could be caused by a volatile compound low in vapor pressure) was only observed in long-life lactose-free milk with 1.5% fat (track/ID 4). Overall, the results were surprising, as such strong antibiotic responses against Gram-positive bacteria were not expected in milk samples. Regardless of whether it is a metabolite, contaminant, degradation product, or veterinary drug residue, the effect indicated on the planar surface will most likely also affect the human microbiome when consumed. Already this very first screening highlighted the full picture of the real levels of active compounds, regardless of the permitted limits of antibiotics. Why become more and more sensitive when we already see intense signals that we

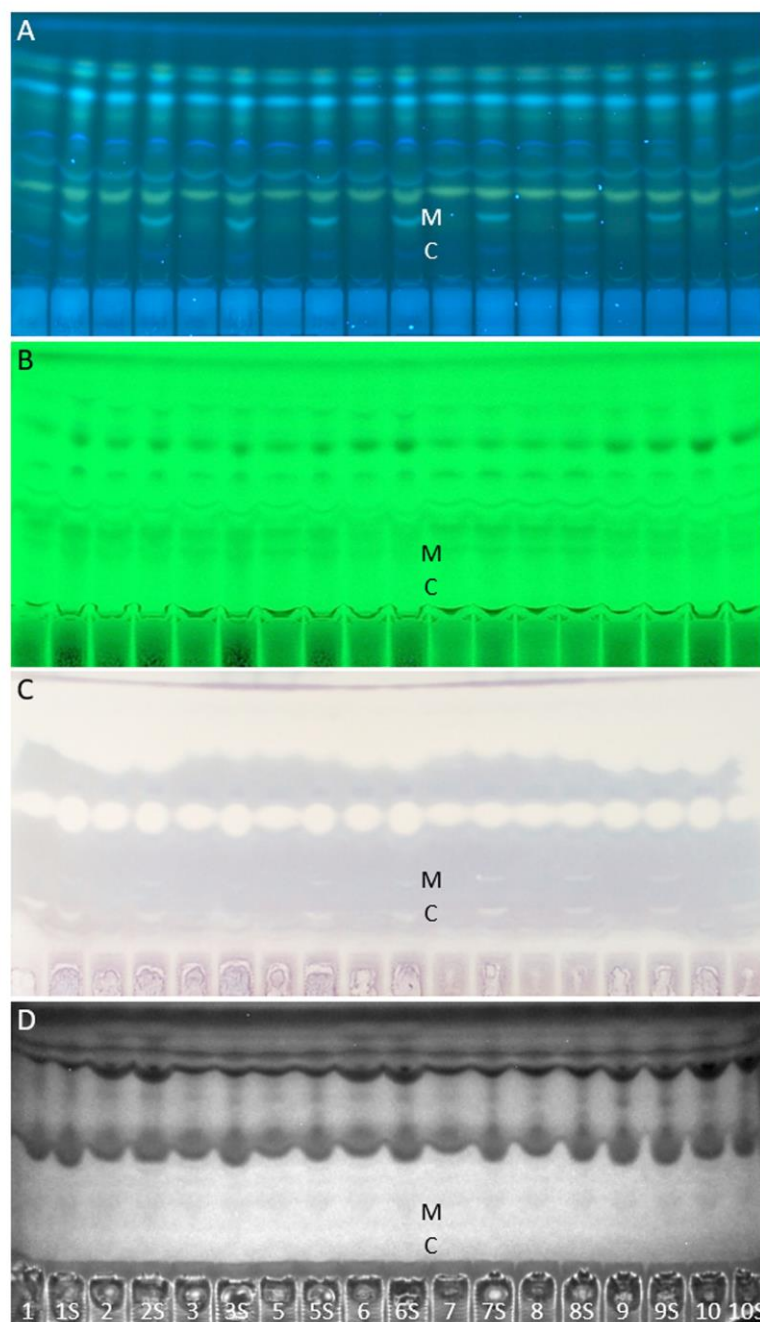


Fig. 4. Planar bioassay screening after HPTLC separation of different milk samples (as in Fig. 3, spiked with ciprofloxacin **C** and marbofloxacin **M**) detected at FLD 366 nm (A), UV 254 nm (B), white light illumination after the *Bacillus subtilis* bioassay (C), and via the greyscale image of the bioluminescence after the *Allivibrio fischeri* bioassay (D).

do not care about, but show the same antibiotic effect that is even much stronger than fluoroquinolones at the limit value?

3.4. Improved planar bioassay screening of milk samples

It has been challenging to meet the low MRLs of fluoroquinolones in milk. However, further ideas came up to lower the detection limits. Using plates with fluorescence indicator was noticed to be more sensitive in the biological detection via *Bacillus subtilis*. If the application geometry (12 mm × 7 mm) was used vice versa (7 mm × 12 mm), biological detectability could be improved (smaller band length after focussing) and the sample throughput could be increased, exploiting the full potential of the method. Hence, different milk samples (IDs as in Table 1) and the same samples spiked with ciprofloxacin at 200 µg/kg and marbofloxacin at 800 µg/kg (18 samples instead of 14) were applied on HPTLC silica gel 60 F₂₅₄ plates with the changed geometry (Fig. 3). To reduce the application time to a minimum, only 100 µL of the sample solutions were applied, which required only one syringe filling cycle (instead of two for 120 µL), and thus, the application took 55 min in total, which equals 3.1 min per sample (which was only diluted). To avoid distortion during development, later evident in the chromatogram, the blank silica gel on the left and right-hand sides was scraped off. Because of the increased height of the application areas, the focusing procedure was even more important and had to be modified to be efficient. Isopropyl acetate was replaced by acetonitrile as in a recently published high-throughput planar solid-phase extraction method for antibiotics in food [17]. But solvents high in elution strength (able to focus to a sharp band in one go) were not able to penetrate the extracts evenly due to missing solubility in the high matrix load. A mixture of acetonitrile – methanol – ammonia 5.5:2.5:2 (V/V/V) was chosen as a compromise between elution strength and penetrability (Fig. 3). The focusing had to be carried out four times up to 30 mm to focus the analytes in a sharp start zone; after the second pre-focusing, the lower plate edge was cut off at 15 mm because the analytes had already reached a sufficient *hR_F* (Fig. 3), and this shortened the following two focusing steps (10 min for

all four steps) and development (38 min). Development was carried out with a modified mobile phase ratio of isopropyl acetate – methanol – ammonia 5:3.5:1.4 (V/V/V) up to 1 cm below the upper plate edge. Using this separation system, the spiked standards were sufficiently separated from the application zone and the interfering matrix compounds and formed sharp bands, as evident in the fluorescence detection (Fig. 4A) as well as biological detection via the *Bacillus subtilis* bioassay (Fig. 4C). Thus, the fluoroquinolone antibiotics could be biologically detected down to 200 µg/kg for ciprofloxacin and 800 µg/kg for marbofloxacin. Higher application volumes should improve detectability but were not applied given a fast sample throughput (100 µL equal to one syringe filling was applied faster). Detectability can also be improved when integrating a high-throughput planar solid-phase extraction clean-up, or when using other more sensitive bacterial strains, which should be part of future investigations. Still, strong antibacterial activity against the Gram-positive *Bacillus subtilis* bacteria exhibited by the milk matrix compounds was observed in the upper plate region, which confirmed previous results (Figs. 3/4). Comparison between the *Bacillus subtilis* bioautograms of the same samples on an HPTLC plate silica gel 60 and an HPTLC plate silica gel 60 F₂₅₄ revealed a higher sensitivity of plates with fluorescence indicator for ciprofloxacin, but not for marbofloxacin. The plate with fluorescence indicator required an extended incubation time of 1.5 h instead of 30 min (for substrate) and showed a bluish rather than rose background, which can be explained by the incorporated inorganic fluorescence indicator (manganese activated zinc silicate) that interacts with the bioassay detection.

The developed bioassay screening method was transferred to another bioassay, which acted against Gram-negative bacteria and thus was orthogonal in the antibacterial effect. Thus, another chromatogram of milk samples was subjected to the Gram-negative *Aliivibrio fischeri* bioassay (Fig. 1). Antibacterial zones active against the naturally luminescent marine bacteria were observed as dark zones, whereby the native bioluminescence was depicted as a greyscale image (Fig. 4D). Ciprofloxacin, the main metabolism product of enrofloxacin, was more sensitively detected than marbofloxacin in the previous Gram-positive *Bacillus subtilis* bioassay,

Table 2

Calculation of the analysis time for 54 samples in total and also per sample as well as manual operating time for the daily screening using one effect-directed HPTLC working station; steps in italics can be performed during the pre-step.

Analysis step	Analysis time (min) 18 samples/plate			Manual operating time (min)
	Plate 1	Plate 2	Plate 3	Plates 1 – 3
Preparation of cell suspension	5	–	–	5
Dilution of milk samples	8	8	8	24
Centrifugation	13	13	13	9
Transfer to vials	10	10	10	30
Sample application	55	55	55	10
<i>Mobile phase preparation</i>	<i>(8)</i>	–	–	8
Focusing, plate drying	10	10	10	30
Development	38	–	–	2
Plate drying	32	–	–	2
<i>Cell OD adjustment</i>	<i>(15)</i>	–	–	15
Spraying cell suspension	10	8	8	26
Incubation cells	120	–	–	2
<i>Cleaning Derivatizer</i>	<i>(20)</i>	–	–	20
Spraying substrate solution	5	4	4	13
Incubation substrate	30	–	–	2
<i>Cleaning Derivatizer</i>	<i>(10)</i>	–	–	10
Plate drying, documentation	13	–	–	3
Sum	349 min	108 min	108 min	
per 54 samples	5.8 h	1.8 h	1.8 h	
In total		565 min		211 min
per 54 samples		9.4 h		3.5 h
per 1 sample	19 min	6 min	6 min	
Mean per sample		10 min		4 min

whereas as expected, both fluoroquinolones did not show an effect against Gram-negative bacteria in the *Aliivibrio fischeri* bioautogram. Nevertheless, dark antibacterial zones and thus a substantial impact of the milk samples on the Gram-negative *Aliivibrio fischeri* bacteria was observed. Up to five more or less intense antibacterial zones were detected in the upper bioautogram, apart from the start zone. The effect indicated on the planar surface will most likely also affect the human microbiome when milk is consumed, regardless of whether it is a metabolite, contaminant, degradation product, or veterinary drug residue. These results challenge the current prevailing understanding of food safety and antimicrobial resistance based on the results of target (rather than effect) analyses.

3.5. Benefits of the planar bioassay screening

Tedious sample preparation was avoided for the newly developed workflow. Only dilution and centrifugation were necessary (dilute & screen strategy). Thus, samples were kept as native as possible to avoid any change or loss of compounds which is the best precondition for a comprehensive non-target screening. The resulting high matrix load on the HPTLC plate did not generate any interference or memory effect with subsequent analyses, as the layer material was used only once. The non-target planar bioassay screening provided the full picture of the real levels of active compounds. Including sample preparation, the manual operating time per sample analysis was about 4 min (Table 2). The operating costs were about 0.5 Euro per sample. This allows for a faster, less expensive, more eco-friendly, and more comprehensive screening than the current target analysis. With only one effect-directed HPTLC working station, the planar bioassay screening of 54 milk samples per 9.4-h day was possible. Directly from the bioautogram containing still the bioassay medium [30], high-resolution mass spectra can optionally be recorded (only in case of positive results occurring in the per mil range), which substantially reduces costs, contaminations, and cleaning cycles of the HRMS system.

4. Conclusions

The newly developed planar bioassay screening is simple, rapid, eco-friendly, low-cost, and sensitive down to the trace level. The results explained the inconsistencies observed between chromatographic/spectrometric and microbiological data. The currently practiced target analysis by HPLC-MS/MS does only inform about the target analytes and is expensive for society, as positive findings are only in the per mil range. Instead, this non-target planar bioassay screening, requiring only sample dilution, provided the full picture of the real levels of active compounds. Any active metabolites, degradation products, and contaminants not previously in the focus were detected along with the targeted antibiotics. It revealed that compounds with substantial antibacterial effects are present in the milk, regardless of the permitted limits of antibiotics. If strong effects are observed on the surface of the planar bioassay, it will most likely also impair and affect the human microbiome, when the milk is consumed. The question is whether only the regulated antibiotic residues or generally all antibacterial compounds in a sample count. It was a challenge to meet the low maximum residue levels, but why focus only on the weak antibacterial signals of the regulated compounds while ignoring much stronger other antibacterial signals? The prevailing understanding of food safety, consumer protection, and antimicrobial resistance based on the results of target (rather than effect) analyses is being challenged. In the future, a sample cleanup step can be integrated at the detriment of a longer workflow when the sensitivity does matter. The study can be expanded to further structural classes of antibiotics in further matrices as well as different strains of *Bacillus subtilis* and

other bacterial strains such as *Enterobacteria*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* to widen the application range and detectability of antibiotics.

CRedit authorship contribution statement

Gertrud E. Morlock: Conceptualization, Methodology, Supervision, Writing – Original draft, Writing – Reviewing & Editing. **Jan A. Morlock:** Investigation, Writing – Reviewing and Editing. **Ayşe Deniz Cardak:** Investigation (initial experiments). **Annabel Mehl:** Investigation, Writing – Reviewing & Editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2022.463392.

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Supplementary data

Potential of simple, rapid, and non-target planar bioassay screening of veterinary drug residues

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S-1

Table S-1 Method performance for ciprofloxacin and marbofloxacin spiked at 600 µg/kg in five different milk samples, which were applied on the HPTLC plate silica gel 60 as 6-mm bands (4-mm measurement slit), focused with isopropyl acetate – methanol – ammonia 3.8:2:2 (V/V/V), separated with the same mixture but in the ratio 7:7:2, and detected via fluorescence measurement at 313/> 400 nm. The mean recovery for both antibiotics was 115%.

Milk spiked (600 µg/kg)	Ciprofloxacin			Marbofloxacin		
	Recovery (%)	Repeatability (%RSD, n = 2/4)	Reproducibility (%RSD, n = 2)	Recovery (%)	Repeatability (%RSD, n = 2/4)	Reproducibility (%RSD, n = 2)
1	133	2.9/0.8	0.8	120	1.0/1.8	0.9
2	117	0.5/1.2	2.5	107	0.9/0.7	0.7
3	122	1.5/0.6	1.1	120	0.3/1.3	3.8
4	122	3.0/0.5	0	102	0.9/0.7	1.0
5	113	2.6/0.4	1.8	91	0.4/0.9	0.4
Mean	121	2.1/0.7	1.2	108	0.7/1.1	1.4

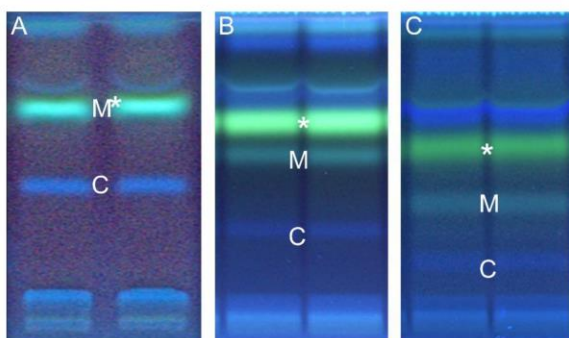


Fig. S-1 HPTLC–FLD chromatograms obtained for separation of marbofloxacin **M** and ciprofloxacin **C** in a 1.5% fat fresh milk sample using different solvent ratios of isopropyl acetate – methanol – ammonia (V/V/V), *i.e.* (A) 4:4:2, (B) 6:6:2 and (C) 7:7:2 in 10 cm × 10 cm twin-trough chamber; the proper elution power has to be selected to avoid coelution of **M** with the upper natively yellow fluorescent milk zone (*).

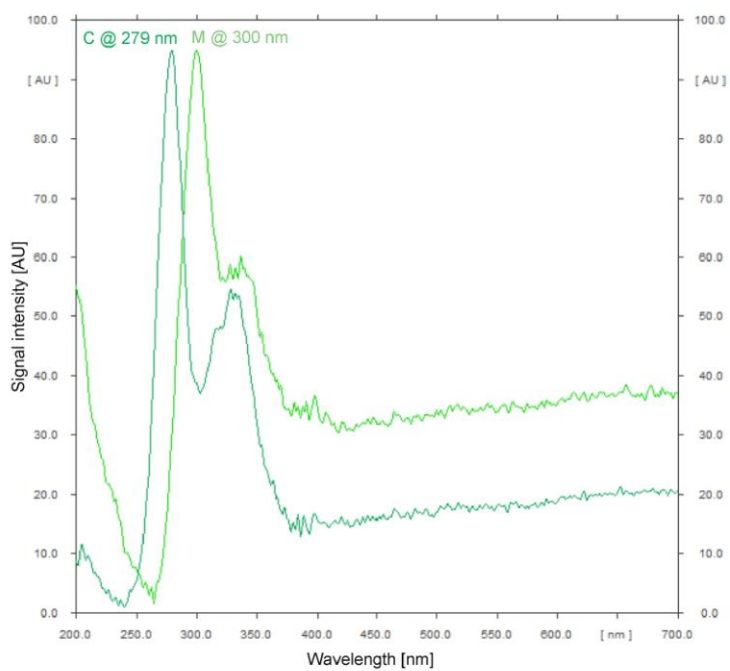


Fig. S-2 Recorded HPTLC-UV/Vis spectra (200–700 nm) of ciprofloxacin **C** and marbofloxacin **M**, 25 ng/band each.

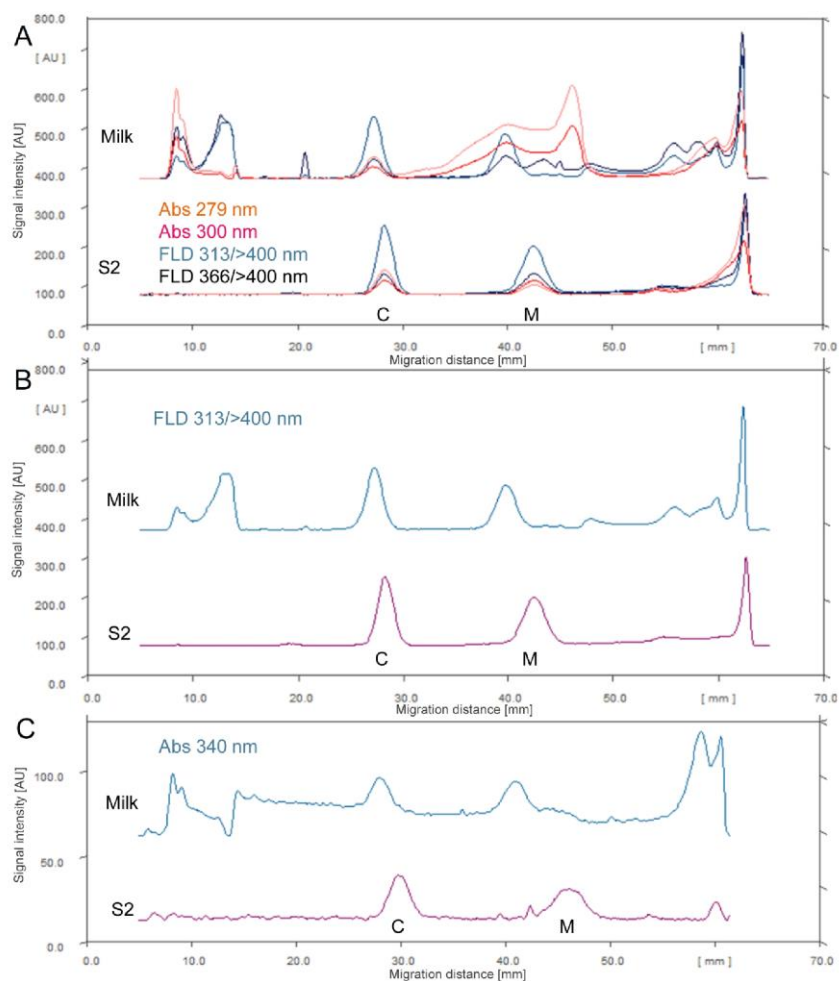


Fig. S-3 Densitograms showing matrix interference of milk spiked at 600 µg/kg observed for absorbance measurement of marbofloxacin **M** at 279 nm or its maximum wavelength at 300 nm; whereas ciprofloxacin **C** was not influenced (A); without any matrix interferences, fluorescence measurement at 313/>400 nm (B) resulted in more pronounced signals, if compared to absorbance measurement at 340 nm (C).

S-5

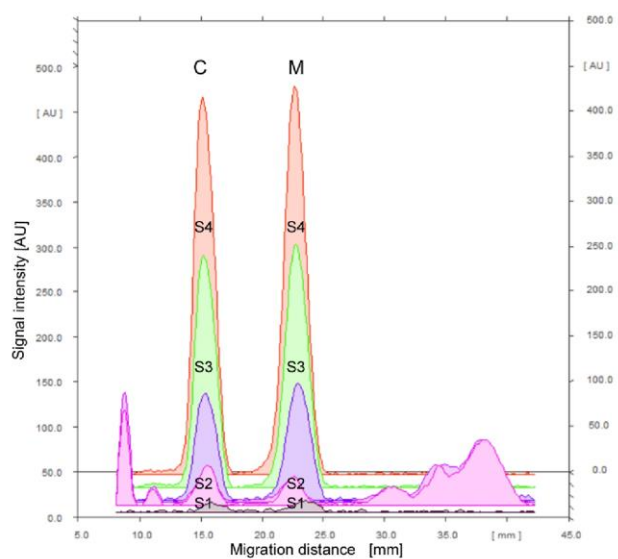


Fig. S-4 3D densitograms of fluorescence measurement at 313/>400 nm showing the 1:30 working range (S1–S4: 2.5–75 ng/zone) of ciprofloxacin **C** and marbofloxacin **M** along with two milk samples in between (S2 is hidden by the samples).

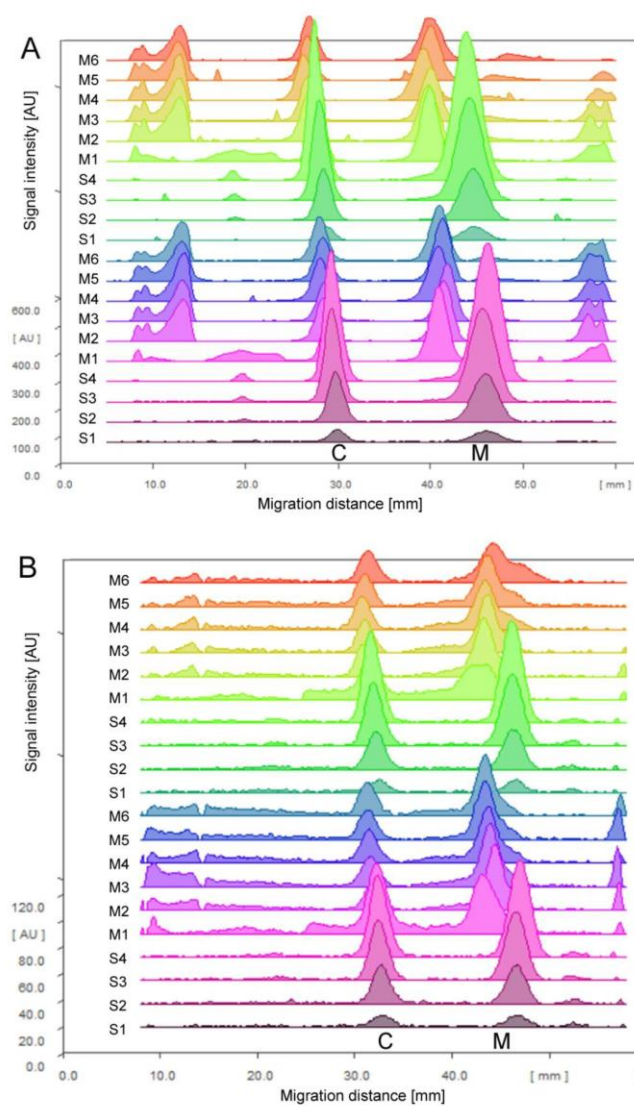


Fig. S-5 3D densitograms of fluorescence measurement at 313/>400 nm (A) versus absorbance measurement at 340 nm (B) showing standard mixture (S1–S4, 7.5–75 ng/zone) and milk samples (M1–M6) spiked at 600 µg/kg with ciprofloxacin **C** and marbofloxacin **M**.

S-7

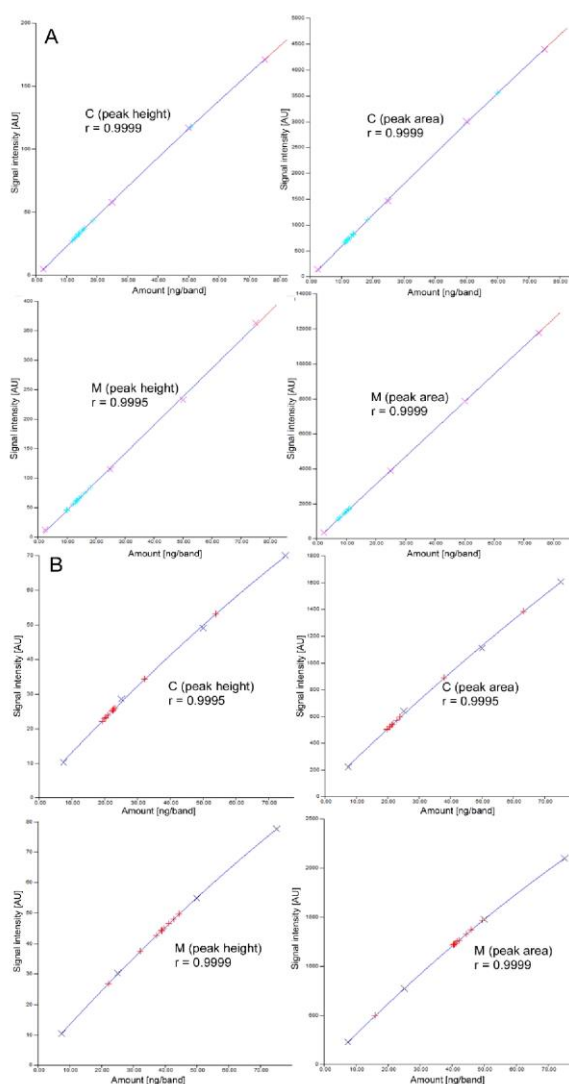


Fig. S-6 Linear calibrations of ciprofloxacin **C** and marbofloxacin **M** via fluorescence measurement at 313 nm versus polynomial calibrations obtained for absorbance measurement at 340 nm (A, 2.5–75 ng/band) versus polynomial calibrations obtained for absorbance measurement at 340 nm (B, 7.5–75 ng/band); the four standard levels are marked as (x), whereas samples are marked as (+) on the regression line.

S-8

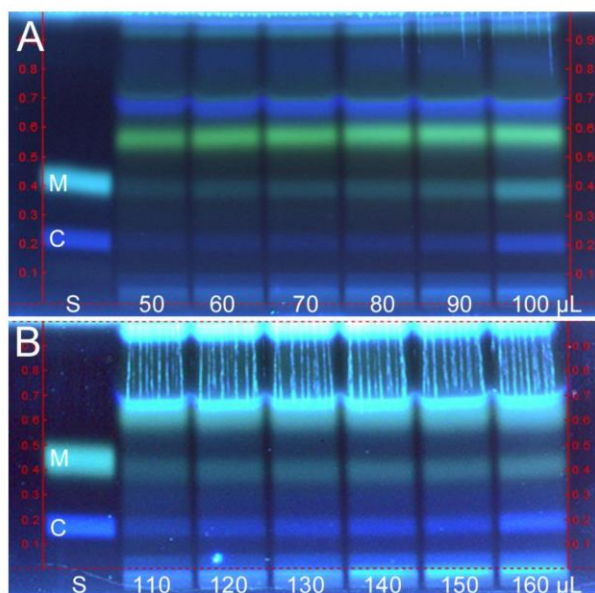


Fig. S-7 Chromatogram at FLD 366 nm showing ciprofloxacin **C** and marbofloxacin **M** added to milk diluted 1:3 with acetonitrile, of which 50 to 160 µL was applied as area (12 mm × 5 mm) and developed without any focusing of the start zone, resulting in broader analyte zones.

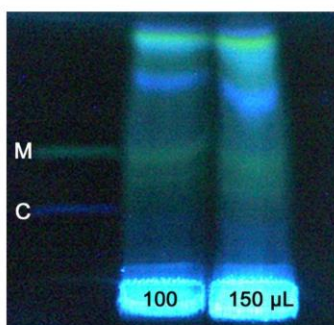


Fig. S-8 Application as 12-mm x 5-mm area of 100 μL and 150 μL of milk sample diluted 1:2 in reference to ciprofloxacin **C** and marbofloxacin **M** using a dosage speed at 1200 nL/s and spray nozzle heating at 60 °C, followed by focusing of the start area.

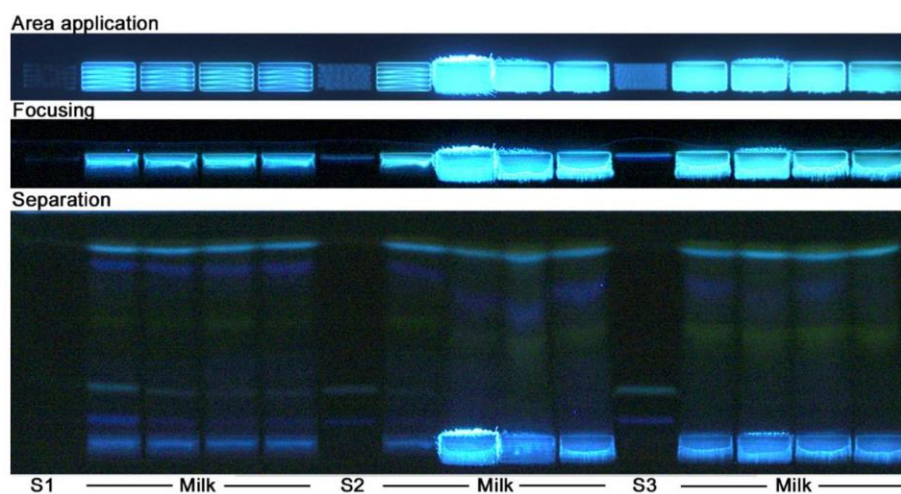


Fig. S-9 Images after application and focusing as well as HPTLC chromatogram at FLD 366 nm of milk samples (1:2 diluted, 120 μ L, 12 mm x 7 mm) in reference to ciprofloxacin **C** and marbofloxacin **M** on the standard mixture tracks (**S1–S3**; 5, 25 and 50 ng each). The mixture of isopropyl acetate – methanol – ammonia was used in the ratio of 3.8:2:2 (V/V/V) for focusing, and 10:7:2 (V/V/V) for separation (lower elution power) on the HPTLC plate silica gel 60.

9. Publication V

Strong antibacterial effects in animal-derived food

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Abstract

The current analytical tools in food safety are questioned. Are only the targeted antibacterial residue compounds of interest or all antibacterial effects in food that can have an impact on the human microbiome upon consumption? Screening for antibiotic residues in food of animal origin is largely done by *in vitro* assays that provide sum values. However, a non-negligible number of positive screening results remains with no confirmation by high-performance liquid chromatography coupled to high-sophisticated mass spectrometers. To investigate this inconsistency, a new strategy was sought that combines chromatography with specific antibacterial bioassay detection, *i.e.*, high-performance thin-layer chromatography hyphenated with multi-imaging and effect-directed detection. Strong antibacterial activity against Gram-positive and Gram-negative bacteria was revealed in four residue-free animal-derived foods (pig muscle, cow milk, chicken eggs, and honey). The antibacterial effect of the food extracts even exceeded the effect of a mixture of 81 veterinary drug standards. The unknown antibacterial zones were online-eluted to a high-resolution mass spectrometer to obtain information on the molecular formulas. Endogenous fatty acids and lipids were identified to be the cause of the strong antibacterial effects, which was confirmed by co-analyzed standard compounds. These results resolved the methodological inconsistency and challenge the current understanding of food safety.

Keywords: Bioassay screening, Effect-directed analysis, Antibiotic residues, Matrix effects, Food of animal origin

1. Introduction

Screening of antibacterial substances in food of animal origin has been largely performed by biological assays that provide only sum parameter values and are rather time-consuming (incubation of up to 24 h) and thus expensive. Each bioassay is preferentially sensitive for one or two antibiotic classes, and thus a combination of microorganisms is used for screening. For example, the four-plate test is based on *Bacillus subtilis*, *Sarcina lutea*, *Escherichia coli*, and *Micrococcus luteus* NCTC 8340, and the five-plate test (so-called STAR protocol) on *Bacillus cereus*, *Escherichia coli*, *Bacillus subtilis*, *Kocuria varians*, and *Geobacillus stearothermophilus* (Gaudin et al., 2010; Gaudin et al., 2009b; Laurentie & Gaudin, 2009; Gaudin et al., 2008; Gaudin et al., 2009a), whereas the Brilliant Black Reduction Test (BRT) uses only *Geobacillus stearothermophilus* var. *calidolactis* C953 (AiM, 2021). Screening of drug residues by planar chromatography has also been demonstrated but did not play a major role (Abjean, 1993a; Abjean, 1994, 1993b, 1993c, 1997; Abjean & Lahogue, 1997; Juhel-Gaugain & Abjean, 1998; Gaugain & Abjean, 1995; Gaugain & Abjean, 1996; Choma et al., 2008; Grzelak et al., 2011) despite its benefits (Morlock & Schwack, 2007, 2008). In the last decade, high-performance liquid chromatography has been coupled with sophisticated mass spectrometers (e.g., HPLC–ESI–MS/MS or -MSⁿ) to confirm the measured biological effect by identifying the antibiotics by mass spectrometric analysis (Gaugain-Juhel et al., 2009; Delatour et al., 2018; Desmarchelier et al., 2018; Desmarchelier et al., 2021). This approach seemed to be promising in the field of veterinary drug residue analysis, however, revealed its limits because the results obtained by microbial and physicochemical methods differed considerably.

In 2019, an exceptionally high number of 87% positive screening cases in meat remained without confirmation by HPLC–ESI–MS/MS and lacked mass spectrometric identification. Most antibiotic drug residue effects indicated to be present in the biological screening in Germany's official control laboratories could not be clarified (German Federal Office of Consumer Protection and Food Safety, 2020). False positive results from bioassay detection have also

been described as problematic in the literature (Hoff et al., 2012). Target analysis by HPLC–MS/MS is blind to off-target antibiotics, misused or unknown unapproved antibiotics, and active degradation products, metabolites, and contaminants. To avoid matrix effects, samples must undergo a clean-up procedure to reduce co-extractives that could lead to ion suppression or signal enhancement, resulting in inaccurate quantification, false-negatives or false-positives (Taylor, 2005; Delatour et al., 2018). Important antibacterial compounds can also be overlooked, since a portion of the sample is discarded during the clean-up procedure. Hence, sample preparation methods for HPLC–MS/MS methods are designed to remove as many matrix compounds as possible and exclude all compounds other than the target compounds from the analytical focus. In contrast, samples are often used in their native form in biological screening methods or simply extracted without purification, often including a concentration step (Gaudin et al., 2010; Gaudin et al., 2009b; Laurentie & Gaudin, 2009; Gaudin et al., 2008; Gaudin et al., 2009a; AiM, 2021). However, these methods are not selective enough to identify antibacterial residues. Non-target HPLC–high-resolution mass spectrometry (HRMS) workflows are not a satisfactory solution to these problems, as the analyst cannot prioritize the thousands of unknown mass signals acquired without information about the effect of the compound in question.

Thus, for the unconfirmed positive screening results, a new strategy is being sought that can resolve this methodological inconsistency. We hypothesize that thin-layer chromatography or high-performance thin-layer chromatography hyphenated with multi-imaging and effect-directed analysis (TLC/HPTLC–UV/vis/FLD–EDA) is a promising tool to investigate pig muscle, cow milk, chicken eggs, and honey for unknown antibacterial compounds. Up to 24 samples can be analyzed in parallel on the same plate surface, and chromatographic separation is independent of biological detection because development solvents are evaporated and have no influence on biological detection (Morlock & Schwack, 2010b). In particular, antibacterial substances that are not in analytical focus could be easily found thanks to the non-targeted antibacterial detection. For example, substances against Gram-positive and Gram-negative

bacteria can be discovered using the *Bacillus subtilis* and *Aliivibrio fischeri* bioassays, respectively. Antibacterial zones of interest can be online eluted from the adsorbent plate surface and transferred to a HRMS instrument without cost-intensive isolation and purification processes (Morlock & Schwack, 2010a, 2010b). Within a minute, respective mass signals are acquired. Hence, on the same surface, important information should be obtainable from parallel chromatographic screening of samples (applied as raw or as original as possible), individual antibacterial effect detection and HRMS recording of detected antibacterial zones to clarify which samples have how many antibacterials of which molecular formulas.

2. Material and methods

2.1 Chemicals and materials

Acetonitrile (ACN, LC-MS grade, $\geq 99.95\%$), *o*-phosphoric acid (85% puriss.), and yeast extract powder (Chemsolute, for microbiol.) were purchased from Th. Geyer, Renningen, Germany. Methanol (LC-MS grade, $\geq 99.9\%$, Riedel-de Haën), sodium acetate (NaOAc, $>99\%$), and anhydrous sodium sulfate (Na_2SO_4 , $\geq 99\%$) were delivered by Honeywell, Seelze, Germany. Ethanol ($\geq 99.8\%$), ACN ($\geq 99.9\%$, used for extraction), aniline ($\geq 99.5\%$), diphenylamine ($\geq 99\%$), caffeine (waterfree, ReagentPlus), 1-oleoyl-*rac*-glycerol ($\geq 99\%$), dioleoylglycerol (mixture of 1,3- and 1,2-isomers, $\geq 99\%$), glyceryl trioleate ($\geq 99\%$), lauric acid (98%), myristic acid ($\geq 99\%$), palmitic acid ($>99\%$), linolenic acid (60–74%), ninhydrin (for amino acid detection), diammonium hydrogen phosphate (99%), ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA, 99–101%), D(+) lactose monohydrate ($\geq 99.95\%$), magnesium sulfate heptahydrate ($\geq 99.0\%$), primuline (50%), rhodamine B ($\geq 95\%$), Müller-Hinton broth, and peptone from casein (tryptone, for microbiol.) were purchased from Fluka or Sigma-Aldrich, Steinheim, Germany. Acetone (HPLC grade), water (LC-MS grade), sodium chloride (NaCl, $\geq 99.5\%$), and anhydrous magnesium sulfate (MgSO_4 , 99%) were obtained from VWR, Darmstadt, Germany. Caprylic acid ($>99\%$), capric acid ($>98\%$), stearic acid ($>98\%$), oleic acid

(>99%), molecular sieve (4 Å), dipotassium hydrogen phosphate (>99%), glycerol (86%), sodium dihydrogen phosphate monohydrate (≥98%), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, ≥98%), and bovine serum albumin (BSA, ≥98%) were ordered from Carl Roth, Karlsruhe, Germany. Polypropylene falcon tubes (15 and 50 mL) were delivered by Sarstedt, Nümbrecht, Germany. Double-distilled water (used for extraction) was produced by a Heraeus Destamat Bi-18E (Fisher Scientific, Schwerte, Germany). Ammonia solution (Suprapur, 25%), glacial acetic acid (HAc, 100%), TLC silica gel 60 F₂₅₄ MS-grade plates 20 cm × 20 cm with a layer thickness of 0.21 mm (cut to 20 cm × 10 cm), Dulbecco's phosphate-buffered saline (PBS) as well as *B. subtilis* spore suspension (BGA, DSM 618 strain) were purchased from Merck, Darmstadt, Germany. *A. fischeri* bacteria (NRRI-B11177, strain 7151) were delivered by Leibniz Institute, DSMZ, German Collection of Microorganisms and Cell Cultures, Berlin, Germany. Tetracycline-HCl (reagent grade) was obtained from Serva Electrophoresis, Heidelberg, Germany, and linolenic acid (99%) was bought from Acros Organics, Morris Plains, NJ, USA. Pig muscle, raw cow milk, chicken eggs, and honey were purchased at local farms or supermarkets and were either organically produced and/or known to be free from antibiotic residues.

2.2 Stock solutions and standard mixtures

Veterinary drug (VD) stock solutions at 0.1 or 1 mg/mL in different solvents (Table S1) were stored at -20 °C in the dark for up to one year, except for penicillins, which were prepared freshly every four weeks. An 81-VD standard mixture comprising all standard compounds (0.5 µg/mL each) was prepared by combining 10 µL of each 1-mg/mL VD stock solution and 100 µL of each 0.1-mg/mL VD stock solution in a 10-mL volumetric flask and making up to volume, followed by 1:2 dilution, both with ACN. A rhodamine B stock solution (1 mg/mL) was prepared and diluted with ACN (1 µg/mL). A fatty acid (FA) mixture (10 µg/mL) was prepared by combining 1 mL of respective 10-mg/mL stock solutions of caprylic (C8:0), capric (C10:0), lauric (C12:0), myristic (C14:0), palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2),

and linolenic acid (C18:3) in a 10-mL volumetric flask and making up to volume with *n*-hexane, followed by a 1:100-dilution with *n*-hexane. The 10-mg/mL stock solutions of monoacylglycerols (MAGs), diacylglycerols (DAGs), and triacylglycerols (TAGs) were 1:10 diluted with *n*-hexane to a concentration of 1 mg/mL.

2.3 Sample preparation and extraction

Samples were prepared and extracted according to the latest protocol (Mehl et al., 2022). Briefly, pig muscle, cow milk, chicken eggs, and honey samples were each pooled by homogenizing 10 g of five individual samples of the same matrix and stored at -20°C until use. For 2-min homogenization, a Multi Moulinette (Moulinex, Alençon, France) was used for meat samples, a Vortex Genie 2 with multi-tube holder (Scientific Industries, New York, NY, USA) was used for milk samples, and an Ultra-turrax T25 (IKA, Staufen, Germany) for egg samples, all at the highest speed. Honey samples were stored at 4°C , gently warmed to 40°C , and then homogenized by stirring thoroughly. Each pooled sample (2.5 g) was weighed into a 50-mL polypropylene tube and 200 μL ACN were added and the tubes were vortexed for 30 s. Then, 2.5 mL of an aqueous 0.1 M EDTA solution were added to the samples and vortexed for 30 s. In a second extraction step, 2.5 mL of a 1% HAc solution in ACN were added to the pig muscle, cow milk, and chicken egg samples, whereas 5% HAc in ACN was used for the honey samples. After 15 min end-over-end shaking at 70 rpm using a Reax 2 shaker (Heidolph Instruments, Schwabach, Germany), the samples were centrifuged at $3,000 \times g$ for 5 min (Rotanta 460 R, Hettich, Tuttlingen, Germany). The supernatants were decanted into 15-mL polypropylene tubes containing 1 g MgSO_4 and 0.25 g NaOAc for pig muscle and honey, and 1.5 g Na_2SO_4 and 0.25 g NaCl for cow milk and chicken eggs, and were immediately shaken vigorously for 15 s. The 15-mL tubes were centrifuged at $3,000 \times g$ for 10 min, and the supernatants were transferred into autosampler vials. Accordingly, reagent blanks were prepared without the sample matrices.

2.4 HPTLC and chemical derivatization

The extracts were applied as 4 mm × 20 mm area (50 or 25 µL) with an Automatic TLC Sampler 4 (ATS 4, CAMAG, Muttenz, Switzerland) on TLC silica gel 60 F₂₅₄ MS-grade plates that were prewashed twice with methanol/water 3:1 (V/V) and dried for 15 min at 110 °C in a clean oven after each prewashing step. The settings were: dosage speed 1200 nL/s, predosage volume 200 nL, filling speed 15 µL/s, filling vacuum time 4 s, retraction volume 200 nL, rinsing solvent ACN, rinsing vacuum time 0 s, rinsing cycles 2, filling cycles 1, fill only programmed volume, and nozzle heating to 60 °C, with the following layout: application position Y 10.0 mm, first track position X 10.3 mm and track distance 15.6 mm. The samples were oversprayed with 5 µL of a rhodamine B solution (1 µg/mL). Depending on the experiment, different volumes of standard solutions were additionally applied: 10 or 15 µL of the 81-VD standard mixture (0.5 µg/mL) were applied with the same settings, whereas 15 µL of the FA mixture (10 µg/mL), 5 µL of MAGs (1 mg/mL), and 20 µL of DAGs and TAGs (1 mg/mL) were applied with nozzle heating to 40 °C and *n*-hexane as rinsing solvent. After a 5-min humidity control of the layer (0–5% relative humidity using dry molecular sieve of 4 Å), the plates were first developed with ACN/ammonia/ethanol 13:4:3 (V/V/V) up to 80 mm (from the lower plate edge), and then, depending on the experiment, also in the reverse direction with methanol/ACN/ethanol/ammonia 6:2:1:1 (V/V/V/V) up to 45 mm (Automatic Developing Chamber ADC 2, CAMAG). After each step, the plates were automatically dried in a cold stream of air for 10 min. Images were recorded at UV 254 nm and fluorescence light detection (FLD) 366 nm (automatic exposure, TLC Visualizer 2, CAMAG). Instrumentation was controlled by visionCATS 3.1.21109.3 (Ultimate software package, CAMAG).

For qualitative visualization of lipids and FAs after a single front-elution with ACN/ammonia/ethanol 13:4:3 (V/V/V) up to 80 mm, the plates were piezoelectrically sprayed (Derivatizer, CAMAG; yellow nozzle, level 6) with 4 mL primuline reagent (500 mg/L primuline in acetone/water 4:1, V/V). The TAG solution was applied as positive control (1–3 µL/band, 1 mg/mL). As reagent sequence for detection of amino acids, 4 mL ninhydrin reagent (1.5 g/L

ninhydrin + 10 mM NaCl in ethanol/HAc 47:2, V/V) were sprayed (blue nozzle, level 4) onto the same plate with bovine serum albumin (2–4 µL/band, 0.1 mg/mL) as positive control. Subsequently, the plate was heated at 110 °C on the TLC Plate Heater III (CAMAG) for 5 min. Last, 4 mL aniline diphenylamine phosphoric acid reagent (20 g/L diphenylamine in methanol/*o*-phosphoric acid (85%)/aniline 44:5:1, V/V/V) was sprayed onto the plate (blue nozzle, level 6) and it was then heated at 110 °C for 10 min for detection of saccharides with lactose (1–4 µL/band, 1 mg/mL) as positive control.

2.5 Planar *B. subtilis* bioassay

For preparation of the *B. subtilis* suspension, 100 µL cryostock were cultivated in 20 mL Müller-Hinton broth (23 g/L) in a 125-mL baffled conical flask at 37 °C and 130 rpm for 14–18 h. Tetracycline (0.4–1.2 µL/band, 10 µg/mL in ethanol) was applied (ATS 4) on the plate as positive control. When the optical density at 600 nm (M501, Spectronic Camspec, Leeds, UK) was in a range of 0.8–1.2, 3.5 mL bacteria suspension were piezoelectrically sprayed onto the plate (Derivatizer, CAMAG, red nozzle, level 6). The plate was then horizontally incubated at 37 °C in a moistened plastic box (22 cm × 14 cm × 8 cm, KIS, ABM, Wolframs-Eschenbach, Germany) with filter paper lining at nearly 100% relative humidity for 2 h. Then, the plate was sprayed with 500 µL of a 0.2% PBS-buffered MTT solution (Derivatizer, blue nozzle, level 6), incubated at 37 °C and 100% relative humidity for 1.5 h, and dried for 10 min at 50 °C. Colorless antibacterial bands were obtained on a bluish-purple background.

2.6 Planar *A. fischeri* bioassay

The *A. fischeri* suspension was prepared according to DIN EN ISO 11348-1 (DIN EN ISO, 2007). In short, 150 µL cryostock were cultivated in 20 mL medium in a 125-mL baffled conical flask at room temperature and 130 rpm for 18–24 h. Caffeine (0.5–3 µL/band, 1 mg/mL in methanol) was applied (ATS 4) on the plate as positive control. When the green-blue bioluminescence of the bacteria was brilliant (observed via vessel shaking in a dark room), 4.5 mL of the suspension were sprayed onto the plate (Derivatizer, red nozzle, level 6) and

immediately placed inside the cabinet of the BioLuminizer (CAMAG). Ten images were taken over 30 min with a trigger interval of 3.0 min and an exposure time of 60 s. Antimicrobials were visible as dark zones on a bioluminescent background.

2.7 HPTLC–HESI–HRMS

Samples were applied and separated as described (section 2.4) without the second front-elution. A duplicate plate was prepared accordingly and subjected to the *B. subtilis* bioassay. On the first plate, zone positions analogous to the antibacterial zones were online eluted and transferred (95% methanol, flow rate 0.2 mL/min, provided by an Ultimate HPG-3200SD standard binary pump, Dionex Softron, Germering, Germany) using the open-source modified autoTLC–LC–MS interface with an elution time 60 s, rinsing time 30 s, and oval elution head 4 mm × 2 mm (Mehl et al., 2021b; Mehl et al., 2021a). It was coupled to a Q Exactive Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, Dreieich, Germany) equipped with an Ion-Max HESI-II probe with sheath gas 20 AU (arbitrary units), aux gas 10 AU, spray voltage 3.5 kV, capillary temperature 270 °C, probe heater temperature 200 °C and S-lens RF level 50 AU. Between the interface and the mass spectrometer, a filter frit (Upchurch Scientific A-356 and PEEK-Frit Blue UPA-703, Techlab, Erkerode, Germany) was installed to protect the heated electrospray ionization (HESI) source from particles. Full scan spectra were acquired at a resolution of 280,000 (full width at half-maximum at m/z 200) in the range of m/z 100–1000 in positive and negative ionization mode. The spectrometer was operated and spectra were recorded with Xcalibur 4.2.47 with Foundation 3.1.261.0 and SII for Xcalibur 1.5.0.10747 (Thermo Fisher Scientific) software. External mass calibration was performed daily with Pierce™ LTQ Velos ESI positive ion calibration solution and Pierce™ ESI negative ion calibration solution (Thermo Scientific). In between each zone elution, a blank position above the elution front was transferred to clean the elution head from residual analyte amounts. A background spectrum obtained from reagent blanks at a migration distance comparable to the active zones in the food extracts was subtracted from each spectrum. After

the measurement, the plates with the eluted zone imprints were subjected to the *B. subtilis* bioassay to confirm the proper elution of the zones.

3. Results and discussion

3.1 Unexpected detection of very intense antibiotic zones in blank food samples

Analogously to the high-throughput planar solid-phase extraction (HTpSPE) protocol (Mehl et al., 2022), samples free of 81 VD residues were extracted first with an EDTA solution and then with acidified ACN, followed by salting out-induced partitioning, and application of the supernatants on a TLC plate. The target analytes were front-eluted on the plate, separating them from matrix compounds with higher adsorption energy, and again front-eluted in the reverse direction, focusing them in a sharp target analyte zone. The target analyte zones were then automatically eluted into an HPLC column using a fully automated autoTLC–LC–MS interface and detected via orbitrap-HRMS/MS. The plates with the eluted zone imprints were subjected to the *B. subtilis* bioassay. Thus, colorless zones on a purple background indicated the inhibition of bacterial growth. Although the HTpSPE–UV/Vis/FLD–HPLC–HRMS/MS method showed no positive screening results for the target VDs, the *B. subtilis* bioassay revealed a surprisingly strong antibacterial activity of all four extracts as a white area spread over the entire mid-part of the plate (Fig. 1A). For further investigation, pooled blank matrices and reagent blanks (with 5% HAc, which was the highest acid content used for honey) were freshly extracted simultaneously. These were applied side-by-side with the 81-VD standard mixture and an overspray of the 81-VD standard mixture (5 ng/area each) over the blank extracts on the plate, but with twice the track distance than previously used to prevent zone diffusion of different areas into each other (Fig. 1B). The reagent blanks exhibited a very strong antibacterial response in the lower part of the plate (hR_F 20–40), whereas the blank extracts and the oversprayed blank extracts revealed very strong and partly diffuse white zones around the target analyte zone at hR_F 65. At this position, the response to the 81-VD standard mixture

differed little from the response to the reagent blanks. No relevant difference was observed between the blank extracts and the blank extracts with oversprayed VDs, and thus, the detection of VD residues in the matrices via bioassay was proven to be impossible.

These results led to the hypothesis that the strong antibacterial response could be caused by other contaminants outside the target analyte range or by endogenous compounds of the matrices. Hence, further experiments were carried out to investigate these assumptions.

3.2 Characterization of antibiotic zones by chemical derivatization and planar bioassays

For further characterization of the found antibiotic zones, reagent blanks and pooled blank extracts were applied with half the sample concentration (46.5 mg sample/area) to avoid overloading of the plate. Instead of performing a full HTpSPE procedure, the plates were only developed once up to 80 mm to separate the zones instead of focusing them. At UV 254 nm, especially the pig muscle and honey extract showed diffuse UV light absorbing zones (Fig. 2A). The matrices pig muscle, cow milk, and chicken eggs revealed green and blue fluorescent zones at UV 366 nm from hR_F 30–60, whereas honey exhibited a strong blue fluorescence over the entire migration distance (Fig. 2B). At both illumination modes, no UV light absorbing or fluorescent zones were visible in the reagent blanks (except for the orange fluorescence of rhodamine B, which was oversprayed to serve as a marker for the position of the target analytes after a full HTpSPE procedure). Under white light illumination, slightly greyish zones were observed below hR_F 50 not only in the extracts but also in the reagent blanks (Fig. 2C).

Three different chemical derivatizations were carried out on the same plate as reagent sequence via piezoelectric spraying. First, primuline reagent was sprayed onto the plate for detection of acylglycerols and FAs, which were visible as bright blue fluorescent zones in the extracts, but not in the reagent blanks (Fig. 2D). In pig muscle, a bright blue fluorescent zone was observed at hR_F 55 and rather greenish fluorescent zones at hR_F 40–50 and hR_F 80–100. The milk and egg extract showed similar weakly fluorescent zones in the mid part of the plate, whereas close to the elution front, there was a bright blue fluorescent zone in the milk extract,

but not in the egg extract. The honey extract revealed only very weak fluorescent zones. Thus, the lipid content in the muscle and milk extract was higher than in the other extracts. Then, ninhydrin derivatization was carried out for the detection of primary amino acids and proteins, visible as dark orange bands on a light orange background (Fig. 2E). The muscle and egg extract showed dark orange zones from hR_F 20–45 and an additional sharp orange band at hR_F 60 in the muscle extract. The milk extract revealed only weakly colored zones, but the honey extract showed a dark orange color close to the application area. Hence, the protein content in the muscle and egg extract was higher than in the milk extract, and the strong response in the honey extract was most probably caused by free amino acids (Morlock et al., 2022a). The last chemical derivatization step was the detection of saccharides with aniline diphenylamine phosphoric acid reagent, visible as dark bluish bands on a yellow background (Fig. 2F). As expected, the muscle, milk, and egg extract showed only weak bluish bands below hR_F 30, whereas the high sugar content of the honey extract was visible as a dark bluish-brown area at hR_F 15–35.

Two additional plates were developed once and subjected to the Gram-positive *B. subtilis* and Gram-negative *A. fischeri* bioassay, respectively. For the reagent blanks with 5% HAc (as used for the honey extract), the *B. subtilis* bioautogram (Fig. 2G) revealed an antibacterial zone at hR_F 20–40. This was caused by the HAc used for extraction, because it was observed for the reagent blanks, for 5% HAc in ACN without salting out and also in the honey extract, but not as strong in the other extracts with 1% HAc content. Reddish zones were observed in the reagent blanks at hR_F 95, which could have been caused by contaminations (*i.e.* slipping agents) of the salts used for partitioning and were present in all food extracts as well. These assumptions were confirmed by the *A. fischeri* bioautogram (Fig. 2H), in which dark zones on a bright, bioluminescent background indicated antibacterial activity. A diffuse dark zone at hR_F 20–40 was observed for the solutions containing 5% HAc and dark, sharp bands were visible close to the elution front in the reagent blanks and samples, but not for the 5% HAc in ACN. In all food extracts, there were antibacterial zones at hR_F 60 in both bioautograms (Fig. 2G and

H), that were more pronounced in the muscle and milk extract than in eggs and honey. Since the primuline derivatization (Fig. 2D) showed bright fluorescing zones in this region for muscle in particular, but also for milk and to a lesser extent for eggs and honey, this suggests that lipids were responsible for this effect. This hypothesis was supported by the fact that the milk extract showed strong antibacterial activity at hR_F 85 and 95 in the *B. subtilis* bioautogram (Fig. 2G). Additionally, a dark band in the *A. fischeri* bioautogram (Fig. 2H) was observed at hR_F 80. This position corresponded to the bright blue fluorescent zone observed for the milk extract after primuline derivatization (Fig. 2D). The antibacterial effect of FAs and MAGs is known and they were recently discussed as alternative antimicrobials (Churchward et al., 2018). Other HPTLC studies suggested that the antibacterial activity observed in milk extracts might have been caused by FAs and lipids (Morlock et al., 2022b) and have already demonstrated the antibacterial activity of FAs in plant extracts (Chandana & Morlock, 2021b, 2021a; Gawande & Morlock, 2021). For muscle, there was an intense orange band at hR_F 60 after ninhydrin derivatization, which might have contributed to the strong antibacterial activity observed in both bioautograms. The egg extract did not exhibit as strong antibacterial activity as muscle and milk, but still, there were antibacterial zones at the same hR_F positions (60 and 85). The same holds for the honey extract in the *B. subtilis* bioautogram (Fig. 2G), but in the *A. fischeri* bioautogram, there were dark bands over the whole track length, which could not be explained by any chemical derivatization. All antibacterial zones observed required further clarification and were therefore subjected to HPTLC–HRMS.

3.3 Characterization of antibacterial zones by HPTLC–HRMS

Especially for any unknown zones, the recording of HRMS spectra is an essential option to obtain molecular formulas. Reagent blanks and food extracts were applied (46.5 ng/area) on two different prewashed plates, one was subjected to the *B. subtilis* bioassay, and the other was kept clean for HPTLC–HRMS. The open-source modified, fully automated autoTLC–LC–MS interface (Mehl et al., 2021a; Mehl et al., 2021b) was chosen. The elution-head based

interface yielded detectability comparable to HPLC–MS due to the complete elution of the substance bands from the plate (Jautz & Morlock, 2006). It rapidly eluted the active zones from the clean plate into the HRMS with 100% methanol at a 0.2-mL/min flow rate based on clicks on antibacterial zones in the image of the *B. subtilis* bioautogram used as a template (depicted in the autoTLC–LC–MS software screen). No acid was added to the eluent to ensure sufficient ionization of potential FAs also in the negative mode. Ionization settings were chosen as previously used in the HPTLC–HRMS analysis of FAs (Chandana & Morlock, 2021a, 2021b; Gawande & Morlock, 2021). For each eluted zone, there were numerous mass signals detected with high or very high intensity, which is exemplarily shown for pig muscle in Fig. 3 (for milk in Fig. S1, for egg in Fig. S2, and for honey in Fig. S3). Spectra obtained from reagent blanks at the same elution position were subtracted from the spectra obtained from the food extracts. All mass signals found were then numbered, assigned the respective molecular formula, and tentatively matched to possible structures (Table 1) using mainly the SciFinderⁿ substance search (<https://scifinder-n.cas.org>) and the LIPID MAPS bulk structure search (www.lipidmaps.org/resources/tools/bulk-structure-search/create?database=COMP_DB).

As expected, zone 1 (hR_F 20–40), which was observed in the 5% HAc in ACN reagent blanks and all extracts, showed strong HAc clusters in the positive and negative mode and thus proved HAc to be responsible for the antibacterial activity against *B. subtilis* and *A. fischeri* bacteria. In honey, hexoses with the molecular formula $C_6H_{12}O_6$, *i.e.*, glucose and fructose, were detected, which is in agreement with the derivatization via the aniline diphenylamine phosphoric acid reagent (Fig. 2F) and not surprising, as saccharides are the main components of honey. The reddish zone 2 (hR_F 45) in muscle also showed mainly HAc clusters and background signals. In zone 3 (hR_F 60) with the strongest antibacterial effect, the found mass signals matched mostly with unsaturated FAs and some of their oxidation products, as well as MAGs, monoacylglycerophosphates (LPAs), monoalkylglycerols (MAG-Os), alkylacylglycerols (DAG-Os), monoalkylglycerophosphates (LPA-Os), alkylacylglycerophosphates (PA-Os), ceramides (CEs), and sterol lipids (STs). The most complex lipid composition in zone 3 was

observed in the muscle extract. Only FAs, but no other lipid classes, were detected in milk and eggs. Interestingly, in honey, the FAs 10:0, 16:0, 18:1, 18:3, the oxidized FA 12:2, and the FA degradation product 9-oxononanoic acid were evident. In zone **4** (hR_F 85), which was most prominent in milk, followed by muscle, mass signals of various FAs, MAGs, DAGs, TAGs, MAG-Os, and alkydiacylglycerols (TAG-Os) and their oxidation products were found in these two matrices. The egg extract revealed only a few mass signals of oxidized FAs and some MAGs, TAGs, and ST, whereas in honey, a larger number of FAs, DAGs, oxidized TAGs, MAG-Os, and TAG-Os was found. The reddish zone **5** (hR_F 95), observed in the muscle and milk extract, revealed compounds with a sulfonate moiety in pig muscle. A mass signal was found that matched with oxidation products of either oxidized DAGs, TAGs, or TAG-Os, as well as further unidentified mass signals. As expected, the reddish and in the case of milk, also antibacterial zone **6** in the elution front (hR_F 100) showed the largest number of mass signals of all investigated zones. Especially in milk, a large number of masses of oxidized FAs, DAGs, TAGs, DAG-Os, and TAG-Os was evident, which was also the case for the other matrices, but to a lesser extent. In eggs, the detected mass signals were partly matched to STs, and there may have also been pesticide residues in honey. The mass signals found in the reddish zone **7** (hR_F 50), which was only observed for the egg extract, could not be tentatively identified. Zone **8** (hR_F 90), which was evident in both reagent blanks and especially in the egg and honey extracts, revealed no identifiable mass signals and some oxidized FAs.

Although the exact mechanism of action of the various lipid classes is unknown, it has been hypothesized that FAs and MAGs may destabilize bacterial bilayers by acting as detergents due to their amphiphilic nature. This may induce morphological changes of bacteria, such as loss of cell shape, cell disorganization and disruption. They may also interfere with cell division and other essential cell processes. (Churchward et al., 2018)

In a recent study (Meyer & Morlock, in submission), the genotoxic activity of "healthy" oils was demonstrated using a planar SOS-Umu-C genotoxicity assay. The longer the oils were stored and the more the oils were exposed to oxygen during storage, the stronger the effects on the

test organism. This was mainly due to the epoxidation of (poly)unsaturated lipids. Therefore, it can be assumed that epoxidized lipid species may be responsible for the antibacterial effects observed in our study. This assumption is supported by the HRMS results, as a large number of oxidized lipid species were identified (Table 1).

3.4 Confirmation of the HRMS results using lipid standards

To further confirm the results obtained by HPTLC–HRMS, reagent blanks, food extracts, and standard solutions of 81 VDs, FAs, MAGs, DAGs, and TAGs were applied side-by-side on one TLC plate, separated with a single development, and then subjected to the *B. subtilis* (Fig. 4A) and *A. fischeri* (Fig. 4B) bioassays. The 81-VD standard mixture was applied in higher amounts (7.5 ng each/area) than to be expected in trace analysis. It was visible that the antibiotics were distributed between hR_F 60–100 in both assays, but the antibacterial effect was more dominant in the *B. subtilis* bioassay. If a full HTPSPE procedure was performed, the distributed antibiotic zones would be focused in the target analyte zone at hR_F 60. The FA mixture on the other hand completely coeluted at hR_F 65, where the most prominent antibiotic activity was evident in all food extracts. This corresponded to the HRMS results, as most mass signals found in zone 3 could be matched to various FAs. The MAG standard migrated up to hR_F 90 and showed antibacterial activity as a diffuse pale blue zone in the *B. subtilis* bioautogram, and a diffuse, dark zone at hR_F 90 as well as an additional dark band at hR_F 70 in the *A. fischeri* bioautogram, possibly due to a standard impurity or degradation products. In zones 4 and 5 in the same region on the plate, mass signals of MAGs and MAG-Os were found in the HRMS spectra, whereas in zone 6 at hR_F 100, mostly DAGs, DAG-Os, TAGs, and TAG-Os were detected. The DAG standard migrated almost up to the elution front and exhibited a zone with a purple center and diffuse bluish edges, as was also observed for zone 6 in the *B. subtilis* bioautogram, particularly in the muscle and milk extract. In the *A. fischeri* bioautogram, the DAG standard appeared as a dark spot with a lighter center. The TAG standard migrated with the solvent front (despite some tailing), but did not show a substantial effect in the *B. subtilis*

or *A. fischeri* bioautograms, respectively. In the HRMS spectra, mass signals of TAGs or TAG Os were mainly found in zones 4–6 above hR_f 80.

Hence, these results confirmed the tentative assignment of different lipid classes to the mass signals found. The lipids present in the food extracts were proven to be the main contributors to the antibacterial effects observed in the two bioassays, and additionally, some chemicals used during the extraction, such as acetic acid. The antibacterial effect of FAs and MAGs has been known for over 100 years and they have been discussed as alternative antimicrobials recently (Churchward et al., 2018). Depending on the FA and lipid composition in the food, different effects on the human gut microbiota can be expected. Although beneficial effects of n -3 (ω -3) polyunsaturated FAs on the human microbiome have been reported (Costantini et al., 2017), latest research has shown the genotoxic potential of unsaturated lipids (Meyer & Morlock, in submission). Further research in this field is necessary to investigate this discrepancy.

4. Conclusions

Hyphenated HPTLC–UV/vis/FLD–EDA–HRMS provided new insights into the antibacterial power of animal-derived food. The Gram-positive *B. subtilis* and the Gram-negative *A. fischeri* bioassay revealed strong antibacterial zones caused by extracts of pig muscle, cow milk, chicken eggs, and honey. Multiple chemical derivatizations, HRMS recording, and comparison to standard compounds proved endogenous FAs and various lipid classes to be mainly responsible for the antibacterial activity. These findings explain the up to 87% positive results obtained from biological screening for antibiotic residues without confirmation by HPLC–MS/MS. Depending on the animal species, sex, and type of feed, animal-derived foods may have different lipid compositions, resulting in varying degrees of antibacterial activity. Targeted HPLC–MS/MS methods are unable to detect these compounds, as they are blind to contaminants or active metabolites outside the analytical focus and are also designed to

reduce co-extractives of endogenous matrix compounds that might cause ion suppression. Non-target HPLC–HRMS methods are designed to detect off-target compounds, but one can get lost in the thousands of mass signals caused by unknowns because effect-directed prioritization of the important compounds is missing. Current non-target biological assays do not represent a solution for such problems, as they cannot selectively detect the compounds responsible for the antibacterial effect, only providing sum parameters. The rapid, non-target planar screening strategy proved to be an ideal tool to clarify this issue because it directly links the separation of all extract compounds to their bioactive effect and consequently to their identity via HRMS spectra recording and comparison with standard compounds, thus resolving the methodological inconsistency. In this study, the antibacterial activity by endogenous FAs and lipids present in the food matrices was much stronger than the antibacterial effect of a mixture of 81 VDs. The influence of these endogenous compounds on the human microbiome still needs to be clarified. Our findings challenge the current understanding of food safety based on target analysis often combined with precedent positive bioassay screening. The question arises which compounds are important for food safety, only exogenous target compounds or all compounds present that have a specific effect.

CRedit authorship contribution statement

Annabel Mehl: Methodology, Investigation, Formal analysis, Writing – Original draft; **Gertrud E. Morlock:** Conceptualization, Supervision, Resources, Writing – Review & Editing

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at...

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Table 1. Complete list of analytes found in the food extracts and their tentative assignment to possible (lipid) structures.

Zone	Mass signal <i>m/z</i>	Adduct	Molecular formula	Δ ppm	Tentative assignment	Observed in
1	203.0526	[M105+Na] ⁺		-0.14		
	179.0560	[M105-H] ⁻	C ₆ H ₁₂ O ₆	0.08	Hexose, pentose	E4
	359.1196	[2M105-H] ⁻		-0.65		
	136.0482	[M1+Na] ⁺	C ₄ H ₇ N ₃ O	-0.27	-	E1
	195.0992	[M2+Na] ⁺		-0.12		
	217.0812	[M2-H+2Na] ⁺	C ₉ H ₁₆ O ₃	0.19	9-oxononanoic acid (FA degradation product)	E1, E2; E3, E4
	171.1028	[M2-H] ⁻		-0.71		
	235.1304	[M3+Na] ⁺		0.37		
	211.1339	[M3-H] ⁻	C ₁₂ H ₂₀ O ₃	0.23	FA 12:2;O	E1, E3, E4
	321.2399	[M4+Na] ⁺	C ₁₈ H ₃₄ O ₃	0.39	FA 18:1;O	E1
	319.2243	[M5+Na] ⁺		0.27		
	295.2279	[M5-H] ⁻	C ₁₈ H ₃₂ O ₃	-0.21	FA 18:2;O, MAG-O 15:3	E1
	325.2112	[M6-H+2Na] ⁺		0.54	FA 18:2	
	279.2331	[M6-H] ⁻	C ₁₈ H ₃₂ O ₂	-0.63	FA 18:2	E1, E2, E3
	327.2268	[M7-H+2Na] ⁺		0.81		
	281.2487	[M7-H] ⁻	C ₁₈ H ₃₄ O ₂	-0.31	FA 18:1	E1, E2, E3, E4
	335.2190	[M8+Na] ⁺	C ₁₈ H ₃₂ O ₂	0.87	FA 18:2, MAG 15:2, MAG-O 15:3;O	E1
	605.4516	[M9+H] ⁺	C ₃₃ H ₆₅ O ₇ P	4.06	LPA 30:1, LPA-O 30:2;O, PA-O 30:1	E1
	629.1359	[M10+Na] ⁺	C ₃₃ H ₆₇ O ₇ P	3.98	LPA 30:0, LPA-O 30:1;O, PA-O 30:0	E1
3	301.1113	[M11-H+2Na] ⁺		0.32		
	255.2332	[M11-H] ⁻	C ₁₆ H ₃₂ O ₂	-0.89	FA 16:0	E1, E2, E3, E4
	303.2331	[M12-H] ⁻	C ₂₀ H ₃₂ O ₂	-0.48	FA 20:4	E1
	305.2488	[M13-H] ⁻	C ₂₀ H ₃₄ O ₂	-0.71	FA 20:3	E1
	307.2645	[M14-H] ⁻	C ₂₀ H ₃₆ O ₂	-0.64	FA 20:2	E1
	308.2801	[M15-H] ⁻	C ₂₀ H ₃₈ O ₂	-0.50	FA 20:1	E1
	535.4737	[M16-H] ⁻	C ₃₄ H ₆₄ O ₄	-0.87	FA 34:2;O2, MAG 31:2, MAG-O 31:3;O, DAG-O 31:2	E1
	561.4893	[M17-H] ⁻	C ₃₆ H ₆₆ O ₄	-0.74	MAG 33:3, MAG-O 33:4;O, DAG-O 33:3,	E1
	583.4715	[M18-H] ⁻	C ₃₈ H ₆₄ O ₄	2.94	DAG-O 35:6, CE 11:1;O2	E1
	223.1304	[M48+Na] ⁺	C ₁₁ H ₂₀ O ₃	0.25	FA 11:1;O	E2
	227.1253	[M49+Na] ⁺	C ₁₀ H ₂₀ O ₄	0.40	FA 10:0;O2	E2, E4
	227.2018	[M50-H] ⁻	C ₁₄ H ₂₈ O ₂	-1.04	FA 14:0	E2
	277.2175	[M51-H] ⁻		-0.56	FA 18:3, ST 18:0;O2	E2, E4
	544.2680	[M84+Na] ⁺		-0.64	-	E3
	283.2643	[M85-H] ⁻		0.01	FA 18:0	E3
	498.2898	[M86-H] ⁻		-	-	E3
	209.0818	[M106-H] ⁻		0.50	-	E4
	171.1027	[M2-H] ⁻	C ₉ H ₁₆ O ₃	-0.30	9-oxononanoic acid	E1
	269.1359	[M19+Na] ⁺		0.32		
	245.1395	[M19-H] ⁻	C ₁₂ H ₂₂ O ₅	-0.13	FA 12:1;O3	E1, E2, E3
	301.1621	[M20+Na] ⁺	C ₁₃ H ₂₆ O ₆	0.34	FA 13:0;O4	E1, E2
	353.2660	[M21+Na] ⁺	C ₁₉ H ₃₈ O ₄	0.51	FA 19:0;O2, MAG 16:0, MAG-O 16:1;O	E1, E2
	379.2817	[M22+Na] ⁺	C ₂₁ H ₄₀ O ₄	0.48	FA 21:1;O2, MAG 18:1, MAG-O 18:2;O	E1
	661.4650	[M23+Na] ⁺	C ₃₇ H ₆₆ O ₈	-0.04	TAG 34:2;O2, TAG-O 34:3;O3	E1, E3
	689.4962	[M24+Na] ⁺	C ₃₉ H ₇₀ O ₈	0.09	TAG 36:2;O2, TAG-O 36:3;O3	E1
	773.6264	[M25+Na] ⁺	C ₄₆ H ₈₆ O ₇	0.27	DAG 43:2;O2, TAG 43:1;O, TAG-O 43:2;O2	E1
	291.1452	[M26-H] ⁻	C ₁₃ H ₂₄ O ₇	-0.83	-	E1
	305.1608	[M27-H] ⁻	C ₁₄ H ₂₆ O ₇	-0.66	-	E1, E2
	375.2756	[M28-H] ⁻	C ₂₀ H ₄₀ O ₆	-1.11	FA 20:0;O4	E1, E2
	389.2915	[M29-H] ⁻	C ₂₁ H ₄₂ O ₆	-1.45	FA 21:0;O4	E1, E2
4	415.3071	[M30-H] ⁻	C ₂₃ H ₄₄ O ₆	-1.39	FA 23:1;O4, DAG 20:0;O, DAG-O 20:1;O2	E1
	297.2036	[M52+Na] ⁺	C ₁₅ H ₃₀ O ₄	0.51	FA 15:0;O2, MAG 12:0, MAG-O 12:1;O	E2
	325.2348	[M53+Na] ⁺	C ₁₇ H ₃₄ O ₄	0.86	FA 17:0;O2, MAG 14:0, MAG-O 14:1;O	E2
	333.2285	[M54-H] ⁻	C ₁₇ H ₃₄ O ₆	-0.71	FA 17:0;O4	E2
	361.2599	[M55-H] ⁻	C ₁₉ H ₃₈ O ₆	-1.04	FA 19:0;O4	E2
	675.5170	[M75+Na] ⁺	C ₃₉ H ₇₂ O ₇	-0.11	DAG 38:2;O2, TAG 38:1;O, TAG-O 38:2;O2	E4
	309.1671	[M87+Na] ⁺	C ₁₅ H ₂₆ O ₅	0.50	FA 15:2;O3, MAG 12:2;O	E3
	437.1934	[M88+Na] ⁺	C ₂₄ H ₃₀ O ₆	0.07	ST 18:5;O;Hex, ST 24:6;O6	E3, E4
	305.1607	[M89-H] ⁻	C ₁₄ H ₂₆ O ₅	-0.36	-	E3
	149.0210	[M108+Na] ⁺		-0.70	-	E4
	207.0992	[M109+Na] ⁺	C ₁₀ H ₁₆ O ₃	-0.36	FA 10:2;O	E4
	209.1149	[M110+Na] ⁺	C ₁₀ H ₁₈ O ₃	-0.21	FA 10:1;O	E4
	247.1305	[M111+Na] ⁺	C ₁₃ H ₂₀ O ₃	0.02	FA 13:3;O	E4

	263.1254	[M112+Na] ⁺	C ₁₃ H ₂₀ O ₄	0.00	FA 13:3;O2	E4
	367.3182	[M113+Na] ⁺	C ₂₁ H ₄₄ O ₃	0.10	MAG-O 18:0	E4
	395.3495	[M114+Na] ⁺	C ₂₃ H ₄₈ O ₃	0.04	MAG-O 20:0	E4
	703.5485	[M115+Na] ⁺	C ₄₁ H ₇₆ O ₇	-0.09	DAG 38:2;O2, TAG 38:1;O, TAG-O 38:2;O2	E4
	153.0921	[M116-H] ⁻	C ₉ H ₁₄ O ₂	0.03	-	E4
	182.0459	[M117-H] ⁻	C ₈ H ₈ NO ₄	-0.20	-	E4
	243.1237	[M118-H] ⁻	C ₁₂ H ₂₀ O ₅	0.28	-	E4
	264.0490	[M119-H] ⁻	C ₉ H ₁₃ O ₃	-1.20	-	E4
	283.1552	[M120-H] ⁻	C ₁₅ H ₂₄ O ₅	-0.32	-	E4
	299.1500	[M121-H] ⁻	C ₁₅ H ₂₄ O ₆	-0.05	-	E4
	320.2559	[M31+H] ⁺	C ₁₇ H ₃₅ O ₅	-0.11	-	E1, E2
	297.1532	[M32-H] ⁻	C ₁₆ H ₂₆ O ₇ S	-0.27	sulfonate	E1
	311.1690	[M33-H] ⁻	C ₁₇ H ₂₆ O ₇ S	-0.64	sulfonate	E1
	325.1845	[M34-H] ⁻	C ₁₈ H ₃₀ O ₃ S	-0.37	sulfonate	E1
	194.1152	[M56+H] ⁺	C ₈ H ₁₇ O ₅	-1.82	2,3,5-tri- <i>o</i> -methyl-arabinopyranose	E2
	208.1309	[M57+H] ⁺	C ₉ H ₁₉ O ₅	-1.41	3-methoxy-2-(methoxymethyl)-2-methyl-propan-1-ol acetate	E2
5	222.1464	[M58+H] ⁺	C ₁₀ H ₂₁ O ₅	-1.19	-	E2
	236.1620	[M59+H] ⁺	C ₁₁ H ₂₃ O ₅	-0.91	-	E2
	250.1777	[M60+H] ⁺	C ₁₂ H ₂₅ O ₅	-0.74	-	E2
	605.4389	[M61+Na] ⁺	C ₃₄ H ₆₂ O ₇	-0.17	DAG 31:2;O2, TAG 31:1;O, TAG-O 31:2;O2	E2
	391.1978	[M62-H] ⁻	C ₁₈ H ₃₂ O ₉	-1.15	-	E2
	419.2655	[M63-H] ⁻	C ₂₁ H ₄₀ O ₈	-0.99	-	E2
	447.2968	[M64-H] ⁻	C ₂₃ H ₄₄ O ₈	-0.93	-	E2
	475.3279	[M65-H] ⁻	C ₂₅ H ₄₈ O ₈	-0.62	-	E2
	661.4650	[M23+Na] ⁺	C ₃₇ H ₆₆ O ₈	-0.07	TAG 34:2;O2, TAG-O 34:3;O3	E4
	423.2354	[M35+Na] ⁺	C ₂₁ H ₃₆ O ₇	-0.15	ST 21:0;O7	E1, E3
	455.2617	[M36+Na] ⁺	C ₂₂ H ₄₀ O ₈	-0.42	-	E1, E3
	507.3660	[M37+Na] ⁺	C ₂₈ H ₅₂ O ₆	-0.75	FA 28:2;O4, DAG 25:1;O, DAG-O 25:2;O2 FA 30:3;O4, DAG 27:2;O, DAG-O 27:3;O2, ST 30:0;O6	E1, E3
	533.3816	[M38+Na] ⁺	C ₃₀ H ₅₄ O ₆	-0.73	MAG 34:2;O, DAG 34:1; DAG-O 34:2;O, TAG-O 34:1	E1
	617.5119	[M39+Na] ⁺	C ₃₇ H ₇₀ O ₅	-0.51	-	E1, E4
	233.0854	[M40-H] ⁻	C ₁₀ H ₁₈ O ₄ S	-0.24	-	E1
	293.1794	[M41-H] ⁻	C ₁₄ H ₃₀ O ₄ S	-0.80	-	E1
	409.3115	[M42-H] ⁻	C ₂₈ H ₄₂ O ₂	-0.85	FA 28:7, ST 28:4;O2	E1
	473.2829	[M43-H] ⁻	C ₂₈ H ₄₂ O ₄ P	-0.63	-	E1, E3
	543.3908	[M44-H] ⁻	C ₃₀ H ₅₆ O ₈	-1.24	-	E1, E2
	569.4066	[M45-H] ⁻	C ₃₃ H ₅₄ N ₄ O ₄	1.39	-	E1
	625.5059	[M46-H] ⁻	C ₃₇ H ₇₀ O ₇	-1.18	DAG 34:1;O2, TAG 34:0;O, TAG-O 34:1;O2	E1
	639.5216	[M47-H] ⁻	C ₃₈ H ₇₂ O ₇	-1.11	DAG 35:1;O2, TAG 35:0;O, TAG-O 35:1;O2	E1
	653.5373	[M48-H] ⁻	C ₃₉ H ₇₄ O ₇	-1.16	DAG 36:1;O2, TAG 36:0;O, TAG-O 36:1;O2	E1
	339.2142	[M66+Na] ⁺	C ₁₇ H ₃₂ O ₅	-0.04	FA 17:1;O3, MAG 14:1;O	E2
	423.3080	[M67+Na] ⁺	C ₂₃ H ₄₄ O ₅	-0.13	FA 23:1;O3, MAG-20:1;O DAG 20:0, DAG-O 20:1;O	E2
	465.2824	[M68+Na] ⁺	C ₂₄ H ₄₅ O ₇	-0.61	DAG 21:2;O2, ST 24:0;O7	E2
	493.3500	[M69+Na] ⁺	C ₂₇ H ₅₀ O ₆	-0.42	DAG 24:1;O, TAG 30:0, DAG-O 24:2;O2	E2
6	521.3814	[M70+Na] ⁺	C ₂₉ H ₅₄ O ₆	-0.57	FA 29:2;O4, DAG 26:1;O, DAG-O 26:2;O2	E2
	549.4127	[M71+Na] ⁺	C ₃₁ H ₅₈ O ₆	-0.42	FA 31:2;O4, DAG 28:1;O, DAG-O 28:2;O2	E2
	577.4439	[M72+Na] ⁺	C ₃₃ H ₆₂ O ₆	-0.33	FA 33:2;O4, DAG 30:1;O, TAG 30:0, DAG-O 30:2;O, TAG-O 30:1;O	E2
	603.4598	[M73+Na] ⁺	C ₃₅ H ₆₄ O ₆	-0.43	DAG 32:2;O, TAG 32:1, DAG-O 32:3;O2, TAG-O 32:2;O	E2
	631.4910	[M74+Na] ⁺	C ₃₇ H ₆₈ O ₆	-0.36	DAG 34:2;O, TAG 34:1, DAG-O 34:3;O2, TAG-O 34:2;O	E2
	675.5170	[M75+Na] ⁺	C ₃₉ H ₇₂ O ₇	-0.18	DAG 36:2;O2, TAG 36:1;O, TAG-O 36:2;O2	E2
	701.5329	[M76+Na] ⁺	C ₄₁ H ₇₄ O ₇	-0.32	DAG 38:3;O2, TAG 38:2;O, TAG-O 38:3;O2	E2
	187.0071	[M77-H] ⁻	C ₇ H ₈ O ₄ S	0.29	-	E2, E3
	201.0227	[M78-H] ⁻	C ₈ H ₁₀ O ₄ S	0.02	-	E2
	217.0176	[M79-H] ⁻	C ₈ H ₁₀ O ₅ S	0.28	-	E2
	377.0702	[M80-H] ⁻	C ₁₈ H ₁₈ O ₇ S	-0.50	-	E2
	393.2975	[M96+Na] ⁺	C ₂₂ H ₄₂ O ₄	0.10	FA 22:1;O2, MAG 19:1, MAG-O 19:2;O	E3, E4
	463.2666	[M97+Na] ⁺	C ₂₄ H ₄₆ O ₇	0.01	DAG 21:3;O2, ST 18:0;O2;Hex, ST 24:1;O7	E3
	549.3037	[M98+Na] ⁺	C ₂₈ H ₄₆ O ₉	-0.54	ST 22:1;O4;Hex	E3
	577.3350	[M99+Na] ⁺	C ₃₀ H ₅₀ O ₉	-0.47	ST 24:1;O4;Hex	E3, E4
	609.3612	[M100+Na] ⁺	C ₃₁ H ₅₄ O ₁₀	-0.54	ST 25:0;O5;Hex, MGDG 22:2	E3, E4
	239.0676	[M101-H] ⁻	Unknown	-	-	E3
	381.2288	[M102-H] ⁻	C ₂₁ H ₃₄ O ₆	-1.43	FA 21:4;O4, ST 21:1;O6	E3
	459.2606	[M103-H] ⁻	C ₂₃ H ₄₀ O ₉	-1.44	-	E3
	491.2867	[M104-H] ⁻	C ₂₄ H ₄₄ O ₁₀	-1.17	-	E3
	679.5526	[M105-H] ⁻	C ₄₁ H ₇₆ O ₇	-1.13	DAG 38:2;O2, TAG 38:1;O, TAG-O 38:2;O2	E3

	223.1304	[M128+Na] ⁺	C ₁₁ H ₂₀ O ₃	0.12	FA 11:1;O	E4
	335.2791	[M129+Na] ⁺	C ₁₈ H ₃₂ O ₄	0.51	FA 18:2;O2, MAG 15:2, MAG-O 15:3;O	E4
	447.3803	[M130+Na] ⁺	C ₂₇ H ₅₂ O ₃	0.17	FA 27:1;O, MAG-O 24:2	E4
	521.4178	[M131+Na] ⁺	C ₃₀ H ₅₈ O ₅	-0.33	FA 30:1;O3, MAG 27:1;O, DAG 27:0, DAG-O 27:1;O	E4
	549.4492	[M132+Na] ⁺	C ₃₂ H ₆₂ O ₅	-0.37	FA 32:1;O3, MAG 29:1;O, DAG 29:0, DAG-O 29:1;O	E4
	239.0674	[M133-H] ⁻	C ₁₀ H ₁₂ N ₂ O ₅	-0.26	dinoseb, dinoterb, dinoprop	E4
	650.4395	[M81+H] ⁺	C ₃₇ H ₆₁ O ₉	-1.08	-	E3
	672.4215	[M81+Na]⁺		-0.99	-	E3
7	712.4526	[M82+H] ⁺	C ₃₅ H ₆₈ O ₁₂ P	-0.69	-	E3
	758.5697	[M83+H] ⁺	C ₄₆ H ₇₇ O ₈	-0.78	-	E3
	780.55156	[M83+Na]⁺		-0.66	-	E3
	236.1620	[M55+H] ⁺	C ₁₁ H ₂₃ O ₅	-1.41	-	E4
	675.5170	[M75+Na] ⁺	C ₃₉ H ₇₂ O ₇	-0.33	DAG 36:2;O2, TAG 36:1;O, TAG-O 36:2;O2	E4
	194.1153	[M90+Na] ⁺	C ₉ H ₁₇ NO ₂	-0.62	-	E3
	226.1413	[M91+Na] ⁺	C ₁₀ H ₂₁ NO ₃	0.11	-	E3
	236.1621	[M92+Na] ⁺	C ₁₂ H ₂₃ NO ₂	0.13	-	E3
	278.2454	[M93+H] ⁺	C ₁₅ H ₃₄ O ₄	-0.82	-	E3
8	320.2559	[M94+H] ⁺	C ₁₇ H ₃₅ O ₅	-0.54	-	E3, E4
	352.2457	[M95+H] ⁺	C ₁₇ H ₃₅ O ₇	-0.38	-	E3, E4
	222.1465	[M122+H] ⁺	C ₁₁ H ₂₁ NO ₂	-0.41	-	E4
	250.1778	[M123+H] ⁺	C ₁₂ H ₂₆ O ₅	-1.10	-	E4
	277.1776	[M124+H] ⁺	C ₁₅ H ₂₆ O ₃	-0.34	FA 15:2;O, MAG-O 12:3	E4
	221.1184	[M125-H] ⁻	C ₁₃ H ₁₈ O ₃	-0.23	FA 11:2;O	E4
	237.1133	[M126-H] ⁻	C ₁₃ H ₁₈ O ₄	-0.45	FA 11:2;O2	E4
	281.1396	[M127-H] ⁻	C ₁₅ H ₂₂ O ₅	-0.61	-	E4

FA = fatty acid, MAG = monoacylglycerol, DAG = diacylglycerol, TAG = triacylglycerol, LPA = monoacylglycerophosphate, MAG-O = monoalkylglycerol, DAG-O = alkylacylglycerol, TAG-O = alkyltriacylglycerol, LPA-O = monoalkylglycerophosphate, PA-O = alkylacylglycerophosphate, CE = ceramide, ST = sterol lipid, MGDG = monogalactosyldiacylglycerol; the first number after the lipid class indicates the number of carbons in the acyl/alkyl chain, the number behind the colon indicates the number of double bonds in the chain; O = additional oxygens, Hex = additional hexyl ring; E1 = pig muscle, E2 = cow milk, E3 = chicken egg = E4 = honey; main mass signals in bold

Figure legends

Fig. 1. HTPSPE–*B. subtilis* bioautograms of blank extracts (93 mg/area) after HTPSPE–autoTLC–LC–HRMS/MS measurement (A) according to the latest protocol (Mehl et al., 2022), and reagent blanks compared to pooled blank extracts and overspray of the 81-standard mixture (5 ng each/area) over the latter (B), both after two-fold front-elution with first ACN/ammonia/ethanol 13:4:3 (V/V/V) up to 80 mm, and second with methanol/ACN/ethanol/ammonia 6:2:1:1 (V/V/V/V) up to 45 mm in the reverse direction on TLC silica gel 60 F₂₅₄ MS-grade plates at white light illumination. ACN: acetonitrile, B1: reagent blank MgSO₄/NaOAC, B2: reagent blank Na₂SO₄/NaCl, 1%: 1% HAc in ACN, 5%: 5% HAc in ACN, E1: pig muscle, E2: cow milk, E3: chicken eggs, E4: honey, 81: 81-standard mixture.

Fig. 2. HPTLC–UV/vis/FLD chromatograms of reagent blanks compared to pooled blank extracts (46.5 mg/area) after a single development with ACN/ammonia/ethanol 13:4:3 (V/V/V) up to 80 mm (A–C), after derivatization with primuline (D), ninhydrin (E), aniline diphenylamine phosphoric acid (F) as reagent sequence, and respective bioautograms after *B. subtilis* (G) and *A. fischeri* bioassay (H) on TLC silica gel 60 F₂₅₄ MS-grade at UV 254 nm (A), FLD 366 nm (B, D), white light illumination (C, E–G), and native bioluminescence (F). 5%: 5% HAc in ACN, B1: reagent blank MgSO₄/NaOAC, B2: reagent blank Na₂SO₄/NaCl, E1: pig muscle, E2: cow milk, E3: chicken eggs, E4: honey; orange band: marker rhodamine B.

Fig. 3. HPTLC–*B. subtilis* bioautograms of reagent blanks compared to pooled blank extracts (46.5 mg/area) after a single development with ACN/ammonia/ethanol 13:4:3 (V/V/V) up to 80 mm on TLC silica gel 60 F₂₅₄ MS-grade; template for the HPTLC–autoTLC–HRMS measurement (A) and imprinted plate duplicate after the measurement and subsequent *B. subtilis* bioassay (B). Molecular formulas of antibacterial zones 1–8 were assigned to the

respective mass signals found (Table 1) in pig muscle (E1). 5%: 5% HAc in ACN, B1: reagent blank $\text{MgSO}_4/\text{NaOAc}$, B2: reagent blank $\text{Na}_2\text{SO}_4/\text{NaCl}$.

Fig. 4. HPTLC bioautograms of reagent blanks compared to pooled blank extracts (23.25 mg/area) and 81-standard mixture (7.5 ng/area each), FA mixture (150 ng/area each), MAGs (5 $\mu\text{g}/\text{area}$), DAGs and TAGs (20 $\mu\text{g}/\text{area}$) after development with ACN/ammonia/ethanol 13:4:3 (V/V/V) up to 80 mm on TLC silica gel 60 F_{254} MS-grade plates, after *B. subtilis* bioassay at white light illumination (A), and native bioluminescence of *A. fischeri* bacteria (B). B1: reagent blank $\text{MgSO}_4/\text{NaOAc}$, B2: reagent blank $\text{Na}_2\text{SO}_4/\text{NaCl}$, 5%: 5% HAc in ACN, E1: pig muscle, E2: cow milk, E3: chicken eggs, E4: honey, 81: 81-standard mixture, FA: fatty acid, MAG: monoacylglycerols DAG: diacylglycerols, TAG: triacylglycerols.

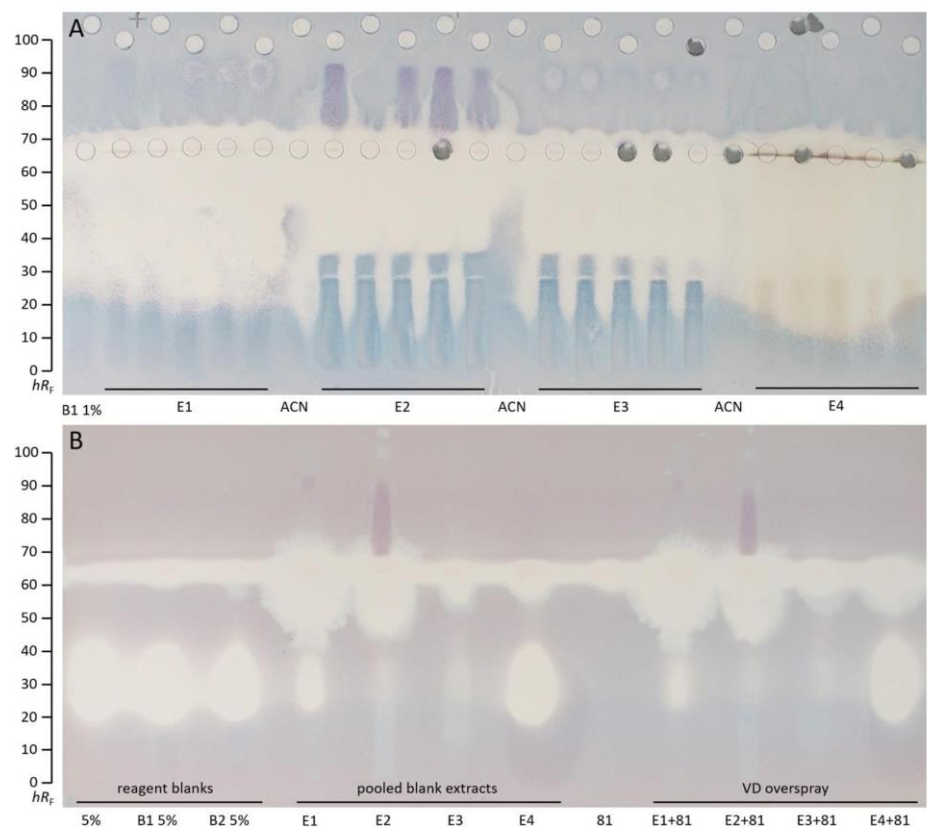


Fig. 1

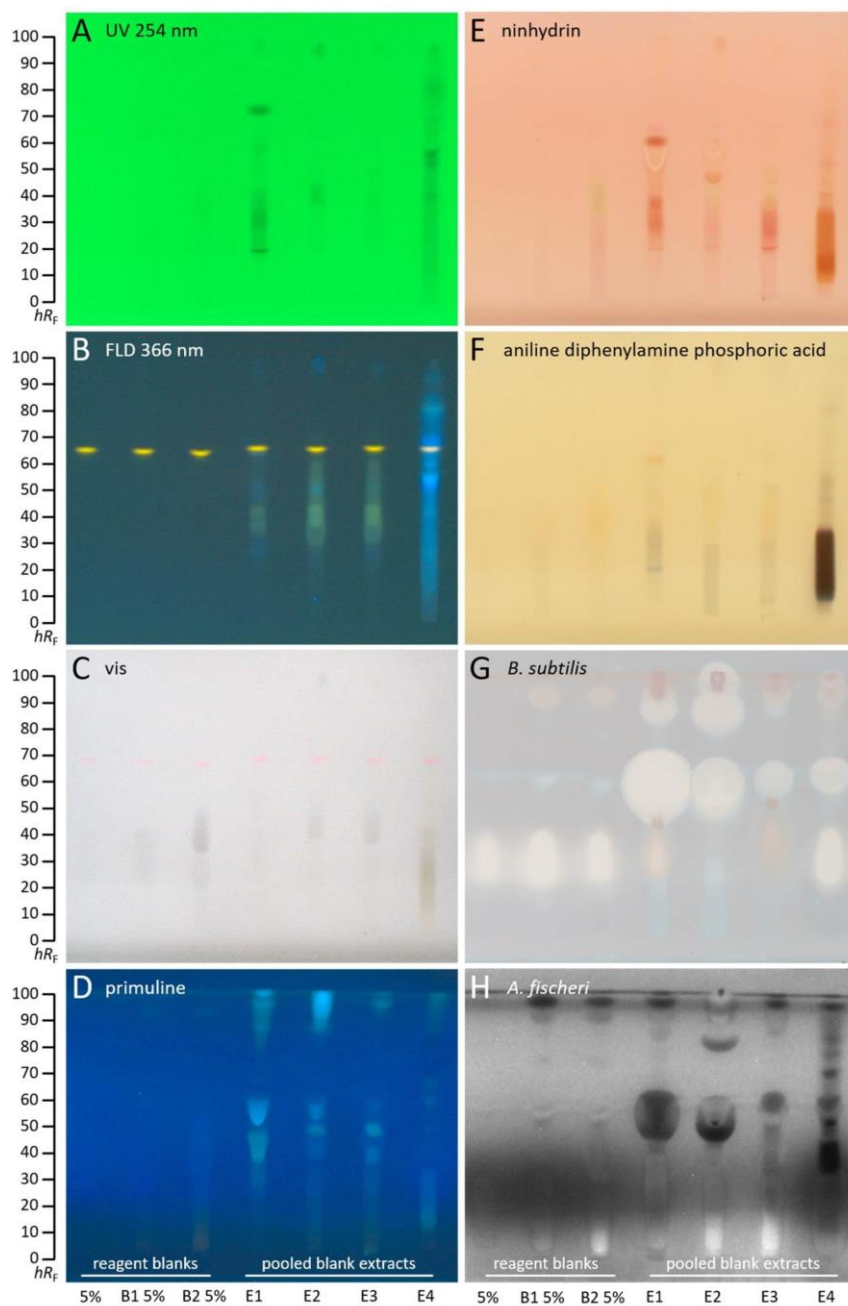


Fig. 2

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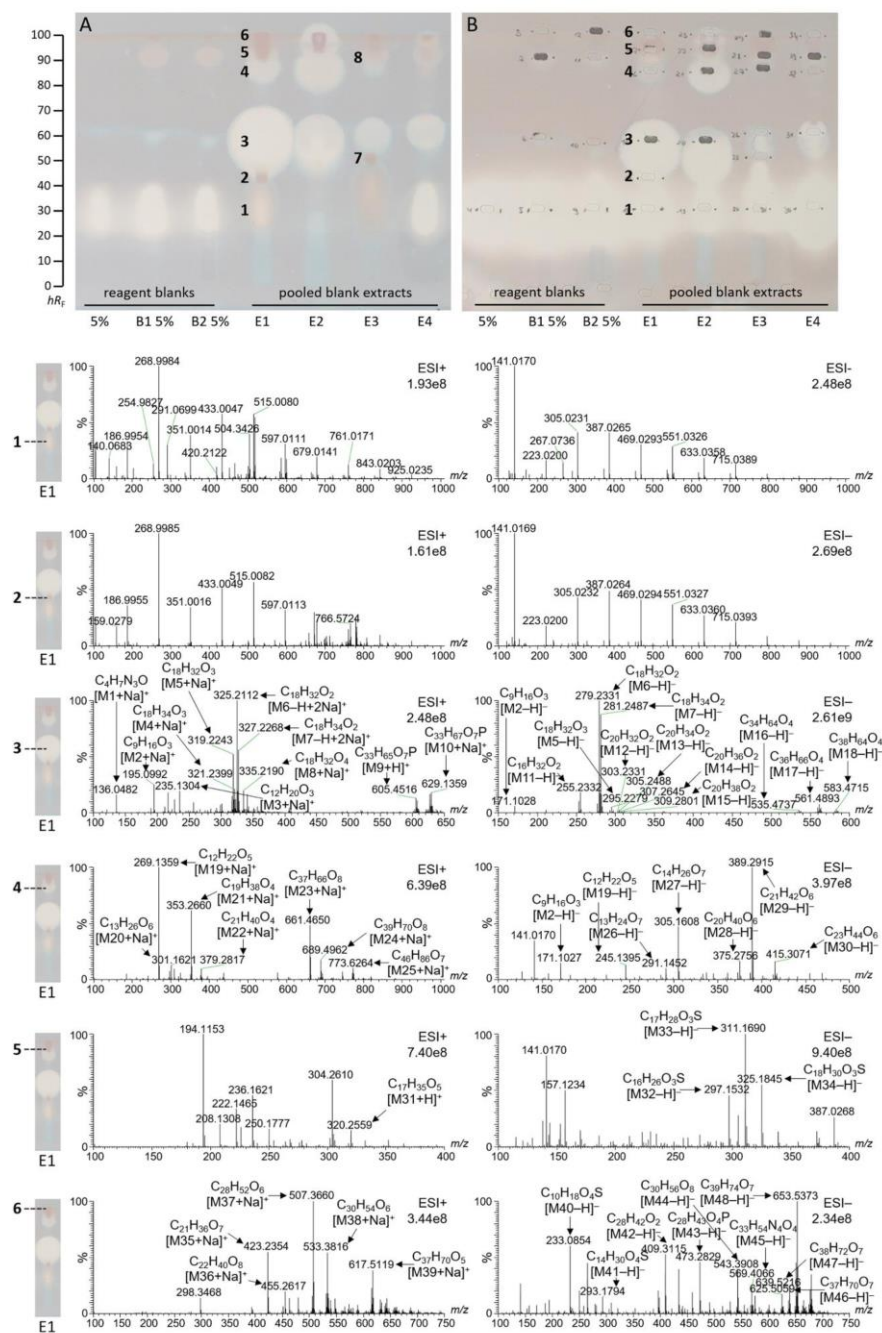


Fig. 3

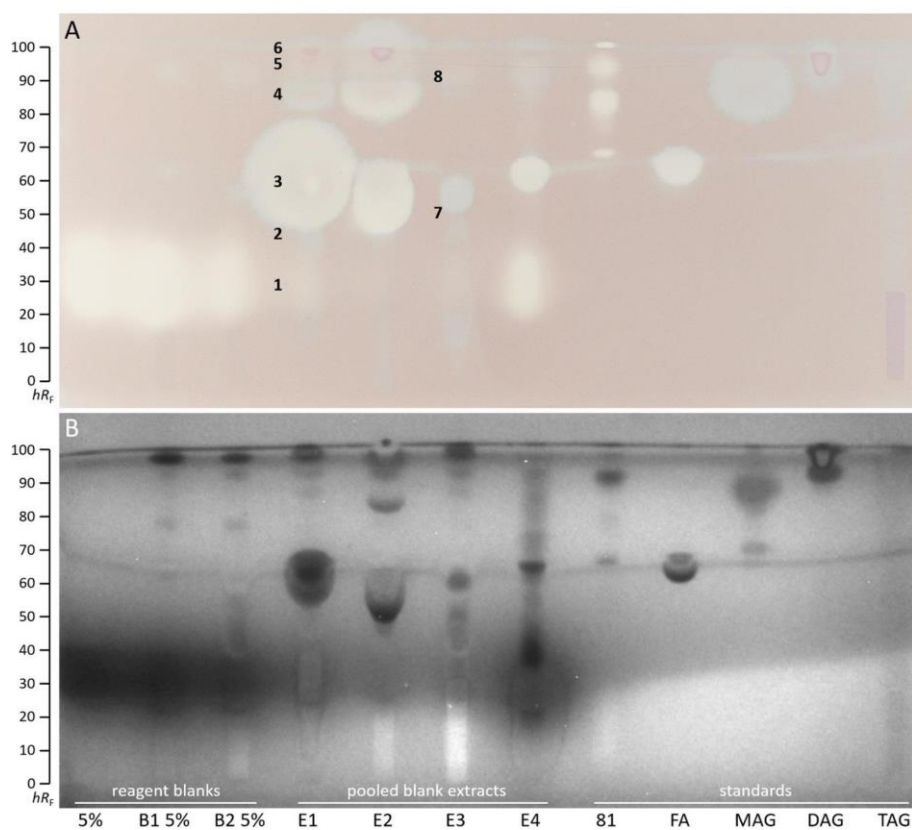


Fig. 4

Supplementary material

Strong antibacterial effects in animal-derived food

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Page S-6	Fig. S2. HPTLC– <i>B. subtilis</i> bioautograms of reagent blanks compared to pooled blank extracts (46.5 mg/area) after a single development with ACN/ammonia/ethanol 13:4:3 (V/V/V) up to 80 mm on TLC silica gel 60 F ₂₅₄ MS-grade; template for the HPTLC–autoTLC–HRMS measurement (A) and imprinted plate duplicate after the measurement and subsequent <i>Bacillus subtilis</i> bioassay (B). Molecular formulas of antibacterial zones 1–8 were assigned to the respective mass signals found (see also Table 1) in chicken egg (E3). 5%: 5% HAc in ACN, B1: reagent blank MgSO ₄ /NaOAc, B2: reagent blank Na ₂ SO ₄ /NaCl.
Page S-7	Fig. S3. HPTLC– <i>B. subtilis</i> bioautograms of reagent blanks compared to pooled blank extracts (46.5 mg/area) after a single development with ACN/ammonia/ethanol 13:4:3 (V/V/V) up to 80 mm on TLC silica gel 60 F ₂₅₄ MS-grade; template for the HPTLC–autoTLC–HRMS measurement (A) and imprinted plate duplicate after the measurement and subsequent <i>Bacillus subtilis</i> bioassay (B). Molecular formulas of antibacterial zones 1–8 were assigned to the respective mass signals found (see also Table 1) in honey (E4). 5%: 5% HAc in ACN, B1: reagent blank MgSO ₄ /NaOAc, B2: reagent blank Na ₂ SO ₄ /NaCl.

Table S1. Complete list of VD standards and solutions prepared.

Therapeutic class	Structural class	VD standard	Manufacturer	Purity [%]	Concentration [mg/mL]	Solvent* (V/V)
Antibiotic	Sulfonamide	Dapsone	Sigma-Aldrich	97.2	1	Acetonitrile
		Sulfacetamide	Sigma-Aldrich	99.9		
		Sulfachloropyridazine	Sigma-Aldrich	99.7		
		Sulfaclozine	Chemos ^a	99.8		
		Sulfadiazine	Sigma-Aldrich	99.8		
		Sulfadimethoxine	Sigma-Aldrich	99.9		
		Sulfadimidine	Sigma-Aldrich	99.8		
		Sulfadoxine	Sigma-Aldrich	98.9		
		Sulfamerazine	Sigma-Aldrich	99.7		
		Sulfamethoxazole	Sigma-Aldrich	99.9		
		Sulfamethoxypyridazine	Sigma-Aldrich	99.8		
		Sulfanilamide	Sigma-Aldrich	99.9		
		Sulfaquinoxaline	Sigma-Aldrich	97.8		Dimethylformamide
		Sulfathiazole	Sigma-Aldrich	99.9		Acetonitrile
	Diaminopyrimidine	Baquiloprim	Witega ^b	99.89	1	Methanol
		Trimethoprim	Sigma-Aldrich	99.7		
	Lincosamide	Clindamycin	Sigma-Aldrich	99	1	Methanol
		Lincomycin	Sigma-Aldrich	n. a.		
	Pleuromutilin	Tiamulin	Sigma-Aldrich	99.9	1	Methanol
		Valnemulin	Sigma-Aldrich	96.2		
	Macrolide	Clarithromycin	Sigma-Aldrich	98.4	1	Methanol
		Erythromycin	Sigma-Aldrich	93		
		Gamithromycin	Sigma-Aldrich	93.2		
		Josamycin	Sigma-Aldrich	n. a.		
		Neospiramycin	TRC ^c	96		
		Oleandomycin	SantaCruz ^d	98		
		Spiramycin	Sigma-Aldrich	87.7		
		Tilmicosin	Sigma-Aldrich	94		
		Tulathromycin 2	SantaCruz ^d	99		
		Tylosin A	Sigma-Aldrich	89.7		
		Tylosin B	TRC ^c	95		
	Cephalosporin	Cefalexin	Dr. Ehrenstorfer ^e	99	0.1	Acetonitrile/water 3:1 (V/V)
		Cefalonium	Sigma-Aldrich	99.4		
		Cefapirin	Sigma-Aldrich	98		
		Cefazolin	Sigma-Aldrich	97.4		
		Cefoperazone	Sigma-Aldrich	98.2		
		Cefquinome	Sigma-Aldrich	98.3		
		Ceftiofur	Sigma-Aldrich	97.4		
		Desacetyl-cefapirin	TRC ^c	95		

Table S1. continued.

Therapeutic class	Structural class	VD standard	Manufacturer	Purity [%]	Concentration [mg/mL]	Solvent (V/V)
Antibiotic	Penicillin	Amoxicillin	Sigma-Aldrich	98.7	1	Water
		Ampicillin	Sigma-Aldrich	98.8		
		Cloxacillin	Sigma-Aldrich	96		
		Dicloxacillin	Sigma-Aldrich	99		
		Methicillin	Sigma-Aldrich	97.1		
		Nafcillin	Sigma-Aldrich	98.7		
		Oxacillin	Sigma-Aldrich	99.2		
		Penicillin G	Dr. Ehrenstorfer ^a	99.8		
		Penicillin V	Sigma-Aldrich	98.7		
	Amphenicol	Chloramphenicol	Sigma-Aldrich	100	1	Methanol
		Florfenicol	Sigma-Aldrich	98		
		Florfenicolamine	Sigma-Aldrich	99.3		
		Thiamphenicol	Sigma-Aldrich	>99.9		
	Nitroimidazole	Camidazole	Witega ^b	99.4	0.1	Methanol
		Dimetridazole	Sigma-Aldrich	n. a. ³		
		Hydroxydimetridazole	Witega ^b	99.9		
		Hydroxyipronidazole	Witega ^b	99.7		
		Hydroxymetronidazole	Witega ^b	99.37		
		Ipronidazole	Witega ^b	99.4		
		Metronidazole	Sigma-Aldrich	100		
		Omidazole	Sigma-Aldrich	>99		
		Ronidazole	Sigma-Aldrich	>99		
		Secnidazole	Witega ^b	99.8		
		Ternidazole	Witega ^b	99.8		
		Tinidazole	Sigma-Aldrich	99.7		
Anti-inflammatory	Glucocorticoid	Dexamethasone	Sigma-Aldrich	99.6	1	Methanol
		Flumethasone	Sigma-Aldrich	99.6		
		Methylprednisolone	Sigma-Aldrich	98.3		
		Prednisolone	Sigma-Aldrich	99		
Anthelmintic	Salicylate	Oxyclozanide	Sigma-Aldrich	99.6	1	Methanol
		Rafoxanide	Sigma-Aldrich	99.53		
	Imidazothiazole	Levamisole	Sigma-Aldrich	99.9	1	Dimethylformamide
	Benzimidazole	Mebendazole	Sigma-Aldrich	99.7	1	Dimethylformamide
	Benzolsulfonamide	Clorsulon	Sigma-Aldrich	99.5	1	Methanol
Antiparasitic	Avermectin	Avermectin B _{1a}	Sigma-Aldrich	98.7	1	Methanol
		Ivermectin B _{1a}	Sigma-Aldrich	96		
Coccidiostat	Quinolone	Decoquinat	Sigma-Aldrich	99.2	1	Acetonitrile/formic acid 9:1 (V/V)
Non-steroidal anti-inflammatory drug	Arylpropionic acid	Carprofen	Sigma-Aldrich	99.7	1	Acetonitrile/methanol 9:1 (V/V)
		Ibuprofen	Sigma-Aldrich	100		
	Fenamate	Flunixin	Sigma-Aldrich	99.8	1	Acetonitrile/methanol 9:1 (V/V)
	Oxicam	Meloxicam	Sigma-Aldrich	99.9	1	Acetonitrile/methanol 9:1 (V/V)
	Pyrazolidindione	Phenylbutazone	Sigma-Aldrich	99.9	1	Acetonitrile/methanol 9:1 (V/V)

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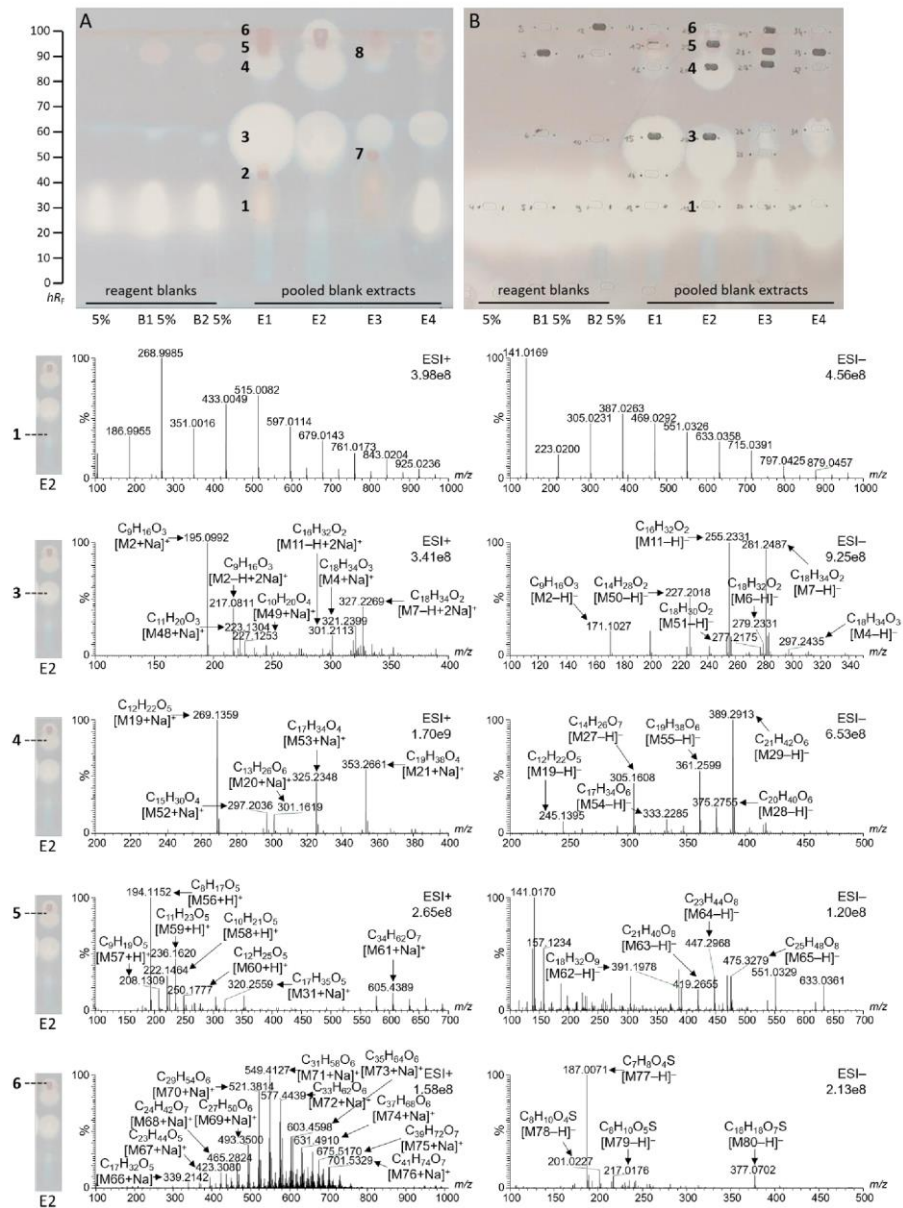


Fig. S1. HPTLC–*B. subtilis* bioautograms of reagent blanks compared to pooled blank extracts (46.5 mg/area) after a single development with ACN/ammonia/ethanol 13:4:3 (V/V/V) up to 80 mm on TLC silica gel 60 F₂₅₄ MS-grade; template for the HPTLC–autoTLC–HRMS measurement (A) and imprinted plate duplicate after the measurement and subsequent *Bacillus subtilis* bioassay (B). Molecular formulas of antibacterial zones 1–8 were assigned to the respective mass signals found (see also Table 1) in cow milk (E2). 5%: 5% HAc in ACN, B1: reagent blank MgSO₄/NaOAc, B2: reagent blank Na₂SO₄/NaCl.

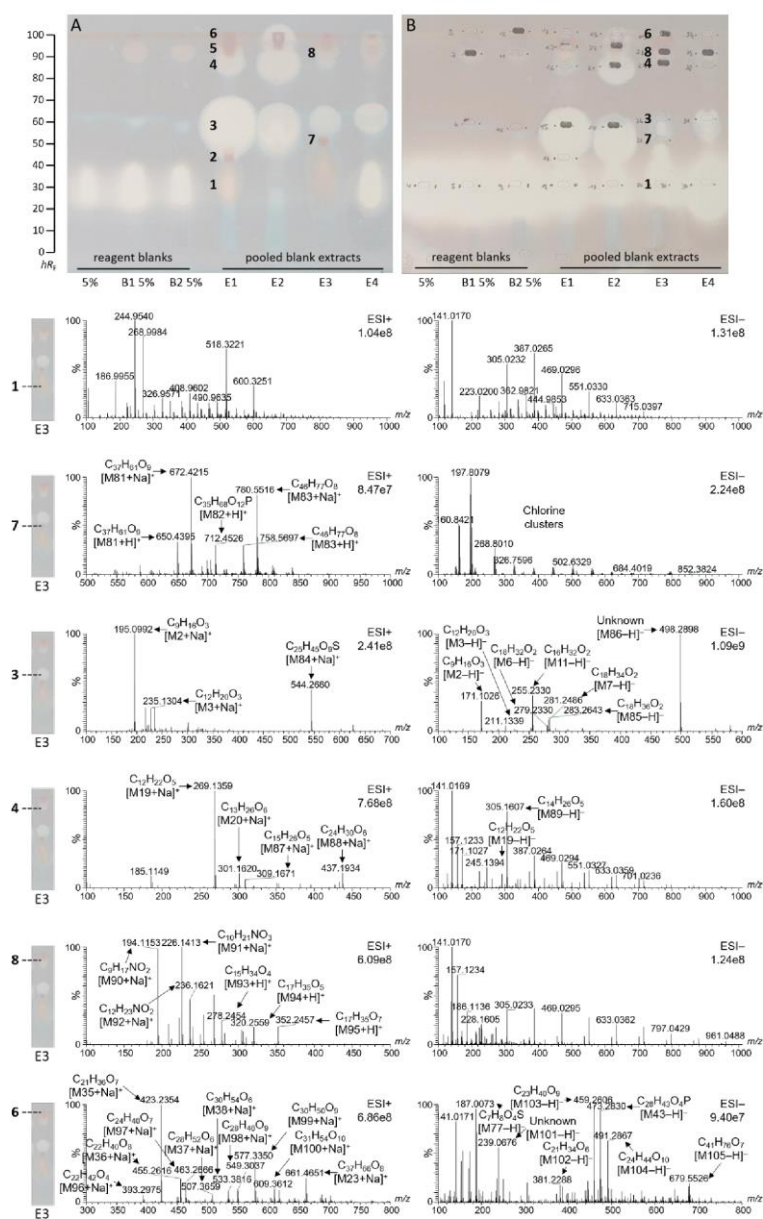


Fig. S2. HPTLC–*B. subtilis* bioautograms of reagent blanks compared to pooled blank extracts (46.5 mg/area) after a single development with ACN/ammonia/ethanol 13:4:3 (V/V/V) up to 80 mm on TLC silica gel 60 F₂₅₄ MS-grade; template for the HPTLC–autoTLC–HRMS measurement (A) and imprinted plate duplicate after the measurement and subsequent *Bacillus subtilis* bioassay (B). Molecular formulas of antibacterial zones 1–8 were assigned to the respective mass signals found (see also Table 1) in chicken egg (E3). 5%: 5% HAc in ACN, B1: reagent blank MgSO₄/NaOAc, B2: reagent blank Na₂SO₄/NaCl.

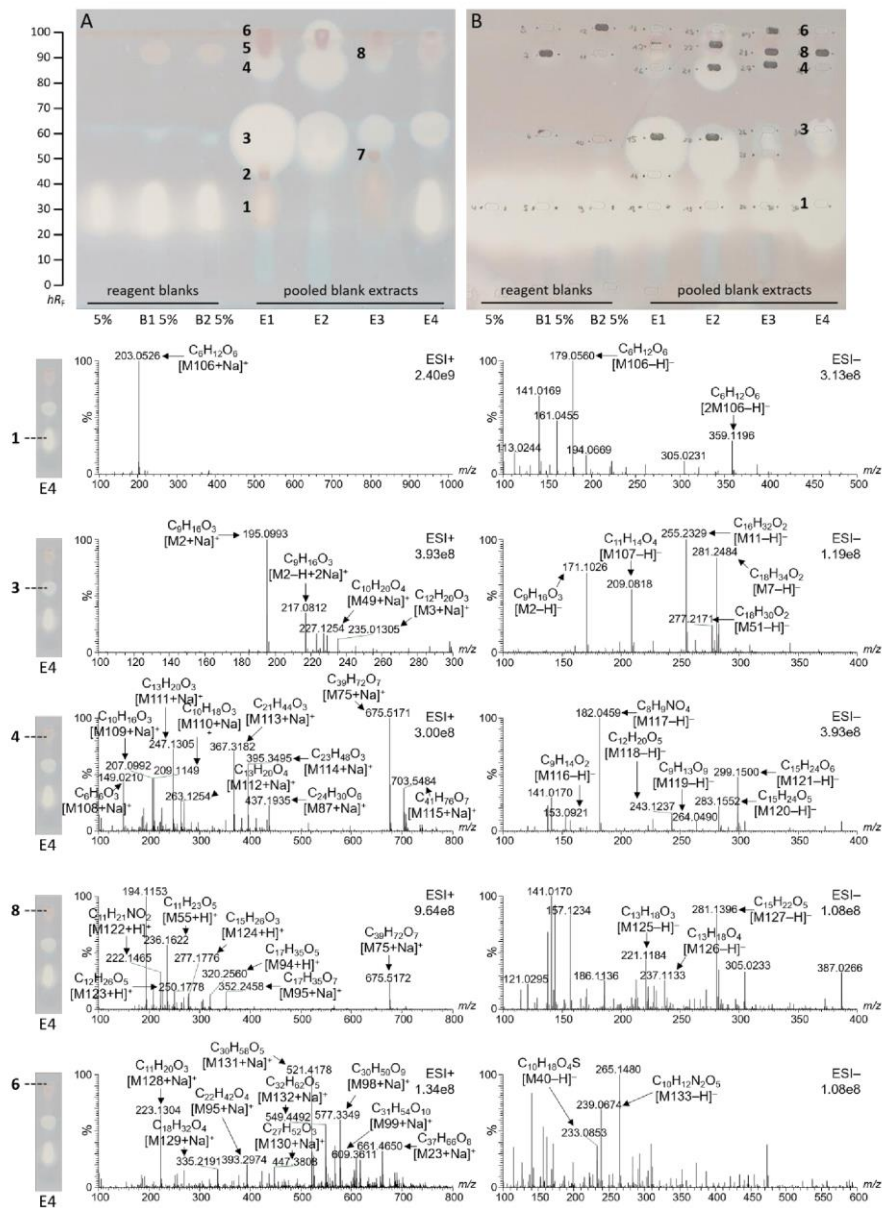


Fig. S3. HPTLC–*B. subtilis* bioautograms of reagent blanks compared to pooled blank extracts (46.5 mg/area) after a single development with ACN/ammonia/ethanol 13:4:3 (V/V/V) up to 80 mm on TLC silica gel 60 F₂₅₄ MS-grade; template for the HPTLC–autoTLC–HRMS measurement (A) and imprinted plate duplicate after the measurement and subsequent *Bacillus subtilis* bioassay (B). Molecular formulas of antibacterial zones 1–8 were assigned to the respective mass signals found (see also Table 1) in honey (E4). 5%: 5% HAc in ACN, B1: reagent blank MgSO₄/NaOAc, B2: reagent blank Na₂SO₄/NaCl.

10. Publication VI

Evidence that Indo-Pacific bottlenose dolphins self-medicate with invertebrates in coral reefs

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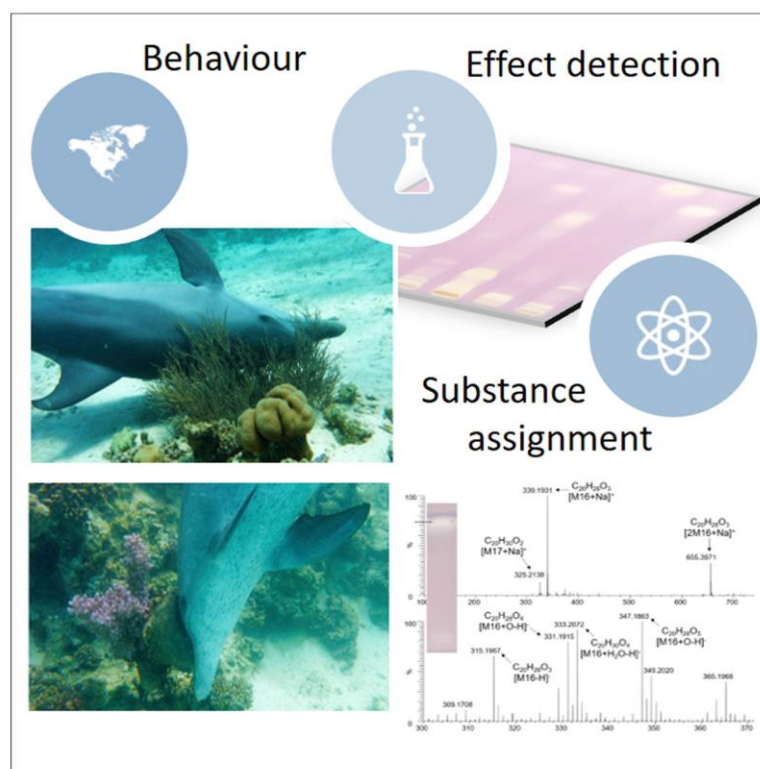
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Article

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Gertrud E. Morlock, Angela Ziltener, Sascha Geyer, ..., Tamara Schreiner, Tamer Kamel, Franz Brümmer

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Highlights

Dolphins rubbed body parts against specifically selected corals and sponges

Behavioral studies were linked with hyphenated bioanalytical technique

Vertebrate-invertebrate interaction in coral reefs may serve self-medication

Molecular formulae were assigned to known and unknown bioactive molecules

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Article

Evidence that Indo-Pacific bottlenose dolphins self-medicate with invertebrates in coral reefs

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SUMMARY

Indo-Pacific bottlenose dolphins (*Tursiops aduncus*) have been observed queueing up in natural environments to rub particular body parts against selected corals (*Rumphella aggregata*, *Sarcophyton* sp.) and sponges (*Ircinia* sp.) in the Egyptian Northern Red Sea. It was hypothesized that the presence of bioactive metabolites accounts for this selective rubbing behavior. The three invertebrates preferentially accessed by the dolphins, collected and analyzed by hyphenated high-performance thin-layer chromatography contained seventeen active metabolites, providing evidence of potential self-medication. Repeated rubbing allows these active metabolites to come into contact with the skin of the dolphins, which in turn could help them achieve skin homeostasis and be useful for prophylaxis or auxiliary treatment against microbial infections. This interdisciplinary research in behavior, separation science, and effect-directed analysis highlighted the importance of particular invertebrates in coral reefs, the urgent need to protect coral reefs for dolphins and other species, and calls for further vertebrate-invertebrate interaction studies.

INTRODUCTION

Rubbing behavior on distinct substrates is part of the natural physical contact behavior in cetaceans and has only been observed in a few odontocetes, for example in killer whales (*Orcinus orca*) (Ford et al., 2000) and beluga whales (*Delphinapterus leucas*) (Smith et al., 1992). The repeated rubbing behavior of wild Indo-Pacific bottlenose dolphins (*Tursiops aduncus*) around Hurghada and El Gouna in the Northern Red Sea, Egypt, against three distinct marine invertebrates is reported here. The dolphins glide toward and rub their skin against the following invertebrates accessed selectively and preferentially: the gorgonian coral *Rumphella aggregata*, the leather coral *Sarcophyton* sp., and the sponge *Ircinia* sp. They use distinct substrates for particular body parts due to the unique properties of the invertebrates (i.e. texture) and differences in the sensitivity of their body parts (i.e. strong head rubbing against the harder sponge structure). Up to now, the intention behind this rubbing behavior has been unclear. We hypothesized that this behavior serves the purpose of self-medication. Similar *Rumphella* species are known to produce antimicrobial as well as cytotoxic secondary metabolites (Sung et al., 2007b; Alarif 2012; El-Ezz et al., 2017), and also *Ircinia* sp. (Bifulco et al., 1995; Hardoim and Costa 2014; Mioso et al., 2017) and *Sarcophyton* sp. (Gomaa et al., 2016) are reported to contain bioactive metabolites. Thus, our hypothesis that dolphins utilize these secondary metabolites against dermal disease-causing pathogens (Harzen and Brunnick 1997; van Bressem et al., 2008; Kiszka et al., 2009; Hart et al., 2012) in the sense of preventative or curative self-medication (zoopharmacognosy) (Huffman 1997) appears plausible. Dolphins can suffer from viruses (van Bressem and van Waerebeek 1996; Esperón et al., 2008) and bacterial infections (Palmer et al., 1991; Russo et al., 2018); however so far, zoopharmacognosy has never been reported in any cetacean (Ansari et al., 2013). Reasons may be due to the paucity of systematic underwater observations of cetaceans, and otherwise limited on-boat surveys providing little behavioral data. Through a unique combination of boat and underwater surveys using SCUBA diving, a resident population of about 360 Indo-Pacific bottlenose dolphins in the Northern Red Sea has systematically been studied by the Dolphin Watch research team since 2009 (Kleinertz et al., 2014). So far, only the gorgonian rubbing behavior (gorgoning) has been highlighted in the film Blue Planet II Episode 1 of the BBC Natural History Unit (Attenborough 2017). Similar reports in the Bahamas (Herzing, 2015) and in Florida (Precht et al., 2018) substantiate our observations. Given that

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Figure 1. Rubbing behavior of dolphins on three specific marine organisms and underwater photo documentation of sampling

Rubbing behavior on (A) gorgonian coral *Rumphella aggregata*, (B) leather coral *Sarcophyton* sp., and (C) sponge *Ircinia* sp.; (D) underwater photo documentation of sampling and location, exemplarily shown for the leather coral. Respective scale bars and further details are given in [Figures S3–S5](#).

the dolphins rub particular body parts against specific invertebrates, we postulate that bioactive metabolites incorporated in the invertebrates are explicitly sought out by them for targeted self-medication.

RESULTS AND DISCUSSION

Rubbing behavior on selectively accessed invertebrates

Indo-Pacific bottlenose dolphins around Hurghada and El Gouna in the Northern Red Sea ([Figure S1](#)) glide toward and rub their skin against the selectively accessed gorgonian coral *Rumphella aggregata*, the leather coral *Sarcophyton* sp., and the sponge *Ircinia* sp. ([Table S1](#) Compilation of the organisms, related to [Figures 1](#) and [S1](#)). On the gorgonian coral, dolphins slide into the branches of the coral and often repeat this behavior, so several body parts are rubbed ([Videos S1](#) and [S2](#) Gorgonian rubbing behavior, related to [Figure 1A](#) and [S2](#)). Upon rubbing, the gorgonian coral polyps start to secrete mucus and to close and the mucus secreted by the corals can then be transferred to the skin of the dolphin. Through the closed polyps and resultant harder and rougher surface of the coral, skin contact via abrasion and subsequent absorption might be even more efficient. Leather corals and sponges are more compact and harder in their texture than the soft gorgonian coral branches, so the dolphins push one isolated body part strongly into the substrates. For example, the dolphin rubs its ventral, lateral, or dorsal body part on the leather coral ([Figure 1B](#)). Its head and fluke often touch the coral, too or as a further example, the dolphin rubs its ventral or dorsal body part and fluke on the sponge and pushes its head strongly against it and twists it around ([Figure 1C](#)).

When in groups, dolphins are often observed queueing up behind each other to wait their turn to approach the invertebrate. This group event has been observed for the gorgonian coral and sponge, but not for the leather coral. The more sensitive calves aged under one year have not been observed engaging in the group rubbing on these particular organisms, instead they watch the adults doing the rubbing ([Video S2](#) Gorgonian rubbing behavior, related to [Figure 1A](#)). As they start to become integrated into the group rubbing event, the calves carefully rub themselves on the substrate by touching it with certain body parts quickly ([Video S3](#) Gorgonian rubbing behavior in group with calf, related to [Figure 1A](#)). This would imply that the behavior and any potential knowledge about the effects of the bioactive compounds contained



within the invertebrates is not innate but acquired through processes of social learning (Box and Gibson 1999) and therefore socially transmitted to next generations (Krützen et al., 2005; Sargeant and Mann 2009; van Schaik and Burkart 2011). If this learned behavior serves the purpose of self-medication, these specifically accessed invertebrates must contain bioactive compounds.

If dolphins rub intensively on the substrate, the substances are released (Video S4 Sponge rubbing behavior, related to Figure 1C). Sometimes dolphins will open their mouths during rubbing, making the direct contact with the invertebrate compounds obvious. An even more intense contact behavior was observed during leather coral rubbing. Dolphins sometimes extract the invertebrate from the ground and carry it in their mouths for a few minutes, the dolphin may then swing the leather coral around, causing compounds to visibly leak out of the coral and spread around the head and rostrum (Video S5 Dolphin carrying a leather coral, related to Figure 1B). These particular body parts of the dolphin can subsequently be stained a striking yellowish or greenish coloration due to the compounds excreted by the invertebrates.

Sampling of the accessed invertebrates

Interference with nature was kept to a minimum during the sampling of the substrates, and the amount of sample taken from its natural habitat was as minimal as possible. Underwater photo documentation precisely traced the natural habitats and environment around the El Gouna and Hurghada area (Figures S3–S5). The collected samples at the sites visited by the dolphins (Figure S1) were transported aboard on a mixture of ice cubes and salt and stored at -20°C in the laboratory. The metabolite profile can alter during transportation, so methanol was added immediately after the dive to one set of samples. Despite potential reaction with aldehydes to hemiacetals, methanol was preferred based on previous bio-profiling studies which allowed the most comprehensive view on active compounds present. Besides methanol, *n*-hexane was also chosen as an extraction solvent to widen the range of extractable compounds in the apolar range. The 48 extracts of the collected samples had a volume of 0.2–1.5 mL, depending on the substrate and solvent (Table S2 Sample preparation, related to STAR Methods, 0.2–1 mL for methanol extracts and 1.1–1.5 mL for *n*-hexane extracts). Such small sample volumes were challenging for analysis aimed at the detection of bioactive compounds.

Outline of the bioanalytical screening

One of the main advantages of the high-performance thin-layer chromatography (HPTLC) method used (Figure S6) is the efficient use of costly samples (Klöppel et al. 2008, 2013; Mahran et al., 2019). Not even a microliter of the limited sample volume was lost during instrumental operations. As compound isolation was skipped, the direct analysis avoided any artifacts (Czarkie et al., 1985). Another advantage of the HPTLC method is the multi-detection and subsequent on-surface application of assays (Morlock 2021), if compared to status quo procedures (Kongstad et al., 2015). A mobile phase system was developed that spreads the compounds present in the extracts along the migration distance (data not shown). Ten effect-directed assays of different mechanisms (i.e., seven biological assays, two enzymatic assays, and a radical scavenging assay, Figure 2) were exploited to figure out which of the on-surface-separated metabolites had bioactive properties due to their interaction with the applied microorganism or enzyme. Depending on the assay, the generated specific response was based on color formation, fluorescence generation, fluorescence reduction (for antagonistic effects), or change in the intrinsic bioluminescence, which indicated the respective bioactive properties of a substance (Table S3 Ten different assays applied, related to Figure 2). All assays were repeated, and responses were confirmed several times. The combination of the parallel sample separation with the assay response led to expressive effect-profiles (Video S6 Bioanalytical workflow, related to Figure 2). As an alternative instrumental system, the miniaturized open source OCLab3 system (<https://github.com/OfficeChromatography/OCLab3>), which comprises a 2LabsToGo system both the chemistry and biology laboratory, can be used (Morlock, 2021). The subsequent targeted recording of heated electrospray ionization–high-resolution mass spectra (HESI–HRMS) of bioactive zones allowed to assign molecular formulae. Note that a fully automated workflow was reported recently (Mehl et al., 2021), which makes this hyphenation highly convenient (Video S7 Hyphenation to MS, related to Figures 2–4).

Effect-directed profiling

After centrifugation, the supernatants of the 48 extracts were applied (1–10 μL /band). The higher methanol volume added to the boat samples was compensated by a respective higher application volume, so that the sample amount was equivalent to the respective laboratory sample in the HPTLC image. Bioactive

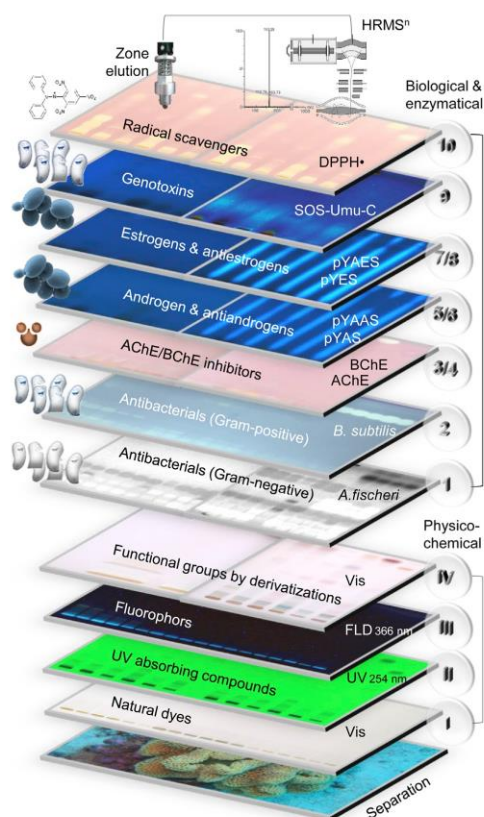


Figure 2. Scheme of effect-directed profiling

Planar chromatographic separation of substrates was combined with (I–IV) physicochemical multi-detection at Vis/UV/FLD including chemical derivatizations, (1–10) ten different effect-directed on-surface assays (abbreviations in supplemental information), and HRMS.

compounds were detected in both extraction polarities in all substrates. Both extractions were complementary, as evident via the antibacterial metabolite patterns of the *Aliivibrio fischeri* bioautograms (Figure 3). The methanol extracts contained more polar bioactive compounds ($hR_F < 20$), which were lost in the *n*-hexane extracts, which in turn contained the middle to apolar bioactive compounds. The metabolite pattern of the methanol extracts obtained on board differed to a certain extent from that obtained in the laboratory. The differences were minor for the gorgonian corals, moderate for the leather coral, and highest for the sponge extracts. This shift in the individual intensities of bioactive compounds within a substrate can be explained by the lability of metabolites (e.g., due to oxidation) during boat transportation. Across all assays performed, the boat-methanol and *n*-hexane extracts showed comparatively stronger responses than the laboratory-methanol extracts.

The two bacterial assays were selected for the antibacterial effect detection to represent the two different bacterial cell wall types (Figure 3). The Gram-negative *Aliivibrio fischeri* bioassay was used as a starter assay, as it immediately indicates most bioactive compounds based on our experience. A rough indication

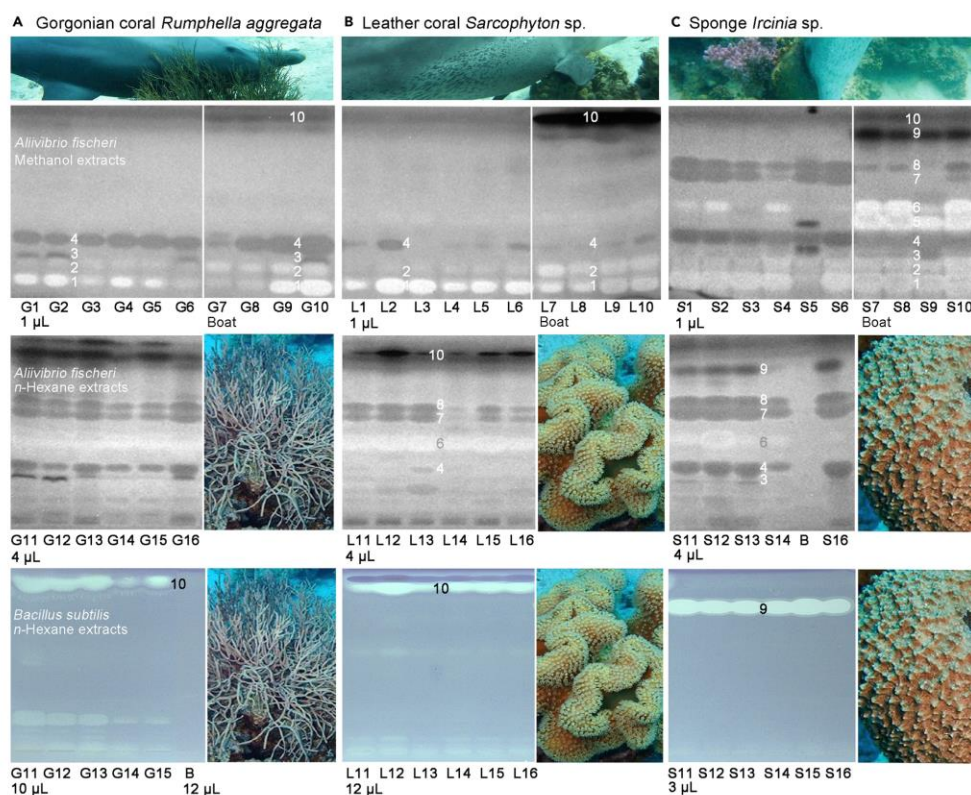


Figure 3. Antibacterial compounds against Gram-negative as well as Gram-positive bacteria

Bioprofiling of the methanol and n-hexane extracts of the (A–C) three distinct substrates revealed the antibacterial compound zones 1–10 (evident as dark or bright bands), developed on HPTLC plates silica gel 60 with ethyl acetate – methanol – water 15:3:1 (V/V/V) and detected via the bioluminescence (bioautogram after 27 min depicted as grayscale image) of the applied Gram-negative *A. fischeri* and at white light illumination after the applied planar Gram-positive *B. subtilis* bioassay (B: solvent blank; respective pre-assay FLD 366 nm chromatograms in Figure S7).

of the required sample volumes to be applied was obtained as well as information on the quality of the separation regarding the distribution of bioactive zones along the separated sample track. In the first *A. fischeri* bioautogram, up to 10 different bioactive zones (zones 1–10) were observed. On the one hand, up to six compounds (evident as dark bands) reduced the native bioluminescence of the bacteria, which is related to the decrease of the energetic cell metabolism and of the vitality of the bacteria. On the other hand, up to four compounds, evident as bright bands, increased the bioluminescence related to an improved energetic cell metabolism. The comparison between boat and laboratory extracts revealed only slight differences for the gorgonian corals. However, for the leather coral, the more polar bioactive compound zone 2 (hR_F 20) and the very strong lipophilic bioactive compound zone 10 (hR_F > 90) were more pronounced in the boat-methanol extracts. The difference was highest for the sponge extracts, for which the bioactive compound zones 5 and 6 (hR_F 40–50) as well as 9 and 10 (hR_F > 90) were more dominant in the boat-methanol extracts, whereas zones 7 and 8 were less intense in their response.

The second bioassay detected antibacterial compounds against Gram-positive *Bacillus subtilis* bacteria. The antibacterial responses were more pronouncedly detectable in the n-hexane than methanol



extracts. The applied volumes were adjusted depending on the three distinct substrates. Approximately at the same upper position as in the previous bioassay, the two apolar antibacterial zones **9** and **10** were evident as colorless (white) bands in the bioautogram of the *n*-hexane extracts of the three distinct substrates (Figure 3). The latter two zones were strongest in their response and prominently acted against both bacterial cell wall types, whereby zone **9** was predominantly present in the sponges and zone **10** in the corals. Because in the first two bioassays and in the multi-detection imaging, the intra-species responses were similar, and due to the limited volume of sample extracts, further analyses were not performed with all but only with selected representatives. These were also replaced during assay repetitions and resulted in the same responses, confirming that similar results were obtained within a species.

The third and fourth enzymatic assays detected inhibitors of the acetyl- and butyrylcholinesterases (AChE/BChE). Activity responses were more pronounced in the *n*-hexane and boat-methanol extracts than in the methanol extracts (Figures S8, S9A and S9B). At the same upper position as in the previous two bioassays, the two zones, zones **9** and **10**, were observed as colorless (white) inhibition bands. Only the leather corals revealed a pronounced response for zone **10** in both inhibition assays. The much weaker inhibiting zone **9** was only observed in the sponge extracts. The fifth planar yeast estrogen screen (pYES) bioassay detected the blue fluorescent estrogen-like compound zones **11–13**, mainly in the methanol boat extract of the leather coral L8 (Figures S9C). A much weaker estrogenic response was observed in the *n*-hexane extract of the gorgonian coral G11 (zone **13**) or leather coral L13 (zone **12**). In the sixth planar yeast antiestrogenic screen (pYAES) bioassay, the compound zones **14** and **15** were observed in the boat-methanol (Figure S9D, L8) and *n*-hexane extracts (L13) of the leather corals only. These zones were evident through fluorescence reduction of the estrogen 17 β -estradiol, which was applied as a vertical rectangle along each separated sample track. The seventh planar yeast androgen screen (pYAS) bioassay detected androgen-like compounds as blue fluorescent zones. However, no androgen was observed for the given amounts applied (up to 10 μ g/band, Figure S9E). The eighth planar yeast antiandrogen screen (pYAAS) bioassay revealed two pronounced antiandrogenic zones, as evident in the fluorescence reduction of the androgen testosterone which was applied as a vertical rectangle along each sample track. These antiandrogenic responses were at the same hR_F values as the previously detected antiestrogenic compound zones **14** and **15** (Figure S9F, L8/L13), again observed only in the leather corals. The ninth genotoxicity (SOS-Umu-C) bioassay using *Salmonella typhimurium* bacteria was performed not only on the normal phase but also on the reversed phase plate. The application of both allowed a complementary perspective on the samples. The more apolar genotoxic compound zone **16** was only detected in the leather corals (Figure S9G, L8/L13) on the normal phase plate. This normal phase system detects the zones more sensitive, and hence, this weak genotoxic zone was not observed in the reversed phase system. On the reversed phase plate (Figure S9H), a further weak genotoxic response (zone **17**) was observed for all tested substrates. Although the RP-HPTLC system leads to much sharper zones than the NP-HPTLC system for long incubation times in polar bioassay media (Klingelhöfer and Morlock 2014), this already diffused apolar zone is even more prone to diffusion on the normal phase bioassay plate, on which it was thus not detectable. The tenth 2,2-diphenyl-1-picrylhydrazyl (DPPH[•]) assay revealed up to six different radical scavenging (antioxidant) compound zones (Figure S10A). Among these, the previously discovered more apolar bioactive zones **9** and **10** in the leather coral and sponge extracts, respectively, stand out, whereas the gorgonian coral extracts contained several intense polar antioxidants.

Across the ten assays and the multi-imaging performed (Figures 2 and 3, and S7–S11), the obtained effect-profiles of the 48 extracts were similar between species or within a phylum, as evident for the gorgonian coral and leather coral. They differed more between different phyla (corals versus sponge). Bridging the gap between chromatography and toxicology, the effect-directed profiles provided information on biological activity. Such responses could be explained by the developed antimicrobial defense strategies of the sessile marine microorganisms, but it can also contribute to a certain extent to the homeostasis of the skin of the dolphins in case of parasite infestation. The bioactive metabolites discovered in the three substrates, which were exclusively accessed by the dolphins, can explain the purpose of self-medication by rubbing on the substrates. These metabolites can come into contact with the skin of the dolphins and could be useful for skin protection or as an auxiliary treatment against bacterial infections or for balancing the hormonal status or homeostasis of the skin.

Physicochemical profiles of the sampled substrates

Additional information on spectral characteristics and on polarity of the compound zones was obtained by chemical derivatization reactions. For example, the FLD 366 nm chromatograms recorded before application of the assays (Figures S7 and S8) showed the start zone as the main fluorescent zone. The Vis



chromatogram did not show any visible responses (not shown). However, the physicochemical detection of the derivatized chromatograms revealed the bioactive zones **9** and **10** after derivatization with the diethylamine aniline sulphuric acid reagent (used for detection of glycosides or lactones, Figure S10B) and with the vanillin sulfuric acid reagent (used for a more universal detection of organic compounds, Figure S10C). The natural product reagent for detection of molecules with adjacent keto/hydroxy groups did not show any response (not shown), whereas both zones were detected by the primuline reagent, which points to lipophilic compounds (Figure S11). Both zones did also absorb at UV 254 nm (Figure S11). Although dedicated structure elucidation experiments are needed to identify molecular structures, the here obtained information can confirm tentative assignments. For example, zones **9** and **10** ($hR_F > 90$) were not natively visible or fluorescent, but UV-active (Figure S11). Both were assigned a glycosidic or lactonic (Figure S10) and lipophilic (Figure S11) structural moiety, as proven by the use of derivatization reagents. Both zones showed a pronounced antibacterial (Figure 3) and radical scavenging (antioxidative) activity (Figure S10A). Zone **10** was a comparatively stronger inhibitor of AChE and BChE than zone **9** (Figures S9A and S9B). Based on these data, it was hypothesized that both zones were a lipolactone due to their high hR_F value in the given chromatographic system, UV-activity, and responses in both antibacterial bioassays as well as in the radical scavenging assay. Antibacterial and antioxidative properties of lactones are known (Mar'in et al., 2002; Shoaib et al., 2017; Zhang et al., 2020).

Characterization of antibacterial zones 1–10 by HPTLC–HESI-HRMS–bioassay–bioluminescence

The *A. fischeri* bioluminescence enhancing or decreasing zones 1–10 (Figure 3) were further characterized by online elution to HESI-HRMS. The plate was prepared twice. One plate was directly subjected to the bioassay used for zone marking on the other plate. The latter plate was used for recording of HRMS spectra and subsequent bioassay application to prove the proper positioning and zone transfer by the match of the stamped oval elution head imprint with the post-HRMS bioautogram (Figure 4A–4C). The targeted recording of HRMS spectra led to pronounced mass-over-charge signals (m/z) and respective molecular formulae were assigned after a detailed literature study (Table S4 Compilation of activities related to Figure 4, and Table S5 Assignment of the mass signals, related to Figure 5). The formation of multiple adducts allowed the multiple confirmation of the assigned molecular formulae, and is explained by the zone elution from the bioautogram containing salt-rich bioassay media. Recently, it was reported that inorganic sulfur can be assimilated from the seawater by corals (Higuchi et al., 2021) even in the absence of symbionts (Erwin et al., 2012). Apart from natural halogenated products, also xenogenic chlorinated molecules were found in corals (van der Schyff et al., 2021). Both references support the assignment of the sulfur- or chlorine-containing molecular formulae obtained (Table S5 Assignment of the mass signals, related to Figure 4).

In accordance with literature (Table S5 Assignment of the mass signals, related to Figure 4), the HRMS signals of zone **9** (basepeak 397.2383 $[M15-H]^+$, $C_{25}H_{34}O_4$, Δppm 0.03) in the sponge were tentatively assigned to the structural isomers fasciculatin/variabilin/palinurin (Ishibashi et al., 1993), all furanosesterterpene tetrionic acids and thus lipolactones. Fasciculatin has been isolated from *Ircinia fasciculata* (Cafieri et al., 1972), present in digestive glands, mucus, and mantle sections (Marin et al., 1997), in the Tyrrhenian Sea (Rosa et al., 1997) and Atlantic Coast of Morocco reporting its moderate cytotoxicity as defense strategy (Rifa'i et al., 2005). Variabilin has been isolated from *Ircinia variabilis* showing antibiotic activity (Faulkner 1973), also against Gram-positive *Sarcina lutea* (minimum inhibitory concentration of 66 pg/mL). Palinurin has been isolated from *Ircinia variabilis* (Alfano et al., 1979) and was proven to be mainly responsible for its antimicrobial activity (Martí et al., 2003). These reports are in full agreement with the strong effects against Gram-positive and Gram-negative bacteria of zone **9** in the sponge extracts in the *A. fischeri* bioautogram (Figures 3 and 4). The HRMS signals of zone **10** (basepeak m/z 339.1931 $[M16 + Na]^+$, $C_{20}H_{28}O_3$, Δppm 0.2) in the leather corals were tentatively assigned to the isomers sarcophine/sarcophytolide/sarcophytolide B or C (Figures 3, 4 and S12). Sarcophytolide was reported to act as an antimicrobial (Gomaa et al., 2016), which corresponded to our results. Though isomers were not distinguished, both tentative zone assignments were considered as proof of the bioprofiling. The mass signal (basepeak 203.0526, $[M6 + Na]^+$, $C_6H_{12}O_6$) obtained from the bioluminescence enhancing zone **2** (more or less present in the three substrates) pointed to glucose. This finding is in agreement with the rise in blood glucose levels after intake of sponge extracts (Ajabnoor et al., 1991), or the glucose uptake by corals (Stephens 1960). The obtained mass signal ($C_6H_{14}O_6$, basepeak 181.0718 $[M5-H]^+$) of the bioluminescence enhancing zone **1** can be explained by reduction of glucose to a sugar alcohol (hexane-hexol).

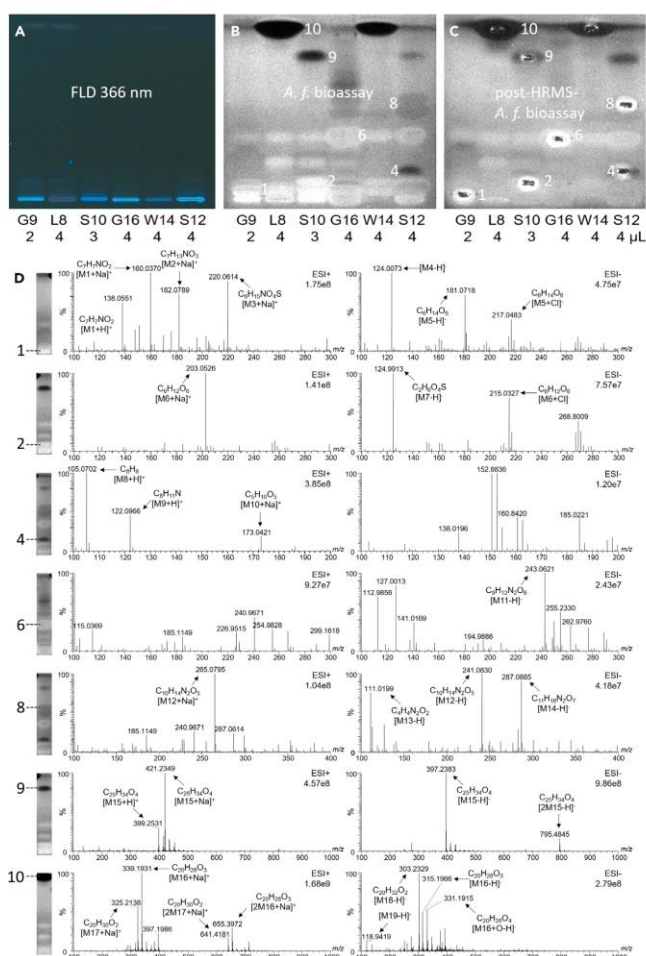


Figure 4. Characterization of antibacterial zones 1–10 by HPTLC-HESI-HRMS-bioassay-luminescence detection (A) Chromatogram at FLD 366 nm on HPTLC plate silica gel 60 with ethyl acetate – methanol – water 15:3:1 (V/V/V) of the methanol boat and *n*-hexane extracts of the three distinct substrates, (B) respective *A. fischeri* bioassay (bioluminescence depicted as grayscale image), and (C) duplicate plate used for recording of mass spectra of zones 1–10, followed by post-HRMS *A. fischeri* bioassay application to verify the proper positioning of the elution head zones. (D) Tentative assignment of molecular formulae.

Characterization of hormone-like zones 11–15 by NP-HPTLC – bioassay – RP-HPLC – PDA – HESI-MS

A recently developed 8D hyphenation (Schreiner and Morlock 2021) was alternatively used to further characterize the hormonal-effective compound zones. This hyphenation allowed eluting the bioactive zone from the bioautogram into the second separation and photodiode array (PDA) detection dimension (RP-HPLC-PDA-HESI-MS). By the fully automated workflow, hormonal-effective compound zones were eluted directly out of the highly salted bioautogram, which still contained the genetically modified yeast cells with

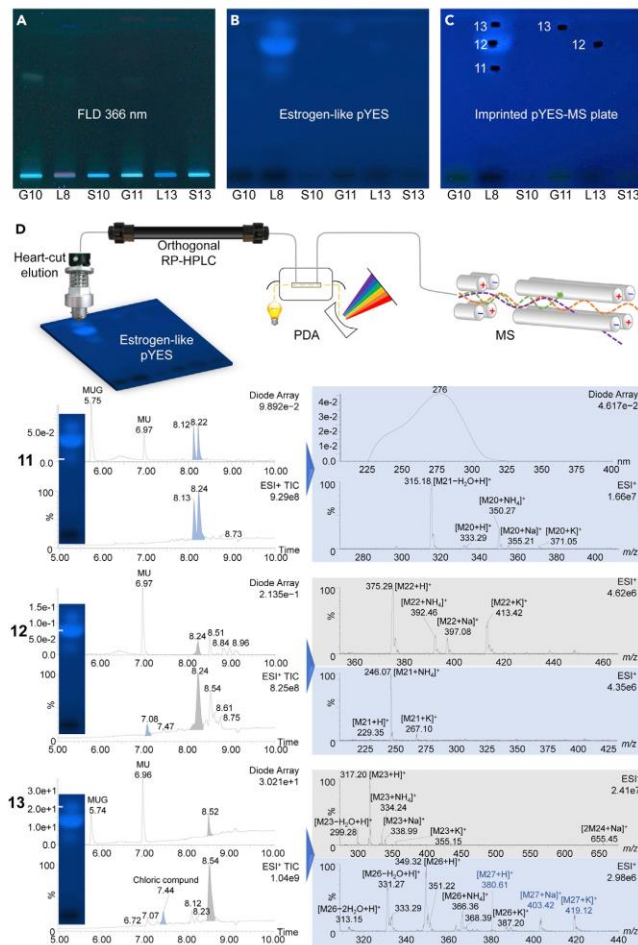


Figure 5. Characterization of estrogenic zones 11–13 directly out of the pYES bioautogram

(A) Chromatogram at FLD 366 nm on HPTLC plate silica gel 60 with ethyl acetate – methanol – water 15:3:1 (V/V/V) of the extracts of the three distinct substrates, (B) pYES bioautogram, and (C) elution head imprint, which verifies the proper positioning of the elution head on the zones. (D) Scheme of the zone characterization by NP-HPTLC-pYES bioassay-RP-HPLC-DAD-HESI-MS showing recorded PDA, MS-TIC chromatograms and extracted mass spectra (same colour) with assigned mass signals.

the human estrogen/androgen receptor incorporated. The zone eluate was automatically transferred via online desalting to RP-HPLC–PDA–HESI-MS (Figures 5 and S13). The connection to the HRMS system (to assign molecular formulae) would have been the best choice, but was not available. Predominantly in the leather corals, zone 12 was strongest in the estrogen-like response, whereas zones 11 and 13 were comparatively weaker (Figures 5B and S9C). Although less intense than in the leather coral L8, zone 12 was also detected in the leather coral L13, and zone 13 in the gorgonian coral G11. Androgenic compounds were not detected for the amounts applied (up to 10 µg/band, Figures S9E and S13B). The elution head was



properly positioned on the zones, as proven by the remaining oval elution head imprint (Figures 5B and 5C).

The RP-HPLC–PDA–HESI-MS data were obtained (Figure 5D). The substrate MUG and the fluorescent cleavage product MU were also observed and assigned in the RP-HPLC–PDA chromatogram. Zone 11 (Figure 5D) was separated into two peaks at RT 8.13 and 8.24 min, which were proven to be isomers based on the same mass signals observed. Both isomers showed a maximal absorbance at 276 nm. The mass spectral data obtained by RP-HPLC–PDA–HESI-MS revealed multiple adduct formations (Table S6 Assignment of the mass signals, related to Figure 5), all belonging to the same neutral molecule of 332 Da. Zone 12 (Figure 5D) was separated into two peaks at RT 7.08 and 8.24 min. The observed mass signals for the first peak at RT 7.08 min showed multiple adduct formations (Table S6 Assignment of the mass signals, related to Figure 5), which evidenced a neutral molecule of 228 Da. In the planar chromatogram, neither a UV/Vis absorbance nor fluorescence at 366 nm was detected. This lack in a chromophore was confirmed by the following photodiode array detection (Figures 5A and 5D). The mass spectral data of the second peak at RT 8.24 min revealed multiple adduct formations (Table S6 Assignment of the mass signals, related to Figure 5), which evidenced the neutral molecule of 374 Da (a different molecule was clearly obtained despite the same retention time as for zone 11).

The estrogen-like zone 13 (Figure 5D) and the antiandrogenic zone 14 (Figure S13D), both located at the same hR_F value on the pYES and pYAAS bioautograms, respectively, showed the same two peaks with the same spectral data. The first peak at RT 7.44 min contained two different compounds, which pointed to the neutral molecules of 348 Da and 380 Da based on their pronounced multiple adduct formations (Table S6 Assignment of the mass signals, related to Figure 5). The molecule of 348 Da showed a distinct chlorine isotope pattern and clearly contained one chlorine atom in the molecule (Figure 5D). The second peak at RT 8.54 min evidenced the neutral molecule of 316 Da, which was proven by the multiple adduct formations (Table S6 Assignment of the mass signals, related to Figure 5). The same mass signals indicating the neutral molecule of 316 Da were determined in the antiestrogenic/antiandrogenic zone 15 (Figure S13D), which was considered to be a structural isomer of zone 14. A further peak at RT 7.09 min pointed to the positively charged molecule of 366 Da, evidenced by its multiple adducts (Table S6 Assignment of the mass signals, related to Figure 5). Note that the clarification of the effect-responsible HPLC peak needs an individual collection after the PDA detection, followed by the HPTLC–bioassay. The MS recording of bioactive compound zones directly out of the bioautogram was found to be advantageous, as all signals obtained were confirmed by the multiple adduct formations (caused by the assay salts present). This corroborated the assignment of each mass signal.

Limitations of the study

This interdisciplinary study provides evidence of self-medication where dolphins rub on specific invertebrates in the natural environment and shows the relationship between this behavior and the presence of bioactive metabolites. Meaningful effect profiles were obtained by linking behavioral studies with a hyphenated bioanalytical technique, performed at a minimally exploitative environmental level. We combined the disciplines of biology, biochemistry, separation science, and high-resolution mass spectrometry on a single adsorbent surface to collect information on the activity of compounds and to identify them. Sampling was performed according to Egyptian regulations, and a major challenge for the bioanalytical procedure was the limited small sample volume of only 0.2–1.5 mL per sample. From such small sample volumes, ten effect-directed assays of different mechanisms and then the characterization of the most important bioactive compounds had to be performed. To the best of our knowledge, any other technique would have required a much larger sample volume to achieve the same results obtained. The 17 bioactive compounds detected in the three invertebrates selectively accessed by the dolphins showed antibacterial (against Gram-positive and Gram-negative bacteria), antioxidative, acetyl-/butyrylcholinesterase inhibiting, estrogenic, antiestrogenic, antiandrogenic, and genotoxic activities. In a straightforward way, molecular formulae were obtained from these bioactive zones. The identification of some molecules that were already known (such as isosarcophytolide, sarcophytol B, and the isomers of fasciculatin/variabilin/palinurin and sarcophine/sarcophytolide/sarcophytolide B/sarcophytolide C) was considered as proof of principle for properly detecting further unknown ones, which could not be assigned to substances described in the literature and still need to be identified or structure elucidated. However, the latter would require the collection of much larger quantities of invertebrate samples. Nevertheless, the detected effects and assignments in turn have provided an explanation (medication purpose) for the astonishing rubbing behavior



of the dolphins. Ultimately, this study shows the relationship between the selective rubbing behavior of Indo-Pacific bottlenose dolphins and the presence of bioactive substances, which may provide evidence for their self-medication. Moreover, it calls for further research on vertebrate-invertebrate interaction in coral reefs, and draws attention to the social intelligence of dolphins, the need for interdisciplinary and hyphenated bioanalytical thinking, and to the importance of efforts to conserve this important habitat for marine life.

DATA AVAILABILITY

All data are available in the main text or [supplemental information](#). The datasets generated during the study are available from the corresponding author on reasonable request. New codes were not created.

ETHICAL STATEMENT

We are committed to climate change harm prevention, integrity of scientific data and evidence, solidarity, gender balance, sustainability, justice and equity, and a precautionary attitude.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.isci.2022.104271>.

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AUTHOR CONTRIBUTIONS

Conceptualization: A.Z., F.B., T.K. (sampling), G.E.M. (analysis); Methodology: A.Z., J.T., F.B. (sampling), G.E.M. (analysis); Sampling: A.Z., J.T.; Investigation: S.G. (all HPTLC-assay experiments), A.M. (HRMS), T.S. (hormonal-effective bioassays and respective MS), J.T. (HPTLC–A. *fischeri* assay); Funding acquisition: A.Z., G.E.M., F.B.; Project administration: A.Z., G.E.M.; Supervision: F.B. (dive protocol), A.Z. (sampling), G.E.M. (analysis); Writing – original draft: G.E.M., A.Z.; Writing – review & editing: G.E.M., A.Z., F.B., J.T.



DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCE TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
<i>Rumphella aggregata</i>	Shaab El Erg and Shaab El Fanous, Northern Red Sea, Egypt	Red Sea Marine Parks Authority, El Sakalla – Marina Square, Hurghada, Red Sea Governorate, Egypt
<i>Sarcophyton</i> sp.	Shaab El Erg and Shaab El Fanous, Northern Red Sea, Egypt	Red Sea Marine Parks Authority, El Sakalla – Marina Square, Hurghada, Red Sea Governorate, Egypt
<i>Ircinia</i> sp.	Shaab El Erg and Shaab El Fanous, Northern Red Sea, Egypt	Red Sea Marine Parks Authority, El Sakalla – Marina Square, Hurghada, Red Sea Governorate, Egypt
Chemicals, peptides, and recombinant proteins		
Methanol, 99.9%	Merck, Darmstadt, Germany	CAS No. 67-56-1
n-Hexane, 95%	Tedia Company, Fairfield, OH, USA	CAS No. 110-54-3
Methanol, chromatography grade	MS-grade, Honeywell, Morristown, NJ, USA	CAS No. 67-56-1
Acetone, chromatography grade	Merck, Darmstadt, Germany	CAS No. 67-64-1
Ethyl acetate, chromatography grade	Sigma-Aldrich-Fluka, Steinheim, Germany	CAS No. 141-78-6
Müller-Hinton broth, for microbiology	Sigma-Aldrich-Fluka, Steinheim, Germany	www.sigmaaldrich.com/DE/en/product/sial/70192
Lysogeny broth (LB, Lennox) powder, including 5 g/L sodium chloride	Sigma-Aldrich-Fluka, Steinheim, Germany	www.sigmaaldrich.com/DE/en/product/sigma/13522
Dimethyl sulfoxide (DMSO), ≥99.8%	Carl Roth, Karlsruhe, Germany	CAS No. 67-68-5
3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), ≥98%	Carl Roth, Karlsruhe, Germany	CAS No. 298-93-1
Tris-HCl buffer, for molecular biology	Sigma-Aldrich-Fluka, Steinheim, Germany	CAS No. 1185-53-1
Citrate phosphate buffer: 6 g/L citric acid monohydrate and 10 g/L disodium hydrogen phosphate in double-distilled water, adjusted to pH 12 by solid sodium hydroxide	Sigma-Aldrich-Fluka, Steinheim, Germany	Morlock and Klingelhöfer (2014)
Diphenyl-1-picrylhydrazyl (DPPH•), 95%	Alfa Aesar, Schwerte, Germany	CAS No. 1898-66-4
α-Naphthyl acetate, ≥98%	Sigma-Aldrich-Fluka, Steinheim, Germany	CAS No. 830-81-9
Fast Blue B salt, 95%	MP Biomedicals, Eschwege, Germany	CAS No. 14263-94-6
Caffeine, 99%	Sigma-Aldrich-Fluka, Steinheim, Germany	CAS No. 58-08-2
Rivastigmine tartrate, 98%	Sigma-Aldrich-Fluka, Steinheim, Germany	CAS No. 123441-03-2
Testosterone, ≥99%	Sigma-Aldrich-Fluka, Steinheim, Germany	CAS No. 58-22-0
Gallic acid, ≥98%	Carl Roth, Karlsruhe, Germany	CAS No. 149-91-7
Tetracycline hydrochloride, reagent grade	Serva Electrophoresis, Heidelberg, Germany	CAS No. 60-54-8
17β-Estradiol (E2), 98.5%	Dr. Ehrenstorfer, Augsburg, Germany	CAS No. 50-28-2
Acetylcholinesterase (AChE) from <i>Electrophorus electricus</i> , ≥245 U/mg, 10 kU/vial	Sigma-Aldrich-Fluka, Steinheim, Germany	CAS No. 9000-81-1
Butyrylcholinesterase (BChE) from equine serum, ≥140 U/mg	SERVA, Heidelberg, Germany.	CAS No. 9001-08-5
<i>Aliivibrio fischeri</i> NRRL-B11177	Leibniz Institute, DSMZ, Braunschweig, Germany	Strain DSM-7151
<i>Bacillus subtilis</i> subsp. <i>spizizenii</i>	Merck, Darmstadt, Germany	Strain DSM-618

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Recombinant DNA		
<i>Saccharomyces cerevisiae</i> BJ1991, containing the hAR expression plasmid	Xenometrix, Allschwil, Switzerland	www.xenometrix.ch/shop/XenoScreen-YAS-Strain-Screening-for-Endocrine-Disruptive-Chemical-with-Androgenic-Activity
<i>Saccharomyces cerevisiae</i> BJ3505, containing the hER expression plasmid	McDonnell et al. (1991)	https://doi.org/10.1016/0960-0760(91)90038-7
PTM™ <i>Salmonella typhimurium</i> TA1535/pSK1002	Trinova Biochem, Giessen, Germany	https://trinova.de/components_for_assay_kits.php
Software and algorithms		
FreeMode option of winCATS software	CAMAG, Muttentz, Switzerland	version 1.4.7.2018
VisionCATS software	CAMAG, Muttentz, Switzerland	version 3.1.21109.3
Xcalibur 3.0.63 with Foundation 3.0 SP2 software	Thermo Fisher Scientific, Bellefonte, PA, USA	version 3.0.63
Other		
Scientific diving, SCUBA diving	Confédération Mondiale des Activités Subaquatiques (CMAS) standards	www.cmas.org
<i>Aliivibrio fischeri</i> medium	Klöppel et al. (2008)	https://doi.org/10.1556/jpc.21.2008.6.7
<i>Bacillus subtilis</i> medium and substrate	Jamshidi-Aidji and Morlock (2016)	https://doi.org/10.1021/acs.analchem.6b02648
<i>Salmonella typhimurium</i> medium and substrate	Meyer et al. (2020)	https://doi.org/10.14573/altex.2006201
<i>Saccharomyces cerevisiae</i> BJ3505 medium and substrate	Morlock and Klingelhöfer (2014)	https://doi.org/10.1021/ac501723j
<i>Saccharomyces cerevisiae</i> BJ1991 medium and substrate	Klingelhöfer et al. (2020)	https://doi.org/10.1016/j.aca.2020.05.057
Acetyl-/butyrylcholinesterase solution and substrate	Jamshidi-Aidji and Morlock (2018)	https://doi.org/10.1021/acs.analchem.8b03407
Heraeus Pico 17 Centrifuge	Thermo Scientific, Waltham, MA, USA	www.thermofisher.com
HPTLC system consisting of Automated TLC Sampler 4, Automated Developing Chamber 2, TLC Visualizer, Derivatizer, TLC Immersion Device and BioLuminizer	CAMAG, Muttentz, Schweiz	www.camag.com
Alternative open source OCLab3 system	open source	https://github.com/OfficeChromatography/OCLab3
KIS polypropylene box, 26.5 cm × 16 cm × 10 cm	ABM, Wolframs-Eschenbach, Germany	https://bauhaus.ch/de/kis-bi-box-xs-schwarz-66745701
Laboratory oven	Memmert, Schwabach, Germany	www.memmert.com
Standalone HPLC pump 515	Waters, Eschborn, Germany	www.waters.com
Dionex Ultimate HPLC system equipped with binary pump (HPG-3200SD), autosampler (WPS-3000TXRS), column oven (TCC-3000RS) and diode array detector (DAD-3000RS)	Dionex Softron, Germering, Germany	www.thermofisher.com
Q Exactive Plus Hybrid Quadrupole-Orbitrap	Thermo Fisher Scientific, Bellefonte, PA, USA	www.thermofisher.com
50-mL Centrifuge tubes	Isolab, Wertheim, Germany	https://isolab.de/default
2-mL Laboratory tubes, Protein LoBind Tube	Eppendorf, Hamburg, Germany	www.eppendorf.com

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Gertrud E. Morlock (gertrud.morlock@uni-giessen.de).

**Materials availability**

This study did not generate new unique reagents.

Data and code availability

This study did not generate new codes. Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

The microorganisms *A. livivrio fischeri* NRRL-B11177, *Saccharomyces cerevisiae* BJ1991, containing the hAR expression plasmid, *Saccharomyces cerevisiae* BJ3505, containing the hER expression plasmid, PTM™ *Salmonella typhimurium* TA1535/pSK1002 and *Bacillus subtilis* subsp. *spizizenii* were used for the planar on-surface assays as described.

METHOD DETAILS

We confirm that the following experiments were conform to relevant regulatory standards.

Study sites

Along the coastline of Hurghada and El Gouna, Northern Red Sea, Egypt, the research area of approximately 600 km² ([Figure S1](#)) ranges from the reefs of Shaab Umm Usk in the North (27°35.001 N, 33°52.272 E) to the Abu Hashish reefs in the South (27°1.453 N, 33°55.644 E). Large reef complexes are typical for this region, which is marked by islands and a variety of coastal and offshore coral reefs either isolated or grouped ([Riegl and Velimirov 1994](#); [Costa 2015](#)). Belonging to one of the most diverse ecosystems worldwide, these Red Sea reef complexes provide a diversity of substrates to rub on (e.g., hard and soft corals, sponges, sand, sea grass beds). Large parts of the Red Sea comprise a shallow coastal shelf. Next to it, the bottom slopes gradually to depths of 500 m average depth is 491 m and the maximum depth is 2850 m ([Edwards and Head, 1987](#)). The salinity ranges from 38–39 ppt, but can also rise to 42 ppt ([Behairy et al., 1993](#)). For the Gulf of Aqaba an average summer temperature of 21–27°C is given for the upper 200 m ([Manasrah et al., 2006](#)). It offers optimal conditions for the emergence of coral reefs and contains a high number of endemic species ([Fine et al., 2019](#)). The organism samples were taken by SCUBA diving on the reefs Shaab El Erg and Shaab El Fanous which are valuable meeting points for the dolphins, especially for resting ([Kreicker and Ziltener 2017](#); [Fumagalli et al., 2019](#)), but also for socializing ([Orbach et al., 2019](#)) and rubbing on selected substrates ([Ziltener et al., 2015](#)). Since it is obvious that the reefs with the occurring organisms are regularly visited by the dolphins, it is important to protect these areas. For this reason, the areas have now been declared an Important Marine Mammal Area. However, further work is needed to ensure the full protection of these sites ([Marine mammal, 2019](#)).

Behavioural data collection

Photo-identification of dolphins by using the mark-recapture method was conducted in a research boat for at least 5 min ([Würsig and Würsig 1977](#)). Data were collected including group behaviour, group composition and structure, GPS location, water depth and features of the accessed organism. Secondly, in appropriate situations, a minimum of two SCUBA divers then joined the dolphins underwater and conducted a group follow using video and photo cameras. Individual identification data was completed with whole body photographs that allowed gender and age classifications, the assessment of reproductive state and physical condition (e.g., skin lesions, ectoparasites, etc.).

Substrate collection

The project was authorized by the Egyptian Ministry of Environment on 22 July 2019 and supervised by the Red Sea National Parks Authorities. The dives off the coast of El Gouna and Hurghada, Egypt, were conducted from 30 July 2019 to 1 August 2019 according to the standards of recreational and scientific diving (VDST, CMAS) and according to Egyptian regulations. Boat surveys were conducted for a minimum of 5 min *ad libitum*. When the conditions were given, two SCUBA divers started to follow a group of dolphins to continuously record underwater behaviour on video. In addition to the dolphin behaviour surveys, independent dives were conducted to collect the three distinct marine organisms visited by the dolphins. During the dives, only those organisms were sampled that were explicitly accessed by the dolphins for rubbing ([Table S1](#) Compilation of the organisms, related to [Figures 1](#) and [S1](#)). The initial dive protocol



(Brümmer et al., 2009) was adapted to the present sampling, so that it contained the following described steps. First, a photo of the labelled sample tube was taken with an underwater camera (TG-4 with PT-056, Olympus Corporation, Tokyo, Japan) and an underwater torch (DS-21000 NWRUV, DiveSun LED Tauchlampen, Chemnitz, Germany), so it was possible to assign each sample to the corresponding organism. For each sample the depth at which it was taken was noted (Table S1 Compilation of the organisms, related to Figures 1 and S1). For underwater photo documentation, several overview images and several detailed images were taken, both with and without a scale (Figures S3–S5). In this way, each collection position was specified in terms of the exact location visited by the dolphins. Depending on the organism, small pieces of about 3–5 cm each were cut off with scissors or a scalpel (Figure 1 and S3–S5) and transferred to a 50-mL centrifuge tube (Isolab, Wertheim, Germany) filled with sea water. To obtain another specimen for each individual organism, a second sample was imaged taken, and documented in the same way. Additional samples were taken in labeled 2-mL laboratory tubes (Protein LoBind Tube, Eppendorf, Hamburg, Germany). In order to close these small tubes under water, a small air bubble had to be in the tubes. For these samples, the extraction solvent was already added on the boat directly after the dive, without freezing the samples in between. Therefore, the remaining water was removed with a micropipette (Eppendorf) and 1 mL methanol (99.9%; Merck, Darmstadt, Germany) was added (IDs 7–10). The other samples were drained. All sample tubes were put into a cooling box half-filled with ice and salt to freeze them as fast as possible aboard. The box was tightly sealed with a lid. Afterwards the drained samples were prepared in the laboratory at the El Gouna campus of the Technical University of Berlin. Each collected gorgonian coral (G), leather coral (L) and sponge (S) sample was cut with a scalpel into very small pieces and divided with tweezers as homogeneously as possible in 2-mL laboratory tubes. Either methanol (99.9%; IDs 1–6) or *n*-hexane (95%; Tedia Company, Fairfield, OH, USA; IDs 11–16) was added according to the structural consistency of the marine organism as follows: 200, 400 and 800 μ L for leather coral, sponge and gorgonian coral, respectively (Table S2 Sample preparation, related to STAR Methods). Note that the volumes were kept as low as possible to avoid concentration/reconstitution steps and to allow small application volumes for analysis. All tubes were stored at -20°C and transported chilled to Germany.

Sample preparation

Although the fresh organisms were cut into comparably small pieces for extraction, the surface area of the differently structured substrates and their intrinsic water contents varied, which could influence the extraction yield. Hence, the effect-directed profiling was used for a qualitative comparison only. The gorgonian coral extract was quite clear and dark brown due to the natural dyes contained therein. The leather coral extract was comparatively brighter and more of a suspension. The sponge extract was light-brownish grey and very turbid due to its fine structural consistency. Methanol samples were vortexed and ultrasonicated at almost 100% humidity at $20\text{--}25^{\circ}\text{C}$ for 10 min (Sonorex Digiplus, Bandelin, Berlin, Germany). Each solution was transferred to a 1.5-mL Eppendorf tube (Eppendorf, Hamburg, Germany) and centrifuged for 5 min at 13,000 rpm (Heraeus Pico 17 Centrifuge, Thermo Scientific, Waltham, Massachusetts, USA). The supernatant was transferred into sample vials for HPTLC. Since *n*-hexane was almost evaporated during the transport to Germany, a 200- μ L portion *n*-hexane (95%; Chemsolute, Th. Geyer, Renningen, Germany) was added for volume compensation to these samples. As the marine organism contained water, two phases were formed, which were united by the 1:1 dilution with acetone (Merck, for chromatography). In detail, 500, 700 and 750 μ L acetone were added to the gorgonian coral, leather coral and sponge extracts, respectively (Table S2 Sample preparation, related to STAR Methods), which were prepared as mentioned.

Effect-directed profiling

The extracts were weighed back and the approximate substrate amounts were calculated to be around $1\text{ mg} \pm 0.4\text{ mg}$ depending on the structure of the organism (Table S2 Sample preparation, related to STAR Methods). The samples (1–10 μ L depending on the assay) were automatically applied (ATS 4, CAMAG, Muttenz, Switzerland). The settings “fill only programmed volume” and “vacuum time 0 s” were crucial to avoid any volume loss of the sample during the application. The application volume of the boat samples was comparatively higher depending on the substrate (2.4, 3.2 and 3.6 μ L instead of 1 μ L) to compensate for and adjust to the higher methanol addition (1 mL for all). The development on HPTLC plates silica gel 60 was automatically performed (ADC 2, CAMAG) with ethyl acetate – methanol – water 15:3:1 (V/V/V). For separation of apolar zones, *n*-hexane – ethyl acetate 3:4 (Figures S10C–S10F) and 1:4 (Figure S10G) were used. Each chromatogram was multi-detected at white light illumination (Vis), 254 and 366 nm (TLC Visualizer 2, CAMAG). For each assay, a respective positive control (PC) was applied on the upper plate edge. The assay suspensions or solutions were piezoelectrically sprayed; if



not stated otherwise (Derivatizer, CAMAG). The chromatogram was horizontally placed for incubation in a polypropylene box (27 × 16 × 10 cm, KIS, ABM, Wolframs-Eschenbach, Germany). It was pre-moistened for 30 min, using 35 mL water spread on filter papers aligned on walls and bottom at room temperature. Images at FLD 366 nm or white light illumination were recorded depending on the assay. The bioluminescence images were taken in the BioLuminizer (CAMAG). As an alternative instrumental system, the miniaturized open source OCLab3 system (<https://github.com/OfficeChromatography/OCLab3>) can be used (Morlock 2021). The following ten assays were applied:

- 1) For the Gram-negative *Aliivibrio fischeri* bioassay (Klöppel et al., 2008; DIN EN ISO 2009; Klöppel et al., 2013), 150 μ L cryostock (NRRL-B11177, strain 7151; Leibniz Institute, DSMZ, Braunschweig, Germany) were cultivated in 20 mL medium in a 100-mL culture flask at 75 rpm and room temperature for 18–24 h according to DIN EN ISO 11348–1. When the green–blue bioluminescence of the bacteria was brilliant, 4 mL bacterial suspension were sprayed on the plate (blue nozzle, level 6). The still humid plate placed in the cabinet of the BioLuminizer (CAMAG). Ten images were recorded over 30 min with an exposure time of 60 s and trigger interval of 3.0 min. Antimicrobials were detected as a dark or bright band on the instantly bioluminescent plate background. Caffeine (0.5, 1.5 and 3 μ L/band, 1 mg/mL in methanol) was used as a positive control.
- 2) For the Gram-positive *Bacillus subtilis* bioassay (Jamshidi-Aidji and Morlock 2016; Chandana and Morlock 2021), 100 μ L cryostock (BGA, DSM 618 strain, spores, Merck, Darmstadt, Germany) were cultivated in 20 mL 2.3% Müller–Hinton broth with an optical density of ca. 0.8 at 600 nm. Thereof, 3.5 mL bacteria suspension were sprayed (red nozzle). Incubation at 37°C followed for 2 h in an oven (Memmert, Schwabach, Germany). Then, 500 μ L 0.2% DPBS-buffered MTT solution was sprayed and incubated at 37°C for 45 min, followed by drying (50°C, 5 min, Plate Heater, CAMAG). Colourless (white) antibacterial bands were obtained on a purple background. Tetracycline (0.5, 1.5 and 3 μ L/band, 0.005 mg/mL in ethanol) was used as positive control.
- 3/4) For the acetyl-/butyrylcholinesterase inhibition assays (Jamshidi-Aidji and Morlock 2018; Morlock et al., 2021), 1 mL Tris–HCl buffer (pH 7.8, 0.05 M, for prewetting) were sprayed (green nozzle), followed by 3 mL enzyme solution (6.6 U/mL acetylcholinesterase from *Electrophorus electricus*, or 3.3 U/mL butyrylcholinesterase from equine serum, both in Tris–HCl buffer, Sigma–Aldrich–Fluka, Steinheim, Germany). Incubation at 37°C followed for 25 min. Piezoelectric spraying of 0.75 mL of a 1:2 mixture of ethanolic α -naphthyl acetate solution and aqueous Fast Blue B salt solution (each 3 mg/mL, red nozzle) led to colourless (white) inhibition bands on a purple background. Rivastigmine (2, 4 and 8 μ L/band, 0.1 mg/mL in methanol) was used as a positive control.
- 5/6) For the planar yeast estrogen screen (pYES) bioassay (Morlock and Klingelhöfer 2014), 1 mL cryostock (1×10^8 *Saccharomyces cerevisiae* BJ3505 cells genetically modified by McDonnell et al., 1991) was added to 29 mL culture medium in a 100-mL glass culture flask, followed by incubation at 30°C by shaking at 100 rpm for 20–22 h. The cell culture adjusted to 0.8×10^8 mL⁻¹ cells (2.8 mL) was piezoelectrically sprayed (Derivatizer, CAMAG, red nozzle, spraying level 6) and incubated at 30°C and 100% humidity for 3 h. After drying in a cold stream of air for 4 min, the substrate solution (2 mg 4-methylumbelliferyl- β -D-galactopyranoside, 100 μ L dimethyl sulfoxide, 3 mL citrate buffer pH 12) was sprayed on. After the second incubation (1 h, 37°C), the detection at FLD 366 nm (Reporter 3, CAMAG) showed estrogen-like compounds as blue fluorescent bands. The estrogen 17 β -estradiol (E2, 1 μ L, 100 ng/mL in ethanol) was used as a positive control. For the planar yeast antagonistic estrogen screen (pYAES) bioassay, the estrogen 17 β -estradiol (2 ng/mL; 5 μ L applied) was applied, partially overlapping (e.g., at 7–8 mm of the 12-mm band) along each track as 1×70 mm area (FreeMode option, winCATS software), before the assay application.
- 7/8) For the planar yeast androgen screen (pYAS) bioassays (Klingelhöfer et al., 2020), the workflow was analogously performed to the pYES bioassay, except for the use of *Saccharomyces cerevisiae* BJ1991 cells containing the human AR expression plasmid (Xenometrix, Allschwil, Switzerland) and an incubation of only 4 h. Testosterone (1 μ L, 0.75 μ g/mL in methanol) was used as a positive control. For the planar yeast antagonistic androgen screen (pYAAS) bioassay, the androgen testosterone (4 μ L, 1.5 μ g/mL in methanol) was applied, partially overlapping (e.g., at 7–8 mm of the 12-mm band) along each track as a 1×70 mm area (FreeMode option, winCATS software) before the assay application.



- 9) For the genotoxic bioassay (Meyer et al., 2020), 20 μ L cryostock (PTM™ *Salmonella typhimurium* TA1535/pSK1002, Trinova Biochem, Giessen, Germany) were cultivated in 35 mL lysogeny broth for 16 h to reach an optical density of ca. 0.2 at 660 nm after re-suspension. The chromatogram was immersed (immersion speed 3.5 cm/s, for 3 s) in the *Salmonella* suspension and incubated at 37°C for 3 h. Alternatively, spraying was used. After plate drying, the workflow was the same as for the pYES bioassay. 4-NQO (1 ng/ μ L, 1 μ L in methanol) was manually applied as a positive control.
- 10) For the 2,2-diphenyl-1-picrylhydrazyl (DPPH•) assay (Krüger et al., 2018), 4 mL 0.04% methanolic DPPH• solution was sprayed (green nozzle, level 4) to instantly generate yellow bands on a purple background. The plate image was inspected again on the next day due to signal intensity increase. Gallic acid (0.5, 1.3 and 2 μ L/band, 0.1 mg/mL in methanol) was used as a positive control.

Characterization by HPTLC–HESI-HRMS

Higher sample volumes were applied twofold (as listed in respective figure legend). Plates were prewashed (Morlock 2014). After development, each HPTLC plate was cut in two identical halves, one was subjected to the respective assay and the other was used for the HRMS recording. For the latter, the positions were marked on the chromatogram with reference to the active zones on the other plate half. The marked zones were eluted with methanol for 1 min at a flow rate of 0.2 mL/min provided by a Dionex Ultimate LPG-3400XRS quaternary pump (Dionex Softron) using an elution head-based interface (TLC–MS Interface 2, CAMAG). The latter was coupled to a Q Exactive Plus Hybrid Quadrupole Orbitrap mass spectrometer (Thermo Fisher Scientific) equipped with an Ion Max HESI II source operated with the following settings: spray voltage $\pm$ 3.5 kV, capillary temperature 270°C, sheath gas 20 arbitrary units, aux gas 10 arbitrary units, S–Lens RF level 50 and probe heater temperature 200°C. Full scan mass spectra (m/z 100–1000) were recorded with a resolution of 280,000 (FWHM at m/z 200), AGC target $1e6$, maximum inject time 200 ms and the lock masses 413.26623 (diisooctyl phthalate) and 301.14103 (dibutyl phthalate) in the positive ionization mode, and 255.23295 (palmitic acid) and 283.26425 (stearic acid) in the negative ionization mode. From each analyte spectrum, a plate background spectrum was subtracted using Xcalibur 3.0.63 with Foundation 3.0 SP2 software (Thermo Fisher Scientific).

Characterization by NP-HPTLC–bioassay–RP-HPLC–PDA–HESI-MS

According to the latest workflow (Schreiner and Morlock 2021) bioactive zones were eluted (TLC–MS Interface, CAMAG) with 10% aqueous methanol (MS-grade, Honeywell, Morristown, NJ, USA) provided by a standalone pump (515 HPLC pump, Waters, Eschborn, Germany) at a flow rate of 0.1 mL/min. The eluted zone was transferred through a biocompatible inline-filter (IDEX Health & Science, Oak Harbor, WA, USA) containing a PEEK frit (0.5 μ m, Techlab, Braunschweig, Germany) to a two-position switching-valve (MXT-Series PD715-000, Rheodyne IDEX Health & Science), where the analytes were trapped on a defender guard/pre-column (Accucore RP-MS, 10 mm $\times$ 2.1 mm, 2.6 μ m, Thermo Scientific, Bellefonte, PA, USA). By switching, trapped analytes were guided to an Acquity UPLC System (Waters, Eschborn, Germany) equipped with a quaternary solvent manager, sample manager, column oven (thermostated at 40°C), photodiode array detector and a single quadrupole mass spectrometer. Binary gradient (A: methanol, B: 2.5 mM ammonium acetate, pH 4.5 adjusted with acetic acid) flushed the precolumn and transferred the analytes to the main RP column (Accucore RP-MS, 100 mm $\times$ 2.1 mm, 2.6 μ m, Thermo Scientific) for orthogonal separation. The photodiode array detector (190–400 nm) was operated with a deuterium lamp, and the MS instrument according to the following conditions: polarity switching mode with cone voltage $\pm$ 10 V, sampling frequency 5 Hz, ESI probe temperature 600°C, source temperature 120°C and scan range m/z 50–1000. Instruments were controlled by MassLynx V4.2 software (Waters). Mass spectra were depicted after a plate blank subtraction, recorded at a position comparable to the analyte.

QUANTIFICATION AND STATISTICAL ANALYSIS

All experiments were repeatedly performed and confirmed the imaging results or mass signals. Quantification and statistical analysis are not applicable.

ADDITIONAL RESOURCES

Additional resources were not applicable.

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Supplemental information

**Evidence that Indo-Pacific
bottlenose dolphins self-medicate
with invertebrates in coral reefs**

Gertrud E. Morlock, Angela Ziltener, Sascha Geyer, Jennifer Tersteegen, Annabel Mehl, Tamara Schreiner, Tamer Kamel, and Franz Brümmer

Supplementary Information

Table S1 Compilation of the organisms explicitly accessed by the dolphins for repeated rubbing behaviour. Sampling data on the three different organisms collected from 30th July 2019 to 1st August 2019 according to the standards of recreational and scientific diving (VDST, CMAS) and according to Egyptian regulations, Related to Figure 1.

Marine organism	ID	Sample place	Depth [m]
<i>Rumphella aggregata</i>	G1	Shaab El Fanous West	9.5
	G2	Shaab El Fanous East	12.0
	G3	Shaab El Erg	10.5
	G4	Shaab El Erg	10.6
	G5	Shaab El Erg	10.8
	G6	Shaab El Erg	10.0
	G7	Shaab El Erg	10.5
	G8	Shaab El Erg	10.6
	G9	Shaab El Erg	10.8
	G10	Shaab El Erg	10.0
<i>Ircinia</i> sp.	S1	Shaab El Fanous East	12.7
	S2	Shaab El Fanous East	12.8
	S3	Shaab El Erg	8.3
	S4	Shaab El Erg	9.7
	S5	Shaab El Erg	7.8
	S6	Shaab El Erg	7.1
	S7	Shaab El Erg	8.3
	S8	Shaab El Erg	9.7
	S9	Shaab El Erg	7.8
	S10	Shaab El Erg	7.1
<i>Sarcophyton</i> sp.	L1	Shaab El Erg	10.5
	L2	Shaab El Erg	12.0
	L3	Shaab El Erg	9.2
	L4	Shaab El Erg	9.4
	L5	Shaab El Erg	9.8
	L6	Shaab El Erg	9.2
	L7	Shaab El Erg	9.2
	L8	Shaab El Erg	9.4
	L9	Shaab El Erg	9.8
	L10	Shaab El Erg	9.2

Table S2 Sample preparation of the three different organisms collected. Related to Figure 1.

Sponge ID	Treated in	Same as	Tube no.	Extraction solvent	Solvent volume [μL]	ad volume ¹ [μL]	Sample weight [mg] ²
S1	Lab		1	Methanol	400	-	1352
			2	Methanol	400	-	1242
S11			3	<i>n</i> -Hexane	400	1350	1465
S2	Lab		1	Methanol	400	-	1738
			2	Methanol	400	-	1389
S12			3	<i>n</i> -Hexane	400	1350	1122
S3	Lab	S7	1	Methanol	400	-	1216
			2	Methanol	400	-	1463
S13			3	<i>n</i> -Hexane	400	1350	1240
S4	Lab	S8	1	Methanol	400	-	875
			2	Methanol	400	-	1088
S14			3	<i>n</i> -Hexane	400	1350	949
S5	Lab	S9	1	Methanol	400	-	1139
			2	Methanol	400	-	1314
S15			3	<i>n</i> -Hexane	400	1350	1131
S6	Lab	S10	1	Methanol	400	-	1369
			2	Methanol	400	-	1452
S16			3	<i>n</i> -Hexane	400	1350	1463
S7	Boat			Methanol	1000	-	600
S8	Boat			Methanol	1000	-	897
S9	Boat			Methanol	1000	-	970
S10	Boat			Methanol	1000	-	897
Gorgonian coral ID	Treated in	Same as	Tube no.	Extraction solvent	Solvent volume [μL]	After addition ³ [μL]	Sample weight [mg] ²
G1	Lab		1	Methanol	800	-	1224
			2	Methanol	800	-	1258
G11			3	<i>n</i> -Hexane	800	1500	892
G2	Lab		1	Methanol	800	-	1285
			2	Methanol	800	-	1090
G12			3	<i>n</i> -Hexane	800	1500	671
G3	Lab	G7	1	Methanol	800	-	1088
			2	Methanol	800	-	1138
G13			3	<i>n</i> -Hexane	800	1500	730
G4	Lab	G8	1	Methanol	800	-	1231
			2	Methanol	800	-	1379
G14			3	<i>n</i> -Hexane	800	1500	685
G5	Lab	G9	1	Methanol	800	-	1146
			2	Methanol	800	-	1016
G15			3	<i>n</i> -Hexane	800	1500	605
G6	Lab	G10	1	Methanol	800	-	1043
			2	Methanol	800	-	1049
G16			3	<i>n</i> -Hexane	800	1500	458
G7	Boat			Methanol	1000	-	813
G8	Boat			Methanol	1000	-	967
G9	Boat			Methanol	1000	-	735
G10	Boat			Methanol	1000	-	1046

Leather coral ID	Treated in	Same as	Tube no.	Extraction solvent	Solvent volume [μl]	After addition ⁴ [μL]	Sample weight [mg] ²
L1	Lab		1	Methanol	200	-	853
			2	Methanol	200	-	793
L11			3	<i>n</i> -Hexane	200	1100	936
L2	Lab		1	Methanol	200	-	899
			2	Methanol	200	-	952
L12			3	<i>n</i> -Hexane	200	1100	915
L3	Lab	L7	1	Methanol	200	-	925
			2	Methanol	200	-	887
L13			3	<i>n</i> -Hexane	200	1100	823
L4	Lab	L8	1	Methanol	200	-	786
			2	Methanol	200	-	984
L14			3	<i>n</i> -Hexane	200	1100	915
L5	Lab	L9	1	Methanol	200	-	1002
			2	Methanol	200	-	933
L15			3	<i>n</i> -Hexane	200	1100	1180
L6	Lab	L10	1	Methanol	200	-	770
			2	Methanol	200	-	933
L16			3	<i>n</i> -Hexane	200	1100	722
L7	Boat			Methanol	1000	-	972
L8	Boat			Methanol	1000	-	994
L9	Boat			Methanol	1000	-	687
L10	Boat			Methanol	1000	-	843

¹200 μL *n*-Hexane and 750 μL acetone were added.

²The extractant volumes in the Eppendorf tubes were 200, 400, 800 and 1000 μL of *n*-hexane or methanol as specified. In Germany, the weight of the samples was determined approximately: three empty Eppendorf tubes with the respective extractant were weighed and averaged. This mean value was subtracted from the respective sample tube weight.

³200 μL *n*-Hexane and 500 μL acetone were added.

⁴200 μL *n*-Hexane and 700 μL acetone were added.

Table S3 Ten different assays applied for effect-directed profiling. Organisms used in the effect-directed assays and respectively generated responses for detection of bioactive metabolites in the three selected substrates. Related to Figure 2.

Biological assay		Final response of the band	Interpretation
Gram-negative bacteria	<i>Aliivibrio fischeri</i>	Dark	Antimicrobials and bioactive compounds
		Bright	- Reduction of bioluminescence related to decrease of energetic cell metabolism
	<i>Salmonella typhimurium</i> TA1535/pSK1002	Blue fluorescent	- Increase of bioluminescence related to improved energetic cell metabolism
Gram-positive bacteria	<i>Bacillus subtilis</i>	Blue fluorescent	Genotoxic effect
Yeast cells	<i>Saccharomyces cerevisiae</i> BJ3505	Colourless	Antimicrobials
		Blue fluorescent	Estrogen-like effect
	<i>Saccharomyces cerevisiae</i> BJ1991	Reduction of blue fluorescence	Antiestrogen-like effect
		Blue fluorescent	Androgen-like effect
		Reduction of blue fluorescence	Antiandrogen-like effect
Biochemical assay			
Enzymes	Acetylcholinesterase inhibition assay	Colourless	Neurotoxins or inhibitors of the degrading of the neurotransmitter acetylcholine in the nerve synapses, thus improving cholinergic neurotransmission
	Butyrylcholinesterase inhibition assay	Colourless	Neurotoxins or inhibitors decreasing the increased number of neuritic plaques in demented brains, acting non-specific in plasma and tissue
Chemical assay			
Chemical	2,2-Diphenyl-1-picrylhydrazyl (DPPH•)	Yellow	Radical scavengers

Table S4 Compilation of activities described in literature. Bioactive metabolites already isolated and described for the three different marine organisms. Related to Figures 3–5.

Species	Substance	Molecular formula	Weight (Da)	Effect	Literature
<i>Ircinia</i> sp.	7-Methyl-9-oxo-dec-7-eneoic acid	C ₁₁ H ₁₈ O ₃	198.26	Active in Alzheimer's diseases	(Tatli et al. 2008)
	2-Hexaprenylhydroquinone	C ₃₆ H ₅₄ O ₂	518.81	Reverse transcriptase and HIV-integrase inhibitor, bacteriostatic	(Bifulco et al. 1995; Loya et al. 1997)
	Pentaprenylhydroquinone 4-sulfate	C ₃₁ H ₄₆ O ₅ S	530.77	Neuropeptide Y receptor, tyrosine protein kinase and HIV-integrase inhibitor	(Bifulco et al. 1995)
	Hexaprenylhydroquinone 4-sulfate	C ₃₆ H ₅₄ O ₅ S	598.89	Neuropeptide Y receptor, tyrosine protein kinase and HIV-integrase inhibitor	
	Heptaprenylhydroquinone 4-sulfate	C ₄₁ H ₆₂ O ₅ S	667.01	Neuropeptide Y receptor, tyrosine protein kinase and HIV-integrase inhibitor	
	Variabilin	C ₂₅ H ₃₄ O ₄	398.54	Antibacterial	(Faulkner 1973)
	Fasciculatin	C ₂₅ H ₃₄ O ₄	398.54	Inosine monophosphate dehydrogenase inhibitor Moderately cytotoxic	(Cafieri et al. 1972; Rifai et al. 2005)
	Palinurin	C ₂₅ H ₃₄ O ₄	398.54	Cytotoxic	(Martí et al. 2003)
	Tedanolid C	C ₃₂ H ₅₀ O ₁₁	610.73	Cytotoxic	(Blunt et al. 2008)
	Irciniasulfonic acid B1	C ₃₆ H ₆₅ NO ₆ S	639.97	Reversing multi-drug resistance in cancer cells	
	Irciniasulfonic acid B2	C ₃₄ H ₆₃ NO ₆ S	613.93	Reversing multi-drug resistance in cancer cells	
	Irciformonin C	C ₂₃ H ₃₄ O ₇	422.51	Moderately cytotoxic	
	Irciformonin D	C ₂₃ H ₃₄ O ₇	422.51	Moderately cytotoxic	
	Ircinolin A	C ₂₁ H ₃₄ O ₆	382.49	Cytotoxic	(Mioso et al. 2017)
	15-Acetylirciformonin B	C ₂₄ H ₃₄ O ₆	418.52	Cytotoxic	
	10-Acetylirciformonin B	C ₂₄ H ₃₄ O ₆	418.52	Cytotoxic	
	Irciformonin B	C ₂₂ H ₃₂ O ₅	376.49	Cytotoxic	
	Irciformonin F	C ₂₂ H ₃₂ O ₅	376.49	Cytotoxic	
<i>Rumphella</i> sp.	Fucoxanthin	C ₄₂ H ₅₈ O ₆	658.91	Cytotoxic	(Alarif 2012)
	5 α ,8 α -Epidioxysterosta-6,9(11)-diene-3 β -ol	C ₂₈ H ₄₄ O ₃	428.33	Moderately cytotoxic	(Yin et al. 2020)
	Antipacid A	C ₁₅ H ₂₄ O ₃	252.17	Anti-inflammatory	(Chang et al. 2020)
	Rumphellolide L	C ₃₀ H ₄₈ O ₄	472.36	Anti-inflammatory	
	Rumphellaic acid A	C ₁₅ H ₂₄ O ₂	236.35	Anti-inflammatory	(Chung et al. 2014a)
	Rumphellol A	C ₁₅ H ₂₄ O ₂	236.35	Anti-inflammatory	(Chung et al. 2014c)
	Rumphellol B	C ₁₇ H ₃₀ O ₂	266.22	Anti-inflammatory	
	Rumphellaone C	C ₁₄ H ₂₄ O ₄	268.17	Anti-inflammatory	(Chung et al. 2014b)
	2 β -Acetoxyclovan-9 α -ol	C ₁₇ H ₂₈ O ₃	280.20	Anti-inflammatory	(Chung et al. 2013)
	9 α -Acetoxyclovan-2 β -ol	C ₁₇ H ₂₈ O ₃	280.20	Anti-inflammatory	
	Rumphellclovane B	C ₁₅ H ₂₄ O ₃	252.17	Anti-inflammatory	(Chung et al. 2011)
	Rumphellaone A	C ₁₅ H ₂₄ O ₃	252.17	Moderately cytotoxic	(Chung et al. 2010)

	Rumphellolide I	C ₁₄ H ₂₂ O ₃	238.32	Anti-inflammatory	(Sung et al. 2009)
	Rumphellatin B	C ₁₄ H ₂₃ ClO ₃	274.78	Antibacterial - <i>Staphylococcus aureus</i>	(Sung et al. 2007a)
	Rumphellolide A	C ₁₅ H ₂₄ O ₃	252.17	Antibacterial - <i>Pseudomonas aeruginosa</i>	(Sung et al. 2007b)
	Rumphellolide D	C ₁₄ H ₂₂ O ₃	238.32	Antibacterial - <i>Pseudomonas aeruginosa</i> - <i>Vibrio parahaemolyticus</i>	
	Rumphellolide E	C ₁₄ H ₂₂ O ₃	238.32	Antibacterial - <i>Escherichia coli</i> , - <i>Pseudomonas aeruginosa</i>	
	Rumphellolide F	C ₁₄ H ₂₂ O ₂	222.23	Antibacterial - <i>Staphylococcus aureus</i> - <i>Vibrio parahaemolyticus</i>	
Sarcophyton sp.	Sarcassin A	C ₂₂ H ₃₂ O ₅	376.49	Moderately cytotoxic	(Blunt et al. 2008)
	Sarcassin B	C ₂₂ H ₃₂ O ₆	392.47	Moderately cytotoxic	
	Sarcassin C	C ₂₃ H ₃₆ O ₆	408.53	Moderately cytotoxic	
	Sarcassin D	C ₂₃ H ₃₂ O ₆	404.50	Moderately cytotoxic	
	Sarcassin E	C ₂₁ H ₂₈ O ₅	360.44	Moderately cytotoxic	
	Bisglaucumlid C	C ₄₃ H ₆₂ O ₁₀	738.95	Moderately cytotoxic	
	Bisglaucumlid D	C ₄₃ H ₆₂ O ₁₀	738.95	Moderately cytotoxic	
	Alloaromadendrene	C ₁₅ H ₂₄	204.35	Cytotoxic	(El-Ezz et al. 2017)
	Sarcophine	C ₂₀ H ₂₈ O ₃	316.44	Cytotoxic, antifungal	
	Sarcophytolide	C ₂₀ H ₂₈ O ₃	316.44	Cytotoxic, antimicrobial properties - <i>Staphylococcus aureus</i> - <i>Pseudomonas aeruginosa</i> - <i>Candida albicans</i> - <i>Saccharomyces cerevisiae</i>	
	Sarcophytolide B	C ₂₀ H ₂₈ O ₃	316.44	Cytotoxic	
	Sarcophytolide C	C ₂₀ H ₂₈ O ₃	316.44	Cytotoxic	
	Sarcophytolol	C ₂₀ H ₃₄ O ₂	306.48	Cytotoxic	
	Isosarcophytoxide	C ₂₀ H ₃₀ O ₂	302.45	Antimicrobial	(Bowden et al. 1979)
	Sarcophytol B	C ₂₀ H ₃₂ O ₂	304.47	Cytotoxic	(Kobayashi et al. 1979)
	24ε-Methylcholestane-3β,5α,6β,25-tetraol 25-monoacetate	C ₃₀ H ₅₂ O ₅	492.38	Cytotoxic	(Zubair et al. 2016; Ahmed, M.M.A., Albadry, M.A., Ragab, E.A., El-Ghaly, E.M., Kotb, S.E., Khan, S.I., Chittiboyina, A.G. & Khan, I.A. 2019)
	(24S)-Methylcholestane-3β,5α,6β,12β,25-pentol 25-monoacetate	C ₃₀ H ₅₂ O ₆	508.38	Cytotoxic	
	(24S)-Methylcholestane-3β,5α,6β,25-tetraol 25-monoacetate	C ₃₀ H ₅₂ O ₅	492.38	Cytotoxic	
	(24S)-24-Methylcholestane-3β,5α,6β-triol	C ₂₈ H ₅₀ O ₃	434.38	Cytotoxic	
	(24S)-Ergostan-3β,5α,6β,18,25-pentaol,18,25-diacetate	C ₃₁ H ₅₂ O ₇	536.37	Cytotoxic	
	Sarcoaldestero A	C ₃₀ H ₅₂ O ₄	476.39	Antibacterial - <i>Escherichia coli</i> - <i>Bacillus megaterium</i> and antifungal - <i>Microbotryum violaceum</i> - <i>Septoria tritici</i>	

Sarcoaldestero B	C ₂₈ H ₅₀ O ₄	450.37	Antibacterial - <i>Escherichia coli</i> - <i>Bacillus megaterium</i> , and antifungal - <i>Microbotryum violaceum</i> - <i>Septoria tritici</i>
11 α -Acetoxy-16 β -methoxy- 23,24-dimethylcholest-17 (20)-en-3 β ,5 α ,6 β -triol	C ₃₂ H ₅₄ O ₆	534.39	Cytotoxic
(24R)-Gorgost-25-en- 3 β ,5 α ,6 β ,11 α -tetraol	C ₃₀ H ₅₀ O ₄	474.37	Cytotoxic
(24S)-11 α -Acetoxy-ergost- 3 β ,5 α ,6 β -triol	C ₃₀ H ₅₀ O ₅	490.37	Cytotoxic
(24R)-Methylcholest-7-en- 3 β ,5 α ,6 β -triol	C ₂₀ H ₄₈ O ₃	432.36	Moderately cytotoxic, antiviral - H1N1 IAV
(24S)-24-Methylcholestan- 1b,3b,5a,6b,25-pentaol 25-monoacetate	C ₃₀ H ₅₂ O ₆	508.38	Cytotoxic, anti-inflammatory antibacterial - <i>Staphylococcus aureus</i>
Gorgostane-1 α ,3 β ,5 α ,6 β , 11 α -pentaol	C ₃₀ H ₅₂ O ₅	492.38	Moderately antibacterial, antifungal
11 α -Acetoxycholest-24-en- 1 α ,3 β ,5 α ,6 β -tetraol	C ₂₉ H ₄₈ O ₆	492.35	Cytotoxic
Sarcopanol A	C ₃₀ H ₅₀ O ₆	506.36	Anti-inflammatory
Sarcomilasterol	C ₂₈ H ₄₈ O ₄	448.68	Anti-osteoporotic
(24S)-24-Methylcholestan- 3 β ,6 β ,25-triol-25-O-acetate	C ₃₀ H ₅₂ O ₄	476.39	Antibacterial - <i>Staphylococcus aureus</i>

Table S5 Assignment of the NP-HPTLC–HESI–HRMS signals of bioactive zones 1–10. Main signals in bold, Related to Figures 4 and S12.

Zone	Sample	Bioactivity	Fig.	Mass signal <i>m/z</i>	Assignment	Molecular formula	Δ ppm	Tentative molecule
1	G9	Metabolism enhancing	5D	138.0551	$[M1+H]^+$	$C_7H_7NO_2$	-0.36	
				160.0370	$[M1+Na]^+$		-0.31	
				182.0789	$[M2+Na]^+$	$C_7H_{13}NO_3$	-0.44	
				220.0614	$[M3+Na]^+$	$C_6H_{15}NO_4S$	0.14	Cyclohexylamine sulfate
				124.0073	$[M4-H]^-$	Unknown	-	
				181.0718	$[M5-H]^-$	$C_6H_{14}O_6$	0.00	Hexane-hexol (e.g., sorbitol)
				217.0483	$[M5+Cl]^-$		0.51	
2	S10	Metabolism enhancing	5D	203.0526	$[M6+Na]^+$	$C_6H_{12}O_6$	-0.25	Glucose
				215.0327	$[M6+Cl]^-$		0.29	
				124.9913	$[M7-H]^-$	$C_2H_6O_4S$	0.56	Ethyl sulfate
4	S12	Antibacterial	5D	105.0702	$[M8+H]^+$	C_8H_8	-3.52	
				122.0966	$[M9+H]^+$	$C_8H_{11}N$	-1.64	
				173.0421	$[M10+Na]^+$	$C_5H_{10}O_5$	-0.52	
6	G16	Metabolism enhancing	5D	243.0621	$[M11-H]^-$	$C_9H_{12}N_2O_6$	0.53	
8	S12	Antibacterial	5D	265.0795	$[M12+Na]^+$	$C_{10}H_{14}N_2O_5$	0.08	
				241.0830	$[M12-H]^-$		-0.12	
				111.0199	$[M13-H]^-$	$C_4H_4N_2O_2$	0.54	
				287.0885	$[M14-H]^-$	$C_{11}H_{16}N_2O_7$	-0.14	
9	S10	Antibacterial, weakly AChE/BChE inhibiting	5D	399.2531	$[M15+H]^+$	$C_{25}H_{34}O_4$	-0.35	Fasciculatin, variabilin, palinurin
				421.2349	$[M15+Na]^+$		-0.05	
				397.2383	$[M15-H]^-$		-0.03	
				795.4845	$[2M15-H]^-$		-0.25	
10	L8	Antibacterial, AChE/BChE inhibiting	5D S12	339.1931	$[M16+Na]^+$	$C_{20}H_{28}O_3$	0.24	Sarcophine, sarcophytolide, sarcophytolide B, sarcophytolide C
				655.3972	$[2M16+Na]^+$		-0.40	
				315.1966	$[M16-H]^-$		-0.29	
				331.1915	$[M16+O-H]^-$	$C_{20}H_{28}O_4$	0.03	
				333.2072	$[M16+H_2O-H]^-$	$C_{20}H_{30}O_4$	-0.11	
				347.1863	$[M16+2O-H]^-$	$C_{20}H_{28}O_5$	0.26	
				325.2138	$[M17+Na]^+$	$C_{20}H_{30}O_2$	0.28	Isosarcophytoxide
				641.4181	$[2M17+Na]^+$		-0.41	
				303.2329	$[M18-H]^-$	$C_{20}H_{32}O_2$	0.03	Sarcophytol B
				118.9419	$[M19-H]^-$	Unknown	-	

Table S6 Assignment of the NP-HPTLC– pYES/pYAAS–RP-HPLC–PDA–HESI-MS signals of bioactive zones 11–15. Main signals in bold, Same signals same colour, Related to Figures 5 and S13.

Zone	Sample	Bioactivity	Fig.	Mass signal <i>m/z</i>	Assignment	RT [min]	$\lambda_{\max}$ [nm]
11	L8	Estrogen-like	6E	315.18	$[M20-H_2O+H]^+$	8.13/8.24	276
				333.29	$[M20+H]^+$		
				350.27	$[M20+NH_4]^+$		
				355.21	$[M20+Na]^+$		
				371.05	$[M20+K]^+$		
				331.38	$[M20-H]^-$		
				377.32	$[M20+HCOO]^-$		
				391.32	$[M20+H_3C-COO]^-$		
12	L8/L13	Estrogen-like	6F	229.35	$[M21+H]^+$	7.07	-
				246.07	$[M21+NH_4]^+$	8.24	226
				267.10	$[M21+K]^+$		
				375.29	$[M22+H]^+$	8.54	231
				392.46	$[M22+NH_4]^+$		
				397.08	$[M22+Na]^+$		
				413.42	$[M22+K]^+$	8.75	-
				299.28	$[M23-H_2O+H]^+$		
				317.20	$[M23+H]^+$		
				334.43	$[M23+NH_4]^+$	8.75	-
				339.12	$[M23+Na]^+$		
				355.15	$[M23+K]^+$		
				315.24	$[M23-H]^-$	8.75	-
				333.29	$[M24-H_2O+H]^+$		
13	L8/G11	Estrogen-like	6G	349.32	$[M24+Na]^+$	8.75	-
				389.23	$[M24+K]^+$		
				331.27	$[M25-2H_2O+H]^+$	6.72/7.07	-
				349.32	$[M25-H_2O+H]^+$		
				366.36	$[M25]^+$		
				384.29	$[M25+NH_4]^+$		
				389.16	$[M25+Na]^+$		
				405.19	$[M25+K]^+$		
				365.35	$[M25-H]^-$		
				401.20	$[M25+Cl]^-$		
				411.27	$[M25+HCOO]^-$		
				425.27	$[M25+H_3C-COO]^-$		
				313.15	$[M26-2H_2O+H]^+$	7.44	-
				331.27	$[M26-H_2O+H]^+$		
				349.32	$[M26+H]^+$		
				366.36	$[M26+NH_4]^+$		
				387.20	$[M26+K]^+$		
				347.23	$[M26-H]^-$		
				393.28	$[M26+HCOO]^-$		
				380.61	$[M27+H]^+$	7.44	-
				403.42	$[M27+Na]^+$		
				419.12	$[M27+K]^+$		
				415.20	$[M27+Cl]^-$		
				425.08	$[M27+HCOO]^-$		
				439.01	$[M27+H_3C-COO]^-$		

14	L8	Antiandrogenic	7E	297.00	[M20-2H ₂ O+H] ⁺	8.12/8.23	275
				315.05	[M20-2H ₂ O+H] ⁺		
				332.15	[M20]⁺		
				350.27	[M20+NH ₄] ⁺		
				355.27	[M20+Na] ⁺		
				370.98	[M20+K] ⁺		
				299.28	[M23-H ₂ O+H] ⁺	8.54	225
				317.20	[M23+H]⁺		
				334.24	[M23+NH ₄] ⁺		
				338.99	[M23+Na] ⁺		
				355.15	[M23+K] ⁺		
				655.45	[2M23+Na] ⁺		
				331.33	[M27-H ₂ O+H] ⁺	7.44	-
				349.38	[M27+H]⁺		
				366.36	[M27+NH ₄] ⁺		
				387.26	[M27+K] ⁺		
				347.36	[M27-H] ⁻		
				403.16	[M28+Na] ⁺		
15	L13	Antiandrogenic	7F	419.44	[M28+K] ⁺	8.12/8.23	275
				415.32	[M28+Cl] ⁻		
				425.02	[M28+HCOO] ⁻		
				439.33	[M28+H ₃ C-COO] ⁻		
				315.18	[M21-2H ₂ O+H] ⁺		
				332.34	[M21]⁺		
				350.08	[M21+NH ₄] ⁺	8.53	225
				355.08	[M21+Na] ⁺		
				371.11	[M21+K] ⁺		
				299.28	[M23-H ₂ O+H] ⁺		
				317.14	[M23+H]⁺		
				334.31	[M23+NH ₄] ⁺		
				339.18	[M23+Na] ⁺		
				355.34	[M23+K] ⁺		
				655.45	[2M23+Na] ⁺		
				331.20	[M25-2H ₂ O+H] ⁺	6.73/7.09	-
				349.26	[M25-H ₂ O+H] ⁺		
				366.23	[M25]⁺		
				384.29	[M25+NH ₄] ⁺		
				389.35	[M25+Na] ⁺		
				405.32	[M25+K] ⁺		
				365.22	[M25-H] ⁻	8.51	208
				401.33	[M25+Cl] ⁻		
				411.27	[M25+HCOO] ⁻		
				425.14	[M25+H ₃ C-COO] ⁻		
				299.28	[M23-H ₂ O+H] ⁺		
				317.14	[M23+H]⁺		
				334.31	[M23+NH ₄] ⁺		
				339.25	[M23+Na] ⁺		
				355.34	[M23+K] ⁺		
				655.51	[2M23+Na] ⁺		



Figure S1. Study site of sampling

Study map of the Indo-Pacific bottlenose dolphins around Hurghada in the Northern Red Sea, Egypt ranges from the reefs of Shaab Umm Usk in the North to the Abu Hashish reefs in the south (yellow boxes). Organism samples were taken at the two reef sites Shaab El Erg and Shaab El Fanous (red circles), which are regularly visited by the dolphins for resting, socializing and rubbing, Related to Figure 1.

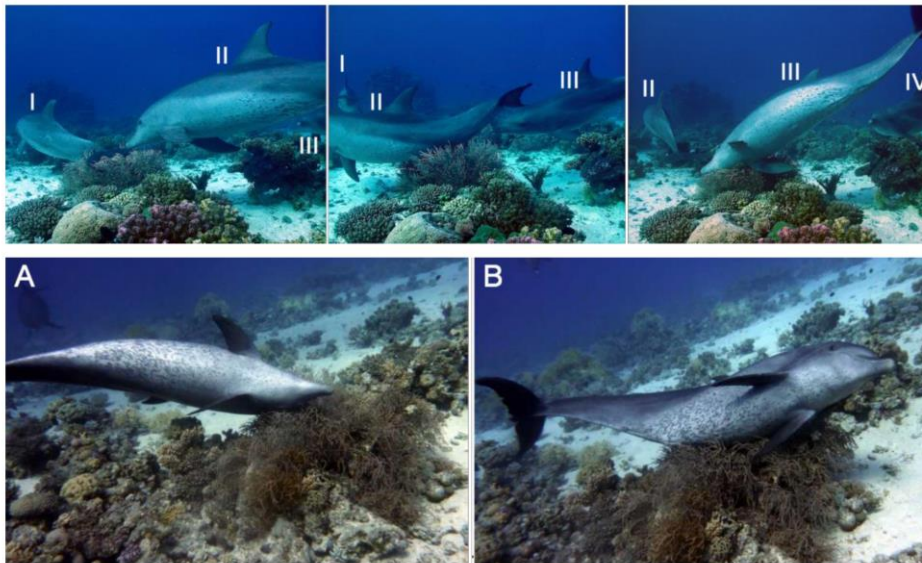


Figure S2. Rubbing behaviour of a group of Indo-Pacific bottlenose dolphins (I–IV), queuing up to rub against the gorgonian coral (gorgoning)

Dolphins queue up behind each other to wait their turn: (A) An Indo-Pacific bottlenose dolphin glides towards and (B) rubs its skin on the gorgonian coral *Rumphella aggregata* as documented in Shaab El Erg and Shaab El Fanous, Red Sea, Egypt (details in Videos [S1](#) and [S2](#)). The individual either rubs its ventral, lateral or dorsal body part on the gorgonian coral; its head, pectoral fins and fluke often touch the gorgonian too. This is often repeated so that all body areas are rubbed. When in groups, dolphins queue up behind each other to wait their turn for their next approach. In general, the soft gorgonian coral polyps start to close and retract when dolphins rub on them. This inherent coral protection mechanism can support abrasion, as it allows the dolphins' skin to come into contact with secondary metabolites. A larger mucus secretion from the branches of the coral during the rubbing behaviour has been observed, Related to Figure 1.

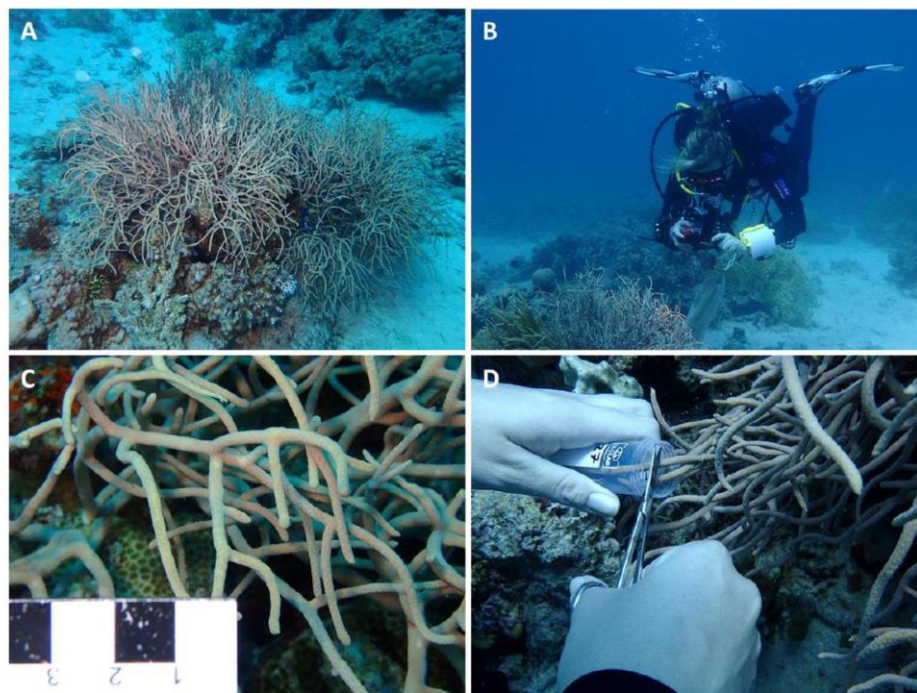


Figure S3. Underwater photo documentation of the gorgonian coral sampling

(A) Individual gorgonian coral *Rumphella aggregata*, (B) overview image, (C) detail image with scale bar and (D) sampling process, Related to Figure 1. Scientific diver Jennifer Tersteegen consents to the use of the image.

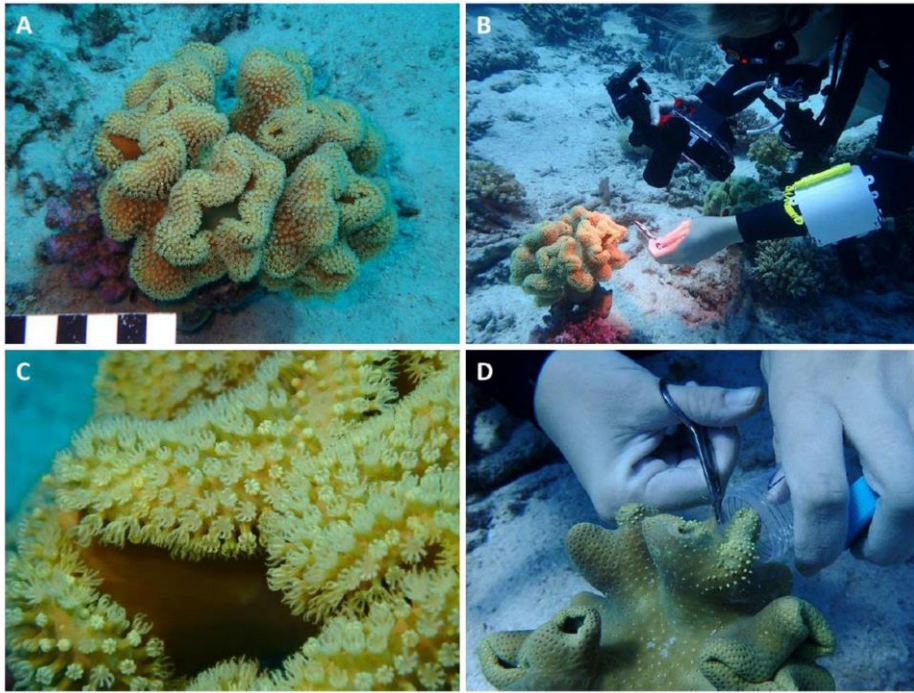


Figure S4. Underwater photo documentation of the leather coral sampling

(A) Individual leather coral *Sarcophyton* sp. with scale bar, (B) overview image, (C) detail image and (D) sampling process, Related to Figure 1. Scientific diver Jennifer Tersteegen consents to the use of the image.

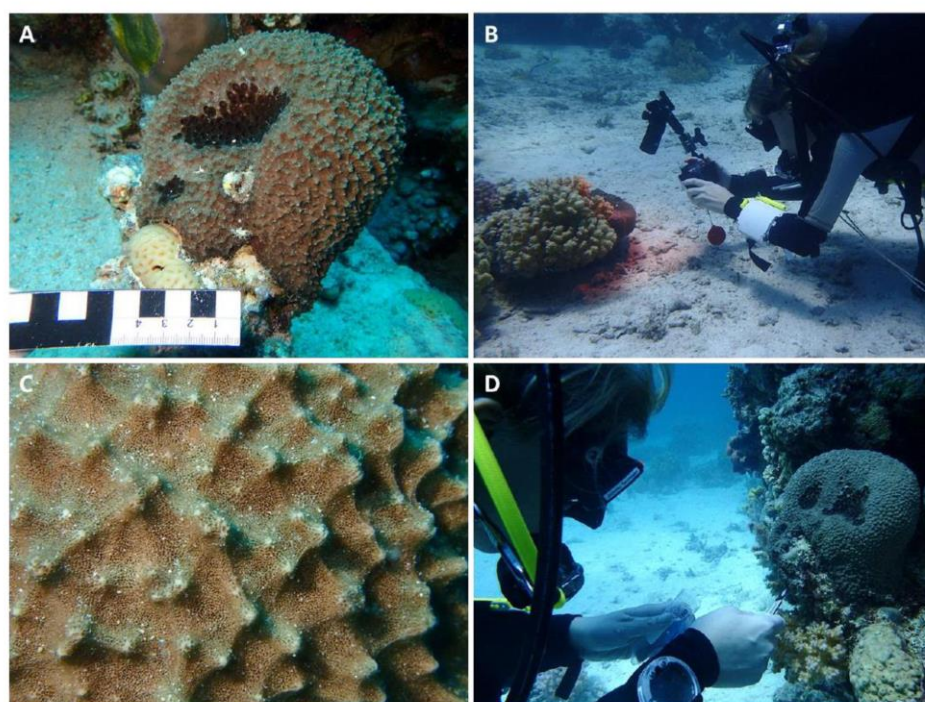


Figure S5. Underwater photo documentation of the sponge sampling

(A) Individual sponge *Ircinia* sp. with scale bar, (B) overview image, (C) detail image and (D) sampling process, Related to Figure 1. Scientific diver Jennifer Tersteegen consents to the use of the image.

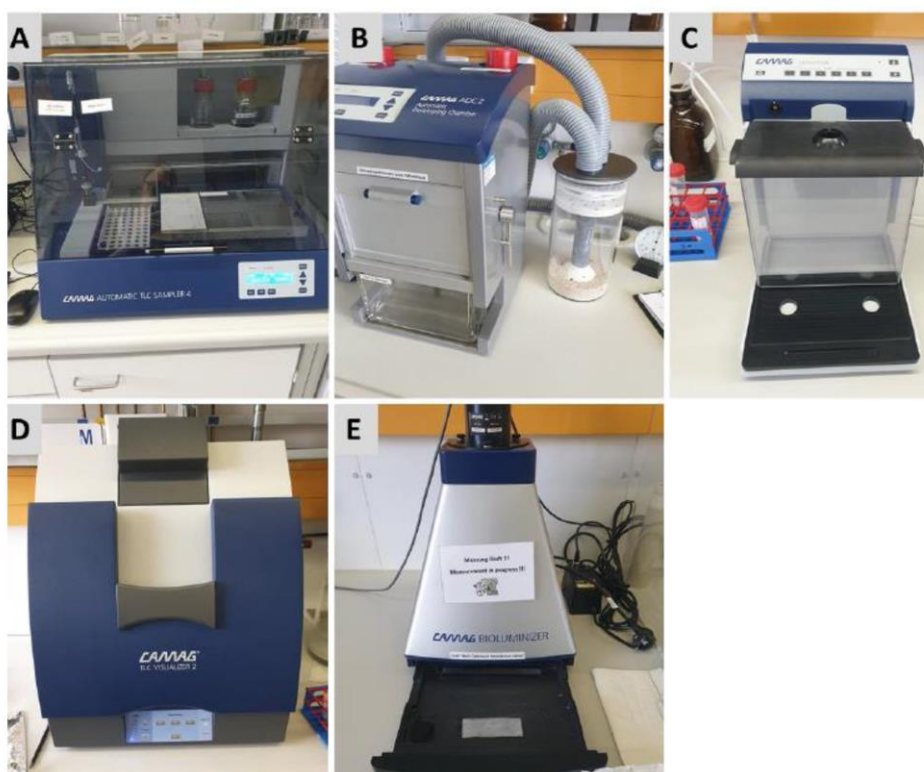


Figure S6. HPTLC instrumentation used for the effect-directed profiling

(A) Application, (B) development, (C) piezoelectric spraying of assay solutions or suspensions, (D) UV/Vis/FLD detection and (E) bioluminescence detection; all in operation in Video [S6](#), Related to Figure 2.

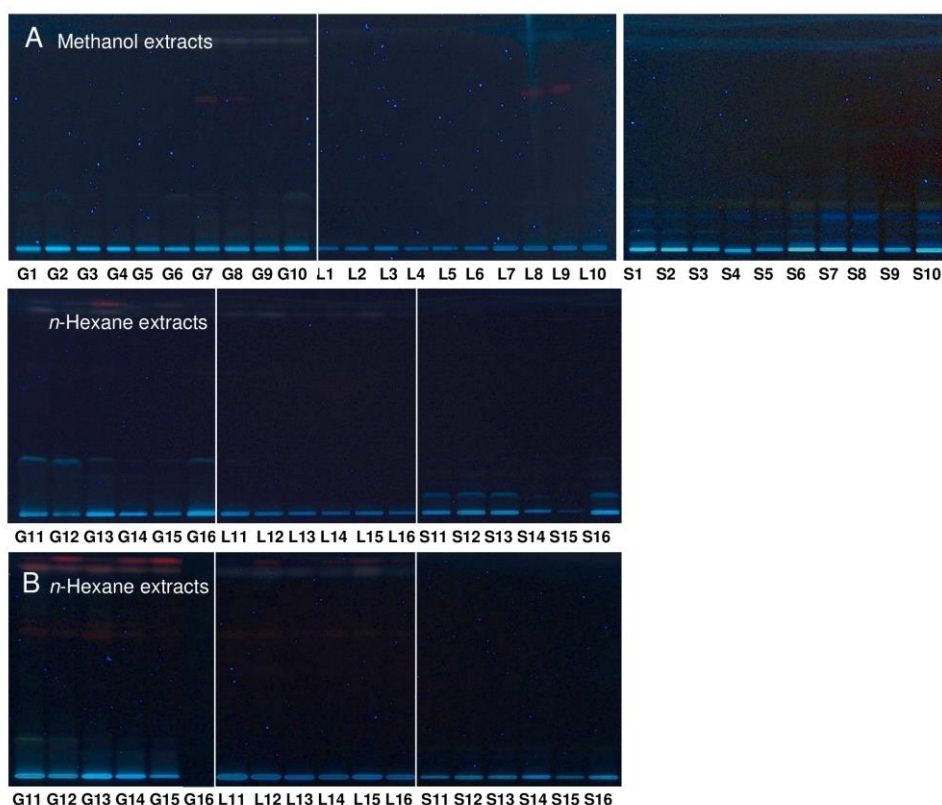


Figure S7. Chromatograms at FLD 366 nm before the *A. fischeri* (A) and *B. subtilis* bioassays (B)

Chromatograms of the three different substrate extracts developed on HPTLC plates silica gel 60 with ethyl acetate – methanol – water 15:3:1 (V/V/V) and detected at FLD 366 nm before the bioassay performance, Related to Figures 3 and 4.

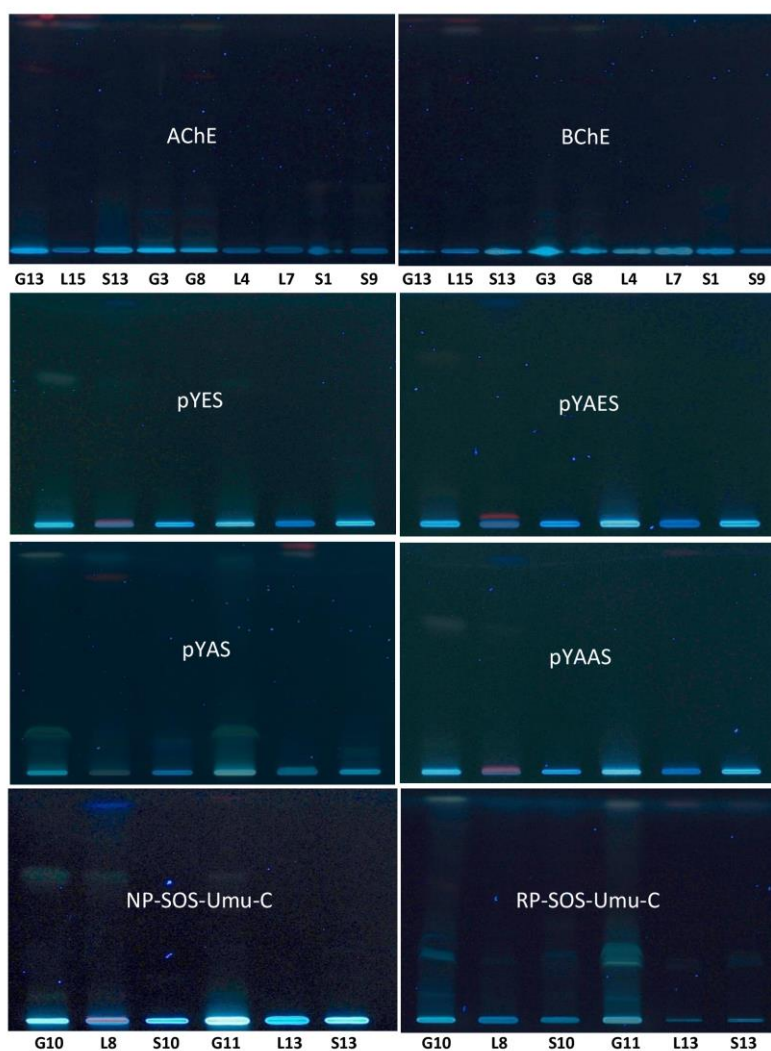


Figure S8. Chromatograms at FLD 366 nm before application of the respective assays

Separation of the *n*-hexane and methanol boat extracts of the three different substrates; respective UV 254 nm chromatograms did not show additional compounds and Vis chromatograms showed orange chlorophylls near the front only (both not depicted), Related to Figures 5 and S9.

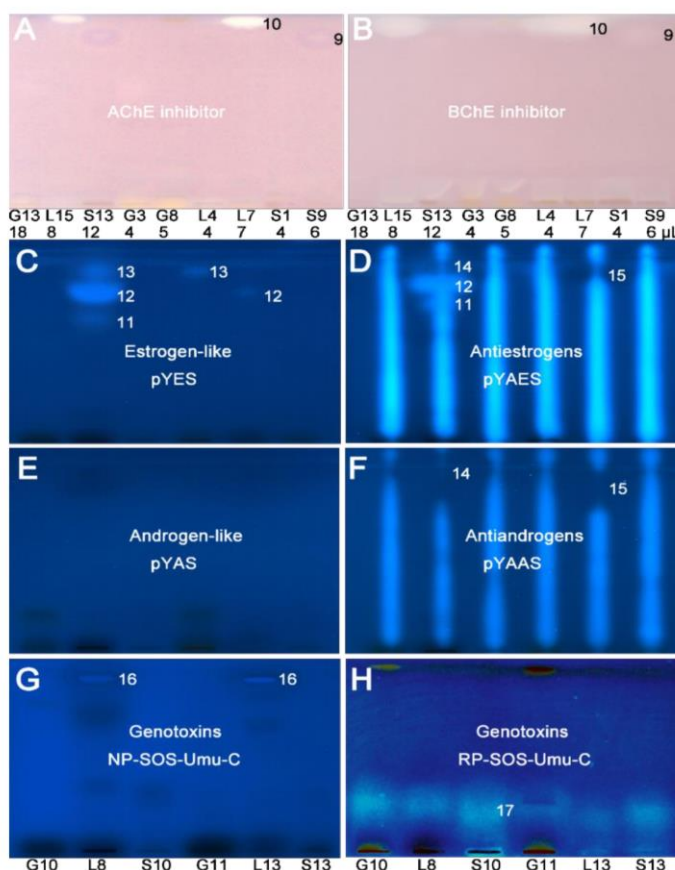


Figure S9. AChE/BChE inhibiting, hormonal and genotoxic compounds

(A) AChE and (B) BChE inhibiting compound zones **9** and **10** evident as colourless/white bands (L7/15 and L4/7, respectively; zone 9 with halo-effect), (C/D) estrogenic (**11–13**), (D) antiestrogenic (**14/15**), (E/F) androgenic (none), (F) antiandrogenic (**14/15**), and (G/H) genotoxic (**16/17**) compound zones, evident as (C–H) blue fluorescence or (D/F) fluorescence reduction in the *n*-hexane and methanol boat extracts of the three different substrates (C–H 10 μ L/band each as listed at bottom; up to 10 μ g/band) developed on HPTLC plates silica gel 60 (except for **H** on RP-18 W plates) with A/B/H ethyl acetate – methanol – water 15:3:1 (V/V/V), C–F *n*-hexane – ethyl acetate 3:4 (V/V) and G 1:4 (V/V), detected at A/B white light illumination and C–H FLD 366 nm (respective pre-assay FLD 366 nm chromatograms), Related to Figures 5 and S8.

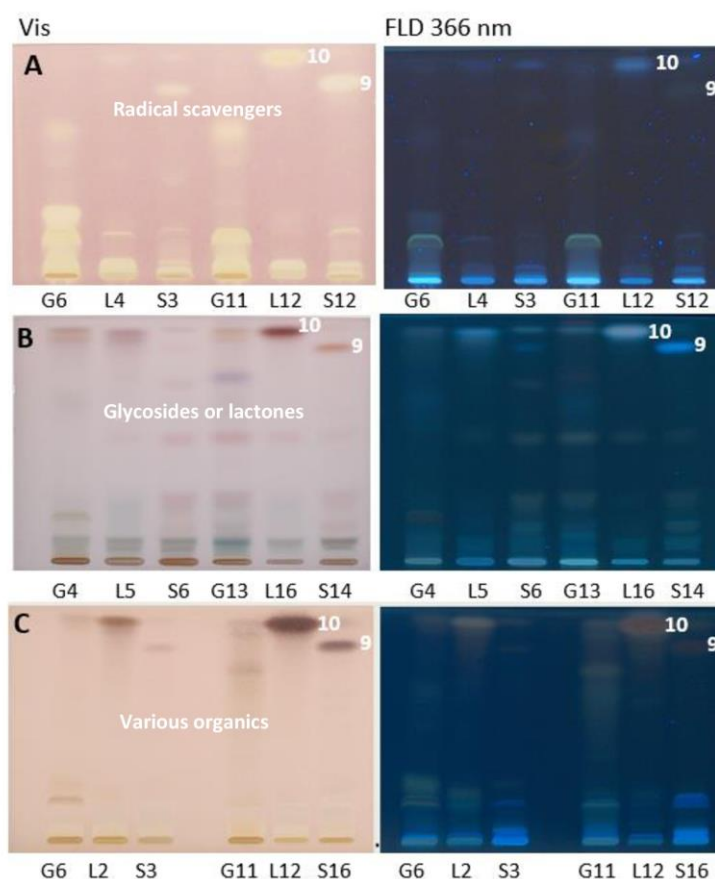


Figure S10. Radical scavenging assay and physico-chemical detection of the chromatograms including derivatization

Chromatograms of the methanol and *n*-hexane extracts of the three different substrates (10 μ L each) developed on HPTLC plates silica gel 60 with ethyl acetate – methanol – water 15:3:1 (V/V/V) and detected mainly the more apolar bioactive zones **9** and **10** at Vis and FLD 366 nm after the (A) 2,2-diphenyl-1-picrylhydrazyl (DPPH•) assay for detection of radical scavengers (antioxidants), as well as after derivatization with (B) 2% diethylamine aniline sulphuric acid reagent for detection of glycosides or lactones; (saccharides are supposed to be in the start region for the given mobile phase); (C) 1% vanillin sulfuric acid reagent for more universal detection of organic compounds like sesquiterpene derivatives. Related to Figure 2.

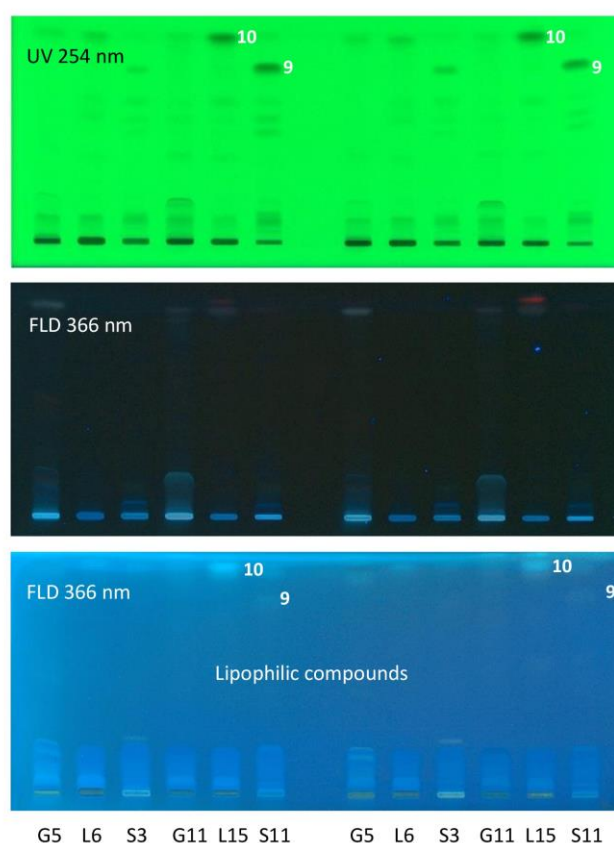


Figure S11 Physico-chemical detection of the chromatograms after primuline reagent

Chromatograms of the methanol and *n*-hexane extracts of the three different substrates (10 μ L each) developed on HPTLC plates silica gel 60 with ethyl acetate – methanol – water 15:3:1 (V/V/V) and detected at UV 254 nm (native UV-absorbance of the bioactive zones **9** and **10**) and at FLD 366 nm before and after physisorption of the primuline reagent for detection of lipophilic compounds as blue fluorescent bands. Related to Figure 2.

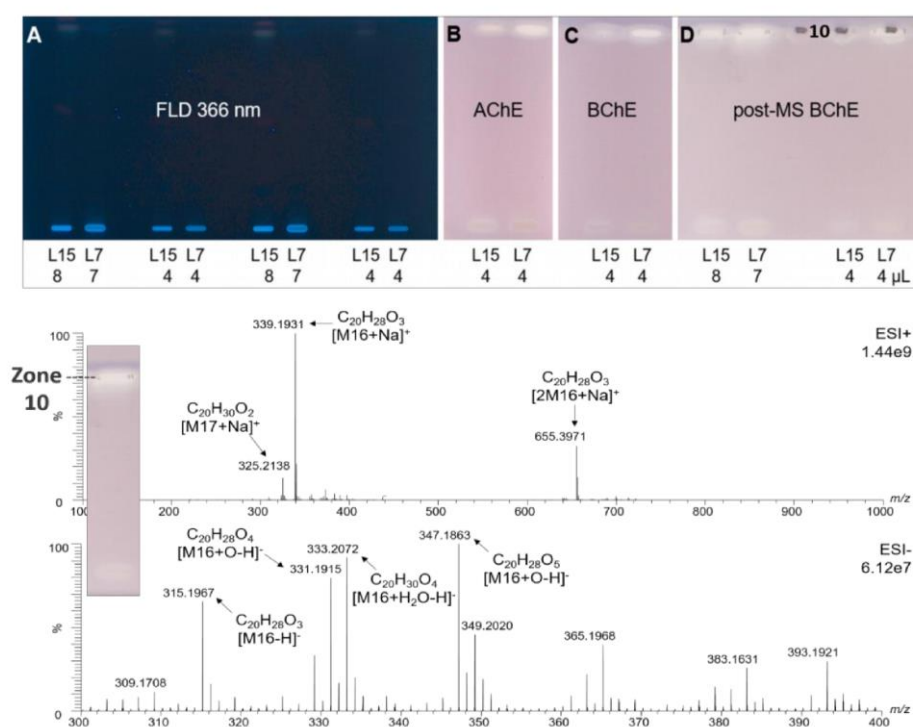


Figure S12. Proof of proper positioning and mass spectra recorded for zone 10

(A) FLD 366 nm chromatogram developed on HPTLC plates silica gel 60 with ethyl acetate – methanol – water 15:3:1 (V/V/V) each of a methanol boat (L15) and *n*-hexane extract (L7) of the leather coral, (B) respective acetyl- and butyrylcholinesterase (AChE/BChE) inhibition autogram, and (C) plate duplicate used for the recording of mass spectra followed by post-MS BChE assay application to prove the proper positioning of the elution head on the zones (fully automated operation in Video [S7](#)). (D) Mass signal assignment of zone 10. Related to Figure 3.

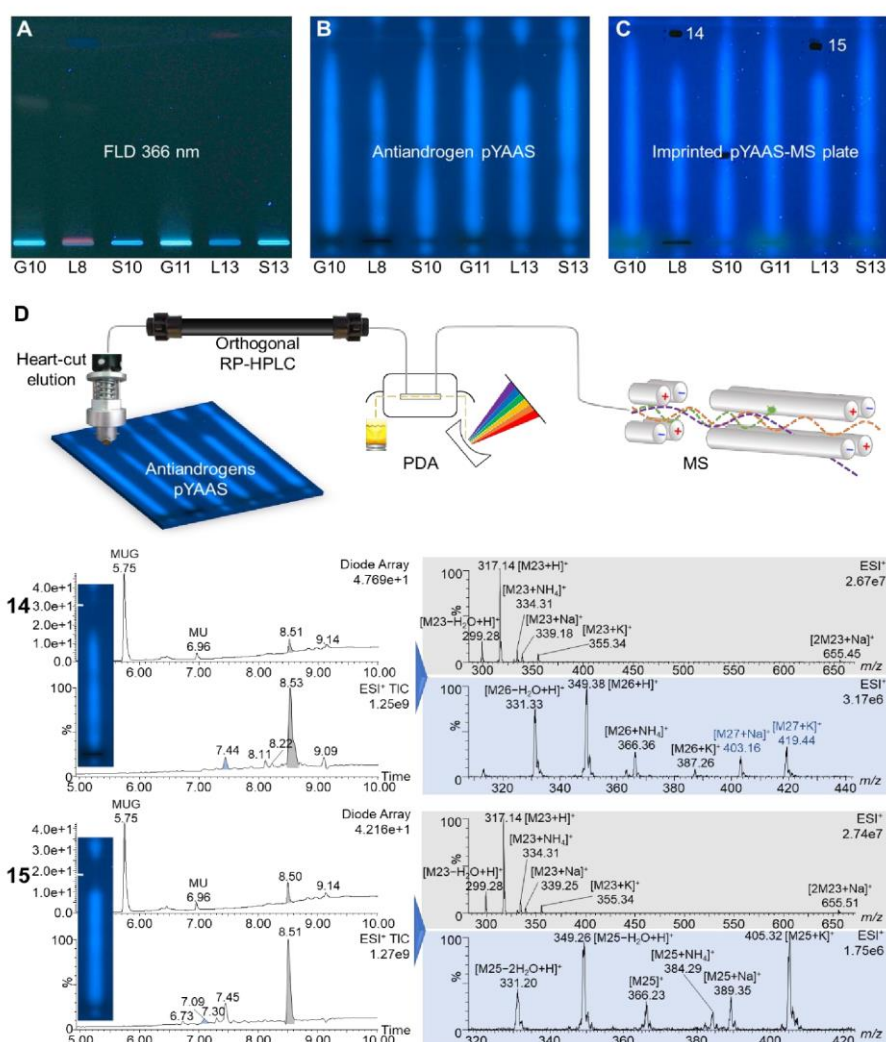


Figure S13. Characterization of the antiandrogenic zones 14 and 15 directly from the pYAAS bioautogram

(A) Chromatogram at FLD 366 nm on HPTLC plate silica gel 60 with ethyl acetate – methanol – water 15:3:1 (V/V/V) of the methanol boat and *n*-hexane extracts of the three distinct substrates, (B) respective pYAAS bioautogram and (C) elution head imprint, verifying proper positioning of the elution head on the zones. (D) Schematic overview of the zone characterization by NP-HPTLC-pYAAS bioassay-RP-HPLC-DAD-HESI-MS and recorded PDA or MS-TIC chromatograms as well as extracted mass spectra (same colour) with assigned mass signals. Related to Figures 5 and S9.

11. Publication VII

Effect-Directed Profiling of Monofloral Honeys from Ethiopia by High-Performance Thin-Layer Chromatography and High-Resolution Mass Spectrometry

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Article

Effect-Directed Profiling of Monofloral Honeys from Ethiopia by High-Performance Thin-Layer Chromatography and High-Resolution Mass Spectrometry

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Abstract: Ethiopian honey is used not only as food but also for treatment in traditional medicine. For its valorization, bioactive compounds were analyzed in nine types of monofloral Ethiopian honey. Therefore, a non-target effect-directed profiling was developed via high-performance thin-layer chromatography combined with multi-imaging and planar effect-directed assays. Characteristic bioactivity profiles of the different honeys were determined in terms of antibacterial, free-radical scavenging, and various enzyme inhibitory activities. Honeys from *Hypoestes* spp. and *Leucas abyssinica* showed low activity in all assays. In contrast, others from *Acacia* spp., *Becium grandiflorum*, *Croton macrostachyus*, *Eucalyptus globulus*, *Schefflera abyssinica*, *Vernonia amygdalina*, and *Coffea arabica* showed more intense activity profiles, but these differed depending on the assay. In particular, the radical scavenging activity of *Croton macrostachyus* and *Coffea arabica* honeys, the acetylcholinesterase-inhibiting activity of *Eucalyptus globulus* and *Coffea arabica* honeys, and the antibacterial activity of *Schefflera abyssinica* honey are highlighted. Bioactive compounds of interest were further characterized by high-resolution mass spectrometry. Identifying differences in bioactivity between mono-floral honey types affects quality designation and branding. Effect-directed profiling provides new insights that are valuable for food science and nutrition as well as for the market, and contributes to honey differentiation, categorization, and authentication.

Keywords: HPTLC-direct bioautography; radical scavenging assay; antioxidative assay; antibacterial assay; enzyme inhibition assay; HPTLC–HRMS

1. Introduction

Honey is a complex natural food that has been consumed worldwide since ancient times. It is produced by bees that ingest and concentrate the nectar of flowers. It is an aqueous but viscous concentrate of different saccharides, mainly glucose and fructose, produced without food additives or technical adjuvants. Apart from the saccharides, honey also contains phytochemicals (derived from the plant nectar taken up by the bees), enzymes (added by the bees, when the nectar is stored in the honey stomach during transport), and metabolites (resulting from digestive metabolic processes during transport and concentration during mouth-to-mouth transport in the beehive). In this way, the flower nectar changes and matures into honey. In the case of honeydew, sweet insect excretes (mainly from aphids, leaf fleas, and cicadas) are collected and further metabolized by bees. The minor components therefore change depending on the honeybee species, geographical origin, and available floral nectar or insect source. Honeys contain, among others, amino acids, organic acids, phenolic acids and further (poly) phenolic compounds,

flavonoids, carotenoid-like substances, terpenes, Maillard reaction products such as 5-(hydroxymethyl)furfural, vitamins (ascorbic acid, tocopherols, carotenes), minerals, aroma compounds, pigments, waxes, pollen, and enzymes [1]. Honey is also used in traditional medicines due to the bioactive compounds present in the complex mixture [2,3].

In Ethiopia, different types of monofloral blossom honey as well as honeydew honey are produced [4,5], which is important for the bee economy of the country [6]. Products were reported from *Acacia* species, *Becium grandiflorum*, *Croton macrostachyus*, *Eucalyptus globulus*, *Hypoestes* species, *Leucas abyssinica*, *Schefflera abyssinica*, *Syzygium guineense*, and *Harennia* forest [7–10]. These were analyzed according to different standardized protocols described in the *Codex Alimentarius* by the International Honey Commission and Association of Official Analytical Chemists [11]. Ethiopian monofloral blossom honeys have a moisture content of 14–21% and contain mainly fructose (35–43%), glucose (29–37%), sucrose (1.1–2.8%), maltose (0.6–2.0%), turanose (0.3–1.7%), and isomaltose (0.0–1.5%). Three amino acids were largely found, i.e., phenylalanine (5–119 mg/100 g), proline (16–74 mg/100 g), and aspartic acid (8–22 mg/100 g). Their 5-(hydroxymethyl)furfural content ranged from 0.5 to 3.4 mg/kg, diastase activity ranged from 4.9 to 13.5 Schade units, and invertase activity ranged from 11.6 to 36.5 IN [7–10]. In addition to determining basic properties, there is growing interest in fingerprinting techniques that are associated with characteristic constituents for authentication [12–16]. Information on the bioactivity of honey is also gaining interest [17–20] to add value to food beyond the purpose of sweetening. For example, the antioxidative effect and total phenolic content of *Eucalyptus* honey from Portugal [16] or *Schefflera abyssinica* honey from Ethiopia [10] was measured as a sum parameter, and the characteristic profile of different flavonoids of *Eucalyptus* honeys from Southern Europe was determined [15].

By combining a chromatographic separation with a non-target assay, it is possible to truly measure the activity of bioactive compounds and, in particular, to differentiate opposing effects. This would provide more understanding of honey quality than is possible with simple in vitro assays (providing only sum parameters for complex samples) or nuclear magnetic resonance spectroscopy (without actual proof of activity). Such a non-target effect-directed profiling was shown for plant-based food [21–24], but has not yet been applied for Ethiopian honey samples. In this study, a profiling method was developed to detect individual bioactive compounds in honey. It was considered important to recognize differences in bioactivity between monofloral honeys, which can affect quality designation and branding. In particular, the non-target analysis of bioactive compounds could provide new insights, also regarding differentiation, categorization, and authentication of the types of honey. Nine types of monofloral Ethiopian honey were screened side by side for radical scavenging, antimicrobial, and enzyme inhibition activities, using high-performance thin-layer chromatography combined with multi-imaging and planar effect-directed assays (HPTLC–UV/Vis/FLD–EDA) and (heated) electrospray high-resolution mass spectrometry (HPTLC–HESI–HRMS) for further characterization of zones of interest.

2. Results and Discussion

2.1. Development of the Physico-Chemical Profiling

Ethiopian honey types originating from herbs, shrubs, and trees were collected at the farm gate, directly from the beekeepers in the honey-harvesting period. The honey colors were mostly brown but also bright, ochre, and blackish (Table 1). The samples were proven via pollen analysis by melissopalynology [4,5,10] and categorized into the respective monofloral honey types. The honey samples were freed from saccharides by solid-phase extraction using a spherical, hydrophobic polystyrene–divinylbenzene adsorbent and methanol as eluent. Different mobile-phase mixtures were tested for separation of the extracts on HPTLC silica gel 60 F₂₅₄ plates. A mixture of ethyl acetate and methanol in the ratio of 3:2, V/V, was found to be suitable, since it spread the components along the migration distance. No particular analytes were selected at this stage for non-target

profiling. The parallel separation of honey samples took only 14 min. The detection via multi-imaging at white light illumination (Vis), 254 nm (UV), and 366 nm (FLD) took 3 min.

Table 1. Harvest period and Ethiopian sampling site of the collected floral honey samples with respective color depicted (ID numbers indicate individual samples taken).

Honey Color	ID	Floral Honey Source	Pollen Count (Out of 500)	Pollen Dominancy (%)	Harvested in 2018	Sampling Site
	A	<i>Acacia</i> spp.	310	62	September, October	Zikwala
	B1	<i>Becium</i>	370	74	August, September	Maychew Wukro
	B2	<i>grandiflorum</i>				
	C1/2	<i>Croton macrostachyus</i>	300	60	June, July	Dello Mena
	E	<i>Eucalyptus globulus</i>	430	86	April, May, June	Addis Ababa, Yeka
	H	<i>Hypoestes</i> spp.	315	63	September, October	Wukro
	L	<i>Leucas abyssinica</i>	430	86	September, October	Maychew
	S	<i>Schefflera abyssinica</i>	450	90	April, May	Sheka, Bonga
	V	<i>Vernonia amygdalina</i>	390	78	January, February	Becho, Anfillo, Gida-Ayana
	Y1–4	<i>Coffea arabica</i>	375	75	February, March	Yayu
	G1–4					Goma
	M1–4					Mana

Next, the different monofloral honey sample extracts (Table 1) were subjected to physico-chemical profiling by HPTLC–UV/Vis/FLD to obtain information on the differences in their phytochemical patterns and their diversity. Visible zones were not observed under white light illumination (data not shown). The honey extracts from tree or shrub sources, i.e., *Acacia* spp. (A), *Croton macrostachyus* (C), *Schefflera abyssinica* (S), *Eucalyptus globulus* (E), and *Vernonia amygdalina* (V), showed comparatively more UV-active (Figure 1, UV 254 nm chromatogram) and bluish fluorescent substances (FLD 366 nm chromatogram) than honey extracts from herb sources, i.e., *Becium grandiflorum* (B), *Hypoestes* spp. (H), and *Leucas abyssinica* (L). Among these Ethiopian honeys, *Croton macrostachyus* was richest in compounds. Two additional derivatization reagents were tested, since derivatization can readily be performed on the planar chromatogram and provides additional helpful information for further characterization [24]. However, the derivatization using the natural product reagent for the detection of flavonoids did not show additional fluorescent zones at FLD 366 nm at the given amounts applied, whereas when using the primuline reagent for the detection of lipophilic substances, faint blue fluorescent zones were observed at the start zone of *Hypoestes* spp. as well as in the solvent front for some samples (data not shown). These results and the impression of the samples obtained by this physico-chemical profiling were satisfactory in informative value to start the non-target effect-directed profiling.

2.2. Effect-Directed Profiling of Nine Different Types of Monofloral Honey

The same chromatogram was prepared several times and subjected to six different planar effect-directed assays targeting antibacterials (against Gram-negative bacteria), 2,2-diphenyl-1-picrylhydrazyl (DPPH•) scavengers (antioxidants), and inhibitors of acetylcholinesterase (AChE), α -glucosidase, β -glucosidase, and α -amylase. In the case of the immersion technique, minor zone shifts (1–2 mm) were observed, as reported in [21], which needed to be taken into account for zone matching on the different images. The effect-directed profiling via HPTLC–UV/Vis/FLD–EDA (Figures 1 and 2) showed characteristic fingerprints of the nine different types of monofloral honey that differed in number and position as well as the intensity of the bioactive zones. Similar to the physico-chemical profiling, honey extracts from tree or shrub sources showed comparatively more bioactive

reactions than honey extracts from herb sources, and *Croton macrostachyus* was richest in bioactive compounds across all assays. Comparable effect-directed profiles were obtained for the two *Croton macrostachyus* honey extract samples, which was expected because it was a repeated analysis of the same sample (Table 1). This highlighted the repeatability of the profiling. In contrast, the two *Becium grandiflorum* honey extracts, which were collected at two different sites in Ethiopia, showed the same activity pattern in the same assays but differed strongly in intensity. This clearly shows that nominally, the same monofloral honey originating from different sites can differ in the intensity of the inherent bioactive zones.

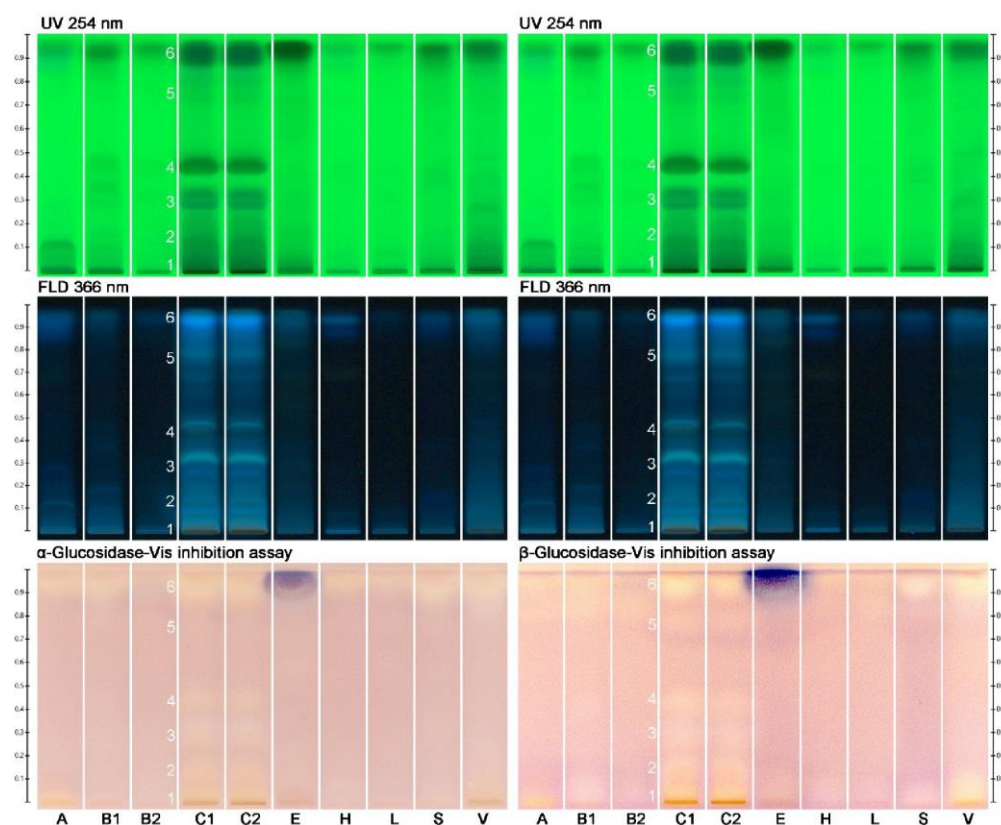


Figure 1. Physico-chemical profiling at UV 254 nm and FLD 366 nm of 10 monofloral honey extract samples (assigned as in Table 1; 3 μ L/band each) on HPTLC silica gel 60 F₂₅₄ plates with ethyl acetate–methanol 3:2, V/V, as well as effect-directed profiling via the α - and β -glucosidase inhibition assays, detecting the glucosidase inhibiting zones 1–6 at white light illumination (Vis).

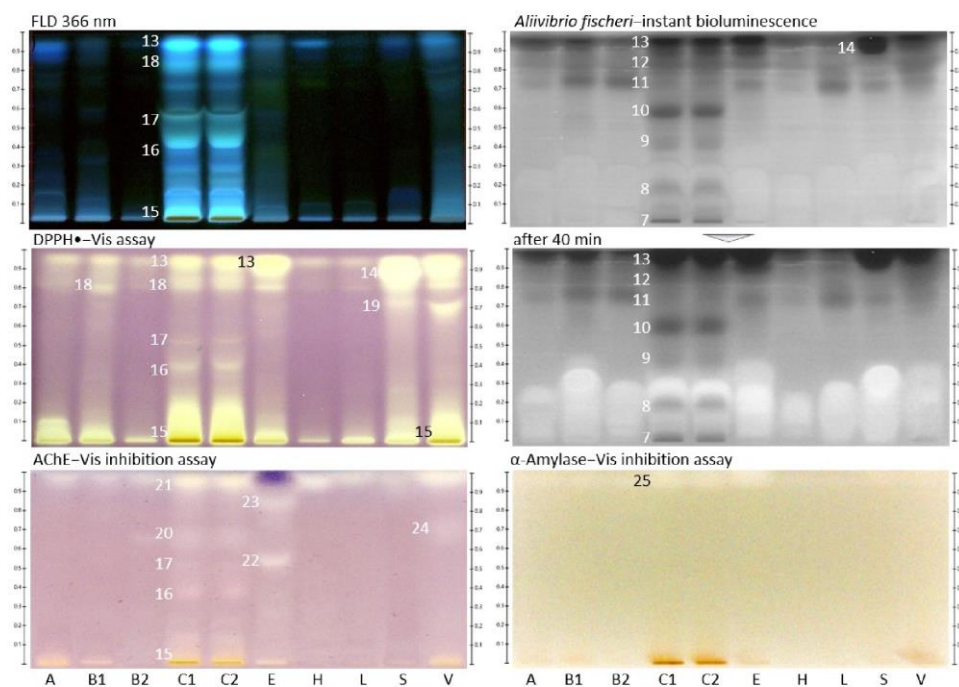


Figure 2. Chromatogram at FLD 366 nm and respective effect-directed profiling of 10 monofloral honey extract samples (assigned as in Table 1; 5 μ L/band for *Aliivibrio fischeri* bioassay, others 3 μ L/band each) developed on HPTLC silica gel 60 F₂₅₄ plates with ethyl acetate–methanol 3:2, V/V, detected via the *Aliivibrio fischeri* bioassay (bioluminescence recorded as greyscale image instantly and after 40 min) as well as DPPH•, AChE, and α -amylase inhibition assays (white light illumination, Vis), revealing the bioactive zones 7–25.

The α - and β -glucosidase inhibition autograms (Figure 1) showed weak to moderate inhibition of the α - and β -glucosidase when 9 μ L/band (equivalent to 90 mg honey) were applied. Almost the same zones were evident in both enzyme assays, although the inhibition of β -glucosidase was stronger. *Croton macrostachyus* showed the most pronounced responses and revealed glucosidase-inhibiting compounds 1–6 (evident as colorless or bright zones) not only near the solvent front and in the start zone, as evident in other scrub/tree honey samples, but also in further zones in between. The honey extracts from herb sources (*Becium grandiflorum*, *Hypoestes* spp., and *Leucas abyssinica*) were comparatively weaker in the glucosidase-inhibiting response. The UV 254 nm and FLD 366 nm chromatograms (Figure 1) of both glucosidase inhibition assays highlight the good reproducibility of the profiling.

Several compounds that were active against Gram-negative bacteria were detected as dark zones in the HPTLC-*Aliivibrio fischeri* bioautogram (Figure 2). The bioluminescence image was monitored over a period of 40 min, wherefrom the instantly recorded bioautogram is depicted as well as one after 40 min. The latter bioautogram was found to be more important since it indicated strong longer-lasting effects. All investigated honey extract samples showed antibacterial activity; however, they were quite different in number and intensity of the individual responsible compounds. *Croton macrostachyus* honey extract revealed the darkest zones, numbered 7–13 (Figure 2). The most pronounced antibacterial zone (14, near the solvent front) was evident in *Schefflera abyssinica* honey extract, which

was instantly detected and indicated a strong acute effect. Interestingly, the longer the monitoring period, the more bright zones were found, as evident in the bioluminescence image recorded after 40 min. Observing changing effects and distinguishing between individual enhancing and darkening effects is very important for understanding. Only then can one recognize and distinguish whether it is an acute or gentle and slower-reacting or even reversing (bacteria do recover) or a strong long-lasting effect. This provides hints for bacteriostatic or bactericide mechanisms of action. Such a deep understanding cannot be achieved with the commonly used in vitro tests because these provide an incorrect sum parameter value for opposing effects in complex samples such as honey (Figure 2, *Aliivibrio fischeri* bioautogram after 40 min; the opposing effects increased in intensity over time, images between 0 and 40 min are not depicted).

The radical scavenging (antioxidative) activity was investigated using the HPTLC-DPPH• assay. All tested honey extract samples showed radical scavenging activity (visible as a yellow zone against a purple background) with a varying intensity and number of active zones (Figure 2, zones 15–19). In the DPPH• autogram, the horizontal intensity pattern for zones 13 and 14 (both near the solvent front) across the samples was comparable to that in the *Aliivibrio fischeri* bioautogram. Hence, the zones were found to be the same (same numbers as before).

All investigated honey extract samples revealed an inhibition of the AChE (visible as a colorless or bright zone against a purple background) near the solvent front (Figure 2, zone 21). In particular, *Croton macrostachyus*, *Eucalyptus globulus*, and *Vernonia amygdalina* showed AChE inhibiting zones 15–17 and 20–24. Above zone 23, the same lilac zone was observed as in the previous anti-glucosidase assays (Figure 1). It was proven (using the same workflow but without enzyme) to be a color reaction with the Fast Blue B salt and assumed to be a phenolic compound. It is known that reactions of sample compounds with substrates/chromogenic reagents can occur [25].

Honey extracts from *Croton macrostachyus* and *Eucalyptus globulus* in particular showed moderate inhibiting effects on the α -amylase (visible as a colorless or bright zone against a yellow background), whereas it was weaker in the response for *Schefflera abyssinica* and *Vernonia amygdalina*. These α -amylase-inhibiting activities were caused by a more lipophilic compound near the solvent front (Figure 2, zone 25). Comparing the different assay results, it could have been the same substance as in zone 6, but it could not be confirmed for *Eucalyptus globulus* due to the coeluting lilac zone, as discussed. Hence, a new number was given. Note that bioactive zones were assigned to a previous number based on similarity to a previous horizontal activity pattern (at the same R_F position across the different samples) but also newly numbered, if there was no pattern in common (or it was uncertain). The numbering of bioactive zones was also transferred to the images of the physico-chemical profiling, which gave an additional hint regarding native spectral properties as UV-absorber or fluorophore (e.g., Figure 1, zone 6), or in case the horizontal pattern did not match, it was helpful for orientation (e.g., Figure 2, zone 13). Hence, bands numbered in the physico-chemical profiling are not necessarily meant to be bioactive zones.

In agreement with our results (high bioactivity of *Croton macrostachyus*), high antioxidant and antibacterial bioactivity was shown for another *Croton* species (*Croton lechleri*) of the same Euphorbiaceae family, whose essential oil was discussed as a new functional food ingredient [26]. The intensities of the antioxidative zones in the autogram were in accordance with results of a photometric method reporting ascorbic acid equivalents (AAE) [27]. Therein, the honey extract of *Hypoestes* ssp. showed the weakest activity, with 9 mg AAE/kg, whereas the *Croton macrostachyus* honey extract showed the highest activity, with 166 mg AAE/kg, followed by the *Vernonia amygdalina* honey extract, with 149 mg AAE/kg. The same ranking was evident in our DPPH• autogram (Figure 2). The honey extracts from herb sources (*Becium grandiflorum*, *Hypoestes* ssp., and *Leucas abyssinica*) were comparatively weaker in the radical scavenging response. Interestingly, the antioxidant activity of monofloral honeys could not be correlated to high values of flavonoids therein [17].

2.3. Effect-Directed Profiling within the Same Type of Monofloral Honey

Ethiopian coffee honey is attracting special interest and was also subjected to effect-directed profiling. Twelve *Coffea arabica* honeys were collected from the Ethiopian regions of Yayu (Y), Goma (G), and Mana (M), each at four different locations (Table 1). The chromatograms at UV 254 nm and FLD 366 nm of the 12 *Coffea arabica* honey extract samples (2 μ L/band each, equivalent to 20 mg honey) showed a characteristic pattern that was very similar between the 12 samples (Figure 3) but quite different to the previous monofloral honey extract samples (Figures 1 and 2). Each respective effect-directed profiling (via the DPPH• radical scavenging assay, AChE and β -glucosidase inhibition assays, and *Aliivibrio fischeri* bioassay) showed highly comparable bioactivity patterns between the 12 samples. In particular, AChE inhibition and DPPH• radical scavenging activities were determined in the *Coffea arabica* honey extract samples. These bioactivity profiles of *Coffea arabica* honey were quite different when compared to those of *Croton macrostachyus*, which was the most active candidate in the effect-directed profiling of the previous monofloral honeys.

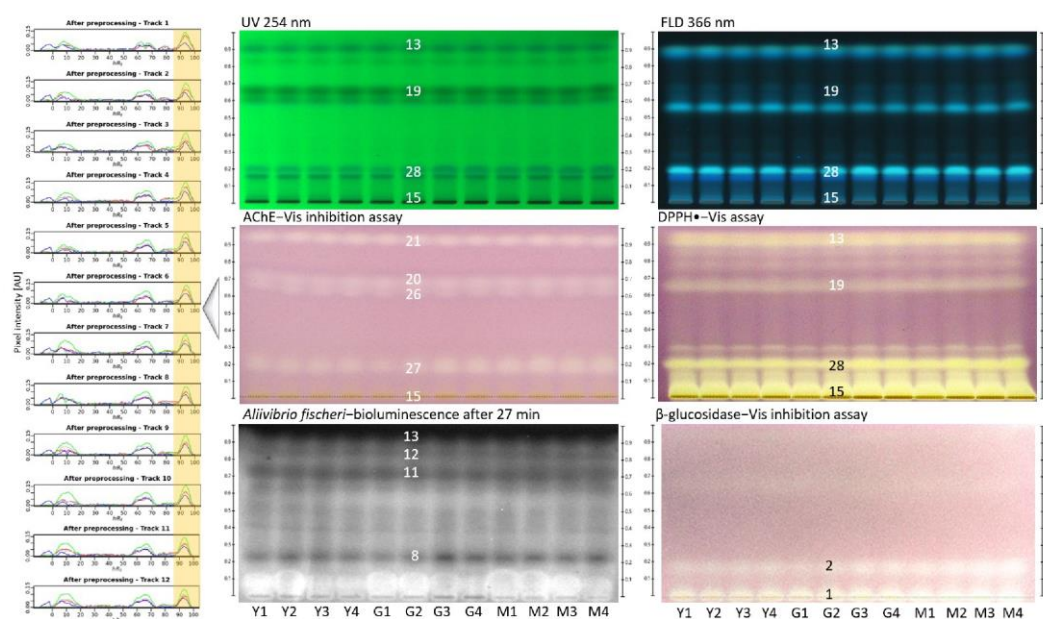


Figure 3. Chromatograms at UV 254 nm and FLD 366 nm and respective effect-directed profiling of 12 *Coffea arabica* honey extract samples (2 μ L/band each; samples 1–4 collected at different sites in the Yayu (Y), Goma (G), and Mana (M) regions, Table 1 developed on HPTLC silica gel 60 F₂₅₄ plates with ethyl acetate–methanol 3:2, V/V, detected via the DPPH• radical scavenging assay, AChE and β -glucosidase inhibition assays (white light illumination, Vis), and *Aliivibrio fischeri* bioassay (bioluminescence after 27 min depicted as greyscale image); AChE inhibition autogram evaluated via quanTLC software to measure the variation of the pixel signal intensity of zone 21 (framed in yellow).

As an example, the AChE inhibition autogram was evaluated using the open-source quanTLC software [28]. The variation of the activity of the AChE inhibiting compound zone 21 across all 12 different *Coffea arabica* samples (Figure 3, framed in yellow) was 6.3% (%RSD, $n = 12$, green channel signal). This variance is in agreement with the visual impression in the autogram, which showed similar AChE inhibitory responses for zone 21 across the samples. This example of digital evaluation of the autogram shows the potential

to use the enzymatic or biological responses for drawing quantitative conclusions about the activity depending on the region or harvest time.

2.4. Bioactivities Found in Monofloral Honeys

The main bioactivity responses obtained across the investigated honey samples are summarized in Table 2. It indicates that *Coffea arabica* (Table 1, Y, G, M; here YGM) and *Croton macrostachyus* (C) honeys were richest and strongest in bioactivity. The two are the most promising candidates among the investigated Ethiopian monofloral honey types, taking into account the investigated effect-directed profiles.

Table 2. Main bioactive zones 1–28 (Figures 1–3) observed in the monofloral honeys (as assigned in Table 1; honey with pronounced effect in bold).

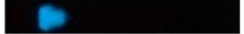
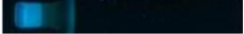
Zone	Radical Scavenger	Glucosidase Inhibitor	AChE Inhibitor	Amylase Inhibitor *	Antibacterial
1		A C V YGM			
2		C YGM			
3		C			
4		C			
5		C			
6		A B C S V			
7					C
8					C YGM
9					C
10					C
11					B L YGM
12					C V YGM
13	A B C E V YGM				C E V YGM
14	S				S
15	A B C E S V YGM		C YGM		
16	C		C		
17	C		C		
18	B C E S V				
19	S V YGM				
20			C YGM		
21			C YGM		
22			E		
23			E		
24			V		
25				A B C E S V	
26			YGM		
27			YGM		
28	YGM				

* Assay not performed for *Coffea arabica* (Y, G, M).

2.5. Optional On-Surface Extraction and Separation on the Same Plate

For non-target profiling, sample preparation should be minimal, as compounds may be lost or altered at each step. In addition, the solid-phase extraction step is time-consuming and expensive. Hence, a simpler solution was targeted. Sample preparation could be performed on the surface of the adsorbent [29,30]; however, the high saccharide content might restrict the sample load. This challenge was explored exemplarily for three different monofloral honeys, i.e., *Becium grandiflorum*, *Coffea arabica*, and *Vernonia amygdalina* honeys. Different dissolution/dilution solvents for the honeys were compared with regard to the saccharide load on the adsorbent. As a compromise between saccharide load and loss of polar compounds, methanol was selected. The methanolic honey suspension was only ultrasonicated and centrifuged. Each supernatant (25 µL) was applied as an area of 8 mm × 10 mm on the HPTLC silica gel 60 F₂₅₄ plate. The focusing of the applied area to a sharp start band was studied using single solvents or solvent mixtures, also with small portions of formic or acetic acid (Table 3).

Table 3. On-surface extraction and separation on the same plate: Investigated solvent systems on HPTLC silica gel 60 F₂₅₄ plate for focusing of the *Coffea arabica* honey applied as an area to a sharp start band and for subsequent separation of the compounds, as well as an example of a combination.

Focusing of the Start Area	Ratio (V/V)	Chromatogram at UV/Vis/FLD
Methanol	1	
Acetonitrile	1	
2-Propanol	1	
Acetonitrile–methanol–ammonium hydroxide	5:3:2	
Acetonitrile–ethanol–ammonium hydroxide	8:1:1	
Acetonitrile–ethanol–ammonium hydroxide	1:3:1	
Acetonitrile–water	9:1	
Ethanol–ammonium hydroxide	9:1	
Methanol–ammonium hydroxide	10:1	
Ethanol–formic acid–water	7:1:2	
Mobile Phase System for Separation		
Ethyl acetate–ethanol–water	3.8:1:0.2	
Toluene–ethyl acetate–ethanol	2.4:1.8:0.8	
Petroleum ether–ethyl acetate–acetone	3.8:0.6:0.7	
Ethyl acetate–ethanol–ammonium hydroxide	4.2:0.6:0.2	
Toluene–ethyl acetate–formic acid–water	2:6:1:0.6	
Toluene–ethyl acetate–formic acid–water	1.6:7:0.8:0.6	
On-Surface Extraction and Separation		
1. Focusing twice with acetonitrile–ethanol–ammonia to 3 cm, then plate cut at 1.5 cm	8:1:2	
2. Separation with toluene–ethyl acetate–formic acid–water	1.6:7:0.8:0.6	

It was interesting to observe how the different solvent polarities migrated through the heavy saccharide load on the adsorbent. For example, methanol or ethanol, either solely or at a higher portion in the mixture, caused an A-shaped zone distortion. As another example, a V-shaped zone distortion was caused by addition of formic acid when focusing with ethanol–formic acid–water 7:1:2 (V/V/V). These zone deformations were reproducible.

For separation on the HPTLC silica gel 60 F₂₅₄ plate, solvent systems were investigated based on polar–acidic, polar–basic, and polar-to-mid-polar properties (Table 3). As an example of a combined on-surface extraction and separation on the same plate, the start areas were focused twice with acetonitrile–ethanol–ammonia 8:1:2, V/V/V, up to 30 mm, and after, the plate was cut at 15 mm and developed with toluene–ethyl acetate–formic acid–

water 1.6:7:0.8:0.6, V/V/V/V, up to a migration distance of 70 mm (Table 3). As another even simpler example, the start areas were just separated with toluene–ethyl acetate–formic acid–water 2:6:1:0.6, V/V/V/V. However, still compounds were retained at the start area. These attempts showed that on-surface extraction and separation on the same plate has potential, but is challenging due to the high saccharide load.

Further targeted optimization for selective front elution and thus separation of polar compounds (which are currently still trapped) from the saccharide-rich start area is necessary for this new idea of on-surface extraction and separation in the field of fast honey analysis.

2.6. Characterization of Selected Bioactive Compounds by HPTLC–HESI–HRMS

Prominent bioactive zones of the different monofloral honey extracts (Figures 4 and 5) were chosen for further characterization by HPTLC–HESI–HRMS. In addition, honey zones that were fluorescent at FLD 366 nm after on-surface extraction and separation were recorded by HPTLC–HESI–HRMS (Figure 6). Each elution position is marked in Figures 4–6 according to the numbering in Figures 1–3 for orientation purposes, but should not be misunderstood as equivalent to the actual bioactive zones. For example, radical scavenging zone 13 showed significant differences in the mass signals for *Croton macrostachyus* and *Eucalyptus globulus* at the same elution position, indicating different constituents in honeys of different plant origins. In such a case, zones detected at the same hR_F position in different samples cannot be assigned to the same number. Hence, different monofloral honeys can show different mass signals even for the same hR_F positions on the plate, since the bees had collected it from different floral sources, whose secondary metabolites differ. Note that the separation system chosen for on-surface extraction (Figure 6) completely differs from the separation system used for the samples purified using solid-phase extraction (Figures 4 and 5). Thus, the same zones cannot be expected to be at the same hR_F position, i.e., compound zones do not correspond to previous positions on the plate and mass signals should be different. Hence, these recorded zones were marked with Roman numerals to make evident this difference between solid-phase extraction and on-surface extraction.

Corresponding molecular formulas were assigned to the mass signals obtained (Table 4, Figures 4–6). Most zones revealed complex mass signal patterns, especially in the positive ionization mode, resulting in 69 different analytes. These predominantly contained only oxygen as a heteroatom. The analytes closer to the start zone (Figure 4, zone 15, and Figure 5, zones 19 and 28) contained nitrogen, whereas some analytes also contained sulfur (Figure 4, zones 14 and 18). In general, the solid-phase extracts of the honeys (Figures 4 and 5) showed many more different mass signals than the on-surface extracts, which supports the assumption that most compounds were still trapped at the start area due to the high saccharide load.

Some of the bioactive compounds found were matched to possible structures already described for other types of honey in the literature (Table 4). For example, the very prominent analyte M6 was assigned the molecular formula C_9H_7NO , which could tentatively be matched to 2-hydroxyquinoline (Table 4). It was detected in all zones of the on-surface extracts (Figure 6), either in positive mode as m/z 146.0601 $[M6 + H]^+$ and m/z 168.0420 $[M6 + Na]^+$ or in the negative mode as m/z 144.0455 $[M6 - H]^-$. It was detected not only in the on-surface extracts, but also in the solid-phase extracts. The corresponding mass signals were distributed over the whole length of the track; thus, it was considered to be a strongly tailing compound. It can be assumed that the slightly brighter color (when compared to the purple background) over the length of all tracks in the effect-directed profiling via the DPPH• assay (Figures 2 and 3) was caused by C_9H_7NO . Thus, M6 could also contribute to a synergistic radical scavenging effect with other compounds, as observed in other studies [22]. Interestingly, the mass signals of C_9H_7NO were not found in all zones. For example, a comparison of zone 13 from *Croton macrostachyus* and *Eucalyptus globulus* (Figure 4) showed no corresponding mass signals in *Eucalyptus globulus*, but very intense signals in *Croton macrostachyus*, which was considered to be caused by signal suppression

by the other analytes. In zone 13 from *Coffea arabica* (Figure 5), not only C_9H_7NO but also C_9H_9NO was found in the positive and negative ionization mode, which was considered to be related to M6 because it contained one double bond fewer.

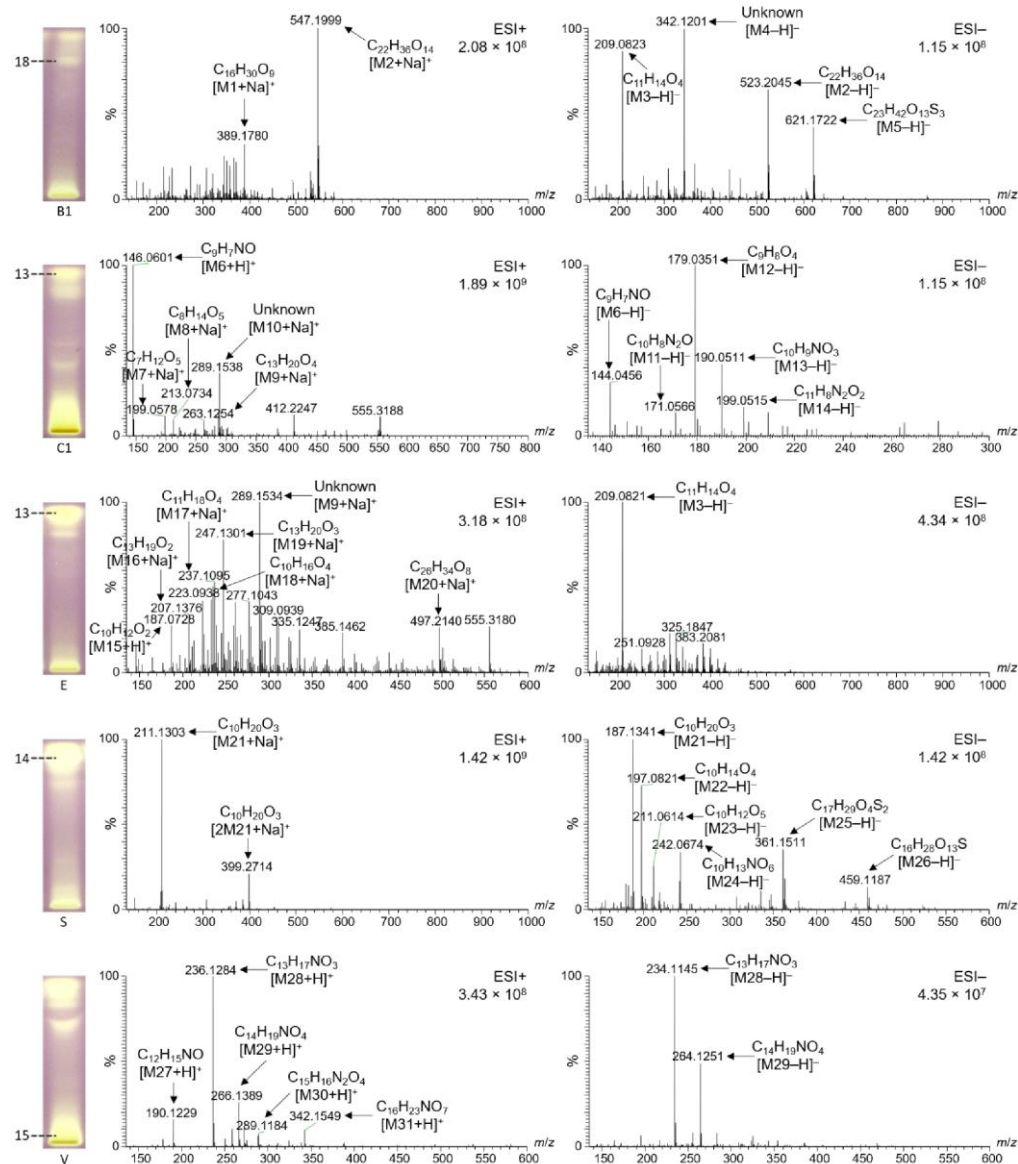


Figure 4. HPTLC-HESI-HRMS spectra in the positive and negative ionization mode of prominent bioactive zones detected via the DPPH• radical scavenging assay (for orientation, zones are marked as in Figure 2) of different monofloral honeys (IDs below the chromatogram stripe, as given in Table 1).

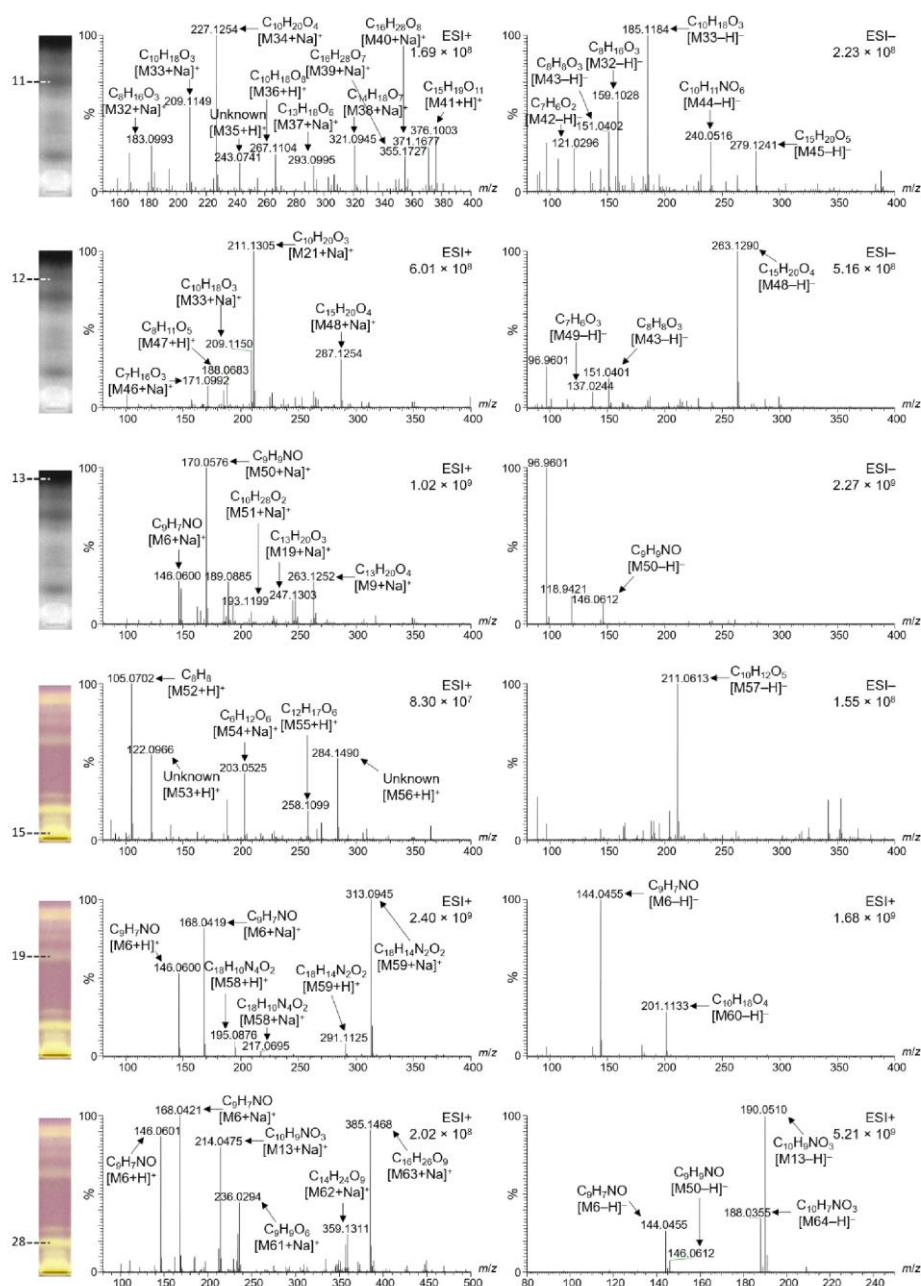


Figure 5. HPTLC-HESI-HRMS spectra in the positive and negative ionization mode of prominent bioactive zones of *Coffea arabica* honey detected via the *Aliivibrio fischeri* bioassay or DPPH• radical scavenging assay (for orientation, zones are marked as in Figure 3).

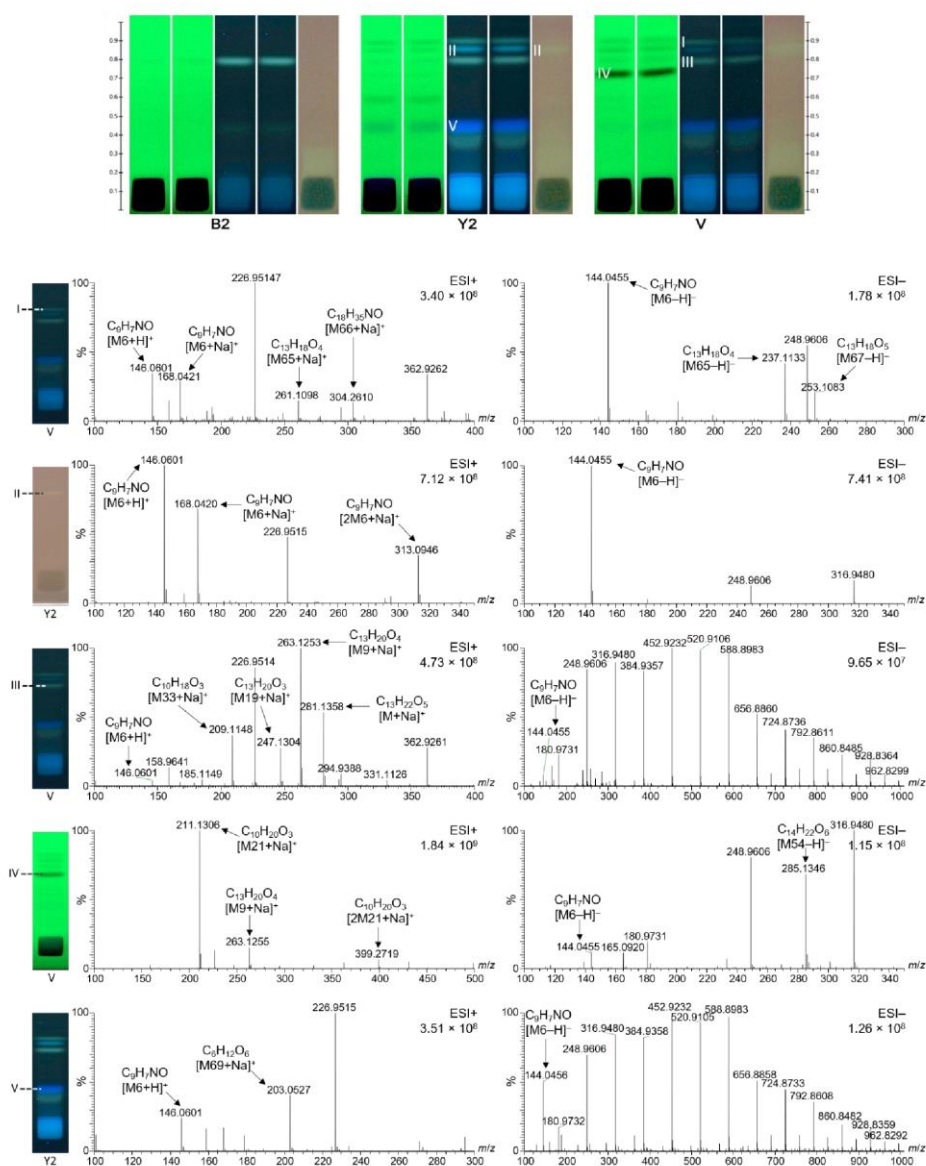


Figure 6. On-surface extraction and separation on the same plate: HPTLC chromatograms of *Becium grandiflorum* (B2), *Coffea arabica* from Yayu (Y2), and *Vernonia amygdalina* (V) honey extracts (25 μ L each, note that here the start area focusing was skipped) at UV 254 nm, FLD 366 nm, and at white light illumination after the DPPH• scavenging assay, separated on HPTLC silica gel 60 F₂₅₄ plates with toluene–ethyl acetate–formic acid–water 2:6:1:0.6, V/V/V/V, and HPTLC–HESI–HRMS spectra in the positive and negative ionization mode of selected zones (for orientation, marked with Roman numerals, as the separation system is different to previous ones).

Table 4. Complete list of analytes found in the various honey samples and their tentative assignment to possible structures according to the literature.

Zone	Mass Signal <i>m/z</i>	Adduct	Molecular Formula (Neutral Molecule)	Δ ppm	Tentative Assignment According to the Literature	Lit.
18 (B1), Figure 4	389.1780	[M1 + Na] ⁺	C ₁₆ H ₃₀ O ₉	0.50		
	547.1999	[M2 + Na] ⁺	C ₂₂ H ₃₆ O ₁₄	−0.29		
	523.2045	[M2 − H] [−]		−3.26		
	209.0823	[M3 − H] [−]	C ₁₁ H ₁₄ O ₄	−2.61		
	342.1201	[M4 − H] [−]	Unknown	-		
13 (C1), Figure 4	621.1722	[M5 − H] [−]	C ₂₃ H ₄₂ O ₁₃ S ₃	−0.76		
	146.0601	[M6 + H] ⁺	C ₉ H ₇ NO	−0.62	2-Hydroxyquinoline in various Iranian honeys	[31]
	144.0456			−0.71		
	199.0578	[M7 + Na] ⁺	C ₇ H ₁₂ O ₅	−0.47		
	213.0734	[M8 + Na] ⁺	C ₈ H ₁₄ O ₅	−0.16		
	263.1254	[M9 + Na] ⁺	C ₁₃ H ₂₀ O ₄	−0.07		
	289.1538	[M10 + Na] ⁺	Unknown	-		
	171.0566	[M11 − H] [−]	C ₁₀ H ₈ N ₂ O	−1.01		
	179.0351	[M12 − H] [−]	C ₉ H ₇ O ₄	−0.87	Caffeic acid in various Iranian honeys	[31]
	190.0511	[M13 − H] [−]	C ₁₀ H ₉ NO ₃	−0.85		
13 (E), Figure 4	199.0515	[M14 − H] [−]	C ₁₁ H ₈ N ₂ O ₂	−0.89		
	209.0821	[M3 − H] [−]	C ₁₁ H ₁₄ O ₄	−1.27		
	289.1534	[M10 + Na] ⁺	Unknown	-		
	187.0728	[M15 + H] ⁺	C ₁₀ H ₁₂ O ₂	0.91		
	207.1376	[M16 + Na] ⁺	C ₁₃ H ₁₉ O ₂	1.48		
	237.1095	[M17 + Na] ⁺	C ₁₁ H ₁₈ O ₄	1.19		
	223.0938	[M18 + Na] ⁺	C ₁₀ H ₁₆ O ₄	1.13	Succinic acid monocyclohexyl ester in various Iranian honeys	[31]
					(E)-2-Decenedioic acid in various Italian honeys	[32]
	247.1301	[M19 + Na] ⁺	C ₁₃ H ₂₀ O ₃	1.48		
	497.2140	[M20 + Na] ⁺	C ₂₆ H ₃₄ O ₈	1.15		
14 (S), Figure 4	211.1303	[M21 + Na] ⁺	C ₁₀ H ₂₀ O ₃	−1.05	Hydroxy decenoic acid in various Iranian honeys	[31]
	399.2714	[2M21 + Na] ⁺		0.88		
	187.1341	[M21 − H] [−]		0.75		
	197.0821	[M22 − H] [−]	C ₁₀ H ₁₄ O ₄	−0.74		
	211.0614	[M23 − H] [−]	C ₁₀ H ₁₂ O ₅	−1.05	Methyl syringate in various Iranian honeys	[31]
	242.0674	[M24 − H] [−]	C ₁₀ H ₁₃ NO ₆	−1.39		
	361.1511	[M25 − H] [−]	C ₁₇ H ₂₉ O ₄ S ₂	0.60		
	459.1187	[M26 − H] [−]	C ₁₆ H ₂₈ O ₁₃ S	−2.00		

Table 4. Cont.

Zone	Mass Signal <i>m/z</i>	Adduct	Molecular Formula (Neutral Molecule)	Δ ppm	Tentative Assignment According to the Literature	Lit.
15 (V), Figure 4	190.1229	[M27 + H] ⁺	C ₁₂ H ₁₅ NO	−1.16		
	236.1284	[M28 + H] ⁺	C ₁₃ H ₁₇ NO ₃	−1.31		
	234.1145	[M28 − H] [−]		−3.94		
	266.1389	[M29 + H] ⁺	C ₁₄ H ₁₉ NO ₄	−0.51		
	264.1251	[M29 − H] [−]		−3.74		
	289.1184	[M30 + H] ⁺	C ₁₅ H ₁₆ N ₂ O ₄	−0.64		
11 (G), Figure 5	342.1549	[M31 + H] ⁺	C ₁₆ H ₂₃ NO ₇	−0.53		
	183.0993	[M32 + Na] ⁺	C ₈ H ₁₆ O ₃	−0.84		
	159.1028	[M32 − H] [−]		−0.51		
	209.1149	[M33 + Na] ⁺	C ₁₀ H ₁₈ O ₃	−0.59	Royal jelly acid in various Iranian honeys	[31]
	185.1184	[M33 − H] [−]		−0.49		
	227.1254	[M34 + Na] ⁺	C ₁₀ H ₂₀ O ₄	−0.22		
	243.0741	[M35 + H] ⁺	Unknown	-		
	267.1104	[M36 + H] ⁺	Unknown	-		
	293.0995	[M37 + Na] ⁺	C ₁₃ H ₁₈ O ₆	0.14		
	321.0945	[M38 + Na] ⁺	C ₁₄ H ₁₈ O ₇	−0.11		
	355.1727	[M39 + Na] ⁺	C ₁₆ H ₂₈ O ₇	0.07		
	371.1677	[M40 + Na] ⁺	C ₁₆ H ₂₈ O ₈	−0.13		
	376.1003	[M41 + H] ⁺	C ₁₅ H ₁₉ O ₁₁	−0.70		
	121.0296	[M42 − H] [−]	C ₇ H ₆ O ₂	−0.55	Benzoic acid in honeydew honey from Brazil	[33]
	151.0402	[M43 − H] [−]	C ₈ H ₈ O ₃	−0.60	Mandelic acid in various honeys	[34]
					Vanillin in various Czech honeys	[35]
	240.0516	[M44 − H] [−]	C ₁₀ H ₁₁ NO ₆	−1.03		
	279.1241	[M45 − H] [−]	C ₁₅ H ₂₀ O ₅	−0.90		
12 (G), Figure 5	211.1305	[M21 + Na] ⁺	C ₁₀ H ₂₀ O ₃	−0.26	Hydroxy decanoic acid in various Iranian honeys	[31]
	209.1150	[M33 + Na] ⁺	C ₁₀ H ₁₈ O ₃	−0.93	Royal jelly acid in various Iranian honeys	[31]
	151.0401	[M43 − H] [−]	C ₈ H ₈ O ₃	−0.21	Mandelic acid in various honeys	[34,35]
					Vanillin in various Czech honeys	
	171.0992	[M46 + H] ⁺	C ₇ H ₁₆ O ₃	−0.43		
	188.0683	[M47 + H] ⁺	C ₈ H ₁₁ O ₅	−2.04		
	287.1254	[M48 + Na] ⁺	C ₁₅ H ₂₀ O ₄	0.04		
	263.1290	[M48 − H] [−]				
	137.0244	[M49 − H] [−]	C ₇ H ₆ O ₃	−0.15	Salicylic acid in honeydew honey from Brazil	[33]
					4-Hydroxybenzoic acid in <i>Agastache</i> honey	[36]

Table 4. Cont.

Zone	Mass Signal <i>m/z</i>	Adduct	Molecular Formula (Neutral Molecule)	Δ ppm	Tentative Assignment According to the Literature	Lit.
13 (G), Figure 5	146.0600	[M6 + Na] ⁺	C ₉ H ₇ NO	0.07	2-Hydroxyquinoline in various Iranian honeys	[31]
	263.1252	[M9 + Na] ⁺	C ₁₃ H ₂₀ O ₄	0.76		
	247.1303	[M19 + Na] ⁺	C ₁₃ H ₂₀ O ₃	0.71		
	170.0576	[M50 + Na] ⁺	C ₉ H ₉ NO	0.26		
	146.0612	[M50 – H] [–]		0.07		
15 (G), Figure 5	193.1199	[M51 + Na] ⁺	C ₁₀ H ₂₈ O ₂	0.21	Hexose	
	105.0702	[M52 + H] ⁺	C ₈ H ₈	–3.37		
	122.0966	[M53 + H] ⁺	Unknown	-		
	203.0525	[M54 + Na] ⁺	C ₆ H ₁₂ O ₆	0.05		
	258.1099	[M55 + H] ⁺	C ₁₂ H ₁₇ O ₆	–0.89		
19 (G), Figure 5	284.1490	[M56 + H] ⁺	Unknown	-	Methyl syringate in various Iranian honeys	[31]
	211.0613	[M57–H] [–]	C ₁₀ H ₁₂ O ₅	–0.24		
	146.0600	[M6 + H] ⁺	C ₉ H ₇ NO	0.07		
	168.0419	[M6 + Na] ⁺		0.15		
	144.0455	[M6 – H] [–]		–0.01		
28 (G), Figure 5	195.0876	[M58 + H] ⁺	C ₁₈ H ₁₀ N ₄ O ₂	0.27	Decanedioic acid in various Italian honeys	[32]
	217.0695	[M58 + Na] ⁺		0.22		
	291.1125	[M59 + H] ⁺	C ₁₈ H ₁₄ N ₂ O ₂	0.67		
	313.0945	[M59 + Na] ⁺		0.67		
	201.1133	[M60 – H] [–]	C ₁₀ H ₁₈ O ₄	–0.23		
I (V), Figure 6	146.0601	[M6 + H] ⁺	C ₉ H ₇ NO	–1.57	2-Hydroxyquinoline in various Iranian honeys	[31]
	168.0421	[M6 + Na] ⁺		–1.52		
	144.0455	[M6 – H] [–]		–0.22		
	214.0475	[M13 + Na] ⁺	C ₁₀ H ₉ NO ₃	–1.14		
	190.0510	[M13 – H] [–]		–0.22		
I (V), Figure 6	146.0612	[M50 – H] [–]	C ₉ H ₉ NO	–0.22		
	236.0294	[M61 + Na] ⁺	C ₉ H ₉ O ₆	–2.05		
	359.1311	[M62 + Na] ⁺	C ₁₄ H ₂₄ O ₉	–0.68		
	385.1468	[M63 + Na] ⁺	C ₁₆ H ₂₆ O ₉	–0.82		
	188.0355	[M64 – H] [–]	C ₁₀ H ₇ NO ₃	–1.02		
I (V), Figure 6	146.0601	[M6 + H] ⁺	C ₉ H ₇ NO	–1.57	2-Hydroxyquinoline in various Iranian honeys	[31]
	168.0421	[M6 + Na] ⁺		–1.52		
	144.0455	[M6 – H] [–]		–0.22		
	261.1098	[M65 + Na] ⁺	C ₁₃ H ₁₈ O ₄	0.00		
	237.1133	[M65 – H] [–]		–0.40		
I (V), Figure 6	304.2610	[M66 + Na] ⁺	C ₁₈ H ₃₅ NO	0.31		
	253.1083	[M67 – H] [–]	C ₁₃ H ₁₈ O ₅	–0.40		

Table 4. Cont.

Zone	Mass Signal <i>m/z</i>	Adduct	Molecular Formula (Neutral Molecule)	Δ ppm	Tentative Assignment According to the Literature	Lit.
II (Y2), Figure 6	146.0601	[M6 + H] ⁺	C ₉ H ₇ NO	−0.48	2-Hydroxyquinoline in various Iranian honeys	[31]
	168.0421	[M6 + Na] ⁺		−0.33		
	313.0946	[2M6 + Na] ⁺		0.35		
	144.0455	[M6 − H] [−]		0.06		
III (V), Figure 6	146.0601	[M6 + H] ⁺	C ₉ H ₇ NO	−0.48	2-Hydroxyquinoline in various Iranian honeys	[31]
	144.0455	[M6 − H] [−]		0.06		
	263.1253	[M9 + Na] ⁺	C ₁₃ H ₂₀ O ₄	0.12	Royal jelly acid in various Iranian honeys	[31]
	247.1304	[M19 + Na] ⁺	C ₁₃ H ₂₀ O ₃	0.27		
	209.1148	[M33 + Na] ⁺	C ₁₀ H ₁₈ O ₃	−0.02		
	281.1358	[M68 + Na] ⁺	C ₁₃ H ₂₂ O ₅	0.41		
IV (V), Figure 6	144.0455	[M6 − H] [−]	C ₉ H ₇ NO	0.12	2-Hydroxyquinoline in various Iranian honeys	[31]
	263.1255	[M9 + Na] ⁺	C ₁₃ H ₂₀ O ₄	0.12	Hydroxy decenoic acid in various Iranian honeys	[31]
	211.1306	[M21 + Na] ⁺	C ₁₀ H ₂₀ O ₃	−0.21		
	399.2719	[2M21 + Na] ⁺		−0.05		
V (Y2), Figure 6	146.0601	[M6 + H] ⁺	C ₉ H ₇ NO	−0.48	2-Hydroxyquinoline in various Iranian honeys	[31]
	144.0455	[M6 − H] [−]		0.12		
	203.0527	[M69 + Na] ⁺	C ₆ H ₁₂ O ₆	−0.39	Hexose	

Another very intense signal at *m/z* 211.1303 [M21 + Na]⁺ was found in zone 14 from *Schefflera abyssinica* (Figure 4), whereas other related compounds were present in zones 11, 12, and 17 from *Coffea arabica* (Figure 5) and in zones III and IV from *Vernonia amygdalina* (Figure 6). M21 was assigned to the molecular formula C₁₀H₂₀O₃ and was tentatively matched to hydroxy decanoic acid (Table 4). The mass signal at *m/z* 263.1254 [M9 + Na]⁺ was assigned to C₁₃H₂₀O₄. M9 and some related compounds were detected in zone 13 from *Croton macrostachyus*, *Eucalyptus globulus* (Figure 4), and *Coffea arabica* (Figure 5), and in zones III and IV from *Vernonia amygdalina* (Figure 6). In most cases, the HRMS spectra were free from residual sugars of the honey matrix, except for zone 15 close to the application zone (Figure 5) and zone V at *hR_F* 45 in the on-surface extracts (Figure 6), both at similar intensity. This clearly verified the high potential of on-surface extraction to sufficiently remove matrix interferences.

2.7. Limitation of the Developed Effect-Directed Profiling Method

Honeys from very different botanical origins were analyzed. The straightforward workflow developed is helpful for identifying differences in the bioactivity between monofloral honey types and can contribute to quality designation and branding. The effect-directed profiling provided new insights that are valuable for food science and nutrition. A wealth of 28 different bioactive compound zones were observed via effect-directed profiling. The effect-directed assays (Figures 1–3) together with the HPTLC–HRMS analysis of selected zones (Figures 4–6) thus revealed 69 different analytes of potential importance (Table 4). Since no data have been available in the literature on the identity of bioactive compounds in these nine different honey types studied, matching of HPTLC–HESI–HRMS signals and molecular formulas as well as tentative assignment to molecule structures was not possible. Nevertheless, a wealth of molecular formulas was successfully assigned due to the high-resolution feature of the MS system, and possible structural candidates were assigned (Table 4) according to the literature data from other honey types. This is how far one can go from effect-directed profiling to characterization of bioactive zones using

the newly developed profiling method. In view of the number of samples and analytes, identification of all bioactive compound zones was not possible in the current study, which was mainly aimed at the development of bioactivity profiling.

For identification of the bioactive compounds, molecule fragmentation by HRMS/MS, co-chromatography with supposed standard substances, and nuclear magnetic resonance spectroscopy analyses would be further necessary options. For future structure elucidation, the separation system can be adjusted to the zone region of interest. For example, a very apolar mobile phase will better resolve the apolar compound zones (in comparison to our compromise-guided selection of the solvent system). After effect detection, the number of assignable mass signals in the recorded HRMS spectra from a better resolved zone should be reduced. As another option, a 2D separation using orthogonal mobile phases [37] or an orthogonal 8D hyphenation [22] can be studied for honey samples to differentiate the mass signals and to highlight the mass signals responsible for the individual effects.

3. Materials and Methods

3.1. Chemicals and Reagents

Methanol, ethanol, and 2-propanol (all HPLC grade), as well as formic acid and acetic acid (99%, LC-MS), were obtained from VWR, Darmstadt, Germany. Acetonitrile (HPLC grade) was received from Honeywell Specialty Chemicals, Seelze, Germany. Toluene (HPLC grade) was delivered by Promochem, LGC Standards, Wesel, Germany. Gram-negative, bioluminescent marine *Aliivibrio fischeri* bacteria (DSM-7151) were obtained from the German Collection of Microorganisms and Cell Cultures, Berlin, Germany. α -Glucosidase from *Saccharomyces cerevisiae* (1000 U/vial), α -amylase from hog pancreas (50 U/mg), 2-chloro-*p*-nitrophenyl- α -D-maltotriose, acetylcholinesterase (AChE) from *Electrophorus electricus* (≥ 245 U/mg solid, 10 kU/vial), sodium acetate, di-sodium hydrogen phosphate, magnesium sulfate heptahydrate, sodium chloride, and ammonium hydroxide (>98) were delivered by Sigma-Aldrich, Steinheim, Germany. β -Glucosidase from almonds (3040 U/mg) and 2-naphthyl- β -D-glucopyranoside were provided by ABCR, Karlsruhe, Germany. Fast Blue B salt (95%) was from MP Biomedicals, Eschwege, Germany. 2,2-Diphenyl-1-picrylhydrazyl (DPPH $\bullet$, 95%) was delivered by Alfa Aesar, Schwerte, Germany. 2-Naphthyl- α -D-glucopyranoside was from Fluorochem, Hadfield Derbyshire, UK. Tris(hydroxymethyl)aminomethane (Tris, $\geq 99.9\%$), dipotassium hydrogen phosphate ($\geq 99\%$), sodium dihydrogen phosphate monohydrate ($\geq 98\%$), sodium hydroxide ($\geq 98\%$), hydrochloric acid (HCl, 37%), ethyl acetate (HPLC grade), and petroleum ether (40–60 °C) were purchased from Carl Roth, Karlsruhe, Germany. HPTLC silica gel 60 F₂₅₄ plates (20 cm $\times$ 10 cm) were provided by Merck, Darmstadt, Germany. 1-Naphthyl acetate ($\geq 98\%$) was obtained from AppliChem, Darmstadt, Germany. Bi-distilled water was produced with a Heraeus Destamat Bi-18E, Thermo Fisher Scientific, Dreieich, Germany. The polypropylene box (KIS, 27 cm $\times$ 16 cm $\times$ 10 cm, for plate incubation) was from ABM, Wolframs-Eschenbach, Germany. Honey samples were collected from the forest and honey-producing areas in the honey-harvesting period in Ethiopia, Africa.

3.2. Solid-Phase Extraction

Each honey sample (10 g) was dissolved in 100 mL of bi-distilled water, centrifuged (3000 $\times$ g), and applied to spherical, hydrophobic polystyrene-divinylbenzene resin solid-phase extraction columns (CHROMABOND[®] HR-X, 3 mL, 200 mg, 85 μ m particle size, REF 730931) according to the product instruction Appl. No. 304310 (Macherey-Nagel, Düren, Germany). After elution with 10 mL methanol, the eluent solvent was concentrated to 1 mL, centrifuged (17,000 $\times$ g, 5 min), and filled in a sampler vial for analysis.

3.3. HPTLC–UV/Vis/FLD Method

Each honey extract was applied (1–9 μ L/band, as listed in the respective figure) in an 8 mm band (track distance 15 mm, distance from left edge 15 mm, distance from bottom edge 8 mm, and dosage speed 150 nL/s; Automatic TLC Sampler ATS 4, CAMAG, Muttenz,

Switzerland), dried in a stream of cold air (2 min, hair dryer), and developed with ethyl acetate–methanol 3:2 (V/V) up to a migration distance of 60 mm (which took 14 min) in a twin trough chamber (20 × 10 cm, CAMAG), dried (3 min), and documented at white light illumination (Vis), UV 254 nm, and FLD 366 nm (TLC Visualizer, CAMAG). For derivatization, the plate was immersed in the respective reagent as described elsewhere [21,24,38,39]. Instruments were operated and data were processed with winCATS, version 1.4.7.2018, or visionCATS, version 3.1.21109.3 software (CAMAG).

3.4. Optional On-Surface Extraction and Separation on the Same Plate

This approach was only used for Section 2.5. Each honey sample (2 g) was diluted in 10 mL methanol, vortexed, ultrasonicated (15 min), and centrifuged (3000× g, 15 min). An aliquot of each supernatant was applied (25 µL) in an area of 8 mm × 10 mm (track distance 15 mm, distance from left edge 15 mm, distance from bottom edge 8 mm, and dosage speed 600 nL/s) and dried (2 min, stream of cold air). As an example of a combined on-surface extraction and separation on the same plate, the start areas were focused twice with acetonitrile–ethanol–ammonia 8:1:2, V/V/V, up to 30 mm, and after the plate was cut at 15 mm (TLC Plate Cutter, CAMAG) and developed with toluene–ethyl acetate–formic acid–water 1.6:7:0.8:0.6, V/V/V/V, up to a migration distance of 70 mm.

3.5. Effect-Directed Profiling

Six chromatograms were prepared in parallel. Depending on the assay, the sample volume was selected (listed in the figure caption) and respective positive controls were applied above the solvent front at the upper chromatogram edge [39]. Then, the plate was subjected to the respective assay solutions or suspensions. Each assay was repeated and the results were confirmed. For incubation in the incubator (at 37 °C, if not stated otherwise), each plate was placed horizontally in a humid polypropylene box (moistened a priori for 30 min with 35 mL water spread on filter papers aligned on walls and the bottom). Drying was performed in a stream of cold air (hair dryer) or on a TLC Plate Heater (CAMAG). If not stated otherwise, documentation was performed at white light illumination in the reflectance mode (TLC Visualizer, CAMAG).

3.5.1. α- and β-Glucosidase Inhibition Assays

The chromatogram was piezoelectrically sprayed (green nozzle, level 6, Derivatizer, CAMAG) with 2 mL substrate solution (12 mg 2-naphthyl-α- (or β)-D-glucopyranoside in 9 mL ethanol and 1 mL 10 mmol sodium chloride solution) and dried (2 min). For prewetting, 1 mL sodium acetate buffer (10 g sodium acetate in 250 mL water, adjusted to pH 7.5 with 0.1 M acetic acid) was sprayed on the plate, followed by 2 mL enzyme solution (10 U/mL α-glucosidase, or 1000 U/mL β-glucosidase in buffer). The incubation took 15 min for α-glucosidase, or 30 min for β-glucosidase. Then, the plate was sprayed with 0.75 mL Fast Blue B salt solution (2 mg/mL in bi-distilled water) and dried [40,41]. Colorless (bright) spots on a purple background indicated α- or β-glucosidase inhibitors.

3.5.2. Radical Scavenging Assay

The chromatogram was immersed (immersion speed 3.5 cm/s, immersion time 2 s) in a 0.05% methanolic DPPH• solution and dried (60 °C, 1 min) [42]. Image capture under white light illumination was carried out directly, but also repeated the next day, as the zone response increased over time. Radical scavenging compounds appeared as yellow zones against a purple background.

3.5.3. *Aliivibrio fischeri* Bioassay

The chromatogram was immersed (immersion speed 3.5 cm/s and immersion time 2 s, TLC Immersion Device, CAMAG) in an *Aliivibrio fischeri* culture. The readiness of the bacterial growth prepared according to [43,44] was visually controlled for an emitted brilliant green-blue light by shaking the culture flask in a dark room. The bioluminescence

was recorded with an exposure time of 50 s (time interval 3 min, BioLuminizer, CAMAG). Dark zones indicated antibacterial compounds.

3.5.4. AChE Inhibition Assay

The AChE inhibition assay was performed as described [45,46]. The chromatogram was prewetted with 1 mL Tris-HCl buffer solution (pH 7.8, 0.05 M), then sprayed with 3 mL AChE solution (6.66 U/mL) and incubated at 37 °C for 25 min. The plate was sprayed with 0.75 mL of the substrate/chromogenic reagent mixture (ethanolic 1-naphthyl acetate solution 4.5 mg/1.5 mL and aqueous Fast Blue B salt solution, 9 mg/3 mL) and dried (3 min). Colorless (bright) spots on a purple background indicated AChE inhibitors.

3.5.5. α -Amylase Inhibition Assay

The chromatogram was immersed in the substrate solution (1.4 mg/mL 2-chloro-*p*-nitrophenyl- α -D-maltotriose in ethanol; immersion speed 3.5 cm/s and immersion time 2 s, TLC Immersion Device). The plate was dried (2 min), then immersed in the freshly prepared enzyme solution (1.2 mg/mL α -amylase in sodium acetate buffer) and incubated for 15 min at 37 °C [39]. Colorless (bright) spots on a yellow background indicated α -amylase inhibitors.

3.6. HPTLC–HESI–HRMS

The extracts (3 μ L/band) or sample solutions (25 μ L/area each) were applied in duplicate on the same HPTLC plate prewashed twice with methanol–water 3:1 (V/V) [47]. After development with toluene–ethyl acetate–formic acid–water 2:6:1:0.6, V/V/V/V, up to a migration distance of 70 mm, the plates were cut (smartCut Plate Cutter, CAMAG) into two identical halves; one was subjected to the DPPH• assay, and the other was kept clean for HRMS. The zones of interest were online eluted for 60 s with methanol at 0.2 mL/min into a Q Exactive Plus Hybrid Quadrupole Orbitrap system (Thermo Fisher Scientific, Dreieich, Germany) using an open-source modified fully automated elution head-based interface [48]. In between the interface and the mass spectrometer, a filter frit (Upchurch Scientific A-356 and PEEK frit Blue UPA-703, Techlab, Erkerode, Germany) was installed to prevent the HESI source from particles. The ionization settings were a spray voltage of 3.5 kV, capillary temperature of 270 °C, sheath gas of 20 (arbitrary units), aux gas of 10 (arbitrary units), probe heater temperature of 200 °C, and S-lens RF level of 50 (arbitrary units). Full scan spectra were recorded at a resolution of 280,000 (FWHM at m/z 200), AGC target $1e6$, and maximum inject time 200 ms with lock masses 301.14103 (dibutyl phthalate, $[M + H]^+$) and 413.26623 (diisooctyl phthalate, $[M + H]^+$) in the positive and 112.98563 (formic acid, $[2M + Na - 2H]^-$) in the negative ionization mode. In between each analyte zone elution, the elution head and the tubing were cleaned via elution of a blank plate background (at a similar R_F position as the target zone) to avoid cross-contamination. The blank spectrum was subtracted from the analyte mass spectrum. The instrument was controlled and the data were processed with Xcalibur 4.2.47 SP1 with Foundation 3.1.261.0 SP6 and SII for Xcalibur 1.5.0.10747 (Thermo Fisher Scientific).

4. Conclusions

The developed non-target effect-directed profiling of nine different types of monofloral Ethiopian honey using HPTLC–UV/Vis/FLD–EDA provided new insights that are helpful for the valorization of Ethiopian honeys. Highlighting the physiological activity of Ethiopian honeys based on bioactivity profiles is valuable for food science and nutrition as well as for the market. It is important to understand the differences in the bioactivity profiles between monofloral honeys since it can affect quality designation and branding. The effect-directed profiles of radical scavenging compounds, antibacterial compounds against Gram-negative bacteria, and compounds inhibiting AChE, α -glucosidase, β -glucosidase, and α -amylase, contributed to the differentiation, categorization, and authentication of the honey types. Given the global trade in honey and the associated vulnerability to fraud,

contamination, and pesticide or antibiotic residues, non-target profiling is also able to detect other bioactive ingredients that have not previously been in focus. The non-target effect-directed profiling developed could provide more comprehensive information on honey quality and safety compared to conventional target analysis or microtiter plate assays, which only provide a sum parameter. It was also shown that the further characterization of bioactive zones of interest and the assignment of molecular structures was possible using HPTLC–HESI–HRMS, although structural assignment was limited.

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12. Publication VIII

Metabolic reprogramming of hepatocytes by *Schistosoma mansoni* eggs

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27 † The authors dedicate this publication to Laura Hehr, who deceased in December 2021.

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47 experiments. T.Q., S.H., B.S., G.S., F.H.F., G.M., K.B., P.W., and C.G.G. supervised
48 experiments and contributed samples, materials, methods and instrumentation. A.B., L.H., S.G.,
49 V.W., L.R., S.W., A.M., J.P.-K., and M.R. were involved in statistical analysis. All authors
50 analyzed, interpreted, discussed the data, and reviewed the manuscript.

Abstract

Background & Aims: Schistosomiasis, is a parasitic infection, which globally affects more than 200 million people. *Schistosoma* eggs, but not the adult worms, are mainly responsible for schistosomiasis-specific morbidity in the liver. It is unclear if *S. mansoni* eggs consume host metabolites, and how this compromises the host parenchyma.

Methods: Metabolic reprogramming was analyzed by MALDI-MSI imaging, LC- and HPTLC-HRMS, metabolite quantification, confocal laser scanning microscopy, live cell imaging, quantitative real-time PCR, western blotting, assessment of DNA damage, and immunohistology in hamster models and functional experiments in human cell lines. Major results were validated in human biopsies.

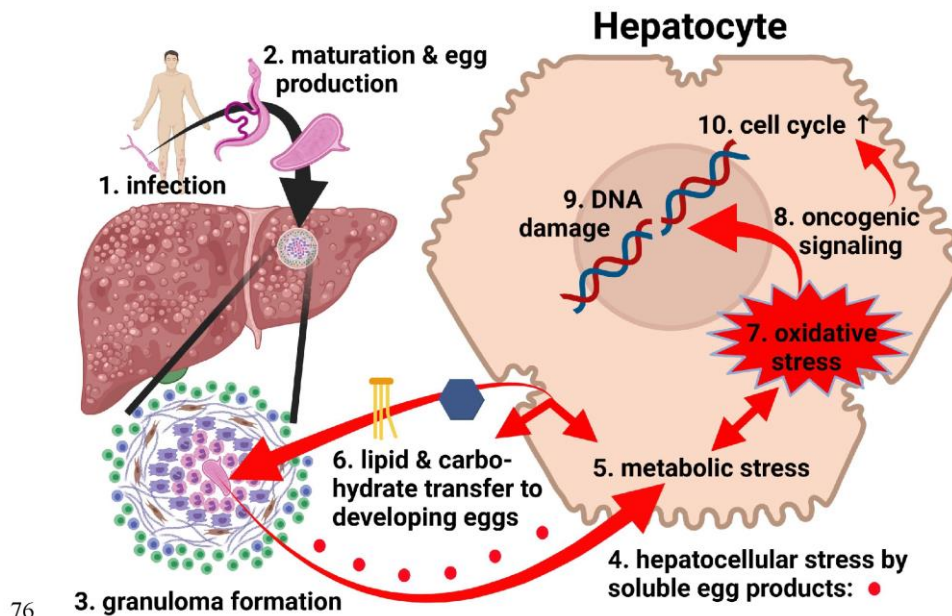
Results: Notably, eggs mobilize, incorporate, and store host lipids, while the metabolic reprogramming causes oxidative stress-induced DNA damage in hepatocytes. Administration of ROS scavengers ameliorates these deleterious effects.

Conclusions: Our findings indicate that *S. mansoni* eggs completely reprogram lipid and carbohydrate metabolism by soluble factors, which results in oxidative stress-induced cell damage in the host parenchyma.

Lay summary

The authors demonstrate that soluble egg products of the parasite *S. mansoni* induce hepatocellular reprogramming causing metabolic exhaustion and a strong redox imbalance. Notably, eggs mobilize, incorporate, and store host lipids, while the metabolic reprogramming causes oxidative stress-induced DNA damage in hepatocytes, independent of the host's immune response.

75 Graphical abstract



76

77

78 Highlights

- 79 -*S. mansoni* soluble egg products induce hepatocellular metabolic reprogramming
- 80 -*S. mansoni* eggs mobilize, incorporate, and store host lipids
- 81 -*S. mansoni* induced parenchymal reprogramming causes metabolic exhaustion in the liver
- 82 -the parasite induced metabolic crisis associated redox imbalance provokes DNA damage
- 83 -the egg induced processes can harm hepatocytes independent of the host's immune response
- 84
- 85 All relevant data is contained within the manuscript and supporting information. Raw data are
- 86 available upon request.

87 **Introduction**

88 Schistosomiasis, one of the most important parasitic infections worldwide, is caused by
89 trematodes of the genus *Schistosoma*. It is accompanied by severe clinical symptoms as well as
90 socioeconomic problems, and more than 200,000 deaths per year. According to the World
91 Health Organization (WHO), schistosomiasis is a neglected tropical disease, and at least 236.6
92 million people required preventive treatment in 2019 [1]. Climate change and globalization will
93 inevitably influence both, the distribution of the parasites and the incidence of schistosomiasis,
94 even in areas where it is currently not endemic. Reports about an outbreak of urogenital
95 schistosomiasis in Corsica (France), demonstrate the potential risk for schistosomiasis to spread
96 into other areas [2].

97 In intestinal schistosomiasis schistosome eggs cause inflammatory responses leading to
98 granuloma formation [3]. The process of egg excretion is driven by an immune-dependent
99 formation of granulomatous inflammation in the gut. A similar immunopathology is caused in
100 the hepatic dead-end [4]. In contrast to intestinal granulomas, liver granulomas become fibrotic
101 over time, which often leads to obstructive portal lesions and portal hypertension, resulting in
102 gastrointestinal bleeding, hepatic encephalopathy, or liver failure [4].

103 There is compelling evidence that schistosome eggs, but not the adult worms, are mainly
104 responsible for schistosomiasis-specific morbidity [5]. Unusually for trematodes, *S. mansoni*
105 eggs are not self-contained. As central part of the eggs, the supporting vitelline cells are unable
106 to drive embryonic development, which also depends on host factors. Taking into account
107 density and volume changes between deposition and maturation, it has been estimated that an
108 egg increases in mass > threefold [6]. This means that more than two-thirds of tissue
109 constituents in a mature egg are externally derived, while even neglecting energy requirements
110 during embryogenesis [6].

111 In endemic areas, *S. mansoni* infection is associated with anemia and chronic malnutrition [7].
112 It is yet unclear if schistosomiasis is a cause of anemia and malnutrition or if both are
113 threatening consequences of the poverty-related living conditions in endemic areas.
114 Nevertheless, malnutrition is one of the major factors driving schistosomiasis [8]. The current
115 study illuminates the physiologic consequences of an *S. mansoni* infection, thus improving our
116 understanding of the connections between malnutrition and schistosomiasis.

117 In contrast to *S. mansoni*-associated malnutrition in endemic areas, also positive metabolic
118 effects of infection have been shown in humans suffering from the metabolic syndrome [9].
119 Furthermore, the injection of *S. mansoni* eggs into mice indicated that the cholesterol-lowering
120 effect observed in the serum during infection is mediated by factors released from eggs, while
121 adult worms seemed to have little or no effects [10]. *S. japonicum* infection induced host genes
122 involved in catabolism including glucose uptake, glycolysis, fatty acid oxidation, and
123 suppression of anabolism (glycogen synthesis) in the liver. This might be regulated by
124 macrophage metabolic states involving AMPK (AMP-activated protein kinase), AKT (Protein
125 kinase B), and TORC1 (target of rapamycin kinase complex I) pathways, as shown by *in vitro*
126 stimulation with SEA (representing the total soluble complement of homogenized eggs) [11].
127 A recently published study demonstrated reprogramming of the metabolic signature of
128 macrophages after *S. mansoni* infection [12]. Of note, this reprogramming paralleled the
129 establishment of a longevity memory-like phenotype in myeloid cells of infected mice [12].
130

131 Imbalances of nutrition supply, *i.e.* undernutrition as well as overnutrition, have been associated
132 with enhanced hepatic oxidative stress that promotes liver disease [13]. Increased oxidative
133 stress during *S. mansoni* infection has been attributed to the inflammatory response of
134 granulomatous immune cells within the liver [14]. High ROS levels induce oncogenic signaling
135 and may promote cancer by increasing DNA mutations [15]. Previously, we discovered that

136 SEA, including IPSE/alpha-1 of *S. mansoni*, activates oncogenic signaling in liver and colon
137 [16, 17].
138 In this study we provide first evidence that *S. mansoni* eggs are capable of inducing metabolic
139 reprogramming of the host parenchyma, in order to take up host metabolites. These processes
140 involve hepatic exhaustion, finally leading to hepatic DNA damage.

141 **Methods (Details in supplementary CTAT Table)**

142 **Human Material**

143 Pseudonymized human colon samples were kindly provided by Dr. Senckenberg Institute of
144 Pathology, University Hospital Frankfurt. The use of pseudonymized human residual samples
145 that were routinely taken for pathologic assessment was approved by the local ethics committee
146 (AZ 05/19). According to the ethics vote, an informed consent was not required for our
147 retrospective analyses of archived tissues.

148

149 **Animal Experimentation**

150 *Biomphalaria glabrata* snails served as intermediate hosts and Syrian hamsters (*Mesocricetus*
151 *auratus*) as final hosts for maintaining the life-cycle of a Liberian strain of *S. mansoni*[18]. Bs
152 (= mixed sex) and ms (= monosex) worm populations were generated by polymiracidial and
153 monomiracidial intermediate host infections, respectively[19]. Bs infections(n=36 ♀) were
154 carried out at the age of 8 weeks and were maintained for 46 days, and ms infections (n=17 ♀)
155 67 days[20] to ensure a complete maturation of the worms; females need longer to grow and
156 develop in the absence of male partners. Untreated hamsters (n=6 ♀) were used as
157 supercontrols. All animal experiments were performed in accordance with the European
158 Convention for the Protection of Vertebrate Animals used for experimental and other scientific
159 purposes (ETS No 123; revised Appendix A) and were approved by the Regional Council
160 Giessen (V54-19 c 20/15 c GI 18/10 Nr. A26/2018).

161

162 **MALDI MSI analysis and identification by LC-MS/MS**

163 MALDI MSI and LC-MS/MS experiments were performed as described elsewhere[21]. For
164 data analysis, "Lipid Match Flow"[22] was used for identification, "Perseus"[23] for statistical
165 analysis and "Mirion"[24] for image generation. Detailed description of the method:

166 Supplemental material.

167

168 **Isolation of soluble egg antigens**

169 *S. mansoni* eggs were obtained from livers of bs-infected hamsters at day 46 post infection, and

170 SEA was isolated as described earlier[25].

171

172 **Isolation of *S. mansoni* eggs**

173 Liver eggs and *in vitro*-laid eggs of *S. mansoni*-infected hamsters were isolated as described

174 elsewhere with minor modifications[25, 26].

175

176 **Cell culture experiments**

177 HepG2 cells (stock ordered in 2019, CLS # 330198, expanded and stored as cryostocks for

178 consistent quality in culture for up to 10 passages per cryostock) were stimulated with

179 15 µg/mL SEA and/or 10 mM reduced L-gluthatione (GSH) for the indicated time points. For

180 co-culture assays, HepG2 cells were treated with 400 µM fluorescently labeled oleic acid

181 (Avanti Polar Lipids, Alabaster, AL, USA, #810259C) for 24 h and washed three times with

182 PBS prior to co-culturing with 100 *S. mansoni* eggs per well (24-well plate) for further 24 h. *In*

183 *vitro*-laid or liver-extracted eggs were co-cultured in the same compartment or in transwells

184 with a permeable polycarbonate membrane (Corning, New York, USA), as indicated in the

185 figure legends. Subsequently, eggs were washed three times and analyzed directly or after

186 fixation with 1% formaldehyde by confocal laser scanning microscopy (CLSM).

187

188 **Confocal laser scanning microscopy and live cell imaging**

189 For lipid staining of eggs, fluorescently-labeled oleic acid (fLOA; TopFlour Oleic Acid, Avanti

190 SKU 810259C) was applied and uptake analyzed by CLSM. Liver eggs or *in vitro*-laid eggs

191 were cultured up to 24 h with 50 µg/mL fLOA (mixed with 200 µM oleic acid) or with HepG2
192 cells pretreated with 50 µg/mL fLOA (mixed with 200 µM oleic acid) 24 h prior to co-culture
193 with eggs. Negative controls received oleic acid only. Image acquisition was done on a Leica
194 TCS SP5 VIS using a 488 nm argon laser. Autofluorescence of eggs was excited with a DPSS
195 laser at 561 nm to visualize egg contours. Live imaging of fLOA uptake was conducted by image
196 acquisition every 20 sec over a period of 30 min. Confocal z-stacks were composed of 100-122
197 images with a step size of 0.5 µm and further processed in IMARIS imaging software
198 (Bitplane). Quantification of fLOA fluorescence per egg was achieved by acquiring 15-25 z-
199 stacks and averaging the mean grey values of the egg in all images using Image J.

200

201 **Histochemistry and immunohistochemistry**

202 Periodic acid-Schiff (PAS) reaction was applied for visualization of glycogen distribution in
203 hepatic tissue. For analyzing the hepatic distribution of neutral triacylglycerides (TAGs) and
204 lipids, liver tissue was stained by the fat-soluble Oil red O dye (Serva, Heidelberg, Germany,
205 #31170). Immunohistochemical detections were performed as described previously[27].

206

207 **TAG Assay**

208 The TAG concentration of homogenized hamster liver lysates was determined as recommended
209 by the manufacturer (Triacylglyceride Assay Kit – Quantification, Abcam, Cambridge, UK,
210 #ab65336).

211

212 **High-performance thin-layer chromatography (HPTLC)**

213 Liver tissue extracts were prepared ten times more concentrated than described[28]. Instrumen-
214 tation used was from CAMAG, Muttenz, Switzerland. Samples and calibration standards
215 (1 mg/mL in chloroform/methanol 3:1, *V/V*) were applied as 8-mm bands (ATS4) on HPTLC
216 silica gel 60 F₂₅₄ MS grade plates (Merck, Darmstadt, Germany) and dried (40 °C, 5 min).

217 Separation of polar lipids was performed with chloroform/methanol/ammonia (25%)/water
218 60:30:3:1 (*V/V/V/V*) up to 50 mm, and of non-polar lipids with *n*-hexane/diethyl ether/acetic
219 acid 40:10:1 (*V/V/V*) up to 65 mm migration distance after 20 min pre-saturation (via filter
220 paper, Twin-Trough Chamber). The dried plate (5 min, cold air stream) was immersed into the
221 primuline reagent (0.05 % in acetone/water 4:1, *V/V*). Fluorescence detection (FLD) was at
222 366/>400 nm (TLC Scanner 4). For visualization of saccharides and amino acids per reagent
223 sequence, the primuline-treated polar lipid plate was immersed into the ninhydrin and then
224 aniline-diphenylamine-*o*-phosphoric acid reagents[29]. The primuline-treated non-polar lipid
225 plate was sprayed with phosphomolybdic acid reagent (20 mg/mL solution in ethanol), heated
226 (110°C, 3 min) and detected at white light illumination. For high-resolution mass spectrometry
227 (HRMS), samples and standards were applied in duplicate on prewashed plates[30], which were
228 cut after development. One plate part was used for detection via the primuline reagent, and the
229 other via HPTLC–HRMS as described[28].

230

231 **Reporter gene assay**

232 Analysis of AP-1 promotor-activity in SW620 cells has been described previously[16].

233

234 **Western blot analysis**

235 Western blotting was performed as described before[31].

236

237 **Glycogen assay**

238 Liver tissue and glycogen standard samples were dissolved in 2 M hydrochloric acid, boiled for
239 1 h and neutralized with 2 M sodium hydroxide. After centrifugation, the supernatant was
240 analyzed with the Glucose Assay Kit (Merck) according to the manufacturer's protocol.

241

242 **Malondialdehyde (MDA) assay**

243 Lipid-peroxidation as a marker for oxidative stress was measured in liver samples and HepG2
244 cells by the MDA Assay Kit (Merck), according to the manufacturer's protocol.

245

246 **Pyruvate assay**

247 The pyruvate content of HepG2 cells was determined by the Pyruvate Assay Kit (Merck)
248 according to the manufacturer's protocol.

249

250 **Quantitative realtime polymerase chain reaction (qRT-PCR)**

251 mRNA isolation, transcription, quantitative real-time PCR, and data analysis was performed as
252 described before[32].

253

254 **Quantification of *S. mansoni* eggs**

255 Liver tissue (100 mg) of bs-infected hamsters ($n=36$) was digested in 5 % potassium hydroxide
256 at 37°C for 16 h[33]. Subsequently, eggs were counted independently by two persons three
257 times per sample. The number of eggs was calculated per mg of liver tissue.

258

259 **Comet assay**

260 The alkaline comet assay (Abcam #ab238544) for assessing DNA damage in HepG2 cells was
261 performed according to the manufacturer's protocol.

262

263 **Statistic analysis**

264 The present study is of an exploratory nature. Therefore the group size were not estimated in
265 advance by pre-specified effect size. The study was started with an existing number of
266 cryopreserved organs that were not required for the maintenance of the parasite life cycle.

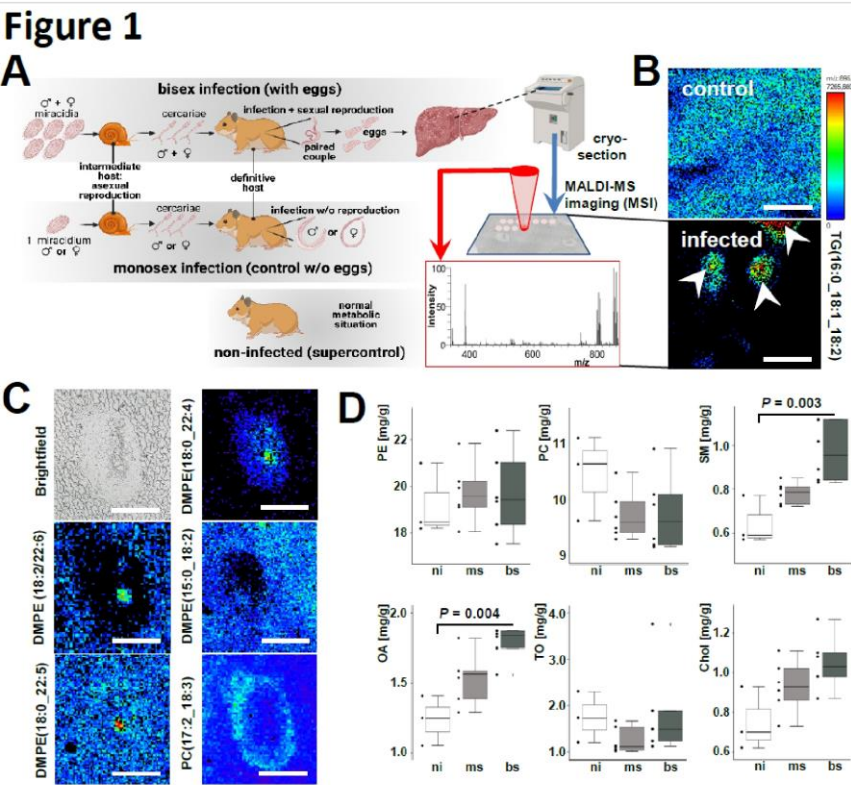
267 Statistic analysis was performed using SPSS version 26.0 (SPSS Inc., IBM corporation,
268 Armonk, NY, RRID:SCR_002865). Subsequent comparison of groups within each Kruskal-

269 Wallis test were Bonferroni-corrected. Because of the exploratory nature of the study, no
270 further adjustment for p-values was performed. Densitometrically assessed data from western
271 blots (hamster colon) were depicted as mean or median $\pm$ 95% confidence intervals.

Results

S. mansoni eggs disrupt hepatic lipid metabolism

To study the role of *S. mansoni* eggs in the liver *in vivo*, we used a hamster model and infections with bisexual (bs)- and monosex (ms)-cercariae. This allowed to discriminate between worm-induced and egg-mediated effects *in vivo* (Fig. 1A). For analyzing the hepatic distribution of certain lipid species in livers of bs-infected hamsters, we performed MALDI-MS imaging [34], revealing the relative abundance and locations of hepatic lipids (Fig. 1A). MS imaging experiments revealed that all detected triacylglycerides accumulated in the granuloma area with highest levels inside the eggs (Fig. 1B arrowheads) while they were depleted in the surrounding liver tissue compared to ni controls (Fig. 1B, SFig. 1 and 2). Please note, that the color encodes for the concentration of the target molecule shown in Fig. 1B and SFig. 1 and 2.



284 In total, we detected 77 triacylglycerides with altered concentrations - 33 were depleted and 43
285 enriched in livers of bs-infected hamsters compared to the controls (SFig. 3). Adult
286 schistosomes modify fatty acids from their host for biosynthetic purposes and incorporate those
287 in phospholipids and neutral lipids [35]. Here, we demonstrate that distinct isoforms of
288 dimethyl-phosphatidylethanolamine (DMPE) were enriched in the eggs while they were
289 depleted in the granuloma and in the surrounding liver parenchyma (Fig. 1C). Representative
290 MS images of different lipid classes are shown for the three animal groups (SFig. 4, Fig. 1C
291 focuses on the distribution of the lipids in and around the granuloma). We detected
292 characteristic distributions of lipids in the liver of bs-infected versus ms-infected and ni
293 animals. DMPE (18:2/22:6) was evenly distributed in the parenchyma of bs-infected hamster
294 liver but depleted in granuloma and enriched in eggs (SFig. 4B). On the other hand, DMPE
295 (18:0/22:5) was found in parenchyma and enriched in granuloma and eggs (SFig. 4C). DMPE
296 (18:0/22:4) was not detected in hepatic parenchyma but in granulomas, with enrichment in eggs
297 (SFig. 4D). DMPE (15:0/18:2) was found evenly distributed in all samples despite the
298 granulomatous regions of bs-infected samples (SFig. 4E). A different lipid species,
299 phosphatidylcholine (PC) (17:2/18:3) was enriched in the outer borders of granulomas but
300 evenly distributed in samples from both controls (SFig. 4F). The distribution of other lipid
301 species, like lysophosphatidylethanolamines (LPE), phosphatidylethanolamines (PE), and
302 Ceramides (Cer) are shown in SFig. 5 and here exemplarily demonstrated as overlay of different
303 lipid species in SFigs. 6 and 7 (please note, that the colors depicts different lipid species in
304 SFigs. 6 and 7, while the color encodes for the concentration of one target molecule in Fig. 1B
305 and SFigs. 1-2 and SFigs. 4-5). While the dimethyl-phosphatidylethanolamine
306 DMPE(18:3_20:4) (SFig. 6B and in SFig. 6E in blue) was depleted in the granulomatous area
307 (dashed lines), the phosphatidylcholine lipid PC(15:0_22:6) was enriched (in red in SFig. 6C
308 and E, red arrowheads). Granulomas and surrounding tissues were differentiated, and a
309 substructure was detected within the granuloma. Plasmeyl-PE(O-18:0_20:4) (green arrows)

was only found in the outer regions of the granulomas. All lipids detected in this region were identified as plasmalogens. Further phosphatidylinositol lipid PI(18:0_22:4) (SFig. 7A, red arrowheads) was enriched in distinct parts of granulomas while plasmenyl-PE (P-16:0_18:1) (green arrows) was found in the center and around the periphery of the granulomas (SFig. 7). Next, we quantified spatioregionally occurring lipids by HPTLC–FLD using the primuline reagent. The chromatogram and the respective densitogram of the non-polar lipids (SFig. 8A/B) showed well-separated blue fluorescence of cholesterol (hR_F 13), oleic acid (hR_F 22), triolein (hR_F 45) and cholesteryl oleate (hR_F 79). A reagent sequence of: first of primuline and then phosphomolybdic acid on the same plate resulted in dark green lipid zones (SFig. 9). The tentative assignment of the different lipid species (based on hR_F and reference substances) was confirmed by HPTLC–HRMS, exemplarily shown for the liver samples of *S. mansoni* ms- and bs-infected hamsters (SFig. 10, track 5 versus 15). Here, we unambiguously confirmed the presence of oleic acid (m/z 281.2486), triolein (m/z 907.7727) and cholesteryl oleate (m/z 673.5896) (SFig. 10A/B and D–G). Oleic acid, stearic acid (m/z 283.2642), linoleic acid (m/z 279.2329), and linolenic acid (m/z 277.2171) were present in liver samples of ms-infected hamsters (SFig. 10D). Cholesterol (SFig. 10C) showed the mass signals of two oxidation products at m/z 425.3391 ($[M+O+Na]^+$) and 441.3340 ($[M+2O+Na]^+$), and only a very weak signal at m/z 409.3443 $[M+Na]^+$, all in accordance with the mass signals of liver samples of ms-infected hamsters. In ms-infected hamsters, triolein or similar triacylglycerides (TAGs) were absent (SFig. 10A/E). However, we confirmed a strongly fluorescing zone of cholesteryl oleate (SFig. 10A/F/G), which was absent in liver samples of bs-infected hamsters. The comparatively more complex mass spectra of phosphatidic acid, sphingomyelin, phosphatidylcholine, and phosphatidylethanolamine were assigned to each respective lipid class (SFig. 11), whose presence we confirmed in the samples, except for phosphatidic acid. The low phosphatidic acid content (SFig. 8B, weakly blue fluorescent zone at hR_F of 9) was most likely masked by the mass signals of saccharides (glucose) and amino acids (SFig. 9C),

336 whose coelution was proven by derivatization (SFig. 9B, red zone at hR_F 5 and SFig. 9C, blue-
337 grey zone at hR_F 6). The quantitative analysis of the eight lipid compounds in the 15 samples
338 (STable 1) showed good reproducibility (mean %RSD 7.9%, $n = 3$, ranged 0–29%). The
339 performance of the linear calibration lines was sufficient (coefficients of determination >0.997
340 and reproducibilities %RSD $<10\%$, $n = 3$). The quantitative results revealed enhanced hepatic
341 levels of sphingomyelins and oleic acid in bs-infected animals, while global hepatic levels of
342 PE, PC, triolein, and cholesterol showed no alteration (Fig. 1D and SFigs. 8–11).

343

344 ***S. mansoni* eggs exhaust hepatocellular neutral lipids**

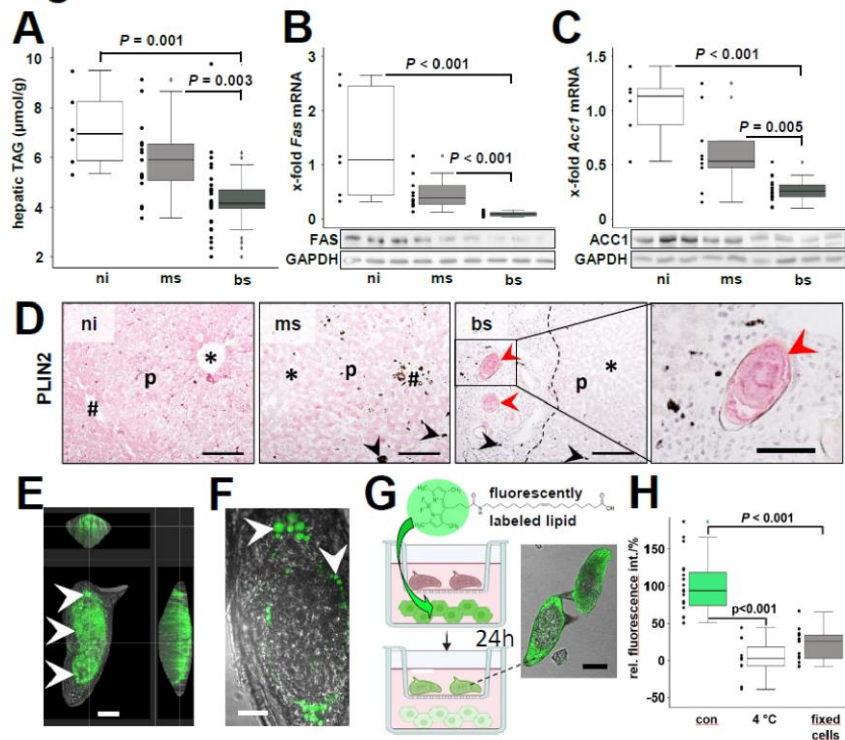
345 Next, we analyzed the concentration of TAGs in whole liver lysates of bs-, ms- and ni-hamsters
346 (Fig. 2A). In line with a significant reduction of whole hepatic TAGs in bs-samples compared
347 to ms and ni, we detected the corresponding downregulation of hepatic mRNA and protein
348 expression levels of rate-limiting enzymes for lipid synthesis, namely fatty acid synthase (FAS)
349 and acetyl-CoA-carboxylase (ACC1), in liver lysates of bs-infected hamsters (Fig. 2B and C).
350 Notably, the differences between ms-infected- and bs-infected hamsters underline that the
351 bisexual infection with egg-production caused pronounced effects (Fig. 2A-C).
352 Immunohistochemistry revealed that infection with *S. mansoni* reduced parenchymal FAS
353 expression with the exception of perigranulomatous hepatocytes (red arrow SFig. 12).

354

355 ***S. mansoni* eggs mobilize and take up lipids from hepatocytes**

356 Furthermore, we observed a strong reduction of PLIN2, a marker for neutral lipid storage, in
357 the liver parenchyma compared to ni- and ms-infected liver specimens (Fig 2D). Notably, eggs
358 were positively stained for PLIN2 (red arrowheads, Fig. 2D). The accumulation of neutral lipids
359 in the eggs was confirmed by Oil-Red staining (red arrowhead, SFig. 13). Please note that some
360 perigranulomatous hepatocytes were positive for Oil-Red staining (red arrow, SFig. 13).

361

Figure 2

362

363 Next, we investigated whether the eggs of *S. mansoni* were able to actively take up lipids. We
 364 found fLOA uptake by freshly isolated eggs using confocal live-cell imaging (suppl.
 365 Films 1 and 2) and confocal fluorescence microscopy (Fig. 2E-F, SFig. 14A-C). The uptake
 366 occurred rapidly in some living eggs, starting after 10 min (SFig. 14A/B). We also observed
 367 fLOA in fully developed miracidia (SFig. 14C). Next, we analyzed the uptake of fLOA into in
 368 vitro-laid eggs of different maturity over 18 hours (SFig. 15). Eggs incorporated lipid
 369 independent of their maturity stage. Depending on their maturity, however, lipids accumulated
 370 in distinct structures of the egg. Young eggs (SFig. 15A) incorporated fLOA into their vitelline
 371 cells. The oocyte remained negative. During embryo development, fLOA-positive vitelline-cell
 372 content was found inside the embryo as small granular structures as well as in the sub-shell area
 373 (SFig. 15B). When the miracidium matured, the sub-shell envelopes (Reynolds' layer and van-

Lichtenberg's envelope) took up fIOA (SFig. 15C). Additionally, we questioned whether eggs mobilize and take up lipids from human hepatocytes. As shown schematically in Fig. 2G, we co-cultured *in vitro*-laid eggs with HepG2 cells, which had been pretreated with fIOA (SFig. 16) 24 h prior to the coculture. Nearly all pre-matured *in vitro*-laid eggs took up fIOA from HepG2 in transwell cocultures (Fig. 2G) but also in direct cocultures (SFig. 14D-E). Based on a previous study design [36], we performed two additional control experiments and demonstrated that the fixation of fIOA-fed HepG2 cells or co-culturing of fIOA-fed HepG2 cells with *in vitro*-laid eggs at 4°C impaired fIOA uptake by the eggs, suggesting that we were measuring a process that requires an active cellular metabolism (Fig. 2H).

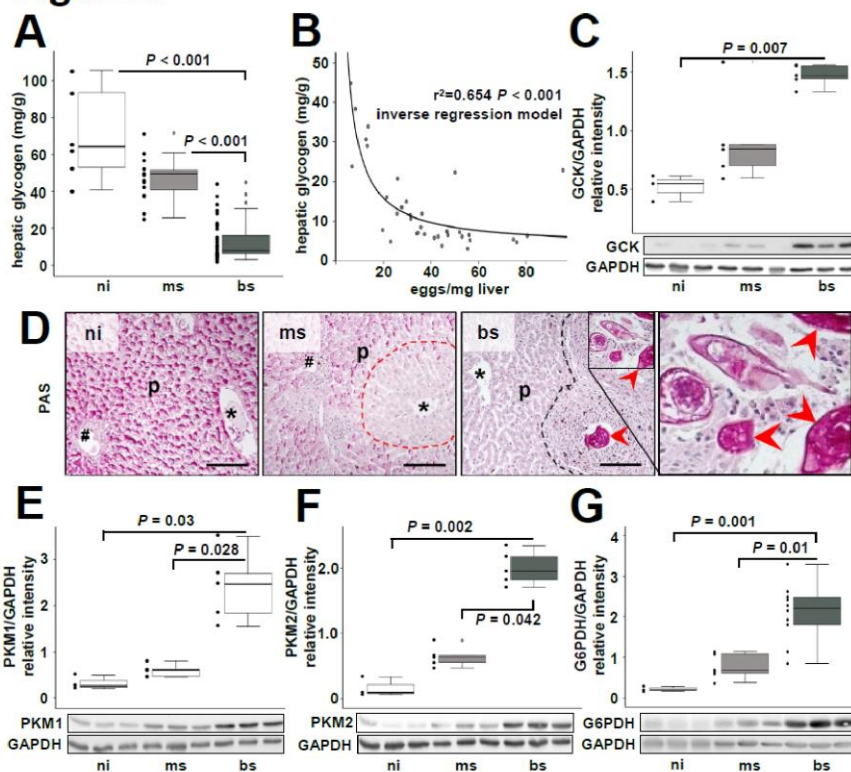
383

***S. mansoni* eggs dysregulate the carbohydrate-metabolism**

Next, we analyzed in detail how *S. mansoni* infection affects the hepatic carbohydrate metabolism of the host. As depicted in Fig. 3A, we found hepatic glycogen to be significantly reduced in livers of bs- compared to ms-infected and ni-hamsters. Furthermore, the amount of hepatic glycogen inversely correlated with the number of eggs per mg hamster liver (Fig. 3B). In line with glycogen exhaustion, we found a significant downregulation of rate-limiting enzymes responsible for the glycogen turnover such as glycogen synthase (GS) and glycogen phosphorylase (PYGL) in liver lysates of bs-infected hamsters (SFig. 17). Remarkably, hepatic protein levels of the rate-limiting glycolysis-initiating enzyme glucokinase (GCK) were significantly increased in the bs group (Fig. 3C). PAS staining visualized a deprivation of intracellular glycogen in parenchymal cells, while the eggs were strongly stained and thus appeared to be rich in glycogen (Fig. 3D). Western blot and immunohistochemistry demonstrated a significant increase of the hepatic protein levels of the glycolytic enzymes pyruvate kinase 1 and 2 (PKM 1 and 2), both in bs-infected animals (Fig. 3E/F). Notably, PKM1 and PKM2 occurred in different areas and cells in the livers of bs-infected hamsters, *i.e.* PKM1 in the hepatic parenchyma (SFig. 18A) and PKM2 in the granulomas (SFig. 18B). The

opposite regulation of elevated glycolysis and reduced glycogenesis rate-limiting enzymes was confirmed by stimulating human hepatoma cell lines with SEA (SFig. 19). Pyruvate dehydrogenase (PDH) catalyzes the conversion of pyruvate, the end-product of glycolysis, into acetyl-CoA, which links glycolysis and the citric acid cycle. Hepatic PDH was induced by *S. mansoni* infection (SFig. 20). In addition, we observed an elevation of glucose-6-phosphate dehydrogenase (G6PDH), which catalyzes the transition of glucose-6-phosphate into 6-phosphogluconate, the first step in the PPP (Fig. 3G, SFig. 21).

Figure 3



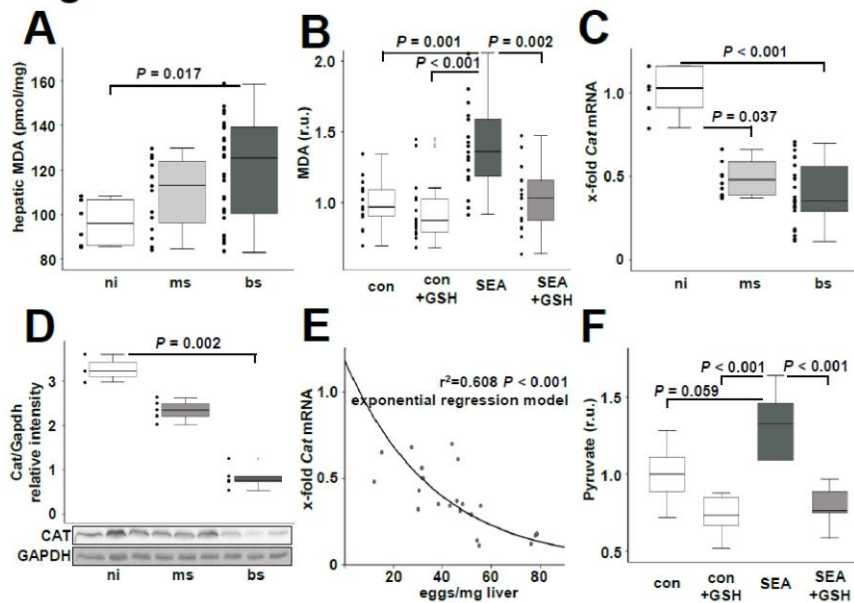
407

Finally, we wondered which evolutionary mechanisms might have promoted the ability of eggs to reprogram the host's metabolism in order to utilize the host's lipid and glucose reserves. This question led to the idea, that the hepatic effects might have their evolutionary origin in the egg's

translocation through the bowel wall into the gut lumen. Remarkably, we found a similar regulation of PKM2, GK2, and PEPCK2 expression in the colon of bs-infected hamsters (SFig. 22 A-D).

***S. mansoni* eggs induce oxidative stress**

G6PDH is a key regulator of the oxidative branch of the PPP and indispensable for maintaining the cytosolic pool of NADPH, thus being essential for the cellular redox balance [37]. We observed a significant augmentation of MDA (malondialdehyde), a secondary reaction product of lipid peroxidation, in livers of *S. mansoni*-infected hamsters (Fig. 4A). GSH (reduced L-glutathione) significantly decreased the SEA-induced MDA in human hepatoma cells (Fig. 4B). Catalase is one of the crucial antioxidant enzymes that mitigates oxidative stress by destroying cellular hydrogen peroxide to produce water and oxygen. We detected both, mRNA and protein levels of catalase significantly downregulated in hamster livers (Fig. 4C/D). The downregulated mRNA level of the host's catalase in the bs-group correlated with an increasing number of eggs in the liver (Fig. 4E). Notably, catalase mRNA levels also decreased by stimulation with soluble egg products in human hepatoma cells (SFig. 23).

Figure 4

427

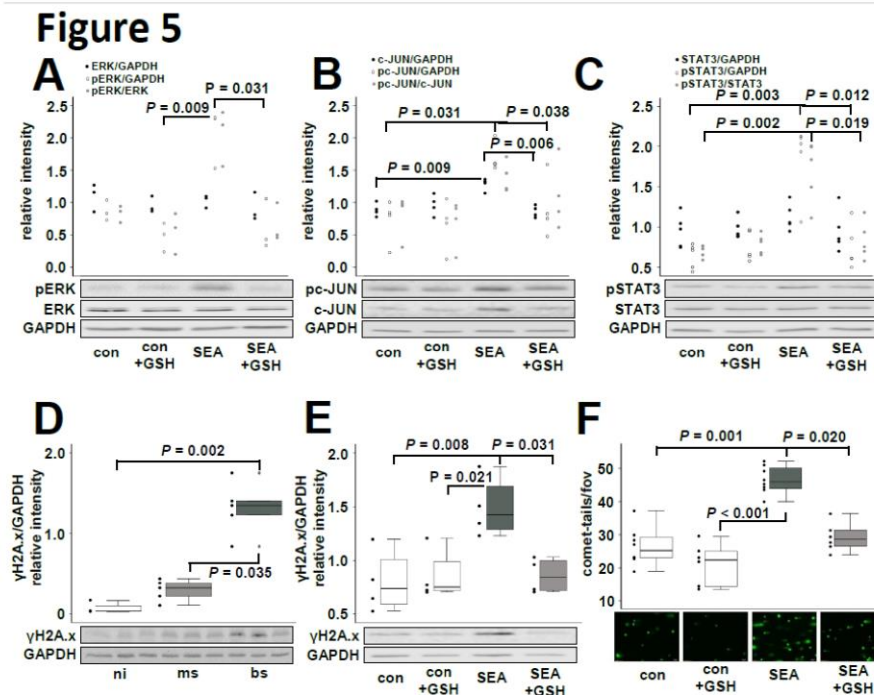
428 Surprisingly, the infection with *S. mansoni* increased hepatic mRNA levels of Mn^{2+} -dependent
 429 superoxide dismutase (*MnSod*), while *CuZnSod* mRNA levels decreased (SFig. 24A/B). In the
 430 bs group, glutathione peroxidase, *Gsh-Px*, was not regulated (SFig. 24C). SEA treatment
 431 induced the accumulation of pyruvate in HepG2 cells, which was reversed by GSH (Fig. 4F).
 432 Strikingly, also fIOA transfer from fIOA-fed HepG2 cells to eggs was reduced in the presence
 433 of GSH in co-cultures (SFig. 25). Accordingly, we found a similar regulation of *Gsh-Px*
 434 expression in the colon of bs-infected hamsters (SFig. 26).

435

436 *S. mansoni* egg-induced oxidative stress activates the protooncogen c-Jun and DNA 437 damage

438 We investigated whether the exhaustion of hepatocytes and the associated oxidative stress
 439 might trigger the activation of oncogenic signaling and DNA damage. In human hepatoma cells,
 440 GSH inhibited the egg product-induced activation of ERK, c-JUN, and STAT3 (Fig. 5 A-C).

441 In parallel, we observed significantly elevated protein expression levels of the DNA damage
 442 marker γ -H2A.x in bs-infected hamsters and in HepG2 cells following SEA treatment, which
 443 could be reversed *in vitro* by the ROS scavenger GSH (Fig. 5D/E).



444

445 In line with these findings, we demonstrated an SEA-induced DNA damage *in vitro* by comet
 446 assay. This effect was reversed by reduced GSH (Fig. 5F). As the antagonization of oxidative
 447 stress abolishes the SEA-induced c-Jun activation (Fig. 5B), we further analyzed whether the
 448 functional activation of AP1 promotor activity is influenced by GSH. The SEA induced
 449 functional activation of AP1 promotor activity was also reduced by GSH in the human colon
 450 cell line SW620 (SFig. 27).

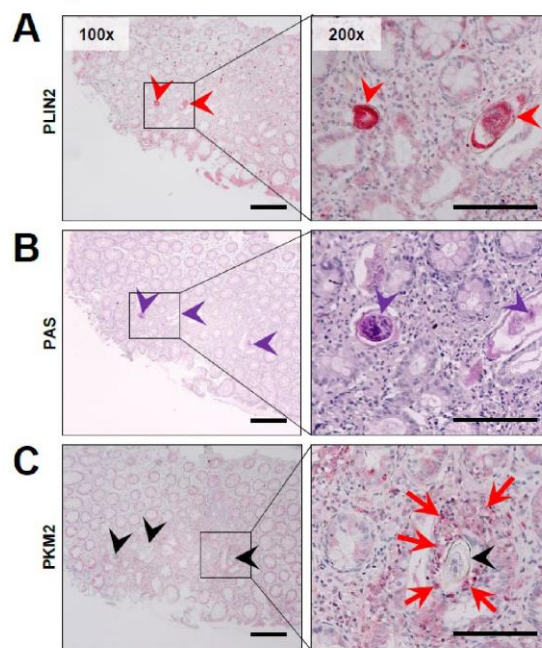
451

452 Proof of clinical relevance in human biopsies

453 In order to obtain a model-independent proof of the most important results from hamster and
 454 human cell lines, some major targets were visualized in histological slices colon biopsies that

455 were routinely taken for diagnosis from a 23 years old male patient suffering from
456 schistosomiasis (Fig. 6). *S. mansoni* eggs were positively stained for PLIN2 (Fig. 6A) and PAS
457 (Fig. 6B) indicating enhanced accumulation of neutral lipids (A) and glycogen (B) in eggs
458 passing the rectal wall. The staining for PKM2 demonstrated enhanced glycolysis in
459 inflammatory cells around the eggs (Fig. 6C). A visual comparison of PKM2 in infected vs
460 non-infected human tissue is depicted in SFig. 28.

Figure 6



461

Discussion

Genes encoding the beta-oxidation pathway are lacking in *S. mansoni* at any stage of its life cycle [35]. Therefore, *S. mansoni* may not be capable of catabolizing fatty acids for energy production. We demonstrated that eggs mobilized and took-up fLOA from well-differentiated human hepatoma cells. The development of larvae requires energy resources but also structural components for the synthesis of cell membranes like lipids that cannot be synthesized by the parasite itself. A single ovum in a newly laid egg does not have high nutritional requirements. After 2-3 days, however, the developing embryo will take more nutrients from its host environment. Moreover, we identified distinct phospholipid species that were enriched in hepatic eggs as previously proposed [35] but depleted in granulomas and the surrounding liver parenchyma. Furthermore, lipid mobilization from host cells requires an active cellular metabolism and can be suppressed by the addition of a ROS scavenger GSH. During the migration of eggs through host tissues the uptake of lipids and other molecules might be facilitated by cribriform-like pores inside the eggshell [38, 39].

Our data strengthen the idea that nutrient uptake from the host is a basic prerequisite for the increase of the eggs' biomass and dimensions during development [6, 40]. This may explain why we found exhausted hepatic glycogen stores in the presence of eggs. However, the glycogen content of eggs increases with the embryonic development and is highest at the miracidium stage [40]. It has been observed that *S. mansoni* eggs directly absorb glucose *in vitro* [35]. Acetyl-CoA and glycerol-3-phosphate, both products of the glycolytic breakdown, serve as components for the *de novo* fatty-acid synthesis [35]. It has been hypothesized that *S. mansoni* eggs store neutral lipids among others for developmental processes of miracidia, which require phospholipids e.g., for synthesizing new membranes [35].

In *S. japonicum*-infected mice, changes in gene expression of catabolism and anabolism in the liver were affected or could occur via the macrophage M2 phenotype state [11]. Our data however, clearly demonstrate that soluble egg products modulate the metabolic reprogramming

488 of the host's hepatic carbohydrate and lipid metabolism, independently of any immune
489 response. In line with our findings, maturation of *S. japonicum* eggs depends on cholesteryl
490 ester uptake from HDL and occurs via a CD36-related protein from the egg developmental
491 stages [41]. Thus, depletion of host lipids/glycogen storage during *S. mansoni* infection is likely
492 effected by the eggs itself.

493 Most of the factors we analyzed indicate that the hepatic metabolism of ms-infected hamsters
494 is different to that of bs-infected hamsters, which underlines the role of eggs for these processes.
495 Remarkably, some parameters like PDH or catalase expression are also different between ni-
496 and ms-infected animals, which presumably reflects the effects on hepatic metabolism and
497 oxidative stress that are even caused by adult worms alone. Nevertheless, biomolecular insights
498 into reprogramming of the host's metabolism by *S. mansoni* might also be important when
499 considering the two sides of the same coin – detrimental malnutrition of infected patients in
500 endemic countries versus beneficial anti-obesity effects that have been explored in the context
501 of obesity.

502 Metabolic dysregulation as well as DNA damage induced by *S. mansoni* SEA was restored by
503 the addition of a ROS scavenger *in vitro*. We demonstrated by *in vitro* assays that SEA is
504 capable of inducing oxidative stress, subsequently resulting in the activation of oncogenic
505 signaling and DNA damage. In addition, we found a metabolism-associated dysregulation of
506 the antioxidant system, which is in line with previous observations [14].

507 The phenomenon of exploitation the host's energy reserves constitutes a parasitic principle. In
508 the case of schistosome eggs, however, for the first time we describe metabolic deprivation and
509 effects on carbohydrate as well as lipid regulation. Nutrient deficiency resulted in oxidative
510 stress in the livers of bs-infected hamsters as well as in SEA-stimulated human hepatoma cells
511 *in vitro*. SEA significantly inhibited the expression of catalase *in vitro*, which inversely
512 correlated with elevated egg load *in vivo*. Furthermore, we demonstrated that oncogenic
513 signaling and DNA damage are consequences of egg-mediated metabolic deprivation, and that

514 the ensuing oxidative stress can be neutralized by the addition of the ROS scavenger GSH. Our
515 data suggest metabolic reprogramming of the host in order to acquire the hosts lipid and
516 carbohydrate reserves, absolutely essential for miracidial development within the egg.
517 In summary, our results shed new light on the schistosome parasite – host parenchyma
518 interaction, at the level of the egg stage. We demonstrate that the *S. mansoni* egg take advantage
519 of its host environment through metabolic reprogramming of hepatocytes and enterocytes. Eggs
520 ending up in the liver cause oxidative stress-induced- and malignancy-associated signaling as
521 well as DNA damage, which in combination might precondition or promote hepatocellular
522 damage.
523

524 **Abbreviations:** AKT Protein kinase B, AMPK AMP-activated protein kinase, DAG
 525 diacylglyceride, DMPE dimethyl-phosphatidylethanolamine, ERK extracellular-signal
 526 regulated kinases, G6PDH glucose-6-phosphate dehydrogenase, GAPDH glyceraldehyde 3-
 527 phosphate dehydrogenase, GCK glucokinase, GSH reduced L-glutathione, GS glycogen
 528 synthase, H2A.x H2a histone family member x, HCC hepatocellular carcinoma, hR_F retention
 529 factor * 100, IPSE IL-4-inducing principle from *Schistosoma mansoni* eggs, JNK c-JUN N-
 530 terminal kinase, MALDI matrix assisted laser desorption ionization, MDA malondialdehyde,
 531 MSI MALDI-MS imaging, OA oleic acid, PARP poly(ADP-ribose) polymerase, PC
 532 phosphatidylcholine, PDH pyruvate dehydrogenase, PE phosphatidylethanolamine, PI
 533 phosphatidylinositol, PKM pyruvate kinase in mammals, PLIN2 perilipin 2, PPP pentose
 534 phosphate pathway, PYGL hepatic glycogen phosphorylase, ROS reactive oxygen species,
 535 SEA soluble egg antigens, *S. japonicum* *Schistosoma japonicum*, SM sphingomyelin,
 536 *S. mansoni* *Schistosoma mansoni*, SP600125 JNK inhibitor, STAT3 signal transducers and
 537 activators of transcription 3, TAG triacylglyceride, Th1 T-helper type 1, TORC1 target of
 538 rapamycin kinase complex I, WNT wingless and Int-1
 539

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 544

545 **Figure Legends:**

546 **Fig. 1 Infection with *S. mansoni* induces alterations in the composition and distribution of**
 547 **hepatic lipids. (A)** Infection of hamster with *S. mansoni* cercariae of both sexes (bisex, bs
 548 infection) or with clonal cercariae of one sex (monosex, ms infection) in order to compare egg-
 549 induced vs worm-only effects. The distribution of lipids was analyzed by MALDI-MS imaging
 550 in three biological replicates of bs-infected- and ms-infected hamster, and ni (non infected)
 551 control cryosections, respectively. **(B)** *S. mansoni* infection causes an accumulation of
 552 triacylglycerides in the granuloma area with highest levels inside the eggs (arrowheads).
 553 Triacylglycerides were depleted in the surrounding liver tissue compared to ni controls. The
 554 panels depict the distribution of TG(16:0_18:1_18:2)+K, m/z 895.714876 in the liver of bs-
 555 infected-hamster and ni controls. **(C)** The distribution of lipid species differs characteristically
 556 in eggs, granuloma, and the surrounding of granulomas. Upper left: Brightfield image. Middle
 557 left: Dimethylphosphatidylethanolamine DMPE (18:2/22:6) was found depleted in granulomas
 558 and enriched in eggs. Lower left: Dimethylphosphatidylethanolamine DMPE (18:0/22:5) was
 559 also detected with higher intensity in the eggs, but no depletion was observed for the granuloma
 560 regions. Upper right: DMPE (18:0/22:4) was mainly found in granulomas with slight
 561 enrichments in eggs. Middle right: DMPE (15:0/18:2) was found depleted in some part of
 562 granulomatous regions. Lower right: phosphatidylcholine m/z 810.532050 PC(17:2_18:3)
 563 [M+HCO₂]⁺ was found enriched in the outer borders of granulomas. A second set of MSI-
 564 pictures demonstrating altered lipid distribution in *S. mansoni*-infected hamsters is depicted in
 565 SFig. 5. These experiments were performed at least three times independently. **(D)** The
 566 quantification of selected lipid species revealed enhanced hepatic levels of SM and oleic acid
 567 (OA) in bs infected animals. TO triolein, Chol cholesterol, ni non-infected, ms monosex-
 568 infected, bs bisexual-infected hamsters. These experiments were performed at least three times

569 independently. Levels of significance are indicated in the figure (Kruskal-Wallis-test).

570

571 **Fig. 2 *S. mansoni* infection exhausts the host's hepatic neutral lipid depots while parasite**

572 **eggs accumulate lipids. (A)** Hepatic TAG content was reduced in bs-infected hamsters. **(B and**

573 **C)** Hepatic mRNA and protein levels of fatty acid synthase (*Fas*) and acetyl-CoA-carboxylase 1

574 (*Acc1*), both rate limiting enzymes for lipid synthesis, were reduced in bs-infected hamsters.

575 Representative western blots are depicted. ni non-infected, ms monosex-infected, bs bisex-

576 infected hamsters. **(D)** PLIN2-staining visualized the reduction of neutral lipids in liver

577 parenchyma (p) of bisex-infected (bs) hamsters and the accumulation of neutral lipids in

578 *S. mansoni* eggs (red arrowheads). Black arrowheads depict *S. mansoni*-infection-specific

579 hepatic hemosiderin deposits, *central vein, #portal tract, 200 x, bar 100 µm, black dotted line

580 indicates granuloma. **(E and F)** Confocal microscopy clearly demonstrated the sites of lipid

581 accumulation (white arrowheads) inside the eggs (liver) cultured with fluorescently labeled

582 oleic acid (fLOA, in green), bars 20 µm **(E)** and 10 µm **(F)**. A live video showing the active

583 uptake of fLOA by an egg (liver) was deposited online ([suppl. Film 1](#)) and also a 3D video

584 demonstrating the lipid distribution inside liver-extracted eggs can be found online ([suppl.](#)

585 [Film 2](#)). **(G)** To prove if *S. mansoni* eggs mobilize and take up lipids from hepatocytes, HepG2

586 cells were fed with fLOA, washed three times and subsequently co-cultivated with eggs in a

587 transwell system. Around 3% of the eggs freshly isolated from the host liver took up fLOA from

588 HepG2 cells after 24 h of co-culture. The workflow of the experiment depicted schematically

589 (left panel) and representative pictures of a liver-derived egg that took up fluorescently labeled

590 oleic acid (bar 25 µm, right panel). Remarkably, nearly 100% of pre-matured *in vitro* laid eggs

591 took up fLOA from HepG2 cells. These experiments were performed at least three times

592 independently. **(H)** CLSM-based quantification of fluorescence intensity of individual eggs

593 from coculture performed at 4 °C (white bar) or with 4% PFA fixed HepG2 cells (grey bar) in

594 comparison to the conventional experiment as a control (con, green bar). At least 10 eggs per
595 condition were analyzed in each of two independent experiments. Levels of significance are
596 indicated in the figure (Kruskal-Wallis-test).

597

598 **Fig. 3 *S. mansoni* infection exhausts the host's hepatic carbohydrate storage.** (A) The
599 hepatic glycogen content was diminished approximately 6-fold in livers of bs-infected
600 hamsters. (B) Hepatic glycogen content inversely correlated with the number of eggs per mg
601 liver tissue. (C) Glucokinase, the first rate limiting enzyme of glycolysis, was upregulated in
602 livers of bs-infected hamsters. A representative western blot is shown. (D) PAS staining of
603 histologic liver sections revealed homogenous hepatic glycogen storage in ni animals (left
604 panel), zoned glycogen storage in livers of ms-infected hamsters with a reduction of glycogen
605 deposits around the central veins* (dotted red line indicates zonation), as well as a complete
606 absence of glycogen in the liver parenchyma (p) of bs-infected hamsters, while eggs were
607 strongly positive stained (red arrowheads). *central vein, #portal tract, 200 x, bar 100 μ m, black
608 dotted line indicates granuloma. (E and F) Western blot analysis demonstrated the induction
609 of rate-limiting enzymes of glycolysis like PKM1 (E) and PKM2 (F) in livers of bs-infected
610 hamsters. (G) G6PDH, the rate-limiting enzyme of the PPP of glycolysis, was also upregulated
611 in livers of bs-infected hamsters. $n=3-5$, ni non-infected, ms monosex-infected, bs bisex-
612 infected hamsters. These experiments were performed at least three times independently.
613 Levels of significance are indicated in the figure (Kruskal-Wallis-test and non-linear
614 regression).

615

616 **Fig. 4 *S. mansoni* egg-induced hepatocellular oxidative stress.** (A) Hepatic MDA levels
617 were increased in hamster liver upon bs infection. (B) SEA stimulation induced MDA in HepG2
618 cells. The induction was abolished by the addition of reduction equivalents in form of GSH

619 (n=6). (C and D) Hepatic catalase expression is reduced in hamster liver of the bs group (C,
620 mRNA, D, protein level). (E) Hepatic catalase mRNA levels and the number of eggs per mg
621 liver tissue inversely correlated with exponential trend. Data were normalized to the control
622 group. (F) Pyruvate quantification demonstrated SEA-induced glycolysis in HepG2 cells,
623 which was reduced to unstimulated levels by the addition of reduction equivalents in form of
624 GSH. The experiment was repeated three times. Data were normalized to the control group.
625 Kruskal-Wallis test was performed to asses group differences. ni non-infected, ms monosex-
626 infected, bs bisex-infected hamsters. These experiments were performed at least three times
627 independently. Levels of significance are indicated in the figure (Kruskal-Wallis-test).

628

629 **Fig. 5 *S. mansoni* eggs induce malignant signaling and oxidative stress-dependent DNA**
630 **damage in HepG2 cells.** (A-C) Western blot analyses revealed the activation of ERK, c-JUN,
631 and STAT3 in SEA-stimulated HepG2 cells. The addition of GSH diminished SEA-induced
632 HCC-associated signaling to control levels. Representative western blots are depicted. ni non-
633 infected, ms monosex-infected, bs bisex-infected hamsters. (D) γ H2A.x, a marker for DNA
634 double strand breaks, is induced in the bs group. (E) Stimulation with SEA induced γ H2A.x,
635 while the addition of GSH abolished this effect. (F) Comet assay demonstrated that SEA-
636 induced DNA damage in HepG2 cells, and that DNA damage was reduced by addition of GSH.
637 Kruskal-Wallis test was performed to asses group differences. These experiments were
638 performed at least three times independently. Levels of significance are indicated in the figure
639 (Kruskal-Wallis-test).

640

641 **Fig. 6 Validation of metabolic implications with tissue samples of a patient infected with**
642 ***S. mansoni*.** (A) Histologic specimen of a colon biopsy from a 23 year old patient with
643 schistosomiasis was stained for PLIN2. The signal for PLIN2 indicated an accumulation of
644 neutral lipids inside the eggs (red arrowheads). (B) The positive PAS reaction demonstrated an

645 accumulation of glycogen in most of the eggs passing the bowel wall (purple arrowheads). (C)
646 Enhanced staining for PKM2 (red arrowheads) in granulomatous infiltrates around the eggs
647 (black arrowhead). Representative stainings are depicted, bars 100 (right) and 200 μ m (left).

648

649 **Graphical abstract.** Following *S. mansoni* infection (1), the worm couples start egg production
650 in the host (2). A proportion of eggs get trapped in the liver leading to granuloma formation (3).
651 Secretion of soluble egg products (4) induce lipid and carbohydrate exhaustion, while
652 metabolites concentrate in perigranulomatous hepatocytes. These metabolic changes are
653 accompanied by metabolic stress in the host parenchyma (5). Mobilized lipids and probably
654 carbohydrates are transferred from the host parenchyma to the eggs (6). Developing eggs rely
655 on the host for the bulk of their nutrient needs for embryogenesis, and very likely for their
656 energetics as well. The metabolic deprivation goes along with dysregulation of the energy
657 metabolism inducing oxidative stress (7). As one consequence, oxidative stress activates
658 oncogenic signaling (8) and finally induces DNA damage (9). Created with BioRender.com

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660 **Author names in bold designate shared co-first authorship**

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775

1 **Supplementary Materials**

2

3 **Metabolic reprogramming of hepatocytes by *Schistosoma mansoni* eggs**

4

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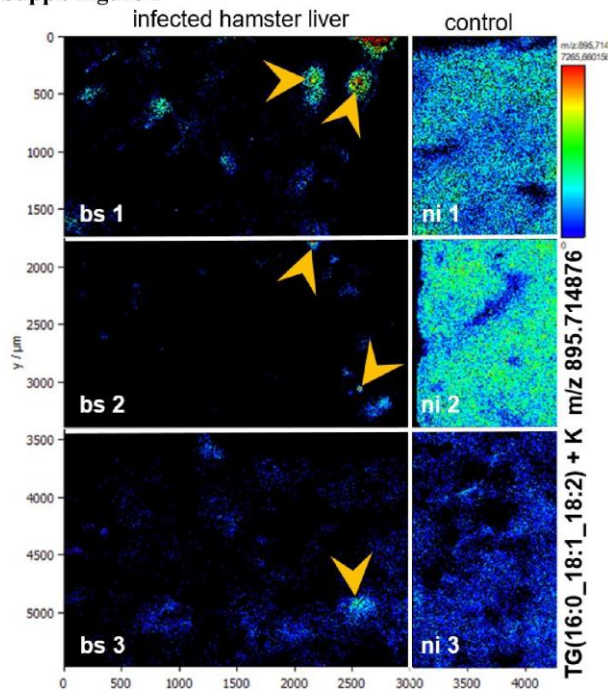
11

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49	and GK2.
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52	markers.
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54	coculture.
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56	P30: SFig. 27: SEA activated promotor activity was reduced by GSH.
57	P31: SFig. 28: Visual comparison of PKM2 in infected vs non-infected tissue.
58	P32-34: Supplementary CTAT Tables
59	P35: STable 1: Quantification results and reproducibilities determined via HPTLC-FLD.

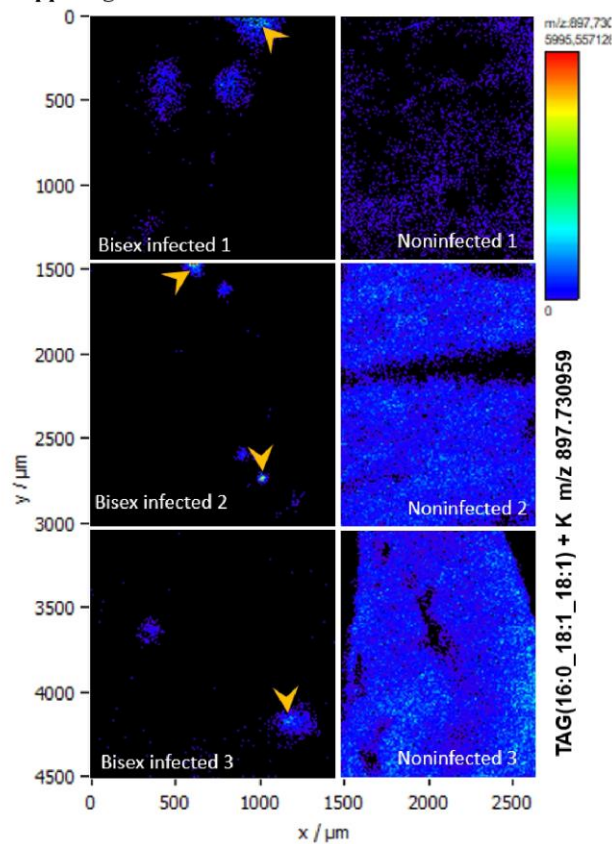
60 Detailed description of MALDI MSI analysis and identification by LC-MS/MS

61 For matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI MSI), an
62 ultrafine pneumatic sprayer (SMALDIPrep, TransMIT, Giessen, Germany) was used to apply
63 matrix on cryosections of 20 μm thickness. 2,5-dihydroxybenzoic acid (DHB) (30 mg/mL in
64 H_2O /acetone (1:1, V/V) with 0.1 vol-% trifluoroacetic acid in the total volume, 100 μL , 10
65 $\mu\text{L}/\text{min}$) and 1,5-diaminonaphthalene (DAN) (3.3 mg/mL in H_2O /Methanol (1:9, V/V), 400 μL ,
66 30 $\mu\text{L}/\text{min}$) were used as matrices for positive- and negative-ion mode, respectively. A high-
67 resolution atmospheric-pressure MALDI imaging ion source (AP-SMALDI5 AF, TransMIT)
68 was coupled to a Q Exactive HF orbital trapping mass spectrometer (Thermo Fisher Scientific).
69 Lipid extractions of liver homogenates were performed for each sample group as described by
70 [Garikapati et al., in revision Analytical and Bioanalytical Chemistry]. For LC-MS/MS, a
71 reversed-phase 1.8 μm column (100 X 2.1 mm, ACQUITY UPLC HSS T3, Waters, Eschborn,
72 Germany) on a UHPLC system (Ultimate 3000 UHPLC, Thermo Fisher Scientific, Dreieich,
73 Germany) was coupled to a Q Exactive HF-X orbital trapping mass spectrometer (Thermo
74 Fisher Scientific, Bremen, Germany). LC gradient and MS instrument settings are described in
75 detail elsewhere [Wiedemann et al., in revision Analytical and Bioanalytical Chemistry].
76 For annotation of lipid species, "Lipid Match Flow" was used and statistical analysis was
77 performed using "Perseus". Comparing bs-infected and ms-infected, respectively, with ni
78 control samples, ANOVA tests with permutation-based false discovery rate followed by
79 posthoc tests were performed, in order to determine infection markers. Resulting marker lists
80 were further used to generate ion images of the MALDI MSI data using the software "Mirion".

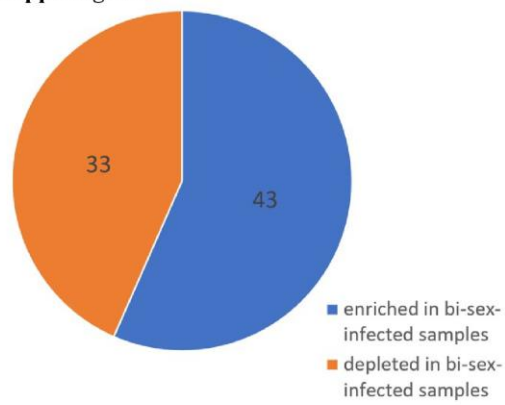
81

82 **Suppl. Figure 1**

83
84 **Suppl. Figure 1: *S. mansoni* infection caused TAG accumulation in eggs and surrounding**
85 **granulomas** (arrowheads) and depletion in the non-affected tissue as it can be seen by
86 comparing bs-infected samples (left) to ni control samples (right), exemplarily shown for
87 TAG(16:0_16:1_18:1) $[C_{53}H_{98}O_6+K]^+$ at m/z 869.699079. A second set of MSI-pictures
88 showing the accumulation of another TAG-species in the eggs and granuloma is depicted in
89 SFig. 2.

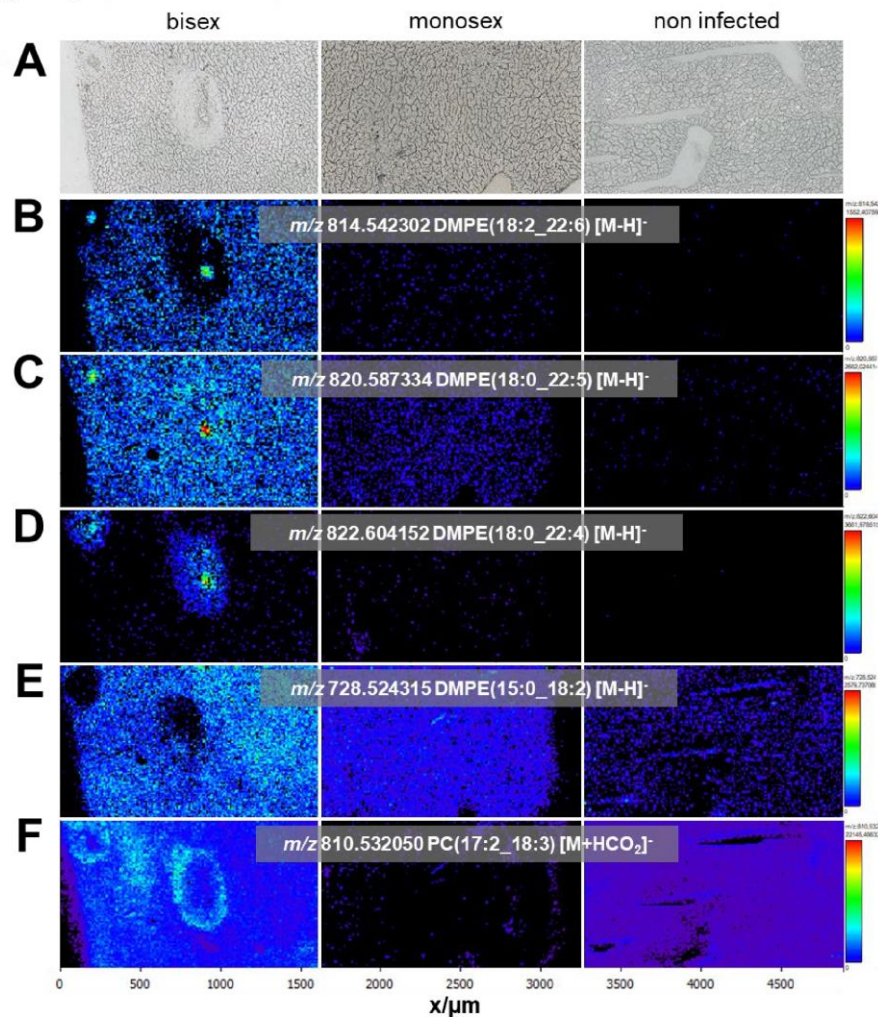
90 **Suppl. Figure 2**

91
 92 **Suppl. Figure 2: *S. mansoni* infection caused TAG accumulation in eggs and surrounding**
 93 **granulomas (arrowheads) and depletion in the non-affected tissue. Bisex-infected samples**
 94 **(left) to non-infected control samples (right), exemplarily shown for TAG(16:0_18:1_18:1)**
 95 **[C₅₅H₁₀₂O₆+K]⁺ at m/z 897.730959. MSI Mass spectrometry imaging, TAG triacylglyceride.**

96 **Suppl. Figure 3**

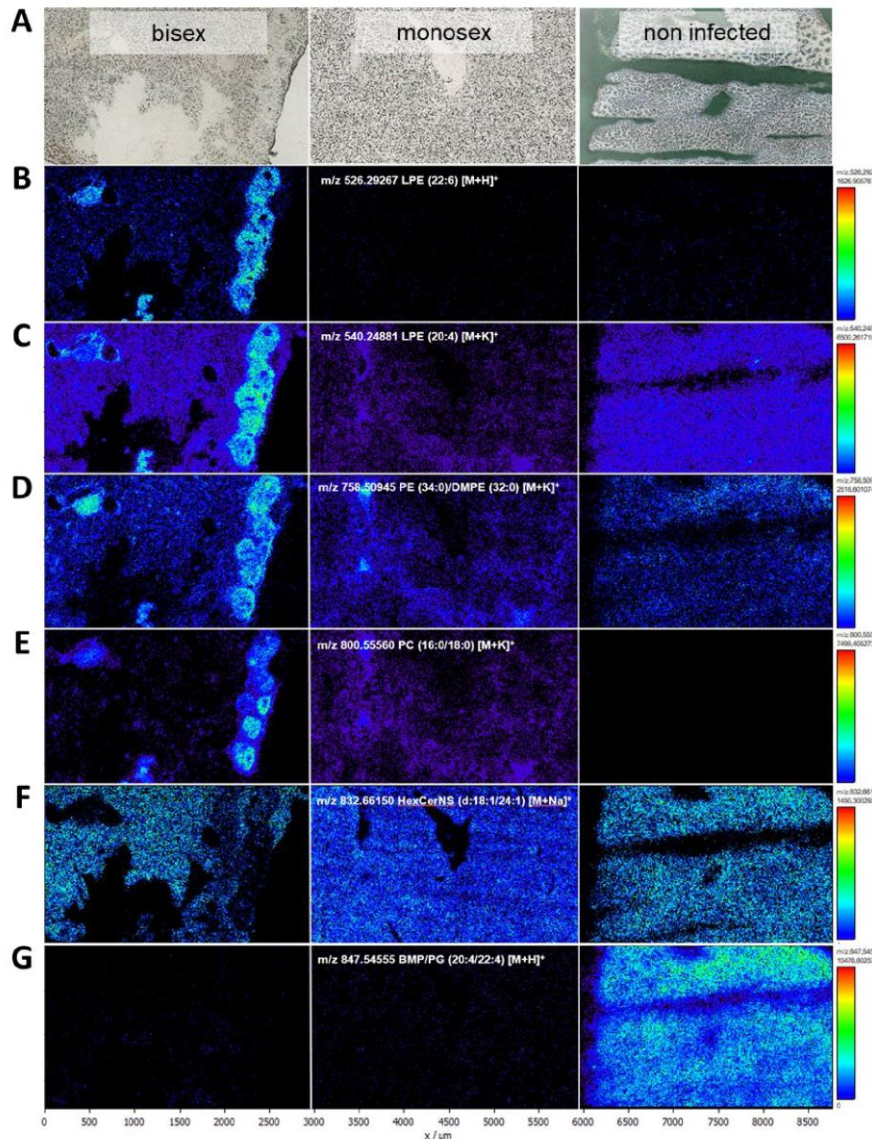
97
98 **Suppl. Figure 3: Numbers of enriched or depleted triglyceride signals, determined as**
99 **markers for infection.**

Suppl. Figure 4

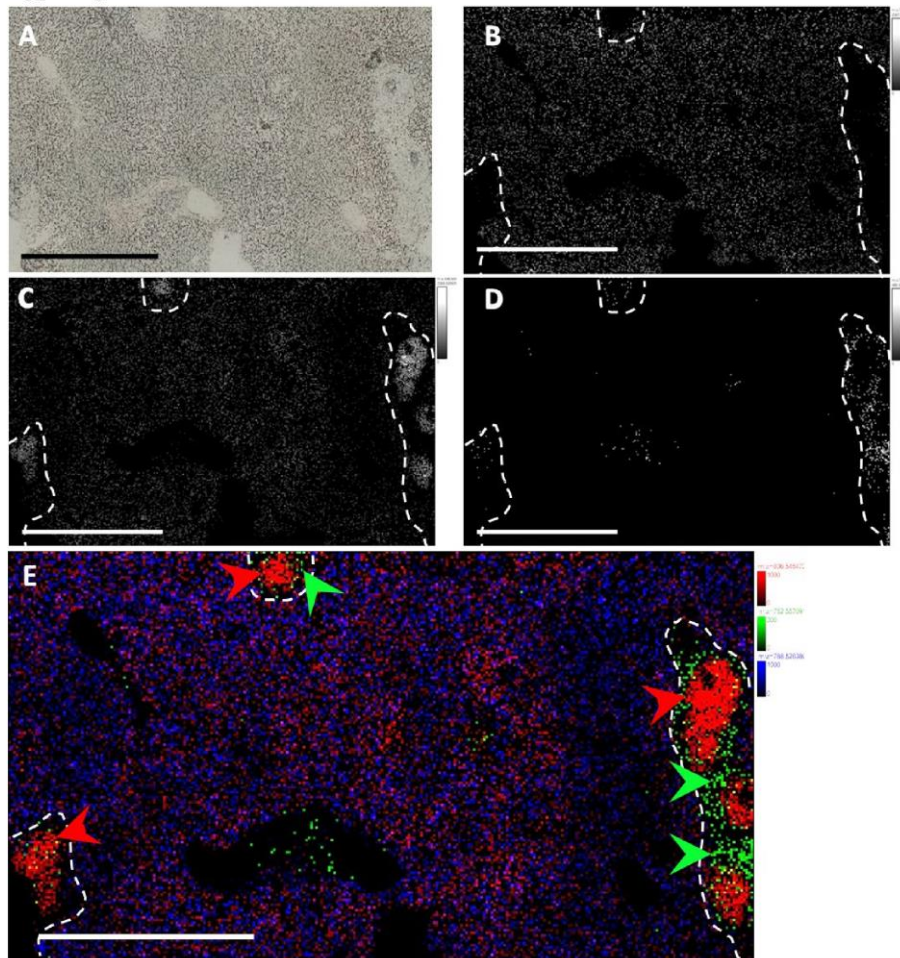


Suppl. Figure 4: Distribution of lipid species differs characteristically in bisex-, monosex-infected, and control samples. (A) Representative brightfield images. (B) DMPE (18:2/22:6) was found depleted in granulomas and enriched in eggs. (D) DMPE (18:0/22:4) was mainly found in granulomas with slight enrichments in eggs, but only rarely in control samples. (C) DMPE (18:0/22:5) was also detected with higher intensities in the eggs, but no depletion was observed for the granuloma regions. (E) DMPE (15:0/18:2) was found evenly distributed in all samples despite the granulomatous regions in bs-infected samples. (F) PC (17:2/18:3) was found enriched in the outer borders of granulomas. A second set of MSI-pictures demonstrating altered lipid distribution in *S. mansoni*-infected mice is depicted in SFig. 5. These experiments were performed at least three times independently.

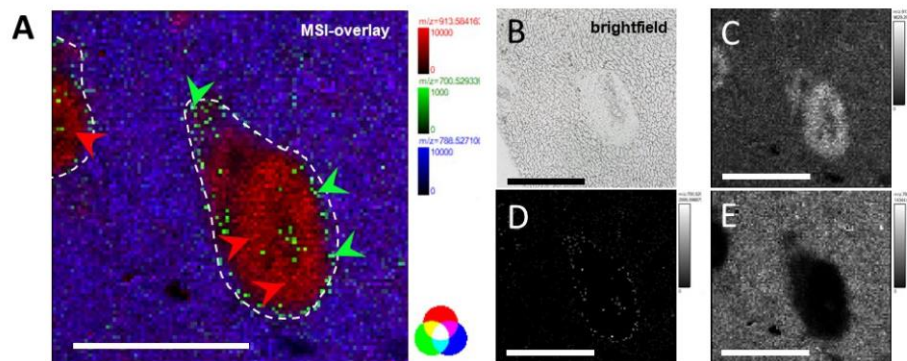
113 Suppl. Figure 5



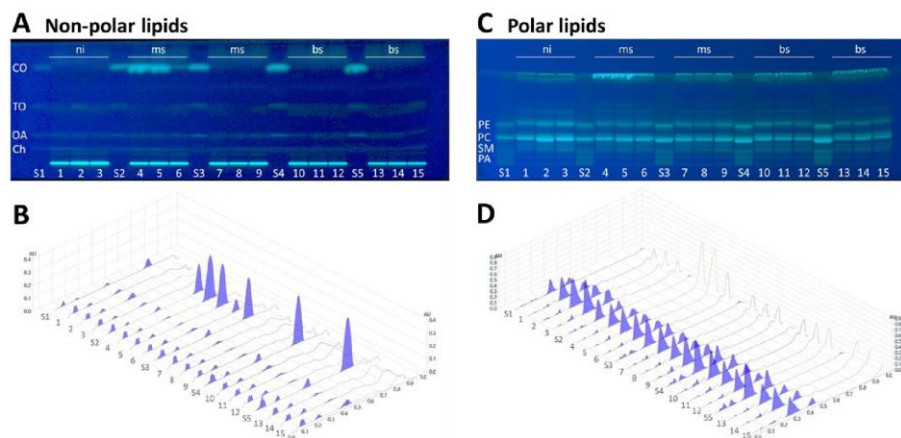
114
 115 **Suppl. Figure 5: Distributions of lipid species differ characteristically in bisex-, monosex-**
 116 **infected, and control samples.** (A) Representative brightfield images. (B) LPE(22:6) was
 117 found only in bisex-infected samples and enriched in granulomas. (C) LPE(20:4) was found in
 118 all samples, but again, enrichment in granulomas was observed. (D) A PE or DMPE lipid
 119 species was detected in all samples (E) PC(16:0/ 18:0) was detected in monosex- and bisex-
 120 infected samples. Signal intensity in control samples was negligible. (F) Ceramide species
 121 HexCerNS(d18:1/24:1) was detected in all samples, with lower signal intensities in
 122 granulomatous areas. (G) This BMP or PG lipid was detected only in control samples.
 123

124 **Suppl. Figure 6**

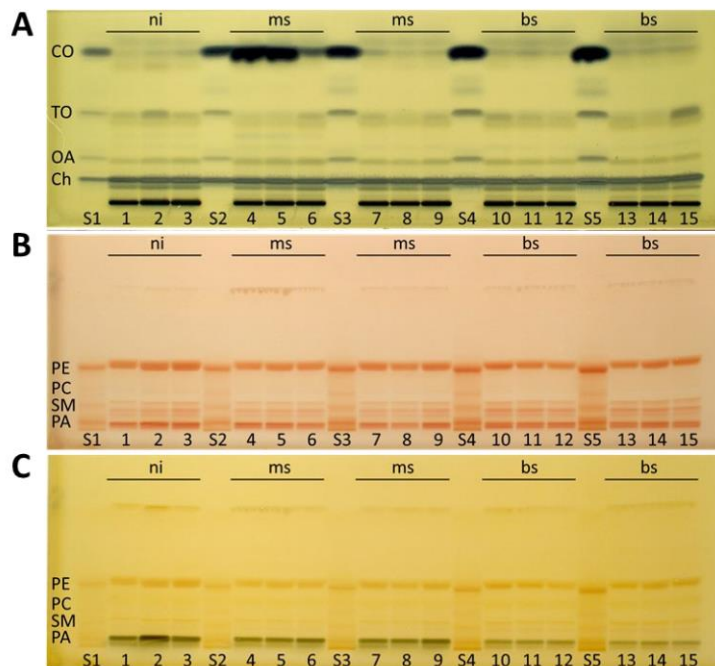
125 **Suppl. Figure 6: Individual MSI-scans of distinct lipids and overlay.** (A) Representative
 126 brightfield picture of the area of interest in the liver of a bisex-infected hamster. (B)-(D)
 127 Representative MSI scans at 10 μm pixel size visualize altered ion distributions of (B) *m/z*
 128 788.528381, identified as N,N-dimethyl phosphatidylethanolamine DMPE(18:3_20:4)
 129 $[C_{45}H_{76}NO_8P-H]^-$, (C) *m/z* 836.545472, identified as phosphatidylcholine PC(15:0_22:6)
 130 $[C_{45}H_{78}NO_8P+HCO_2]^-$, and (D) *m/z* 752.557091, identified as phosphatidylethanolamine
 131 plasmaenyl-PE(O-18:0_20:4) $[C_{43}H_{80}NO_7P-H]^-$. Dashed lines indicate granulomas. Bars
 132 500 μm. (E) Overlay of individual MSI scans for DMPE(18:3_20:4) (blue), PC(15:0_22:6)
 133 (red), and plasmaenyl-PE(O-18:0_20:4) (green). A second example of a representative MSI-scan
 134 focussing on a granuloma is depicted in SFig. 2.
 135
 136

137 **Suppl. Figure 7**

138 **Suppl. Figure 7: *S. mansoni* infection caused an altered distribution of hepatic lipids.** MSI
 139 overlay (A), brightfield (B), and individual MSI-scans (C-E) of distinct lipids. (B)
 140 Representative brightfield picture of the area of interest in the liver of a bisex-infected hamster.
 141 (C)-(E) MSI scans of ions representing distinct lipids (C) m/z 913.584163 (red) PI(18:0_22:4),
 142 [M-H]⁻ (D) m/z 700.529339 (green) plasmalogen-PE(P-16:0/18:1) [M-H]⁻ (E) m/z 788.527100
 143 (blue) DMPE(18:3_20:4) [M-H]⁻. Dashed lines indicate granulomas. Scale bars 500 μm, MSI
 144 Mass spectrometry imaging, DMPE dimethyl-phosphatidylethanolamine, PE
 145 phosphatidylethanolamine, PI phosphatidylinositol.
 146
 147
 148

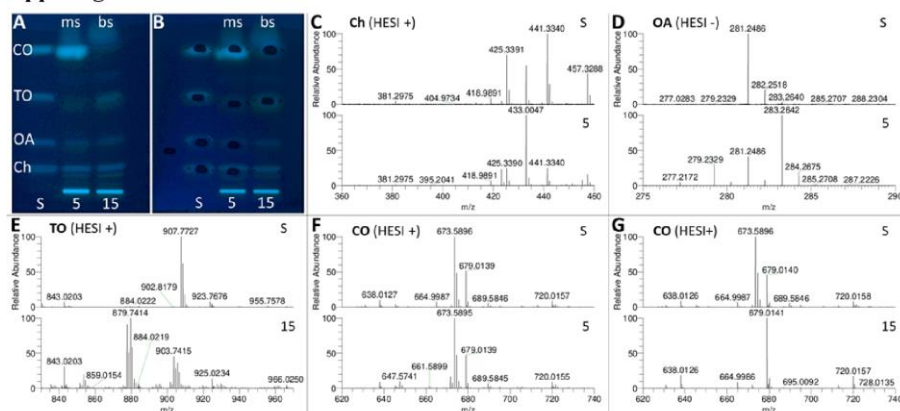
149 **Suppl. Figure 8**

150 **Suppl. Figure 8: Quantification of non-polar and polar lipids in liver samples by HPTLC-**
 151 **FLD.** Chromatograms (A/C) and corresponding 3D densitograms (B/D) of sample tracks no.
 152 1–15 (ni = non-infected, ms = monosex-infected, bs = bisex-infected hamsters) and five
 153 calibration standard levels (S1–S5) of non-polar lipids (A/B: Ch= cholesterol, OA = oleic acid,
 154 TO = triolein, CO = cholesteryl oleate) and polar lipids (C/D; PA = phosphatidic acid, SM =
 155 sphingomyeline, PC = phosphatidylcholine, PE = phosphatidylethanolamine).
 156
 157

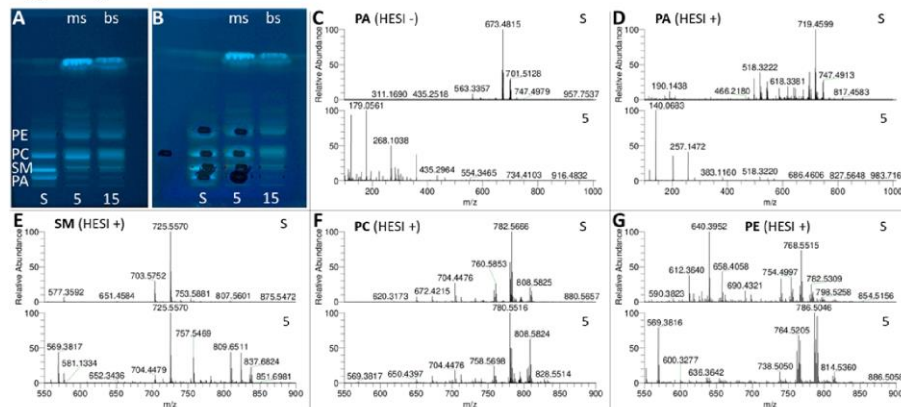
158 **Suppl. Figure 9**

159 **Suppl. Figure 9: Further characterization and detection of liver samples by HPTLC-Vis.**
 160 Chromatograms of non-polar (A; Ch = cholesterol, OA = oleic acid, TO = trioleine, CO =
 161 cholesteryl oleate) and polar lipids (B,C; PA = phosphatidic acid, SM = sphingomyeline, PC =
 162 phosphatidylcholine, PE = phosphatidylethanolamine) in sample tracks no. 1–15 (ni = non-
 163 infected, ms = monosex-infected, bs = bisex-infected hamsters) and five standard calibration
 164 levels (S1–S5). As reagent sequence, the primuline-treated plate was derivatized with
 165 phosphomolybdic acid (A), or for detection of amino acids or derivatives like PE and
 166 saccharides (glucose), with ninhydrin (B) and diphenylamine-aniline o-phosphoric acid reagents
 167 (C), respectively.
 168
 169

170 **Suppl. Figure 10**

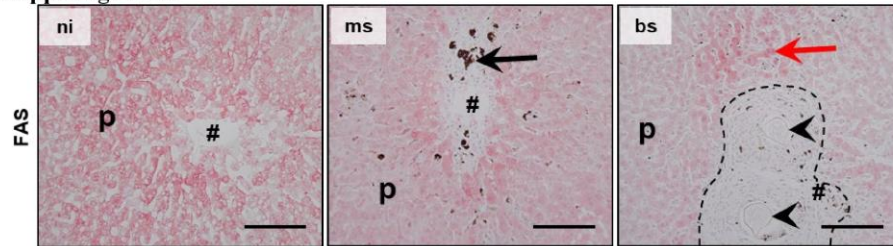


171 **Suppl. Figure 10: Confirmation of assigned non-polar lipids in liver samples by HPTLC-**
 172 **HRMS. HPTLC-FLD chromatograms before (A) and after (B) online elution of the zones of**
 173 **interest via TLC-MS Interface 2 into the HRMS. Mass spectra (C-G) of standard zones (S) and**
 174 **respective sample zones (ms = monosex-infected hamster on track no. 5 and bs = bisex-infected**
 175 **hamster on track no. 15).**
 176
 177

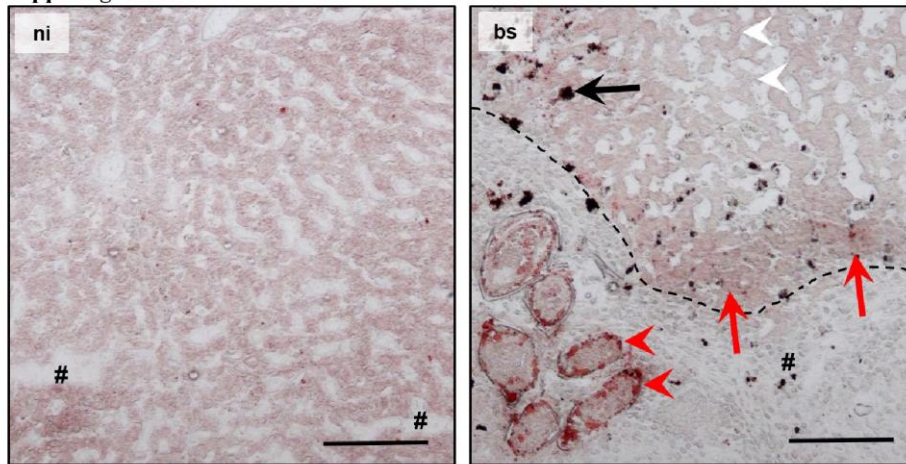
178 **Suppl. Figure 11**

179 **Suppl. Figure 11: Confirmation of assigned polar lipids in liver samples by HPTLC-**
180 **HRMS.** HPTLC-FLD chromatograms before **(A)** and after **(B)** online elution of the zones of
181 interest via TLC-MS Interface 2 into the HRMS. Mass spectra **(C-G)** of standard zones (S) and
182 respective sample zones (ms = monosex-infected hamster on track no. 5 and bs = bisex-infected
183 hamster on track no. 15).
184
185

186 Suppl. Figure 12

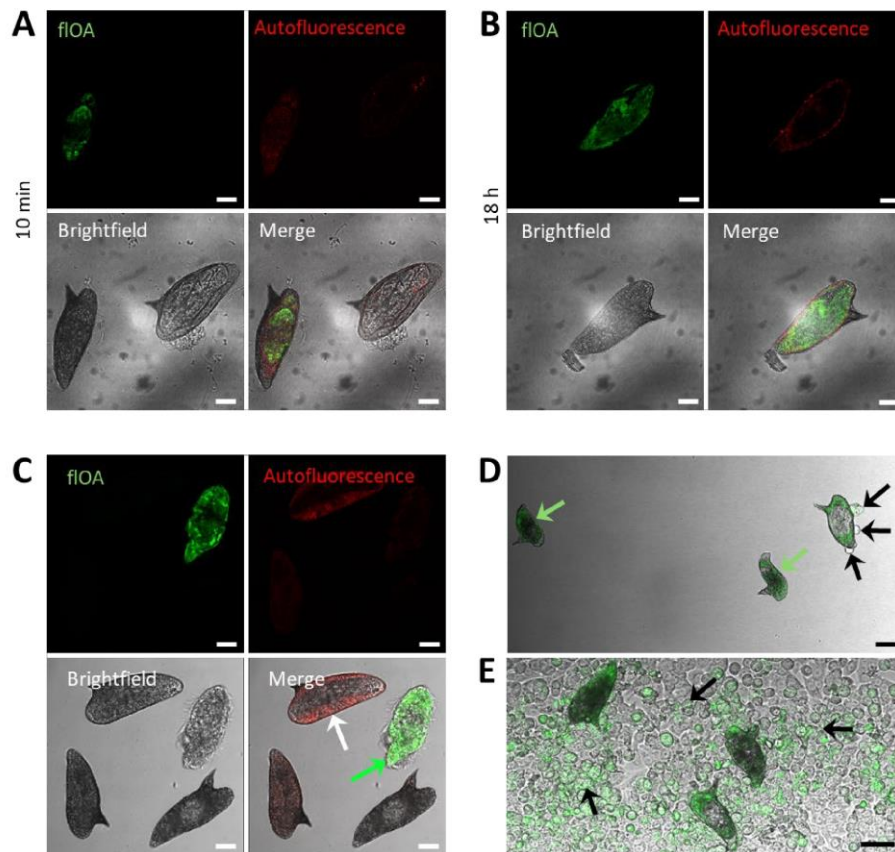


187
188 **Suppl. Figure 12: *S. mansoni* infection reduced parenchymal FAS expression with the**
189 **exception of perigranulomatous hepatocytes.** Fatty acid synthase (FAS) immunostaining
190 (red) in the liver of non-infected (ni), monosex-infected (ms), and bisex-infected (bs) hamsters.
191 Bar 100 μ m, p parenchyma, # portal field, ----- granuloma, arrowhead *S. mansoni* eggs, black
192 arrow hemosiderin deposits, red arrow FAS positive perigranulomatous hepatocytes.
193 Representative immunostainings are shown.
194

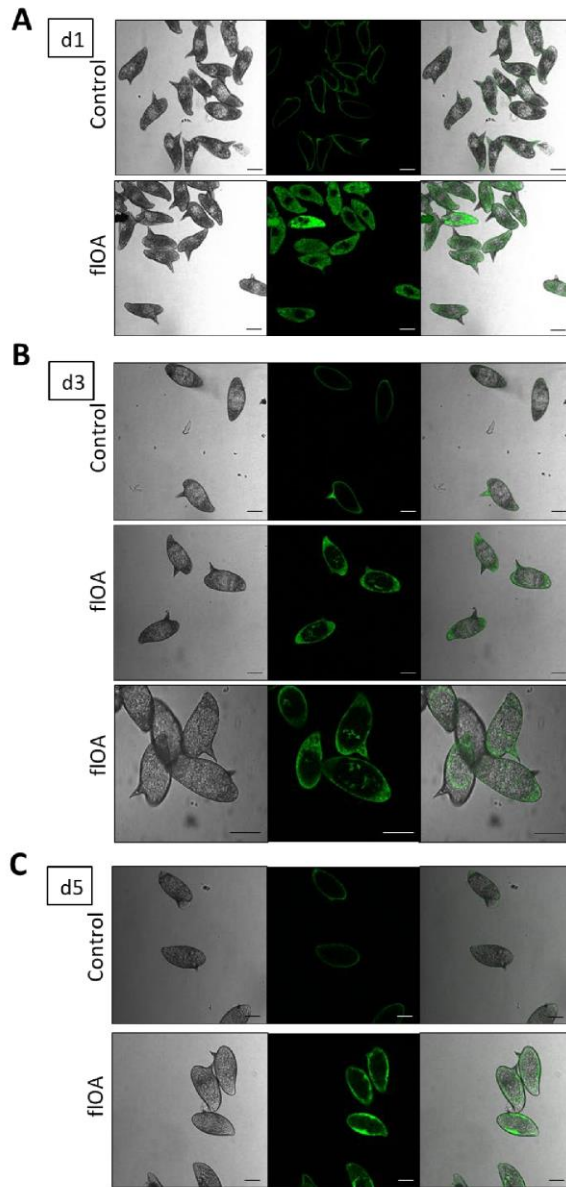
195 **Suppl. Figure 13**

196 **Suppl. Figure 13: *S. mansoni* infection reduced the accumulation of neutral lipids in**
197 **parenchyma with the exception of perigranulomatous hepatocytes.** OilRed staining of
198 neutral lipids on cryosections of livers from non infected and bisex infected hamsters. While
199 all hepatocytes in non infected hamsters were positively stained for neutral lipids (left panel),
200 OilRed staining was completely lost in hepatocytes (white arrowheads) of bisex infected
201 animals except distinct perigranulomatous hepatocytes (red arrows). The arrowhead indicates
202 the accumulation of neutral lipids in eggs, white arrowheads: non stained hepatocytes, red
203 arrows: OilRed positive perigranulomatous hepatocytes. Bar 100 μ m, p parenchyma, # portal
204 field, ----- granuloma, black arrow hemosiderin deposits. Representative stainings are shown.
205
206

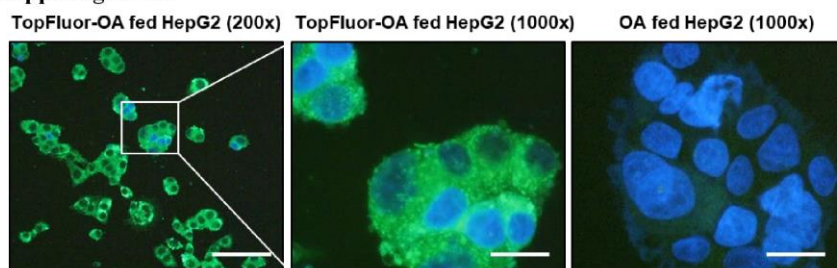
207 Suppl. Figure 14



208
 209 **Suppl. Figure 14: Confocal microscopy revealed the uptake and intracellular storage of**
 210 **TopFluor OA (green) into 2-3% of liver derived *S. mansoni* eggs and nearly 100% in pre-**
 211 **matured *in vitro* laid eggs.** Uptake of fluorescently labeled oleic acid (fIOA) in 2-3% of liver-
 212 derived *S. mansoni* eggs after 10 min (A) and after 18 h (B) of cultivation with fIOA-charged
 213 medium. (C) Interestingly, the observation of fluorescently labeled miracidia (green arrow)
 214 suggested that also miracidia were able to absorb fIOA after hatching. 60 min cultivation with
 215 fIOA, white arrow depicts an egg, autofluorescence of the egg was used as a counterstain to
 216 visualize the contours of eggs: red. (D and E) Nearly all pre-matured *in vitro* laid eggs took up
 217 fIOA from hepatocytes that were fed with the labeled lipid before coculture in the same
 218 compartment (black arrows indicate HepG2 cells, D: area with low number of HepG2 cells, E:
 219 area confluent with HepG2 cells). Please note, that even eggs without direct contact
 220 (D, green arrows) to fIOA fed HepG2 cells accumulated fIOA as we also could show in
 221 transwell experiments (Fig. 3H). Bars 25 μ m (A-C) and 50 μ m (D-E). Representative pictures
 222 are shown.
 223

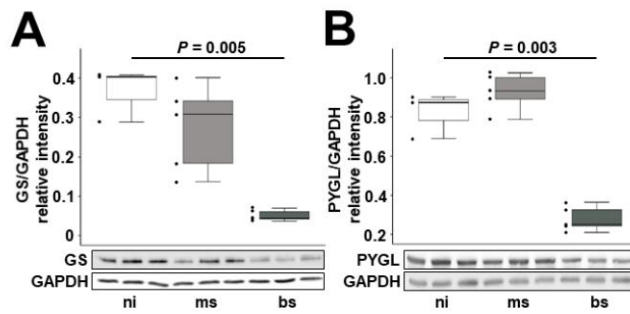
224 **Suppl. Figure 15**

225 **Suppl. Fig. 15: Uptake of fIOA into *in vitro*-laid eggs of different maturity.** Prior to addition
 226 of fIOA, eggs matured *in vitro* for (A) 1 day, (B) 3 days, (C) 5 days. At these different time
 227 points of egg maturation (Jurberg et al. 2009 doi: 10.1007/s00427-009-0285-9), fIOA was
 228 added, and its uptake monitored 18 h later. Untreated eggs served as control and showed
 229 autofluorescence of the egg shell only. Autofluorescence and fIOA fluorescence were excited
 230 at 488 nm and recorded using the identical detection window. Eggs in the images are
 231 representative for 30-50 eggs per condition. Scale bars: 50 μ m.
 232

233 **Suppl. Figure 16**

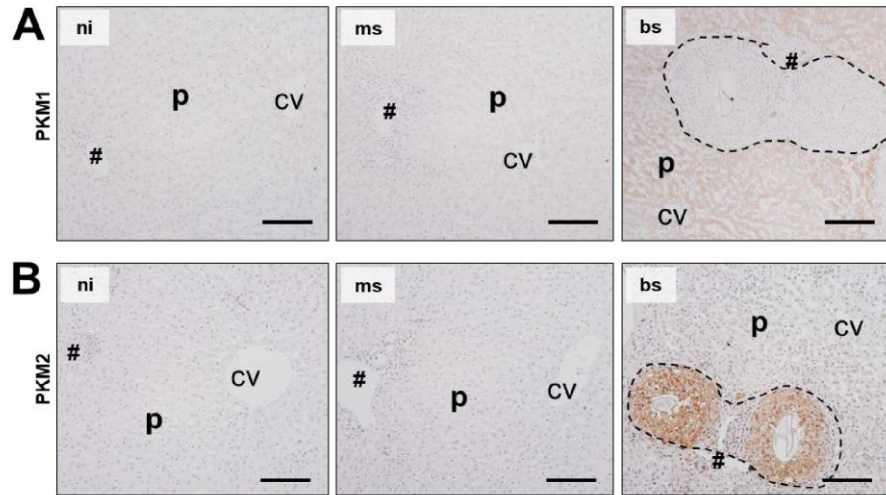
Suppl. Figure 16: Fluorescence microscopy revealed uptake and intracellular storage of TopFluor OA (green) into HepG2 cells. Controls (right) were fed with non-labeled OA. Bars 100 μ m and 20 μ m.

239 Suppl. Figure 17



240 **Suppl. Figure 17: *S. mansoni* bisex infection reduced hepatic glycogen turnover.** Western
241 blot analysis demonstrated lower hepatic expression of glycogen synthase (A) and hepatic
242 glycogen phosphorylase (B) in bisex-*S. mansoni* infected hamsters. Representative blots are
243 shown.
244
245

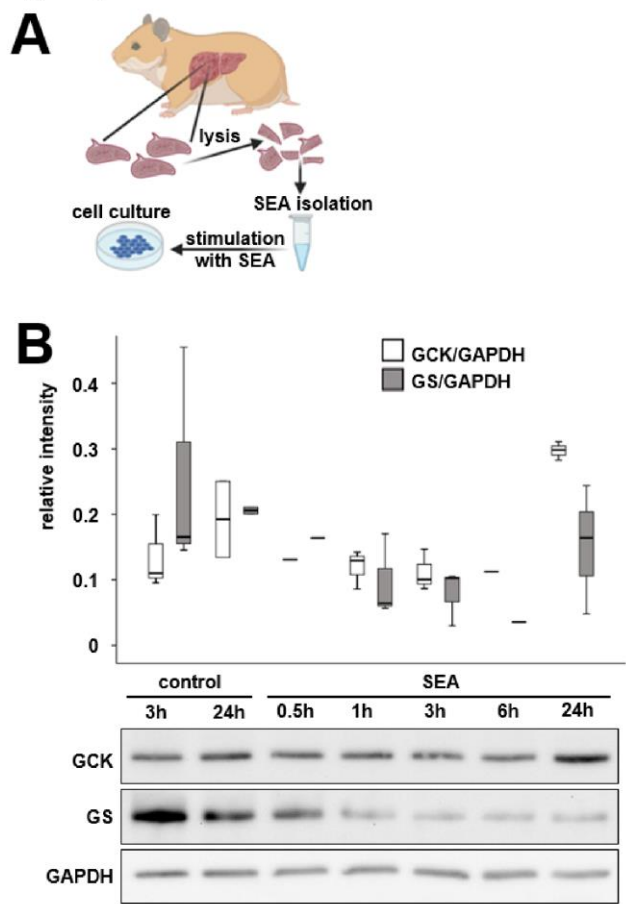
246 Suppl. Figure 18



247 **Suppl. Figure 18: Enhanced hepatic expression pattern of PKM1 and PKM2 in *S. mansoni***
 248 **infected hamsters.** While PKM1 expression was enhanced in hepatocytes (A), PKM2
 249 expression was induced in granulomas (B) in bisex infected hamsters. p parenchyma, # portal
 250 tract, cv central vein, dashed line granuloma. Bars 100 μm.

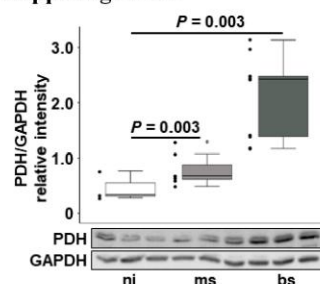
251 Please note, that PKM1 and PKM2 are splicing variants of the same gene. Therefore, the results
 252 in Fig. S17A/B may suggest an alternative splicing of the PKM gene expression products in
 253 immune cells versus hepatocytes.
 254
 255

256 Suppl. Figure 19



257
258 **Suppl. Figure 19: Soluble egg antigen (SEA) induced glycolysis and reduced glycogen**
259 **synthesis in HepG2 cells. (A)** Schematic illustration of SEA isolation. **(B)** Western blot
260 analysis of glucokinase (GCK) and glycogen synthase (GS) in HepG2 cells stimulated with
261 SEA. GAPDH was used as loading control. Representative blots are shown.
262

263 Suppl. Figure 20

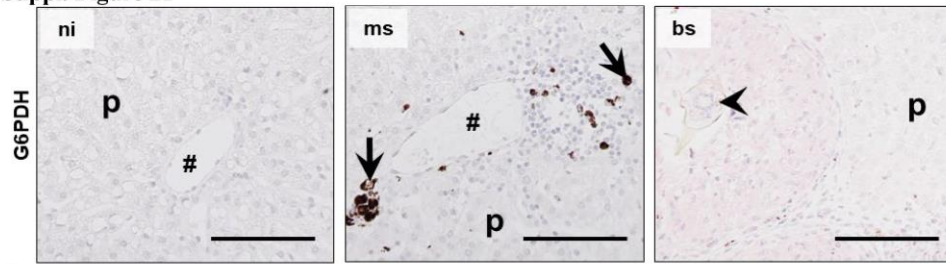


264

265 Suppl. Figure 20: *S. mansoni* bisex infection induced hepatic levels of pyruvate
266 dehydrogenase. Western blots demonstrate the accumulation of PDH in liver tissue of bisex
267 infected hamsters. Representative blots are shown.

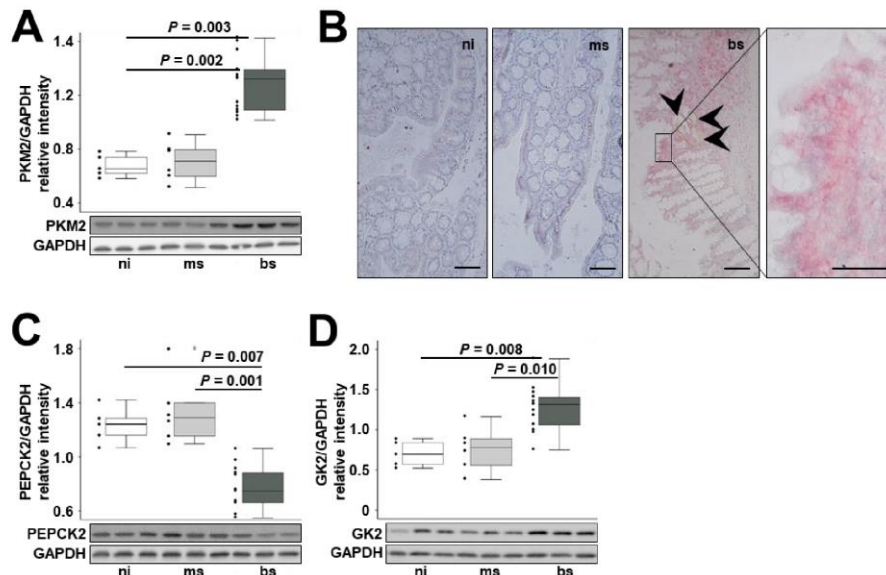
268

269 **Suppl. Figure 21**

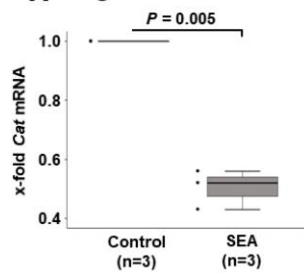


270 **Suppl. Figure 21: Enhanced hepatic expression pattern of G6PDH in *S. mansoni* infected**
 271 **hamster liver.** G6PDH was enhanced inside granuloma and in perigranulomatous hepatocytes.
 273 Arrowhead *S.mansoni* egg, p parenchyma, # portal tract, black arrows hemosiderin deposits.
 274 Bars 100 μm.
 275

276 Suppl. Figure 22

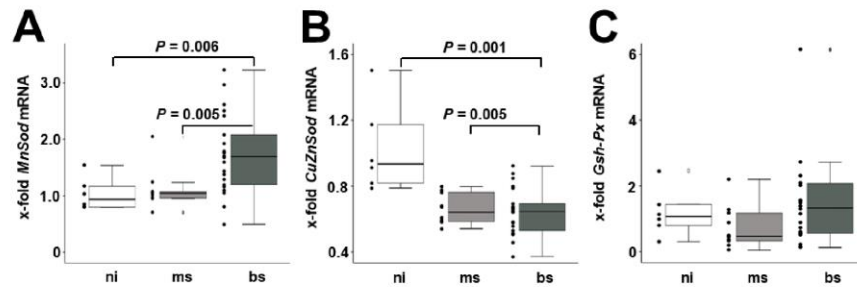


277
 278 **Suppl. Figure 22: *S. mansoni* bisex infection modulated the expression of colonic PKM2,**
 279 **PCK2, and GK2.** Western blot (A) and immunostaining (B) demonstrated the induction of
 280 PKM2 in mucosal and submucosal cells of the colon near *S. mansoni* eggs (arrowheads, bars
 281 200 μ m and 50 μ m in the magnified panel on the right). While protein expression of PEPCK2
 282 (C) was reduced in the colon of bisex infected hamsters, GK2 was induced (D). All experiments
 283 were reproduced at least 3 times.
 284

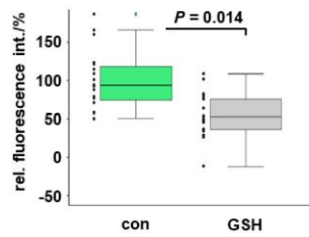
285 **Suppl. Figure 23**

286
287 **Suppl. Figure 23: SEA stimulation decreased *catalase* mRNA level in HepG2 cells.** The
288 presented data represent the mean of three independent experiments. Kruskal-Wallis test.
289

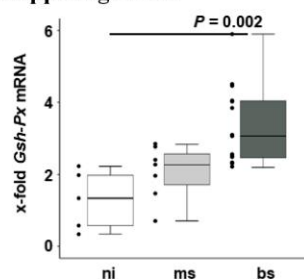
290 Suppl. Figure 24



291 Suppl. Figure 24: *S. mansoni* bisex infection modulated hepatic mRNA of oxidative stress
 292 markers. qRT-PCR demonstrated the accumulation of *MnSod*-mRNA (A) and the decrease of
 293 *CuZnSod*-mRNA (B) in liver tissue of bisex infected hamsters. (C) *Gsh-Px* was not regulated.
 294 Representative mean data of at least three independent experiments is depicted.
 295
 296

297 **Suppl. Figure 25**

298 **Suppl. Figure 25: GSH decreased fIOA uptake of the eggs from fIOA-fed HepG2 cells in**
299 **coculture.** CLSM-based quantification of fluorescence intensity of individual eggs from
300 coculture performed with GSH (10 mM, grey bar) in comparison to the conventional
301 experiment as a control (con, green bar). At least 10 eggs per condition were analyzed in each
302 of two independent experiments.
303
304

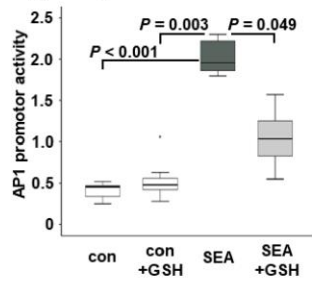
305 **Suppl. Figure 26**

306

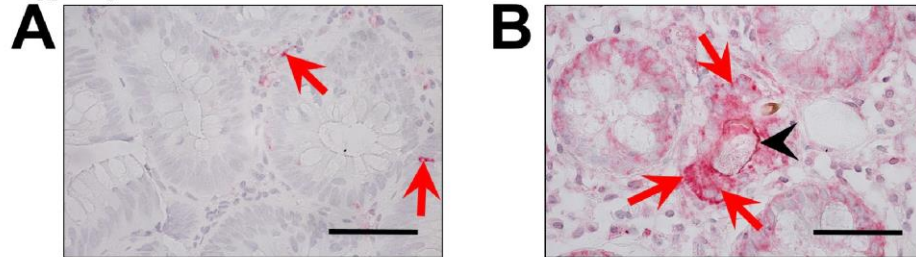
307 **Suppl. Figure 26: *S. mansoni* bisex infection modulated the expression of colonic *Gsh-Px*.**308 qRT-PCR demonstrated the induction of *Gsh-Px* in colon tissue of bisex-infected hamsters. All

309 experiments were reproduced at least 3 times.

310

311 **Suppl. Figure 27**

312
313 **Suppl. Figure 27: SEA activated promotor activity was reduced by GSH.** The reporter gene
314 assay demonstrated, that SEA induced functional activation of the AP-1 promotor was
315 diminished by the addition of GSH. All experiments were reproduced at least 3 times.
316

317 **Suppl. Figure 28**

318
319 **Suppl. Figure 28:** Visual comparison of PKM2 in infected vs non-infected tissue. Histologic
320 specimen of non-infected (A) vs. infected human colon tissue was stained for PKM2 (red
321 arrowheads) in granulomatous infiltrates around the eggs (black arrowhead). Representative
322 stainings are depicted, bars 50 μm.
323

Supplementary CTAT Tables

1.1 Antibodies

Name	Citation	Supplier	Cat no.	Clone no.
ADRP/Perilipin 2	PMID: 31570772	Proteintech	15294-1-AP	Polycl. rabbit antibody
PKM2	PMID: 15908230	CST	4053	D78A4
FAS	PMID: 34855620	CST	3189	Polycl. rabbit antibody
glycogen synthase	PMID: 35563842	CST	3886	15B1
PYGL	PMID: 20957198	Novus	NBP1-86182	Polycl. rabbit antibody
PKM1	PMID: 35610475	CST	7067	D30G6
PKM2	PMID: 35584006	CST	4053	D78A4
GCK	PMID: 34476698	Novus	NBP1-33144	Polycl. rabbit antibody
GAPDH	PMID: 30555521	Proteintech	60004-1-Ig	1E6D9
PDH	PMID: 26823462	CST	3205	C54G1
G6PDH	PMID: 35213227	CST	8866	Polycl. rabbit antibody
PEPCK	PMID: 34954190	B. Christ, Leipzig	homemade	Polycl. rabbit antibody
Phospho-c-Jun	PMID: 35896539	CST	3270	D47G9
c-Jun	PMID: 35896539	CST	9165	60A8
Phospho-STAT3	PMID: 35906210	CST	9145	D3A7
STAT3	PMID: 35906694	CST	4904	79D7
p44/42 ERK	PMID: 35306530	CST	4695	137F5
Phospho-p44/42 ERK	PMID: 35306530	CST	4370	D13.14.4E
Phospho-H2A.X	PMID: 35578032	CST	9718	20E3

1.2 Cell lines

Name	Citation	Supplier	Cat no.	Passage no.	Authentication test method
HepG2	PMID233137	CLS	#330198	10-15	Morphology

1.3 Organisms

Name	Citation	Supplier	Strain	Sex	Age	Overall n number
Syrian hamsters, final hosts	PMID: 18119161	own breeding	<i>Mesocricetus auratus</i>	female	18 weeks	36
Schistosomes	PMID: 7477111	Bayer AG (Monheim, Germany)	<i>S. mansoni</i>	female & male	Worms: 46-67 days	~5,000
Snail, intermediate hosts	PMID: 27499125	own breeding	<i>Biomphalaria glabrata</i>	hermaphrodite	1-6 months	~20

1.4 Sequence based reagents

Name	Sequence	Supplier
<i>Fas</i>	F: 5'-CAC AGA TGA TGA CAG GAG ATG G-3' R: 5'-TCG GAG TGA GGC TGG GTT GAT-3'	Microsynth Seqlab GmbH, Göttingen, Germany
<i>Acc1</i>	F: 5'-ACA CTG GCT GGC TGG ACA G-3' R: 5'-CAC ACA ACT CCC AAC ATG GTG-3'	Microsynth Seqlab GmbH, Göttingen, Germany
<i>Cat</i>	F: 5'-TCC GTC CTT CAT CCA TAG C-3' R: 5'-TGT GAG CCG TAG CCA TTC-3'	Microsynth Seqlab GmbH, Göttingen, Germany
<i>MnSod</i>	F: 5'-CTG GAG CCG CAC ATC AAC-3' R: 5'-AAG AGC AAC CTG AGT CGT AAC-3'	Microsynth Seqlab GmbH, Göttingen, Germany
<i>CuZnSod</i>	F: 5'-TCA GGA GAG CAT TCC ATC ATT G-3' R: 5'-TCC CAG CAT TTC CAG TCT TTG-3'	Microsynth Seqlab GmbH, Göttingen, Germany
<i>GshPx</i>	F: 5'-TTA CAT CGC CAA GTC GTT CTA TG-3' R: 5'-CTG GTT GAA GTC TCT GGT AGT TG-3'	Microsynth Seqlab GmbH, Göttingen, Germany

1.5 Biological samples

Description	Source	Identifier
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Pseudonymized human colon samples	Dr. Senckenberg Institute of Pathology, University Hospital Frankfurt	ethics committee (AZ 05/19), ID19 H1XX1-14
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1.6 Deposited data

Name of repository	Identifier	Link

1.7 Software

Software name	Manufacturer	Version
Lipid Match Flow	Innovative Omics	3.5
Perseus	MPI	2.0.6.0
Mirion	Institute of Inorganic and Analytical Chemistry, Justus Liebig University Giessen, Prof. Dr. Bernhard Spengler	3.3.64.22
IMARIS imaging software	Bitplane	4.8.2
Image J	NIH	1.53a
SPSS	IBM	26.0
Excel Professional Plus	Microsoft, Redmond, WA, USA	2010, 2016
visionCATS	CAMAG (Muttentz, Switzerland)	2.5 (Ultimate software package)
Xcalibur	Thermo Fisher Scientific (Dreieich, Germany)	3.0.152
Foundation	Thermo Fisher Scientific (Dreieich, Germany)	3.0.152

1.8 Other (e.g. drugs, proteins, vectors etc.)

TopFlour Oleic Acid	Avanti	SKU 810259C
Oleic Acid	Sigma	O-1008
1,5-diaminonaphthalene (DAN)	Acros Organics, Geel, Belgium	97%
2,5-dihydroxy benzoic acid (DHB)	Merck, Darmstadt, Germany	for synthesis
2-propanol	Chemsolute, Renningen, Germany	for HPLC
acetone	VWR International, Fontenay-sous- Bois, France	HiPerSolv
ammonium formate	Sigma-Aldrich, Steinheim, Germany	99.995%
acetonitrile	VWR International, Fontenay-sous- Bois, France	HiPerSolv
Eosin Y solution	Sigma-Aldrich, Steinheim, Germany	
ethanol	Merck, Darmstadt, Germany	Uvasol
Eukitt quick hardening medium	Sigma-Aldrich, Steinheim, Germany	
formic acid	Honeywell, Morris Plains, NJ, USA	for mass spectrometry
Mayer's hematoxylin solution	Sigma-Aldrich, Steinheim, Germany	
methanol	Merck, Darmstadt, Germany	LiChroSolv
Methyl-tert-butylether (MTBE)	Sigma-Aldrich, Steinheim, Germany	for HPLC
phosphate buffered saline (PBS)	Gibco, Carlsbad, CA, USA	
trifluoro acetic acid	Merck, Darmstadt, Germany	Uvasol
water	VWR International, Fontenay-sous- Bois, France	HiPerSolv
xylene	Merck, Darmstadt, Germany	for analysis
Cholesterol solution 10 mg/mL	Sigma Aldrich (Darmstadt, Germany)	471274
Glycerol trioleate	Sigma Aldrich (Darmstadt, Germany)	41679
Cholesteryl oleate	Sigma Aldrich (Darmstadt, Germany)	C9253
3-sn-phosphatidic acid sodium salt	Sigma Aldrich (Darmstadt, Germany)	P9511
Sphingomyelin	Sigma Aldrich (Darmstadt, Germany)	85615
L- α -phosphatidylcholine	Sigma Aldrich (Darmstadt, Germany)	P3556
L- α -phosphatidylethanolamine	Sigma Aldrich (Darmstadt, Germany)	P7943
Primuline	Sigma Aldrich (Darmstadt, Germany)	50%
Ninhydrin	Sigma Aldrich (Darmstadt, Germany)	Reagent grade
Aniline	Sigma Aldrich (Darmstadt, Germany)	$\geq 99.5\%$
Diphenylamine	Sigma Aldrich (Darmstadt, Germany)	$\geq 99\%$
Phosphomolybdic acid hydrate	Sigma Aldrich (Darmstadt, Germany)	Reagent grade
n-Hexane	Honeywell Riedel-de Haën (Seelze, Germany)	$\geq 97\%$
Ammonia solution	Honeywell Riedel-de Haën (Seelze, Germany)	25%, p.a.
o-Phosphoric acid	Th. Geyer (Renningen, Germany)	85%
2-Propanol	Th. Geyer (Renningen, Germany)	$\geq 99.8\%$

Chloroform	AppliChem GmbH (Darmstadt, Germany)	> 99.0%
Methanol	VWR (Darmstadt, Germany)	≥ 99.8%
Ethanol	Fisher Scientific (Schwerte, Germany)	≥ 99.8%
Diethyl ether	Acros Organics (New Jersey, NJ, USA)	> 99%
Glacial acetic acid	Carl Roth (Karlsruhe, Germany)	100%, p.a.

1.9 Please provide the details of the corresponding methods author for the manuscript:

PD Dr. Martin Roderfeld
Justus-Liebig University Giessen
martin.roderfeld@innere.med.uni-giessen.de

2.0 Please confirm for randomised controlled trials all versions of the clinical protocol are included in the submission. These will be published online as supplementary information.

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Suppl. Table 1: Quantification results and reproducibilities ($n = 3$) determined via HPTLC-FLD.

		Oleic acid		Triolein		Cholesterol		Cholesteryl oleate		Phosphatidyl-ethanolamine		Phosphatidic acid		Sphingomyelin		Phosphatidyl-choline	
Group	Sample track	Mean content [mg/g]	Reproducibility [%RSD]	Mean content [mg/g]	Reproducibility [%RSD]	Mean content [mg/g]	Reproducibility [%RSD]	Mean content [mg/g]	Reproducibility [%RSD]	Mean content [mg/g]	Reproducibility [%RSD]	Mean content [mg/g]	Reproducibility [%RSD]	Mean content [mg/g]	Reproducibility [%RSD]	Mean content [mg/g]	Reproducibility [%RSD]
ni	1	1.4	12.7	1.7	9.7	0.9	0.5	<LOD		18.5	3.6	<LOQ		0.6	0.1	9.6	1.1
ni	2	1.3	6.7	2.3	4.0	0.6	17.6	<LOD		18.2	1.5	0.3	1.2	0.6	10.9	10.6	1.8
ni	3	1.2	17.5	1.2	6.0	0.7	15.6	-		21.0	3.6	0.2	6.7	0.8	10.5	11.1	2.2
ms	4	1.3	12.7	1.1	19.3	1.0	0.5	21.2	4.9	19.1	6.4	<LOQ		0.7	12.1	9.3	2.6
ms	5	1.6	12.7	1.0	28.8	0.9	0.0	16.4	3.1	19.9	0.1	<LOQ		0.9	6.7	10.0	0.3
ms	6	1.8	5.7	1.7	13.9	0.9	4.3	2.4	4.3	19.2	2.0	<LOQ		0.7	5.5	9.5	5.2
ms	7	1.6	4.4	1.5	12.3	1.1	7.1	<LOD		21.8	5.7	<LOQ		0.8	0.2	10.5	3.7
ms	8	1.5	5.5	1.0	19.8	1.0	9.6	<LOD		20.2	6.4	<LOQ		0.8	6.4	9.7	0.2
ms	9	1.4	5.7	1.1	15.2	0.7	9.7	<LOD		18.1	5.4	0.2	2.8	0.8	3.3	9.4	0.3
bs	10	1.6	9.6	1.9	4.3	0.9	1.9	<LOD		22.4	0.1	0.3	7.0	1.1	2.2	10.9	7.6
bs	11	1.8	9.5	1.5	14.4	1.0	12.1	<LOD		21.0	6.5	0.3	1.6	1.1	4.6	9.9	10.4
bs	12	1.8	10.5	1.2	20.0	1.0	12.6	<LOD		18.4	7.8	0.2	19.6	0.9	6.0	9.2	8.5
bs	13	1.9	7.9	1.5	18.7	1.1	14.7	<LOD		19.4	8.4	0.2	21.9	0.8	15.1	9.3	6.8
bs	14	1.9	7.1	1.1	21.1	1.1	17.2	<LOD		19.5	7.1	<LOQ		1.0	0.5	10.1	6.6
bs	15	1.9	5.1	3.8	3.4	1.3	18.5	<LOD		17.5	14.4	<LOQ		0.8	0.4	9.2	7.2

Summary

Rapid, efficient, cost-effective, and reliable screening methods are needed to cope with the large sample sizes in VD residue analysis to ensure food safety. Therefore, the potential of different hyphenations of HPTLC techniques for the trace-level analysis of VDs in animal-derived food was investigated.

An HTpSPE method was developed for the clean-up of 66 antibiotics in muscle meat, cow milk, and chicken eggs as an alternative to routine methods for the first time. The analytes were automatically eluted via the autoTLC–MS interface into an orbitrap-HRMS instrument for detection. The hardware and software of the autoTLC–MS interface were improved for full automation of HPTLC–HPLC–MS hyphenations, and it was renamed to autoTLC–LC–MS interface. This improvement was the key to success in expanding the scope of the method to 81 VDs in muscle meat, cow milk, chicken eggs, and honey. The HTpSPE–UV/vis/FLD–HPLC–HRMS/MS screening proved its ability to detect VD residues down to the trace level by validation. For 21 samples in parallel, the clean-up was five times faster and consumed less solvent than routine methods, and thus it will enhance current screening capacities as a more ecological option.

As an alternative to MS, non-target planar bioassay screening was investigated for its potential to detect VD residues on the example of two fluoroquinolones in milk. This approach showed promising results and also revealed strong antibacterial activity outside the analytical focus. For further investigation of the observed effects, the same approach was applied to HTpSPE chromatograms of pig muscle, milk, eggs, and honey. Very prominent antibacterial zones evident in all matrices were characterized by direct coupling to HRMS. This proved fatty acids and lipids from the sample matrix to be mainly responsible for the antibacterial effect and clarified the inconsistency of positive bioassay screening results that remain unconfirmed by subsequent HPLC–MS/MS analyses.

In addition, HPTLC–HRMS as a promising tool for the identification of unknown bioactive compounds was applied for the characterization of coral samples, Ethiopian honey, and the lipid composition in the liver of *Schistosoma mansoni* infected hamsters.

Zusammenfassung

Zur Gewährleistung der Lebensmittelsicherheit sind schnelle, effiziente, kostengünstige und zuverlässige Screening-Methoden erforderlich, um den großen Probenumfang bei der Analyse von Tierarzneimittelrückständen zu bewältigen. Daher wurde das Potenzial verschiedener Kopplungen von HPTLC-Techniken für die Spurenanalytik von Tierarzneimitteln in Lebensmitteln tierischen Ursprungs untersucht.

Erstmals wurde eine HTPSPE-Methode für 66 Antibiotika in Muskelfleisch, Kuhmilch und Hühnereiern als Alternative zu Routinemethoden entwickelt. Zur Detektion wurden die Analyten automatisch mittels autoTLC-MS Interface in ein Orbitrap-HRMS-Gerät eluiert. Zur vollständigen Automatisierung von HPTLC-HPLC-MS-Kopplungen wurden Hard- und Software des autoTLC-MS Interfaces verbessert und dieses wurde in autoTLC-LC-MS Interface umbenannt. Diese Verbesserung war der Schlüssel zum Erfolg bei der Erweiterung des Anwendungsbereichs der Methode auf 81 VDs in Muskelfleisch, Kuhmilch, Hühnereiern und Honig. Die HTPSPE-UV/vis/FLD-HPLC-HRMS/MS-Screening-Methode bewies durch Validierung ihre Fähigkeit, Tierarzneimittelrückstände bis in den Spurenbereich nachzuweisen. Für 21 Proben gleichzeitig war die Aufreinigung fünfmal schneller und verbrauchte weniger Lösungsmittel als Routinemethoden und wird die derzeitigen Screening-Kapazitäten als umweltfreundlichere Option verbessern.

Alternativ zur MS wurde das planare Non-Target-Bioassay-Screening auf sein Potenzial zum Nachweis von Tierarzneimittelrückständen am Beispiel von zwei Fluorchinolonen in Milch untersucht. Dieser Ansatz zeigte vielversprechende Ergebnisse und offenbarte auch eine starke antibakterielle Aktivität außerhalb des analytischen Fokus. Zur weiteren Untersuchung der beobachteten Wirkungen wurde derselbe Ansatz auf HTPSPE-Chromatogramme von Schweinemuskel, Milch, Eiern und Honig angewendet. Die sehr ausgeprägten antibakteriellen Zonen in allen Matrices wurden durch direkte Kopplung mit HRMS charakterisiert. Dies bewies, dass Fettsäuren und Lipide aus der Probenmatrix hauptsächlich für die antibakterielle Wirkung verantwortlich waren und erklärte die Unstimmigkeit positiver Bioassay-Screening-Ergebnisse, die durch nachfolgende HPLC-MS/MS-Analysen unbestätigt bleiben.

HPTLC-HRMS als vielversprechendes Instrument zur Identifizierung unbekannter bioaktiver Verbindungen wurde außerdem für die Charakterisierung von Korallenproben, äthiopischem Honig und der Lipidzusammensetzung in der Leber von mit *Schistosoma mansoni* infizierten Hamstern eingesetzt.

Danksagung

Zuallererst möchte ich ganz herzlich Prof. Dr. Gertrud Morlock danken für die Möglichkeit, meine Dissertation in ihrer Arbeitsgruppe anzufertigen. Danke für die sehr lehrreiche Zeit, für die Vergabe des interessanten Promotionsthemas, für die stete Unterstützung und Betreuung während dessen Bearbeitung sowie die Begutachtung dieser Arbeit.

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Abschließend möchte ich meiner Familie danken, die mir diesen Weg ermöglicht und mich stets unterstützt hat und ganz besonders meinem Freund Alexander – für alles.