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Determination of quinolones of veterinary use in bee products by ultra-high performance liquid chromatography–tandem mass spectrometry using a QuEChERS extraction procedure

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ABSTRACT

A reliable and rapid ultra high performance liquid chromatography tandem mass spectrometry (UHPLC–MS/MS) method has been developed for the determination of the eight quinolones of veterinary use regulated by European Union (marbofloxacin, ciprofloxacin, danofloxacin, enrofloxacin, sarafloxacin, difloxacin, flumequine and oxolinic acid). Chromatographic conditions were optimized in order to increase sample throughput and sensitivity. The antibiotics were detected by electrospray ionization in positive ion mode with multiple reaction monitoring (MRM) and MS/MS conditions were optimized in order to increase selectivity, selecting the corresponding product ions for quantification and identification. The separation was achieved in 3 min, using a Zorbax Eclipse Plus C18 column (50 mm × 2.1 mm, 1.8 μm), with a mobile phase of 0.02% aqueous formic acid solution and acetonitrile. A dispersive solid phase extraction methodology, often referred to as the "QuEChERS" (quick, easy, cheap, effective, rugged, and safe) method, was optimized for extraction of the quinolones from honey and also it was evaluated for other bee products such as royal jelly and propolis. The method was validated for each matrix in terms of linearity, trueness, precision, limits of detection (LODs) and quantification (LOQ). LODs ranged between 0.2 and 4.1 μg kg^{-1} with precision lower than 12% and satisfactory recoveries in most cases. The method was also applied for studying the occurrence of these antibiotics in several market samples.

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1. Introduction

Quinolones (Qns) constitute one of the main groups of antibiotics used both in human and veterinary medicine for therapeutic purposes. The wide application range and the extensive use of Qns in veterinary medicine represent a potential hazard for human health; they can produce residues in foodstuffs [1], causing allergic reactions or antibiotic resistance in humans. Veterinary use of these compounds had been regulated by European Union (EU) and maximum residue limits (MRLs) have been established for eight Qns: marbofloxacin: MARBO; ciprofloxacin: CIPRO; danofloxacin: DANO; enrofloxacin: ENRO; sarafloxacin: SARA; difloxacin: DIFLO; flumequine: FLUME; and oxolinic acid: OXO; (see structures in Fig. 1) in different food matrixes of animal origin [2]. Antibiotic drugs are not authorized for the treatment of honey bees in the EU; thus, there are no MRLs established. However, it is certainly the case that antimicrobial drugs are authorized for the treatment of honey bees in many third countries [3]. Also, the incurrence of Qns in bee products could be produced by a wrong or illegal use of these antibiotics to treat bees. Despite the fact that their use are not allowed by EU, Qns, ENRO and CIPRO had been found in honey from third countries [4,5]. Therefore, sensitive methods for their determination in bee products are necessary.

Different methods have been published for the determination of several families of antibiotics in honey and royal jelly (e.g. sulphonamides [6,7], tetracyclines [8–10] or macrolides [11–13]), mainly using liquid chromatography tandem mass spectrometry (HPLC–MS/MS). In the case of Qns, some methods based on HPLC–MS have been developed for the analysis in honey of 16 Qns by using turbulent flow chromatography automated online extraction [14], 4 Qns by combining HPLC–MS with a stir rod sorptive extraction with monolithic polymer as coating [15] and, mainly of human use, 19 Qns using SPE [16] or 7 Qns in royal jelly by ultrasonic assisted extraction and HPLC with fluorescence detection [17]. There are no applications of the analysis of Qns in propolis. Also, multiclass/multiresidue LC–MS/MS methods have been proposed for the analysis of different veterinary drug residues in honey, such as nitrofurans [18] or 42 veterinary drugs, including tetracyclines, macrolides, aminoglycosides, β-lactams, amphenicols and sulphonamides [19]. Other multiclass methods included also some veterinary Qns, such as CIPRO, DANO, DIFLO, ENRO and SARA [20]. Recently, ultra high performance liquid

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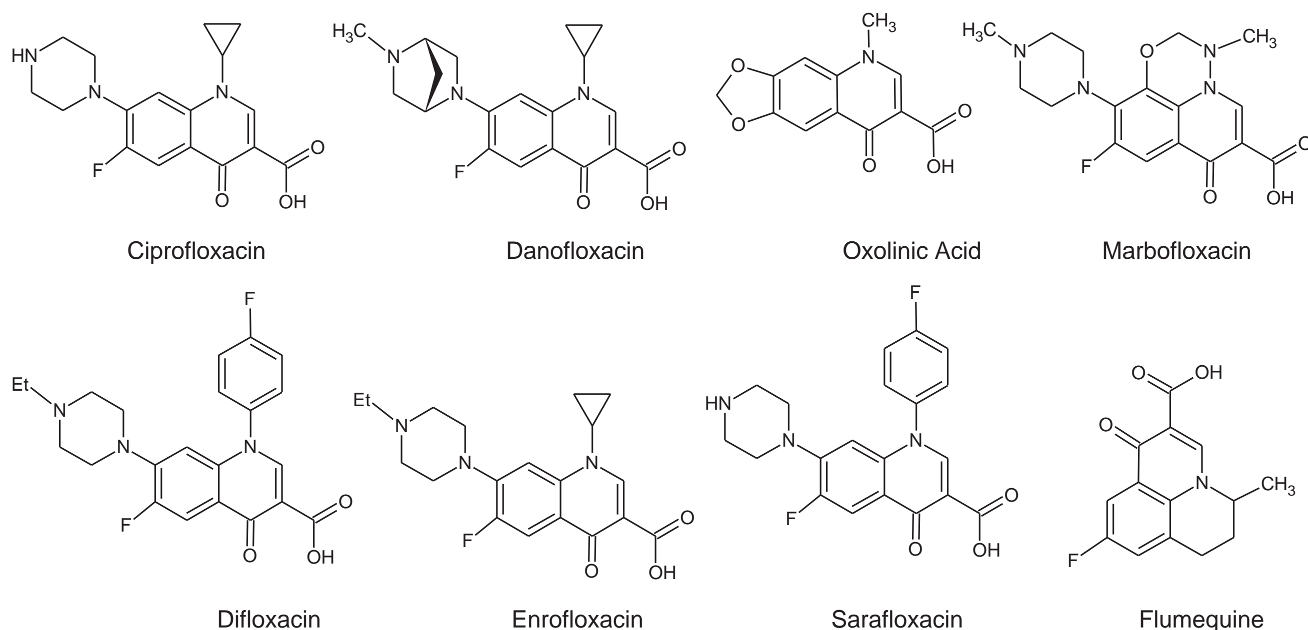

Fig. 1. Chemical structure of the different quinolones studied.

chromatography tandem mass spectrometry (UHPLC–MS/MS) methods for the determination of antibiotics in honey have been reported, including six macrolides [12] and a mixture of macrolides, tetracyclines, sulfonamides and Qns (MARBO, ENRO, DANO, DIFLO and SARA) [21]. However, as far as we know, no UHPLC–MS/MS methods specifically for the determination of the eight regulated Qns for veterinary use neither the analysis of these compounds in royal jelly and propolis have been proposed.

UHPLC technique shows several advantages compared to conventional HPLC, associated with the use of columns of less than 2.0 μm porous stationary phase able to withstand very high pressures, which allows an increased efficiency with a shortened analysis. UHPLC provides higher peak capacity, greater resolution, increased sensitivity and a higher speed of analysis and it is recommended especially to reduce analysis time and sample preparation [22,23], mainly in combination with MS/MS.

Concerning sample treatment, several methods have been proposed for the determination of Qns in different sample matrixes, being solid phase extraction (SPE) the most common methodology [24], reported also for the analysis of Qns in honey [16,21]. More recently, new methodologies showing higher selectivity and efficiency, being less time-consuming or environmentally friendly have been proposed for the determination of Qns in different matrixes, such as molecular imprinted polymers in milk [25–27] and kidney [27], dispersive liquid–liquid microextraction in water [28], turbulent flow chromatography automated online extraction in honey [14] or ultrasonic-assisted extraction combined with SPE for clean-up in royal jelly [17]. In the last few years, a fast and inexpensive extraction method named QuEChERS (quick, easy, cheap, effective, rugged and safe) has shown its usefulness in the analysis of residues in foods, presenting some advantages, such as its simplicity, minimum steps, and effectiveness for cleaning up complex samples. QuEChERS methodology involves two steps: the first one is an extraction step based on partitioning via salting-out extraction involving the equilibrium between an aqueous and an organic layer, and the second one is a dispersive SPE step that involves further clean-up using combinations of $MgSO_4$ and different sorbents, such as C18, primary–secondary amine (PSA) or graphitized carbon (GCB) to remove interfering substances [29,30]. This sample treatment has been extensively used for extraction of pesticides residues in vegetables, but it has been extended to other residues and matrixes [31]. QuEChERS has been used for the determination of veterinary residues (including Qns) in water [32], animal tissues [33,34], milk [25,35,36] and eggs [37]. However, as far as we know, it has not been used for the analysis of bee products.

The purpose of this work is the development of a simple, sensitive, selective and efficient UHPLC–MS/MS method for the simultaneous determination of the eight Qns of veterinary use regulated by EU (MARBO, CIPRO, DANO, ENRO, SARA, DIFLO, FLUME and OXO) using a simple and fast extraction procedure (QuEChERS methodology), optimized for honey and evaluated in other bee products such as royal jelly and propolis, that reduces sample handling and increase sample throughput.

2. Materials and methods

2.1. Chemicals and reagents

Solvents were LC–MS grade and Qns were analytical standard grade. Ultrapure water (Milli-Q Plus system, Millipore Bedford, MA, USA) was used to prepare buffer and standard solutions. Sodium hydroxide and sodium dihydrogen phosphate monohydrate were obtained from Panreac-Química (Madrid, Spain). Formic acid eluent additive for LC–MS, acetonitrile (ACN) and water were obtained from Sigma Aldrich (St. Louis, MO, USA). Formic acid (analysis grade) was supplied by Merck (Darmstadt, Germany). DANO, SARA and DIFLO were supplied by Riedel-de Haën (Seelze, Germany), FLUME by Sigma Aldrich and MARBO, CIPRO, ENRO and OXO by Fluka (Steinheim, Germany).

Individual stock standard solutions ($100\,mg\,L^{-1}$) of each Qn were prepared by dissolving the appropriate amount of each analyte in ACN/0.02% formic acid aqueous solution (50/50, v/v) and were stored in the dark at 4 °C. Formic acid (analysis grade) was added to each standard to increase solubility of analytes in this solvent mixture. Under such conditions, they were stable for at least 1 month. Working solutions (containing all Qns) were prepared daily from dilution of the individual stock solutions with Milli-Q water.

A 30 mM phosphate buffer solution pH 7.1 was prepared by dissolving 2.07 g of dihydrogen phosphate monohydrate in 500 mL of water and the pH was adjusted with 4 M NaOH solution. A 0.02%

formic acid aqueous solution was prepared by adding 20 μL of formic acid (eluent additive for LC–MS) to 100 mL of water (LC–MS grade). The 5% formic acid solution in ACN was obtaining by adding 25 mL of formic acid (analysis grade) to 500 mL of ACN.

SampliQ QuEChERS kits (Agilent Technologies Inc., Wilmington, DE, USA) consisted on 50 mL tube and buffered QuEChERS extraction kit (4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate, 0.5 g disodium citrate sesquihydrate) and dispersive tubes (15 mL, 150 mg C18 and 900 mg $MgSO_4$).

Filters of 13 mm with 0.2 μm nylon membrane (Bulk Acrodisc®, Pall Corp., MI, USA), were used for filtration of the final extracts before analysis.

2.2. Instrumentation

Separation was performed on an extreme pressure liquid chromatography (XLC) system (two pumps, oven, auto sampler, mixer and degasser unit) from Jasco (Easton, MD, USA). The mass-spectrometer measurements were performed on a triple quadrupole mass spectrometer API 3200 (Applied Biosystems, Darmstadt, Germany) with electrospray ionization (ESI). The instrument data were collected using the Analyst® Software version 1.5 with Schedule MRM TM Algorithm (AB SCIEX). Different chromatographic columns were tested to achieve the separation: Zorbax Eclipse Plus HHRD (50 mm × 2.1 mm, 1.8 μm), Kinetex (50 mm × 2.1 mm, 1.7 μm) and Varian (50 mm × 2.1 mm, 1.9 μm).

A pH-meter with a resolution of ±0.01 pH unit (Crison model pH 2000, Barcelona, Spain), a Universal 320R centrifuge (Hettich Zentrifugen, Tuttlingen, Germany), a Visiprep TM DL vacuum manifold for SPE (Supelco), a vortex-2 Genie (Scientific Industries, Bohemia, NY, USA) and a evaporator System (System EVA-EC, from VLM GmbH, Bielefeld, Germany) were also used.

2.3. Procedures

2.3.1. Extraction procedure

Honey, royal jelly and propolis samples were purchased in local markets from Granada (Spain) and stored at room temperature. The QuEChERS procedure was modified from that described by Agilent Technologies for the determination of Qns in bovine liver [38], and previously reported for the analysis of milk [25]. In all cases, preliminary analyses were performed to the selected matrixes in order to check that they were free from analytes. These samples were used as blank samples in the preparation of calibration standards and during the validation study.

Samples were placed into 50 mL centrifuge tubes and spiked by adding the proper volume of a solution containing each Qns at a concentration of 1 mg L^{-1} (FLUME and OXO) or 10 mg L^{-1} (for the rest of Qns) to portions of 1 g of honey, a 10 mL vial of royal jelly (with an equivalent quantity of 1 g of fresh royal jelly), or 1.5 mL of commercial propolis extract. In order to achieve a proper homogenization of samples, honey was warmed before spiking and vortexed, while royal jelly and propolis extract samples were directly spiked and vortexed without warming, since their density is much lower than this of honey. Then, 8 mL of 30 mM NaH_2PO_4 buffer pH 7.0 were added and the sample was dissolved in this media. Subsequently, 10 mL of 5% formic acid in ACN were added and the mixture was homogenized in vortex. Agilent SampliQ EN QuEChERS extraction kit was added and the tube was shaken vigorously for 1 min. The sample was centrifuged at 9000 rpm for 5 min and 4 mL of the upper ACN layer was transferred into the SampliQ QuEChERS dispersive tube, stirred in vortex for 1 min and centrifuged (9000 rpm for 2 min). An aliquot of 1 mL of supernatant was transferred to a vial, dried under a stream of nitrogen and the residue was redissolved with 1 mL of H_2O/ACN/formic acid (88/10/2), filtered and analyzed by UHPLC–MS/MS.

2.3.2. UHPLC–MS/MS analysis

UHPLC separations were performed in a C18 column (Zorbax Eclipse Plus HHRD 50 mm × 2.1 mm, 1.8 μm) using a mobile phase consisting of 0.02% aqueous formic acid solution (solvent A), and ACN (solvent B) at a flow rate of 0.4 mL min^{-1}. The gradient profile started at 15% of B until 1.5 min; then it went to 55% B in 0.1 min and kept until 3 min; then it went to 90% B in 0.1 min and kept until 4 min. Finally it was back to 15% B in 0.1 min. The run time for each injection was 5.5 min, the temperature of the column was 35 °C and the injection volume was 5 μL (full loop). Under optimum conditions, all analytes were eluted in 3 min. The mass-spectrometer was working with an electro spray ion source (ESI) in positive mode under the multiple reaction monitoring (MRM) conditions shown in Table 1. The ionization source parameters were: source temperature 500 °C; curtain gas (nitrogen) 35 psi, ion spray voltage 5500 V; and GAS 1 and GAS 2 (both of them nitrogen) were set to 60 psi.

3. Results and discussion

3.1. Optimization of MS/MS detection and chromatographic separation

For each individual Qns, the mass spectrometer was optimized to provide the best responses for quantification. In order to get high sensitivity, each analyte was individually infused as a standard solution of 1 mg L^{-1} mixture of 0.1% aqueous formic acid solution/ACN (50/50, v/v), directly into the mass spectrometer. All compounds were tested using ESI positive/negative mode. As it was expected from previous data [32,35,37,39] ESI operating in positive mode showed the best results in term of sensitivity. During the infusion, the parameters declusterin potential (DP), entrance potential (EP), collision cell entrance potential (CEP), collision cell exit potential (CXP) and collision energy (CE) were optimized for each compound in order to obtain the maximum sensitivity (see results in Table 1). Each compound was characterized by its retention time and by two precursor-product ion transitions. The most intense product ion was used for quantification (see Fig. 2), whereas the second one was used to complete the identification. The dwell time established for each transition was 0.1 s. Under the experimental conditions, protonated molecules $[M+H]^+$, were observed for all the compounds and no sodium adducts were observed. Ion source parameters, source temperature, curtain gas, ion spray voltage, and GAS 1 and GAS 2, were optimized once chromatographic conditions were established, obtaining the optimum values indicated in Section 2.3.2.

In relation to the chromatographic conditions, aqueous standard solutions of Qns were used during the optimization of chromatographic separation. The mobile phase consisted of 0.02% aqueous formic acid solution (solvent A) and ACN (solvent B). The gradient was studied in order to get the best separation, peak shape and sensitivity. According to previous papers [25,39] aqueous and ACN phases were selected as solvents for the separation of Qns by HPLC. The gradient was studied to get the best separation in the shorter time and finally was found that a rising gradient until 55% of ACN was necessary to get a good separation and elute the most retained analyte (FLUME). The ACN percentage was increased until 90% after the elution of FLUME to elute other possible components included in the final sample extract. The use of acid in the mobile phase is required to improve the ionization step in ESI (+). Therefore, different acids (formic and acetic acid) in solvent A were tested. Formic acid provided better results than acetic acid and it was selected for the rest of the experimental work, evaluating different percentages

Table 1
Monitored ions of the target analytes and MS/MS parameters.

	Precursor ion (m/z)	DP[a]	EP[a]	CEP[a]	Product ions	CE[a]	CXP[a]
SARA	386.0	45	5	19.5	299.1 (Q)[b]	35	2.2
					368.1 (I)[b]	29	6
DANO	358.0	48	5	18.8	340.0 (Q)	30	5.5
					255.2 (I)	31	4.2
CIPRO	332.0	43	5	18.2	231.2 (Q)	29	6
					245.0 (I)	32	2
ENRO	360.0	45	5	18.9	316.0 (Q)	26	5.5
					245.0 (I)	36	2
DIFLO	400.0	50	5	19.9	356.3 (Q)	24	6
					299.3 (I)	43	4.6
FLUME	262.0	37	5.5	16.3	244.2 (Q)	23	3
					202.3 (I)	43	3
MARBO	363.0	38	4.8	18.9	72.0 (Q)	42	2.5
					320.2 (I)	19	6
OXO	262.0	36	4.5	18.0	244.2 (Q)	19	6
					216.0 (I)	43	4

[a] Declusterin potential (DP); entrance potential (EP); collision cell entrance potential (CEP); collision cell exit potential (CXP); and collision energy (CE).
[b] Product ions: (Q) transition used for quantification; (I) transition employed to complete the identification.

(0–0.1%). Aqueous mobile phase with 0.02% formic acid gave the higher signals and peak shape. The addition of acid in both phases (solvents A and B) was studied, but worst signal were observed when acidic percentage was increased.

Once selected the mobile phase and gradient, three different reversed-phase (C18) chromatographic columns were studied: Zorbax Eclipse Plus HHRD (50 mm × 2.1 mm, 1.8 μm), Kinetex (50 mm × 2.1 mm, 1.7 μm) and Varian (50 mm × 2.1 mm, 1.9 μm). The first one gave slightly better separation of SARA and DIFLO, and much better peak shapes, for which it was selected for the rest of the experimental work. The flow rate was studied from 0.3 mL min^{-1} to 0.5 mL min^{-1} and finally 0.4 mL min^{-1} was selected as a compromise between signal, peak shape and run time. The temperature of the column was studied between 25 °C and 45 °C and 35 °C was selected for giving the best results, as it provided the higher peak height and area with the best resolution and good analysis time. The injection volume was 5 μL for all the experiments (full loop). Using these conditions, only two peaks (SARA and DIFLO), were not completely resolved but the use of MS/MS enabled their accurate analysis.

3.2. Optimization of sample preparation

For the extraction procedure, QuEChERS methodology was selected in order to achieve a quick and effective extraction method. This methodology has been previously used for the extraction of Qns of veterinary use from liver [38] and milk [25] and some of these Qns with other antibiotics in eggs [32], but, to the best of our knowledge, it has not been tested for bee products. Thus, our purpose was to investigate if this methodology is suitable for extracting Qns from honey, royal jelly and propolis.

Honey was selected as sample for the optimization of the QuEChERS procedure. Initially, 2 g of honey were spiked to get a concentration of 10 μg L^{-1} for FLUME and OXO and 100 μg L^{-1} for the other Qns and treated using the procedure previously described [25], but the final extract was too much dirty and almost all the peaks were split in two, so we used only 1 g of honey. In this case the final extract obtained was clean enough to continue working, splitting peaks disappeared and the extraction was achieved satisfactorily. So, 1 g of honey was placed into 50 mL centrifuge tubes, then 8 mL of 30 mM NaH_2PO_4 buffer pH 7.0 was added, shaking by hand. Subsequently, 10 mL of 5% formic acid in ACN was added to the tube, shaking by hand for 30 s and Agilent SampliQ EN QuEChERS extraction kit was added and the tube was shaken vigorously for 1 min and centrifuged, then 4 mL of the upper ACN layer was transferred to another tube containing the dispersive SPE and stirred in vortex for 1 min. In order to get the cleanest extract, different possibilities in the second step procedure (dispersive tube composition) were tested: C18 + $MgSO_4$ (150 mg + 900 mg); C18 + PSA + $MgSO_4$ (150 mg + 150 mg + 900 mg); C18 + PSA + $MgSO_4$ (400 mg + 400 mg + 1200 mg); C18 + PSA + GCB + $MgSO_4$ (400 mg + 400 mg + 400 mg + 1200 mg) (PSA = primary–secondary amine, GCB = graphitized carbon), all of them supplied by Agilent Technologies. When GCB was included in the dispersive tube, no signals for the analytes were obtained; thus, this dispersive phase composition was discarded. The other three options showed different results; when PSA was included the recoveries percentages vary between 41.9 and 88.5% but dispersive tube with only C18 gave the highest recoveries for all the compounds, comprised between 70.1 and 93.7%, so it was selected.

After clean-up, 1 mL aliquot of the obtained extract from the dispersive tube was dried under a N_2 stream. The recomposition media was studied and different possibilities were tested: water; 1% formic acid aqueous solution; 2% formic acid aqueous solution; water/ACN (90/10, v/v); water/ACN/formic acid (89/10/1); water/ACN/formic acid (88/10/2); and water/ACN/perchloric acid (88/10/2). The highest recoveries were obtained when 1% and 2% formic acid aqueous solution were used, but slightly higher signal were obtained with the second one, so this was selected for the rest of the work. Finally, the extracts were filtered with a 0.2 μm filter before injection.

3.3. Performance characteristics

Each compound was analyzed in MRM mode, selecting the two highest precursor ion/product ion transitions, which, with retention times, were used to ensure adequate analyte identification. Performance characteristics of the method (linearity, trueness, intra and interday precision, and limits of detection (LOD) and quantification (LOQ)) were established with fortified samples, previously analyzed to ensure the absence of Qns. Matrix–matched calibration curves were established for the three kinds of samples selected, fortifying samples at five concentration levels (from 5 to 30 μg kg^{-1} for OXO and FLUME and from 50 to 300 μg kg^{-1} for the other Qns). Samples of 1 g of multifloral honey, 10 mL of royal jelly (containing 1000 mg of royal jelly, water, fructose, and preservatives) and 1.5 mL of commercial propolis extract (containing 68.5% of propolis, water and alcohol) were used to establish the calibration curves. LODs and LOQs were provided by the software Analyst, as 3xS/N and 10xS/N, respectively. Determination coefficients, and LODs and LOQs are shown in Table 2, showing that the method is enough sensitive for the determination of

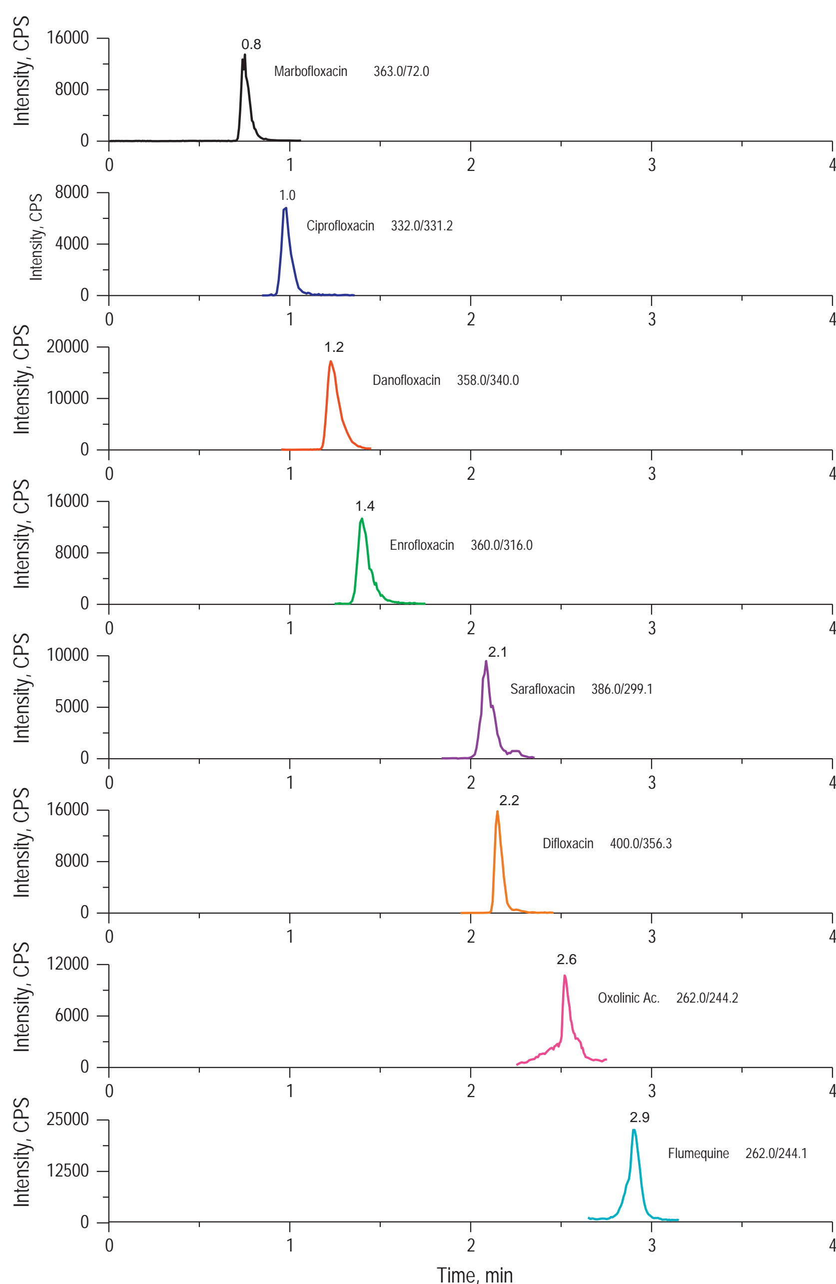

Fig. 2. UHPLC–MS/MS extracted ion chromatograms of a spiked honey sample at 20 μg kg^{-1} for FLUME and OXO and 200 μg kg^{-1} for the other quinolones.

very low levels of these compounds in the selected matrixes. The LOQ obtained for the analysis of the eight Qns in honey from the proposed method (0.8–5.5 μg kg^{-1}) were in some cases lower or the same order than other obtained from other methods such as those based on LC–MS/MS (5 μg kg^{-1}) [14] or UHPLC–MS/MS [21] for MARBO, ENRO, DANO, DIFLO and SARA (0.3–3.3 μg kg^{-1}) using SPE. For royal jelly, the obtained LOQs (0.6–8.3) were lower than the obtained from a previous HPLC-FLD method (2–40 μg kg^{-1}) [17].

Table 2
Linearity, detection and quantification limits of the QuEChERS-UHPLC–MS/MS proposed method for the different samples.

	Honey			Royal jelly			Propolis		
	R^2	LOD (μg kg^{-1})	LOQ (μg kg^{-1})	R^2	LOD (μg kg^{-1})	LOQ (μg kg^{-1})	R^2	LOD (μg kg^{-1})	LOQ (μg kg^{-1})
SARA	0.991	1.6	5.5	0.992	0.9	2.9	0.991	2.1	7.0
DANO	0.994	1.4	4.6	0.995	0.9	2.9	0.992	4.1	13.0
CIPRO	0.990	1.7	5.5	0.994	2.5	8.3	0.990	4.0	13.4
ENRO	0.995	1.1	3.7	0.995	1.6	5.5	0.992	3.5	11.6
DIFLO	0.994	1.2	3.9	0.993	0.8	2.7	0.991	1.6	5.4
FLUME	0.984	0.2	0.8	0.990	0.2	0.6	0.993	0.7	2.4
MARBO	0.994	0.7	2.2	0.996	0.5	1.5	0.990	1.0	3.4
OXO	0.990	0.5	1.6	0.993	0.3	0.8	0.992	1.2	3.9

Table 3
Intraday ($n = 9$) and interday precision ($n = 15$) expressed as RSD (%).

	Intraday/interday (low spiking level)[a]			Intraday/interday (medium spiking level)[b]			Intraday/interday (high spiking level)[c]		
	Honey	Royal jelly	Propolis	Honey	Royal jelly	Propolis	Honey	Royal jelly	Propolis
SARA	7.3/3.2	5.6/6.0	4.0/13.8	6.5/9.2	4.4/2.6	5.6/12.9	5.6/7.3	2.9/3.2	6.6/8.2
DANO	5.0/8.6	3.3/7.2	7.9/9.7	4.9/10.7	3.7/8.8	7.3/12.6	4.1/10.7	3.5/9.6	8.4/7.1
CIPRO	7.7/9.0	5.2/7.3	5.1/13.2	5.4/6.3	5.5/6.0	9.2/12.3	6.2/6.7	3.7/2.9	7.7/10.0
ENRO	4.9/11.9	8.2/8.3	4.6/11.1	3.7/10.5	3.0/7.9	6.1/7.4	5.4/11.3	2.2/7.6	8.2/11.3
DIFLO	3.7/6.0	3.6/11.8	5.1/11.2	1.7/5.1	2.8/4.4	4.7/11.5	4.4/5.9	1.8/5.6	5.4/8.6
FLUME	17.1/6.3	4.9/6.3	5.2/12.9	6.5/13.9	2.0/5.9	4.9/5.0	1.5/12.9	1.7/7.1	4.2/5.6
MARBO	5.4/7.4	5.7/2.8	4.4/8.1	4.7/6.4	3.9/4.3	8.0/9.1	6.2/7.6	2.9/4.2	8.0/14.7
OXO	3.4/6.0	3.2/3.7	7.7/8.9	8.5/8.5	1.7/6.5	5.2/6.3	2.9/8.1	7.2/9.2	2.6/7.7

[a] Low spiking level 10 $\mu g\,kg^{-1}$ for FLUME and OXO and 100 $\mu g\,kg^{-1}$ for the others quinolones.
[b] Medium spiking level 15 $\mu g\,kg^{-1}$ for FLUME and OXO and 150 $\mu g\,kg^{-1}$ for the others quinolones.
[c] High spiking level 20 $\mu g\,kg^{-1}$ for FLUME and OXO and 200 $\mu g\,kg^{-1}$ for the others quinolones.

Table 4
Recovery study and RSD ($n = 9$) for the different studied samples.

	%*R* (RSD%) at low spiking level[a]			%*R* (RSD%) at medium spiking level[b]			%*R* (RSD%) at high spiking level[c]		
	Honey	Royal jelly	Propolis	Honey	Royal jelly	Propolis	Honey	Royal jelly	Propolis
SARA	74.0 (7.3)	74.5 (5.5)	59.1 (4.0)	85.5 (6.5)	80.8 (4.4)	48.2 (5.6)	79.3 (5.6)	90.7 (2.9)	56.0 (6.6)
DANO	72.2 (5.0)	74.8 (3.3)	55.8 (7.9)	79.1 (4.9)	73.7 (3.7)	40.2 (7.3)	72.9 (4.1)	80.4 (3.5)	51.2 (8.4)
CIPRO	61.3 (7.7)	56.7 (5.2)	61.7 (5.1)	77.1 (5.4)	61.7 (5.5)	45.0 (9.2)	61.2 (6.2)	63.2 (3.7)	63.2 (5.9)
ENRO	72.7 (4.9)	77.9 (8.2)	57.7 (4.6)	86.8 (3.7)	86.2 (3.0)	42.9 (6.1)	73.2 (5.4)	88.4 (2.2)	55.0 (8.2)
DIFLO	76.8 (3.7)	81.0 (3.6)	52.7 (5.1)	88.8 (1.7)	84.7 (2.8)	48.1 (4.7)	78.1 (4.4)	94.6 (1.8)	51.9 (5.4)
FLUME	99.8 (3.1)	90.2 (4.9)	39.7 (5.2)	92.3 (6.5)	92.9 (2.0)	55.2 (4.9)	93.9 (1.5)	95.2 (1.7)	55.5 (4.2)
MARBO	70.0 (5.3)	68.9 (5.7)	64.0 (4.4)	78.3 (4.7)	63.7 (3.9)	60.3 (8.0)	70.4 (6.2)	68.3 (2.9)	69.2 (8.0)
OXO	76.0 (3.4)	82.0 (3.2)	65.2 (7.7)	82.5 (8.5)	85.5 (1.7)	49.5 (5.2)	75.6 (2.9)	96.5 (7.2)	62.8 (2.6)

[a] Low spiking level: 10 $\mu g\,kg^{-1}$ for FLUME and OXO and 100 $\mu g\,kg^{-1}$ for the other quinolones.
[b] Medium spiking level: 15 $\mu g\,kg^{-1}$ for FLUME and OXO and 150 $\mu g\,kg^{-1}$ for the other quinolones.
[c] High spiking level: 20 $\mu g\,kg^{-1}$ for FLUME and OXO and 200 $\mu g\,kg^{-1}$ for the other quinolones.

Intraday precision was performed fortifying honey, royal jelly and propolis samples at three concentration levels (10, 15 and 20 $\mu g\,kg^{-1}$ for OXO and FLUME and 100, 150, and 200 $\mu g\,kg^{-1}$ for the other Qns), using three replicates for each concentration level, analyzed by triplicate the same day. Interday precision was evaluated in a similar way, but the samples were analyzed by triplicate during five consecutive days (Table 3). Trueness was evaluated by analyzing fortified blank samples by triplicate with concentrations similar to those used in the precision study (Table 4). As can be seen, satisfactory values of recoveries and precision were obtained for honey at the three studied levels, which was the target matrix selected in the optimization of the proposed method. Also, using the same procedure, good results in term of accuracy and sensitivity can be obtained for the analysis of royal jelly. However, for propolis samples lower recoveries and LODs have been obtained. This fact could be due to the high complexity of propolis samples. Thus, the extracts were dirtier than for the other samples, because most of the components of propolis showed a high solubility in the organic solvent (ACN) used for extraction. Moreover, the clean-up step was not as effective as for the other samples and other strategies could be checked for further work in relation to this matrix.

3.4. Analysis of real samples

Different samples of commercial honey, royal jelly and propolis were analyzed in order to demonstrate the applicability of the method.

Six different samples of honey were checked, three different multifloral honeys, all of them from different regions of Spain – Valencia, Badajoz and Las Alpujarras (Granada), and three monofloral honeys: rosemary and orange blossom honey from Valencia, and heather honey from Burgos. None of the samples gave a positive result for Qns by using the proposed method.

Four different samples of royal jelly were analyzed. The first and second one containing 1 g of fresh royal jelly, water, fructose, and preservatives; the third one contained 0.6 g of royal jelly, water, fructose, citric acid and preservatives, while the fourth one was composed by royal jelly, alcoholic extract of propolis (0.6 g), water, fructose, vitamin C, citric acid and preservatives. No Qn residues were found in the samples.

Also, three different propolis samples were analyzed to find Qns, the first one containing propolis (68.5%), water and alcohol; the second one dewaxed propolis (20%), water, fructose, sodium lactate, aroma, lactic acid, ascorbic acid, soybean lecitine, xanthan gum, neoesperidine, and potassium sorbate. Once again, Qns were not detected below the LOD in the samples.

4. Conclusions

A new method, based on a QuEChERS extraction procedure and UHPLC–MS/MS, has been developed for the simultaneous determination of the eight Qns of veterinary use regulated by EU. The extraction procedure is quick, effective and cheap showing high throughput. The separation of the compounds is achieved in only 3 min and the full analysis (including sample treatment) takes no more than 40 min for a batch of six samples. The results show the suitability of this procedure for the monitoring of the eight Qn residues in bee products in a single run and for providing data of the occurrence of these compounds in a wide range of bee products.

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