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ABSTRACT

In mass spectrometry imaging (MSI), ion suppression can lead to a misinterpretation of results. Particularly phospholipids, most of which exhibit high gas-phase basicity (*GB*), are known to suppress the detection of metabolites and drugs. This study was initiated by the observation that the signal of an herbicide, i.e., atrazine, was suppressed in MSI investigations of earthworm tissue sections. Herbicide accumulation in earthworms was investigated by time-of-flight secondary ion mass spectrometry and matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI). Additionally, earthworm tissue sections without accumulation of atrazine but with a homogeneous spray deposition of the herbicide were analyzed to highlight region-specific ion suppression. Furthermore, the relationship of signal intensity and *GB* in binary mixtures of lipids, amino acids, and atrazine was investigated in both MSI techniques. The *GB* of atrazine was determined experimentally through a linear plot of the obtained intensity ratios of the binary amino acid mixtures, as well as theoretically. The *GB*s values for atrazine of 896 and 906 kJ/mol in ToF-SIMS and 933 and 987 kJ/mol in MALDI-MSI were determined experimentally and that of 913 kJ/mol by quantum mechanical calculations. Compared with the *GB* of a major lipid component, phosphatidylcholine (*GB*_{PC} = 1044.7 kJ/mol), atrazine's experimentally and computationally determined *GB*s in this work are significantly lower, making it prone to ion suppression in biological samples containing polar lipids.

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I. INTRODUCTION

Understanding ionization mechanisms is and has been a major research focus in the field of mass spectrometry. This is because analyte ionization ultimately dictates ion yields and influences all mass spectrometric figures of merit. In secondary ion mass spectrometry (SIMS) and matrix-assisted laser desorption/ionization (MALDI), a desorption/ionization (DI) mechanism is usually postulated for organic analytes.^{1–3} Hereby, after an impact of high-energetic primary ions in SIMS or laser irradiation in MALDI, the molecules first desorb and are ionized by different mechanisms in the seldge close to the surface before they follow trajectories toward the mass analyzer. For organic analytes, cationization takes place most probably by protonation via proton transfer from other molecules.⁴ Alternatively, alkali adducts can be formed. The period for ionization is short in the range of pico- to nanoseconds. The ionization mechanism and efficiency usually depend on the chemical properties of the analyte molecule, the chemical surrounding, and possible surface coverage. The last two factors are called “matrix effect.” Despite several decades of research, the distinct ionization mechanisms have not been fully understood.

Ionization efficiency depends on many factors but is mostly affected by the chemical environment, i.e., the matrix of the sample. In biological samples, chances for encountering substances that interfere in the ionization process are high. Sterner *et al.* showed that macromolecules such as large peptides, proteins, and enzymes may suppress the signals of smaller molecules.⁵ In addition to macromolecules, some phospholipid classes have been shown to cause the so-called ion suppression of less basic phospholipids in binary mixtures.⁶ To overcome ion suppressive effects (ISEs) in quantitative mass spectrometry of liquid samples, liquid chromatography (LC) is used as a separation tool before ionization to separate interfering substances from analytes.^{7–11} Despite the advanced chromatographic separation methods for biological samples, interferences between sample components still occur during the ionization process.¹² However, problems of this kind can often be eliminated by suitable sample preparation and adjustment of the settings of chromatography and ionization source.¹³

For solid samples, MSI has become an important tool to localize and identify drugs and metabolites in tissue sections.^{14,15} In these experiments, the influence of the matrix on ionization is of particular importance because the sample components cannot be separated by chromatography before ionization.^{16,17} To get rid of interfering substances, washing steps can be used, but these might alter the sample and dislocate analytes. Therefore, ISEs in all areas of the pristine tissue sections should be investigated and corrected by appropriate methods.^{18,19} A common approach is the preparation of untreated samples that are homogeneously spiked with the targeted analyte to identify differences between ionization efficiency in different sample areas. These reference values can be used to correct region-specific differences in ionization efficiency.^{20,21}

Phospholipids can cause ion suppression in both positive- and negative-ion modes. In positive-ion mode, one important factor influencing ion suppression is the high gas-phase basicity (*GB*) of phospholipids relative to values for other endogenous and exogenous metabolites, possibly hampering the protonation of analytes.²² The relation between *GB* and signal intensity of binary mixtures in

particle-induced ionization methods was first investigated in 1986 by Sunner *et al.*²³ Lately, matrix effects in ToF-SIMS of amino acid mixtures have been related to *GB*.²⁴ Previous studies about charge distribution in phospholipid samples have shown severe ion suppression due to the presence of phosphatidylcholine.⁶

The theoretical basis of the correlation of *GB* and ion suppression stems from the hypothesis that a competition between two bases in the gas phase after desorption/ionization will determine the charge carrier distribution. As already stated, the dwell time of molecules and especially of ions in the seldge of the sample is comparatively low. If ions are formed, they will be accelerated by the electric field and extracted from the seldge region. There is usually not enough time for chemical equilibrium to occur. In MALDI, there is definitely more time for proton exchange since the extraction voltage is usually applied with a time delay with respect to the desorption process. In addition, the fact that MALDI is carried out at atmospheric pressure in contrast to ultrahigh vacuum (SIMS) allows a longer time to achieve equilibrium in the MALDI laser plume. However, for the commonly used *GB* model, chemical equilibrium is assumed for the following proton competition equation between analyte molecule *B*₁ and suppressive molecule *B*₂ [Eq. (1)]. The model assumes second-order reactions for the forward and the reverse reactions,



With respect to the law of mass action, the equilibrium constant *K*_{eq} of this equation is as follows:

$$K_{eq} = \frac{[B_1H^+] \cdot [B_2]}{[B_1] \cdot [B_2H^+]} = \frac{k_2}{k_1}. \quad (2)$$

The Gibbs free energy of this proton exchange is given by the difference of the *GB*s of the two reactants [Eq. (3)]. Hereby, we assume equal starting amounts of *B*₁ and *B*₂ and a quasistatic process for the proton exchange,

$$GB_{B_1} - GB_{B_2} = -\Delta rG^\circ = RT \ln K_{eq} = RT \ln \frac{k_2}{k_1}. \quad (3)$$

The ratio of the rate constants *k*₂ and *k*₁ of protonation is set equal to the ratio of the observed signal intensities of the two analytes in experiment 2 [Eq. (4)]. Here, we assume that the proportion of protonated species is low overall and that the unprotonated analyte molecules are correspondingly in excess,

$$RT \ln \frac{k_2}{k_1} = RT \ln \frac{I_{B_1H^+}}{I_{B_2H^+}} = GB_{B_1} - GB_{B_2}. \quad (4)$$

This results in a linear relationship between the *GB*s and the natural logarithm of the two protonated competing molecules' intensity ratio. If the measurements reveal a linear relationship between those two values, proton exchange and thermodynamic equilibrium inside the desorption plumes of both ionization methods are likely.

In this work, we investigate the uptake of the herbicide atrazine in earthworms via SIMS and MALDI-MSI as a test case to (a)

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identify the impact of the presence of phospholipids on the analyte signal in both MSI methods; (b) study differences in ion suppression caused by phospholipids in MALDI and SIMS for the same sample; and (c) estimate whether the ion suppression in biological samples is in line with *GB* differences between the phospholipids and the model analyte. For this purpose, an MSI analysis of samples homogeneously covered with atrazine and earthworms exposed to atrazine was conducted to reveal the ionization efficiency of the different areas of the earthworm thin sections. To estimate the impact of *GB* differences between phospholipids and atrazine on the ion suppression effect, theoretical and experimental *GB* values were determined and connected to the analyte signal suppression in earthworm samples.

II. EXPERIMENT

A. Preparation of earthworm samples with accumulation of atrazine

Atrazine (2 mg, PESTANAL[®] analytical standard, Merck KGaA, Darmstadt, Germany) in acetonitrile (2 mL, solvent standard) was placed in Petri dishes and the acetonitrile was removed by evaporation. Two adult earthworms (*Eisenia fetida*) with well-developed clitellum were gently rinsed with demineralized water and then placed in the atrazine-prepared Petri dishes after addition of 2 mL demineralized water. After 20 h of potential uptake of atrazine by the earthworms, the worms were removed from the Petri dishes, rinsed again with demineralized water, and sacrificed at -20°C in a freezer. Afterward, a ~ 2 mm section was cut from the middle part of an earthworm and embedded in an 8 vol. % gelatine solution at -20°C . Sections of $20\text{ }\mu\text{m}$ thickness of the embedded piece were prepared with a cryotome (Thermo Scientific Microm HM 525 Kryostat, Microm International GmbH, Walldorf, Germany) and thawed on glass slides at -20°C . Afterward, the sections were stored at -80°C .

B. Preparation of earthworm samples with homogeneous deposition of atrazine

For the second experiment, adult *E. fetida* were kept in glass Petri dishes for 20 h in 2 mL demineralized water, but in this case without prior addition of atrazine. Afterward, thin sections of the earthworms were prepared in the same way as described in this section and stored at -80°C . Before MSI measurements, a homogeneous layer of atrazine was deposited on the worm sections that had not previously been exposed to atrazine. For this purpose, $100\text{ }\mu\text{L}$ of a 1 mM atrazine solution was applied at a flow rate of $10\text{ }\mu\text{L}/\text{min}$ using a home-built pneumatic spraying system under nitrogen flow (similar to SMALDIPrep, TransMIT GmbH, Giessen, Germany).²⁵

C. Preparation of amino acid and phospholipid standards

Solutions of amino acids alanine, serine, threonine, tryptophan, lysine, and arginine (Sigma-Aldrich, St. Louis, Missouri, USA) with a concentration of 1 mM were prepared in ethanol/water mixtures (1:1, solvent standard) with 2% acetic acid. In addition, 1 mM phospholipid solutions of phosphatidic acid PA (18:0/18:0) (sodium salt),

phosphatidylethanolamine PE (18:0/18:0), and phosphatidylcholine PC [16:0/20:4(5Z,8Z,11Z,14Z)] (Avanti Polar Lipids Inc., Alabaster, AL, USA) were prepared in chloroform (solvent standard). Subsequently, individual amino acid solutions were mixed with respective lipid solutions in a ratio of 1:1. Furthermore, mixtures in a ratio of 1:1 of the individual lipid solutions were prepared with a 1 mM atrazine solution.

D. ToF-SIMS

All SIMS measurements were performed on an M6 Hybrid SIMS (IONTOF GmbH, Münster, Germany) using the ToF analyzer. Earthworm sections were thawed in a desiccator and then placed on a temperature-controllable SIMS sample holder using a double-sided copper tape. Before evacuation, the prechamber was flooded with nitrogen and the sample was cooled to -100°C . The subsequent measurements in the main chamber were carried out at -155°C . Spectrometry (hybrid) mode was selected as the analyzer setting and measurements were performed in positive-ion mode. More details on the mode can be found in Kern *et al.*²⁶ Analysis was carried out with the bismuth nanoprobe with 60 keV Bi_3^{+} ions, 0.28 pA primary ion current, $200\text{ }\mu\text{s}$ cycle time, and $700\text{ }\mu\text{m}$ aperture. For the depicted earthworm section of the first series of experiments (accumulation of atrazine over 20 h in living earthworms), a stage scan of an area of $4 \times 4\text{ mm}^2$ with a total of 800×800 pixels and a resulting pixel size of $5\text{ }\mu\text{m}$ was acquired. The mass resolution of the protonated atrazine signal $[\text{M} + \text{H}]^{+}$ at m/z 216.10 was $R > 11\,000$ (FWHM). For the second series of experiments (homogeneous distribution of atrazine on untreated earthworm sections), the primary ion current of the primary ion beam was 0.22 pA at a cycle time of $180\text{ }\mu\text{s}$. The stage scan of the second series of experiments resulted in an area of $3.2 \times 2.8\text{ mm}^2$ containing 640×560 pixels. The mass resolution of the protonated atrazine signal $[\text{M} + \text{H}]^{+}$ at m/z 216.10 was $R > 14\,000$ with ± 10 ppm mass deviation. All data analyses were performed with SURFACE LAB 7.3 software (IONTOF GmbH, Münster, Germany).

Amino acid and phospholipid standard solutions were deposited on PTFE-coated glass slides using pipette tips (volume $100\text{--}1000\text{ }\mu\text{L}$, Eppendorf AG, Hamburg, Germany) and dried. Subsequently, a 4 nm-thick layer of platinum was deposited on the slide using a Leica EM ACE600 high-vacuum sputter coater (Leica Microsystems GmbH, Wetzlar, Germany). The glass slide with the prepared standards was placed on a temperature-controllable SIMS sample holder using a double-sided copper adhesive tape. Before evacuating the antechamber of the SIMS instrument, the antechamber was flooded with nitrogen and the sample was cooled to -100°C . The subsequent measurements in the main chamber of the instrument were conducted at -155°C . Spectrometry mode was selected as the analyzer setting and measurements were carried out in the positive-ion mode. Ions with 60 keV Bi_3^{+} with 0.31 pA primary ion current were used as analysis species in the spectrometry mode of the primary ion gun. The cycle time was $175\text{ }\mu\text{s}$ and the measurements were terminated when a primary ion dose of 3×10^{11} primary ions/ cm^2 was reached to maintain static conditions. The average mass resolution of the protonated atrazine signal $[\text{M} + \text{H}]^{+}$ at m/z 216.10 was $R > 8000$ with ± 20 ppm mass deviation. Areas of $200 \times 200\text{ }\mu\text{m}^2$ with 128×128 pixels were measured.

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For the sake of improved statistics, the measurement pixels were binned to 8×8 pixels and a pixel-by-pixel analysis was performed for each measurement.

E. MALDI-MS

Earthworm sections were thawed in a desiccator and covered with 100 μ L of a 2,5-dihydroxybenzoic acid solution (DHB, Merck KGaA, Darmstadt, Germany) with a concentration of 30 mg/ml in a 1:1 acetone/water ratio with 0.1% trifluoroacetic acid (Merck KGaA, Darmstadt, Germany). The matrix was deposited with a flow rate of 10 μ L/min under nitrogen flow using a self-constructed pneumatic sprayer. All MALDI-MS measurements of earthworm sections and standards were performed on an AP-SMALDI5 AF ion source (TransMIT GmbH, Giessen, Germany) attached to a Q Exactive HF mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) in positive-ion mode. For the sections of the first series of experiments (accumulation of atrazine over 20 h in living earthworm), a measurement area of 4×4 mm² was defined with a lateral resolution of 20 μ m and a resulting pixel number of 200×200 pixels. The measurement was performed in the positive-ion mode with 3D autofocus and a mass range of m/z 80–900 with a mass resolution of $R = 240\,000$ at m/z 200.²⁷ Internal mass calibration was performed with DHB clusters and mass deviations were in the range of ± 2 ppm. The laser energy was adjusted by a 20% filter and a variable attenuator module (setting 15°). For the section of the second series of experiments (homogeneous distribution of atrazine on untreated earthworm sections), an area of 3.0×3.5 mm² was measured with 120×140 pixels and a resulting pixel size of 25 μ m. The measurement was performed in the 2D mode of the ion source.²⁷ The data were visualized with MIRION software version 3.3.64.15 (TransMIT GmbH, Giessen, Germany).

Amino acid and phospholipid standard solutions were mixed with the MALDI matrix solution in a 7:3 ratio and dropped onto PTFE-coated diagnostic slides as described in Sec. II D. Sample

areas of 600×300 μ m² with a number of 20×10 pixels were measured in 2D mode.²⁷ The laser energy was regulated by inserting a 20% filter in the laser beam path and setting the attenuator to 10°. Data analysis was performed with the software XCALIBUR™ (version 4.3, Thermo Fisher Scientific Inc., Waltham, USA). Similar to ToF-SIMS data, a pixel-by-pixel evaluation was performed to increase the statistical value of the data. Due to the detection limit of the Orbitrap analyzer, only those pixels that showed a signal for both analytes were evaluated.

F. Computational methods

Conformers of neutral and protonated amino acids and atrazine, respectively, were generated using Grimme's conformer-rotamer ensemble sampling tool (CREST, v2.11.3). Either the ten energetically most favorable conformers or all conformers within a window of 2 kcal/mol was taken and their geometry optimized at the DSD-PBEP86/def2-TZVPP level of theory as available in ORCA v5.0.3. Furthermore, enthalpic and entropic contributions to the Gibbs energy were computed for the gas phase under standard conditions of 1013 hPa and 298 K. The so-obtained Gibbs energies were subjected to a Boltzmann weighting scheme for the conformer equilibrium and the weighted results were then used for the calculation of mean gas-phase basicities and corresponding errors.

III. RESULTS

A. Earthworm sections with accumulation of atrazine in living earthworm

ToF-SIMS measurements of earthworm sections, which were exposed to atrazine for 20 h, are shown in Fig. 1. Elevated intensities of protonated atrazine ($C_8H_{15}N_5Cl^+$; m/z 216.10) are associated with the inner worm area [Fig. 1(b)]. However, the analyte signal is present only in the central region of the tissue and not in the skin or outer musculature of the worm. Moreover, no atrazine signal

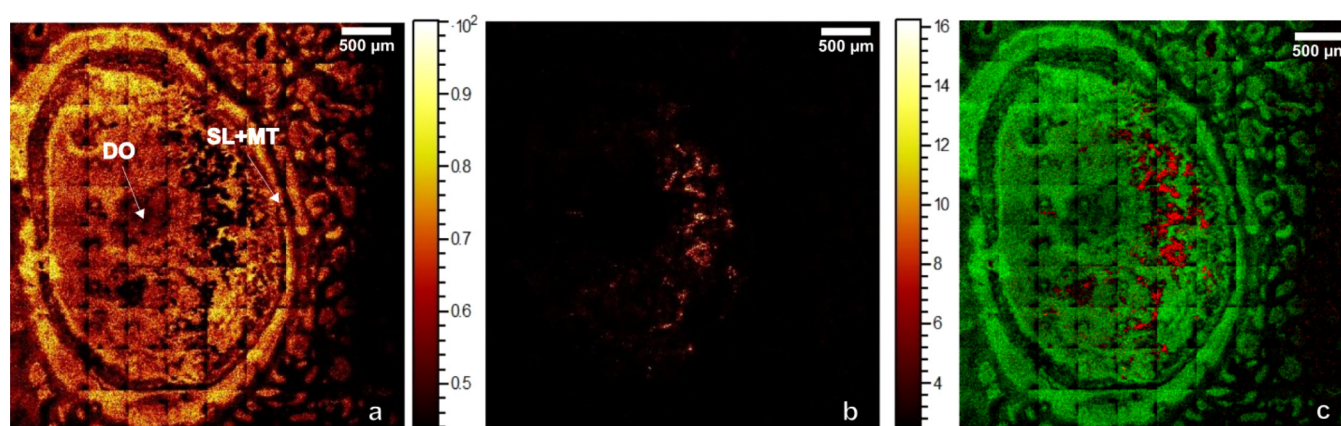


FIG. 1. ToF-SIMS mass images of an atrazine accumulation experiment over 20 h. (a) Distribution of $C_8H_{19}NO_4P^+$ signal (m/z 224.10). DO, digestive organ, MT, muscle tissue, and SL, skin layer. (b) Distribution of the protonated atrazine signal ($C_8H_{15}N_5Cl^+$; m/z 216.10) in an earthworm section. (c) RG image of an earthworm section showing an atrazine signal (red: $C_8H_{15}N_5Cl^+$; m/z 216.10) and $C_8H_{19}NO_4P^+$ signal (green: m/z 224.10).

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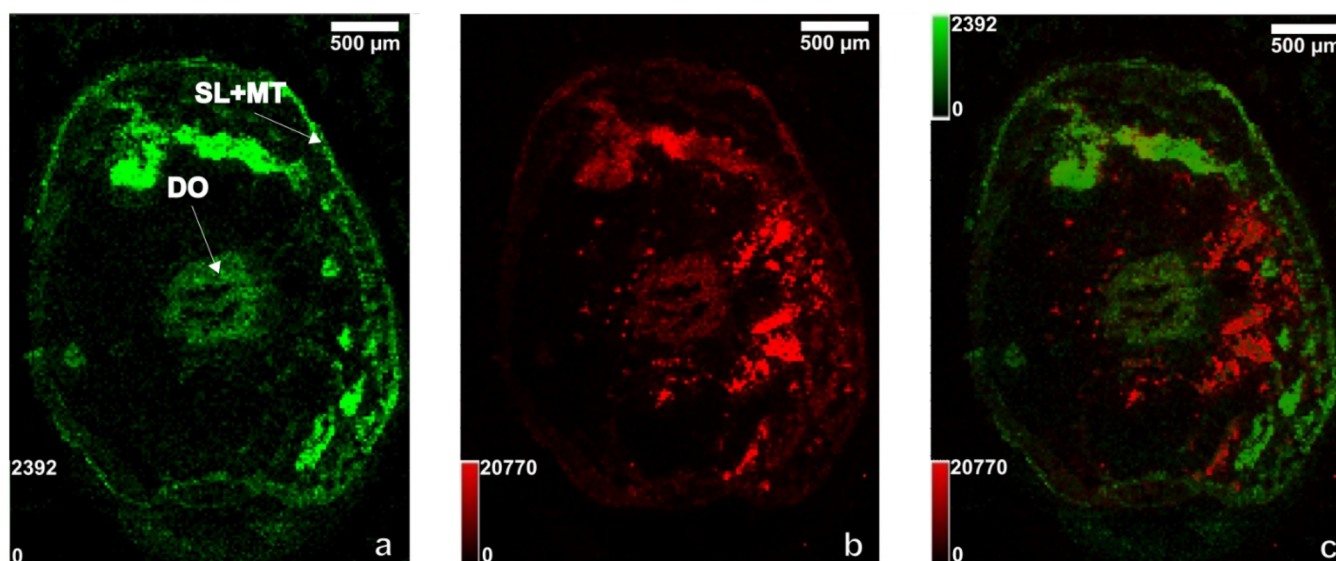


FIG. 2. MALDI mass images of an atrazine accumulation experiment over 20 h. (a) Distribution of the $C_8H_{19}NO_4P^+$ signal (m/z 224.1046) in an earthworm section. DO, digestive organ, MT, muscle tissue, and SL, skin layer. (b) Distribution of an atrazine signal ($C_8H_{15}N_5Cl^+$ at m/z 216.1011). (c) RG image of an atrazine signal (red: $C_8H_{15}N_5Cl^+$, m/z 216.1011) and a PC headgroup (green: $C_8H_{19}NO_4P^+$, m/z 224.1046).

was detected in the region of the worm's digestive organ. Complementary to the atrazine signal is the signal of the phosphatidylcholine (PC) fragment $C_8H_{19}NO_4P^+$ at m/z 224.10, indicating the presence of phospholipids. Inside the earthworm section, certain areas²⁷ exhibited no or only low ion signal intensity of $C_8H_{19}NO_4P^+$ [conf. Fig. 1(a)]. A comparison with Fig. 1(b) shows that these sites with missing m/z 224.10 signal coincide with regions in which protonated atrazine was detected [Fig. 1(c)]. The PC signal detected in areas around the earthworm is most probably

related to surface diffusion of the lipid and the high surface sensitivity of ToF-SIMS analysis. These results indicate that the presence of phospholipids potentially affects the signal intensity of atrazine because we expect the analyte to be present in the digestive tract and/or skin due to atrazine exposure, but the analyte signal is observed only in the regions of no or low PC head group signals.

In addition to SIMS imaging, thin worm sections were analyzed by MALDI-MSI (see Fig. 2). Compared with the ToF-SIMS results, the herbicide was detected not only in areas of low PC head

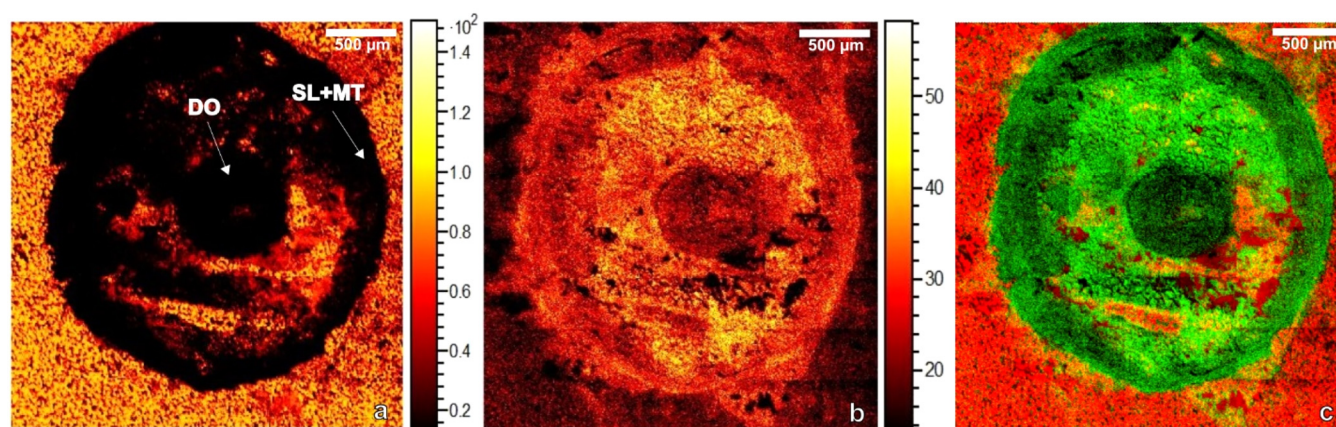


FIG. 3. ToF-SIMS images of an untreated worm section after a homogeneous spray deposition of atrazine. (a) Distribution of a protonated atrazine signal ($C_8H_{15}N_5Cl^+$; m/z 216.10) on an earthworm section. DO, digestive organ, MT, muscle tissue, and SL, skin layer. (b) Distribution of a PC headgroup $C_8H_{19}NO_4P^+$ signal (m/z 224.10). (c) RG image of an earthworm section showing an atrazine signal (red: $C_8H_{15}N_5Cl^+$; m/z 216.10) and a $C_8H_{19}NO_4P^+$ signal (green: m/z 224.10).

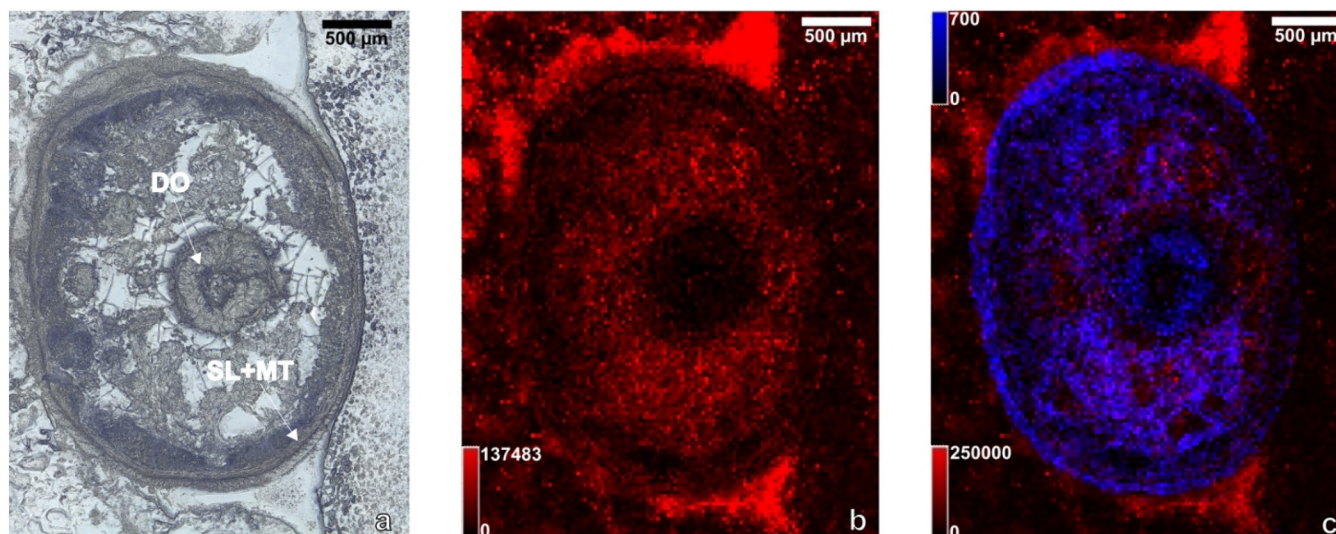


FIG. 4. MALDI results of an untreated worm section after a homogeneous spray deposition of atrazine. (a) Digital microscopy image of an earthworm section. DO, digestive organ, MT, muscle tissue, and SL, skin layer. (b) Distribution of an atrazine signal (red: $C_8H_{15}N_5Cl^+$; m/z 216.1011). (c) RB image of an earthworm section showing an atrazine signal (red: $C_8H_{15}N_5Cl^+$; m/z 216.1011) and a phospholipid signal (blue: $C_{40}H_{78}NO_7P^+$, PC O-32:2, m/z 716.5591).

group abundance but also in the skin and digestive tracts of the section. Still, highest intensities of the protonated atrazine signal were detected in the regions without PC abundance. When comparing the ToF-SIMS and MALDI-MSI results, it must be noted that significantly more sample material was ablated in MALDI, leading to higher ion currents and therefore possible higher sensitivities in MALDI measurements. However, in MALDI measurements, a low PC signal (green) was detected in the gelatine area around the earthworm in comparison with the ToF-SIMS measurements

(Fig. 1). In contrast to the SIMS MSI data, PC was not detected in the region of the gelatine embedding. This supports our hypothesis of surface diffusion of PC on gelatine. In contrast to real surface imaging with ToF-SIMS, MALDI probes the sample volume.

These results suggest that atrazine signal suppression is due to endogenous PCs and potentially other phospholipids in MSI experiments. To provide further evidence that the presence of phospholipids influences the signal intensity of atrazine, experiments with tissue homogeneously covered with atrazine were performed.

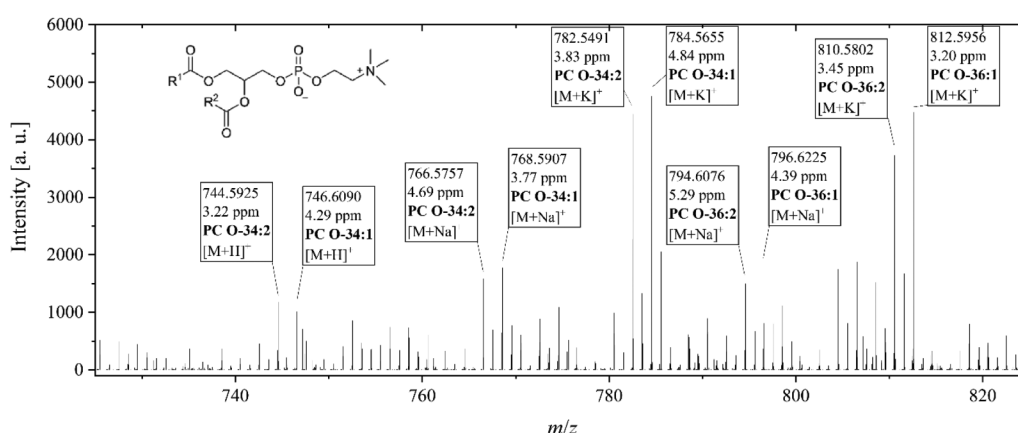
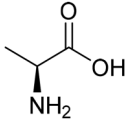
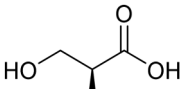
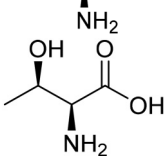
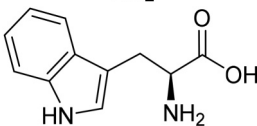
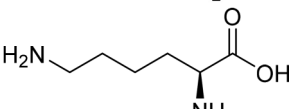
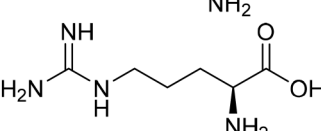
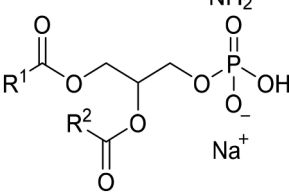
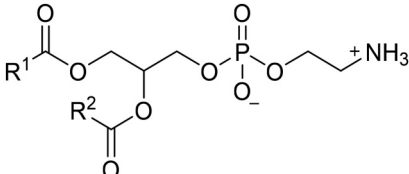
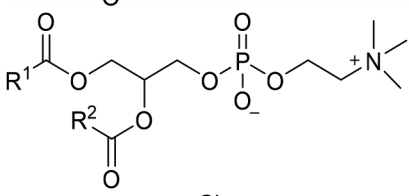
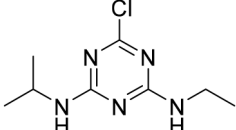


FIG. 5. MALDI mass spectrum of earthworm tissue in positive-ion mode. For selected signals, the m/z value, ion type, annotated lipid, and corresponding mass error are included. Annotation was performed with the LipidMaps database. (Ref. 28).

TABLE I. Substances with known GBs used in the experiments to determine the GB of atrazine.

Substance	Structure	GB (kJ/mol) ^{30,31}
Alanine (ALA)		868
Serine (SER)		878
Threonine (THR)		886
Tryptophan (TRP)		909
Lysine (LYS)		952
Arginine (ARG)		1007
PA(18:0/18:0) (phosphatidic acid)		867.5
PE(18:0/18:0) (phosphatidylethanolamine)		946.4
PC (16:0/20:4) (phosphatidylcholine)		1044.7
Atrazine (ATR)		Unknown

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B. Earthworm sections with homogeneous spray deposition of atrazine

Despite the homogeneous distribution of the herbicide on the tissue section, no or only a very low signal of protonated atrazine was detected in selected areas of the earthworm tissue using a surface-sensitive ToF-SIMS analysis (Fig. 3). The absence of signals in the outer skin layer and muscle tissue in the left part of the sample is particularly striking. Furthermore, no [atrazine + H]⁺ signal was detected in the area of the digestive organ and ventral nerve cord of the sample [Fig. 3(a)]. Compared with the accumulation experiments of Sec. I, the homogeneously deposited atrazine samples showed a similar distribution pattern of the protonated atrazine signal in the worm area. High atrazine signal intensities were detected in areas with lower PC signal intensity.

In MALDI-MSI, the herbicide signal was detected in the entire sample, but local signal intensities varied, even though the analyte was homogeneously deposited (see Fig. 4). A signal decrease from the inner tissue part of the worm to the digestive organ as well as to the outer skin layers could be observed. A fundamental difference in the SIMS results lay in the signal intensity of protonated atrazine in gelatine that was used as embedding material. While the ionization efficiency of atrazine in the SIMS measurements was excellent in gelatine areas, the presence of gelatine in the MALDI experiment led to a suppression of the herbicide ion signal. Surprisingly, the signal intensity of atrazine in inner tissue parts of the worm was even higher in some places than in gelatine areas. The best ionization efficiency for atrazine in MALDI-MSI was detected in regions without tissue or gelatine on the top and bottom right sides of the sample. These regions emanate from the embedding process.

A further analysis of a homogenized earthworm tissue mixture with MALDI-MS revealed highest signal intensities for PCs in the samples (Fig. 5). Phospholipids are known to cause ion suppression in mass spectrometry, leading to a signal loss of targeted analytes and false-negative results.²² Particularly, PCs can impact protonation ion yields of analytes most likely by affecting proton partitioning within the desorption plumes caused by the high *GB* of the phosphocholine headgroup.²⁹ It has to be noted that phospholipids also tend to have a high affinity for alkali metal cations, but in our case, we assume a competition of protons in the gas phase. To rationalize the depletion of the protonated atrazine signal in tissue, in the following, we focus on the *GB* of analytes and related ion suppression effects. Based on these results, we hypothesized that the ion suppression is caused by the presence of polar lipid molecules and that the extent of ion suppression can be correlated to *GB* differences between the lipids and atrazine.

C. Experimental estimation of the GB for atrazine

To investigate the relationship between *GB* and the propensity to form ions of atrazine in SIMS and MALDI mass spectrometry imaging, simplified binary mixtures of solutions of selected amino acid standards listed in Table I and the phospholipids phosphatidic acid (PA), phosphatidylethanolamine (PE), and PC standards were spotted onto glass slides and measured. As described in Sec. I, we propose a correlation between the *GB* and the observed signal intensity ratio of the protonated analytes in the binary mixtures. By

fitting the known *GB*s versus the experimental intensity ratios, we obtain a calibration line to estimate the *GB* of compounds with unknown *GB*s. Therefore, mixtures of the phospholipid solutions with an atrazine solution were prepared to classify the *GB* of the herbicide.

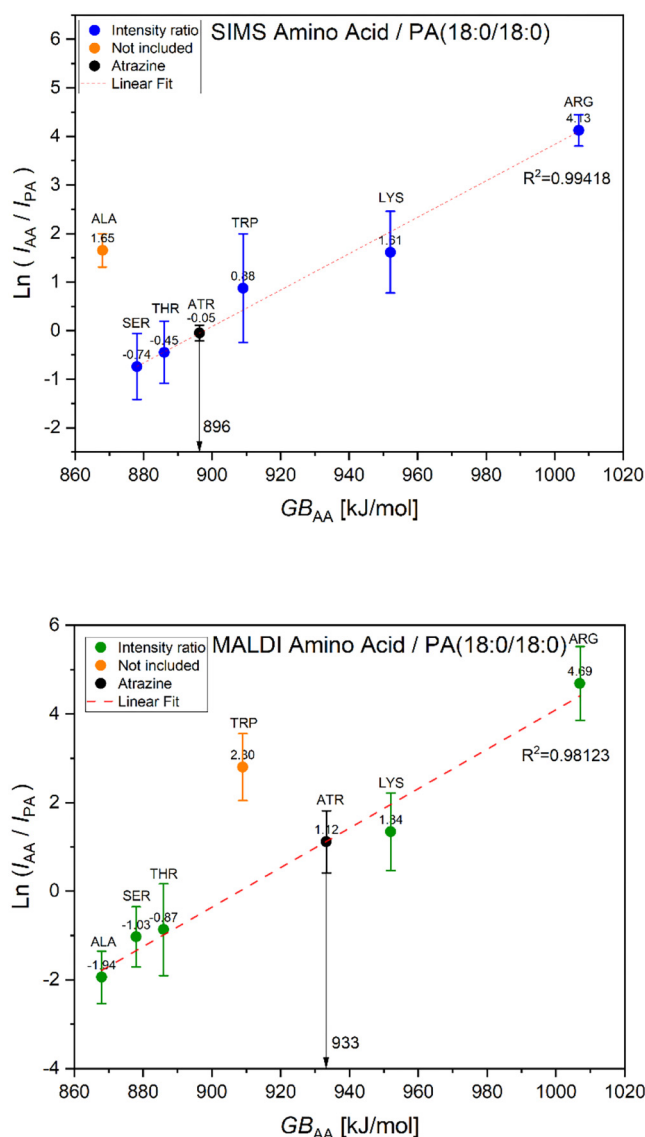


FIG. 6. ToF-SIMS (top) and MALDI (bottom) results of amino acid solutions combined with PA (18:0/18:0) solution. A plot of the natural logarithm of the intensity ratio of the two analytes against the *GB* of the respective amino acids (literature values taken from Table I). Amino acids are assigned with the respective three-letter symbols. The error bars show the standard deviation of the respective measurements. Orange measurement points were not included in the linear regression due to a systematic deviation from the linear model. Determination of the *GB* of atrazine using the measured intensity ratio with PA (18:0/18:0).

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To obtain a calibration line, the intensity ratios of amino acid and phospholipid signals from the SIMS and MALDI measurements were plotted versus GB s values taken from literature reports. Here, the recommended GB values of amino acids by Bouchoux were used (Table I).³⁰ The GB values of phospholipid headgroups were taken from Miller *et al.*³¹ The GB of atrazine has not been determined previously. The logarithmic intensity ratios of the binary mixtures in ToF-SIMS plotted against the literature GB values of amino acids showed a linear dependence (Fig. 6). Systematic deviations from the linear fits were determined by box plots of residual values (Fig. S4 in the supplementary material).⁴⁰ The respective measurement points were, therefore, excluded from the linear fits. Due to its high surface sensitivity, ToF-SIMS can detect the smallest changes in surface composition, which might lead to deviations in the measured intensity ratios. The SIMS measurements including PE (18:0/18:0) (Fig. S5 in the supplementary material)⁴⁰ deviated significantly from the linear trend, hence the large error bars in Fig. 7. For the most basic phospholipid, PC (16:0/20:4), the SIMS measurements did not result in a linear change of the logarithmic intensity ratios with the GB values (Fig. S5 in the supplementary material).⁴⁰ This is most likely caused by ion suppression and consequently diminishing amino acid signals complicating the evaluation of intensity ratios. As a result, only traces of the amino acids were detected in most cases in SIMS.

Plots of the MALDI results show a linear trend in all binary mixtures. However, in the PA and PC measurement series, the intensity ratio of tryptophan deviated significantly from that of the linear model (Figs. 6 and S7 in the supplementary material).⁴⁰ One possible reason might be the different crystallization efficiency of amino acids and lipids in the MALDI matrix. Additionally, tryptophan is known to efficiently take up the UV laser wavelength, acting as an absorbing matrix by itself.³² The determined GB s for atrazine from the linear regressions in SIMS and MALDI deviate up to 20% from each other and inherit error values between 10% and 108% (Fig. 7). Particularly striking was the high signal intensity of atrazine in combination with the phospholipid PE (18:0/18:0) in MALDI, which led to a high value of the determined GB of atrazine. However, the results of the other four phospholipid test series shown in Fig. 7 led to comparable GB values in the range of 896–988 kJ/mol, with a mean value of 931 ± 41 kJ/mol. Atrazine is, therefore, likely to be less basic than common phospholipids such as PCs, with its GB value of 1044.7 kJ/mol.³¹ This suggests the suppression of the atrazine signal in MSI experiments of biological samples due to the presence of phospholipids, especially when atrazine is present in low concentrations and phospholipids in high concentrations.

D. Theoretical estimation of the CB for atrazine

This work's computational determined amino acid GB values that are plotted in Fig. 8 deviated on average by 1.3% from experimental literature values.³⁰ A linear regression of the experimental and computational values showed a favorable correlation. The correlation did not improve noticeably when employing DLPNO-CCSD(T)/def2-TZVPP single-point electronic energies for conformer Gibbs energies, thus implying that any residual deviations from experimental GB s of amino acids are likely due to

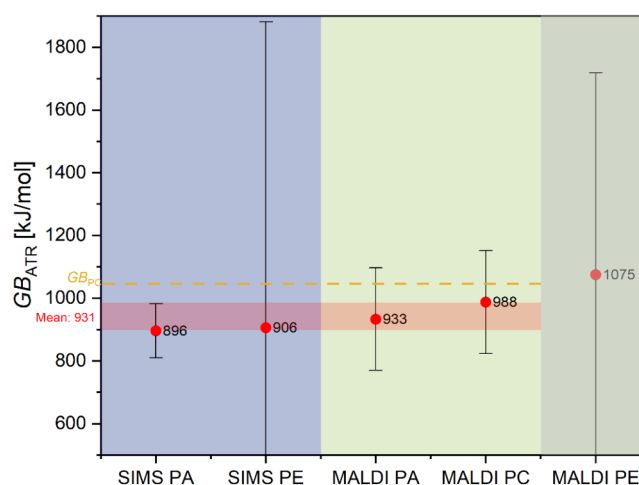


FIG. 7. Determined GB s for atrazine in binary mixtures with phospholipids in SIMS and MALDI. The MALDI measurement of the PE-containing mixture was not included due to its systematic deviation from the other sample series. The range for the GB of atrazine is marked in red with a mean value of 931 kJ/mol. The GB of one of the most basic phospholipids (PC) commonly found in biological samples is indicated by yellow dashes.

inaccuracies of the computational enthalpic and entropic contributions. In the case of atrazine, with its experimentally determined GB value in this work lying between 896 and 988 kJ/mol and with a mean value of 931 ± 41 kJ/mol, the computational values were 923.6 and 912.7 ± 44.1 kJ/mol for the corrected computational value. The corrected value was determined by correlating the computed values with the known experimentally determined values of amino acids.

IV. DISCUSSION

ToF-SIMS imaging of worm sections with homogeneously deposited atrazine confirmed good ionization efficiency of atrazine in the gelatine matrix [see Fig. 3(a)]. Areas containing worm tissue showed significantly less or no signal of the herbicide. This spatially restricted ion suppression is most likely caused by components within the worm tissue. While gelatine is mostly composed of structural proteins called “collagens,” worm tissue also contains other biological substance classes in addition to collagen. Phospholipids, for example, are known to cause ion suppression in mass spectrometry imaging between members of this substance class.²² In our case, the presence of phospholipids seems to cause the suppression of the protonated atrazine signal. The MSI results for the sprayed atrazine samples using a surface-sensitive ToF-SIMS analysis were particularly surprising. One would have expected the formation of a homogeneous surface layer of atrazine molecules after the spray deposition, showing an equal distribution of the atrazine signal in the ToF-SIMS measurements. However, atrazine signal in most of the areas containing earthworm tissue was almost extinguished. Therefore, mixing of the spray-deposited analyte with ion-suppressing molecules of the earthworm tissue is

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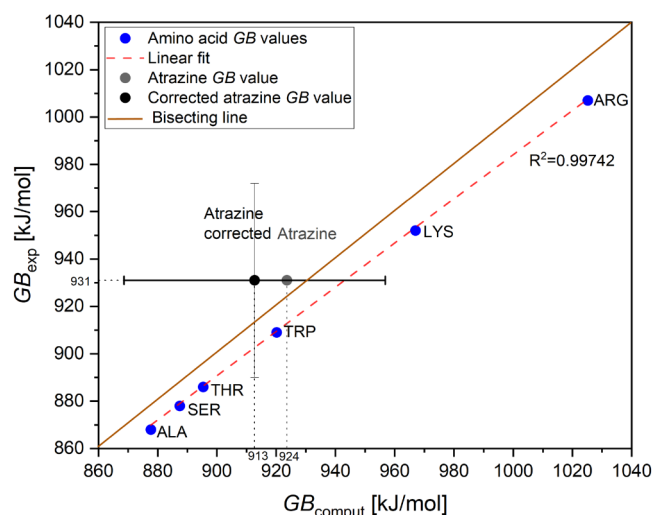


FIG. 8. Plot of computationally determined GB values (in this work) vs experimentally determined GB literature values of amino acids (Refs. 30 and 33) and atrazine (in this work). The perfect correlation of computational and experimental GB values is shown by the bisecting line (beige). The linear fit of computationally determined amino acid GB values is shown in red dashes. The corrected computational GB value of atrazine and its error was determined by correlating the linear fit with the bisecting line.

a possible explanation either during spray deposition or in desorption processes during ion bombardment.

Because we cooled down the sample in the load lock chamber of the SIMS machine to -100°C before vacuum generation, mixing of the spray-coated analyte occurred either before the SIMS measurement or within the collision cascade after the impact of the high-energetic primary ions. The SIMS analysis itself was carried out at -150°C in order to prevent diffusion. This is an advantage over MALDI, where measurements under cryogenic conditions are not possible and an interdiffusion of analyte molecules and the matrix can occur.³⁴

Looking at the MALDI-MSI results in comparison, the signal loss of protonated atrazine in regions containing earthworm tissue was not as pronounced as in ToF-SIMS. A possible explanation for this observation is the addition of trifluoroacetic acid to the deposited matrix layer in MALDI-MSI, leading to a higher proton concentration on the surface. In contrast to ToF-SIMS, gelatine environment in MALDI-MSI suppressed atrazine signal intensity, which was particularly visible in the blank spaces of the sections that originate from holes in the embedding process. In those areas, atrazine was detected in highest intensities, therefore demonstrating that gelatine has an ISE on atrazine ionization in MALDI.

The influence of GB on the signal intensity in fast atom bombardment mass spectrometry (FAB-MS) was investigated in the year 1986, revealing ion suppression based on GBs in binary mixtures of organic low-molecular-weight compounds.²³ In 2007, Jones *et al.* published similar results regarding SIMS analysis of binary mixtures of organic molecules with different GBs.³⁵ In MALDI-MS, the role of proton transfer reactions and ion

formation in desorption plumes was studied in the early 2000s, showing that thermodynamic equilibrium between ion species after desorption is possible.^{36,37} In our case with SIMS and MALDI, deviations from linearity and error margins up to 108% of the resulting GBs for atrazine indicate that there are certainly additional influences on the observed intensity ratios besides GB. Still, measured logarithmic intensity ratios of binary amino acid and phospholipid mixtures revealed in most measurement scenarios a linear dependence of intensity ratios on GB or the difference between GB values. In both ionization methods, MALDI at atmospheric pressure and ToF-SIMS in ultrahigh vacuum, competition for and exchange of charge carriers is likely to occur before, during, or after desorption. However, some parameters need to be addressed when comparing the ion intensities of the two MSI techniques. Even if only a mixture of two pure analyte solutions is dropped onto glass slides for the measurements, the desorption and ionization efficiency of the two molecules can differ significantly in both ionization methods. Additionally, the analytes can show different tendencies to fragment during the impact of the primary ions in SIMS analysis. In most cases, the resulting concentrations of the two analytes in the desorption plume will not be equal, in which case our assumption to have a 1:1 analyte ratio in the gas phase no longer applies. In addition, for sample preparation in MALDI analysis, the dried droplet method was used. Binary analyte mixtures were mixed with the MALDI matrix solution and then dropped onto PTFE-coated glass slides. As the solvent evaporates and the 2,5-DHB matrix slowly crystallizes, the two analytes can incorporate into matrix crystals with different efficiencies, resulting in an inhomogeneous distribution of the targeted molecules in the measurement area. Furthermore, the pressure conditions during analysis in SIMS and MALDI differ significantly. The atmospheric pressure in MALDI will slow down the dissolution of the desorption plume, allowing thermodynamic equilibrium to be established. In the SIMS desorption process, thermodynamic equilibrium in the seldge region is not very likely to be achieved. Taking the previously mentioned facts into account, our simple experiment setup still showed a correlation between GB and signal intensity.

The experimentally determined GB value of atrazine of 931 ± 41 kJ/mol lies within the error margin of the calculated value of 912.7 ± 44.1 kJ/mol. The large error range can stem from many factors that influence the resulting intensities, as described in the last paragraph. Due to the high experimental GB value of 931 ± 41 kJ/mol, atrazine seems to be efficiently desorbed and ionized in both ionization methods from amino acid mixtures. Nevertheless, its GB was found to be significantly lower than the GBs of phospholipids ubiquitous in nature. Therefore, ion suppression of atrazine based on GB differences toward phospholipids is very likely, especially when lipids are present in higher concentrations, e.g., in biological samples. A recent study has revealed summed PC concentrations in biological tissue in the range of 1 nmol/mm^2 , with the highest PC concentration reported to be about 250 pmol/mm^2 .³⁸ Assuming a homogeneous spraying of the atrazine solution onto the sample, we deposited 33 pmol/mm^2 atrazine onto the sample. This means without differences in GB values, we would expect atrazine to be about seven times lower in intensity than the most intense PC signal. However, this ratio is further lowered due to GB differences that will, based on our estimated GB values, lower the intensity of atrazine by a factor of 1000

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for PEs and diminish the analyte signal for PCs. Apart from GB, other properties of phospholipids are additionally responsible for ISEs, as those are also encountered in negative-ion mode.⁶ Still, the linear correlation observed in our measurements between GB and signal intensity implies a significant role for GB in the occurrence of ISEs.

Concerning the specific effects of the sample system, we did not investigate possible metabolism mechanisms for atrazine inside the earthworms. However, there are studies showing that mainly bacteria are responsible for atrazine degradation in soil. When earthworms were added to the soil, the amount of atrazine-degrading bacteria was instead reduced by the presence of the earthworms, leading to less degradation of atrazine in the soil.³⁹

V. CONCLUSION

In ionization processes of both mass spectrometric techniques, the ion suppression of atrazine in earthworm samples seems to be a direct consequence of the presence of phospholipids. When investigating analyte distributions in biological samples, ISEs can lead to false-negative results in MALDI and in SIMS measurements. The results of the MSI experiments using the example of atrazine in earthworm tissue sections show, as other model systems showed previously, the importance of a comparison with control tissue sections for each analyte of interest.^{20,21} As shown in the experiments, the investigation of a homogeneously distributed target analyte on untreated samples can provide a good estimate of the ionization efficiency for the analyte of interest in different sample areas. With regard to the reason for ion suppression in biological samples, GB seems to play a major role due to the presence of ubiquitous phospholipids with high GBs. In general, analyte molecules compete for protons, and molecules with higher GBs succeed. In our case, the target analyte atrazine shows a lower GB than phospholipids that are both experimentally and computationally determined. A better understanding of the influence of GB in MSI measurements can certainly help estimate possible ISEs in future sample systems.

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AUTHOR DECLARATIONS

Conflict of Interest

The authors have no conflicts to disclose.

Ethics Approval

According to Directive 2010/63/EU, ethical approval is not required for experiments with earthworms.

Author Contributions

T.W. carried out earthworm experiments, performed ToF-SIMS and MALDI-MS analyses, data evaluation, and interpretation, and drafted the manuscript. M.R., A.H., and S.H. assisted in

the experiments' design, data evaluation, and revised the manuscript. D.G. performed computational calculations of GBs. R.A.D. and J.J. provided earthworms and assisted in the setup of earthworm accumulation experiments. All authors proofread the manuscript and approved the final version.

Timo Weintraut: Data curation (lead); Formal analysis (lead); Investigation (lead); Writing – original draft (lead). **Sven Heiles:** Conceptualization (equal); Formal analysis (supporting); Project administration (equal); Writing – review & editing (equal). **Dennis Gerbig:** Data curation (equal); Formal analysis (equal); Writing – review & editing (supporting). **Anja Henss:** Conceptualization (supporting); Writing – review & editing (equal). **Johannes Junk:** Conceptualization (supporting); Writing – review & editing (supporting). **Rolf-Alexander Düring:** Conceptualization (supporting); Writing – review & editing (supporting). **Marcus Rohnke:** Conceptualization (equal); Formal analysis (supporting); Project administration (equal); Writing – review & editing (equal).

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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