Simultaneous determination of estrogens and progestogens in honey using high performance liquid chromatography-tandem mass spectrometry

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1 **Abstract**

2 This work describes the development and validation of a method for the simultaneous
3 determination of 13 estrogens and progestogens in honey by high performance liquid
4 chromatography-tandem mass spectrometry. The hormones were preconcentrated by
5 solid phase extraction. Pretreatment variables were optimized for a better compatibility
6 with electrospray ionization interfaced mass spectrometry. The analytes were analyzed
7 in multiple-reaction monitoring mode with two pairs of precursor product ion transitions.
8 The proposed method was validated with method detection limits of 0.01–0.33 ng/g and
9 good linearities ($r^2 > 0.9901$) throughout the studied concentration range. The
10 recoveries of analytes at the spiking levels (5 ng/g and 25 ng/g) ranged from 71.2% to
11 99.7%, with relative standard deviations below 20%. The method was used to
12 determine the target compounds in honey samples (orange blossom, clover and
13 multiflower) obtained from supermarkets. Two samples of honey were found to contain
14 trace amounts of estrone (< MQL) or progesterone (0.2 ± 0.1 ng/g), respectively.

15 **Keywords**

16 Estrogens; Progestogens; LC-MS/MS; Solid phase extraction

1. Introduction

The presence of steroid hormones in environmental matrices has raised worldwide concern due to their endocrine disrupting effects on aquatic organisms [1]. Steroid hormones mainly consist of estrogens, androgens and progestogens which are excreted by living creatures via urine or feces as biologically inactive conjugates or as active precursors [2]. These inactive conjugates can be converted into their estrogenic active forms during the wastewater treatment process. Persistent hormones that are not fully degraded in wastewater treatment plants, together with those from concentrated animal feeding operations, are released to surface waters, posing potential risks to wildlife and humans. Moreover, emerging contaminants may enter food chains through soil via the field application of hormone-containing fertilizer or reclaimed water [3]. Thus, monitoring the concentrations of potent steroid hormones and their conjugates in foodstuffs is a crucial step for risk control.

Due to its nutritional properties, honey is a healthy food that is consumed worldwide. A number of studies demonstrated the presence of phenolic endocrine disruptors [4], pesticides [5], and antibiotics [6] in honey, highlighting a possible risk for consumers. So far, there is limited research on the determination of steroid hormones in honey. To ensure the safe consumption of honey, there is a need to evaluate its levels of highly potent contaminants, which entails the development of an applicable analytical method for a complex matrix.

High performance liquid chromatography coupled with triple quadrupole mass system (HPLC-MS/MS) has become a preferred technique for the determination of steroid hormones in various environmental matrices [7-9]. However, the loss of sensitivity due to complex matrices poses a significant challenge for trace analysis. Due to lack of

availability, internal surrogate addition and isotope dilution approaches may not always be adopted to compensate for recovery losses. Therefore, the development of an efficient extraction and purification procedure helps enhance the sensitivity of the method. The aim of the study was to develop a sensitive, robust and instrument friendly method for the simultaneous determination of 13 steroid hormones, including six estrogen conjugates, five estrogens and two progestogens in honey based on solid phase extraction (SPE) and HPLC-MS/MS. Solid phase extraction was used to preconcentrate the target analytes and selective washing steps were used to remove the unwanted matrix compounds. For the HPLC-MS/MS, the multiple reaction monitoring (MRM) was employed for the simultaneous determination of multiple compounds. The validated method was applied to honey matrices of different floral origins.

2. Experimental

2.1. Chemicals and materials

Analytical standards of estriol (E3), estrone (E1), 17 β -estradiol (E2), 17 α -estradiol (α -E2), 17 α -ethynylestradiol (EE2), progesterone (P), medroxyprogesterone (MP) and estrone-3-sulfate (E1-3S) were purchased from Sigma Aldrich (St. Louis, MO, USA); 17 β -estradiol-17-glucuronide (E2-17G), 17 β -estradiol-3-glucuronide sodium salt (E2-3G), estrone-3-glucuronide (E1-3G), 17 β -estradiol-17-sulfate sodium salt (E2-17S) and 17 β -estradiol-3-sulfate sodium salt (E2-3S) were obtained from Steraloids Inc. (Newport, RI, USA). The purity of all compounds was $\geq 98\%$. Selected characteristics of the compounds are listed in Table 1. To prepare stock standard solutions, 10 mg of each standard was dissolved in 10 mL methanol (MeOH) to give a concentration of 1000 $\mu\text{g/mL}$. The stock standard solutions were stored in amber glass vials at -20°C for up to

6 months. A 10 µg/mL intermediate composite of all compounds was prepared weekly from the stock solution. Working solutions for calibration curves were made daily by diluting the intermediate composite with MeOH-H₂O (20:80, v/v).

An ACS grade ammonium hydroxide solution (NH₄OH; 14.8 M) was obtained from Mallinckrodt (St.Louis, MO, USA). Acetonitrile (ACN) and MeOH were obtained from Burdick & Jackson (Morristown, NJ, USA). Ethyl acetate (EtOAc), tetrahydrofuran (THF), acetic acid, sodium hydroxide (NaOH) and ultrapure water were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Unless otherwise specified, the organic solvents and ultrapure water were of HPLC grade.

The SPE was carried out using a 12-port Supelco vacuum manifold. Oasis hydrophilic-lipophilic balance (HLB) cartridges (200 mg, 6 mL) were acquired from Waters (MA, USA). Polytetrafluoroethylene syringe-driven filters (0.45 µm, I.D. 31 mm and 0.22 µm, I.D. 17 mm) were purchased from Fisher Scientific (Rockwood, TN, USA).

2.2. Sample preparation and purification

Preconcentration and cleanup were carried out according to the procedure depicted in Figure 1. Briefly, 5 g of commercially obtained pure honey was weighed into a 45 mL Teflon centrifuge tube and diluted with 25 mL ultrapure water. An appropriate amount of standard solution was added to the diluent. The centrifuge tube was mixed thoroughly on a vortex mixer, centrifuged at 10,000 RPM for 10 min, and then filtered through 0.45 µm filter. A 20 mL aliquot of the filtrate was loaded onto an Oasis HLB cartridge preconditioned sequentially with 6 mL MeOH and 12 mL ultrapure water. The cartridges were washed with 20 mL ultrapure water. Thereafter, to minimize the interferences, the cartridges were rinsed using the following solution combinations: 1) 4 mL of MeOH-H₂O

(10:90, v/v) + 4 mL of MeOH-H₂O-NH₄OH (14.8 M) (10:83.1:6.9, v/v/v); 2) 4 mL of MeOH-H₂O (20:80, v/v) + 4 mL of MeOH-H₂O-NH₄OH (14.8 M) (20:73.1:6.9, v/v/v); 3) 4 mL of MeOH-H₂O (30:70, v/v) + 4 mL of MeOH-H₂O-NH₄OH (14.8 M) (30:63.1:6.9, v/v/v); and 4) 4 mL of MeOH-H₂O (10:90, v/v) + 4 mL of MeOH-H₂O-NH₄OH (14.8 M) (10:83.1:6.9, v/v/v) + 3 mL of THF-H₂O (25:75, v/v). After the rinse, the cartridges were dried under vacuum for 30 min. Elution of the analytes was conducted by passing 4 mL of EtOAc-MeOH (9:1, v/v) and 4 mL of MeOH-NH₄OH (14.8 M) (93.1/6.9, v/v). The eluent was collected in a graduated centrifuge tube and evaporated under a gentle stream of nitrogen gas to approximately 0.2 mL. This solution was then brought to 1 mL with MeOH-H₂O (20:80, v/v). The reconstituted solution was filtered through a 0.22 µm syringe filter and transferred to a 2 mL autosampler vial.

2.3. Instrument analysis

The determination of estrogens and progestogens was conducted using an Agilent 1100 HPLC coupled with an Agilent 6410 triple quadrupole MS/MS system equipped with an electrospray ionization (ESI) interface. Chromatographic separation was achieved on an Agilent ZORBAX Extend-C18 (3.0 × 150 mm, 3.5 µm) column at a flow rate of 0.35 mL/min. The column temperature was set at 40°C. The injection volume was 20 µL. The mobile phase consisted of (A) H₂O-NH₄OH (77 mM) and (B) ACN-MeOH (80:20, v/v). The gradient and MS/MS parameters are included in Table 2. The total run time for each injection was 28 min. Collection and treatment of data was performed using Mass Hunter software (Version B.01.04). All analytes were analyzed in MRM mode. Nitrogen gas from the nitrogen generator was used as a drying and nebulizing gas. High purity nitrogen gas (99.999% purity) was used as collision gas. In our

previous work, we developed a HPLC-MS/MS method to investigate the occurrence of estrogens and progestogens in river water [10]. In the current work, we used some of the optimized instrument parameters from the previous work (Table 1).

3. Results and discussion

3.1. Extraction and cleanup

For SPE extraction, Oasis HLB was chosen over other sorbents owing to its versatile sorption capacity for hydrophilic and lipophilic compounds. It has been extensively employed for environmental matrices, to extract compounds with a wide range of properties, and it has yielded good recoveries [7-9]. The amphiprotic property of HLB sorbent renders an easier retention of interferences on the cartridges; therefore, the development of a method that deals with the cleanup procedure is paramount for mitigating matrix effects and improving method precision. The effect of pH, adjusted by adding NaOH or acetic acid to the diluted honey samples, on the extraction efficiency was evaluated at pH values of 3, 5 and 7. Recovery was not improved across the tested pH range; therefore we subsequently used the diluted honey samples without pH adjustment.

Honey is characterized by a high content of sugar [11], which was removed by the initial rinse of 20 mL water due to its hydrophilic nature. Without further cleansing, the absolute recoveries of the target compounds ranged from about 58% to 90% (Figure 2A). The absolute recoveries were determined as the detected concentration, calculated by external standard calibration, expressed as percentages of the actual concentration at which the sample was spiked and underwent the whole SPE procedure. Matrix effects in the honey were evaluated by comparing peak areas from the spiked honey extract (after subtracting the peak areas corresponding to the native analytes present in

the honey matrix) with the peak areas of the standard solution containing the same concentration of analytes. The matrix effect values, expressed as matrix %, however, were comparable to those of the recovery, suggesting that the recovery losses most likely resulted from matrix-induced ion suppression, which is a common pitfall encountered in quantitative LC-MS/MS analysis. Compared with less polar progestogens and parent estrogens, estrogen conjugates and E3 were susceptible to matrix effects to a larger extent, which may be ascribed to the presence of a large amount of acidic interferences. Similar results were observed in a study by Rodriguez-Gonzalo et al. who reported a high correlation ($r^2 = 0.954$) between the acidity of the analytes and the extent of ion suppression [12]. Another possible explanation for this is that the high acidity (lower pK_a value) or polarity of the conjugates and E3 limit their effective competition with interferences for charging, desolvation and evaporation in the ESI process, due to their smaller surface-active non-polar area, which facilitates ionization, compared with the unconjugated precursors [13].

In this work, we assessed the efficiencies of various solvent mixtures for SPE cleanup. The stepwise cleansing prior to elution of the target compounds over the HLB cartridges helps reduce the ion suppression resulting from isobaric interferences and decrease the method detection limit (MDL) to a level close to the instrument detection limit (IDL). The dark brown color of the HLB sorbent was possibly due to the presence of caramel, pigment and polyphenols [14, 15]. Upon sugar removal, interferences co-extracted on the column, including organic acids, phenolic compounds and pigments, were subject to cleaning using washing solvents with percentages of MeOH in water between 10% and 30%. The first rinse (MeOH in H₂O) of the four solvent combinations

removed weakly bound substances from the cartridge, such as proteins, amino acids, and low molecular organic acids. The base-wash solution (MeOH solution mixture containing NH_4OH) removed labile acidic substances. These organic acids are partially responsible for the color of the honey; a light brown HLB sorbent was observed after the second rinse. Figure 2B shows the recoveries obtained for estrogens and progestogens when using different washing solution combinations. As is indicated, no substantial improvement was afforded as the proportion of MeOH in water increased; however, further increases in this percentage could enhance the breakthrough of analytes. As an alternative, high strength THF- H_2O (25:75, v/v) was used following the MeOH- H_2O (10:90, v/v) rinse to remove the remaining hydrophobic substances (acidic or neutral) on the column, leaving the HLB sorbent colorless. Although using THF- H_2O (25:75, v/v) solution yielded satisfactory recoveries for most of the compounds, poor recoveries were obtained for E2-3G (36%) and E1-3G (58%). Oasis HLB has the greatest capacity for electron lone pair interactions, resulting in a strong retention of electron-rich compounds such as aromatic compounds [16]. The lowered electron density of E2-3G and E1-3G, due to steric hindrance of the 3-position glucuronide substituent on the aromatic ring, weakens their affinity for the HLB sorbent compared with either their sulfate substitution counterparts (E2-3S and E1-3S) or their 17-position substitution counterparts (E2-17G), possibly accounting for their low recoveries. On the other hand, using THF- H_2O (20:80, v/v) reduced the breakthrough of E2-3G and E1-3G and maintained the recovery of other compounds within an acceptable range, as discussed in the next section. In light of the results, the washing solvent combination [4 mL of MeOH- H_2O (10:90, v/v) + 4 mL of MeOH- H_2O - NH_4OH (14.8 M) (10:83.1:6.9, v/v/v) + 3

mL of THF-H₂O (20:80, v/v)] was selected as optimal for the conditions under which method performance was assessed. With respect to the elution solution, 4 mL of nonpolar solvent EtOAc-MeOH (9:1, v/v) and 4 mL of polar solvent MeOH-NH₄OH (14.8 M) (93.1/6.9, v/v) effectively eluted the parent hormones and estrogen conjugates, respectively.

3.2. Method validation

To evaluate the matrix effect, 1 mL of 100 ng/mL standard solution was dissolved in the honey extract after SPE. The matrix % values at the fortified level of 100 ng/mL ranged from 81.4% to 106.4%, suggesting that ion suppression, though persistent for some of the compounds, was improved substantially compared with unpurified samples (Figure 2A). Recovery experiment was carried out by spiking honey samples at two concentration levels (5 ng/g and 25 ng/g) prior to the entire SPE procedure. Unfortified honey matrix was also investigated for background subtraction. As shown in Table 3, good absolute recoveries were achieved for most of the compounds, ranging from 80.3% to 99.7% for both concentration levels. E2-3G, E1-3G and P showed relatively low recovery, between 71.2% and 82.1%. The overall method precision, expressed by the relative standard deviation (RSD%) was satisfactory (< 16%).

Five-point calibration curves (2 ng/mL, 5 ng/mL, 10 ng/mL, 50 ng/mL and 100 ng/mL) in pure solvent and honey extract were constructed by plotting analyte peak areas against analyte concentrations. Higher concentration standards were not used because high concentrations do not reflect environmentally relevant levels [2]. Good linearities were obtained in the 2–100 ng/mL range, with a correlation coefficient > 0.9901 for the MRM transitions used as quantifiers. The responses of the standard solution calibration

curves were compared with that of the matrix-matched calibration curves using two-tailed paired t-tests with a probability of 95%. The P values acquired were < 0.05 for most of the compounds, suggesting that the data for the pure solvent and honey matrix were not statistically equivalent. Common approaches to compensate for the matrix effect include using matrix-matched calibration curves, internal standard calibration or dilution of sample extracts. In the present study, the external matrix-matched standard calibration was used for quantification in the subsequent experiment.

Regarding sensitivity, the IDL was determined by injecting a series of successively-diluted standard solutions until a concentration level with a signal-to-noise of 3 was achieved. The IDL was lowest for E1-3S (0.02 ng/mL) and highest for EE2 (1 ng/mL), as indicated in Table 3. The MDL was determined by spiking seven replicates of honey matrix with a standard mixture to give a concentration five times the estimated MDL [17]. Each replicate of spiked honey samples was subject to the entire extraction procedure and instrument analysis to determine the final concentration of each compound. The standard deviation was then derived. The MDL was then calculated by multiplying the standard deviation by the appropriate Student's t-value ($t = 3.14$ for seven replicas). The method quantification limit (MQL) is defined as 10 times the standard deviation. The acquired MRM chromatograms of each analyte are shown in Figure 3 and demonstrate a good separation of all the analytes and a clean background. The obtained MDL and MQL values, presented in Table 3, ranged from 0.01 ng/g to 0.33 ng/g and from 0.04 ng/g to 1.05 ng/g, respectively. The MDL of estrogens in the present study was close to previously reported values (0.2 ng/g) for vegetables and fruits [18]. Overall, the present

method was considered highly sensitive, enabling the application of this method to real environmental samples.

3.3. Analysis of real samples

The validated method was applied for the analysis of four representative honey samples of different floral origin (one multiflower honey, one of orange blossom, and two of clover) obtained from supermarkets. All samples were analyzed in duplicate. For quality control, a procedure blank with ultra-pure water substituted for the honey matrix was run at the same time as the real samples to check for background contaminations; this blank underwent the whole pretreatment procedure. A solvent blank was run on the instrument in parallel with the prepared samples to monitor any potential carryover. Background contamination was infrequent and low, but it was subtracted if it was observed. No carryover was observed. Quantification of the target compounds was accomplished using an external matrix-matched standard calibration curve (2 ng/mL, 5 ng/mL and 10 ng/mL). Identification was performed via comparison of the retention time (within 2%) and the ratio (within 20%) of the quantifier to qualifier. The results showed that only E1 (< MQL) and P (0.2 ± 0.1 ng/g) were detected at trace levels in two cases out of the four samples, in orange blossom and multiflora honey, respectively. The detected E1 and P were possibly due to plant uptake from soils amended with biosolids or irrigated with reclaimed wastewater [3]. Plant uptake of estrogens and their translocation to above-ground plant parts has been reported [19, 20]. Estrone was previously found in apple seed (up to 130 ng/g) [21], in date palm seed oil (7.3 ng/g) [22] and in lettuce leaves (0.4–8.4 ng/g) [23]. The results highlighted a need for in-depth

research on the plant uptake of endocrine disrupting compounds, their transformation and translocation to aerial tissue, and the associated human health risks.

In conclusion, the results revealed the efficient removal of interfering constituents, which is important for preserving the system and maintaining the life of the analytical column. The robustness of the method was also demonstrated, testifying to the applicability of the method to a honey matrix. The method provided is potentially useful for the simultaneous determination of compounds of a similar category in other complex matrices.

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Figure legends

Figure 1. Scheme of the determination procedure for estrogens and progestogens in honey samples.

Figure 2. (A) Absolute recoveries and matrix effects (mean $\pm$ standard deviation, $n=2$) obtained for estrogens and progestogens with no further cleansing; (B) Absolute recoveries of target compounds under the four different solution combinations. Spiking level of compounds corresponds to 25 ng/g. The four solution mixtures were 1) 4 mL of MeOH-H₂O (10:90, v/v) + 4 mL of MeOH-H₂O-NH₄OH (14.8 M) (10:83.1:6.9, v/v/v); 2) 4 mL of MeOH-H₂O (20:80, v/v) + 4 mL of MeOH-H₂O-NH₄OH (14.8 M) (20:73.1:6.9, v/v/v); 3) 4 mL of MeOH-H₂O (30:70, v/v) + 4 mL of MeOH-H₂O-NH₄OH (14.8 M) (30:63.1:6.9, v/v/v); and 4) 4 mL of MeOH-H₂O (10:90, v/v) + 4 mL of MeOH-H₂O-NH₄OH (14.8 M) (10:83.1:6.9, v/v/v) + 3 mL of THF-H₂O (25:75, v/v). *Error bars* represent the standard deviation for duplicate determinations.

Figure 3. (A) Stacked and (B) separated multiple reaction monitoring (MRM) chromatograms for the quantification ions under optimized conditions for analytes in honey samples at a fortification level of 5 times the estimated method detection limit.

Tables

Table 1. Characteristics and optimized mass parameters for target compounds.

Analyte	Acronym	Molecular weight (g/mol)	Precursor ionization	MRM ions ^a (m/z)	Fragment energy (V)	Collision energy (eV)
17 β -estradiol-17-glucuronide CAS: 1806-98-0	E2-17G	448.51	[M—H] [−]	<i>447.2 > 271.3</i> 447.2 > 113	170	28 22
17 β -estradiol-3-glucuronide sodium salt CAS: 14982-12-8	E2-3G	470.49	[M—Na] [−]	<i>447.3 > 271.3</i> 447.3 > 113.1	170	42 17
Estrone-3-glucuronide CAS: 2479-90-5	E1-3G	446.49	[M—H] [−]	<i>445.2 > 269.1</i> 445.2 > 113.1	155	40 15
17 β -estradiol-17-sulfate sodium salt CAS: 3233-69-0	E2-17S	374.43	[M—Na] [−]	<i>351.2 > 97</i> 351.2 > 80.1	180	42 100
17 β -estradiol-3-sulfate sodium salt CAS: 4999-79-5	E2-3S	374.43	[M—Na] [−]	<i>351.2 > 271.2</i> 351.2 > 80.1	140	35 30
Estrone-3-sulfate Sodium Salt CAS: 438-67-5	E1-3S	372.41	[M—Na] [−]	<i>349.2 > 269.3</i> 349.2 > 145	140	35 55
Estriol CAS: 50-27-1	E3	288.39	[M—H] [−]	<i>287.1 > 171</i> 287.1 > 145	170	35 40
17 β -estradiol CAS: 50-28-2	E2	272.39	[M—H] [−]	<i>271.1 > 145</i> 271.1 > 183	125	42 42
17 α -estradiol CAS: 57-91-0	α -E2	272.39	[M—H] [−]	<i>271.1 > 145</i> 271.1 > 143	160	42 65
17 α -Ethinylestradiol CAS: 57-63-6	EE2	296.41	[M—H] [−]	<i>295.2 > 145</i> 295.2 > 143	140	42 65
Estrone CAS: 53-16-7	E1	270.37	[M—H] [−]	<i>269.2 > 145</i> 269.2 > 143	140	40 65
Medroxyprogesterone CAS: 520-85-4	MP	344.5	[M+H] ⁺	<i>345.3 > 123.1</i> 345.3 > 97.2	130	25 28
Progesterone CAS: 57-83-0	P	314.47	[M+H] ⁺	<i>315.2 > 109.1</i> 315.2 > 97.1	135	25 25

^a In italic the multiple reaction monitoring (MRM) transition for quantification.

Table 2. Analytical parameters for HPLC/MS/MS.

HPLC gradient			
	Time (min)	A %	B %
	0	88	12
	2	88	12
	10	50	50
	15	10	90
	20	10	90
	20.5	88	12
	28	88	12
MS/MS parameters			
Ion source			
temperature (°C)	325		
Dry gas flow			
(L/min)	12		
Nebulizer (psi)	50		
Capillary voltage			
(V)	4000 (+); 3500 (-)		

Table 3. Results of validation parameters including absolute recovery, matrix effect, instrument detection limit (IDL), method detection limit (MDL) and method quantification limit (MQL)($n=3$).

Analyte	Absolute recovery %				Matrix %		IDL (ng/mL)	5×MDL Honey equivalent ^a	MDL (ng/g)	MQL (ng/g)
	5 ng/g	RSD %	25 ng/g	RSD %	100 ng/mL	RSD %				
E2-17G	89.6	6.3	88.0	3.8	85.1	6.0	0.2	0.25	0.08	0.24
E2-3G	82.1	13.1	72.1	0.1	86.2	5.5	0.2	0.25	0.07	0.22
E1-3G	78.2	4.7	74.1	0.1	81.4	7.3	0.1	0.125	0.04	0.14
E2-17S	93.1	3.5	84.9	1.7	92.4	5.0	0.05	0.0625	0.02	0.07
E2-3S	96.2	1.0	88.7	1.0	97.6	4.4	0.05	0.0625	0.03	0.11
E1-3S	99.7	1.7	90.4	3.0	98.4	4.3	0.02	0.025	0.01	0.04
E3	96.3	3.1	83.6	0.1	100.5	8.1	0.3	0.375	0.07	0.23
E2	89.5	4.1	88.3	6.5	104.6	13.5	0.5	0.625	0.27	0.86
α-E2	90.7	2.1	89.0	6.4	98.9	6.5	0.5	0.625	0.19	0.62
EE2	84.6	4.0	87.2	0.3	100.6	6.0	1	1.25	0.33	1.05
E1	96.1	3.2	92.1	3.0	106.4	5.9	0.2	0.25	0.13	0.42
MP	80.3	11.9	81.6	6.7	91.6	3.7	0.05	0.0625	0.03	0.08
P	71.2	15.6	76.1	8.4	81.5	2.5	0.05	0.0625	0.06	0.20

^a 5×MDL Honey equivalent (ng/g) represents the value corresponding to 5 times the estimated MDL, in which estimated MDL = $\frac{IDL \times 1\text{mL} \times 25\text{mL}}{20\text{mL} \times 5\text{g}}$. Quantification of the on-column concentration (c) of target compounds in the seven fortified samples was carried out using an external matrix-matched standard calibration curve (2, 5, and 10 times estimated MDL). The final concentration C (ng/g) of each compound in honey was determined by the equation $C = \frac{c \times 1\text{mL} \times 25\text{mL}}{20\text{mL} \times 5\text{g}}$

Figure 1. Scheme of the determination procedure for estrogens and progestogens in honey samples.

Figure 2. (A) Absolute recoveries and matrix effects (mean $\pm$ standard deviation, $n=2$) obtained for estrogens and progestogens with no further cleansing; (B) Absolute recoveries of target compounds under the four different solution combinations. Spiking level of compounds corresponds to 25 ng/g. The four solution mixtures were 1) 4 mL of MeOH-H₂O (10:90, v/v) + 4 mL of MeOH-H₂O-NH₄OH (14.8 M) (10:83.1:6.9, v/v/v); 2) 4 mL of MeOH-H₂O (20:80, v/v) + 4 mL of MeOH-H₂O-NH₄OH (14.8 M) (20:73.1:6.9, v/v/v); 3) 4 mL of MeOH-H₂O (30:70, v/v) + 4 mL of MeOH-H₂O-NH₄OH (14.8 M) (30:63.1:6.9, v/v/v); and 4) 4 mL of MeOH-H₂O (10:90, v/v) + 4 mL of MeOH-H₂O-NH₄OH (14.8 M) (10:83.1:6.9, v/v/v) + 3 mL of THF-H₂O (25:75, v/v). Error bars represent the standard deviation for duplicate determinations.

Figure 3. (A) Stacked and (B) separated multiple reaction monitoring (MRM) chromatograms for the quantification ions under optimized conditions for analytes in honey samples at a fortification level of 5 times the estimated method detection limit.

Graphic abstract