Further studies on the signal enhancement effect in laser diode thermal desorption-triple quadrupole mass spectrometry using microwell surface coatings

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Abstract

The laser diode thermal desorption (LDTD) ionization source allows ultra-fast and sensitive analysis of small molecules by mass spectrometry. Signal enhancement in LDTD has been observed when coating the surface of sample microwells with a solution of ethylenediaminetetraacetic acid (EDTA) or nitrilotriacetic acid. Here we present a quantitative analysis of signal enhancement using solutions of diverse commercial proteins (lysozyme, immunoglobulin G, albumin and fibrinogen) as coatings. Results showed that compounds with polar chemical functions such as carboxylic acid, sulfonyl and nitro had signal enhancement factors, in most cases higher than 10, when using any of the tested proteins as coating agent. Analysis of variance revealed that immunoglobulin G and fibrinogen gave the best results. However, the signal enhancement factors obtained with this protein were not superior to those observed with EDTA. To explain the signal enhancement effect of proteins, analysis by scanning electron microscopy of dried samples on the microwell sample plates was carried out. Images showed that salicylic acid, one of the compounds with the highest observed signal enhancement, formed a thick layer when applied directly on the uncoated surface, but it formed small crystals (<1 μm) in the presence of protein or EDTA coatings. Further crystallographic studies using powder x-ray diffraction showed that the crystalline form of salicylic acid is modified in the presence of EDTA. Salicylic acid when mixed with EDTA had a higher percentage of amorphous phase (38.1%) than without EDTA (23.1%). These results appear to confirm that the diminution of crystal size of analytes and the increase of amorphous phase are implicated in signal enhancement effect observed in LDTD using microwell surface coatings. To design better coatings and completely elucidate the signal enhancement effect in LDTD,

more studies are necessary to understand the effects of coatings on the ionization of analytes.

1. Introduction

The advent of high-throughput screening, i.e. techniques capable of analyzing around 10^4 to 10^5 samples per day [1], of small molecules by mass spectrometry has allowed to cut costs and accelerate discoveries in clinical, forensic, pharmaceutical and environmental laboratories. Among the multiple instrumental techniques currently used for fast analysis of small molecules by mass spectrometry [1, 2], laser diode thermal desorption (LDTD) coupled to triple-quadrupole mass spectrometry (QqQMS) is one of the fastest: it allows the analysis of more than 15 000 samples in a single sequence and in less than 35 hours. This technology, introduced in 2004, has been applied to the analysis of : antibiotics in milk [3], honey [4], blood [5], metabolites of pharmaceutical interest [6, 7], as well as contaminants of emerging concern in wastewater [8-10] or surface water [11].

In LDTD, the heat generated by an infrared laser is used to rapidly desorb dried analytes deposited on a stainless-steel plate. Desorbed analytes in the gas phase are then transported by a carrier gas (air) to a corona discharge needle where formation of ions occurs by atmospheric pressure chemical ionization (APCI), generally proton transfer reactions [12]. Thus, ions usually observed are protonated molecules in the positive mode or deprotonated molecules in the negative mode. In some cases, fragmentation in the form of loss of water has been observed in LDTD, but it has only been reported in the case of hormones [8]. Since only traces of water are present during APCI by LDTD, the formation of H_3O^+ as reactive ion species is favored over larger hydrated hydronium ion clusters, i.e. $(H_2O)_2H^+$ and $(H_2O)_3H^+$ observed in liquid chromatography-APCI (LC-APCI) [8, 13]. Therefore, ionization efficiency is superior in LDTD compared to LC-APCI since H_3O^+ has a lower proton affinity than larger hydronium water clusters [14]. Better ionization efficiency in

LDTD compared to APCI was observed by Wu, et al. [6] when developing a fast cytochrome P450 inhibition assay. The authors observed that peak areas of 6β-hydroxytestosterone, 4'-hydroxydiclofenac and acetaminophen were between ≈2 to 90 times higher than those obtained by LC-APCI.

Studies have demonstrated that analyte response in LDTD depends on several parameters such as dilution solvent, laser power, carrier gas flow and sample deposition volume [6, 8]. As other atmospheric pression ionization sources, LDTD is not immune to matrix effects and both signal suppression and enhancement have been observed [8]. In order to improve analyte response in LDTD, several strategies have been employed such as addition of small molecules as a surface coating on the stainless-steel sample plate before analysis [4, 7, 10]. A recent study by Dion-Fortier, et al. [15] demonstrated that surface coatings using iron chelating agents like ethylenediaminetetraacetic acid (EDTA) and nitriloacetic acid improved analyte response of some compounds by a factor of up to 1 000 compared to uncoated microwell plates. The authors explained such signal enhancement effects by changes in the morphology and crystallisation of analytes in the presence of the coatings. Empirical evidence also indicates that precoating the sample microwell plates before analysis with proteins such as bovine serum albumin can also increase the analyte response for some analytes. However, this phenomenon has not been yet explained.

The objective of the present work is to study the effect of microwell surface coatings using proteins and to advance present understanding of the molecular phenomena responsible for signal

enhancement or suppression in LDTD. Such studies are essential to widen the number of compounds analysable by this high-throughput technique and improve the sensibility and detection limits of analytes of interest in LDTD.

2. Material and methods

2.1 Chemicals and reagents

Ammonium hydroxide and analytical standards of small organic molecules of analytical interest (Figure 1) such as pesticides (atrazine, bentazon and metolachlor), pharmaceuticals (acetaminophen, carbamazepine, chloramphenicol, diclofenac, salicylic acid, sulfamethoxazole, trimethoprim), hormones and others (estradiol, ethinylestradiol, benzyl butyl phthalate, caffeine, 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol, tryptophan, ulipristal acetate) as well as microwell plate coating agents ethylenediaminetetraacetic acid (EDTA) disodium dihydrate salt, bovine serum albumin (BSA), fibrinogen from human plasma, immunoglobulin G from human serum (IgG) and human lysozyme were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada). These proteins were chosen as coating agents to investigate whether proteins of human and bovine origin would make better coating agents than EDTA, which is already used as a microwell plate coating in many laboratories using LDTD technology [10, 16].

Methanol (MeOH) and water of LC-MS grade were bought from Fisher Scientific Canada (Ottawa, ON, Canada). Stock solutions of analytical standards were prepared in MeOH at 1000 μg mL^{-1} and stored at -20 °C for 4 months, except for caffeine (H_2O-MeOH, 1:4 *v/v*), trimethoprim (H_2O-

MeOH, 1:9 *v/v*) and tryptophan (H_2O-MeOH, 3:7 *v/v*), which were stored at 4°C. Stock solutions of proteins were prepared in LC-MS grade water at a concentration of 2 µM and were kept at 4 °C for a month.

2.2 Preparation of microwell plate coatings before LDTD-QqQMS analysis

Protein solutions for microwell plate coatings were prepared to obtain a final composition of MeOH-H_2O (1:1.7 *v/v*). The appropriate volume of protein stock solutions was added to 187.5 µL of MeOH. Then, 12.5 µL of NH_4OH (≥ 28 %) was added to the solution. Finally, water was added to reach a final volume of 500 µL. Protein solutions were prepared to obtain a concentration of 750 nM with the highest MeOH proportion possible without causing protein precipitation. Such small quantities of solutions were prepared for environmental and economic reasons, since only 5 µL of coating agent are necessary in each microwell. In the case of BSA, several solutions were prepared at concentrations ranging from 250 nM to 1000 nM. A coating agent solution was prepared in MeOH-H_2O (3:1, *v/v*) with 1.4 % NH_4OH to obtain a concentration of 100 µg mL^{-1} (268 µM) of EDTA for comparison purposes, as this is a widely used coating agent in LDTD.

In order to evaluate the impact of proteins as coating agents on LDTD-QqQMS signal, twelve replicates were prepared as follow: 5 µL of coating agent were transferred on the microwell plate and let to evaporate under a fume hood until dryness. Then, 5 µL of a mix of 17 analytes diluted in MeOH at 0.1 µg mL^{-1} were added to the same wells and let to evaporate as previously described. The same procedure was repeated for each solution of coating containing EDTA, BSA, fibrinogen, IgG and lysozyme.

2.3 LDTD-QqQMS analysis

Thermal desorption and ionization of samples was performed using a LDTD ion source, model Luxon made by Phytronix (Québec, QC, Canada). Mass analysis and detection were done by a TSQ Vantage triple-quadrupole mass spectrometer from ThermoFisher (Waltham, MA, USA) on which the Luxon ion source was mounted. Laser power pattern was as follow: increase from 0 % to 45 % in 3 s; stay at 45 % for 2 s; and return to 0 % immediately. Flow rate of compressed air used as carrier gas was set to 3 L min^{-1}. Six compounds were ionizable by APCI in the negative mode, while the eleven others were ionizable by APCI in the positive mode (Table 1). Therefore, two analyses per sample were required. Acquisition was made using the selective reaction monitoring (SRM) mode, using one transition per compound (Table 1). Data were processed on the software Xcalibur version 4.1 from ThermoFisher. Peak areas of SRM transitions for each analyte were extracted from the acquisition files and used as analyte response (Table 1).

2.4 Statistical analysis of LDTD-QqQMS data

Analysis of variance (ANOVA) at α=0.05 using Origin Pro2019 by OriginLab Corporation (Northampton, MA, USA) was used to compare the means of the data according to a given factor (concentration of BSA, type of coating) on analyte signal. In order to identify which means were different, Tukey post-hoc tests were performed. When the assumption of equal variances of ANOVA was not respected, the nonparametric Kruskal-Wallis ANOVA was performed followed by Dunn’s post-hoc test.

2.5 Powder x-ray diffraction

To study the effect of EDTA coating on the crystalline structure of salicylic acid, three solutions were prepared: SA [10 µg mL^{-1} salicylic acid in MeOH-H_2O (3:1, *v/v*)], EDTA [10 000 µg mL^{-1} of EDTA in MeOH-H_2O (3:1, *v/v*) with 1.4% NH_4OH] and EDTA+SA (same as EDTA solution plus 10 µg mL^{-1} salicylic acid). For each solution, a volume of 180 mL was prepared to obtain enough crystals that could be measured by powder XRD and without significantly changing the experimental conditions of preparation of the coating solutions.

The solutions were dried in a Rocket Evaporator from Thermo Scientific (Waltham, MA) for approximately four hours at 40 °C and 20 mbar in order for the MeOH to evaporate and then approximately six more hours at 40 °C and 0 mbar in order for the residual water to evaporate and the sample to dry completely. The dried samples were then crushed and mixed with paratone oil, cut to approximately 0.3 x 0.3 x 0.3 mm^3, fixed on the goniometer head and mounted at room temperature on a Bruker Apex Duo x-ray diffractometer. Six correlated runs with Phi Scan of 360 degrees and exposure times of 270 seconds were collected with the Cu micro-focus anode (1.54184 Å) and the CCD APEX II detector at 150 mm distance. These runs, from -12 to -72 2-theta and 6 to 36 omega, were then treated and integrated with the XRW^2 Eval Bruker software to produce a WAXD diffraction pattern from 2.5 to 82 degrees 2-theta for each sample. The patterns were analyzed with Diffrac.Eva version 2.0 from Bruker and matched to specific compounds with the database PDF-2 (release 2011) from the International Center for Diffraction Data (ICDD).

2.6 Scanning electron microscopy

Scanning electron microscopy (SEM) using a FEG-SEM S-4700 from Hitachi (Tokyo, Japan) was used to observe the effect of protein and EDTA surface coatings on the morphology and structure of samples deposited on the surface of the microwell plates. Such experiments will be helpful to establish a link between crystallization of analytes and LDTD-QqQMS signal. One of the most and least responsive target analytes to microwell surface coating, salicylic acid and atrazine, respectively, were also selected for this section of the study. For each protein coating agent, 5 µL of protein solution at a concentration of 750 nM was deposited on the stainless-steel surface and let to evaporate until dryness. 5 µL of a 1 µg mL^{-1} salicylic acid or atrazine solution were added into the microwell with or without previous addition of an IgG or lysozyme coating, respectively. Before SEM analysis, samples were sputtered with Au/Pd for 30 s using a Hummer 6.2 instrument from Anatech (Hayward, CA, USA).

3. Results and discussion

3.1 Quantification of the effect of BSA as a microwell plate coating on the LDTD-QqQMS signal of selected analytes

Since the optimal concentration of any protein as microwell plate coating to enhance the signal of analytes in LDTD-QqQMS had never been determined, it was decided to use BSA solutions at various concentrations as microwell plate coating and measure the peak areas of the SRM transitions of four model compounds showing relatively low signals without coating (Figure 2).

These experiments showed that using a BSA solution as a microwell coating agent before the addition of the selected model compounds improved their signal up to 3 orders of magnitude. For all analytes, peaks areas with coatings using BSA concentrations ≥ 250 nM were statistically higher than without BSA coating. It was also determined that for these test analytes, the optimal concentration of BSA was between 750 and 1000 nM. As is shown in Figure 2, in the case of salicylic acid and sulfamethoxazole, a concentration of 750 nM of BSA caused their peak area to increase significantly compared to both 0 nM and 250 nM of BSA (the former is not shown in to avoid overcrowding Figure 2). However, addition of a BSA coating of 1000 nM did not result in a significant increment in the peak area of those two analytes. It is worth noting that no signal was observed for salicylic acid when it was first added on the uncoated surface of the microwell, but that the addition of only 250 nM of the BSA solution as a coating agent drastically increased the peak area to more than 3000 peak area counts. A similar relationship between coating solution concentration and analyte response was observed by Dion-Fortier, et al. [15] when using EDTA as coating agent. The authors of that study observed that the signal of selected analytes (mostly small molecules with carboxylic acid functions) rapidly increased with the presence of a surface coating but did not vary significantly at EDTA concentrations > 100 μg mL^{-1}. The authors also affirmed that the signal enhancement effect of EDTA was due to the formation of homogenous and thin layers of dried analytes that are easier to thermally desorb than the layers of analytes formed in direct contact with the stainless-steel surface. According to the authors, perturbation by EDTA of chemisorption of the analytes to the metal surface was not a major factor since signal enhancement was not observed in microwell plates covered with polytetrafluoroethylene, an inert surface. In the present study, a plateau in the analyte signal was generally observed at concentrations higher than 500 nM of BSA. If the effect of BSA as coating agent is the same as that observed for EDTA,

addition of more BSA on the microwells does not improve analyte desorption since crystallisation is already disrupted and cannot be further modified.

From a molecular perspective, both EDTA and BSA share a common feature: their affinity towards stainless-steel surfaces. Using infrared spectroscopy, Desroches, et al. [17] observed that BSA and fibrinogen spontaneously adsorbed onto stainless-steel surfaces causing changes on the proteins' proportions of α-helices and β-sheets. Consequently, the secondary structure of the proteins was modified after adsorption. Similarly, the chemisorption of organic molecules with carboxylic acid functions on stainless steel has been widely reported [18-20].

3.2 Effect of different protein coating agents on the LDTD-QqQMS/MS signal of analytes and comparison with EDTA

In order to evaluate the performance of various proteins as microwell coatings, experiments with 17 model compounds showing various molecular features and functions were carried out with BSA, fibrinogen, IgG and lysozyme coatings. Every protein solution used was fixed at 750 nM as it was previously determined to be the optimal concentration in such cases (results not shown). Experiments involving the EDTA (100 µg mL^{-1}) coating were also added as a benchmark, since it has already been shown that this type of coating dramatically improves the signal of different categories of compounds such as analytes with carboxylic acid functions [15]. Results of the effect of protein coating agents and EDTA on the signal of analytes in LDTD-QqQMS/MS are shown in Figure 3. The performance of each protein as well as EDTA coating was evaluated by calculating

the enhancement factor for each coating agent paired with every model compound tested. The enhancement factor corresponds to the compound's signal when analysed on a coated microwell divided by its signal when analysed on an uncoated microwell. Therefore, an enhancement factor of 1 suggests that there is no difference between the coated and uncoated microwell for the analyte tested whereas an enhancement factor > 1 suggests that coating the microwell prior to analyte deposition and analysis improves its signal. Similarly, a factor enhancement < 1 implies that the use of microwell coatings reduces analyte signal.

Eight out of the 17 model compounds showed enhancement factors > 1.5 in their signal when using at least one protein coating agent. The same compounds also had higher signals using the EDTA coating compared to no coating. These compounds all displayed polar chemical functions such as carboxylic acids (diclofenac, salicylic acid, THC-COOH, tryptophan), sulfonyl (bentazon, sulfamethoxazole) and nitro (chloramphenicol). Common affinity of EDTA and proteins towards stainless steel and the results presented here suggest that EDTA and the proteins used in this study improve the signal of the same types of compounds using a similar mechanism of action. Other compounds such as atrazine and caffeine showed either no enhancement or even a decrease in their signal when using a coating agent. To further identify the best protein microwell coating agent and compare it to the already existing EDTA coating, ANOVA at $\alpha = 0.05$ of the peak areas of the analytes followed by post-hoc tests showed that, in general, EDTA gave the highest enhancement factors immediately followed by IgG and fibrinogen for the compounds with enhancement factors > 1.5.

3.3 Morphological study of the microwell plate surface using scanning electron microscopy

Qualitative observations of the surface of uncoated and coated microwells made by SEM showed significant differences (Figure 4). These micrographs allowed to assess and explain the performance of the proteins compared to EDTA. In fact, it was observed that EDTA coating produced a thin film on the edges of the microwell that is much more homogenous than the aggregates formed by the various proteins, also on the edges of the microwells (Figure SI-1, Supporting Information).

To investigate the effect of coating on the physical appearance of the model compounds, salicylic acid, which shows a high enhancement factor in presence of coating agents, and atrazine, whose signal slightly decreases when analyzed on a coated microwell, were specifically chosen for further analysis using SEM. Results are shown in Figure 5.

It was observed that salicylic acid formed a dense and layered film of analytes when directly applied to the uncoated surface, which can be difficult to thermally desorb and therefore lead to the low signal previously reported. Salicylic acid crystallized differently in the presence of a coating. Instead of the dense film observed without coating, salicylic acid formed small crystals in the presence of IgG coating and even smaller ones were formed in the presence of EDTA coating. This suggests that the presence of coating reduces analyte-analyte interactions, which decreases

the energy necessary to thermally desorb the analytes on the plate and therefore increases the amount of salicylic acid that reaches the mass spectrometer, which in turn increases its signal. This seems to be confirmed by the fact that EDTA improves the signal of salicylic acid more efficiently than IgG. On the other hand, it was observed that atrazine forms rather large particles on the uncoated microwell and smaller ones on the coated ones. Even though the presence of lysozyme

or EDTA appears to form equally smaller atrazine particles, this difference does not lead to signal enhancement, but rather signal suppression.

At this point, is not clear what other factors caused such unexpected result for atrazine since previous observations showed that a strong correlation between the presence of thin layers of small crystals and high signals in LDTD. It is possible that perturbation of the APCI process by the microwell coating may be involved. For example, it is possible that signal suppression caused by unidentified coating degradation products could affect the ionization of atrazine once it is found in the gas phase. A previous study [15] showed that no thermal degradation products of EDTA could be observed when using LDTD, however the authors observed many ions between *m/z* 50 and *m/z* 215 that could not be identified because of limitations of the experimental setup. Therefore, more studies on the species formed during the LDTD process, and their potential interaction, are necessary to fully understand this source.

3.4 Crystallographic study of dried salicylic acid with and without the presence of EDTA using powder XRD.

Considering the results gathered with SEM on the effect of EDTA, it was hypothesized that the EDTA coating changed the way analytes crystallize on the microwell surface. Therefore, it was decided to study the powder XRD pattern (diffractogram) of salicylic acid and EDTA alone and as a mixture. Changes in the diffractograms of a substance indicate changes in its crystalline structure since x-rays are diffracted differently by the sample.

As expected, salicylic acid and EDTA have a well-defined crystal structure in the solid phase, as shown in Figure 6. Comparison of the experimental diffractogram to the PDF-2 database from the International Center for Diffraction Data (ICDD) showed that both salicylic acid and EDTA samples matched very well to their respective entries in the database (Figures SM-1 and SM-2 in the Supporting Information). However, the diffractogram of the mixture (EDTA+SA in Figure 6), which should be the sum of the diffractograms of EDTA and salicylic acid, did not have the expected peaks and it could not be identified by the database. Thus, these results suggest that the crystalline form of salicylic acid is modified in the presence of EDTA. The diffractograms also showed that the percentage of amorphous phase [21] (i.e. solid in a non-crystalline form) increased from 23.1% to 38.1% when salicylic acid was mixed with EDTA. Such loss of crystallinity and the reduction in crystal size could explain the signal enhancement when salicylic acid is desorbed from LDTD microwell plates coated with EDTA. Amorphous structures are less tightly bound together and require less energy for vaporization. Likewise, small crystals are more easily melted and vaporized.

These results are in agreement with a study of Marsac, et al. [22] on the miscibility of drug-polymer systems. In that paper, the authors estimated that the fraction of a drug available for crystallization decreased with increased weight percent of a polymer in a drug-polymer mixture. Also, they observed that when both polymer and drug are miscible, the melting point of the mixture is lower than that of the pure crystalline compound. For example, for a mixture of indomethacin with

poly(vinylpyrrolidone), a melting point depression of up to 20 °C was observed with a volume fraction of about 0.23 of poly(vinylpyrrolidone) compared to crystalline indomethacin.

4. Conclusion

Given the importance of high throughput in modern laboratories, fundamental studies on LDTD are necessary to improve sensitivity and widen the number of compounds analyzable with this ionization source. The present study showed that microwell surface coatings with commercial proteins such as immunoglobulin G (IgG) and fibrinogen can enhance more than 10 times the signal of organic compounds with polar functions such carboxylic acid, sulfonyl and nitro compared to uncoated microwells. Therefore, proteins can be used as alternative coatings when EDTA or nitrolotriacetic acid cannot be employed.

Studies using scanning electron microscopy (SEM) and powder x-ray diffraction (XRD) confirmed the previous observations that microwell coatings induce the formation of small crystals and also increase the percentage of amorphous phase of the analyte. Such conditions facilitate thermal desorption and subsequent transfer to the gas phase which could explain the signal enhancement observed in LDTD with microwell coatings. However, it was also noticed that reduction of crystal size does not always lead to higher LDTD signals as it was the case of atrazine. Therefore, it is possible that coatings may also affect the atmospheric pressure chemical ionization process in LDTD. More studies are necessary to validate this hypothesis.

5. References

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