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SILVER DOPANTS FOR TARGETED AND UNTARGETED DIRECT ANALYSIS OF UNSATURATED LIPIDS VIA IR-MALDESI

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Abstract

RATIONALE—Unsaturated lipids play a crucial role in cellular processes as signaling factors, membrane building blocks or energy storage molecules. However, adequate mass spectrometry imaging of this diverse group of molecules remains challenging. In this study we implemented silver cationization for IR-MALDESI direct analysis to enhance the ion abundances for olefinic lipids and facilitate peak assignment.

METHODS—Trace amounts of silver nitrate were doped into the electrospray solvent of an IR-MALDESI imaging source coupled to an Orbitrap mass analyzer. Calcifediol was examined as a model compound to demonstrate the effect of silver dopants on sensitivity and assay robustness. Dried human serum spots were subsequently analyzed to compare Ag doped solvents with previously described solvent compositions. Mass difference as well as ion abundance ratio filters were employed to interpret results based on the characteristic isotopic pattern of silver.

RESULTS—Olefinic lipids were readily observed as silver adducts in IR-MALDESI analyses. Silver cationization decreased the limit of detection for calcifediol by at least one order of magnitude and was not affected in complex biological matrices. The ion abundance ratio and mass difference of $[M+^{107}\text{Ag}]^+$ and $[M+^{109}\text{Ag}]^+$ were successfully applied to facilitate the spectral assignment of silver adducts. Overall, silver cationization increased the analyte coverage in human serum by 43% compared with a standard IR-MALDESI approach.

CONCLUSIONS—Silver cationization has been shown to enhance IR-MALDESI sensitivity and selectivity for unsaturated lipids, even when applied to complex samples. Increased compound coverage, enhanced robustness as well as the developed tools for peak assignment and mapping of isotopic patterns will clearly benefit future mass spectrometry imaging studies.

Keywords

MALDESI; mass spectrometry imaging; silver cationization; lipids; untargeted analysis

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INTRODUCTION

Mass spectrometry imaging (MSI) has emerged as a powerful technique for studying spatial distributions of a wide range of biomolecules, including pharmaceuticals^[1], lipids^[2], proteins^[3] and metabolites^[4], within cells or tissue sections. Since the invention of matrix-assisted laser desorption ionization-MSI by Caprioli *et al.*^[5] a variety of ionization techniques have been successfully applied to MSI.^[6] Among these, MALDI and secondary ion mass spectrometry (SIMS) have been of particular interest over the last decade, given their intrinsic high sensitivity and high spatial resolution, which are major goals in imaging experiments.^[7] A recent review by Gode and Volmer highlights the application of MALDI and SIMS for lipid imaging in different tissue types and organs.^[8] An insightful side-by-side comparison of MALDI- and SIMS-TOF imaging was performed by Benabdellah *et al.*^[9] Typically, lower spatial resolutions are achieved in SIMS (2 – 5 μm) than in UV-MALDI (50 – 100 μm) or IR-MALDI (200–250 μm). However, SIMS lacks sensitivity and is mainly restricted to the lower m/z range due to analyte fragmentation. On the contrary, MALDI, as a soft ionization method, is well applicable to higher m/z ranges, but suffers from abundant matrix interferences in the lower m/z range.^[8,9] Moreover, laborious sample preparation steps as well as the high vacuum requirements for MALDI (*ca* 10^{-6} torr) and SIMS (*ca* 10^{-10} torr) are technically challenging and potentially affect the sample integrity.^[10,11] Ambient ionization techniques, including desorption electrospray (DESI), liquid extraction surface analysis (LESA), and matrix-assisted laser desorption electrospray ionization (MALDESI), have shown promise in overcoming these major limitations.^[12,13]

MALDESI refers to a technique that involves resonant excitation of an endogenous or exogenous matrix followed by post-ionization of ablated neutrals through interaction with an electrospray plume.^[14] Thus, MALDESI combines features of both, MALDI and ESI, including the production of multiply charged ions, operation under ambient conditions, and imaging capabilities.^[14,15] A highly engineered IR-MALDESI imaging source has been introduced recently by Robichaud *et al.*^[16] The fundamental ablation event is based on resonant excitation of water O-H stretching at a mid-IR wavelength of 2.94 μm , which has been extensively studied by several research groups for IR-MALDI.^[17–20] However, these techniques inherently suffer from poor ionization efficiencies.^[21] Post-ionization of neutrals through charged droplets from an electrospray plume, as in IR-MALDESI or the analogous laser ablation electrospray ionization (LAESI)^[22,23], therefore boosts the ion yield. Moreover, increased detection frequencies, defined as the fraction of pixels in which the analyte is detected, and enhanced ion abundances were reported when using a thin ice layer as a matrix rather than endogenous water.^[24,25] IR-MALDESI imaging has, *inter alia*, been applied to map pharmaceuticals^[24,26] and lipids^[16,25] in biological tissue sections.

Lipids play a crucial role in cellular and tissue processes, such as signaling and energy storage, and are fundamental building blocks of biological membranes. Their potential role as biomarkers for various diseases is subject to ongoing discussion.^[27,28] Mass spectrometry imaging is particularly enticing in the field of lipidomics as it circumvents tedious sample preparation steps.^[2] However, due to the structural diversity of lipids, the ionization process is often challenging. For instance, (poly)unsaturated lipids such as fatty acids, cholesterol

esters and acyl glycerides often require the presence of metal salts to ionize efficiently in MALDI and ESI.^[29–32]

Among other metal ions, silver ions (Ag^+) are well known for their exceptional affinity toward olefinic compounds. According to a model developed by Dewar^[33], the weak charge-transfer complex can be described as a σ -type interaction between the occupied $2p\pi$ orbitals of the $\text{C}=\text{C}$ double bond and vacant silver $5s$ and $5p$ orbitals. An additional contribution to the bonding arises from π -backbonding between silver $4d$ orbitals and the lowest unoccupied molecular orbital of the olefinic bond ($2p\pi^*$). Similar considerations are valid for silver cation-aromatic π interactions.^[34]

The high affinity of Ag^+ toward π orbitals has been extensively exploited in liquid chromatography^[35], ESI-MS^[32,36,37], and SIMS^[38] as well as MALDI-MS^[39]. Surprisingly, deliberate salt adduct formation has been rarely applied to MSI or direct analysis under ambient conditions. Shrestha *et al.*^[40] reported enhanced structure-specific fragmentation in collision induced dissociation experiments of lithiated lipids in LAESI, and Jackson *et al.*^[41] proposed the use of Ag^+ adducts for the detection of biologically relevant olefins *via* DESI-MS. To the best of our knowledge, no further studies have been conducted in this field.

In this proof-of-concept study, we report the benefits of silver-doped electrospray solvents in IR-MALDESI for improved sensitivity and selectivity of unsaturated lipid analytes. Calcifediol in human serum was chosen as an appropriate model system due to known issues with isobaric interferences and ionization efficiency in conventional approaches.^[42] We further extended our investigation to a more complex system, human serum, to point out the complementary use of regular and silver-doped IR-MALDESI in discovery based approaches. For this, a novel peak finding algorithm based on the characteristic isotopic distribution of silver is introduced.

EXPERIMENTAL

Materials

HPLC grade methanol, chloroform and water were purchased from Burdick and Jackson (Muskegon, MI, USA); and formic acid (FA) and human serum type AB (male) from Sigma-Aldrich (St. Louis, MO, USA). Silver nitrate (99.85 % p.a.), linoleic acid (99 %) and oleic acid (95 %) were purchased from Fisher Scientific (Nazareth, PA, USA). Calcifediol (25-hydroxy vitamin D_3) was purchased from Tocris Bioscience (Minneapolis, MN, USA). All materials were used as purchased without purification.

Sample Preparation

Stock solutions of calcifediol and fatty acids were prepared at a concentration of 1 mg/mL in methanol and chloroform, respectively. Working solutions were obtained by diluting stock solutions to the required concentration levels. Calcifediol standards were prepared in 75 % MeOH in H_2O as the increased surface tension was beneficial to the spotting procedure. Human serum was stored at -80°C until utilized. Spiked serum samples were fortified with 1 $\mu\text{g/mL}$ calcifediol and incubated for 1 h at room temperature to allow equilibration with

vitamin D binding proteins. Typically, 100 nL were spotted on HistoBond adhesive microscopy slides (VWR International, Radnor, PA, USA) using a Hamilton 0.5 μ L syringe (Reno, NV, USA). Spot diameters ranged from 1 to 2 mm. Samples were dried under ambient conditions and subsequently analyzed *via* IR-MALDESI coupled to a Thermo Scientific Q Exactive hybrid quadrupole Orbitrap mass spectrometer (Bremen, Germany).

IR-MALDESI Source

The IR-MALDESI imaging source is described in greater detail elsewhere^[16,25]. Briefly, the sample is placed on a water-cooled peltier stage that is cooled to -10°C while purging the enclosure with nitrogen. Subsequent exposure to ambient environment allows a controlled deposition of a thin ice layer over the sample surface. Once the formation of the ice layer is completed, the source is purged with nitrogen until a relative humidity of *ca* 10 % is reached to maintain a consistent ice layer throughout the course of the experiment and to limit the number of ambient ions. Resonant excitation of the ice matrix with a mid-IR laser tuned to 2.94 μm (IR-Opolette 2371; Oportek, Carlsbad, CA, USA) causes desorption of neutral molecules from the sample. These neutrals partition into charged solvent droplets of an electrospray plume where they are post-ionized in an ESI-like process. Previous studies reported a solvent composition of MeOH/H₂O (1:1) with 0.2 % formic acid to be particularly well suited for small molecules and lipids.^[26] In addition, MeOH/H₂O (1:1) was doped with AgNO₃ at concentrations ranging from 5 to 100 $\mu\text{mol/L}$. Doped solvents were prepared daily from either concentrated stock solutions (1 mg/mL in H₂O) or from the solid and protected from light. The electrospray parameters as well as the solvent flow rates remained unchanged during the entire study. IR-MALDESI analysis of dried sample spots was performed at a spot-to-spot distance of 100 μm .

Mass Spectrometry

All experiments were performed in positive ion mode on a Q Exactive hybrid quadrupole Orbitrap mass spectrometer (^[43]), which was fully synchronized with the MALDESI source to acquire one scan per pixel. A more detailed description of the synchronization can be found elsewhere.^[24] During a fixed injection time of 150 ms, ions from two laser pulses at 20 Hz were accumulated in the C-trap followed by a single Orbitrap acquisition. For direct analysis of calcifediol, linoleic acid and oleic acid the mass range was set to m/z 150 – 600. Human serum analysis was performed at a mass range of m/z 300 – 1200. The mass resolving power was set to 140,000 at m/z 200 unless otherwise stated. Mass accuracies within 2 ppm were obtained by using two ambient diisooctyl phthalate peaks ($[\text{M}+\text{H}]^{+}$ at m/z 391.2843 and $[\text{M}+\text{Na}]^{+}$ at m/z 413.2662) as lock-masses for internal recalibration.^[44]

Data Analysis

Mass spectra were directly exported from raw data employing the Thermo Scientific Xcalibur software package. For ion images, the raw data were converted to the mzXML format using the MSConvert software from Proteowizard.^[45] Stacked ion images required the raw data to be first converted into the mzML format *via* MSConvert and subsequently converted into imzML files, which were then stacked in a single master imzML file using imzMLConverter.^[46] Ion images were then generated in the open source software

MSiReader.^[47] No post-processing, such as background subtraction or interpolation, was performed throughout this study.

The MSiReader built-in ‘peak picking’ tool was employed to extract peaks unique to a user-defined region of interest (ROI). Ion abundance ratios per pixel were visualized in MSiReader using the normalization tool combined with a customized color scale. For a detailed investigation of covariance and colocalization, ion maps were exported as two dimensional intensity matrices and further explored in MatLab R2012b (MathWorks, Natick, MA, USA). The mzXML files were also searched for peak pairs with specified m/z differences using the in-house software GlycoHunter. Advanced filter parameters within GlycoHunter accelerated processing throughput by limiting the search space to scans defined by a ROI and also by discarding peaks that were not identified by MSiReader as unique to the ROI.

RESULTS AND DISCUSSION

The conditions for silver adduct formation during IR-MALDESI were optimized for detection of unsaturated lipids using calcifediol as a reference compound. As a potential analytical application, the direct analysis of calcifediol in human serum was evaluated with respect to sensitivity and robustness. Based on the isotopic pattern of silver, the study was extended to an untargeted approach. For this, the distinctive ion abundance ratio and mass difference of $[M+^{107}\text{Ag}^+]^+$ and $[M+^{109}\text{Ag}^+]^+$ were applied as effective peak filter criteria. The comparison with former optimized conditions for small molecules and lipids^[26] revealed the detection of additional lipids in human serum due to specific silver cationization.

Silver adduct formation in IR-MALDESI

For an initial evaluation of the affinity of Ag^+ toward olefins in IR-MALDESI, standard spots containing 5 ng calcifediol were examined. Calcifediol was readily silver cationized by doping trace amounts of silver nitrate into the electrospray solvent of MeOH/H₂O (1:1). This result is in accord with expectations based on the presence of three double bonds in the molecule, which act as potential binding sites for Ag^+ (Fig. 1). Moreover, the rigid *cis*-configured diene substructure of calcifediol exhibits a “bay” region, which is highly favorable for silver adduct formation in the gas phase due to the increased electron density.^[48]

The extent to which silver adduct formation occurred was directly proportional to the amount of AgNO_3 doped into the solvent (Fig. 2a). The ion abundance of $[\text{Cholesterol}+^{107}\text{Ag}^+]^+$ was increased by about one order of magnitude over a concentration range from 5 to 100 $\mu\text{mol/L}$. At the same time, the detection frequency (f) was significantly increased, indicating a higher stability and reproducibility. However, the signal stability was affected as the AgNO_3 concentration was increased above 100 $\mu\text{mol/L}$. In this regime, the large excess of silver ions caused highly abundant background signals throughout the m/z range investigated. Whereas the signal for a single target analyte, *e.g.* calcifediol, might have been further increased by larger amounts of AgNO_3 , an untargeted experiment would clearly suffer from interfering AgNO_3 clusters and silver-cationized ambient ions. Furthermore, ion

suppression and space-charge effects are likely to occur as the number of accumulated ions increases. Based on these results, a concentration of 100 $\mu\text{mol/L}$ was regarded as a reasonable compromise between ion abundance and background signal.

To further investigate the influence of silver dopants on the limit of detection, calcifediol standards at different concentrations were spotted on a glass slide and analyzed using the above optimized solvent conditions as well as $\text{MeOH}/\text{H}_2\text{O}/\text{FA}$ (1:1:0.2) (Fig. 2b). Silver adducts were observed over a calcifediol range from 10 ng to about 25 pg per spot. With an average number of 150 pixels per spot, this provides a limit of detection below 1 fmol/pixel. The detection limit of silver adducts is thus more than one order of magnitude lower than was achieved using the conventional IR-MALDESI solvent. This result is in accord with the enhanced detection limits reported by others.^[41] The color bars in Fig. 2b illustrate the extended detection range. However, the achieved signal enhancement was not sufficient to detect physiological levels of calcifediol in human serum, as the LOD exceeds the physiological range (*ca* 50 ng/mL)^[49] by a factor of 5 to 10. Having said that, the analysis of physiological concentrations could still be performed *via* up-concentration or derivatization^[49] during sample preparation.

Typical mass spectra of calcifediol obtained using both Ag-doped and conventional IR-MALDESI solvents are shown in Figs. 2c and d. The observed Ag adduct is easily recognized by the characteristic $[\text{M}+^{107}\text{Ag}^+]^+$ (m/z 507) and $[\text{M}+^{109}\text{Ag}^+]^+$ (m/z 509) doublet at an abundance ratio of 0.93 (theoretical value: 0.9291^[50]). The measured mass difference of 1.9995 Da observed between isotopes differs only by 0.15 mDa (30 ppb) from the accurate mass difference of 1.99965 Da.^[50] Furthermore, it is important to notice that silver adduct formation preserved the intact molecule. In contrast, in-source fragmentation occurred when using the conventional IR-MALDESI solvent, and calcifediol was predominantly observed as $[\text{M}+\text{H}^+-\text{H}_2\text{O}]^+$ (m/z 383). With respect to possible artifacts due to in-source decays, the detection of the intact molecule appears advantageous. In both cases, the measured isotopic pattern matched the natural isotope abundances, as indicated in Figs. 2c and d. The mass measurement accuracies were below 2 ppm.

Detection of silver adducts in complex biological matrices

As competitive Ag^+ binding is a known issue,^[41] this becomes particularly important in imaging approaches. In the absence of sample cleanup and separation, multiple olefinic compounds will compete for silver cations. To investigate these matrix effects, calcifediol was spiked into human serum and subsequently analyzed as described above. Figure 3 shows the observed mass spectrum along with the corresponding ion map for $[\text{M}+^{107}\text{Ag}^+]^+$. The mass spectrum exhibits the characteristic 1:0.93 ion abundance ratio for ^{107}Ag and ^{109}Ag isotopes as well as an exact mass difference within 1.7 ppm accuracy ($m/z = 2.0005$). The slightly increased error in the mass difference accuracy compared with the aforementioned values might be traced back to the low absolute ion abundance, which affects the mass measurement accuracy. In spite of the more complex spectra resulting from the serum matrix, two key characteristics of the spectra, ion abundance ratio and mass difference, can be readily combined to simplify peak assignment. As illustrated in the ion map (Fig. 3b), the silver adduct was detected throughout the serum spot. From this, it was

concluded that competitive ionization is negligible in the case of calcifediol. This effect might be more pronounced, however, for molecules with lower gas-phase affinity to Ag^+ . However, we speculate that the selective binding of Ag^+ could be specifically utilized to discriminate against non-olefinic isobaric interferences. As imaging experiments inherently suffer from isobaric interferences, silver cationization could thus increase the assay robustness and the specificity towards a targeted molecule, e.g. in pharmaceutical studies.

Isotopic patterns for advanced peak filtering

In the previous sections, we reported the benefits of silver adduct formation in terms of enhanced sensitivity and selectivity for olefinic compounds. We further hypothesized that silver's unique isotopic pattern could be exploited for automated recognition of silver adducts in complex matrices. Here we have extended the capabilities of the 'GlycoHunter' peak finding algorithm, previously implemented to export peak pairs with a defined mass difference of 1.99965 Da, to determine whether an additional filter based on ion abundance ratios could be applied.

A major concern was the potential overlap of the isotopic pattern of two silver cationized lipids with different degrees of unsaturation as (poly)unsaturated lipids are ubiquitous in biological samples. Jackson *et al.* reported that experimental overlap of the linoleic acid $[\text{C18:2}+^{109}\text{Ag}^+]^+$ and the oleic acid $[\text{C18:1}+^{107}\text{Ag}^+]^+$ peaks results in an overall 1:2:1 profile on a low to mid mass resolving power instrument.^[41] However, the high mass resolving power instrument applied in the present study readily allowed silver isotopes of the two fatty acids to be distinguished (Fig. 4a). Please note that the exact mass difference between the two peaks is as low as 16 mDa, which requires a mass resolving power above 24,000 at m/z 389 to resolve the peaks. Figure 4b elucidates the effect of decreasing mass resolving power. It is worth noting that not only the ion abundance ratio of the ^{107}Ag and ^{109}Ag isotopes is affected, but also the respective mass difference. Although the resolving power of the Orbitrap analyzer decreases with the square root of the m/z value,^[43] a resolving power of 140,000 at m/z 200 is believed to be sufficient to resolve the isotopic pattern over the m/z range investigated.

To further examine the applicability of an isotopic pattern filter, we analyzed human serum *via* Ag-doped IR-MALDESI. In a first step, the acquired mass spectra were searched for peak pairs that match the exact mass difference of the two silver isotopes with an accuracy of ± 2.5 ppm. Advanced filter settings were set to restrict the search to scans that were coincident with the dried serum spot outline, and to exclude background peaks from the electrospray. The histogram in Fig. 4c shows the distribution of the $^{107}\text{Ag}/^{109}\text{Ag}$ ion abundance ratios for the resulting peak pairs. The distribution appears unimodal and slightly skewed towards lower abundance ratios < 0.5 . More importantly, the median is located at the theoretical natural abundance ratio of 0.9291. From this, it was concluded that an additional abundance ratio filter can indeed be applied to exclude peaks that do not match the silver isotopic pattern, *i.e.* "false-positive" mass difference results. For subsequent investigations, we therefore applied an ion abundance ratio filter set to a value of 0.93 ± 0.2 .

Examination of unsaturated lipids in human serum

Applying mass difference and ion abundance ratio filters as outlined above yielded a total of 64 unique ion pairs in the IR-MALDESI mass spectra of human serum (Fig. S1, Supplementary Information). Figure 5 illustrates three examples for silver cationized, unsaturated lipids. Cholesterol, 18:2 cholesteryl ester and triacylglyceride (TAG) 50:2 were tentatively identified based on accurate neutral mass in a METLIN database search.^[51] All three compounds were detected throughout the serum spot and exhibited a more or less pronounced ‘coffee ring’ effect, as indicated by the ion maps of $[M+^{107}\text{Ag}^+]^+$.

Columns 3 and 4 in Fig. 5 highlight the increased level of confidence in peak assignment that is gained when both isotopic peaks, ^{107}Ag and ^{109}Ag , are being considered. By normalizing the $[M+^{109}\text{Ag}^+]^+$ ion abundance with $[M+^{107}\text{Ag}^+]^+$ in each pixel, ion abundance ratio maps can be generated (column 3). These maps are readily interpreted and readily allow silver adducts to be distinguished from non-silver adducts. Ratios close to 0.9291 indicate silver cationization, as discussed in previous sections. As a consequence, this gives rise to a qualitative map of the analyzed compound.

An alternative approach is to visualize the covariance of a given peak pair (column 4). A common statistical measure of covariance, or colocalization, is Pearson’s product-moment correlation coefficient r .^[52,53]

$$r_{A,B} = \text{cov}(A, B) / (\sigma_A \sigma_B) \quad (1)$$

where cov refers to the covariance of two ion abundance matrices A and B , and σ to their corresponding standard deviation. From this definition, we derived a covariance matrix C_i to calculate each pixel’s contribution to the overall correlation coefficient:

$$C_i = \frac{1}{N-1} \frac{(A_i - \langle A \rangle) * (B_i - \langle B \rangle)}{\sigma_A \sigma_B} \quad (2)$$

A_i and B_i refer to the corresponding ion abundances in a pixel i ; and $\langle A \rangle$ and $\langle B \rangle$ are the respective mean values over the number of all pixels in the image, N . The sum of all matrix elements C_i equals Pearson’s correlation coefficient. Values close to 1 and -1 implicate a linear correlation or anti-correlation between A and B , respectively, while a value of 0 implies that there is no linear correlation.

Please note that ^{107}Ag and ^{109}Ag isotopes are *a priori* colocalized and covariant. This is in total agreement with the high correlation coefficients > 0.95 observed in Fig. 5. We therefore propose that covariance maps can be utilized to distinguish silver cationized species from interfering ions. In contrast to visualizing pure abundance ratios, this approach also preserves the relative ion abundance information and is thus equally suited for semi-quantitative studies.

We believe that both approaches, abundance ratio as well as covariance maps, will facilitate the interpretation of imaging experiments and at the same time increase the confidence of peak assignments as they map the isotopic pattern rather than single ion abundances. Although the benefits in the case of dried serum spots might be limited, mapping the

isotopic pattern appears highly advantageous in MSI of complex, heterogeneous samples, *e.g.* brain tissue sections, with a variety of spatially overlapping lipids.

Comparison with conventional IR-MALDESI

Ultimately, we compared Ag doped electrospray solvents with a typical IR-MALDESI solvent (50/50 MeOH:H₂O + 0.2 % FA) for human serum analysis. MSiReader and GlycoHunter peak finding tools were applied as outlined above to determine peaks that were coincident with the serum spots. For silver cations, $[M+H^+]^+$ m/z values were calculated from accurate masses of $[M+^{107}Ag^+]^+$. Mass excesses of the resulting m/z ratios, defined as the difference between nominal and exact mass,^[54] were subsequently compared with those of all entries from the LIPID MAPS Structure Database (LDMS).^[55] A log₂ probability map was then generated by plotting mass excess as a function of m/z of all 25,000 database entries. The patterns observed in the lipid distribution refer to the fact that lipids can be subdivided into different classes with shared backbone structures, *e.g.* glycerolipids, sterol lipids and sphingolipids. Variety within these classes arises from a myriad of fatty acids attached to the particular backbone.^[27] As a consequence, the corresponding distributions will cluster in the mass excess space. Experimental mass excess data for both silver doped and conventional IR-MALDESI of human serum were overlaid on the probability map to reveal the respective lipid distributions (Figs. 6a and b). It appears that most of the ions found in the serum are lipids since they overlap with the lipid distribution. Moreover, we speculate that silver cationization made possible the detection of non-lipid compounds, as a few ions did not match the lipid distribution. However, we were unable to identify these species. Apart from that, both approaches yielded very similar results. Based on the lipid map, the majority of peaks were assigned to sterol lipids, fatty acyls and glycerolipids, which is consistent with more complete studies of serum lipid profiles.^[56] From this, we concluded that silver cationization does not discriminate against specific lipid classes.

However, only 21 compounds were equally detected in both approaches (Fig. 6c). 43 were exclusively silver cationized, while 80 were solely observed in conventional IR-MALDESI. Combining both approaches, a total of 144 compounds were detected in human serum which equates to the overall compound coverage being increased by 43 % using silver dopants in the electrospray solvent. A simple METLIN database search with all detected 144 m/z values yielded 118 tentative identifications based on accurate mass (Table S1, Supplementary Information). Species uniquely observed as Ag⁺ adducts were predominantly identified as unsaturated cholesteryl esters and diacyl- as well as triacylglycerides. In total, the database search suggests that more than 85 % of the silver-cationized species were unsaturated. This is in accord with the enhanced selectivity and specificity toward olefinic compounds found previously in this study. Moreover, in conventional IR-MALDESI, acylglycerides as well as cholesteryl esters were predominantly observed as sodium or potassium adducts. This can become a major issue since the sodium-to-potassium ratio is not consistent throughout biological samples.^[57] As a consequence, the ratio of observed sodiated to potassiated molecules will be affected in the same way and needs to be taken into account when comparing two sample cohorts. However, given the large excess of Ag⁺ ions, it can be assumed that silver cationization is not affected by inter-sample variation and thus, two ion images can be more readily compared.

Furthermore, Grossert *et al.* have recently reported that sodium cations exhibit the highest gas-phase affinity toward unsaturated acylglycerides. However, for unsaturated derivatives, they found silver cationization to be highly preferred.^[32] This is, in fact, in accordance to the increased ion abundances and linear range observed for silver adducts in the present study (Fig. 6d). Please note that the ion current in the case of silver is split up into ¹⁰⁷Ag and ¹⁰⁹Ag isotopes. To further enhance the response for silver adducts, the ion current could be readily concentrated into a single Ag species by implementing monoisotopic silver. This is indicated by the sum of ¹⁰⁷Ag and ¹⁰⁹Ag abundances in Fig. 6d. Clearly, the increased ion abundance would be at the cost of confidence in peak assignment since the distinctive isotopic pattern would be lost. However, a preferential silver cationization of double bonds would still be expected as the chemical nature of the binding affinity is not altered.

CONCLUSIONS

We have demonstrated the enhancement of sensitivity and selectivity in IR-MALDESI for olefinic lipids by silver cationization. Doping trace amounts of AgNO₃ into the electrospray solvent lowered the limit of detection for calcifediol by over one order of magnitude compared with previously reported solvent compositions. Furthermore, we demonstrated the robustness and applicability of this approach in highly complex matrices, such as human serum. Subsequently, we developed enhanced peak filtering methods based on the distinct isotopic distribution of silver isotopes for application in explorative studies. We have also pointed out the benefits of covariance and ion abundance ratio mapping of ¹⁰⁷Ag and ¹⁰⁹Ag isotopes for highly confident peak assignments. Ultimately, we found that Ag dopants increased the overall lipid coverage of IR-MALDESI by 43 %, with ion abundances being enhanced simultaneously. In future studies, we are planning to implement Ag-doped IR-MALDESI together with the advanced data processing tools in comparative MSI studies of different tissue sections.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
Molecular structure of calcifediol. Potential Ag^+ binding sites are highlighted in red.

Figure 2.

(a) IR-MALDESI-MS images 5 ng calcifediol ($[M+^{107}\text{Ag}]^+$) spotted on a glass slide examined using a solvent spray of MeOH:H₂O (1:1) doped with 5 to 100 μM AgNO_3 . (b) Determination of the limit of detection with a solvent spray of MeOH:H₂O (1:1) doped with 100 μM AgNO_3 (top, $[M+^{107}\text{Ag}]^+$) and 0.2 % FA (bottom, $[\text{MH}-\text{H}_2\text{O}]^+$). Detection ranges as well as physiological levels in human serum for a sample volume of 100 nL are indicated by red bars. Representative MALDESI mass spectra of calcifediol for both solvent compositions are depicted in (c) and (d). Full range mass spectra are provided in Figure S1 (Supplementary Information).

Figure 3.

(a) IR-MALDESI mass spectrum of human serum spiked with 1 $\mu\text{g/mL}$ calcifediol and (b) corresponding ion map for $[\text{Calcifediol} + ^{107}\text{Ag}^+]^+$ using $\text{MeOH:H}_2\text{O}$ (1:1) doped with 100 μM AgNO_3 as electrospray solvent. Abundant electrospray background ions are labeled with asterisks.

Figure 4.

(a) IR-MALDESI mass spectra of silver-cationized linoleic (m/z 387/389) and oleic acid (m/z 389/391). (b) Enlarged view of the m/z 389 region acquired at four different resolving powers (at m/z 200). (c) Abundance ratio distribution of peak pairs detected by applying the automated peak picking algorithm to IR-MALDESI-MS analysis of human serum. The natural ion abundance ratio of silver isotopes 109 and 107, 0.9291, is highlighted for clarity.^[50] Filter settings used in subsequent experiments are illustrated by red colored bars.

Figure 5.

Examples for silver-cationized lipids as detected by Ag-doped IR-MALDESI-MS of human serum. Lipids were tentatively identified based on accurate mass. Observed m/z values for $[M+^{107}\text{Ag}]^+$ and $[M+^{109}\text{Ag}]^+$ as well as corresponding ion maps for $[M+^{107}\text{Ag}]^+$ are shown in columns 1 and 3. Column 3 shows the ion abundance ratios for $[M+^{109}\text{Ag}]^+/[M+^{107}\text{Ag}]^+$, whereas the theoretical abundance ratio of 0.9291 is color-coded in red.^[50] Pearson covariance maps in column 4 reveal the high spatial correlation of the detected silver isotopes. Pearson correlation coefficients are indicated on the top right.

Figure 6.

Mass excesses of all serum related peaks detected with IR-MALDESI-MS using $\text{MeOH}:\text{H}_2\text{O}$ (1:1) doped with (a) 0.2 % FA and (b) 100 μM AgNO_3 as spray solvent overlapped with the mass excess distribution for lipids from LIPID MAPS Structure Database ($\log_2$ scale). (c) Overlap of neutral masses detected by using Ag - or FA -doped solvents. (d) Cumulative ion abundance plots for serum related peaks shown in (a) (black circles) and (b) (orange circles). Orange diamonds refer to the summed intensities of $[\text{M} + ^{107}\text{Ag}^+]^+$ and $[\text{M} + ^{109}\text{Ag}^+]^+$.