Elsevier Editorial System(tm) for Journal of Chromatography A
Manuscript Draft

Manuscript Number: JCA-12-354R3

Title: Comprehensive qualitative and quantitative determination of pesticides and veterinary drugs in honey using liquid chromatography-Orbitrap high resolution mass spectrometry

Article Type: Full Length Article

Keywords: Mass exact database; High resolution Orbitrap mass spectrometry; Pesticide; Veterinary drug; Generic extraction; Honey

Corresponding Author: Dr. A. Garrido Frenich,

Corresponding Author's Institution: University of Almeria, Dept. of Hydrogeology and

First Author: Mª Luz Gómez-Pérez

Order of Authors: Mª Luz Gómez-Pérez; Patricia Plaza-Bolaños; Roberto Romero-González; José Luis Martínez Vidal ; A. Garrido Frenich

Highlights

- Creation of a database for the identification of >350 compounds in Orbitrap.
- First comprehensive database for pesticides and veterinary drugs.
- Analysis by UHPLC-Orbitrap-MS.
- Application to honey analysis using a generic approach.
- UHPLC-Orbitrap MS provide adequate quantification/confirmation capabilities.

Comprehensive ~~exact mass database for the analysis~~qualitative and quantitative determination of pesticides and veterinary drugs in honey using liquid chromatography-Orbitrap high resolution mass spectrometry

María Luz Gómez-Pérez[1], Patricia Plaza-Bolaños[1,2], Roberto Romero-González[1], José Luis Martínez-Vidal[1], Antonia Garrido-Frenich[1]*

[1]Research Group "Analytical Chemistry of Contaminants", Department of Hydrogeology and Analytical Chemistry, *Research Centre for Agricultural and Food Biotechnology (BITAL)*, University of Almería, *Agrifood Campus of International Excellence, ceiA3*, E-04120 Almería, Spain

[2]Department of Analytical Chemistry, University of Granada, E-18071, Granada, Spain

* Corresponding author. Tel: +0034 950015985; fax: +0034 950015483.

E-mail address: agarrido@ual.es (A. Garrido Frenich).

ABSTRACT

A database has been created for the simultaneous analysis~~detection~~ of more than 350 pesticides and veterinary drugs (including antibiotics) using ultra-high performance liquid chromatography coupled to high resolution Orbitrap mass spectrometry (UHPLC-Orbitrap-MS). This is a comprehensive exact mass database built using the Exactive-Orbitrap analyzer. The developed database includes ~~accurate~~ exact masses of the target ions and retention time data, and allows the automatic search of the included compounds. Generic chromatographic and MS conditions have been applied. The presented database is suitable for qualitative analysis, and it was also evaluated for quantitative ~~analysis~~ purposes in routine analysis, after the optimization and validation of a generic extraction method in honey samples. Adequate recovery and precision values for most of the studied analytes were obtained and the limits of detection (LOD) ranged from 1 to 50 µg kg^{-1}. For ~~In the case of~~ pesticides, LODs were always lower than the MRLs established by European Union in honey, except for a few compounds. This method was applied to the analysis of 26 real honey samples and some pesticides (azoxystrobin, coumaphos, dimethoate and thiacloprid) were detected in 4 samples. Azoxystrobin and coumaphos were determined in two different samples (organic honey~~s~~) at 1.5 µg kg^{-1} and 5.1 µg kg^{-1}. ~~No v~~Veterinary drugs were not~~was~~ detected in the real samples.

Keywords: mass ~~accuracy~~ exact database, high resolution Orbitrap mass spectrometry, pesticide, veterinary drug, generic extraction, honey

1. Introduction

Pesticides and veterinary drugs (VDs) are chemicals formulations widely used in agriculture and farming to increase production, to treat infections or for prophylactic reasons [1]. However, the potential presence of residues and contaminants is an important issue in the field of food and animal feed safety [2]. The large number of permitted and commercially available pesticides and VDs has caused a steady increase of the number of analytes to be monitored [3]. Therefore, emerging multi-residue methods are capable of monitoring a large number of compounds by the useusing of a single analytical method [4]. The determination of compounds with a wide variety of physical-chemical properties is feasible [5,6] and an important step is the combination of these methods with generic extraction procedures to increase the scope of the analysis [7,8]. For that purpose, several approaches such as the QuEChERS methodology [9] and "dilute and shoot" methods [10] have been applied. To maintain sample throughput and cost-effectiveness ratio, the development of generic liquid chromatography (LC) or ultra-high performance liquid chromatography (UHPLC) coupled to mass spectrometry (MS) screening methods is highly demanded, allowing for the identification of a wide range of these compounds and their metabolites [11,12]. Despite of triple quadrupole (QqQ) has been conventionally used [13,14], it presents some limitations in for comprehensive analysis, in terms of running time, scan speed and sensitivity. These drawbacks can be overcome by the use of high resolution mass spectrometry (HRMS) instruments, such as Orbitrap, which operates in the full scan mode (theoretically, no limitations in number of monitored compounds) [11] and provides accurate mass measurements. Furthermore, comparisons of theoretical and measured isotopic patterns areresult useful additional tools to carry out formula assignment and to facilitate the confirmation process [15]. For this aim, databases

containing retention time, ionization and exact mass information are needed to fully exploit the capabilities of full scan HRMS analyzers such as Exactive Orbitrap.

The utility of full scan UHPLC-Orbitrap MS [16] is sufficient to enable detection and accurate mass measurement of a wide range of residues at low concentration level in complex sample matrices. In this sense, honey is a relatively complex foodstuff, which has been considered as a natural product free of residues and contaminants, although the occurrence of VD and pesticide residues has been reported in this matrix [17-19]. Therefore, several surveillance systems have been established to control the presence of these residues in honey [20].

The European Union (EU) does not accept the use of VDs in beekeeping [21] and there is a zero-tolerance policy of these residues in honey. However, some organizations, such as the United States Food and Drug Administration (US-FDA) [22] and the Canadian Food Inspection Agency [23] allow the use of several VDs for the treatment of bacterial brood diseases. Nevertheless, the EU has been established maximum residue levels (MRLs) of some pesticide in honey, and 10 µg kg^{-1} has been set as the lowest MRL [24].

This study reports the development of an ~~accurate~~ exact mass database for the determination of more than 350 pesticides and VDs by UHPLC-Orbitrap-MS and its application to the multi-residue analysis of honey using generic extraction and chromatographic conditions. Although previous databases have been developed for the determination of pesticides [25] or VDs in several matrices, using several analyzers [3,26,27], this database contains several families of contaminants (pesticides, biopesticides and VDs) using Exactive-Orbitrap as analyzer, including new compounds not evaluated in ~~the~~ previous studies (i. e. chloramphenicol, maduramycin, etc…) and the widest scope for pesticides and veterinary drugs so far. New compounds can be

included and therefore, the database can be easily upgraded. Furthermore, the developed method has been validated and it has been applied for quantification purposes analyzing honey samples.

2. Materials and Methods

2.1. Reagents and chemicals

VDs and pesticide analytical standards were purchased from Riedel-de-Haën (Seelze, Germany), Dr. Ehrenstorfer GmbH (Ausburg, Germany), Sigma-Aldrich (Madrid, Spain), Fluka (Steinheim, Germany), Santa Cruz (Santa Cruz, CA, USA), European Pharmacopoeia (Strasbourg, France), Witega (Berlin, Germany) and LGC Standards (Barcelona, Spain). Individual stock standard solutions (200-400 mg L^{-1}) were prepared in methanol, acetonitrile or acetone, and were stored at 5ºC or -18ºC (VDs). HPLC-grade methanol, acetonitrile and acetone were obtained from Fluka. A solution for each family of VDs was prepared from corresponding individual stock standard solutions in methanol or acetonitrile, whereas two multi-pesticide solutions (corresponding to typical LC and GC-amenable compounds) were prepared in methanol and acetone. Tetracyclines and penicillins solutions were renewed monthly. Then, a multi-compound working solution containing all the analytes (0.31 mg L^{-1}) was prepared by combining suitable aliquots of each individual standard stock solution and diluting them with LC-MS-grade methanol, obtained from Fluka. This solution was kept at -18 ºC. A solution of 0.1 M disodium ethylene diamine tetraacetic acid (Na_2EDTA) was prepared by dissolving Na_2EDTA (Merck, Darmstadt, Germany) in water. Formic acid (purity > 98%) and ammonium formate (purity > 99%) were obtained from Panreac (Barcelona, Spain). LC-MS water was purchased from Scharlau (Barcelona, Spain). For accurate mass calibration, a mixture of caffeine, Met-Arg-Phe-Ala acetate salt (MRFA) and

123 Ultramark 1600 (ProteoMass LTQ/FT-Hybrid ESI positive mode calibration mix) from
124 Sigma-Aldrich was used in the Orbitrap analyzer.
125
126 *2.2. Apparatus*
127 A rotary agitator (mod. Reax-2) from Heidolph (Schwabach, Germany) was used for
128 sample extraction. Centrifugations were performed in a high-volume centrifuge from JP
129 Selecta (mod. Centronic II, Barcelona, Spain). An analytical AB204-S balance (Mettler
130 Toledo, Greinfesee, Switzerland) was also used.
131
132 *2.3. UHPLC-Orbitrap-MS analysis*
133 The separation of the analytes was carried out using a Transcend 600 LC (Thermo
134 Scientific Transcend™, Thermo Fisher Scientific, San Jose, CA, USA) equipped with
135 an analytical column Hypersil GOLD aQ C18 column (100 x 2.1 mm, 1.7 µm particle
136 size) from Thermo (Thermo Fisher Scientific, San Jose, CA, USA). The mobile phase
137 consisted of 0.1% (v/v) formic acid and ammonium formate 4 mM in water (eluent A)
138 and 0.1% (v/v) formic acid and ammonium formate 4 mM in MeOH (eluent B). The
139 analysis started with 95% of eluent A. After 1 min, this percentage was linearly
140 decreased up to 0% in 7.0 min. This composition was held during 4.0 min and increased
141 again up to ~~100~~95% in 0.5 min, followed by a re-equilibration time of 1.5 min (total
142 running time = 14.0 min). The flow rate was 0.3 mL min^{-1} and the column temperature
143 was set at 30 ºC. Aliquots of 10 µL of the sample extract were injected into the
144 chromatographic system.
145 The UHPLC system was coupled to a single stage Orbitrap mass spectrometer
146 (Exactive™, Thermo Fisher Scientific, Bremen, Germany) operating with a heated
147 electrospray interface (HESI-II, Thermo Fisher Scientific, San Jose, CA, USA), in

positive (ESI+) and negative ionization mode (ESI-) using the following operational parameters: spray voltage, 4 kV; sheath gas (N_2, >95%), 35 (adimensional); auxiliary gas (N_2, >95%), 10 (adimensional); skimmer voltage, 18 V (-18 V in ESI-); capillary voltage, 35 V (-35 V in ESI-); tube lens voltage, 95 V (-95 V in ESI-); heater temperature, 305 ºC; and capillary temperature, 300 ºC. The automatic gain control (AGC) was set at a target value of 1 x 10^6. The mass spectra were acquired using four alternating acquisition functions: (1) full MS, ESI+, without fragmentation (the higher collisional dissociation (HCD) collision cell was switched off), mass resolving power = 25000 FWHM; scan time = 0.25 s; (2) full MS, ESI- using the aforementioned settings; (3) MS/MS, ESI+, with fragmentation (HCD on, collision energy = 30 eV), mass resolving power = 10000 FWHM; scan time = 0.10 s ; and (4) MS/MS, ESI- using the settings explained for (3). Considering the scan time ~~for~~ of the four acquisition functions, and the polarity switching (approx. 0.27 s) an ~~an~~ overall scan rate of ~~1.4~~0.56 Hz was obtained. Mass range in the full scan experiments was set at *m/z* 100-1000. All the analyses were performed without lock mass. Mass accuracy was carefully monitored as follows: checked everyday with multi-compound standards; evaluated (once a week) and calibrated when necessary (every two weeks at least) with mass accuracy standards (see Section 2.1.). Data were acquired using external calibration mode. All data were processed using Xcalibur™ version 2.2.1 (Thermo Fisher Scientific, Les Ulis, France) with Qual and Quanbrowser. Genesis peak detection was applied. ToxID™ 2.1.1 (automated compound screening software, Thermo Scientific) was used for screening and LCQuan™ 2.6 software (Thermo Scientific) was used for quantification during method validation and sample analysis.

2.4. Sample preparation

For the extraction of pesticides and VDs, an extraction method developed by Mol et al. was slightly modified [10]. 2.5 g of honey sample were weighed into a 50 mL-propylene tube and 2.5 mL of water were added and mixed using a vortex. Then 7.5 mL of acetonitrile containing 1% of formic acid (v/v) were added, and the sample was shaken end-over-end for 1 h. The tube was centrifuged (10 min, 4500 rpm, 2264 *g*) and 1 mL of extract was transferred into a vial and injected ~~i~~into the chromatographic system.

2.5. Honey samples

A total of 26 different honey samples (including samples from organic production) were analyzed. These samples were purchased from different local markets (Almeria, Spain) and all of them were analyzed following the procedure described above. Those samples showing the absence of the target compounds were used as blank samples during the optimization and validation of the method. In order to avoid errors and ensure the reliability of the results, an internal quality control (IQC) was carried out. This IQC was based on the use of a blank extract that eliminated false positives caused by a contamination in the extraction procedure or by the presence of an interference; a reagent blank (obtained by performing the whole procedure without sample) that removed any possibility of a false positive due to contamination in the instruments or reagents used; a spiked blank sample at 50 µg kg^{-1} to assess the extraction efficiency; and a calibration curve to check linearity and sensitivity. In routine analysis, recovery values between 60 and 120% were accepted extraordinarily, considering that the majority of recoveries of the pesticides and VDs ~~found~~ were within the range 70-120 % [28].

3. Results and Discussion

Firstly, an elevated number of compounds belonging to several families of VDs was selected, such as macrolides, quinolones, tetracyclines, sulphonamides, avermectines, nitroimidazoles, coccidiostats, penicillins, amphenicols, tranquilizers, corticoids, ionophores, non steroidal anti-inflammatory drugs (NSAIDs), β-agonists and estrogens, gestagens and androgens (EGAS). On the other hand, pesticides belonging to several families such as organophosphorus, organochlorines, carbamates, pyrethroids, biopesticides, neocotinoids, triazines, triazoles and ureas were also selected.

3.1. Development of the database and analysis method

In order to develop a database of target compounds, a previous characterization of the analytes must be performed and the essential information includes retention time (RT), ionization mode (including polarity), characteristic ions and possible adducts (i. e. with Na^+ or NH_4^+).

An aliquot of each individual standard solution (250 μg L^{-1}) was injected in the system. A generic chromatographic [29] and API source conditions based on a previous work developed by Romero-González et al. [30] were used, and a MS acquisition in positive and negative mode was performed, without and with fragmentation in the HCD collision cell (higher energy collisional dissociation). In relation to the chromatographic conditions, a generic mobile phase consisting of an aqueous solution of ammonium formate (4 mM) and formic acid (0.1% v/v) and methanol (also containing ammonium formate and formic acid at the same concentrations) was used. Although the UHPLC-Orbitrap-MS method is not as fast as other UHPLC gradients (total running time = 14 min), this is not a key point for generic methods. Considering the high number of monitored compounds in a single injection and using a single extraction, the early

elution of matrix components, minimizing matrix effect, and the reduction of the co-elution of isobaric compounds, which would improve detection/identification, were considered as key factors during the development of the proposed method.

The identification of the target compounds was based on the RT windows (RTWs), which were defined as the RT average plus or minus 3 times the standard deviation (SD) of the RT (RT ± 3 SD) when five spiked samples at 50 µg kg^{-1} were injected. Table S1 (Electronic Supplementary Material, ESI) shows the obtained results. It can be indicated that RT ranged from 1.25 min (sulfaguanidine) to 10.73 (silafluofen). Furthermore, it can be noted that for some compounds the most intense ion is the $[M+Na]^+$ adduct, such as for diphenylamine, monensin, narasin, prosulfuron and salinomycin (Table S1, ESI). Acrinathrin, deltamethrin, esfenvalerate, etofenprox, famoxadone, fenpropathrin, fenvalerate, flucythrinate, isocarbofos, ivermectin B1a, oxamyl, penicillin G, penicillin V, permethrin, propargite, salinomycin, silafluofen, tefluthrin and tetrachlorvinphos mainly formed the $[M+NH_4]^+$ adduct. Up to 20 compounds were monitored in ESI- (Table S1). Furthermore, it can be also indicated that some compounds showed identical formula (and ~~accurate~~ exact mass) but different RT, as is the case of ketoprofen and fenbufen; and methiocarb sulfone and ethiofencarb sulfone, among others. Therefore, the combination of ~~both parameters,~~ RTW and mass accuracy~~,~~ provides a suitable detection of the compounds.

Average mass accuracy was also estimated by injecting matrix-matched standards (*n* = 10) at 100 µg L^{-1} of the selected compounds (Table S1). In general, it can be noted that average mass error was < 3 ppm, except for febantel, malathion, silafluofen and tefluthrin, which presented mass errors ranging from 3 to 5 ppm. Furthermore, ~~no~~ mass errors ~~outside~~ ≥ 5 ppm were not observed for the assayed compounds. Finally, the RSDs values associated to this parameter were always < 25 % (Table S1).

According to the variety of compounds and ionization requirements, full scan acquisition in the Orbitrap instrument was carried out using four acquisition functions (see Section 2.3.). Adequate peak shape and number of points-across-a-peak were obtained (Fig. 1), despite of more scans would be expected considering that the theoretical overall scan rate was 1.4 Hz. It must be considered the time spent on polarity switching (approx. 0.27 s), which ~~would~~ reduces the number of theoretical scans per cycle, providing an overall scan rate (duty cycle) of 0.56 Hz.

For the automatic screening method, characteristic information already registered in the database, such as molecular formula, theoretical exact mass and RT was required to build an Excel spreadsheet compatible with the automatic screening software (ToxID^{TM}). This Excel file was then saved as a ".csv" file to be used by ToxID^{TM}, which generated a ".pdf" report (Fig. 2) for each sample file, showing the identified compounds from the database according to the fixed searching criteria (mass tolerance = ± 5 ppm, RTW = ± 1 min). Then, if no signal was found within the RTW or the accurate mass error was > 5 ppm, the sample was considered negative and it was not re-processed. On the contrary, if a signal was found showing a mass error < 5 ppm within the RTW, this sample was considered as a non-negative sample and it was re-processed to confirm the results.

The identification of the non-negative samples was carried out using isotopic patterns. The relative intensity of the A+1 isotope peak (being A the corresponding $[M+H]^+$), which is mainly due to the presence of ^{13}C, was useful to confirm compounds with a high number of carbons. When characteristic atoms, such as Cl, Br, or S, were present in the molecule, A+2 were observed~~obtained for~~ in the isotopic profile of the accurate mass spectra, and the abundance of the chlorine isotope ^{37}Cl, bromine isotope ^{87}~~^{81}~~Br or sulphur isotope ^{34}S, were used for identification purposes. When halogens are not

present due to the fact that C, H, O and N are basically the elements that make up the molecule, the identification by isotopic patterns was based only on $^{13}C/^{12}C$ ratios (A+1), which were calculated manually. It is important to indicate that this information was obtained in the same injection and therefore, non-negative samples were not re-analyzed but they were re-processed using the data already registered. For instance, Fig. 3 shows the extracted ion chromatogram and the experimental and theoretical spectra of sulfamerazine in a matrix-matched standard (5 µg kg^{-1}). It can be observed that both spectra are similar and A+1 and A+2 ions can be used for identification purposes. Therefore, the risk of false positive has been minimized considering the different criteria (retention time, mass accuracy, isotopic pattern) used to identify the target compounds.

Furthermore, in order to confirm the presence of the compounds, fragment ions obtained after HCD fragmentation could be monitored for each compound by accurate mass measurements. These ions or fragments were generated in the collision cell (straight multipole mounted inside a metal tube), and all the ions generated in the ion source were fragmented.

3.2. Optimization of the extraction procedure

In multi-analyte methods, an important step is the extraction procedure, especially for complex matrixes such as honey, which contains high sugar content. Here, the main purpose was the use of a generic extraction method that allowed the determination of a high number of compounds. To achieve this goal, the extraction procedure developed by Mol et al. was selected as starting point [10]. For the ~~optimisation~~ optimization of the extraction procedure, blank honey samples were spiked at 50 µg kg^{-1}. An aqueous solution to dissolve honey prior to the addition of the extraction solvent was added.

~~Thus~~Then, different solvents like acetone (method A) and acetonitrile (method B) (~~,~~ both with formic acid 1% (v/v)) were tested for the extraction procedure~~;, adding~~ 5 mL of water were also added in both methods ~~as well~~. It must be indicated that during the extraction with acetonitrile, the formation of two phases is observed: an upper liquid phase (acetonitrile) and a gel (water + honey matrix). This phenomenon was considered for recovery calculations and it was previously described by Mol et al [10]. Table 1 shows the results obtained for compounds from the most problematic families whereas in Table 2 a summary of the extracted compounds is shown. It can be observed that acetone (method A) only allowed the extraction of 12 compounds whereas 33 compounds were extracted when acetonitrile (method B) was checked (see Table 2). Furthermore, the majority~~most~~ of the assayed compounds showed~~n~~ recoveries lower than 40 %, whereas ~~there are~~ more than 50 compounds provided~~with~~ recoveries higher than 150 %. Considering that few compounds were extracted, the amount of water and the addition of an aqueous solution of 0.1 M Na_2EDTA (method C) were evaluated, observing that the number of extracted compounds increased (303 compounds). Additionally, different water/~~extractant~~ solvent volume ratios were studied: 5 mL water/10 mL acetonitrile (formic acid 1%, method B), and 2.5 mL water/7.5 mL acetonitrile (formic acid 1%, method D), obtaining better results with method D, which allowed the extraction of more than 380 compounds. The differences between the obtained results for method B and D (33 and 381 extracted compounds respectively), could be due to the different g of matrix/mL sample extract. For method B, this ratio was 0.25 g/mL (considering the formation of the viscous lower phase) whereas for method D, the ratio was 0.33 g/mL. Despite matrix effect is supposed to be more important at higher ratios, the use of lower ratios (use of higher solvent volume) might favour the co-extraction of interferences such as carbohydrates or pigments that may

affect the recovery of the target compounds. Besides, the ratio ACN:water is higher in method B than in method D, observing a higher phase separation in B (5 mL of lower phase in B and 2.5 mL in D), which can reduce the extraction efficiency by "retaining" partiallya ~~part of~~ the target compounds. Comparing methods C and D, it can be observed that for some compounds, such as biopesticides or unexpectedly for tetracyclines, recoveries were higher when water was used instead of the EDTA solution (Table 1), and therefore it was selected to dissolve honey. These results are not in agreement with the general behaviour of tetracyclines since it is considered that the addition of EDTA prevents the formation of chelation complexes with cations present in solution, as described previously [31]. Biopesticides didо not show the best performance when applying the different extraction methods; as possible explanations, problems related to polarity and ionization may be adduced. Some of them have log $K_{o/w}$ higher than 4 (i. e. jasmoline I or rotenone). Therefore, the use of acetonitrile (medium polarity solvent) could not be the best choice for the extraction of these compounds. With respect to highly polar pesticides, low recoveries observed for some of them can be ~~due to~~explained considering the separation phase observed. Therefore, they cannot be properly extracted with acetonitrile and a more polar solvent should be used; ~~so that they are more difficult to extract properly with acetonitrile;~~ besides they may show lower stability in the extract. It must be indicated that some compounds such as lincomycin, natamycin, amoxicillin and ampicillin were not extracted. Finally, method D was selected and validated.

3.3. Method validation

Despite of the capabilities of the developed method for the detection of the compounds included in the database, a validation protocol was carried out in order to establish the

performance characteristics of the method to ensure the adequate detection and quantification of the target compounds. Several parameters such as linearity, intra-day precision, inter-day precision, trueness (expressed as recovery) and limits of detection (LODs) were studied.

Firstly, matrix effect was evaluated by analyzing calibration sets prepared in solvent (from 5 to 100 µg L^{-1}) and in extracted honey (matrix-matched calibration) at the same concentrations. Matrix effects were calculated by comparison of the slope obtained for each compound in the matrix-matched standard with that of the solvent standard, showing in Figure 4a the obtained results. Signal suppression or enhancement effect was considered tolerable if the value was between 80 and 120% (comparison of matrix-matched standard and solvent standard). The values outside this range indicated a strong matrix effect. The matrix-matched calibration was significantly different from the obtained in solvent, indicating a significant matrix effect for a large number of compounds. It can be noted that only 85 compounds showed a tolerable matrix effect and signal enhancement was rarely detected. Thus, only 2 compounds (flonicamid and permethrin) showed matrix enhancement~~effect~~, ranging~~ed~~ from 120 to 150 %, whereas 4 compounds (mevinphos, nicotine, omethoate and penicillin V) showed a significant matrix enhancement (> 150 %). On the other hand, very significant matrix suppression (20-50%) was observed for 4 compounds, whereas most of the included compounds in this study (288) presented significant matrix suppression (50-80%). Therefore, matrix-matched standard calibration was used for quantification purposes. Linearity was then evaluated in the range 5-100 µg L^{-1}, obtaining determination coefficients (R^2) higher than 0.9800 for all compounds and deviations of the individual points from the calibration curve were lower than 20% for most of compounds.

Trueness was estimated in terms of recovery by evaluating three different concentration levels (10, 50 and 250 µg kg^{-1}). Five blank samples were fortified at each level; the results are indicated in Table S2 (Supplementary material), and a summary is shown in Fig. 4b. Thus, 70% of compounds showed adequate recovery (recovery ranging from 70 to 120–%) at the lowest concentration checked (10 µg kg^{-1}). As expected, the percentage of compounds showing acceptable recovery at 50 µg kg^{-1} and 250 µg kg^{-1} was considerably higher: 97% and 90%, respectively. Although some compounds did not show adequate recovery values at the lowest concentration assayed (10 µg kg^{-1}), suitable recoveries were obtained at 50 and 250 µg kg^{-1}. It is important to noticee that the compounds were detected at concentrations not exceeding the MRLs established by EU for pesticides in honey.

Precision was evaluated by performing repeatability (intra-day precision) and reproducibility (inter-day precision) studies. Repeatability was studied by analysing five spiked blank samples at different fortification levels (10, 50 and 250 µg kg^{-1}) extracted on the same day, and a summary of the obtained results is shown in Fig. 4c. RSDs were lower than 25%, except for some compounds at the lowest concentration level (10 µg kg^{-1}) with RSD values higher than 25% (Table S2). Inter-day precision (reproducibility) was studied by analyzing three spiked blank samples at different fortification levels (10, 50 and 250 µg kg^{-1}) extracted in different days. In this case, the range of RSD obtained was lower than 25%, except for some compounds at the low concentration level (10 µg kg^{-1}) with RSD values higher than 25% (Table S2).

Finally, LODs were estimated by analyzing spiked blank samples at six different concentration levels (1, 5, 10, 25, 50 and 100 µg kg^{-1}). Due to the high number of target analytes, a different strategy was used to establish this parameter. LODs were calculated using the information obtained from the ".pdf" file generated by the ToxID™ software,

bearing in mind that lower limit is an important parameter in screening methods, because it is mainly used to distinguish between negative and non-negative samples. Thus, the LOD was set at the concentration that provided a signal detected by the software higher than 1 x 10^3 counts. For instance, if one compound provided a signal higher than 1x10^3 at the lowest level of concentration, it was set as the LOD for that compound. If no signal was provided at that concentration or its sensitivity was lower than 1x10^3, next concentration level was checked.

Most of the selected compounds showed LOD values equal or lower than 10 µg kg^{-1}, although some compounds showed an LOD higher than 10 µg kg^{-1} (Table 3). Most of the pesticides included in Table 3 do not have a set MRL (not included in any of the indicated Annexes), so that a default MRL of 10 µg kg^{-1} has been set. Other pesticides were indeed included in the Annexes of Regulation 396/2005 [24] but the LODs obtained here were higher than the established MRL (acrinathrin, boscalid, clethodim, fonicamid and fluroxipyr). Therefore, for these compounds as well as for the VDs included in the Table, further improvements should be carried out in order to reduce the obtained LODs.

3.4. Sample analysis

In order to show the applicability of the developed method, 26 honey samples were analyzed. For that purpose, the workflow scheme shown in Fig. 5 was followed to detect and identify and confirm positive samples, using several software tools such as ToxID™ for screening and LCQuan™ and Xcalibur (Qualbrowser) for quantification and identification, respectively. Honey samples were purchased from different local markets and they were from honey mixtures from EU and non-EU countries and 8 honeys were organic samples. Furthermore, several types such as multiflower,

422 rosemary, orange blossom and forest honey were analyzed. Table 4 shows the obtained
423 results in positive samples, observing that only 15 % of the studied samples were
424 positive. It must be noted that when automatic screening was performed with ToxID,
425 approximately 20 compounds were detected in each sample, indicating that the number
426 of false positives were lower than 4 % (3.9 %). Therefore, this automatic screening
427 should be improved in order to minimize the false of positives and reduce
428 identification/quantification steps.

429 It can be highlighted that VDs were not found in the samples, whereas some
430 pesticides such as azoxystrobin, coumaphos, dimethoate and thiacloprid were detected.
431 Coumaphos was detected in one sample at 5.1 µg kg^{-1}. It is important to notice that this
432 compound is an organophosphorus pesticide widely used to control parasitic mites
433 (*Varroa jacobsoni*) on bees [32]. Azoxystrobin was detected in one sample at 1.5 µg kg^{-1}.
434 Dimethoate and thiacloprid were also detected at trace levels (< 5 µg kg^{-1}) in one
435 sample each other. Currently, there is not MRL set for coumaphos and dimethoate in
436 honey, whereas for azoxystrobin and thiacloprid ~~the~~ MRLs ~~has~~ have been set at 10 µg
437 kg^{-1} and 200 µg kg^{-1}, respectively and therefore, the concentration values found were
438 below the set MRLs. Azoxystrobin and thiacloprid positive samples are from honey
439 mixtures from EU and non-EU countries. Besides, it is important to point out~~note~~ that
440 these honey samples are organic samples (multiflower), and the presence of these
441 pesticide residues in the analyzed samples can be due to irregular activities or "non-
442 intentional" contamination. Coumaphos and dimethoate positive samples corresponded
443 to~~are from~~ Spanish honeys (non organic samples) from ~~and they were~~ rosemary and
444 orange blossom ~~type~~, respectively.

445 Finally Fig. 6 shows the extracted ion chromatogram of coumaphos (*m/z* 363.02174)
446 in a positive honey sample, solvent and matrix-matched standard, as well as the

theoretical and experimental spectra. It can be observed that the presence of the chlorine in the spectra can result useful ~~–~~as an additional tool for the identification of the compound. Besides, it must be highlighted that the method allows the reliable quantification of the identified compounds at trace levels. Furthermore, several characteristic fragments of coumaphos were monitored and the same chromatographic patterns were obtained in relation to the characteristic ion of this compound, confirming the presence of this pesticide in the ~~analysed~~ analyzed honey.

4. Conclusions

A database has been created for the determination of more than 350 compounds in honey by UHPLC-Orbitrap-MS, studying several types of compounds such as pesticides, biopesticides and VDs. This database includes retention time and characteristic ions of the target compounds. The proposed database allows the automated search of the analytes, and then, the identification and quantification of the detected compounds can be carried out within the same injection. HRMS analyzers can improve the detection/identification process with the information provided by accurate mass measurements. Then, UHPLC-Orbitrap MS allows efficient performance for screening purposes and can also provide adequate quantification/identification values of pesticides and VD residues in positive samples, even at low levels. Generic instrumental and extraction conditions have been used, which facilitate the determination of a wide range of compounds. The developed method showed good quantitative results for most of the studied compounds and in the case of pesticides, LODs were lower than the MRLs established by EU in honey. Finally only pesticides were detected in 15 % of the analyzed samples at trace levels.

Acknowledgements

We gratefully acknowledge Spanish Ministry of Economy and Competitiveness (MINECO) and FEDER for financial support through project AGL2010-21370. MLGP acknowledges her grant (F.P.I.) from the MINECO (Ref. AGL2010-21370). PPB is grateful for personal funding through the Juan de la Cierva Program (MINECO-European Social Fund, ESF). RRG is also grateful for personal funding through the Ramón y Cajal Program (MINECO-ESF). Finally, we also thank the anonymous reviewers for their valuable comments and suggestions.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version.

FIGURE CAPTIONS

Fig. 1. Extracted ion chromatogram of spinosad in a spiked honey sample (5 µg kg^{-1}) showing chromatographic profile and points-across the peak.

Fig. 2. Example of the report provided by ToxID™ software used for screening purposes.

Fig. 3. Extracted ion chromatogram and theoretical and real spectrum of sulfamerazine in a spiked honey sample (5 µg kg^{-1}).

Fig. 4. Summary of some validation results of the proposed method: (a) evaluation of matrix effect; (b) recovery results for spiked samples at different concentration levels and (c) intra-day precision at different concentration levels.

Fig. 5. Workflow scheme used to (a) create the database and (b) apply the database to identify and confirm the analytes in the samples.

Fig. 6. Chromatograms of: (a) theoretical and real spectrum and (b) a real honey sample containing coumaphos (pesticide).

505 **References**

506

507 [1] L. Kantiani, M. Farré, J. M. Grases i Freixiedas, D. Barceló, Anal. Bioanal. Chem.
508 398 (2010) 1195.

509 [2] M. Kellmann, H. Muenster, P. Zomer, H. Mol, J. Am. Soc. Mass. Spectrom. 20
510 (2009) 1464.

511 [3] A. Kaufmann, P. Butcher, K. Maden, S. Walker, M. Widmer, Anal. Chim. Acta 700
512 (2011) 86.

513 [4] A. Kaufmann, P. Butcher, K. Maden, S. Walker, M. Widmer, Analyst 136 (2011)
514 1898.

515 [5] M. Mezcua, O. Malato, J. F. García-Reyes, A. Molina-Díaz, A. R. Fernández-Alba,
516 Anal. Chem. 81 (2009) 913.

517 [6] M. I. Ramón Díaz, J. V. Sancho, F. Hernández, Rapid Commun. Mass Spectrom. 25
518 (2011) 355.

519 [7] Y. Picó, D. Barceló, Trac-Trends Anal. Chem. 27 (2008) 821.

520 [8] A. K. Malik, C. Blasco, Y. Picó, J. Chromatogr. A 1217 (2010) 4018.

521 [9] R. Romero-González, A. Garrido-Frenich, J. L. Martínez Vidal, O. D. Prestes, S. L.
522 Grio, J. Chromatogr. A 1218 (2011) 1477.

523 [10] H. G. Mol, P. Plaza-Bolaños, P. Zomer, Theo C. de Rijk, A. A. M. Stolker, P. P. J.
524 Mulder, Anal. Chem. 80 (2008) 9450.

525 [11] E. van der Heeft, Y. J. C. Bolck, B. Beumer, A. W. J. M. Nijrolder, A. A. M.
526 Stolker, M. W. F. Nielen, J. Am. Soc. Mass. Spectrom. 20 (2009) 451.

527 [12] Y. Picó, M. la Farré, C. Soler, D. Barceló, J. Chromatogr. A 1176 (2007) 123.

528 [13] J. L. Martínez Vidal, M. M. Aguilera-Luiz, R. Romero-González, A. Garrido
529 Frenich, J. Agric. Food Chem. 57 (2009) 1760.

530 [14] G. Tanner, C. Czerwenka. J. Agric. Food Chem. 59 (2011) 12271.

531 [15] S. Ojanperä, A. Pelander, M. Pelzing, I. Krebs, E. Vuori, I. Ojanperä. Rapid
532 Commun. Mass Spectrom. 20 (2006) 1161.

533 [16] A. Makarov, E. Denisov, A. Kholomeev, W. Balschun, O. Lange, K. Strupat, S.
534 Horning, Anal. Chem. 78 (2006) 2113

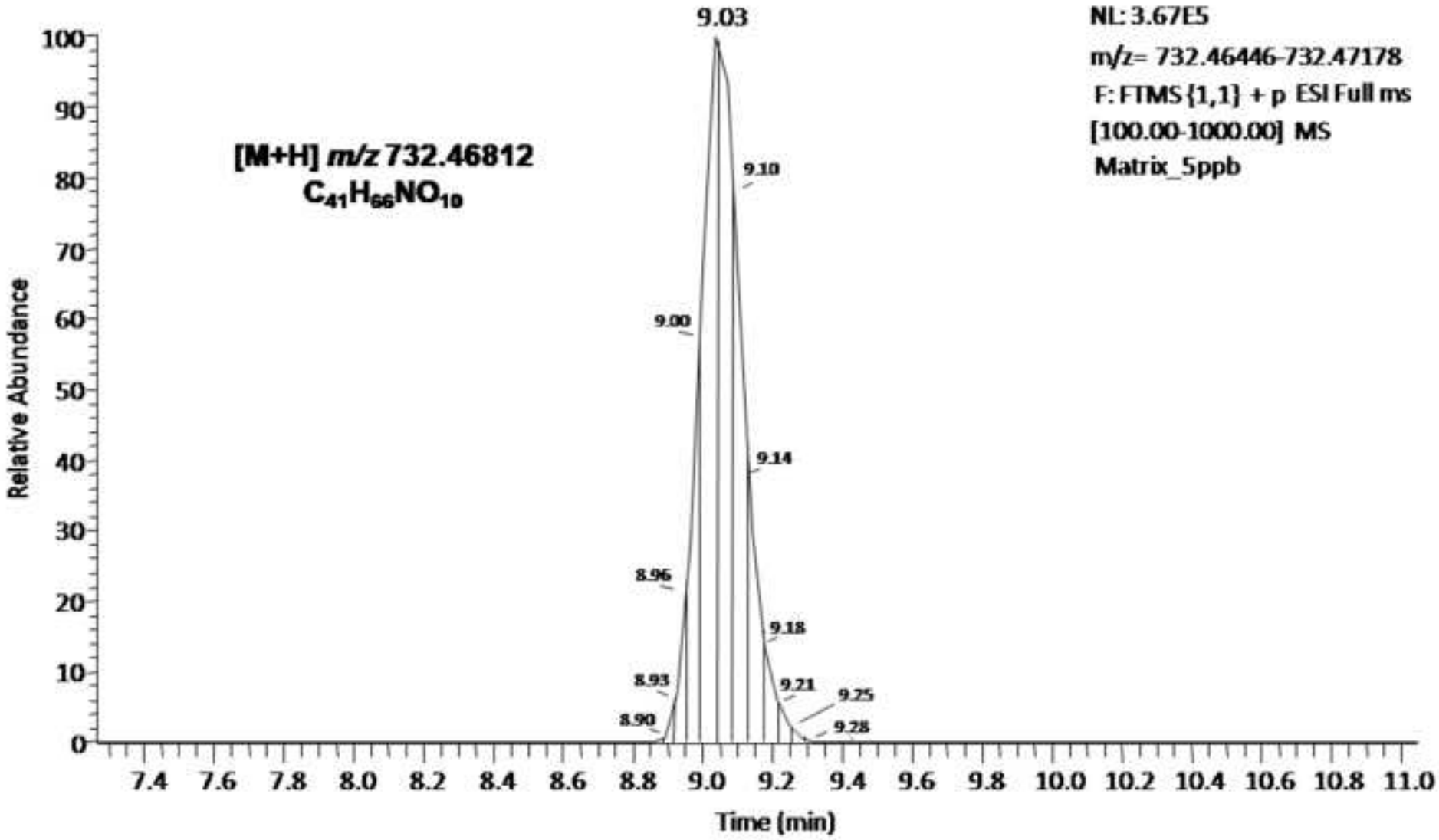
535 [17] J. Wang, J. Agric. Food Chem. 52 (2004) 171.

536 [18] L. Verzegnassi, M. C. Savoy-Perroud, R. H. Stadler, J. Chromatogr. A 977 (2002)
537 77.

538 [19] L. Wiest, A. Buleté, B. Giroud, C. Fratta, S. Amic, O. Lambert, H. Pouliquen, C.
539 Arnaudguilhem, J. Chromatogr. A 1218 (2011) 5743.
540 [20] A. Pena, N. Pelantova, C. M. Lino, M. I. N. Silveira, P. Solich, J. Agric. Food
541 Chem. 53 (2005) 3784.
542 [21] Council Regulation No 37/2010 of 22 December 2009 on pharmacologically active
543 substances and their classification regarding maximum residue limits in foodstuffs of
544 animal origin, Off. J. Eur. Communities L 15 (1), 20.1.2010 (Consolidated version up to
545 14. 07.2011, last accessed January 2012).
546 [22] Tolerances for residues on new animal drugs in food. Code of Federal Regulations,
547 Food and Drugs, Part 556, Title 21; Office of the Federal Register, National Archives
548 and Records Administration: Washington, DC, April 1, 2007 (last accessed January
549 2012).
550 [23] http://www.inspection.gc.ca/english/fssa/honmiel/ind/worfone.shtml (last accessed
551 January 2012).
552 [24] Regulation (EC) No 396/2005 of the European Parliament and of the Council of 23
553 February 2005 on maximum residue levels of pesticides in or on food and feed of plant
554 and animal origin and amending Council Directive 91/414/EEC. Off. J. Eur.
555 Communities L 70(1) 16.3.2005 (available on
556 http://ec.europa.eu/sanco_pesticides/public/index.cfm, last accessed January 2012).
557 [25] L. Alder, A. Steinborn, S. Bergelt, J. AOAC Int. 2011 (94) 1661.
558 [26] D. Hurtaud-Pessel, T. Jagadeshwar-Reddy, E. Verdon, Food Addit. Contam. Part
559 A-Chem. 28 (2011) 1340.
560 [27] R. J. B. Peters, Y. J. C. Bolck, R. Rutgers, A. A. M. Stolker, M. W. F. Nielen, J.
561 Chromatogr. A 1216 (2009) 8206.
562 [28] Method Validation and Quality Control Procedures for Pesticide Residues Analysis
563 in Food and Feed, Document No. SANCO/12495/2011, 01/01/2012.
564 [29] A. Zhang, J. S. Chang, C. Gu, M. Sanders, Thermo Application Note 51878.
565 [30] R. Romero-González, M.M. Aguilera-Luiz, P. Plaza-Bolaños, A. Garrido Frenich,
566 J. L. Martínez Vidal, J. Chromatogr. A 1218 (2011) 9353.
567 [31] M. M. Aguilera-Luiz, J. L. Martínez Vidal, R. Romero-González, A. Garrido
568 Frenich, J. Chromatogr. A 1205 (2008) 10.
569 [32] The e-Pesticide Manual. Version 3.2 2005-2006. Thirteenth edition. BCPC.
570

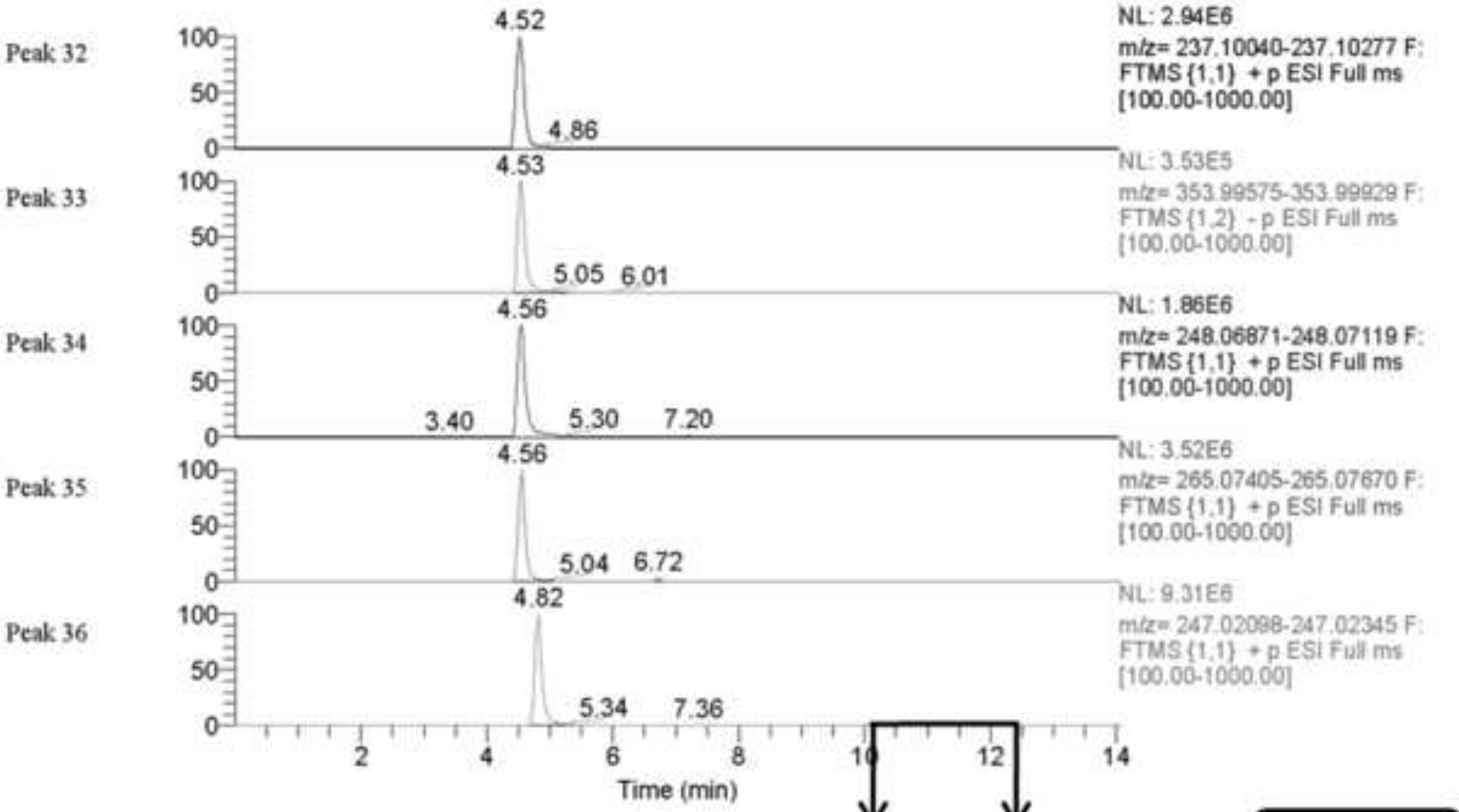
Figure

Click here to download high resolution image



Figure

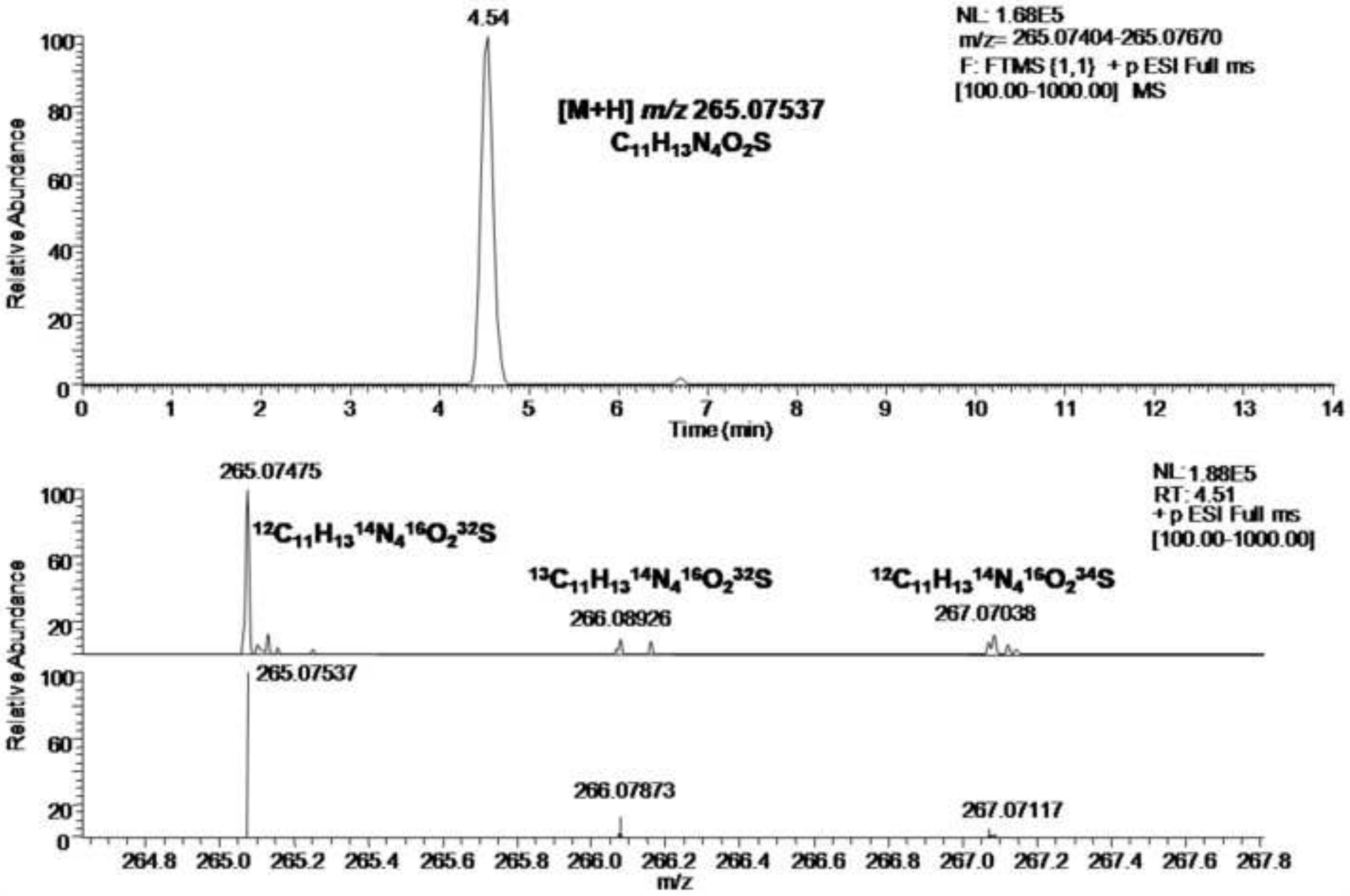
Click here to download high resolution image



#	Comp. Index	Compound Name	Formula	Detected m/z	Delta (ppm)	Expected RT	Actual RT	Intensity	Adducts			Fragments		
									H+	NH4+	Na+	1	2	3
32	36	Oxamyl	C7H13N3O3S	237.10153	-0.2	4.49	4.52	2939266	Y	Y*	Y	-	-	-
33	37	Thianphenicol	C12H15Cl2NO5S	353.99777	0.7	4.53	4.53	353284	Y*	N	N	-	-	-
34	38	Tinidazole	C8H13N3O4S	248.06985	-0.4	4.53	4.56	1864069	Y*	N	Y	-	-	-
35	39	Sulfamerazine	C11H12N4O2S	265.07520	-0.7	4.55	4.56	3515647	Y*	N	Y	-	-	-
36	40	Oxydemeton methyl	C6H15O4PS2	247.02216	-0.2	4.57	4.82	9306686	Y*	N	Y	-	-	-

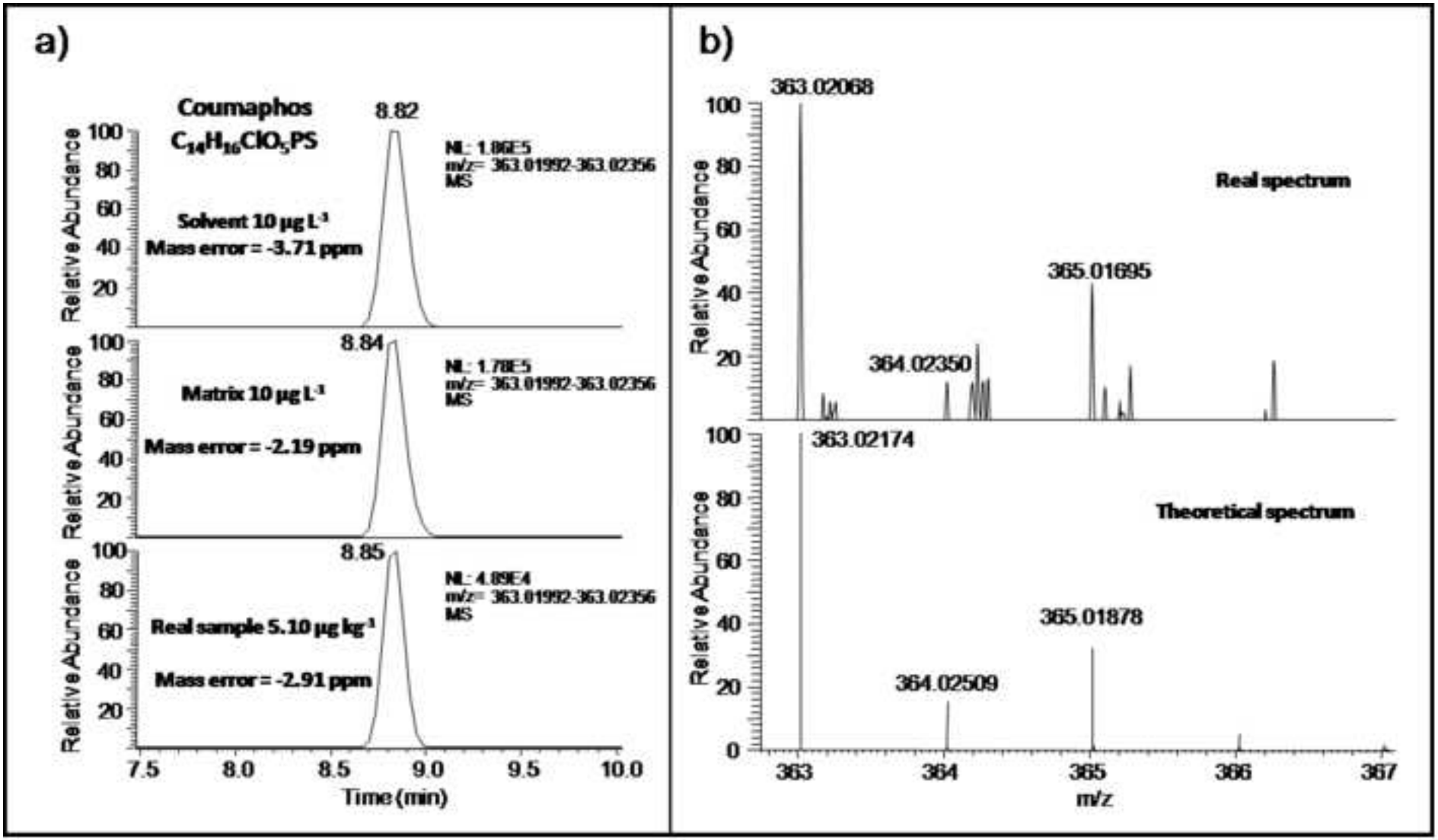
Figure

Click here to download high resolution image



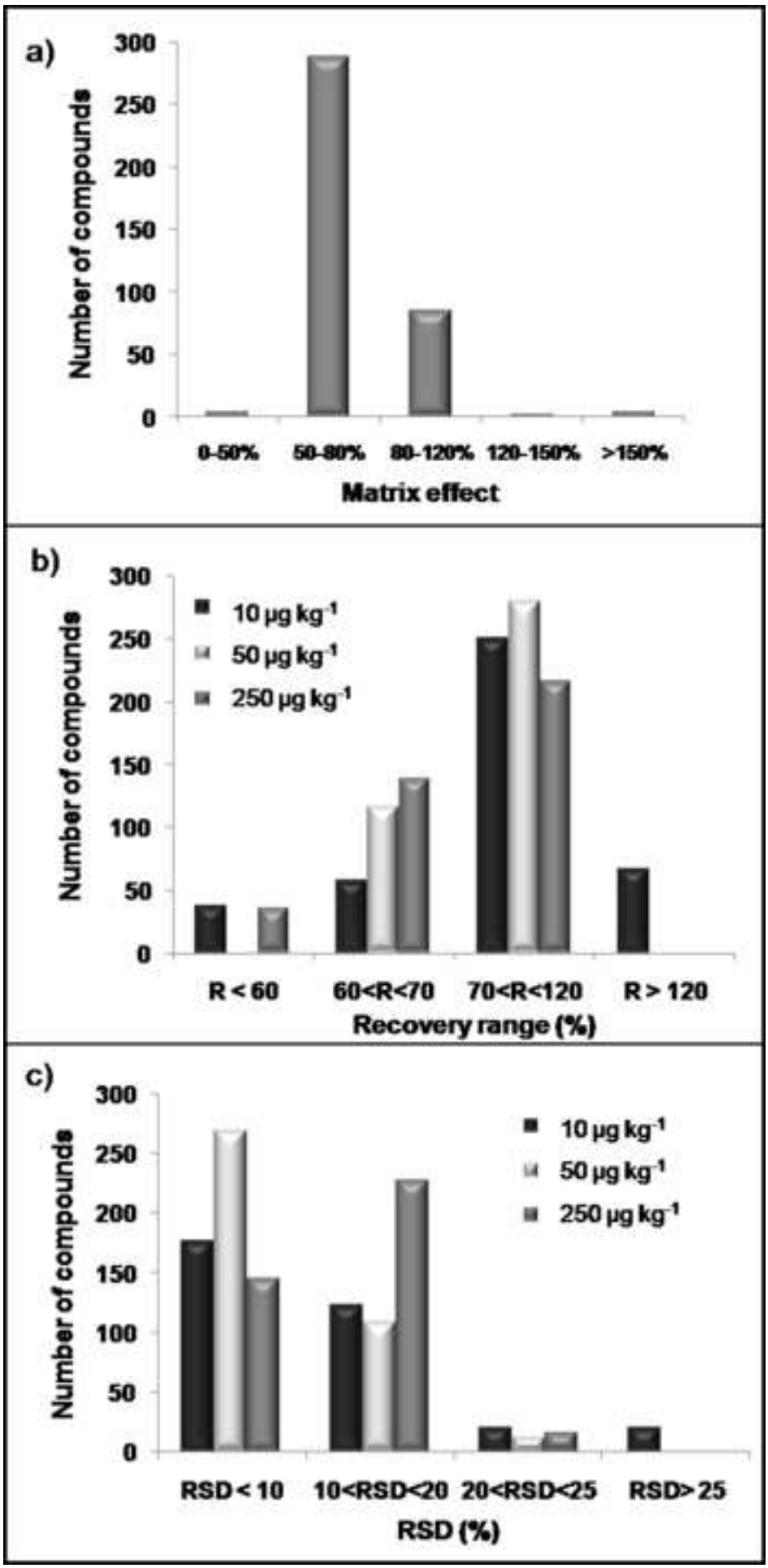
Figure

Click here to download high resolution image



Figure

Click here to download high resolution image



Figure

Click here to download high resolution image

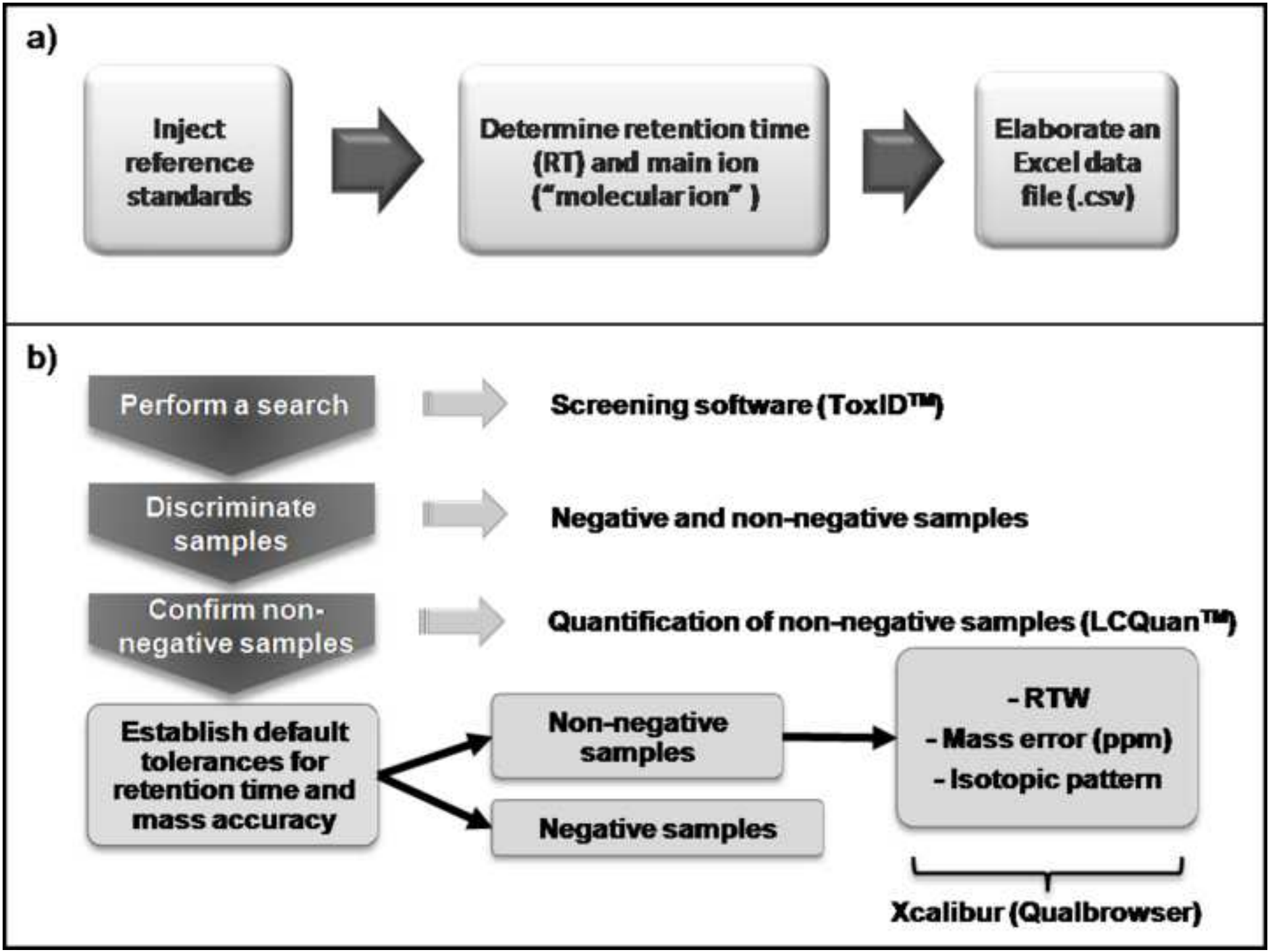


Table 1. Percentage of recovery between 60-120% obtained with the four extraction methods for the most problematic compounds of different families.

Family	Extraction method			
	A[a]	B[b]	C[c]	D[d]
Biopesticides				
Cevadine	N.E[e]	N.E	N.E	75
Deguelin	N.E	N.E	83	79
Jasmoline I	N.E	N.E	N.E	102
Nicotine	N.E	N.E	76	66
Rotenone	N.E	N.E	N.E	79
Spinosad	N.E	101	N.E	76
Veratridine	N.E	N.E	N.E	69
Macrolides				
Erythromycin	N.E	N.E	73	90
Josamycin	N.E	N.E	84	67
Lincomycin	N.E	N.E	N.E	N.E
Natamycin	N.E	N.E	N.E	N.E
Roxythromycin	N.E	N.E	N.E	76
Spiramycin	N.E	N.E	N.E	63
Tiamulin	N.E	N.E	98	81
Tilmicosin	N.E	N.E	N.E	74
Tilosin	N.E	N.E	N.E	73
Valnemulin	N.E	N.E	N.E	76
Virginiamycin M1	N.E	N.E	N.E	75
Penicillins				
Amoxicillin	N.E	N.E	N.E	N.E
Ampicillin	N.E	N.E	N.E	N.E
Penicillin G	N.E	N.E	N.E	77
Penicillin V	N.E	N.E	N.E	61
Highly polar pesticides				
Omethoate	N.E	N.E	N.E	70
Propamocarb	N.E	N.E	N.E	66

[a]Method A (5 mL water + 10 mL acetone).
[b]Method B (5 mL water + 10 mL acetonitrile).
[c]Method C (2.5 mL EDTA + 7.5 mL acetonitrile).
[d]Method D (2.5 mL water + 7.5mL acetonitrile).
[e]N.E.: Not extracted (Recovery < 10 %).

Table 2. Number of extracted compounds applying the different extraction methods.

Recoveries (%)	Extraction method			
	A[a]	**B**[b]	**C**[c]	**D**[d]
R < 40	322	278	65	3
40 < R < 60	6	13	21	7
60 < R < 120	12	33	303	381
120 < R < 150	-	11	-	-
R > 150	51	56	2	-

[a] Method A (5 mL water + 10 mL acetone).
[b] Method B (5 mL water + 10 mL acetonitrile).
[c] Method C (2.5 mL EDTA + 7.5 mL acetonitrile).
[d] Method D (2.5 mL water + 7.5 mL acetonitrile).

Table 3. Compounds showing limits of detection (LOD) higher than 10 µg kg^{-1}.

Compound	LOD (µg kg^{-1})	EU MRL (µg kg^{-1})	Compound	LOD (µg kg^{-1})	Tolerance
Acrinathrin	25	50	Isoxsuprine	50	**10**
Boscalid	50	500	Jasmoline I	25	**10**
Chloropromacine	50	**10**[a]	Maduramycin	25	ZT[b]
Chlorpyrifos	50	**10**	Malaoxon	50	**10**
Clethodim	25	50	Nicotine	50	**10**
Diphenylamine	25	**10**	Penicillin G	25	ZT
Disulfoton	25	**10**	Permethrin	50	**10**
DMST	50	**10**	Phorate	50	**10**
Esfenvalerate	50	**10**	Pirimicarb desmethyl	50	**10**
Etridiazole	50	**10**	Prosulfuron	50	**10**
Febantel	25	**10**	Silafluofen	50	**10**
Fenamiphos sulfone	50	**10**	Sulfadimethoxine	25	ZT
Fenbufen	25	**10**	Sulfadoxin	25	ZT
Fenpropathrin	50	**10**	Tefluthrin	50	**10**
Fenvalerate	50	**10**	Tetracycline	50	ZT
Flonicamid	25	50	Thiometon	50	**10**
Fluroxipyr	25	50	Triadimenol	50	**10**

[a] Default MRL established by EU [24] is shown in bold.
[b] ZT: Zero tolerance policy of veterinary drug residues in honey.

Table 4. Detected compounds in honey samples.

	Sample 1	Sample 8	Sample 10	Sample 21
Compound	Azoxystrobin	Dimethoate	Coumaphos	Thiacloprid
Concentration (µg/kg)	1.5	< LOQ	5.1	< LOQ
Honey origin	EU and non-EU countries	Spain	Spain	EU and non-EU countries
Type	Multiflower	Orange blossom	Rosemary	Multiflower
Organic origin	Yes	No	No	Yes

Electronic Supplementary Material (online publication only)

Click here to download Electronic Supplementary Material (online publication only): Supplementary data_R3.doc