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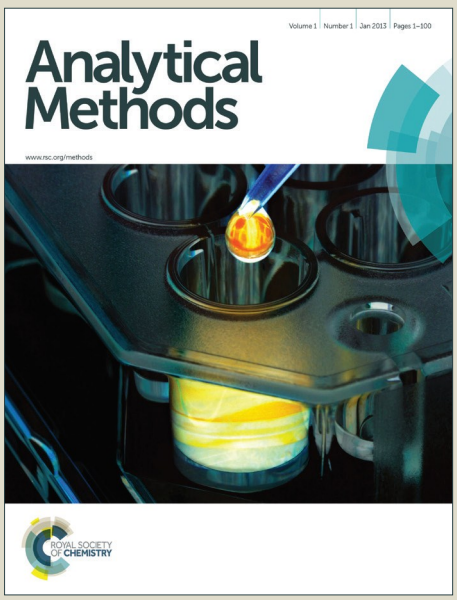

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Rapid determination of multiclass fungicides in wine by low-temperature plasma (LTP) ambient ionization mass spectrometry

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Abstract

Low-temperature plasma (LTP) probe is a plasma-based technique that permits direct and rapid ambient ionization and mass analysis of relatively complex samples in their native environment. It belongs to the ambient desorption/ionization mass spectrometry (MS) techniques. These features map well against the requirements of food quality and safety testing. In this study, the application of LTP-MS for the rapid screening and detection of pesticides in wines has been evaluated. Aliquots of the sample extract (3 μL of each solution) were deposited on a heated (120 ºC) microscope glass slide for analysis by LTP-MS. The analytical performance of LTP-MS has been studied for a set of 10 multiclass fungicides selected according to their relevance and presence in actual wine samples. The compounds included in the study were: azoxystrobin, carbendazim, dimethomorph, fenhexamid, flusilazol, metalaxyl, penconazole, tebuconazole, imazalil and thiabendazole. Two different approaches were examined: (i) direct analyses of wines with no prior treatment besides a simple sample dilution, and; (ii) analyses of sample extracts obtained after a thorough sample preparation step using solid-phase extraction with polymeric cartridges. The proposed approach enabled the detection of the pesticides in wine at low concentration levels in the range from 15 to 300 μg L^{-1} (fulfilling maximum residue levels (MRLs) set in EU regulations in all cases) by means of tandem mass spectrometry experiments with an ion trap operated in the positive ionization mode. The qualitative results obtained with actual red wine market samples compared well against the reference method based on liquid chromatography/mass spectrometry. Different examples shown demonstrate that ambient LTP-MS can be applied for the detection of these chemicals in beverages without sample treatment steps besides dilution.

Introduction

Pesticide testing in foodstuffs is of great interest for the protection of human health and also for international trade and regulatory control. The increasing public concern about the potential health risks posed by the presence of toxic residues in the human diet has focused sight on food quality and safety. Pesticides comprise a large group of substances with a common characteristic of being effective against a pest. Their control represents a challenge for the analyst, since there is not a universal method for their determination, keeping in mind the large number of these substances, which display different physicochemical properties.

Nowadays, different analytical techniques including gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) have been extensively used for trace analysis in complex matrices. Particularly, LC-MS is the mainstream approach for pesticide analysis in food. These techniques often require tedious and time-consuming sample pretreatment prior to analysis. So, it is desirable to develop some simple and efficient methods for analyses of these chemicals in complex samples such as food with minimal or even no sample treatment. Furthermore, the development of fieldable methods enabling accurate, quick and efficient testing of food (composition, nutrition facts, potentially harmful ingredients and allergens) is a challenging endeavor of current analytical science. In the near future, it would be desirable that this type of testing would be performed *on site*, rather than in the laboratory. To achieve this milestone (field analysis), there is a need to further develop portable mass spectrometry technology [1-3] and this equipment should be accompanied by sampling (ionization) methods involving no or little sample preparation as happens with the family of ambient ionization mass spectrometry methods [4] that have emerged in the last decade [5-11].

Ambient ionization refers to the creation of ions for mass spectrometry by examination of native materials in the open environment. In this sense, food quality and safety testing is a field whose requirements map well against the features of ambient ionization mass spectrometric techniques. Different applications dealing with the use of a wide range of ambient MS methods for different food analysis applications have been recently described in the literature [12-14], particularly based on the use of

commercially available ambient MS methods such as Desorption Electrospray Ionization Mass Spectrometry (DESI-MS) [15-17] and Direct Analysis in Real Time (DART) [18-24]

Amongst the available ambient desorption/ionization mass spectrometry (MS) methods developed so far, low-temperature plasma (LTP) [25] is a plasma-based approach [26,27] that permits direct and rapid ambient ionization and mass analysis with minor sample workup. The plasma in an LTP probe is generated by dielectric barrier discharge (DBD), and a discharge gas (typically helium) at a low flow rate (typically 100-300 mL/min) combined with a high AC voltage, are used to ignite and sustain the plasma at ambient pressure. LTP mass spectrometry has been demonstrated as a powerful analytical tool for direct analysis of a wide variety of chemicals from complex samples in particular with small organic molecules with low to moderate polarity [28]. Qualitative and quantitative analysis using LTP probes has been reported for a wide variety of applications [29] including public safety [30], food safety including pesticide analyses in fruit and vegetables [31-33], product quality control and forensics [34].

The Wine Industry is a billion-dollar business in many countries worldwide, with dozens of millions of full-time equivalent jobs. Europe is the main producer of wine in the World, Italy, France and Spain being the larger producing countries [35]. The development of rapid methods for testing quality of wine is of potential interest for these relevant reasons. In the present work, the usefulness of LTP-MS as a quick method to determine the presence of pesticides in wines is examined. To our knowledge this is the first study dealing with ambient mass spectrometry and pesticide testing in alcoholic beverages.

Experimental

Reagents and standard solutions

Pesticide analytical standards were purchased from Fluka, Pestanal® quality (Madrid, Spain) and Sigma–Aldrich (Madrid, Spain). Individual stock solutions of the studied compounds (*ca.* 500 μg mL^{-1} each) were prepared in methanol (MeOH) or acetonitrile and stored at -20 ºC. HPLC-grade MeOH and acetonitrile were obtained from Merck (Darmstadt, Germany). A Milli-Q-Plus ultra-pure water system from Millipore (Milford, MA, USA) was used throughout the study to obtain the HPLC-grade water

used during the analyses. Oasis HLB™ SPE cartridges (200 mg, 6 mL), purchased from Waters (Milford, MA, USA) were used to perform a SPE step to preconcentrate the pesticides in wine. A Supelco Visiprep™ (Bellefonte, PA, USA) SPE vacuum system was also used.

Low-temperature plasma (LTP) tandem mass spectrometry (LTP-MS/MS)

Experiments were performed using a Bruker Esquire HCT ion trap mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). LTP-MS analysis was performed in the positive ionization mode for optimum detection of the precursor ion of interest. The instrument was set to collect spectra for a maximum ion trap injection time of 200 ms and 3 scans per spectrum. The main experimental parameters used were as follows: *m/z* range: 50-450; skimmer: 33.4 V; cap exit voltage: 134.7 V; octopole 1 and 2 voltage: 9.92 and 2.58 V, respectively; trap drive: 32.5 (manufacturer's unit); lens 1 and 2: -4.4 and -89.7 V, respectively. Tandem mass spectrometry experiments (MS/MS) were performed using collision-induced dissociation (CID) in order to confirm the presence and estimate the concentration of the chemicals in the studied samples. These experiments were performed using an isolation window of 1.5 (*m/z* units) and 0.5 - 1 collision energy (manufacturer's unit), the collision gas was helium 6.0 (Air Liquide España S.A., Sevilla, Spain).

The LTP probe (**Figure 1**) described elsewhere [25] consists of a glass tube (O.D. 6.35 mm and I.D. 3.75 mm) with an internal grounded electrode (stainless steel, diameter: 1.57mm) centered axially and an outer electrode (copper tape) surrounding the outside of the tube [25]. The wall of the glass tube serves as the dielectric barrier. An alternating high voltage of 6.2 kV at a frequency of *ca.* 2.5 kHz, is applied to the outer electrode with the center electrode grounded to generate the dielectric barrier discharge. Helium 6.0 was used as a discharge gas and to transport analyte ions to the mass spectrometer at a flow rate *ca.* 0.45 L/min. The sampling plasma torch operates at low temperature (30 ºC) interacting directly with the sample, leading to desorption and ionization of the surface molecules sampled. The standards and samples were placed on the sample holder, typically 0.5 cm away from the mass spectrometer inlet. The LTP probe was placed with its end *ca.* 2-5 mm away from the surface with an angle of ca. 20° from the sample surface. As substrate heating leads to an improvement in sample evaporation and ionization [31,33,34], a heat gun was used in order to set the temperature of the sample substrate to 120 ºC.

<Figure 1>

Samples. Different red wine and soft drink samples were purchased from different local markets. Two main experiments were performed: 1) LTP-MS/MS analysis of wine after dilution; and 2) LTP-MS/MS analysis of acetonitrile extracts after a thorough sample treatment.

Direct LTP-MS/MS analysis of wine. An aliquot of 100 µL of wine was diluted with 400 µL of acetonitrile. Aliquots (3 µL) were deposited on a microscope glass slide and analyzed by LTP-MS/MS.

LTP-MS/MS analyses of wine SPE extracts. The solid-phase extraction procedure was adapted from previous work [36] using polymeric cartridges Oasis HLB. The cartridges were preconditioned with 4 mL of MeOH and 4 mL of ultrapure water at a flow rate of 2 mL min^{-1}. After the conditioning step, an aliquot of 4 mL of wine was passed through the cartridge at a flow rate of 1 mL min^{-1}. Then the cartridge was washed with 4 mL a mixture of MeOH/H_2O (5:95, v/v) and subsequently dried by vacuum during 1 min. The retained analytes were eluted with 2 mL × 4 mL of MeOH at 1 mL min^{-1}. This eluate was then evaporated until near dryness by a gentle nitrogen stream using a TurboVap LV from Zymark (Hopkinton, MA), with a water bath temperature of 37 ºC and a N_2 pressure of 15 psi. The samples were then made up with 1 mL of acetonitrile. Final preconcentration factor attained is 4:1. 3-µL aliquots of the SPE extract (3 µL of each solution) were deposited on a microscope glass slide for analysis by LTP-MS/MS.

Liquid Chromatography Electrospray Mass Spectrometry Reference Method

Wine extracts were analyzed using LC-MS reference method reported by Pérez-Ortega *et al* [36]. The separation of the species from the SPE extracts was carried out in a reversed phase C_{18} analytical column of 50 mm x 4.6 mm and 1.8 µm particle size (Zorbax Rapid Resolution Eclipse XDB-C18) by means of an Agilent HPLC system (Agilent 1290 Infinity, Agilent Technologies, Santa Clara, CA, USA). 20 µL of extract were injected per analysis. Mobile phases A and B were water with 0.1% formic acid and acetonitrile respectively. The chromatographic method held the initial mobile phase composition (10% B) constant for 2 min. Then the content of B was increased up to 50% at 5 min, followed by a linear gradient to 100% B at 15 min and held constant for 3 min at 100% B. The flow-rate used was 0.5 mL min^{-1}. Identification of the analytes was

performed by accurate mass measurements of the protonated ion of the targeted species using a time-of-flight mass spectrometer (Agilent 6220 accurate mass TOF, Agilent Technologies, Santa Clara, CA, USA).

Results and discussion

Qualitative detection of pesticides by LTP-MS/MS and method performance

The studied fungicides (**Table 1**) were selected taking into account the positive findings obtained from a previous monitoring study covering over 70 pesticides in different Spanish red wine samples. [36]. The identification of the targeted pesticides was confirmed with neat standards. Aliquots of 3 µL of each standard solution were pipetted in microscope slides with the plasma probe focused directly towards the sample substrate. The pesticides were ionized and transported inside the mass spectrometer. All the species were detected in the positive ion mode. For identification purposes, different product ion scan MS/MS experiments were accomplished to study the main fragmentation for each species. The parent ion of each pesticide ($[M+H]^+$) was isolated and fragmented in the ion trap, resulting in characteristic fragment ions for each targeted analyte. The data obtained are summarized in **Table 1**. The fragmentation displayed by the pesticides was consistent with previous studies using tandem mass spectrometry [37]. In most cases, it involved neutral losses of small molecules such as methanol to yield even electron fragment ions. For instance, azoxystrobin MS/MS and MS^3 fragmentation yielded two consecutive neutral losses of methanol (*m/z* 404 → 372 → 344). In most cases MS/MS was enough to provide selectivity in terms of absence of chemical background at the measured transitions. Exceptionally, an additional step (MS^3) was necessary for carbendazim when addressing real samples. At least two product ions were found for each analyte using MS/MS or MS^3, except for thiabendazole. **Figure 2** shows a representative example of the transient signals obtained for the LTP-MS/MS analysis of two standards solutions of 100 µg L^{-1} (300 pg) of carbendazim and metalaxyl. Each transient signal corresponds to the LTP-MS/MS analysis of a 3-µL aliquot pipetted on the microscope slide. The RSD (%) obtained (n = 3) were typically in the rage from 5 to 25 %, as in previous studies [31,33,34]. One of the major contributions to these relative high values compared to standard methods such as liquid chromatography/mass spectrometry (LC-MS) is the manually performed sample deposition. The development of automatic devices with automatic feeding of

samples would certainly improve this figure, although at the expense of simplicity, a main feature of these methods in order to expand their applications towards *in situ* (field) analysis.

To estimate the limits of detection (LOD) from standard solutions of the different compounds studied, aliquots of different concentrations were interrogated, using as criterion a signal-to-noise ratio of ca. 5:1. As product ions MS/MS spectra obtained hardly produced any background signal when interrogating solvent standards, the average mass spectrum with a product ion intensity of at least 300 counts was used as default criterion. The results are shown in **Table 1**. Most of the analytes were detected at low concentration levels with LODs below 50 picograms in most cases.

Direct analysis of pesticides in wine by LTP-MS/MS. Sample composition and matrix effects

Firstly, the direct analysis of untreated wine was assayed. 3-µL aliquots spiked with different pesticides spiked at different concentration levels (in the range from 0.01 to 1 mg L^{-1}) were studied. Strong matrix suppression effects were observed compared to the analyses of the same amounts of analytes in the absence of wine matrix. The previous studies (fragmentation and LODs) were performed using the different compounds dissolved in acetonitrile or methanol. In these conditions solvent evaporation and subsequent compound ionization are favored as it has been previous reported [31,34]. Wine samples involve an aqueous environment, and this together with the matrix components was the reason for the matrix suppression issues. To overcome this, different dilutions of wine and acetonitrile were tested (1+1 (v/v), 1+4 (v/v and 1+9 (v/v)). For this purpose, aliquots of 100 µL of wine spiked with 200 µg L^{-1} metalaxyl were mixed with 100, 400 and 900 µL of acetonitrile, yielding a final metalaxyl concentration of 100, 40 and 10 µg L^{-1} respectively (300, 120 and 30 pg of metalaxyl) and they were analyzed by LTP-MS/MS, (MS/MS transition *m/z* 280 > *m/z* 248). As shown in **Figure 3**, regardless the dilution factor, the transient signals obtained from *m/z* 248 did not changed accordingly to the different amount of analyte tested. The same occurs with the product ion spectra for each of the tested dilutions. The same pattern was observed for the rest of analytes, when tested in wine extracts. The 1+4 (v/v) showed the best peak intensity reproducibility within the different tested solutions. Consequently, it was used to dilute the different wine samples used in this work to

facilitate solvent evaporation, analyte desorption and subsequent ionization and mass analysis.

As an example, the analysis of azoxystrobin in spiked wine (60 μg/L) is shown in **Figure 4**. Signals obtained are distinctly higher than the dashed line, which corresponds to the analyses of the same wine before spiking. The sample was also tested using a reference method based on LC-MS [36] and no trace of azoxystrobin was found. Method performance in terms of sensitivity was estimated using 1:5 dilutions selected and the results obtained with spiked samples are shown in **Table 1**. In most cases, the lowest detection level reported for wine keeping in mind the dilution were clearly below the maximum residue level (MRL) set for grapes. The content of pesticide in wine is not regulated with as specific regulation. Actually, (MRLs were calculated on the basis of the corresponding MRLs in grapes, keeping in mind the processing factor of 1 L of wine/1 kg of grape, that should be applied to convert the MRL values to wine according to Commission Implementing Regulation (EU) No 400/2014 [38]. Taking this into consideration, the results demonstrated the usefulness of the approach for rapid testing of pesticides in wine at the levels required by current regulations.

LTP-MS/MS analysis of pesticides in wine extracts obtained using solid-phase extraction

Besides direct analysis of pesticides in wine after dilution, as an alternative, selected red wine samples subjected to the generic SPE procedure using polymeric cartridges described, were also examined. The example of the detection of metalaxyl in two red wine samples is shown in **Figure 5**. According to LC-MS analysis, metalaxyl was detected at 50 and 320 μg L^{-1} respectively for samples A and B. The presence of metalaxyl in these wine samples confirms the results obtained in the previous monitoring study carried out [36]. One of the replicates from sample A yielded an outlier value, due to probably non-reproducible sample spotting. This step (sample spotting) was found the main source of uncertainty regardless the type of extract analysed (SPE or direct dilution), increasing RSDs % in some cases to values higher than 25 %. Besides the transient signals of *m/z* 248 from product ion scan MS/MS experiment, the actual averaged product ion MS/MS spectra is shown also in **Figure 5** (right). Both spectra shown are not exactly the same as those collected with neat

standards. There were additional peaks detected at *m/z* 149/150 and *m/z* 205, with a source different from the analyte itself. This can be attributed to others species presents in the wine. Actually, the contamination is highly likely to be due to the presence of phthalates in the sample extract after SPE step. Di-butyl phthalate in the positive ion mode is detected at *m/z* 279, and its ^{13}C isotope signal corresponds to *m/z* 280. The fragmentation of this phthalate yields *m/z* 149 ($C_8H_5O_3^+$) and 205 ($C_{12}H_{15}O_3^+$). Both fragments were detected in the product ion spectrum of the two samples at relevant concentration, thus altering the fragmentation pattern of the spectra, although not interfering in the detection and identification of metalaxyl. The results shown are consistent with those obtained with LC-MS thus proving the usefulness and performance of the proposed method.

Conclusions

In this study, the usefulness of LTP-MS/MS as a fast method for qualitative and semi-quantitative determination of pesticides in wines has been demonstrated. Only a simple sample dilution with solvent is required to enable the sensitive detection of the chemicals studied at the picogram level, so that the method can be useful for rapid inspection of pesticides and the fulfillment of MRLs levels (considering the processing factor from grapes to wine). Finally, the applicability of the proposed approach can be further extended towards the detection of other relevant chemicals such as food additives like sweeteners, dyes or preservatives, not only in wine but in a wide variety of beverages.

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Figure Captions

Fig. 1 Scheme of the LTP probe used for ambient ionization mass spectrometry.

Fig. 2 LTP-MS/MS analysis of carbendazim (A) and metalaxyl (B) standards (100 µg/L). A 3-µL aliquot was deposited on sample substrate (300 picograms each analyte). Transient signals obtained for carbendazim fragment with *m/z* 160, and metalaxyl main fragment (*m/z* 248), during product ion scan MS/MS analyses (left); and their corresponding product ion mass spectra (right).

Fig. 3 LTP-MS/MS analysis of metalaxyl in spiked wine. Transient signal of metalaxyl at *m/z* 248 in a spiked wine sample diluted with different solvent proportions (left part) and averaged product ion scan MS/MS spectrum (right). A) Dilution 1:1 (100 µL wine and 100 µL acetonitrile, [metalaxyl] = 100 µg/L (300 pg deposited)); B) Dilution 1:5 (100 µL wine and 400 µL acetonitrile, [metalaxyl] = 40 µg/L (120 pg)); C) Dilution 1:10 (100 µL wine and 900 µL acetonitrile, [metalaxyl] = 10 µg/L (30 pg)).

Fig. 4 LTP-MS/MS analysis of azoxystrobin in spiked wine after dilution (60 µg/L, 180 pg). Transient signal at *m/z* 372 obtained for the analysis of 3 µL of spiked wine (continuous line) and blank (dashed line).

Fig. 5 LTP-MS/MS analysis of metalaxyl in two wine samples after SPE extraction in which metalaxyl was detected (50 and 320 µg/L in samples A and B respectively). Transient signals of metalaxyl (*m/z* 248) (left) and averaged product ion scan MS/MS spectra (right).

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Table 1. Identification and analytical performance of studied compounds by low-temperature plasma tandem mass spectrometry.

Compound (Class)[a]	Molecular Formula	M_r[b] (g/mol)	Ion	MS/MS (MS^n)	LOD solvent standards (µg/L)[c]	Lowest detection level in wine (µg/L)	MRL (grape) (mg/kg)
Azoxystrobin[1]	$C_{22}H_{17}N_3O_5$	403.4	$[M+H]^+$	404 → 372 (→344)	20	250	2
Carbendazim[2]	$C_9H_9N_3O_2$	191.2	$[M+H]^+$	192 → 160 (→132)	2	20	0.5
Dimetomorph[3]	$C_{21}H_{22}ClNO_4$	387.9	$[M+H]^+$	388 → 301, 165	30	300	3
Fenhexamid[4]	$C_{14}H_{17}Cl_2NO_2$	302.2	$[M+H]^+$	302 → 142, 178, 266	50	250	5
Flusilazol[5]	$C_{16}H_{15}F_2N_3Si$	315.4	$[M+H]^+$	316 → 165, 187	2	20	0.2
Imazalil[5]	$C_{14}H_{14}Cl_2N_2O$	297.2	$[M+H]^+$	297 → 255, 201, 159	10	50	0.05
Metalaxyl[4]	$C_{15}H_{21}NO_4$	279.3	$[M+H]^+$	280 → 220, 248	2	15	1
Penconazole[5]	$C_5H_{11}NO_2$	284.2	$[M+H]^+$	284 → 159, 173	25	150	0.2
Tebuconazole[5]	$C_{16}H_{22}ClN_3O$	307.8	$[M+H]^+$	308 → 70,125	30	200	2
Thiabendazole[2]	$C_{10}H_7N_3S$	201.2	$[M+H]^+$	202 → 131	2	40	0.05

[a] Fungicide class: 1. stobilurins; 2. benzimidazole; 3. morpholine; 4. anilide fungicides; 5. conazole fungicides.

[b] Molecular mass (Mr) calculated using isotope-averaged atomic masses for the constituent elements.

[c] 3 µL of sample or standards per analysis