Signal enhancement in laser diode thermal desorption-triple quadrupole mass spectrometry analysis using microwell surface coatings

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Abstract

Laser diode thermal desorption (LDTD) is an ionization source usually coupled to triple quadrupole mass spectrometry (QqQMS) and specifically designed for laboratories requiring high-throughput analysis. It has been observed that surface coatings on LDTD microwell plates can improve the sensitivity of the analysis of small polar molecules. The objective of the present study is to understand and quantify the effect of microwell surface coatings on signal intensity of small organic molecules of clinical, environmental and forensic interest. Experiments showed that the peak areas of diclofenac, chloramphenicol, salicylic acid and 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol obtained by LDTD-QqQMS increased by up to 3 orders of magnitude when using microwells coated with ethylenediaminetetraacetic acid (EDTA). Tests with different chelating agents and polytetrafluoroethylene as microwell surface coatings showed that nitrilotriacetic acid gave significantly higher peak areas for five out of the nine compounds that showed signal enhancement using chelating agents as coatings. Scanning electron microscopy studies of EDTA-coated and uncoated microwells showed that analytes deposited in the former formed more uniform and thinner films than in the latter. The enhancement effect of surface coatings in LDTD-QqQMS was explained mainly by the formation of homogenous and thinner layers of nanocrystals of analytes that are easier to desorb thermally than the layers formed when the analytes dry in direct contact with the bare stainless steel surface. Chemisorption of some analytes to the stainless steel surface of the microwell plate appeared to be a minor factor. Surface coatings widen the number of compounds analyzable by LDTD-QqQMS and can also improve sensitivity and limits of detection.

1. Introduction

The increasing demand for fast analysis of organic compounds at trace levels in food safety, toxicology, environmental and clinical laboratories has led to the development of new methodologies that aim to decrease analysis time and do not require sample separation [1]. Given the specificity and sensitivity of mass spectrometry, there have been many interesting developments in the last 15 years to adapt that technique to high throughput analysis. Laser-diode thermal desorption (LDTD), introduced in 2004, was specifically designed for high-throughput laboratories and can analyze one sample in a few seconds and run up to 3840 samples in a single sequence. This technology has been applied to the analysis of diverse small organic molecules of clinical [2, 3], food safety [4, 5] and environmental [6-8] interest.

In LDTD, a low volume (1-10 μL) of sample deposited on a stainless steel microwell plate is dried and then is rapidly thermally desorbed by an infrared laser that hits the back of the microwell plate, therefore there is no contact between the laser and the sample. Desorbed analytes are then transported by a gas to the plasma around a corona needle of an atmospheric pressure chemical ionization (APCI) source. Thus, the ions produced in the LDTD are mainly protonated molecules in the positive mode, or deprotonated molecules in the negative mode. Those ions are sampled by a mass analyzer, usually a triple quadrupole mass spectrometer (QqQMS).

Unpublished studies on the fundamental aspects of this technique [9] indicate that thermal desorption and volatilization at lower temperatures than those observed for bulk compound are explained by the fast heating rate (up to 3000 °C s^{-1}) of analytes present in the form of small crystals in the nanometer to micrometer range. In such state, the authors observed that analyte-surface interactions and molecular cohesive forces showed lower activation energies. Consequently, volatilization of a model compound, sulfadiazine, at temperatures ≈ 100 °C below the bulk melting point was possible.

Nevertheless, many questions about the factors involved in the LDTD process are of interest to further improve performance of this technique. For example, empirical evidence shows that for certain compounds, addition of a small molecule in the sample can enhance the analyte signal. This phenomenon was first reported by Beattie, et al. [10] when applying LDTD-MS/MS to in vitro drug discovery assays. The authors observed that precoating the microwell plate with erythromycin improved both intensity and relative standard deviation of the analytes' signals. Such behavior was also observed during the analysis of chloramphenicol residues in honey [5]. However, the observed increased signal of chloramphenicol caused by the addition of stearic acid appeared to be specific to that compound. Moreover, another study used a mixture of ethylenediaminetetraacetic acid (EDTA), methanol and ammonium hydroxide with the sample to improve thermal desorption of diclofenac [8]. The increased signal of small organic molecules in LDTD, especially carboxylic acids, after addition of EDTA in the sample or spotted on the microwell plate before the addition of the sample as a surface coating has been previously observed [11], but it has not been yet explained or quantitatively measured.

The goal of this study is to improve the understanding of the role of additives used as microwell plate coatings such as EDTA on analyte desorption and vaporization in LDTD-MS/MS. Two working hypotheses were proposed: *i)* Microwell coatings disrupt analyte-surface interactions. Therefore, addition of chelating agents such as EDTA, which can bind metals on the surface of the microwell plate, could decrease analyte-

surface cohesive forces and thus reduce the amount of energy necessary for desorption; *ii)* Microwell coatings disrupt the morphology of analytes after they are deposited on the microwell plates. Previous unpublished findings [9] suggested that the formation of small crystals of analytes on the microwell surface was responsible for lowering their enthalpy of vaporization.

To test these hypotheses a series of experiments with LDTD-QqQMS were performed with different microwell plate coatings and several analytes of clinical, environmental and forensic interest. Also, the surface of the microwell plate was studied using X-ray photoelectron spectroscopy (XPS) and the morphology of dried samples was investigated using scanning electron microscopy (SEM).

2. Material and methods

2.1 Chemicals and reagents

Ammonium hydroxide (concentration ≥ 28 % *v/v*) 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), hormones and others (estradiol, ethinylestradiol, benzyl butyl phthalate, caffeine, 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol, tryptophan) as well as microwell plate coating agents (Figure 2, left) disodium ethylenediaminetetraacetate dihydrate (EDTA), desferrichrome, nitrilotriacetic acid (NTA) and triethylenetetramine hexaacetic acid (TTHA) were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada). These compounds were chosen as coating agents because of their high affinity towards iron, an element present on the microwell plates.

Methanol (MeOH), acetonitrile (ACN) and water of LC-MS grade were bought from Fisher Scientific Canada (Ottawa, ON, Canada). β-glucuronidase and Rapid Hydrolysis Buffer for the urine extraction were bought from Integrated Micro-Chromatography Systems (Irmo, SC, USA). Stock solutions of analytical standards at 1 mg mL^{-1} were prepared in MeOH and stored at -20 °C, except for caffeine (H_2O-MeOH, 1:4, *v/v*) and tryptophan (H_2O-MeOH 3:7, *v/v*) which were stored at 4 °C. All stock solutions were kept for 6 months. Stock solutions of chelating agents were prepared in LC-MS grade water at a concentration of 10 mg mL^{-1} and were kept at 4 °C for a month.

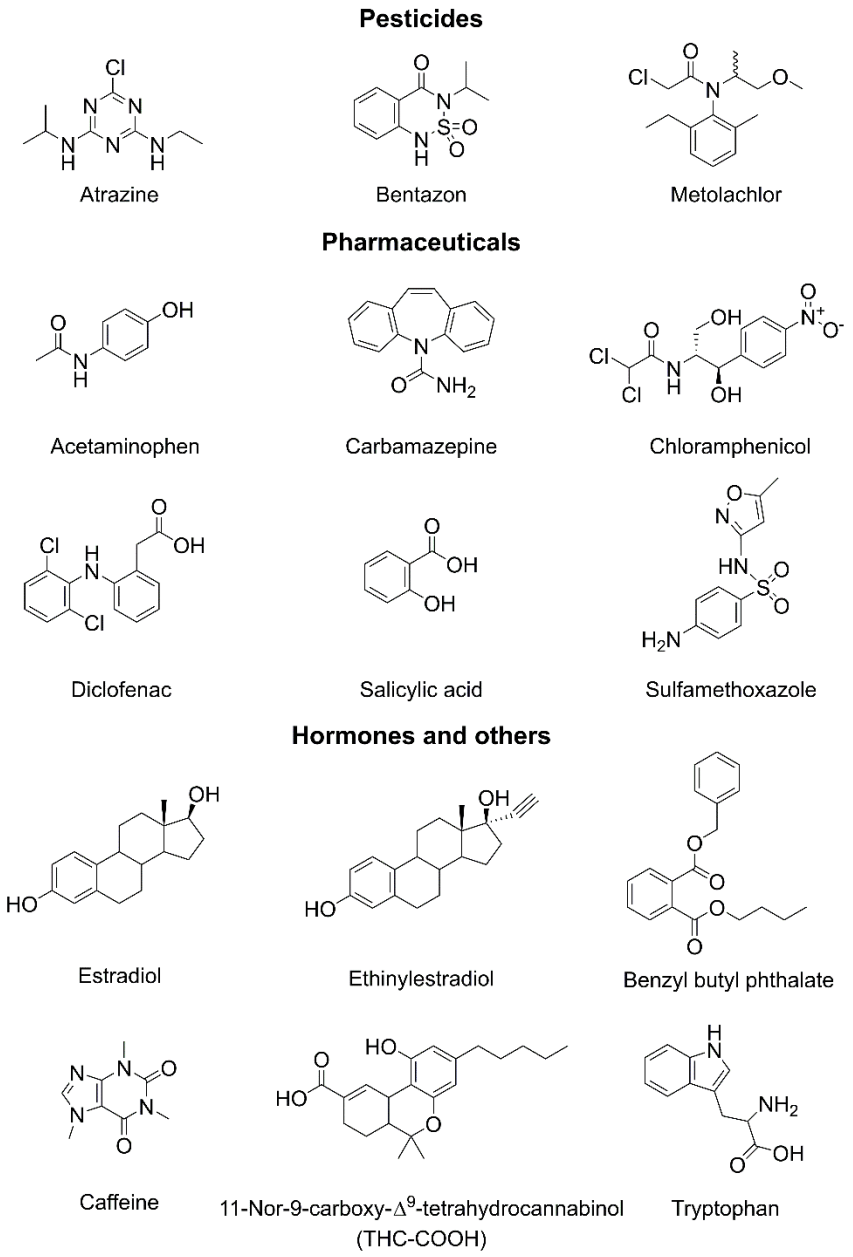

Figure 1. Molecular structures of the target analytes used in this study.

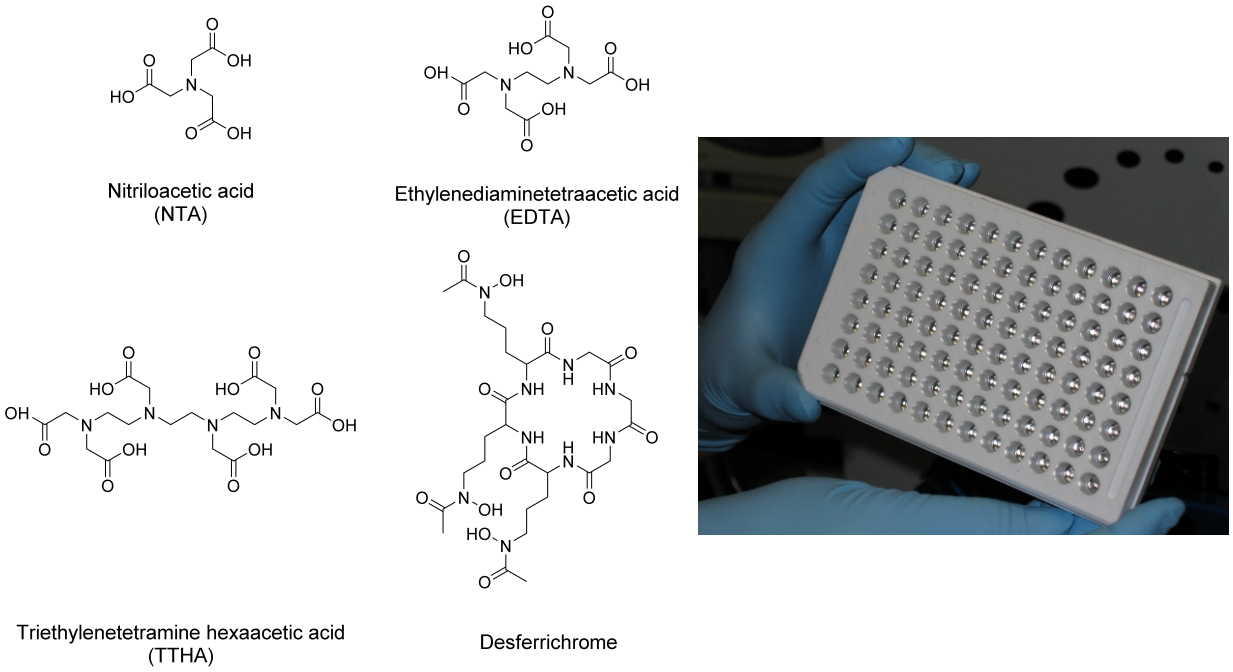

Figure 2. Molecular structures of the chelating agents used as microwell plate coatings in this study (left). Photograph of a LDTD 96-microwell plate (right).

2.2 Preparation of microwell plate coatings before LDTD-QqQMS analysis

LazWell microwell plates made of stainless steel 316L and manufactured by Phytronix (Québec, QC, Canada) were coated using solutions of chelating agents in MeOH-H_2O (3:1, *v/v*). They were prepared to obtain a concentration of coating agent of 100 µg mL^{-1}. In the case of EDTA, several solutions were prepared at concentrations from 10 to 500 µg mL^{-1}. Solutions were prepared by adding the required volume of stock solution of chelating agent and 250 µL of NH_4OH to 3.75 mL of MeOH. Finally, water was added to reach a final volume of 5 mL. Microwell plates with a polytetrafluoroethylene (PTFE) coating (LazwellHDE microwell plates, also made by Phytronix) were also used to evaluate the impact of an inert surface on signal of the analytes in LDTD-QqQMS.

To test the impact of microwell coatings on LDTD-QqQMS signal, five replicates were prepared as follow: 5 µL of coating agent solution were transferred to a single well of a stainless steel microwell plate (Figure 2, right) and were let to evaporate under a fume hood until dryness. Then, 5 µL of analytes diluted in MeOH at 100 ng mL^{-1} were added to the same wells and were let to evaporate as previously described. The same procedure was repeated for each solution of coating containing EDTA, TTHA, NTA or desferrichrome. For the microwell plates with the PTFE coating, only the analytes diluted in MeOH at 100 ng mL^{-1} were added.

2.3 LDTD-QqQMS analysis

Thermal desorption and ionization of samples were performed using a LDTD source model T-960 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 LDTD 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. This laser pattern was optimized to obtain optimal thermal desorption, i.e. higher laser power or longer laser times induced thermal degradation observed as drops in analyte signals. 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 when the nine others were ionizable in APCI in the positive mode. Therefore, two analyses per sample were required. Acquisition was made using the multiple reaction monitoring (MRM) mode, using one transition (Table 1). Data were processed on the software XCalibur version 4.0.27.19 from ThermoFisher. Peak areas of MRM transitions for each analyte were extracted from the acquisition files and used as analyte signal.

Since it has been reported that EDTA decomposes at temperatures > 200 °C, albeit in conditions much different to the ones used in the present study [12], it was hypothesized that EDTA will also decompose during LDTD process and form new compounds that could affect the signal. Therefore, in order to gain additional information about the effect of the EDTA microwell coating on the analytes, full scan mass spectra were acquired with the same QqQMS instrument and LDTD conditions used in the experiments described earlier except that acquisition was done in the full scan mode (*m/z* 50 to *m/z* 220) instead of MRM mode. These experiments could provide new information on the ions formed during the APCI process and possibly the presence of other compounds thermally desorbed by LDTD.

Table 1. Compounds of interest and their parameters for MS/MS analysis using MRM acquisition mode.

Compounds	**APCI polarity**	**MRM transition**			
		Precursor ion (*m/z*)	**Product ion (*m/z*)**	**Collision energy (V)**	**S-Lens (a.u.) [1]**
Acetaminophen	+	152	110	15	60
Atrazine	+	216	174	20	80
Bentazon	-	239	132	25	100
Benzyl butyl phthalate	+	313	149	15	60
Caffeine	+	195	138	25	80
Carbamazepine	+	237	194	20	80
Chloramphenicol	-	321	257	10	80
Diclofenac	+	296	215	20	80
Estradiol	+	255	159	20	80
Ethinylestradiol	-	295	145	45	110
Metolachlor	+	284	252	15	80
Salicylic acid	-	137	93	30	60
Sulfamethoxazole	+	254	156	15	80
THC-COOH [2]	-	343	245	30	123
Tryptophan	+	188	146	15	80

[1] arbitrary units. [2] 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol.

2.4 Surface water extraction

In order to evaluate the effect of microwell plate coatings on the signal of the target analytes in the presence of a real environmental matrix, surface water was sampled in a local river (Sherbrooke, QC, Canada). Several steps of sample preparation were required before solid-phase extraction (SPE). First, samples of 100 mL of surface water were filtered on a 1.2 µm-glass fiber prefilter. Then, pH was adjusted at 6.5 using NaOH or HCl. Finally, 20 mg of EDTA was dissolved in each 100 mL of sample. SPE was carried out using Strata-X cartridges (polymeric reversed phase, 200 mg bed mass, 6 mL cartridge volume) from Phenomenex (Torrance, CA, USA). Cartridges were conditioned with successively 5 mL of the mixture of MeOH-ACN (1:1, *v/v*) and 5 mL of water at pH 6.5. The sample (volume= 100 mL) was then loaded on the cartridge at a flow rate around 2 to 4 mL min^{-1}. After that, the cartridge was washed with 2 × 5 mL of the water at pH 6.5 and was allowed to dry during 10 min. Elution was finally done by 2 × 2.5 mL of the mixture MeOH-ACN (1:1, *v/v*). Extracts were evaporated to dryness with a N_2 flow and the dried sample residues were reconstituted with 10 mL of a solution of the 15 analytes at a concentration of 100 ng mL^{-1} each in MeOH-H_2O (1:1, *v/v*).

2.5 Urine extraction

The effect of microwell plate coatings on the signal of the target analytes in the presence of real human matrices was also investigated using urine samples. Before urine was extracted using SPE, 5 µL of MeOH, 20 µL of β-glucuronidase and 75 µL of Rapid Hydrolysis Buffer were added to 50 µL of urine sample and vortexed directly in the SPE cartridge (IMCStip, Integrated Micro-Chromatography Systems, Irmo, SC, USA). The mixture was incubated at room temperature for at least 30 minutes. The sample was then loaded on the cartridge with a positive displacement pump, washed with 500 µL of HCl 0.1 N and allowed to dry with a 45 psi pressure for 15 minutes. A second wash with 800 µL of a hexane: ethyl acetate (98:2, *v/v*) was also performed on the cartridge before the elution process could begin. Elution was finally done with 400 µL of ethyl acetate. Extracts were evaporated to dryness with an air flow and reconstituted with 17.5 µL of a solution of the 15 analytes, also at a concentration of 100 ng mL^{-1} (except for THC-COOH at 60 ng mL^{-1}) in MeOH:H_2O (1:1, *v/v*).

2.6 Plasma extraction

The effect of microwell plate coatings on the signal of the target analytes in the presence of real human matrices was also investigated using blood plasma samples. Proteins contained in the plasma were first precipitated using acetonitrile in a 4:1 ratio (ACN:plasma, *v/v*). The supernatant was then extracted and evaporated to dryness with an air flow and reconstituted with a solution of the 15 analytes at a concentration of 100 ng mL^{-1} (except for THC-COOH at 60 ng mL^{-1}) in MeOH:H_2O (1:1, *v/v*).

2.7 X-ray photoelectron spectrometry

Semi-quantitative analysis of the stainless steel surface of microwell plates was conducted by X-ray photoelectron spectrometry (XPS) on the Ultra DLD spectrometer from Kratos (Manchester, United Kingdom). Sample excitation was done by the Al Kα monochromatized line (1486.6 eV), with 225 W of applied power. The analyzer was operated in a constant pass energy mode (160 eV for the survey scans and 20 eV for the high-resolution scans). The analyzed area was an oval of dimensions 300 × 700 µm. The work function of the instrument was calibrated to give a binding energy (BE) of 83.96 eV for the Au $4f_{7/2}$ line of

metallic gold. The dispersion of the spectrometer was adjusted to give a BE of 93.62 eV for the Cu $2p_{3/2}$ line of metallic copper. The sample was mounted on a non-conductive tape and a charge neutraliser was used on all samples to compensate for the charging effect. Charge corrections were done using the adventitious carbon peak set at 284.8 eV. The surface of the sample was first analysed at 90° with respect to the analyser. To be able to observe the topmost surface of the sample by decreasing the depth of analysis, the sample was tilted at 30° emission angle relative to the surface. Data analysis was conducted using the Casa XPS software (version 2.3.18) by Casa Software. The relative sensitivity factors used for quantification purposes are the experimental relative sensitivity factors given by Kratos Analytical for their machines. Curve fitted spectra were done using the model given by Biensinger et. al. [13].

2.8 Scanning electron microscopy

Scanning electron microscopy (SEM) using a FEG-SEM S-4700 from Hitachi (Tokyo, Japan) was used to observe the effect of EDTA coating on the morphology and structure of samples deposited on the surface of the microwell plates which will be helpful to establish a link between crystallization of analytes and LDTD-QqQMS signal. The most and least responsive target analytes to microwell surface coating, salicylic acid and atrazine, respectively, were selected for this section of the study. For each compound, a volume of 5 µL at a concentration of 1000 ng mL^{-1} in MeOH was deposited on the stainless-steel surface and let to evaporate until dryness with or without previous addition of an EDTA coating. 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 EDTA as a microwell plate coating on the LDTD-MS/MS signal of selected analytes

Since the effect of EDTA as a microwell plate coating to enhance the signal of analytes in LDTD-MS/MS has not been yet quantified, it was decided to test several concentrations of EDTA coating solutions and measure the peak areas of the MRM transitions of four compounds showing low signals in LDTD-MS/MS (Figure 3).

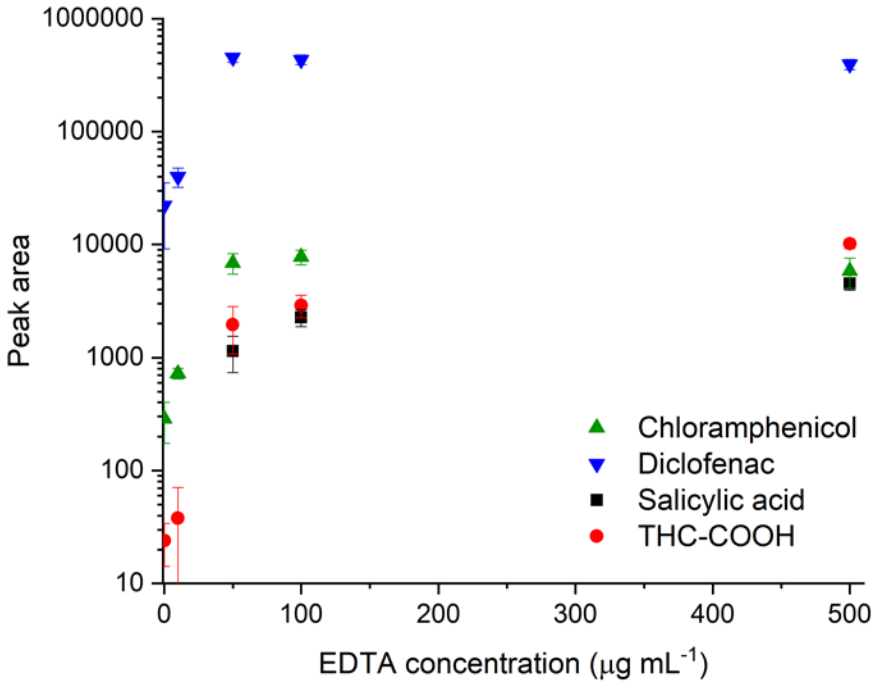

Figure 3. Effect of EDTA concentration in the coating solution on signal enhancement of four analytes in LDTD-MS/MS. Error bars represent ± 1 standard deviation (n = 5). Each compound was added at a concentration of 100 ng mL^{-1}.

These experiments showed that using EDTA as a microwell coating before addition of the sample improved the signal of the analytes up to 3 orders of magnitude. For salicylic acid, no signal was observed when it was added on the uncoated surface of the microwell. However, when the concentration of EDTA coating solution is 100 µg mL^{-1}, the peak area increased to more than 2000 counts. Experiments showed that for the tests compounds, the optimal concentration of EDTA was 100 µg mL^{-1}. Further increase of the concentration of EDTA did not result in a significant improvement on any of the analytes signals. These observations appear to validate the initial hypothesis of the role of surface coatings on the disruption of analyte-surface interactions: carboxylic acid functions of diclofenac, salicylic acid, THC-COOH as well as the nitro group in chloramphenicol could bind strongly to the stainless-steel surface, probably by forming strong coordination bonds with metallic iron on the stainless steel surface of the microwell plate. Chemisorption increases the energy necessary for thermal desorption, consequently less gas phase molecules of analyte reach the APCI source. Addition of an EDTA coating, a strong iron chelator, could reduce or eliminate those strong analyte-surface interactions, thus increasing the amount of analyte thermally desorbed by LDTD. In order to confirm the plausibility of the proposed hypotheses it was decided to study the composition of the surface of the stainless steel microwell plates by XPS. Also, the LDTD-QqQMS signal of the target analytes (Figure 1) deposited on diverse microwell plate coatings (iron chelators and PTFE) were compared to better understand the effect of the surface on the thermal desorption by LDTD.

3.2 Analysis of the composition of the upper layer of stainless steel by X-ray photoelectron spectroscopy

Previous studies have reported the composition of the surface of stainless steel 316L [14-16] but usually a polishing step or chemical treatment is applied before analysis by XPS or the uppermost surface layer is not studied. As electrons can only travel a given distance through a material before an interaction resulting in an energy loss occurs (the inelastic mean free path of an electron), it is possible to enhance contribution of the top layer to the total XPS signal by varying the angle at which these electrons are detected from the surface. Therefore, at angle 30°, the depth of analysis is shallower than at 90°. Comparison of the XPS analysis at 90º and 30º is shown in Table 2. These results indicate that the content of carbon is much higher when the analysis is shallower. This is explained by the presence of adventitious carbon, *i.e.* adsorbed hydrocarbons and carbon oxides on the surface of stainless steel 316L [17].

Table 2. Chemical composition in atomic percentage of stainless steel 316L determined by XPS.

Angle	Fe	Cr	Sn	Ca	Mn	C	Ni	O
90°	20.0	1.7	0.1	0.4	2.4	31.4	0.1	43.7
30°	5.4	0.7	0.1	0.7	0.9	62.6	n.d.	29.6

n.d.: Not detected.

High-resolution scans of the Fe 2p peaks with the curve synthesis for each oxide present (mainly FeO and Fe_2O_3) are shown on Figure SI-1 (Supporting information). As in a previous study[17], mainly Fe_2O_3 is observed at the surface of the sample. Tilting the sample showed that the Fe oxides overlay the metallic Fe (Fe^0). Effectively, the proportion of Fe^0 goes from around 5% of the total iron present at 90° to around 2% at 30°. Figure SI-2 (Supporting information) shows the high-resolution scans of the Cr 2p peaks. Like in the case of Fe, a layer of Cr oxides covers the metallic Cr (Cr^0) since the area of Cr^0 peak decreases by 2.7 % when the sample is tilted from 90° to 30°.

While it is possible that addition of EDTA disrupts the surface chemistry of stainless steel given its industrial use as a chemical treatment to increase corrosion resistance by increasing the Cr/Fe ratio on the surface of the alloy [17], XPS results indicate that metallic forms of Fe and Cr may not be available in sufficient amounts to bind analytes or chelating agents deposited on the microwell plates. However, the chemisorption of carboxylic acids to iron oxides is a phenomenon well known [18, 19] that is explained by a ligand exchange reaction, *i.e.* the metal on the surface layer (a Lewis acid) exchanges the OH^- ligand for another Lewis base, such as an organic acid [20]. Chemisorption of natural organic matter (rich in phenol and carboxylic acid groups), phthalic acid, and salicylic acid has been observed on iron oxides [21]. According to previous reports, small organic acids such as acetic acid [22] and salicylic acid [23] can chemisorb to Fe_2O_3 by acting as mono or bidentate ligands through coordination bonds with iron. These results indicate that chemisorption could explain low signals for small organic molecules with carboxylic acid functions analyzed by LDTD-QqQMS. However, the relative importance of this phenomenon has not been yet evaluated.

3.3 Effect of different coating agents on the LDTD-QqQMS signal of the target analytes

Experiments with different chelating agents as microwell coatings were performed in order to identify better signal enhancement effects and test a larger number of compounds with different molecular features. Analytes were also deposited on a microwell plate covered with PTFE in order to study the effect of an inert surface on thermal desorption by LDTD. Results of these experiments are shown in Figure 4. All test compounds except acetaminophen and estradiol had higher MRM peak areas using at a least one coating compared to no coating (i.e. bare stainless steel surface). Therefore, their average signal enhancement calculated as $\frac{signal_{coating}}{signal_{no\ coating}}$, was > 1. As it can be seen, such enhancement was compound-specific, but some trends were noticed. All the compounds with carboxylic acid functions (diclofenac, salicylic acid, THC-COOH, tryptophan) that are known to chemisorb the surface of stainless steel [15, 24] had average signal enhancement factors between 3 and 11795.

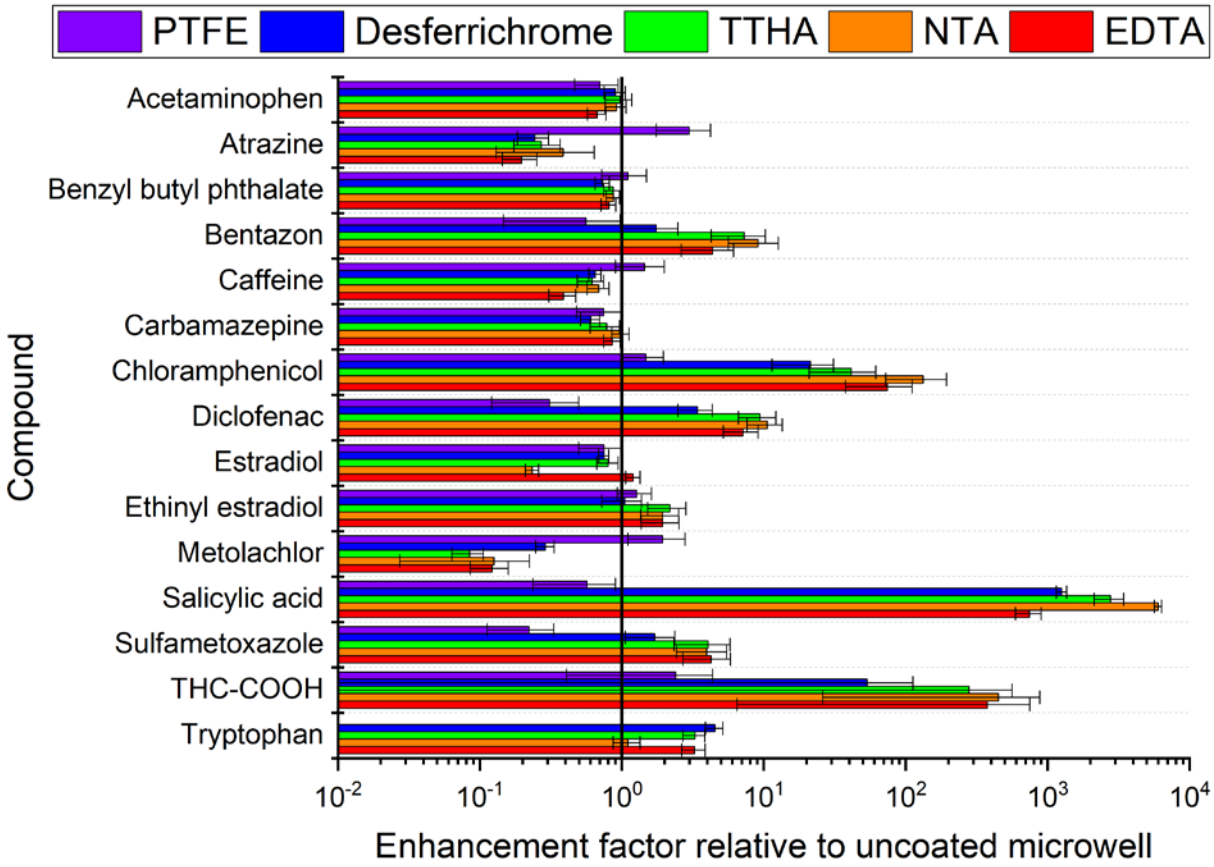

Figure 4. Signal enhancement factor observed with different coatings on the stainless steel microwell plates. Four replicate analyses were carried out for the compounds ionized in the negative mode (bentazon, chloramphenicol, ethinylestradiol, salicylic acid and THC-COOH) and five replicate analyses for the remaining compounds which were ionized in the positive mode. Error bars represent experimental error calculated from the standard deviation of the replicate analyses. Vertical line indicates no signal enhancement (=1).

The four other compounds with high improvement factors in Figure 4 (bentazon, chloramphenicol and sulfamethoxazole) have functional groups (sulfonyl, nitro) that could potentially chemisorb on the surface

stainless steel well since they could act as bidentate ligands [25] on iron oxides, although no experimental evidence of this was found in the literature.

In order to further identify the best chelating agent among those tested to coat microwell surfaces, analysis of variance (ANOVA) of the peak areas of the analytes followed by post-hoc tests at α=0.05 (Tukey's Honestly Significant Difference) were performed. Results showed that, in general, NTA gave the highest enhancement factor for five out of the eight compounds that showed signal enhancement using a coating. Since NTA has the lowest number of chemical functions capable of binding iron (Figure 2) of all the chelating agents used it is possible those functions interact mostly with iron oxides on the microwell surface. Thus, a higher number of binding functions could increase the amount of interactions with the analytes, resulting in increased adsorption to the coating. That explanation appears to be partially validated by the low enhancement factors of desferrichrome which has multiple functional groups (Figure 2) that could augment adsorption of the analytes to the coating. Additional experiments are necessary to further understand how the chelating agents are attached to the iron oxide topmost layer, but such experiments are out of the scope of the present study.

Results of the PTFE coating showed that inertness of the surface is not a major factor on the desorption of the analytes, in fact enhancement factor using PFTE was seldom > 2, and in some cases signal suppression was partial or complete as it was the case of tryptophan. These results suggest that cohesive analyte-surface interactions, i.e. chemisorption of the analytes to the stainless steel surface, does not appear to be the main factor causing the observed signal enhancement since plates covered with PTFE, where analyte-surface cohesive interactions should be very weak or nonexistent, also showed low signals for acidic compounds. In fact, only when chelating agents are added to PTFE (Figure SI-3) strong signals for organic acids are observed. Therefore, the proposed hypothesis that the observed signal enhancement of certain analytes in LDTD-QqQMS when using EDTA (or other chelating agents) as a microwell surface coating is the result of the elimination or reduction of strong analyte-surface interactions was rejected. Therefore, the second hypothesis, disruption of the morphology of the dried samples was tested by analysis of the uncoated and coated microwell plates using SEM.

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

Observation by SEM of the surface of uncoated and coated microwells revealed important differences (Figure 5). EDTA added on the bare stainless steel surface crystalized as a thin film with emerging nano-crystals. It was also observed that salicylic acid crystallized differently with the presence or absence of coating. When salicylic acid was added on uncoated microwells, it did not dry homogenously on the microwell surface, thus forming thick and inhomogeneous films. However, when salicylic acid was added to the microwells with the EDTA coating, the analyte dried more homogenously, covering the surface coated with EDTA, and formed a layer about 100 nm thick (Figure SI-4). On the other hand, atrazine which did not show any signal enhancement effect when coatings were added, appeared to be less drastically affected by the presence of EDTA and both large and small analyte crystals were observed. Therefore, SEM results suggest that addition of a coating on the microwell surface disrupts selectively the morphology of the dried analytes on the microwell plates. The presence of a coating on the microwell surface promoted the formation of smaller analyte crystals and more homogenous and thinner film of analyte on the surface of the microwell which should be easier to desorb thermally than the thick inhomogeneous film observed in the uncoated microwells. Studies on the effect of crystal size on the melting temperature of small organic molecules have demonstrated that a significant depression of the melting temperature of organic compounds are observed when crystal size is reduced to several nanometers [26, 27]. Therefore, chelating agents, which are able to chemisorb very effectively to iron oxides on the microwell surface, seem to form a new chemically modified surface that promotes the formation of a layer of nanosized-crystals on the microwell

plates that is easier to thermally desorb than the layer formed when dried analytes are in direct contact with the stainless steel surface. More studies are necessary to corroborate this hypothesis and to explain why the effect of the coatings shows a certain selectivity towards some chemical functions (e.g. carboxylic acids). These studies will help develop better strategies to improve the sensibility for compounds of interest in LDTD-QqQMS.

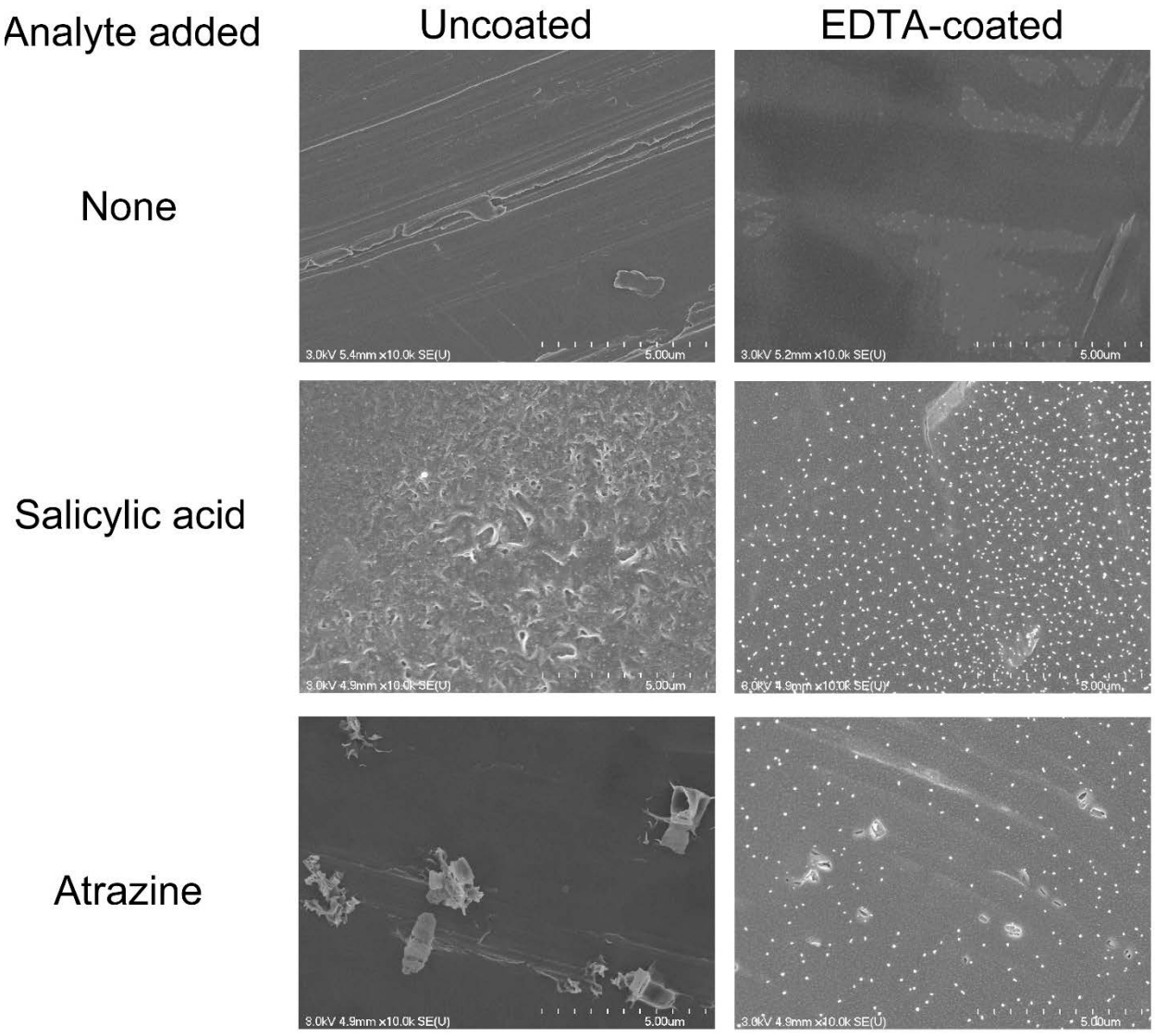

Figure 5. SEM micrographs of uncoated (top) and EDTA-coated stainless steel microwell with dried salicylic acid and atrazine. Deposits were made on the microwells with a 100 μg mL^{-1} EDTA solution and 1000 ng mL^{-1} of analytes.

3.3 Full scan mass spectra of microwells

Initially salicylic acid and atrazine were selected for these experiments, but due to the lower sensitivity of the instrument in the full scan mode, only data for atrazine results were conclusive. Full scan experiments with only atrazine added to the microwell showed that this compound started to desorb at detectable levels around 1.2 s (0.02 min) and reached its highest signal 2.4 s (0.04 min) after the laser was started (Figure SI-5, Supporting information). During this time, numerous compounds were also ionized, as indicated by the high value of the total ion chromatogram (TIC) signal. Therefore, in order to segregate the potential sources of the ions observed in full scan experiments, four different samples were analyzed: an empty well, the coating solution without EDTA, atrazine and atrazine in a well coated with EDTA. The results of these experiments are shown in Figure 6.

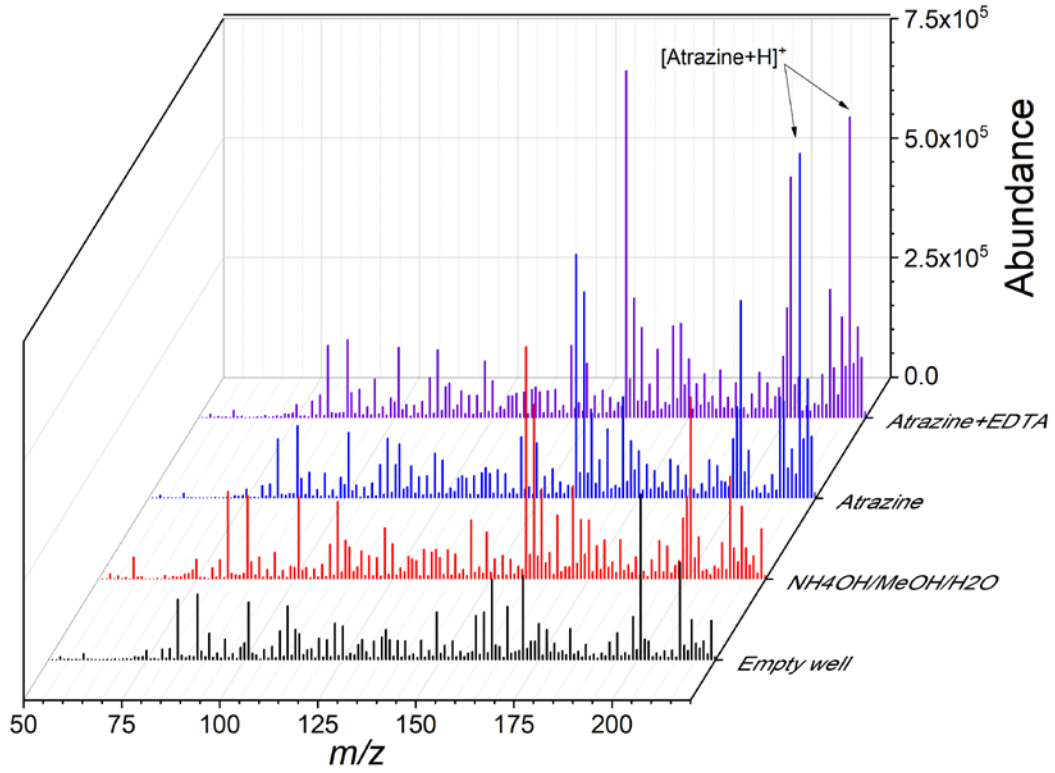

Figure 6. Mass spectra obtained by LDTD-QqQMS in the full scan mode. Only mass spectra obtained between 0.02 and 0.07 min are showed (time of maximum desorption of atrazine). $NH_4OH/MeOH/H_2O$ indicates the coating solution minus EDTA.

Surprisingly, a high number of ions were observed when analyzing an empty microwell, albeit only a few ions (e.g. *m/z* 163, *m/z* 171 *m/z* 201 and *m/z* 211) had intensities > 10^5 counts. The full spectrum of the coating solution minus EDTA ($NH_4OH/MeOH/H_2O$ on Figure 6) showed the presence of few additional ions, the most abundant being *m/z* 159. Spectra of both atrazine (without coating) and atrazine in an EDTA-coated well showed few observable differences, except a drop in the intensity $[M+H]^+$ ion of atrazine (*m/z* 216) of about 13% in the EDTA-coated microwell. In

both samples, none of the ions observed in their mass spectrum between *m/z* 50 and *m/z* 215 corresponds to a product ion of atrazine, which confirms that LDTD does not cause any detectable in-source fragmentation. Additionally, ions that could correspond to known degradation products of EDTA were not observed in the mass range used, therefore these experiments also suggest that the EDTA coating did not affect the ionization process. However, the high number of ions observed in these experiments requires further studies using LDTD coupled to a high-resolution mass spectrometer. Such experiments will be helpful to identify some of the ions observed and improve present understanding of the possible effect of coatings on APCI.

3.4 Analysis of spiked surface water, plasma and urine extracts using EDTA as a microwell coating

In order to evaluate the performance of the EDTA coating in the presence of real matrices, surface water from a local river, human urine and plasma samples were extracted and then spiked with a solution of the 15 compounds of interest. Those extracts were analysed by LDTD-MS/MS using both uncoated and EDTA-coated microwells (Figure 6). Results showed that the signal enhancement factors were much lower than those observed for samples in MeOH, in fact for no compound enhancement factors were > 7 (previously factors > 10 were observed).

Such striking differences with previous experiments could be explained by the presence of natural organic matter in the river water extracts. It is known that river water can contain from 1 to 5 μg mL^{-1} of dissolved humic substances [28] and they contain diverse chemical functions capable of acting as metal ligands [29]. As for the human matrices, the small enhancement factors observed could be explained by the presence of various organic metabolites capable of acting as metal ligands left after the protein precipitation in the plasma [30] or the extraction of urine. Therefore, it is possible that the matrix acted as a coating and no significant signal improvements are observed (compared to an EDTA-coated microwell) since a fraction of the matrix forms the new chemically-modified surface that allows the formation of a layer of sample that is easier to thermally desorb.

In an attempt to verify this hypothesis for the river samples, the same experiment was conducted with a strong anion exchange (SAX) cartridge instead of a reversed-phase cartridge, resulting in an extract of surface water with a significantly reduced amount of humic and fluvic acids [31, 32]. Results showed that when humic and fluvic substances were removed from the matrix (Figure 7), the signal enhancement factors for compounds containing carboxylic acid functions on sp^3 carbons (diclofenac, THC-COOH and tryptophan) were closer to the results obtained previously (Figure 4). No significant change was observed for the other model compounds.

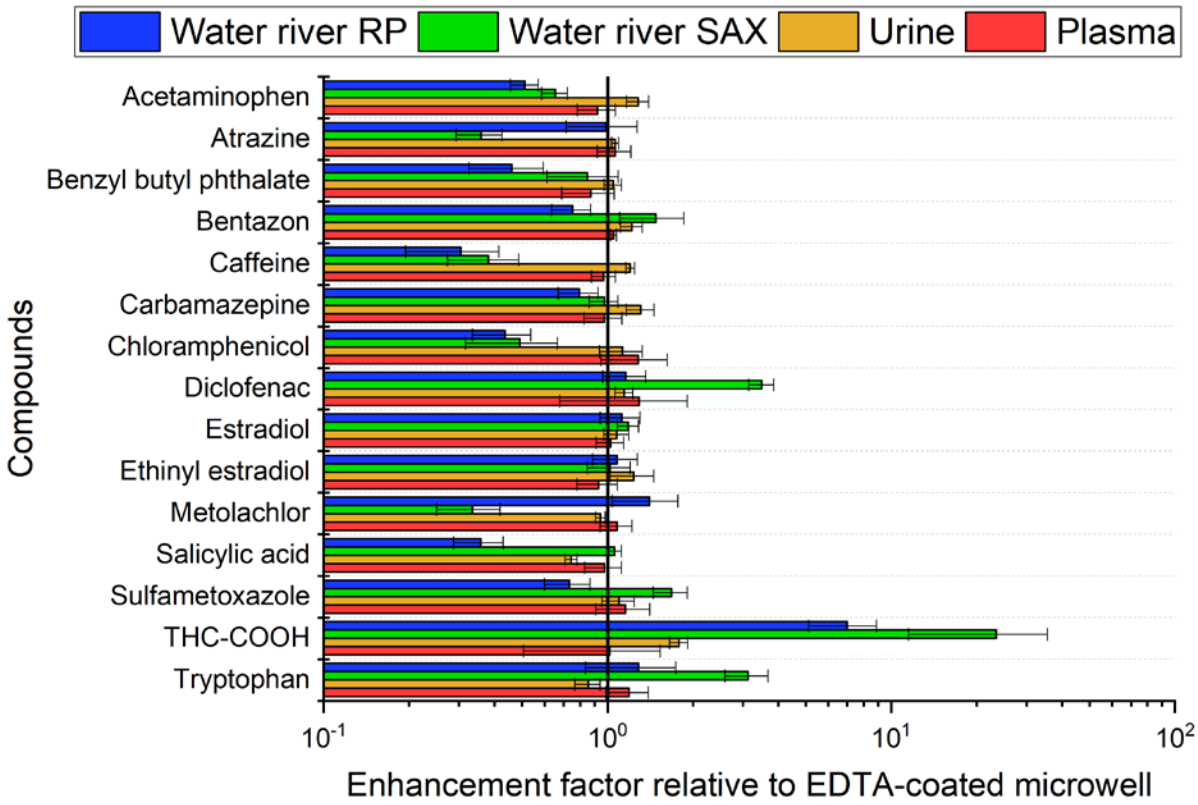

Figure 7. Signal enhancement factor for several organic compounds (each at 100 ng mL^{-1}, except for THC-COOH at 60 ng mL^{-1}) in different environmental and biological matrices analyzed by LDTD-QqQMS. Enhancement was calculated by comparing the signal of the analytes in the sample extracts versus signal of the analytes deposited in EDTA-coated microwells. Error bars represent experimental error calculated from the standard deviation of the replicate analyses. Vertical line indicates no enhancement (= 1).

4. Conclusion

This study quantified and attempted to explain for the first time the effect of surface coatings on LDTD. Results showed that chelating agents such as EDTA and NTA can be used as microwell surface coatings in order to increase the signal of small organic molecules poorly desorbed in LDTD, thus widening the application of this high-throughput ionization source. Experiments suggested that coatings of chelating agents enhanced the signal of several small molecules (especially carboxylic acids) not because of reduction of chemisorption of analytes to the stainless steel but because films of chelating agents appear to promote the formation of a layer of nano-sized crystals that is easier to thermally desorb than the layers formed when analytes are in direct contact with the stainless steel surface of the microwells. Results also showed that NTA is a better coating agent than EDTA, but this must be confirmed with a larger number of compounds. In order to develop new approaches to further improve the performance of LDTD-QqQMS for the analysis of small organic molecules, future studies should further investigate the link between morphology of the solid deposits of analytes on the microwell plates and signal enhancement effect caused by microwell

coatings. Also, it will be crucial to investigate by LDTD coupled to high-resolution mass spectrometry the potential effects of the coatings on the atmospheric pressure chemical ionization process.

5. References

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