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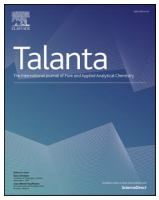

# Concept of a six-fold multiplex planar bioassay to distinguish endocrine agonist, antagonist, cytotoxic and false-positive responses☆

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ABSTRACT

To analyze a complex sample for endocrine activity, different tests must be performed to clarify androgen/estrogen agonism, antagonism, cytotoxicity, anti-cytotoxicity, and corresponding false-positive reactions. This means a large amount of work. Therefore, a six-fold planar multiplex bioassay concept was developed to evaluate up to the mentioned six endpoints or mechanisms simultaneously in the same sample analysis. Separation of active constituents from interfering matrix via high-performance thin-layer chromatography and effect differentiation via four vertical stripes (of agonists and end-products of the respective enzyme–substrate reaction) applied along each separated sample track were key to success. First, duplex endocrine bioassay versions were established. For the androgen/anti-androgen bioassay applied via piezoelectric spraying, the mean limit of biological detection of bisphenol A was 14 ng/band and its mean half maximal inhibitory concentration $IC_{50}$ was 116 ng/band. Applied to trace analysis of six migrate samples from food packaging materials, 19 compound zones with agonistic or antagonistic estrogen/androgen activities were detected, with up to seven active compound zones within one migrate. For the first time, the S9 metabolism of endocrine effective compounds was studied on the same surface and revealed partial deactivation. Coupled to high-resolution mass spectrometry, molecular formulas were tentatively assigned to compounds, known to be present in packaging materials or endocrine active or previously unknown. Finally, the detection of cytotoxicity/anti-cytotoxicity and false-positives was integrated into the duplex androgen/anti-androgen bioassay. The resulting six-fold multiplex planar bioassay was evaluated with positive control standards and successfully applied to one migrate sample. The streamlined stripe concept for multiplex planar bioassays made it possible to assign different mechanisms to individual active compounds in a complex sample. The concept is generic and can be transferred to other assays.

## 1. Introduction

A large number of substance groups can interact with the human steroid receptor system and either mimic the human hormones (agonists), the 17$\beta$-estradiol (E2) and the testosterone, or inhibit their activity (antagonists) [1–3]. It can cause substantial damage as reported for male rats [4] and development of cancer in male reproduction organs [5] and female breasts [6]. Food contact materials can be a potential source of endocrine active substances for humans [7]. The migration of agonistic [8,9] and antagonistic [10] endocrine active substances from packaging into foodstuffs was reported using various *in vitro* yeast estrogen screens. However, in order to obtain sound mechanistic information for understanding the effects from a complex sample mixture, the differentiation of antagonistic responses (biological signal reduction by receptor antagonism) from cytotoxic effects (biological signal reduction by cell death) and from false positive responses (physico--chemical signal reduction) should also be studied as well as metabolic deactivation or activation (de-/toxification). It is obvious that each "antagonist" signal requires clarification and verification whether it is either a true antagonist or cytotoxicant or false positive. Integrating all relevant endpoints (differentiating agonists, antagonists, cytotoxicants, false positives, de-/toxification) means the five-fold experimental effort

☆ Dedicated to the 85th birthday of Dr. Klaus Zieloff, Germany

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for *in vitro* assays for each sample [9,11].

Instead of a mixed sum value from *in vitro* assays, the information on the distinct effects of individual substances in a complex sample mixture is superior for decision-making. Currently, this would require an even lengthier experimental procedure, consisting of bioassay-guided fractionation, analytical separation, fraction collection, compound enrichment, re-solubilization, execution of the various bioassays, and combinatorial evaluation of all sum value results. It is evident that this is critical and not suited for routine food safety analysis. Hence, the identification of endocrine active compounds in complex samples such as food contact materials is performed via column chromatography and mass spectrometry (MS). A high number of mass signals and thus chemicals were detected. Signals found in the MS-spectra that matched database entries were suggested to be responsible for the endocrine activities [2]. This strategy is often used because there is no better idea. However, it might not be very conclusive because active compounds can sometimes be poorly ionizable resulting in small MS signals or even no MS signals (and other more prominent MS signals are wrongly assigned instead). The use of such a strategy brings with it a high degree of uncertainty and may lead to a discrepancy and mismatch between bioassay result and MS assignment, which can lead scientists down the wrong path of reasoning and decision-making. It increases the probability that unknown (not in database, hardly ionizable) but important compounds are overlooked.

Effect-directed analysis using a high-performance thin-layer chromatography (HPTLC) separation combined with a planar bioassay appears comparatively attractive due to differentiated biological effect profiles which are more meaningful. All is performed on the same adsorbent surface. It starts with sample enrichment via application of higher volumes, separation of bioactive components from interfering sample matrix via chromatography, biological effect detection via the on-surface bioassay, effect differentiation via application of stripe(s), and tentative assignment of a molecular formula to the active compound zone via its online elution directly from the bioautogram to high-resolution mass spectrometry (HRMS). The all-on-the-same-surface strategy deciphers the mechanistic source of endocrine activity and streamlines identification of chemicals responsible for the various endocrine responses [12].

For detection of endocrine active compounds, planar bioassays have been used among others for analysis of foods [13–15], cosmetics and thermal paper [16], waste and surface water [17–21], seed oils [22], cannabis [23], plant extracts, herbs and spices [24,25], and packaging material [26]. The planar yeast estrogen screen (pYES) [13,18,19] and planar yeast androgen screen (pYAS) [16,27] bioassays were recently developed further to planar duplex/triplex bioassays. The duplex planar bioassays, *i.e.* the planar yeast antagonist androgen screen (pYAAS) [16,25,28] and the planar yeast antagonist estrogen screen (pYAES) [25,29], detect agonists and antagonists simultaneously in the same analysis. The triplex planar bioassays, *i.e.* the planar yeast antagonist-verified androgen screen (pYAVAS) [28] and the planar yeast antagonist-verified estrogen screen (pYAVES) [29], detect agonists, antagonists and false-positive antagonists in the same sample analysis. Detection of cytotoxicants was also already demonstrated, however, as separate planar cytotoxicity bioassay using the substrate resazurin [27,29] or a tetrazolium salt [30] for dead/alive detection on the planar surface.

In this work, it was hypothesized that all relevant endocrine information about a complex sample can be distinguished on the same planar surface due to the flexibility of the open planar format. In addition to the agonistic responses, the antagonistic responses (biological signal reduction through receptor antagonism) in particular should be distinguishable from cytotoxic effects (biological signal reduction by cell death) or false positive responses (physico-chemical signal reduction). For the first time, the simulated metabolic activation and/or deactivation of endocrine active compounds via the S9 liver enzyme system should be introduced to the planar yeast bioassays. The resulting multiplex planar bioassay concept should be evaluated with positive control standards and applied to verify all relevant endocrine effects in a complex sample such as a migrate sample.

## 2. Material and methods

### 2.1. Materials and chemicals

Dimethyl sulfoxide (≥99.8 %), disodium phosphate (≥99.0 %), solid sodium hydroxide (>99 %), potassium dihydrogen phosphate (≥99 %), and potassium chloride (≥99.5 %) were acquired from Carl Roth (Karlsruhe, Germany). HPTLC plates silica gel 60, citric acid monohydrate (≥99.5 %), and sodium chloride (≥99.5 %) were supplied by Merck (Darmstadt, Germany). Methanol (100 %) was acquired from VWR (Darmstadt, Germany). D-Glucose (≥99.5 %), ethanol (≥99.8 %), copper (II) sulfate (≥98 %), yeast nitrogen base without amino acids, resazurin disodium salt (resazurin), menadione (K3, analytical standard), flutamide (F), testosterone (≥99.0 %), fluorescein disodium salt (fluorescein), Tween 20 and magnesium sulfate heptahydrate (>99 %) were bought from Fluka Sigma Aldrich (Darmstadt, Germany). Toluene (≥99.8 %) was purchased from Fisher Scientific (Schwerte, Germany). Ethyl acetate (≥99.8 %, Chemsolute) was acquired from Th. Geyer (Renningen, Germany). 17$\beta$-Estradiol (≥98.5 %) and methoxychlor (≥98.5 %) was acquired from Dr. Ehrenstorfer (Augsburg, Germany). *Saccharomyces cerevisiae* BJ3505 (YES) were obtained from D. P. McDonnel et al. [31,32]. XenoScreen YAS (*Saccharomyces cerevisiae* BJ1991), phenobarbital/naphthoflavone induced S9-mix, nicotinamide adenine dinucleotide phosphate solution, D-glucopyranose 6-phosphate solution and buffer salt solution were purchased from Xenometrix (Allschwil, Switzerland). *Aliivibrio fischeri* bacteria were provided by Leibniz Institute DSMZ, German Collection of Microorganisms and Cell Cultures (Berlin, Germany). Amino acids for the respective solution were described elsewhere [31,32]. Fluorescein di-(β-D-galactopyranoside, FDG) was supplied by Santa Cruz Biotechnology (Dallas, TX, USA). Dihydrotestosterone (DHT, >99.0 %) and resorufin (>99.5 %) were purchased from TCI (Eschborn, Germany). Bisphenol A (BPA, >97 %) was obtained from Alfa Aesar (Karlsruhe, Germany). Quercetin hydrate (quercetin, 95 %) was acquired from Acros Organics (Geel, Belgium). Degalan P 28 N was donated by Röhm (Darmstadt, Germany). Six tin cans with chemically different R&D coatings were provided by Ceritec SRL, Italy – Metlac Group in collaboration with Nestlé Research, Lausanne, Switzerland. Bidistilled water was produced using the Destamat Bi 18E (Heraeus, Hanau, Germany).

### 2.2. Yeast cell suspensions

Adenine (200 mg), L-arginine (200 mg), L-aspartic acid (1000 mg), L-glutamic acid (1000 mg), L-histidine (200 mg), L-isoleucine (300 mg), L-leucine (1000 mg), L-lysine (300 mg), L-methionine (200 mg), L-phenylalanine (500 mg), L-serine (4000 mg), L-threonine (2000 mg), L-tyrosine (300 mg), and L-valine (1500 mg) were dissolved in 1 L purified water, which was sterile-filtered into a sterile bottle stored at 4 °C until use (amino acid solution). As culture medium, D-glucose (10 g for YES and 5 g for YAS) and yeast nitrogen base without amino acids (6.8 g) were dissolved in purified water (100 mL), which was then sterile-filtered into purified water (800 mL) and the amino acid solution (100 mL) was added. Both individual filtration steps were required, otherwise viscosity was too high. Cells (1 mL cryostock) were suspended in the culture medium (29 mL for YES and 39 mL for YAS) and cultivated (30 °C, 16 h, 100 rpm, rotatory horizontal shaker SM-30, Edmund Bühler, Bodelshausen, Germany). Depending on the cell density determined using a Thoma chamber (VWR, Darmstadt, Germany), a volume of cell suspension was withdrawn, centrifuged (2500 × *g*, 5 min), and resuspended in respective fresh medium (supplemented with 100 µL of an aqueous 200 µM copper (II) sulfate solution per 30 mL medium) to yield a cell density of $8.0 \times 10^7$ cells/mL.

### 2.3. Substrate solutions

For the endocrine response detection, FDG (5 mg) was dissolved in dimethyl sulfoxide (1 mL) and an aliquot (25 μL) was dissolved in 2.5 mL phosphate buffer (40.8 g potassium dihydrogen phosphate, 42.6 g disodium phosphate, 1.2 g magnesium sulfate, and 3.7 g potassium chloride dissolved in 1 L purified water and adjusted to pH 7 with solid sodium hydroxide). For cytotoxicity detection, 2 mg resazurin were dissolved in 4 mL cell suspension.

### 2.4. Standard solutions and fixation solutions

Ethanolic stock solutions of BPA, DHT, quercetin, flutamide, K3, testosterone, E2, fluorescein, and resorufin were prepared (1 mg/mL) and diluted with ethanol to 100 pg/μL DHT, 100 ng/μL flutamide, 10 ng/μL and 100 ng/μL K3 as well as BPA, 1.5 ng/μL testosterone, 50 ng/μL fluorescein, 50 ng/μL resorufin and 2 pg/μL E2. As positive control for metabolization, methoxychlor was dissolved in ethanol (0.04 mg/mL). For substance fixation, Degalan P 28 N was dissolved in *n*-hexane to obtain a 0.25 % solution. Tween 20 was dissolved in a 9:1 mixture of ethanol and 10 mM aqueous sodium chloride solution to obtain a 0.05 % solution.

### 2.5. Migrate preparation

The migrates were prepared by filling the cans with food simulant solvent (300 mL, ethanol 95 %), closed with a 50-μm thick aluminum foil (Korff, Oberbipp, Switzerland), and placed in an incubator at 60 °C for 10 days [33]. The 30-fold migrate concentrates were prepared by evaporating the solvent from a 30-mL aliquot (nitrogen atmosphere) and resolving the residues of each migrate in 1 mL food simulant solvent (ethanol 95 %).

### 2.6. HPTLC–Vis/FLD–multiplex bioassay–Vis/FLD method

The HPTLC plate silica gel 60 was prewashed by front elution using methanol (up to 90 mm, Twin Trough Chamber). If not stated otherwise, HPTLC instrumentation operated by visionCATS software version 3.1.21109.3 was from CAMAG (Muttenz, Switzerland). The migrate concentrates (3, 14, 21, 28, and 35 μL/28-mm band or 5 or 10 μL/8-mm band) and a positive control mixture (generated via overspraying of 1–10 μL/band depending on the compound) were applied (Automatic TLC Sampler 4, visionCATS File S1, file eases the application but is not necessary for reproduction) as 8-mm bands (for 2-fold multiplex bioassay with 1 stripe) or 28-mm bands (for six-fold multiplex bioassay with 4 stripes). The plate was developed with toluene – ethyl acetate (6:1, *V/V*) up to 70 mm (Twin Trough Chamber). After drying in a cold stream of air (8 min), the chromatogram was documented at white light illumination (Vis), fluorescent light detection (FLD) 254 nm, and FLD 366 nm (TLC Visualizer 2) to obtain information about native absorbing or fluorescent compounds in complex samples.

Depending on the intended multiplicity of the bioassay, testosterone (4 μL), E2 (5.7 μL), K3 (3.2 μL), fluorescein (2 μL), and resorufin (2 μL) solutions were applied as vertical stripes (0.1 mm × 80 mm, width × height) on each separated track (using the Freemode option of winCATS software version 1.July 4, 2018, File S2, file eases the application but is not necessary for reproduction). The plate was immersed horizontally into a Degalan solution (5 min) [28,29], let dry at room temperature for 8 min, and Tween 20 solution was sprayed onto the plate (2.5 mL, blue nozzle, level 4, Derivatizer). After drying in a cold stream of air (4 min), the chromatogram was optionally documented again.

For androgens/estrogens/antagonists detection, cell suspension ($8.0 \times 10^7$ cells/mL) of the respective strain was sprayed onto the plate (2.8 mL, red nozzle, level 6, Derivatizer) and incubated in a humid box at 30 °C for 4 h (YAS) or 3 h (YES) and followed by plate drying in a cold stream of air for 4 min. For metabolic activation/deactivation, the positive control methoxychlor (0.04 mg/mL, 0.5, 1.5 and 3 μL) was applied on the top plate part (not used for separation). The cell suspension contained the S9 system, prepared by adding 500 μL S9 liver enzyme suspension mixture, 162 μL nicotinamide adenine dinucleotide phosphate solution, 42 μL D-glucopyranose 6-phosphate solution and 953 μL buffer salt solution to 3334 μL yeast bioassay suspension ($8.0 \times 10^7$ cells/mL) [34]. For the six-fold multiplex assay, the cell suspension ($8.0 \times 10^7$ cells/mL) contained 2 mg resazurin per 4 mL cell suspension, and after the first incubation, the bioautogram was detected at Vis and FLD 254 nm. The FDG substrate solution was piezoelectrically sprayed onto the plate (2.5 mL, yellow nozzle, level 3, Derivatizer), or for the method robustness study, also syringe-dosed (4.0 mL, spray cycles 3, width 200 mm, length 100 mm) using Chromajet DS20 (Biostep, Burkhardtsdorf, Germany). The plate was then incubated in a humid box at 37 °C for 15 min, dried in a cold stream of air for 4 min, and documented at FLD 254 nm (TLC Visualizer 2).

### 2.7. Statistical evaluation

The signals in the green channel (absorbance measurement) were evaluated via peak area using videodensitometry (VideoScan software). Dose–response curves were determined via Excel software (Microsoft, Redmond, WA, USA). Thereof, half maximal inhibitory concentration $IC_{50}$ values were calculated according to AAT Bioquest $IC_{50}$ Calculator [35] as well as limits of biological detection (LOBD) according to ICH guideline (3.3 × residual standard deviation of the calibration curve to slope of the calibration curve) [36].

### 2.8. Zone characterization by HRMS

For characterization, endocrine-active compound zones were eluted from the chromatogram and online transferred to heated electrospray ionization high-resolution mass spectrometry (HPTLC–HESI-HRMS). Briefly, the respective migrate was applied twice on a normal phase plate (10 μL/band each) developed using toluene – ethyl acetate (6:1, *V/V*) up to 70 mm (Twin Trough Chamber). The plate was cut into two pieces. One plate piece was subjected to the pYAAS bioassay. On the other plate piece, each zone of interest (marked corresponding to the respective active zone on the bioassay plate) was eluted (200 μL/s, methanol) into the HESI-HRMS system (QExactive Plus mass spectrometer, Thermo Fisher Scientific, Dreieich, Germany) via the PlateExpress interface (Advion, Ithaca, NY, USA). If signal intensity had to be increased, the sample was applied as mentioned but six times, developed, and cut into two pieces. As a control, the plate piece containing one sample was subjected to the pYAES bioassay. From the other plate piece containing five samples, the zones of interest were then eluted (200 μL/min, 30 s/band) with ethyl acetate (used for good compound solubility and selectivity) and pooled into the same vial. The ethyl acetate solvent was evaporated in a nitrogen stream (room temperature) and the residue was dissolved in methanol (100 μL, used for good compound ionization). Afterward, the sample (20 μL) was injected via flow-injection analysis (FIA, 200 μL/min) into the HESI-HRMS system (HPTLC–FIA–HESI-HRMS). The instrument parameters were +3.5 kV and −3.2 kV, 270 °C capillary and 200 °C probe heater temperature, resolution 280000, *m/z* 100−1500, and automatic maximum injection time 10/200 ms for positive/negative ionization. The data analysis for the tentative assignment of the molecular formulas was performed with Xcalibur 3.0.63 (Thermo Fisher Scientific).

### 2.9. Aliivibrio fischeri culture preparation and bioassay application

The *Aliivibrio fischeri* cell culture medium was prepared according to DIN EN ISO 11348-1 [37]. Medium (20 mL) was inoculated with the bacterial cryostock (150 μL) and agitated in a 100 mL Erlenmeyer flask (ambient temperature, 75 rpm, 24 h). Upon shaking of the culture in a dark room, the brilliance of the bioluminescence was optically

inspected. When judged as brilliantly luminescent, the culture was sprayed onto the HPTLC plate (3 mL, level 6, red nozzle, Derivatizer) which was immediately documented with an exposure time of 60 s and a trigger interval of 1.1 min for 30 min (BioLuminizer) [38].

## 3. Results and discussion

### 3.1. Outlined evolution from duplex to six-fold multiplex bioassay

First, the existing HPTLC–Vis/FLD–pYAAS/pYAES–FLD duplex bioassay methods [28,29] were studied and validated for the first time for their performance in trace analysis of food packaging materials (3.2). As a highlight, the robustness of the planar bioassay method was investigated via an inter-laboratory comparison (using the antagonist bisphenol A). Both HPTLC–Vis/FLD–pYAAS/pYAES–FLD bioassay methods were applied to migrates of six differently coated tin cans, used as a case study in the field of food contact materials to detect any migrated compounds activating the estrogen or androgen receptor (3.3). For the first time, the liver metabolization was simulated on the same surface via the applied S9 liver enzyme system. Thus, the possible role of metabolic activation or deactivation of migrants was investigated (3.4). The observed antagonistic effects of migrants might be mistaken for cytotoxicity, which latter effect was studied as planar yeast cytotoxicity (pYCytotox) bioassay using resazurin as substrate (HPTLC–Vis/FLD–pYCytotox–Vis/FLD), demonstrated for the first time for trace analysis of food packaging materials (3.5). Most prominent endocrine active zones were further characterized by HRMS. The obtained HRMS signals were tentatively assigned to molecular formulas and chemicals were proposed (3.6). Finally, to save costs and time, the tested and successfully applied duplex bioassay methods (HPTLC–Vis/FLD–pYAAS/pYAES–FLD and HPTLC–Vis/FLD–pYCytotox–Vis/FLD) were merged as parts in an advanced, more comprehensive concept for a six-fold multiplex bioassay (3.7) performed on the same surface for the same sample analysis. Up to six endpoints/responses (endocrine agonist, antagonist and false-positives as well as cytotoxicity, anti-cytotoxicity and false-positives) important for verification of all relevant endocrine effects in a complex sample were observed and distinguished in a single bioautogram, which was first proven using positive control standards (3.8) and then successfully applied to one migrate sample (3.9).

### 3.2. Validation of the HPTLC–Vis/FLD–pYAAS–FLD duplex bioassay method

A comparable performance is expected for both HPTLC–Vis/FLD–pYAAS/pYAES–FLD duplex bioassay methods due to the identical procedure, except for the androgen or estrogen receptor in the yeast cell and the 1-h longer incubation for the pYAAS. The HPTLC–Vis/FLD–pYAAS–FLD was chosen as the more challenging example of the two versions, in particular for the longer time for zone diffusion (1 h more) and for the more difficult to evaluate reverse (negative) signal obtained for antagonists (observed via reduction of the fluorescent testosterone stripe) in contrast to agonists (direct positive signal, observed as fluorescent bands). The HPTLC–pYAAS–FLD duplex bioassay was already described in detail elsewhere [16,28]. However, instead of the reversed phase plate, the method was tested here on the cheaper normal phase plate. For validation according to Veyrand et al. [33], BPA was used as model compound, reported to have anti-androgenic properties [39] and to be present in packaging materials [40].

The method robustness was tested on the normal phase plate performed by two different laboratories and two different derivatization devices for six biological replicates. Therefore, dose–response curves of BPA in the range of 10–270 ng/band or 10–490 ng/band were investigated, and the LOBD and $IC_{50}$ values were calculated (Fig. 1 and S1). The mean LOBD was 14 ng/band ($n = 2$; 5 and 23 ng/band) and the mean $IC_{50}$ was 116 ng/band (Fig. 1B, $n = 2$) using piezoelectric spraying technology (Derivatizer) in two different laboratories. The comparison with syringe dosing technology (ChromaJet) used for substrate application in one laboratory showed similar results. A mean LOBD of 23 ng/band ($n = 4$, 12–33 ng/band, *%RSD* 39 %) and a mean $IC_{50}$ of 140 ng/band (Fig. 1A, $n = 4$) were obtained. Even using different instrumental technologies in different laboratories at different periods, performance data for BPA were highly comparable. In literature, a similar $IC_{50}$ value (139 ng/spot) was obtained by an antiandrogenic bioassay, in which testosterone was applied on the whole plate area for detection of the antagonistic response (no duplex bioassay) [27]. In contrast, our duplex bioassay method was much simpler and straightforward, providing the information on agonists and antagonists simultaneously on the same surface in the same sample analysis, saving cost and time, as first described in 2020 [16].

### 3.3. Six differently coated tin can migrates analyzed by both HPTLC–Vis/FLD–pYAAS/pYAES–FLD duplex bioassay methods

The validated duplex bioassay method was applied to food contact materials. Migrates of six differently coated tin cans were prepared using 95 % ethanol as food simulant. The high temperature (60 °C) and long time period (10 days) were chosen to force the migration of the substances. Subsequently, all migrates were concentrated 30-fold to further improve the detectability of the substances. After application and separation of the six different migrate concentrate samples on the HPTLC plate (5 or 10 μL/band applied), respective agonist stripes were applied along each separated sample track. After homogeneous application of the respective cell suspension on the chromatogram, incubation followed. Upon receptor activation by an agonist migrant as well as by the applied agonist stripe, galactosidase was released on such positions on the plate. As enzyme substrate for both androgen/estrogen bioassays, the fluorogenic FDG was applied next, which was cleaved by the galactosidase. The green fluorescence of the formed fluorescein was detected as end-product of the enzyme–substrate reaction. Thus, the detection of agonistic (androgenic/estrogenic) migrants was expected as green-fluorescent agonist bands. The comparatively expensive FDG substrate was selected because the sample showed no native green-fluorescent bands that could interfere with the interpretation of the bioassay results. Antagonist compounds were detected in the bioautogram via suppression (fluorescence reduction) of the green fluorescent testosterone/E2 agonist stripe (producing the biologically induced green fluorescence signal as mentioned).

Altogether 19 agonistic (green-fluorescent) or antagonistic (reducing the green-fluorescent agonist stripe) endocrine active compound bands were detected across all six migrates (Fig. 2A, –S9). In the duplex pYAAS bioautogram, detecting androgen agonists and antagonists at the same time, no agonistic (androgen) compound band was revealed but six antagonistic compound zones (Fig. 2A, –S9, **1**–**6**). In migrate concentrate 38, an androgen antagonist (**1**) was observed. In migrate concentrate 39, four further androgen antagonists (**2**–**5**) were detected. The 5-μL migrate volume resulted in a better resolution of the bands **3** and **4** as compared with the applied 10-μL volume. The migrate concentrate 65 contained only one androgen antagonist zone (**6**).

In the duplex pYAES bioautogram, detecting estrogen agonists and antagonists at the same time, eight agonist bands (Fig. 2A, pYAES, –S9, **7**–**14**) were observed for migrate concentrate 36 but only one (**15**) for migrate concentrate 38. For migrate concentrate 39, several antagonists (labeled as range **16**) were detected and two further agonist bands (**17** and **18**) along with the three agonist bands **12**–**14** already observed in migrate concentrate 36. Migrate concentrate 65 showed one unique agonist band (**19**) and two agonist bands previously detected in migrate concentrates 36 or 39 (**13** and **17**).

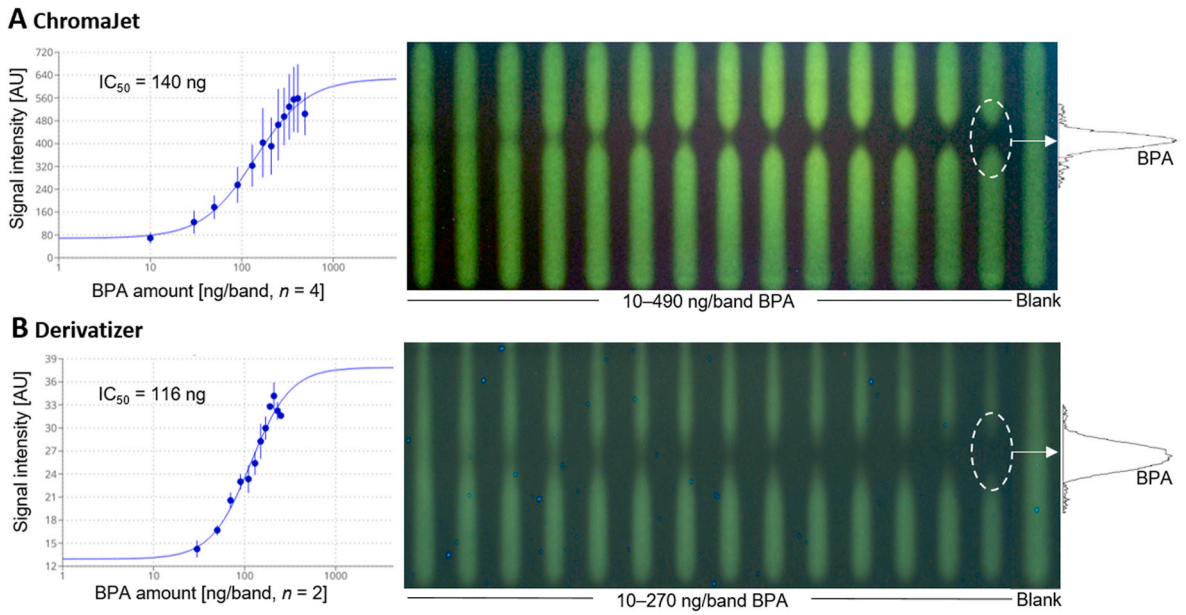


**Fig. 1.** Robustness of the HPTLC–pYAAS–FLD duplex bioassay performance using either the (**A**) ChromaJet or (**B**) Derivatizer device for substrate application: corresponding mean dose-response curves of bisphenol A (BPA, ranged 10–490 ng/band or 10–270 ng/band) and representative example bioautograms at FLD 254 nm (further ones in Fig. S1) and and solvent blank, separated on HPTLC plate silica gel 60 with toluene – ethyl acetate 6:1; testosterone agonist stripe (4 μL) was applied along each BPA track for antagonist (BPA) detection, evident as fluorescence reduction.

### *3.4. Integration of the S9 metabolism (HPTLC–Vis/FLD–S9–pYAAS/pYAES–FLD)*

The simulated metabolic conversion via the S9 liver enzyme system [41] was first integrated and demonstrated on the HPTLC plate surface for a neurotoxicity assay in 2020 [42] and the pYES bioassay in 2021 [25]. Such metabolic activation/deactivation of compounds (migrants) via S9 metabolization on the HPTLC plate was also integrated in the present duplex endocrine bioassay methodology (+S9 system added to the cell culture) to evaluate metabolic conversion observed as changes between the two +/−S9 bioautograms. As positive control for S9 metabolization, methoxychlor was selected as it was reported to undergo hepatic microsomal monooxygenase(s)-mediated activation and to be metabolized *in vivo* into the two demethylated compounds 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane [43,44] and 2,2-bis(*p*-hydroxyphenyl)-1,1-dichloroethane [43] and two *O*-ring methylated compounds [43], which interact with the estrogen receptor [43–45]. The endocrine response of the applied methoxychlor was substantially changed via the on-surface S9 metabolization which verified its proper functioning.

In presence of the S9 liver enzyme system in the duplex pYAAS bioautogram (Fig. 2B, pYAAS, +S9), a decrease of band intensity and thus a metabolic deactivation was observed for the antagonistic responses observed for the three different migrate concentrate samples 38, 39, and 65. Also evident was the overall fluorescence reduction of the testosterone stripe because of the S9 enzyme metabolization and the lower cell number that was reduced by 33 % due to the S9 system added to the cell culture. Next, the simulated metabolic activation/deactivation via the S9 liver system of the six different migrate concentrate samples was applied for the respective duplex pYAES bioassay. The intensities of all fluorescent agonist bands were reduced in presence of S9 metabolization (Fig. 2B, pYAES, +S9). The previously weaker agonist signals were not detectable anymore for the same sample amount applied.

Although the influence of the difference in the cell number has still to be clarified, the comparison for both bioautograms with/without metabolization (+S9/–S9) suggested that the S9 metabolization either converted endocrine active compounds into substances with less activity or induced a detoxification effect, both impacting the transcription activation of the nuclear endocrine receptors. Such a decrease can be caused by deactivation of androgen antagonists or detoxification of cytotoxicants or both. Therefore, integration of cytotoxicity assessment into the given duplex bioassay would add value regarding the mechanistic differentiation between cytotoxicants and antagonists. To prove and uncover the exact mechanism, various cytotoxicants as well as metabolically convertible precursors of agonists/antagonists still need to be studied as positive controls. Hence, these metabolization results should be considered preliminary. As mentioned, also the same cell number is recommended to be used for future +/−S9 bioautogram comparisons.

### *3.5. HPTLC–Vis/FLD–pYCytotox–Vis/FLD bioassay method studied for six differently coated tin can migrates*

With the detected antagonistic responses, cytotoxicity had to be excluded as another possible reason for the fluorescence reduction of the agonist stripe. Because *Salmonella* cells are Gram-negative bacteria, the also Gram-negative bioluminescent *Aliivibrio fischeri* bacteria used as ISO standard in the environmental field [37] were initially applied as planar cytotoxicity bioassay [38,46,47,50], which indicates influences on the cellular energy metabolism. The antiandrogenic bands (Fig. S4, **2**–**5**) matched partially to observed weak dark bands indicating an influence on the bacterial energy metabolism. Since cytotoxicity could not be clearly excluded for all five antiandrogenic bands in the pYAAS bioautogram, a different second approach was tested.

Resazurin was selected as a substrate in the planar yeast bioassay to detect cell viability, indicating a functioning cell metabolism [27, 48–50]. Therefore, resazurin as metabolic indicator was simply added to

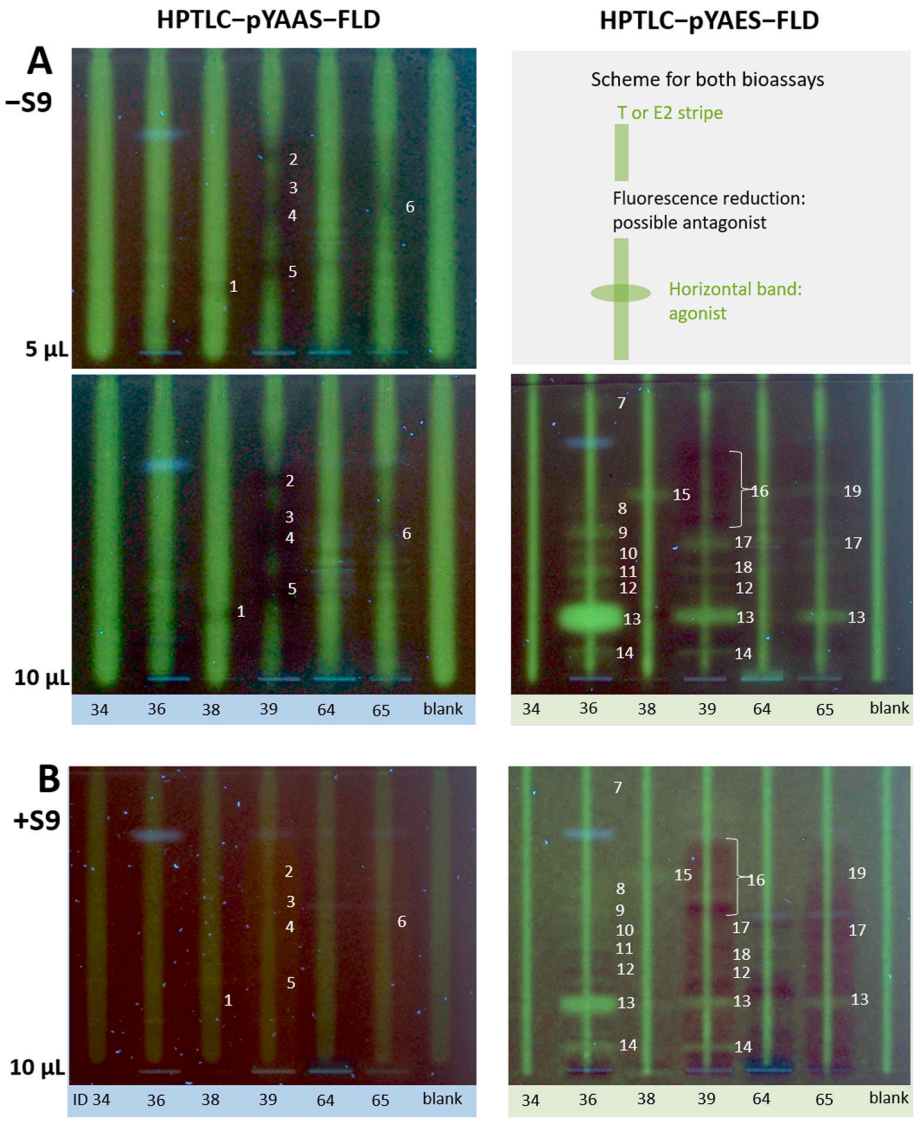


**Fig. 2.** Application of both HPTLC–pYAAS/pYAES–FLD bioassays, each (**A**) without (−S9) and (**B**) with (+S9) liver metabolization, to food contact materials: bioautograms at FLD 254 nm of six different migrate concentrates (IDs 34–65; 5 and 10 µL/band) and a food simulant blank (separated as in Fig. 1) as well as applied testosterone (T, 4 µL) or estradiol (E2, 5.7 µL) agonist stripes to detect respective antagonists.

the yeast cell culture (2.6), which was then applied on the chromatogram to clarify a cytotoxic effect of the separated compounds. Resazurin was orange (pH < 6.5) or purple (pH > 6.5) at Vis (depending on the surrounding pH [49]) as well as dark blue fluorescent at FLD 254 nm. It was reduced by living cells to the rose-colored or orange-fluorescent resorufin at the respective detection mode. Since living cells metabolize the substrate resazurin, a rose-colored or orange-fluorescent resorufin background was expected, whereas cytotoxicants were expected as orange/purple bands at Vis and dark blue fluorescent bands at FLD 254 nm. However, the formed rose-colored resorufin can further be reduced to the colorless dihydroresorufin [27]. Accordingly, on a rose-colored (resorufin) plate background at Vis, orange/purple (resazurin) bands/areas indicated lack of metabolism and thus cytotoxicity, whereas colorless/white (dihydroresorufin) bands/areas indicated oxidizing or oxidative cell stress-provoking compounds (as a kind of precursor of cytotoxicity along with reduction equivalents by active NADPH or NADH dehydrogenases [50]).

Evaluation of the six different migrate concentrate samples via the HPTLC–Vis/FLD–pYCytotox–Vis/FLD bioassay showed no orange/purple cytotoxic bands (Fig. 3B) corresponding to the antagonist bands (Fig. 3A), confirming a true antagonistic effect. As expected, cytotoxicity was excluded for the agonistic estrogen bands (Fig. 3B, *e.g.*, **11**, **13** and **17**). Only the range **16** of estrogen antagonists in concentrate 39 was more orange than rose (assumedly indicating a mixed anti-estrogenic and/or cytotoxic effect). Additionally, BPA known to show also cytotoxicity [51] was studied via the HPTLC–Vis/FLD–pYCytotox–Vis/FLD bioassay. The tested BPA range (50–700 ng/band) exceeded the highest amount used in the previous HPTLC–pYAAS–FLD bioautograms (Fig. 1 and S1). Colorless/white (dihydroresorufin) bands were observed for BPA (Fig. S2), indicating no cytotoxicity but oxidative cell stress in still metabolically active cells. Hence, any influence of cytotoxicity was excluded and the previous antiandrogenic BPA response was verified to be a true antiandrogenic signal.

### 3.6. *Assignment of molecular formulas to prominent endocrine active migrants*

For prominent endocrine active compound bands, as detected in the most active migrate concentrates, the coupling to HRMS was used to assign tentative molecular formulas. The data from the respective bands were compared with a plate blank (showing background signals from the HRMS system, solvents and plate). It was preliminarily assumed that

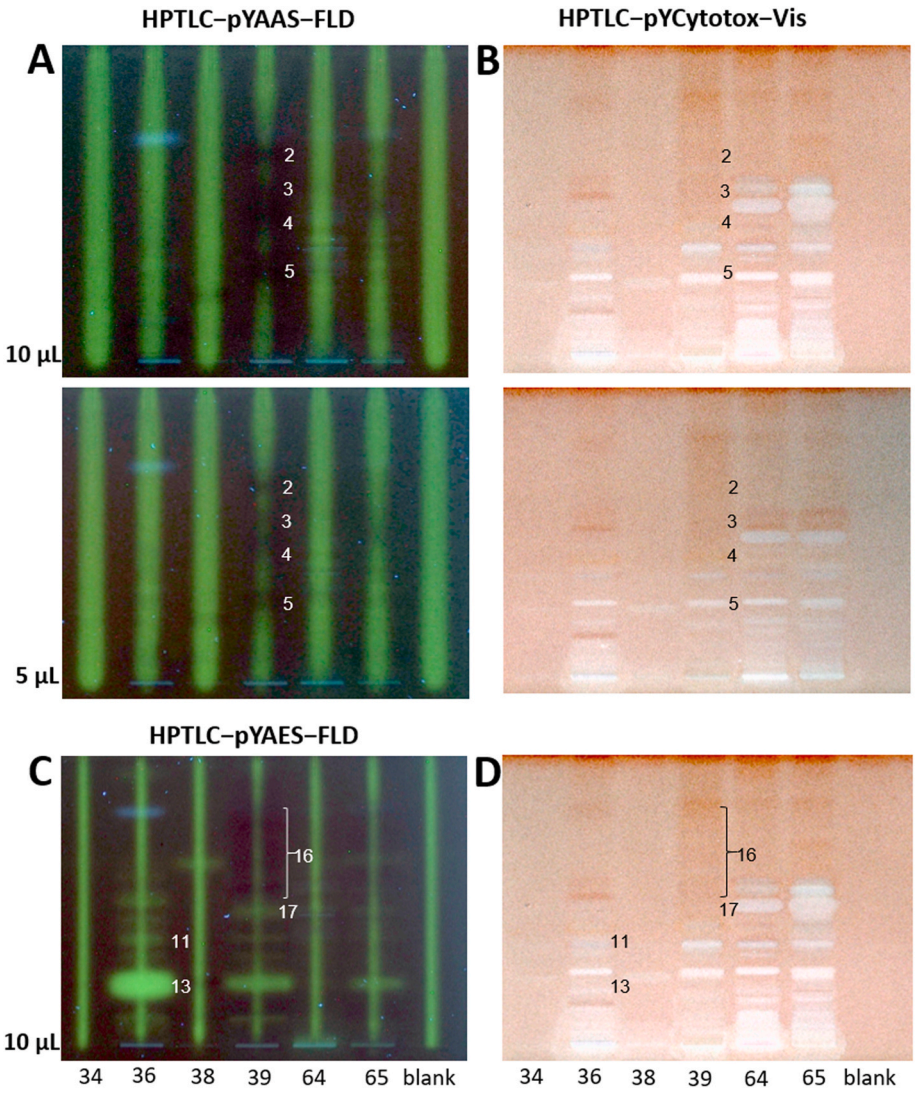


**Fig. 3.** Proof for cytotoxicity: bioautograms of (**A**/**C**) HPTLC–pYAAS/pYAES–FLD at 254 nm (as in Fig. 2) *versus* (**B**/**D**) HPTLC–pYCytotox–Vis at Vis showing the six different migrate concentrates (IDs 34–65; 5 and 10 µL/band) and a food simulant blank (separated as in Fig. 1) with the antagonistic compound bands **1**–**5** marked.

the strongest HRMS signal in the respective background-corrected mass spectrum obtained after chromatographic separation from the biologically active zone pointed to the respective endocrine active substance. The pYAAS bioautogram showed four antiandrogenic compound zones (**2**–**5**) in migrate concentrate 39, and the pYAES bioautogram one strong estrogenic substance zone (**13**). The latter estrogenic zone was assumed to be the same molecule in the three migrate concentrates 36, 39, and 65 due to the similar properties (Fig. 2A). These five endocrine active migrants were further characterized using migrate concentrates 36 and 39, showing the most prominent responses. Both were applied four times (4 sets of pairs), separated and the plate was cut to obtain the four sets, each containing the two migrate concentrates. Since the endocrine active compound bands were not detectable at Vis/FLD (reasons might be insufficient substance amount or missing absorption or fluorescence, nevertheless, which information is helpful for compound assignment), one set was subjected to the pYAAS bioassay and another to the pYAES bioassay to confirm the endocrine responses and obtain reference positions for zone marking on the third and fourth sets, followed by zone elution to HRMS. However, for a successful assignment, the HRMS signals obtained were too weak. It is known that especially the planar yeast-based bioassay can detect estrogens down to femtograms per band [14], which is why it is possible that the amounts of endocrine active substances (assumedly, low picogram amounts) were too low to be detected by HPTLC–HESI-HRMS but enough to be detected biologically via the very sensitive planar bioassay.

To obtain better signal intensity by zone enrichment, both migrate concentrates were applied five times each and the respective zones were eluted five times with ethyl acetate (for zone solubility and selectivity reasons) and pooled in the same vial. After solvent evaporation, the residue was re-dissolved in methanol (for good ionization) and flow-injected into the HRMS system (HPTLC–FIA–HESI-HRMS). This selective elution and enrichment of endocrine active compound zones resulted in the tentative assignment to molecular formulas (using Xcalibur software, Table 1). For band **2**, a deprotonated molecule ($C_{11}H_{13}O_3$) was detected that can tentatively be assigned to the antiandrogen butylparaben which is a well-known additive [53]. Several mass signals with a mass difference of 162 Da standing for a neutral loss of $C_{11}H_{14}O$ were found. A possible explanation of this repetitive neutral loss was the presence of a polymer coating of the can or an oligomer side product [52] that migrated into the food simulant. If it is taken into account that the molecular formula of the fragment can be assigned to an alkylphenol, a chemical from this substance class [54] (*e.g.*, cyclopentylphenol) could cause the observed antiandrogenic activity. For bands **3** and **5**, molecular formulas were tentatively assigned but no molecules could be proposed that were either expected to be in packaging materials or known to be endocrine active. Band **4** was tentatively

**Table 1**
HPTLC–FIA–HESI-HRMS of five endocrine active migrant zones (antiandrogenic compound zones **2**–**5** and estrogenic compound zone **13**) in migrate concentrates 39 and 36, respectively, showing HRMS signals detected and molecular formulas assigned tentatively.

| Migrant band (migrate) | *m/z* | Signal assigned | Proposed molecular formula | Error ± [ppm] | Suggestion |
|---|---|---|---|---|---|
| 2 (39) | 337.2161 | $[M1+H]^+$ | $C_{23}H_{29}O_2$ | 1.94 | |
| | 289.1773 | $[M2+Na]^+$ | $C_{16}H_{26}O_3Na$ | 0.05 | M6 to M2,repetitive neutral loss of Δ162 Da ($C_{11}H_{14}O$), except M3 that is probably a reduced M4 |
| | 435.2873 | $[M3+Na]^+$ | $C_{27}H_{40}O_3Na$ | 0.59 | |
| | 451.2820 | $[M4+Na]^+$ | $C_{27}H_{40}O_4Na$ | 0.02 | |
| | 613.3866 | $[M5+Na]^+$ | $C_{38}H_{54}O_5Na$ | 0.42 | |
| | 775.4912 | $[M6+Na]^+$ | $C_{49}H_{68}O_6Na$ | 0.54 | |
| | 193.0871 | $[M7-H]^-$ | $C_{11}H_{13}O_3$ | 0.89 | butylparaben |
| | 265.1810 | $[M2-H]^-$ | $C_{16}H_{25}O_3$ | 0.42 | M6 to M2, repetitive neutral loss of Δ162 Da ($C_{11}H_{14}O$) |
| | 427.2857 | $[M4-H]^-$ | $C_{27}H_{39}O_4$ | 0.70 | |
| | 589.3907 | $[M5-H]^-$ | $C_{38}H_{53}O_5$ | 1.43 | |
| | 751.4952 | $[M6-H]^-$ | $C_{49}H_{67}O_6$ | 1.88 | |
| 3 (39) | 395.2193 | $[M8+Na]^+$ | $C_{23}H_{32}O_4Na$ | 1.34 | |
| | 355.1918 | $[M9-H]^-$ | $C_{22}H_{27}O_4$ | 0.98 | |
| | 501.3016 | $[M10-H]^-$ | $C_{33}H_{41}O_4$ | 1.21 | |
| 4 (39) | 349.1774 | $[M11+Na]^+$ | $C_{21}H_{26}O_3Na$ | 0.05 | octabenzone |
| | 365.1722 | $[M12+Na]^+$ | $C_{21}H_{26}O_4Na$ | 0.49 | |
| | 463.1362 | $[M13+Na]^+$ | $C_{24}H_{24}O_8Na$ | 0.37 | cyclic butylene terephthalate dimer |
| | 341.1760 | $[M12-H]^-$ | $C_{21}H_{25}O_4$ | 0.37 | |
| | 683.3594 | $[2M11-H]^-$ | $C_{42}H_{51}O_8$ | 1.17 | |
| 5 (39) | 443.1677 | $[M14+Na]^+$ | $C_{22}H_{28}O_8Na$ | 1.12 | |
| | 403.1553 | $[M15-H]^-$ | $C_{25}H_{23}O_5$ | 1.86 | |
| 13 (36) | 282.2790 | $[M16+H]^+$ | $C_{18}H_{36}NO$ | 0.32 | oleamide |
| | 298.2740 | $[M17+H]^+$ | $C_{18}H_{36}NO_2$ | 0.12 | oxidized form |
| | 304.2609 | $[M16+Na]^+$ | $C_{18}H_{35}NONa$ | 0.41 | sodium instead of hydrogen |
| | 314.2685 | $[M18+H]^+$ | $C_{18}H_{36}NO_3$ | 1.43 | di-oxidized form |
| | 330.2641 | $[M19+H]^+$ | $C_{18}H_{36}NO_4$ | 0.92 | tri-oxidized form |
| | 293.1762 | $[M20-H]^-$ | $C_{17}H_{25}O_4$ | 1.19 | |
| | 309.1711 | $[M21+O-H]^-$ | $C_{17}H_{25}O_5$ | 2.75 | |

assigned to the octabenzone sodium adduct ($C_{21}H_{26}O_3Na$) [55], a compound used as an additive in plastic food contact materials, supported by the corresponding oxidized sodium adduct ($C_{21}H_{26}O_4Na$), deprotonated oxidized molecule ($C_{21}H_{25}O_4$) and deprotonated oxidized dimer ($C_{42}H_{51}O_8$). Another molecule in the same zone was tentatively assigned to the sodium adduct of the cyclic butylene terephthalate dimer ($C_{24}H_{24}O_8Na$) included in reactive resin compositions [56]. For band **13**, the protonated molecule $C_{18}H_{36}NO$ was tentatively assigned to oleamide, a compound known as a contaminant in food contact materials [57]. This suggestion was supported by its sodium adduct ($C_{18}H_{35}NONa$), epoxide (C18H36NO2), monohydroperoxide ($C_{18}H_{36}NO_3$) and monohydroperoxide epoxide ($C_{18}H_{36}NO_4$).

Although biological effect detection was superior in sensitivity to HRMS detection, the HPTLC–FIA–HESI-HRMS coupling was considered a straightforward, fast and efficient approach to increase our knowledge on migrants from packaging. The described approach can be combined with information from fragmentation by HRMS/MS in future studies. Molecular ions from already identified characteristic fragments could support tentative assignments.

### 3.7. Concept of a six-fold multiplex bioassay

Existing duplex [16,25,28] and triplex [28,29] planar bioassays have shown that information on several biological endpoints can be obtained on the same HPTLC plate surface from the same (only one) sample analysis. However, in order to comprehensively evaluate complex mixtures and take into account all relevant biological mechanisms, a differentiation of six responses was sought. The addition of biomarkers to assess cytotoxicity was newly introduced in the existing bioassays for endocrine activity. In addition to the two stripes (of agonist and end-product fluorescein) applied in the triplex pYAVAS [28] and pYAVES [29] bioassays, the K3 (cytotoxicant) and resorufin (chemical of the end-product of the enzyme–substrate reaction) were applied as two further stripes to differentiate between anti-cytotoxic effects (biological signal reduction) and false positive responses (physico-chemical response). Thus, altogether four stripes were applied along each separated sample track before the bioassay application (Fig. 4A). Therefore, the sample was applied as 28-mm band each, resulting in the analysis of six samples per 20 cm × 10 cm plate.

All stripes had to remain sharp during the bioassay incubation period to allow for good evaluation. Therefore, substance diffusion was reduced by zone fixation with a Degalan solution [28,29] (Fig. 4). This made the layer apolar and reduced zone diffusion in the polar bioassay environment. However, because the plate was no longer homogeneously water-wettable, a Tween solution was subsequently sprayed onto the plate, which functioned as a surfactant to improve the penetration of the following salt-rich aqueous bioassay into the layer. Note that the use of lower amounts of Degalan (0.1 % instead of 0.25 % Degalan solution) allowed better wettability, but also resulted in increased zone diffusion (Fig. S3).

For cytotoxicity detection (pYAVCytotox), the substrate resazurin indicating cell viability was added to the cell culture and applied as first substrate on the plate, followed by incubation and Vis/FLD detection (Fig. 4B). The second FDG substrate for the endocrine bioassay (pYAVAS or pYAVES) was applied on the same plate, again followed by Vis/FLD detection.

With many different multiplex bioassays performed in parallel for various projects in a laboratory, sound terminology is precondition for clear communication. The resulting six-fold multiplex bioassay workflow (Fig. 4) was termed HPTLC–Vis/FLD–pYAVCytotox–pYAVAS/pYAVES–Vis/FLD (as in already existing terms [28,29], p stands for **p**lanar, Y stands for the **y**east, A for **a**ntiandrogen, and V for **v**erified).

### 3.8. Proof of the six-fold multiplex bioassay concept using positive control standards

In the HPTLC–Vis/FLD–pYAVCytotox–pYAVAS/pYAVES–Vis/FLD six-fold multiplex bioassay workflow, the part of the pYAVAS or pYAVES bioassay were almost identical, except for the 1-h longer incubation for the pYAVAS bioassay (larger zone diffusion expected) and the different

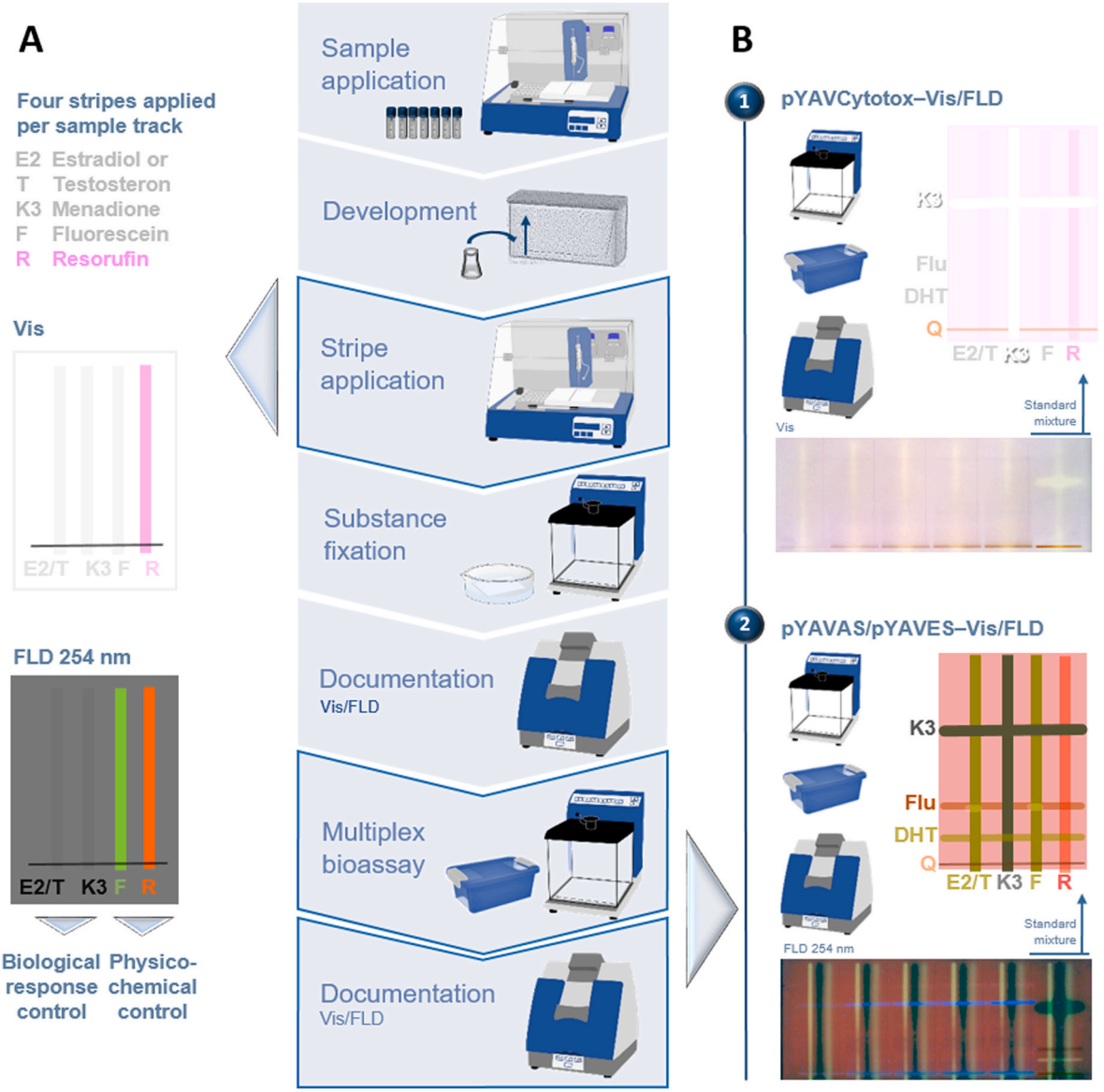


**Fig. 4.** Workflow steps of the HPTLC–Vis/FLD–pYAVCytotox–pYAAS/pYAES–Vis/FLD six-fold multiplex bioassay method, performed on the same planar surface for 6 samples in parallel; (**A**) applied stripe scheme as observed at Vis and FLD 254 nm; (**B**) biological detection after bioassay step **1** (pYAVCytotox–Vis/FLD) and step **2** (pYAVAS/pYAVES–Vis/FLD).

androgen or estrogen receptor in the yeast cell. Although a comparable performance was expected, the more challenging version with the pYAVAS bioassay was chosen for the performance proof. Four positive control solutions (K3, quercetin, DHT, and flutamide) commonly used in the respective *in vitro* bioassays [2, 37−40, 61] were applied in increasing amounts by spraying each solution on the same application zone to generate an oversprayed mixture (Fig. 5). The overspray application mode was used since it allowed individual adjustment of standard amounts depending on the response. After separation of the four positive control standards, the four stripes were applied along each track (Fig. 5A/B), followed by substance fixation via treatment with Degalan/Tween.

*3.8.1. Cytotoxic response evaluation (HPTLC–pYAVCytotox–Vis/FLD)*

The cell suspension containing the resazurin substrate was applied on the chromatogram, which was incubated at 30 °C for 4 h. The resulting first HPTLC–pYAVCytotox–Vis bioautogram was recorded at Vis (Fig. 5C). It showed cell-stressing colorless/white dihydroresorufin bands as well as stripes for the cytotoxicant K3 with a slightly cytotoxic orange resazurin center (Fig. 5C, framed highest K3 amount) on a metabolic active rose resorufin plate background. The cytotoxic potential of K3 can arise from oxidative stress, against which the cells can defend themselves by chemical reduction [58], which explains the reduction of resorufin to the colorless dihydroresorufin (as already observed for BPA in Fig. S2). The high K3 amount tested (30−900 ng/band) can therefore be lowered substantially. It is to mention that the presence of dihydroresorufin alone is not a marker for cytotoxicity, but it can point to cytotoxicity if higher amounts of a substance are applied. This was observed for the cytotoxic orange response (resazurin) in the K3 band center, enhanced in the response after the FDG substrate application (Fig. 5C/E). The respective bioautogram recorded at FLD 254 nm (Fig. 5D) showed the corresponding dark dihydroresorufin bands (indicating cell stress) with the dark blue fluorescent resazurin center (indicating cytotoxicity) on an orange-fluorescent resorufin plate

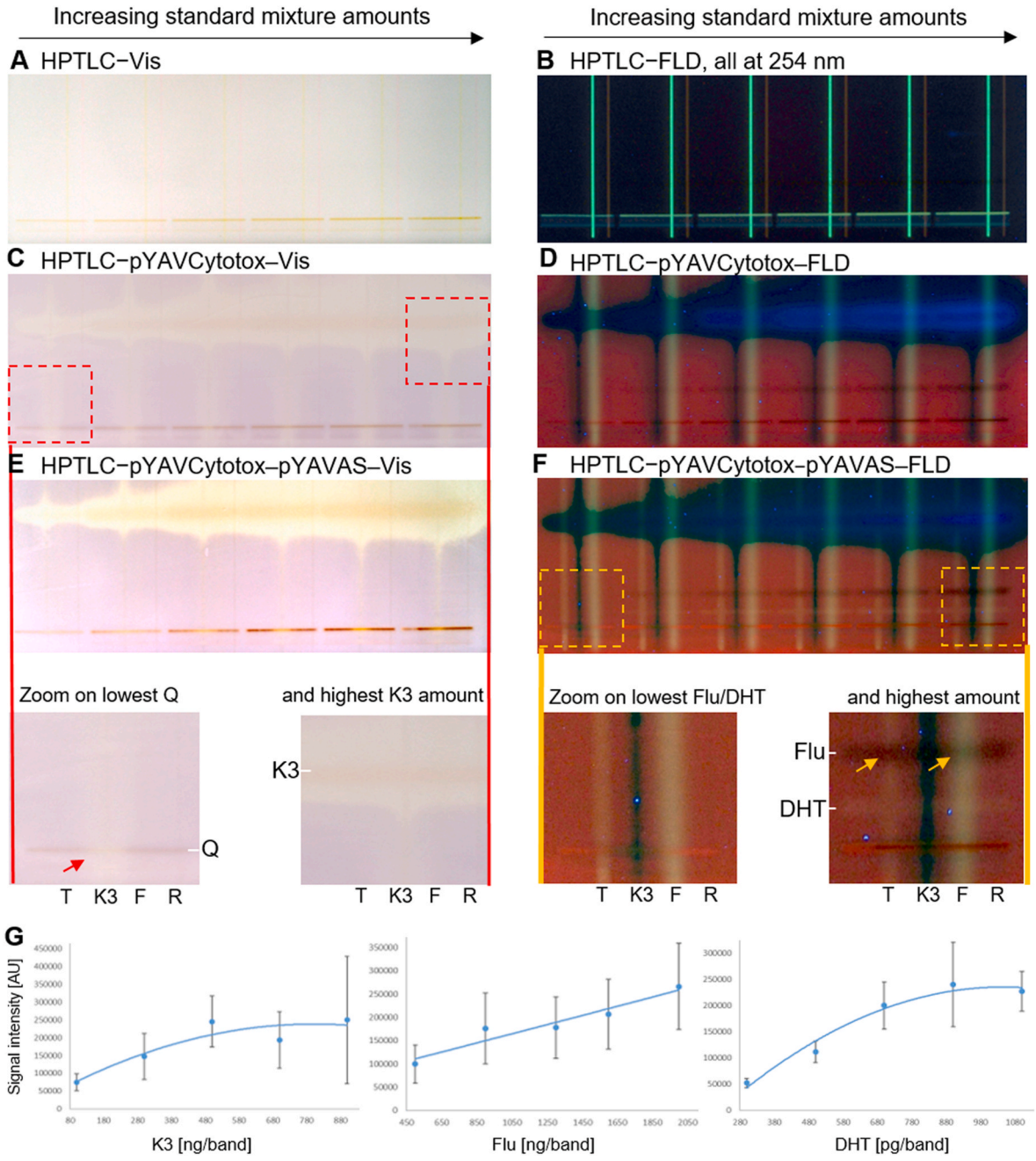


**Fig. 5.** Proof-of-principle of the HPTLC–Vis/FLD–pYAVCytotox–pYAAS/pYAES–Vis/FLD six-fold multiplex bioassay method: Chromatograms/bioautograms at (**A**/**C**/**E**) Vis and (**B**/**D**/**F**) FLD 254 nm of the positive control standards cytotoxicant K3 (30–900 ng/band, 0.3–9 μL, 100 ng/μL), cell viability protective acting quercetin (Q, 700–1700 ng/band, 0.7–1.7 μL), antiandrogen flutamide (Flu, 100–2000 ng/band, 0.1–2.0 μL), and androgen DHT (100–1100 pg/band, 1.0–11 μL) applied (oversprayed on the same start zone) and separated as in Fig. 1; four stripes (testosterone T 4 μL, K3 3.2 μL, fluorescein F 2 μL and resorufin R 2 μL) applied on each separated track, followed by the bioassay performance; zoom on the lowest and highest amount of the standards applied with arrows pointing to region of interest as discussed; (**G**) respective dose–response curves determined via videodensitometry.

background.

Anti-cytotoxic substances able to reduce the cytotoxic effects were detectable via the applied K3 stripe. Any substance capable of protecting against cytotoxic effects, in particular oxidative cell stress (cell viability shelter), should reduce the signal response of the K3 stripe. The substance quercetin was selected as a positive control because it was described as beneficial to protect against cytotoxic effects [59]. At low quercetin amounts (700 ng/band), the colorless cytotoxic K3 stripe was not reduced by quercetin (Fig. 5C, zoom, arrow). However, for increasing amounts, the quercetin increasingly mitigated the oxidative cell stress, and the colorless cytotoxic K3 stripe was reduced by the quercetin. Thus, the effect-dose dependency of the protective effect of quercetin seemed to be evident. However, the originally yellow quercetin (Fig. 5A) showed a brown color in the bioautogram which could mislead the evaluation. In future, another positive control standard should be selected without color formation and eluting to a higher $hR_F$ value in the bioautogram (quercetin is currently located at the start zone with the given mobile phase system).

As proof for false positive responses and *vice versa* for verification of anti-cytotoxicity (*e.g.* protective cell viability effects), the quercetin was evaluated for any physico-chemical influence on the resorufin end-product stripe of the dehydrogenase−resazurin (enzyme−substrate) reaction (Fig. 5C). An influence was not detectable. However, the resorufin end-product stripe (2 μL) was hardly visible, though detectable via image enhancement, and should be increased to 4 μL in future. To conclude, via this first bioautogram at Vis (Fig. 5C), three respective responses were detectable, *i.e.* any cytotoxicants (colorless band with orange center for K3), anti-cytotoxicants (decrease of the colorless/white K3 stripe with increasing quercetin amounts), and false-positive responses (decrease of the resorufin end-product stripe).

#### 3.8.2. Endocrine response evaluation (HPTLC–pYAVCytotox–pYAVAS–FLD)

Subsequently, the FDG substrate solution was applied on the same plate. After a short incubation, the resulting HPTLC–pYAVCytotox–pYAVAS–FLD bioautogram at FLD 254 nm was evaluated for three respective endocrine responses (Fig. 5F). The normally expected green fluorescein fluorescence changed to ochre due to the previously formed orange-fluorescent resorufin end-product already present on the layer (Fig. 4, orange + green → ochre). As expected, the androgen DHT, used as positive control in the androgen screening of packaging migrates [2], was detected as an ochre-fluorescent band. The testosterone agonist stripe for detection of antiandrogens was also ochre-fluorescent. Flutamide, used in *in vitro* assays [60,61], was selected and applied as a positive control for the antiandrogen effect, detected as fluorescence decrease of the testosterone stripe (Fig. 5F, zoom, left arrow). Any false-positive antiandrogenic response (physico-chemical fluorescence reduction) was verified via the vertically applied respective fluorescein end-product stripe. A substantial decrease of the ochre-fluorescent fluorescein stripe was not observed for flutamide in the bioautogram, which confirms the substance being a true anti-androgen according to literature [60,61]. However, flutamide altered the fluorescein hue at higher amounts of 2 μg/band (Fig. 5F, zoom, right arrow), which needs further attention.

These multiplex bioautograms were repeated twice and thus performed as biological triplicate, *i.e.* obtained by three different cell cultures on three different days (Fig. S5). The dose-response curves of the positive control standards (Fig. 5G) showed with increasing amounts an increase in the response of the positive control standards cytotoxicant K3, androgen antagonist flutamide, and androgen agonist DHT with acceptable respective determination coefficients $R^2$ of 0.8431, 0.9197, and 0.9721. The dose-response curve of quercetin was not determined since it remained almost at the start zone In the given mobile phase system. The performance of the new six-fold bioassay was more challenging due to the increased complexity (number of workflow steps). A benchmarking of the performance data of the new six-fold bioassay with simplex [14,18,60,61], duplex [16,25] or triplex [28,29] bioassays of other studies is not possible due to the use of different stationary phases, mobile phases, number of assay steps, and other parameters. All in all, the first proof of concept for the new six-fold multiplex bioassay was considered successful.

### 3.9. Application of the HPTLC–Vis/FLD–pYAVCytotox–pYAVAS–Vis/FLD six-fold multiplex bioassay method to migrate 39

Migrate concentrate 39 showing the antiandrogenic bands **2**−**5** in the duplex HPTLC–pYAAS–FLD bioautogram (Fig. 2A) was selected as sample for application of the six-fold multiplex HPTLC–Vis/FLD–pYAVCytotox–pYAVAS–Vis/FLD method. Five different volumes of this sample were applied on the plate (Fig. 6A/B, Files S1 and S2) as well as the positive controls (standard mixture of quercetin, DHT, flutamide and K3) for proof of the bioassay performance. After development and application of the four vertical stripes (Fig. 4A) along each separated sample track, the plate was treated with Degalan/Tween and the bioassay was performed.

In the HPTLC–pYAVCytotox–Vis bioautogram (Fig. 6C), the positive control K3 a colorless/white band as expected. Its amount (20 ng/band) could be further reduced in the future due to the strong response. No orange or colorless/white cytotoxic band was found for the studied migrate concentrate 39. It means that the antiandrogenic bands **2**−**5** in the migrate concentrate 39 were true antiandrogens and not caused by cytotoxicity in the given dose-response range. Interestingly, the lower half of the colorless/white (dihydroresorufin) K3 stripe, equivalent to the area of bands **2**−**5**, was reduced in intensity, indicating anti-cytotoxic effects, with increasing migrate amounts. This was confirmed by the decrease of the dark (dihydroresorufin) K3 stripe in the bioautogram at FLD 254 nm (Fig. 6D/F). The fluorescence of the red-fluorescent resorufin stripe was not increased at these positions, and by this, any physico-chemical false-positive responses were excluded.

In the HPTLC–pYAVCytotox–pYAVAS–FLD bioautogram (Fig. 6F), the four antiandrogenic bands **2**−**5** were observed as decrease of the ochre-fluorescent T stripe in a dose-response dependence. This was in agreement with the duplex HPTLC–pYAAS–FLD bioautogram (Fig. 2A) used as reference for comparison. In contrast, the ochre-fluorescent F stripe showed no signal decrease, which excluded false-positive responses and thus verified the bands **2**−**5** to be true antiandrogens.

Compared to the status quo, which uses six different *in vitro* assays along with bioassay-guided fractionation, analytical separation by column chromatography, bioassay-testing of each peak, and HRMS recording, the new workflow requires substantially less effort, time, and material. As miniaturized all-on-the-same-surface concept, it is less prone to contamination. However, the six-fold multiplex bioautogram showed a loss in sensitivity (35 μL per 28 mm band) if compared to the duplex bioassay (5 μL per 8 mm band). About the double sample volume was used for the six-fold multiplex bioassay (Fig. 6F, zoom). This was explained by the known signal reduction caused by the Degalan treatment [28,29]. The signal reduction was observed for part of the compounds present in a sample but not necessarily for all [62]. However, the zone fixation via Degalan was crucial and key to success to avoid zone diffusion caused by the increased workflow steps of the six-fold multiplex bioassay.

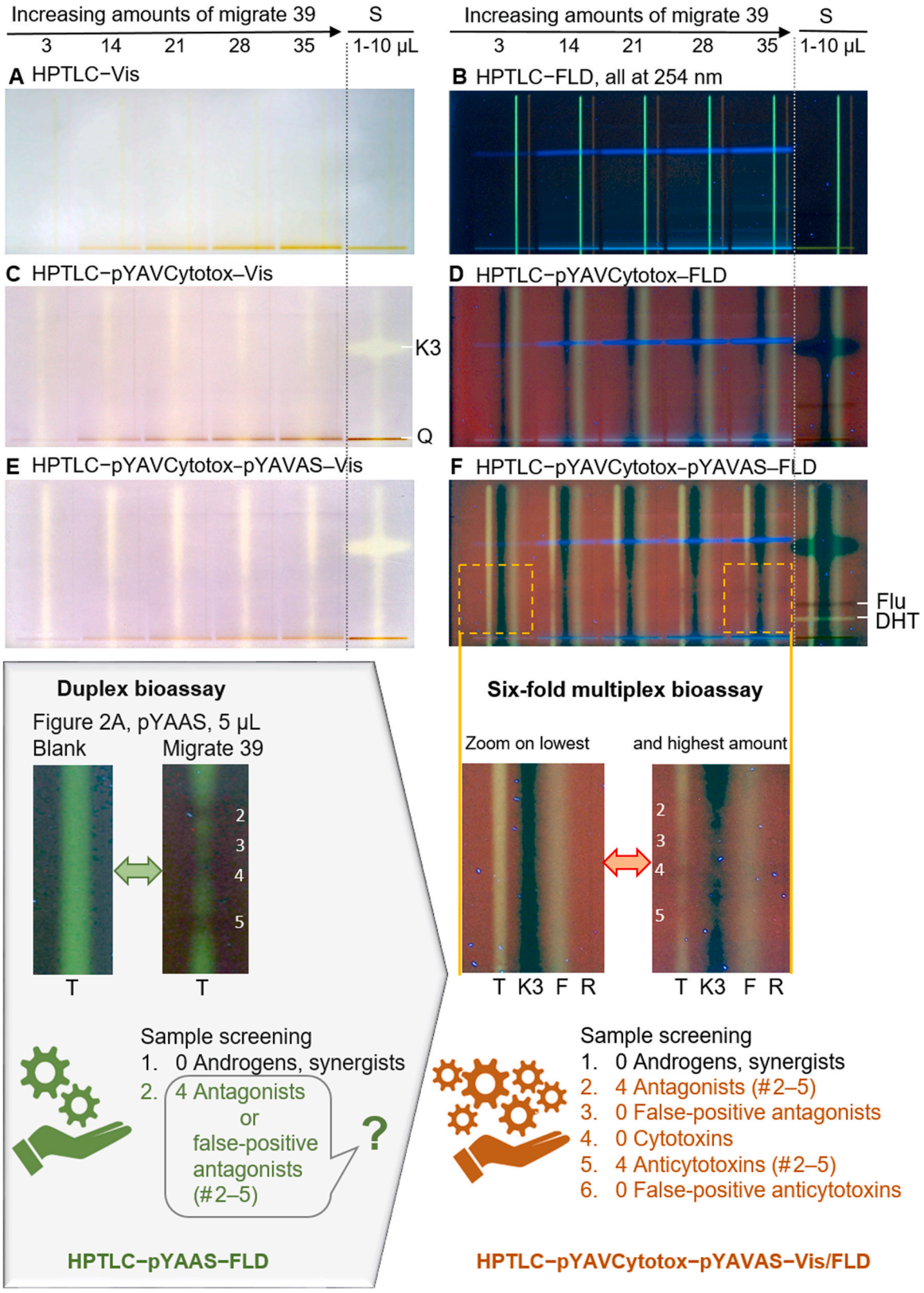


**Fig. 6.** Application of the HPTLC–Vis/FLD–pYAVCytotox–pYAAS/pYAES–Vis/FLD six-fold multiplex bioassay method (Files S1 and S2): Chromatograms/bio-autograms at (**A**/**C**/**E**) Vis and (**B**/**D**/**F**) FLD 254 nm of the migrate concentrate 39 at increasing amounts (3–35 µL/band) and the positive control mixture (S) with quercetin (Q, 1 µg/band, 1 µL), DHT (1 ng/band, 10 µL); flutamide (Flu, 1 µg/band, 10 µL) and K3 (20 ng/band, 2 µL of 10 ng/µL solution), analyzed as in Fig. 5.

## 4. Conclusions

For the first time, a six-fold multiplex bioassay was demonstrated as promising tool in the control of food contact materials for endocrine active compounds. All important endocrine information (agonist, antagonist, cytotoxicant, anti-cytotoxicant and respective false positive responses) was obtained on the same plate surface from the same sample analysis (all-on-the-same-surface concept). The compact bioautogram allowed for the fast differentiation of effect mechanisms in a complex sample, which is important for a profound decision on a sample. The HPTLC–Vis/FLD–pYAVCytotox–pYAAS/pYAES–Vis/FLD six-fold multiplex bioassay method was successfully applied to different positive controls and one tin can migrate. Prominent endocrine active compound zones were detected and tentatively assigned to molecular formulas via HPTLC−FIA−HESI-HRMS of pooled eluted sample zones. In contrast, HPTLC−HESI-HRMS of single sample zones did not provide clear HRMS signals. The tentative assignments can be proven by future HRMS/MS fragmentation and co-elution of standards or standard addition. The structural assignment of poorly ionizable and/or trace-level endocrine disruptors, though strong in the biological signal intensity, remained a challenge. The biological effect detection is often superior in sensitivity compared to sophisticated HRMS detection. It highlights the often overlooked discrepancy in detection mechanisms and mismatch between bioassay result and HRMS assignment. For the first time, the on-surface S9 metabolization was integrated in the pYAAS/pYAES duplex bioassays and showed a reduction of the measured endocrine signals. In the future, the metabolic activation/deactivation via the S9 enzyme system can be performed and integrated in the six-fold multiplex bioassay concept, as the workflow steps are identical whether with or without S9 metabolization. The streamlined stripe concept for multiplex planar bioassays is generic and can be transferred to other assays with other microorganisms, substrates, and positive controls, to extend the range of applications.

## CRediT authorship contribution statement

**Daniel Meyer:** Writing – original draft, Methodology, Investigation, Formal analysis. **Gertrud E. Morlock:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Formal analysis, Conceptualization.

## 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.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2024.126174.

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