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Hybrid quadrupole-orbitrap mass spectrometry analysis with accurate-mass database and parallel reaction monitoring for high-throughput screening and quantification of multi-xenobiotics in honey

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ABSTRACT

This study reports a rapid, automated screening and quantification method for the determination of multi-xenobiotic residues in honey using ultra-high performance liquid chromatography-hybrid quadrupole-Orbitrap mass spectrometry (UHPLC-Q-Orbitrap) with a user-built accurate-mass database plus parallel reaction monitoring (PRM). The database contains multi-xenobiotic information including formulas, adduct types, theoretical exact mass and retention time, characteristic fragment ions, ion ratios, and mass accuracies. A simple sample preparation method was developed to reduce xenobiotic loss in the honey samples. The screening method was validated based on retention time deviation, mass accuracy via full scan-data-dependent MS/MS (full scan-ddMS2), multi-isotope ratio, characteristic ion ratio, sensitivity, and positive/negative switching performance between the spiked sample and corresponding standard solution. The quantification method based on the PRM mode is a promising new quantitative tool which we validated in terms of selectivity, linearity, recovery (accuracy), repeatability (precision), decision limit (CCα), detection capability (CCβ), matrix effects, and carry-over. The optimized methods proposed in this study enable the automated screening and quantification of 157 compounds in less than 15 min in honey. The results of this study, as they represent a convenient protocol for large-scale screening and quantification, also provide a research approach for analysis of various contaminants in other matrices.

1. Introduction

Honey is used worldwide as a sugar substitute and natural preservative in manufactured foods, and is consumed either directly or as an added sweetener in a variety of foodstuffs [1–4]. Depending on the living environment of the surrounding nectariferous plants, honey bees may be exposed to an ever-changing array of xenobiotics from both natural and synthetic sources. As such, honey bees are easily contaminated by environmental pollutants from nectar and pollen via overspray and airborne transmission [5,6] or systemic pesticides drawn from the soil [7,8], surface water [9], or guttation water produced by plants at the leaf margins [10]. Honey bees also are easily attacked by bacteria, viruses, fungi, and exotic parasitic mites under inappropriate environmental conditions, which can result in diseases such as American foulbrood (AFB), European foulbrood (EFB), chalkbrood, nosema, cripaviridae, or varroa mites destructor [11,12]. Various veterinary drugs and pesticides have been employed to control and treat these diseases by adding them to feed or spraying them into hives, but these materials may then result in multi-xenobiotic residues in honey, posing potential hazard to consumers.

There is currently a trend toward the use of high-resolution mass spectrometry (HRMS) coupled with ultra-high performance liquid chromatography (UHPLC), with mass accuracies of <10 ppm and

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resolutions >20,000, as an alternative method to screen and quantify contaminant residues; conventional methods cannot satisfy requirements for quick and high-throughput analysis of multiple contaminants in complex matrices [13,14]. The HRMS technique is effective for the identification and quantification of analytes at trace levels in complex matrices due to its accurate mass analysis capability and high sensitivity in the full-scan mode and selected ion mode (SIM) of acquisition [15]. Various methods have been developed to achieve rapid screening and quantification of multi-class veterinary drugs [13,16], pesticides [13,15], organic contaminants, and illicit substances [17] in, for example, water, baby food, and environmental and human biological samples of various kinds.

For the screening and quantification of multiple contaminants, LC-quadrupole-time-of-flight (LC-Q-TOF) has proven practical for compound analysis (including non-target contaminant identification) [18–21]. There is still a need for simplified sample preparations and fast analysis methods in the field, so mass resolution has an important role in the successful identification and screening of multi-xenobiotics in samples containing high amounts of matrix co-extracts. The hybrid quadrupole-orbitrap mass spectrometer has garnered interest from researchers due to its unique performance, including routinely achieving mass resolutions up to 100,000 with mass accuracy below 5 ppm; this tool, then, can be used to significantly improve the efficiency and accuracy of multi-xenobiotic screening methods, allowing successful screening at trace concentration levels in complex matrices. A previous study evaluated the Q-Orbitrap in comparison to the Q-TOF detector in terms of mass accuracy and relative isotopic abundance with various concentrations of a standard mixture in four complex sample matrices, and results showed that the Orbitrap performed just as well as the Q-TOF [22]. It is important to note that mass accuracy values obtained at insufficient monoisotopic peak intensity on a Q-TOF require a larger m/z window, which may generate incorrect formulae for the detected ions. Q-Orbitrap technology, to this effect, is a promising alternative for the identification and quantification of many toxic compounds in different biological matrices [23–29].

Over the past few years, sample preparation has been a major challenge with regard to analytical methods for the determination of multi-residue compounds in food. It is necessary to remove the impurities from matrices via sample preparation for LC and gas chromatography (GC) with low-resolution detectors. The main groups of chemicals in honey are sugars, organic acids, proteins (albumin and globulin from bee pharyngeal glands) amino acids, enzymes (invertase, amylases, and glucose oxidase from bee glands) acetylcholine, choline, flavonoids, anthocyanins, vitamins, pigments, sterols, phospholipids, and mineral elements [30]. These endogenous substances severely interfere with the qualification and quantification of exogenous substances. The techniques most frequently used for honey sample extraction or clean-up are solvent extraction (SE) [31], liquid–liquid extraction (LLE) [32], solid-phase extraction (SPE) [33], solid-phase microextraction (SPME)/stir bar sorptive extraction (SBSE) [34], or the QuEChERS ("quick, easy, cheap, effective, rugged, and safe") [35] approach. Any of these can greatly reduce interference of the matrix and improve sensitivity to target analytes, however, multi-xenobiotics with different physical and chemical properties pose an unprecedented challenge in terms of the necessity for simultaneous extraction and clean-up in honey samples. These steps are usually the most time-consuming and laborious stages of analysis, and typically result in the loss of some substances. A simple SE method coupled with HRMS that enables simultaneous screening and quantification of these multi-xenobiotics in honey is quite desirable, to this effect.

In this study, we examined the applicability of UHPLC-Q-Orbitrap MS for both the screening and identification of non-target multi-xenobiotics in honey based on a simple extraction process. We first established a home-made accurate-mass database for 157 compounds, including 105 pesticides, 49 antibiotics, and three steroids. We then subjected honey samples to UHPLC-Q-Orbitrap analysis and gathered data by switching between the positive and negative ion mode simultaneously in one run, which included the full scan-ddMS2 and PRM mode. Unknown substances were identified according to the accurate-mass database via mass accuracy (under MS and MS/MS modes) and retention time under the same instrument conditions. Finally, the PRM mode was used to quantify the multi-xenobiotics after the screening procedure was complete. To the best of our knowledge, the UHPLC-Q-Orbitrap technique coupled with this type of simplified extraction procedure has never been used to screen and quantify multi-xenobiotics in honey.

2. Materials and methods

2.1. Reagents and chemicals

The standards for the 105 pesticide, 49 antibiotic, and three steroid targets were purchased from Dr. Ehrenstorfer (Augsburg, Germany), Sigma–Aldrich (Steinheim, Germany), and Witega (Berlin, Germany). All standards were of high-purity grade (>95.0%). Acetonitrile (ACN), methanol, and formic acid were of HPLC grade (DIMA Technology Inc., Richmond, VA, USA). Pure water was prepared using a Milli-Q system (MilliPore, Bedford, MA, USA).

2.2. Standard solutions

Individual standard stock solutions (ca. 1.0 mg/mL) were prepared according to their specific solubility properties by dissolving the appropriate amount of each standard compound in ACN and MeOH or water at different ratios. A mixed standard working solution (1.0 μg/mL) used to spike the control samples, which were prepared by appropriate dilution of an intermediate standard solution (10.0 μg/mL per analyte) with ACN. All stock and working solutions were stored in the dark at −20 °C and 4 °C for 3 months before use.

2.3. Sample collection and preparation

A total of 300 honey samples were collected from beekeepers and supermarkets in 2014 and stored at 4 °C in darkness before use. Prior to analysis, honey samples were allowed to liquefy at room temperature for 1 h and mixed by inversion. If the honey had crystallized, it was first gently heated in a water bath at a temperature not exceeding 50 °C.

Each homogeneous sample (2.0 g honey) was weighed in a 15 mL polypropylene centrifuge tube. Pure water (5.0 mL) containing 200 mg ethylenediaminetetraacetic acid disodium salt (Na_2EDTA) and 100 mg citric acid was added to the polypropylene centrifuge tube, tightly capped, and vigorously shaken for 5 min. Next, 10 mL ACN (containing 1% acetic acid, 4 g anhydrous Na_2SO_4, and 1 g NaCl) was shaken in a tube for 5 min and centrifuged at 8000 rpm for 5 min. The supernatant (5.0 mL) was transferred to a 10 mL graduated test tube, and then dried in a rotary evaporator. The residue was reconstituted in 1.0 mL of the mobile phase with the initial proportions, and then filtered through a 0.22 μm nylon membrane filter prior to injection into the UHPLC-Q-Orbitrap MS.

2.4. LC conditions

Analysis was performed using a Dionex UHPLC system (Dionex Corporation, Sunnyvale, CA, USA) consisting of an Ultimate 3000 rapid separation (RS) pump (HPG-3400RS), an autosampler (WPS-3000), and a column compartment (TCC-3000), all of which were operated using Chromeleon 6.8 software. Chromatographic

separations were performed on a Thermo Accucore C-18 aQ (2.1 mm × 150 mm, 2.6 μm) with a guard column of the same material (10 mm × 2.1 mm). The mobile phase consisted of 2% methanol and 0.01% formic acid in pure water (Solvent A) and 98% methanol with 0.01% formic acid in pure water (Solvent B) formed by gradient elution with the following parameters: 0–4 min, 0–20% B; 4.0–5.5 min, 20–40% B; 5.5–10.5 min, 40–100% B; 10.5–12.9 min, 100% B; 12.9–15 min, 100% B. The total run time was 15 min with a constant flow rate of 400 μL/min and constant column oven temperature of 30 °C.

2.5. HRMS conditions

We used a Q-Orbitrap instrument, Q-Exactive Plus (Thermo Fischer Scientific, Bremen, Germany), operated under Q-Exactive Tune 2.3 Build 1765 and Xcalibur 3.1 software. The voltage of the electrospray ionization (ESI) interface (HESI–II) was 3200 V for both positive and negative switch modes. The heater temperature was adjusted to 350 °C and the heated capillary temperature to 320 °C. The sheath gas, auxiliary gas, and sweep gas flow rate were 40 arb, 10 arb, and 0 arb, respectively, and the S-lens radio frequency (RF) level was 60. All screening data were acquired using the full MS scan/dd–MS^2 mode. The full scan spectra were acquired over an m/z range of 100–1000. Orbitrap resolution was set to 70,000 full-width at half-maximum (FWHM) at m/z = 200, which provided approximately three full scan-ddMS2 (data points) per second. The quadrupole was used as a wide-pass mass filtering device (m/z 100–1000). The automatic gain control (AGC) setting was defined as 100,000 charges and the maximum injection time was set to 50 ms. All extracted mass traces were based on a 4.0 ppm mass isolation window. Stepped normalized collision energies (NCEs) of 30, 50, and 70 v were employed for fragmentation. The dd–MS^2 resolution was set to 17,500 FWHM (m/z = 200). Data processing was performed with Tracefinder 3.1 software; the PRM mode was used to quantify the positive samples, at 17,500 (at m/z = 200) resolution, automatic gain control (AGC) target at 1×10^5, 50 ms maximum fill time, and an individual isolation window of 2 Th. A normalized collision energy of 50 was employed for fragmentation. The fragment ion with the highest real-time abundance was used for multi-xenobiotic quantification.

2.6. Accurate-mass database construction and screening analysis

The 105 pesticides, 49 antibiotics, and three steroids, each at a concentration of 10 ng/mL, were injected into the UHPLC-Q-Orbitrap system. The elemental composition, retention time, theoretical exact mass, characteristic fragment ions, and ion ratio and mass accuracy of individual xenobiotics were collected in an Excel spreadsheet for reference. The honey sample data were first imported into Tracefinder software, then the five parameters observed (retention time, molecular ion, characteristic fragment ions, ion ratio, and mass accuracy) were compared with those of the pre-created accurate-mass database. The defined criteria were as follows: retention time tolerance ≤ ±0.15 min, mass accuracy tolerance of full scan-ddMS2 ≤ ±1.0 ppm, identical molecular ions and MS/MS fragment ions, and ratio range of characteristic fragment ions ≤16%; the sample was believed to be positive if the five parameters of any compound met these criteria. Detailed fragmentation pathways were then elucidated by further dissociation of each fragment ion in the ion spectrum via Mass Frontier software.

2.7. Quantitation method validation

Using the sample preparation procedure and instrument conditions described above, the selectivity, linearity, recovery (accuracy), repeatability (precision), decision limit (CCα), detection capability (CCβ), matrix effects, and carry-over parameters were used to evaluate the performance of the proposed method. Blank samples of honey were collected from our own apiary and tested to confirm each sample was xenobiotic-free via national standard methods (China).

The selectivity of the method was assessed separately by analyzing blank honey using the proposed method and observing the presence of matrix interferences at the retention time of each analyte above a signal-to-noise ratio of three. Mixed calibration standards with concentrations of 0, 0.01, 0.05, 0.1, 1.0, 10.0, 50.0, and 100.0 ng/mL for xenobiotics were prepared by further diluting the individual working solutions with the mobile phase. Calibration curves (Set 1) were constructed by plotting the peak area of the xenobiotics versus their concentrations in pure water using a least squares linear regression with a weighting factor ($1/x^2$). Another set of calibration curves (Set 2) was prepared by adding the working solutions of the positive xenobiotics into blank honey samples that were first subjected to the extraction procedure to yield a series with the same concentrations as the calibration standards (Set 1). Five replicates of each concentration were performed the same day. The matrix effect was evaluated by comparing the PRM responses of xenobiotic standard solutions at 5 μg/kg, 10 μg/kg, and 20 μg/kg in the mobile phase (B) and those of the matrix-matched solutions with the same concentrations (A). Matrix effects were observed by spiking blank honey with known amounts of standard solutions at three concentration levels. For the matrix effect $(A–B)/B$, a negative value (<0%) indicated ionization suppression, whereas a positive value (>0%) indicated an ionization enhancement effect.

CCα was calculated by making a calibration curve with spiked blank honey (0.01, 0.05, 0.1, 1.0, and 10.0 ng/mL). The concentration at the y-intercept plus 2.33 times the standard deviation of the within-laboratory reproducibility (n = 5) of the intercept equaled the decision limit (α = 1%). CCβ was calculated by making a calibration curve with spiked blank honey (0.01, 0.05, 0.1, 1.0, and 10.0 ng/mL). The concentration at the decision limit plus 1.64 times the standard deviation of the within-laboratory reproducibility (n = 5) of the mean measured content at the decision limit equaled the detection capability (β = 5%). Recovery and precision were calculated by analyzing spiked honey samples at three concentration levels (CCβ, 2CCβ and 4CCβ). Precision was determined by analyzing blank honey samples at three different levels (in quintuplicate per batch) using matrix-matched calibrations. Similarly, recovery was determined as the PRM response of honey spiked with a fixed concentration of the xenobiotic standard solution before extraction (C) relative to the response of the blank samples first subjected to the extraction procedure and then spiked with the same amount of xenobiotic (A); thus, recovery was equal to $(C/A) \times 100$. The carry-over effect was measured by alternately testing high-concentration xenobiotic standard solutions (100 ng/mL) and the mobile phase in quintuplicate. Carry-over was considered negligible if the measured concentration was less than the CCα value.

3. Results and discussion

3.1. Sample preparation optimization

The QuEChERS method is useful for the extraction and clean-up of analytes of interest from complex matrices. This method involves an ACN extraction followed by a salting-out step and subsequent subjection of the sample to a dispersive solid-phase extraction (d-SPE) clean-up procedure with one of several available absorbents, including primary secondary amine (PSA), octadecyl (C18), graphitized carbon black (GCB), and others [36]. The QuEChERS approach requires minimal operational steps and a smaller amount of

solvent than standard liquid–liquid extraction and conventional SPE extraction procedures. There inevitably are compounds that are undetected at a concentration of 10 μg/kg after optimizing the contents and proportion of sorbents in the clean-up step of the original QuEChERS method, so the clean-up step has been abandoned due to unstable screening results. The elimination of false negatives, specifically, is one of the most important goals of screening analysis – in effort to achieve this goal, we optimized the extraction procedure of the QuEChERS method to qualify all target analytes in one run.

Pure water was first used to disperse the honey sample because organic solvents (e.g., methanol, acetonitrile and ethyl acetate) are not miscible with honey. Acidified ACN was used as the extraction solvent in our QuEChERS experiment to increase the stability of acidic analytes and improve the protein precipitation of the honey samples. ACN was found to extract most analytes of interest with a wider range of polarities; moreover, we found that the addition of 1% acetic acid in ACN can effectively increase the recoveries of sulfonamides, as the acid hydrolysis step freed the sugar-bound sulfonamides. Different extraction solvents (0.1%, 0.5%, 1%, 2% and 5% acetic acid-ACN) were examined to compare their recoveries. Results indicated that better quantification was obtained at acetic acid concentration of 1% in the ACN. The addition of inorganic salts, including Na_2SO_4, $MgSO_4$, and NaCl, was also shown to benefit the partitioning of target analytes between the organic and aqueous phases. Satisfactory quantification results of all analytes were achieved by adding anhydrous Na_2SO_4 (4.0 g) and NaCl (1.0 g), which effectively absorbed the excess water and increased the ionic strength of the material, enhancing the partition of the analytes from the aqueous phase to the ACN phase. Table S1 (Supporting Information) shows that the comparison of recoveries for the 157 xenobiotics in honey using different extraction conditions.

3.2. Screening method evaluation via accurate-mass database by UHPLC-Q-Orbitrap

Table S2 (Supporting Information) shows the comprehensive information contained in the Excel database of the analyte experimental data. The investigated xenobiotics (pesticides, antibiotics, and steroids, separately) are listed in order of retention time. The database was imported to the Tracefinder software and used to search information according to different parameters – TraceFinder integrated with Xcalibur core software allowed us to develop high-throughput screening and quick, accurate quantitative assays using the Q-Orbitrap instrument.

The total ion chromatograms of selected xenobiotics with accurate mass measurements in the spiked honey extract are shown in Fig. S1 (Supporting Information). The detailed procedure (using the flumequine-spiked honey extract at 5.0 μg/kg as an example) was as follows. First, options on the screening settings page were used to set the method parameters, including peak detection defaults (mass tolerance, retention time, and ion ratio parameters) adducts ($M+NH_4$, M+H, M+Na, M+K, and M−H) to calculate exact masses and to select single or batch sample submission. Next, we created, parameterized, and stored a new Xcalibur Instrument method in the folder. The sample data was then able to be imported to Tracefinder to match the sample information with that of the database. We then matched the mass of the full scan and retention time (Fig. S2A, Supporting Information), followed by the multi-isotope ratio (Fig. S2B, Supporting Information) and the fragment ions of the MS/MS mode (Fig. S2C, Supporting Information). For flumequine, the deviation of retention time between the spiked sample and the corresponding standard solution was 0.05 min, and the mass accuracy via the full scan at m/z 262.08740 was 0.34 ppm, the isotope signature ions and their ratios were consistent for the spiked sample and corresponding standard solution, and the mass accuracies of the MS/MS fragment ions at m/z 220.04068 and 244.07675 were below 1.0 ppm. In brief, this example confirmed the validity, feasibility, and effectiveness of the proposed method. In fact, deviation of the retention time and mass values from the MS and MS/MS spectra completely ensured the accuracy of the screening analysis – so we had no need to apply the multi-isotope ratio for accurate screening.

The instrumental sensitivities with regard to the 105 pesticides, 49 antibiotics, and three steroids included in the database were calculated by the injection of a matrix-matched solution at different concentration levels: 2, 5, and 10 μg/kg. The pesticides, antibiotics, and steroids included in the database were separated into four groups according to their sensitivity: Group 1 (≤2 μg/kg), Group 2 (≥2 μg/kg and ≤5 μg/kg), Group 3 (≥5 μg/kg and ≤10 μg/kg), and Group 4 (≥10 μg/kg). All antibiotics, except tetracyclines (oxytetracycline, tetracycline, chlortetracycline, and doxycycline) and sodium nafcillin, and 70% of the pesticides belonged to Group 1, while oxytetracycline, and tetracycline belonged to Group 2, and 20% of the pesticides, chlortetracycline, doxycycline, penicillin G, and sodium nafcillin belonged to Group 3 and 10% of the pesticides belonged to Group 4. Stepped NCEs (30 v, 50 v, and 70 v) were used to obtain the maximum abundances of the different analytes.

Altogether, our results suggest that the proposed method has potential application to multi-xenobiotic screening in matrices besides honey due to its highly favorable sensitivity under the full scan-ddMS2 mode. We conducted analyses in order to enable a natural comparison of ion ratios of the characteristic fragmentions using both standard substances in pure solvent and matrix-matched solution at the same concentrations, and the results showed that the characteristic fragment ion ratios for all sample xenobiotics were within ±15.4% of their theoretical values. The ion ratio range limit between standard solutions and real samples was set to ±16% from that point forward to ensure accurate identification.

Compounds with similar masses often co-elute during the analysis of complex matrices if the resolution is not high enough, and discrepancies among them can be difficult to identify, so resolution is a key parameter of MS in screening applications. We specified the resolution of the Q-Orbitrap MS in the vicinity of m/z 200, because the multi-xenobiotics analyzed in this study were small molecules. We made a comparison with the sensitivity of monocrotophos at m/z 224.06824 as an example between resolution values of 70,000 and 140,000 with the Q-Orbitrap instrument, and found that there is no significant decrease in the response of monocrotophos as the resolution increases from 70,000 to 140,000 (Fig. 1). It is important to note that a marked decrease in sensitivity was not observed while approaching this unprecedented high resolution.

In non-target screening analysis, the positive/negative switching mode must be used to simultaneously analyze compounds with different ionization modes and adjacent retention times. The Q-Orbitrap instrument has the ability to switch polarities very quickly, allowing the user to perform necessary positive/negative switching without substantial delay in the duty cycle. In this study, sulfamethoxazole and florfenicol served as typical examples to explore the Q-Orbitrap instrument with regard to switching speed. From the centroid mass spectra of the full scan-ddMS2 mode, sulfamethoxazole under the positive mode and florfenicol under the negative mode were able to be simultaneously scanned to implement real-time data acquisition at retention times of 5.82 min and 5.83 min, respectively – HRMS with the Q-Orbitrap increased scan speed at a resolution of 70,000 and performed positive/negative switching to obtain two profiles to the millisecond, without lock mass, based on real-time mass measurement correction (Fig. 2).

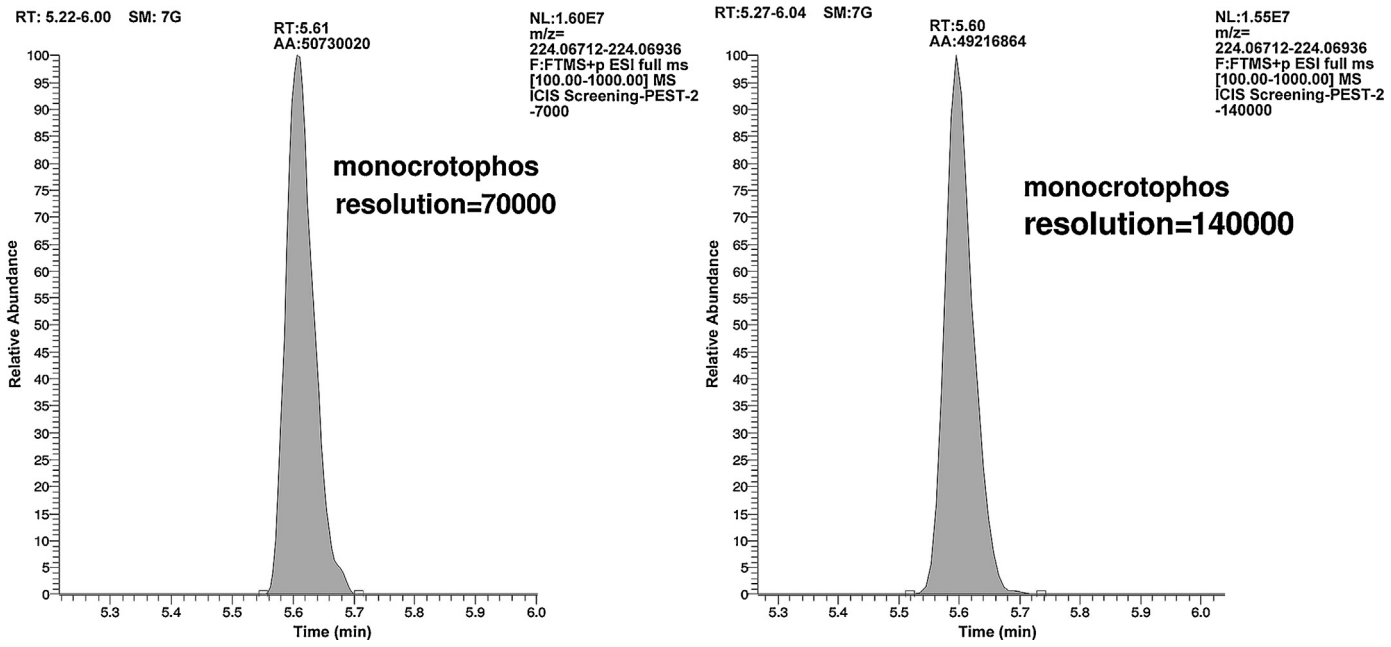

Fig. 1. A comparison with the sensitivity of monocrotophos at *m*/*z* 224.06824 as an example between resolution values of 70,000 and 140,000 used with the Q-Orbitrap instrument.

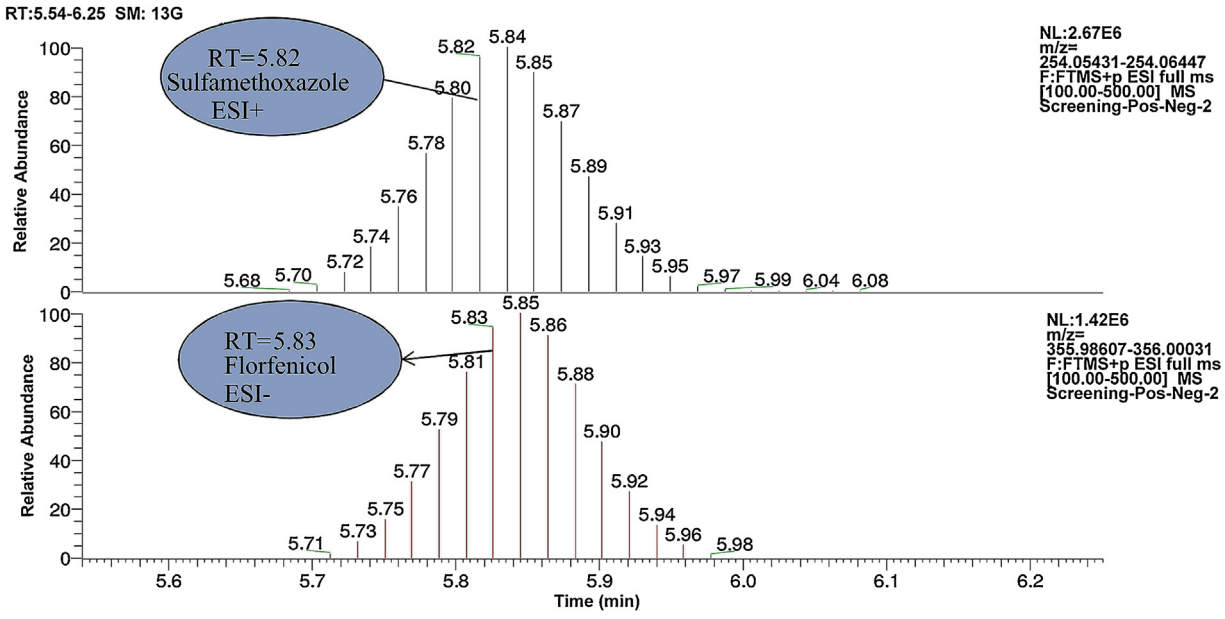

Fig. 2. The effect of positive/negative switching on the scan speed of sulfamethoxazole and florfenicol at retention times of 5.82 min and 5.83 min.

3.3. Screening method application

We tested the proposed method to screen non-target xenobiotics on 300 honey samples collected from beekeepers and supermarkets, and found five compounds: sulfacetamide, norfloxacin, metronidazole, sulfamethoxazole, and carbendazim. A comparison between the positive samples and their corresponding standard solutions at 100 ng/mL in the full scan of the precursor ion and corresponding extracted ion chromatogram, plus their MS/MS fragment ions, was made to reduce any potential for false positives in the screening results. Moreover, we established the pathway from spectra to structure through chemically intelligent structural elucidation with Mass Frontier software to further confirm the characteristics and rationality of the fragment ions.

For sulfacetamide (Fig. S3, Supporting Information), the fitting molecular formula between both the positive sample and the corresponding standard solution was $C_8H_{11}N_2O_3S$, the mass accuracy via the full scan at *m*/*z* 215.04849 and the MS/MS fragment ions at *m*/*z* 92.04948 and 156.01138 was below 0.98 ppm, and the retention time deviation was 0.08 min. For norfloxacin (Fig. S4, Supporting Information), the fitting molecular formula between both the positive sample and the corresponding standard solution was $C_{16}H_{19}FN_3O_3$, the mass accuracy via the full scan at *m*/*z* 320.14050 and the MS/MS fragment ions at *m*/*z* 276.11428 and 234.05609 was less than 0.93 ppm, and the retention time deviation was 0.06 min. For metronidazole (Fig. S5, Supporting Information), the fitting molecular formula between both the positive sample and the corresponding standard solution was $C_6H_{10}N_3O_3$, the mass accuracy via

the full scan at m/z 172.07167 and the MS/MS fragment ions at m/z 128.04543 and 72.0444 was less than 0.96 ppm, and the retention time deviation was 0.04 min. For sulfamethoxazole (Fig. S6, Supporting Information), the fitting molecular formula between both the positive sample and the corresponding standard solution was $C_{10}H_{12}N_3O_3S$, the mass accuracy via the full scan at m/z 254.05939 and the MS/MS fragment ions at m/z 156.0114, 108.04442 and 92.0500 was less than 1.0 ppm, and there was no deviation in retention time. For carbendazim (Fig. S7, Supporting Information), the fitting molecular formula between both the positive sample and the corresponding standard solution was $C_9H_{10}N_3O_2$, the mass accuracy via the full scan at m/z 192.07675 and the MS/MS fragment ions at m/z 160.05054 was less than 0.97 ppm, and the retention time deviation was 0.02 min. The relevant mass spectra and fragmentation pathways are shown in Fig. S8 (Supporting Information).

Altogether, our results showed that the molecular formulae between both the positive samples and the corresponding standard solutions matched well, the mass accuracies at full scan-ddMS2 modes were less than 1.0 ppm, and retention time deviations were less than the threshold (±0.15 min) set in advance for each positive xenobiotic. Moreover, the characteristic fragment ions of positive xenobiotics were interpreted for their structural elucidation using the spectra successfully. Altogether, these analysis results confirmed that the screening method developed in this study is accurate and reliable, and can minimize or eliminate the occurrence of false positives or negatives when analyzing real samples.

3.4. Validation and application of quantification method

The accurate quantification of positive xenobiotics was an obligatory step after samples were subjected to screening, because, of course, the objective of any analytical measurement is to obtain consistent, reliable, and accurate data. Method validation results can be used to judge the quality, reliability, and consistency of the analytical results, which is an integral part of any sound analytical practice. Our results showed that the detection of xenobiotics by PRM in honey was highly selective with no interference. Linear calibrations were obtained for all analytes with correlation coefficients in the range of 0.9990–0.9996 for calibration (Set 1) and 0.9918–0.9991 (Set 2) in honey. The Q-Orbitrap instrument we used offered a linear dynamic range of about four orders of magnitude; the linear dynamic range for most of the compounds was enough for successful quantitative analysis of xenobiotics in real samples. Several honey samples were analyzed, resulting in mean matrix effects of 40.7% ~ 159.2% and 80.7% ~ 95.1% for positive and negative xenobiotics, respectively, representing quite significant matrix effect.

The use of isotopically labeled internal standards can effectively compensate for matrix effects, but it is difficult to acquire internal standards for each analyte in practice. We instead used matrix-matched calibrations to validate the method and to qualify and quantify xenobiotics in the honey samples. The CCα and CCβ values ranged from 0.006 to 4.73 μg/kg and from 0.009 to 6.21 μg/kg for positive xenobiotics, respectively. The sensitivity attained using this method was sufficient to detect the target compounds in real honey samples – given as the relative standard deviation (%), intra-batch and inter-batch precisions were less than or equal to 6.42% and 11.9%, respectively (for three batches).

The main purpose of the recovery calculation method described above was to eliminate the contribution of matrix effect and/or mutual interference among xenobiotics. Recoveries ranged from 78.4% to 103.1% for xenobiotics in honey samples. The carry-over test results showed that the signal response of the mobile phase in the target analyte retention time was lower than that of the CCα value, so we concluded that no carry-over effect was present after applying the developed PRM method.

3.5. Comparison with prior research

Extraction of target analytes with acidic organic solvent is a well-established sample preparation approach for honey samples pre-dissolved in water. We used different solvents, including acetone with 1% formic acid, phosphate buffer, acetonitrile with 1% acetic acid [37], Na_2EDTA (pH 4) [38], and hydrochloric acid [39], for extraction by increasing the stability of acidic analytes and improving the protein precipitation of the honey samples. The extracts were then cleaned up using solid phase extraction or turbulent flow chromatography (TFC) prior to instrument analysis. An effective SPE procedure is challenging to develop, because it is extremely difficult to establish a generic protocol that is efficient for target compounds with different polarity and pK_a values. The most notable contributor to the TFC system was the online extraction of target analytes and Orbitrap-MS, which improved the system's sensitivity. Similar to previous studies, we added acidified ACN, Na_2SO_4, and NaCl to the dissolved honey samples to extract 157 xenobiotics with different physio-chemical characteristics, then we analyzed the filtered extract using UHPLC-Q-Orbitrap. The method we propose allows simple sample preparation but results in high-throughput screening and high sensitivity, as evidenced by our results.

A high number of analytical technologies such as triple quadrupole MS (QqQ), Q-TOF, and Q-Orbitrap, have been developed to allow the determination of xenobiotic residues in many different types of foodstuff. Determination of xenobiotic residues in honey has mostly been performed using the QqQ analyzer; only two studies using TOF [37] and Orbitrap [38] have been published. The results of a xenobiotic identification study using TOF-MS by Spörri et al. showed that the sensitivities of 200 veterinary drugs ranged from 1 to 200 ng/mL (4–800 μg/kg) in honey. In our study, using the Orbitrap analyzer, the limits of detection for 70% of the assessed xenobiotics were below 2 μg/kg; 90% of them did not exceed 10 μg/kg. Mass accuracies were below 1.0 ppm between positive multi-xenobiotics and standard solutions in the honey, as well, and retention time deviations were less than 0.15 min. Aguilera-Luiz et al. [38] also developed an Orbitrap method to identify veterinary drug residues in honey with a mass error of 0.1–4.7 ppm, but the TOF-MS analyzer usually presents mass accuracy from 5 to 10 ppm. In short, Orbitrap exhibits the most powerful identification ability in sample matrices.

The QqQ instrument is a powerful analytical tool that has been widely used for the routine multi-residue screening of xenobiotics in bee products. Hammel et al. [31] developed a multi-screening approach to monitor and quantify 42 antibiotic residues in honey by LC-MS/MS, with a total running time of 45 min and detection limit between 27.1 μg/kg and 80.9 μg/kg. A multi-class residue method for honey samples was also presented by Robert et al. [40], using LC-MS/MS with a running time of 14.0 min with CCβ values between 0.5 μg/kg and 100 μg/kg. In our study, CCβ values ranged from 0.009 to 6.21 μg/kg and recoveries ranged from 92.7% to 103.1% for the xenobiotics in honey samples, and, remarkably, the total running time for UHPLC-Q-Orbitrap was only 15.0 min. The sensitivity of Orbitrap based on the PRM mode is clearly superior to traditional ESI-MS/MS, suggesting that Orbitrap can be considered a very attractive potential instrument for use as a screening and quantification method in the EU legislative context. The Orbitrap analyzer, compared to traditional Q-TOF and QqQ, allows multi-xenobiotic determination at trace level concentrations and can be used to perform screening analysis very rapidly due to short running time and relatively simple sample preparation – that said, wider use of Orbitrap is still limited by the high price of purchase

and elevated running costs in comparison with QqQ and Q-TOF systems.

4. Conclusions

In this study, we developed a simple sample preparation procedure followed by UHPLC-Q-Orbitrap MS with a user-built accurate-mass database and PRM for the automated screening and quantification of multi-xenobiotics in honey. Our screening and quantification method were validated in terms of the parameter threshold, comparison between spiked samples and standard solutions, selectivity, linearity, recovery (accuracy), repeatability (precision), decision limit (CCα), detection capability (CCβ), matrix effects, and carry-over to ensure the accuracy of the results. We not only correctly identified 105 pesticides, 49 antibiotics, and three steroids using the proposed workflow, but were able to simultaneously quantify them based on the PRM mode. Sulfacetamide, norfloxacin, metronidazole, sulfamethoxazole, and carbendazim were detected in the samples at nanogram-per-gram levels; among them, the highest concentrations of sulfacetamide, norfloxacin, metronidazole, sulfamethoxazole, and carbendazim were 5.3 μg/kg, 351.2 μg/kg, 518.4 μg/kg, 281.4 μg/kg, and 75.3 μg/kg, respectively, in the 300 honey samples we tested. The quantification results also confirmed the accuracy of the proposed screening analysis method. We hope that our findings will expand the utility of analytical techniques available for the screening and quantification of xenobiotics for more matrices in the future.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2015.11.075.

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