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Analysis of danofloxacin, difloxacin, ciprofloxacin and sarafloxacin in honey using micellar liquid chromatography and validation according to the 2002/657/EC decision

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A reliable and sensitive method based on micellar liquid chromatography was optimized for the analysis of the fluoroquinolones danofloxacin, difloxacin, ciprofloxacin and sarafloxacin in honey. The sample was 1 : 1 diluted in a 0.05 M sodium dodecyl sulfate solution buffered at pH = 3, thus avoiding an extraction step and the use of toxic chemicals. The fluoroquinolones were resolved in less than 25 min using a C18 column, without interference from the matrix. The mobile phase was a solution of 0.05 M sodium dodecyl sulfate, 1% 1-butanol and 0.5% triethylamine buffered at pH = 3, running under isocratic mode at 1 mL min^{-1}. The excitation and emission wavelengths were 280 and 455 nm, respectively. The method was validated in accordance with the European Union Decision 2002/657/EC in terms of selectivity, sensitivity (limits of detection and quantification, 4 and 10 μg kg^{-1}, respectively), calibration range (10–200 μg kg^{-1}), linearity (r^2 > 0.9990), decision limit (4 μg kg^{-1}), detection capability (4.7–6.2 μg kg^{-1}), intra- and interday accuracy and precision (81.0–103.4% and <12.3%, respectively), and robustness (<8.5%). The method was applied to commercial honey samples purchased from a local supermarket.

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1. Introduction

Fluoroquinolones (FQs) are among the most important antibacterial agents and belong to the current arsenal of antibiotics developed against infections.[1] Therefore, these drugs are extensively used in the treatment of human and veterinary bacterial infections due to their effectiveness and broad spectrum of activity. In veterinary medicine, they are specifically used as prophylactic agents to prevent respiratory diseases and bacterial infections in cattle, swine, broiler, turkey, and aquaculture fish.[2] They have been used as anti-infectious agents to treat foulbrood and nosemosis in bees.[3]

The intensive use of FQ in live animals implies a potential danger for the population. It can stimulate the growth of mutated pathogens resistant to these quinolones, which can lately jump to humans. Besides, drug residues may persist in the edible products of animals, so that there is concern about the possibility of a continuous and long-term exposure of consumers to high levels of these compounds. As a result, they may unknowingly develop resistance to quinolones, and would be unaffected by future antibiotic treatments.[2] In the European Union (EU), the presence of these drugs in foodstuffs has been regulated through the Commission Regulation (EU) no. 37/2010, and maximum residue limits (MRLs) have been established for different food matrices of animal origin.[4] In honey, however, no MRLs have been defined for the fluoroquinolones danofloxacin ($\log P_{o/w}$ = 0.14; pK_a = 6.22/9.43),[5] difloxacin ($\log P_{o/w}$ = 0.77; pK_a = 5.66/7.24),[6,7] ciprofloxacin ($\log P_{o/w}$ = 0.77; pK_a = 6.09/8.09)[7,8] and sarafloxacin ($\log P_{o/w}$ = 0.86; pK_a = 4.12/6.78),[7] the structures of which are shown in Fig. 1. The use of FQs is strictly forbidden, and, consequently, the presence of such residues and their metabolites in bee products must be considered as resulting from illegal beekeeping practices.[4] Thus, a honey sample is declared non-compliant if these compounds are detected, and then the corresponding batch would not be allowed to be distributed within the EU.

Honey is consumed worldwide, especially during breakfast, due to its nutritional and health benefits. It is also largely used in the food industry (bakery and cereal-based goods, baby foods, chocolate, *etc.*). Indeed, on a yearly basis, about 1.2 million tons of honey is produced worldwide and 400 000 tons is traded internationally.[9] In the last few years, the finding of antibiotics in this commodity has had a serious impact on both raw material suppliers and food manufacturers, resulting in rejection and destruction of honey batches and affecting the reputation of the producers. Additionally, this has endangered the image of bee-derived products as healthy and clean. Recently, several FQs have been found in honey originating from China, demonstrating that such broad spectrum antibiotics are used by some beekeepers.[10] Therefore, the

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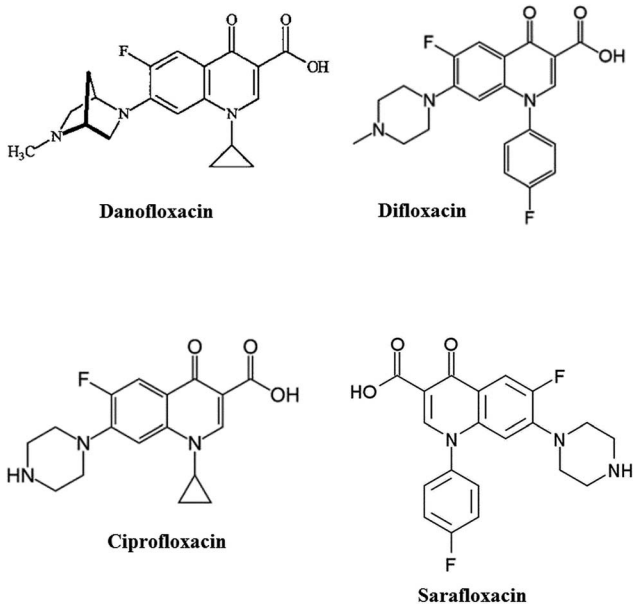

Fig. 1 Structures of the studied fluoroquinolones.

development of screening methods to check the absence of danofloxacin (DAN), difloxacin (DIF), ciprofloxacin (CIP) and sarafloxacin (SAR) in honey before they are sent to markets is of the utmost importance to ensure that the batch complies with the EU regulation and to detect a possible threat to the consumers.

Many methods based on separation techniques, such as capillary electrophoresis,[11] thin layer chromatography, gas chromatography, and liquid chromatography,[12] have been developed for the screening of FQs in edible animal tissues. The latest generation of high performance liquid chromatography/tandem mass spectrometry (HPLC-MS/MS) equipment allows the multiresidue determination of these antibiotics in milk,[13] tilapia,[14] and honey.[15–20] However, this equipment is expensive and not all laboratories can afford it. Besides, due to the current situation of economic crisis, the trend points towards the development of inexpensive analytical procedures. Studies have been published on different liquid chromatographic methods based on fluorescence and UV-Visible absorbance detection of FQ in milk,[13] chicken muscle and egg yolk,[21] tissues of food-producing animals,[22] eggs,[23] feeds,[24] livestock and marine products[25] and royal jelly.[26] However, only a few studies have been published about the analysis of quinolones in honey using LC-FLD.[27,28] Furthermore, most of the extraction procedures applied to analyze honey require clean-up procedures that are tedious and time-consuming, because of the viscosity and the presence of a large amount of sugars. The most usual methods are liquid/liquid[16,18,19] or solid/liquid[16,20,27,28] extraction, or precipitation of matrix compounds.[17,18,20] In some cases, several consecutive clean-up steps[16,18,20] or previous screening by microbiological methods[28] are required. The enlargement of the experimental procedure increases the probability of the loss of analytes, thus reducing the quality of the experimental results. Several authors have proposed the analysis of FQs in honey by automated on-line sample purification, using turbulent flow chromatography coupled with LC-MS.[15]

Micellar liquid chromatography (MLC), using mobile phases containing an aqueous solution of sodium dodecyl sulfate as the surfactant over the critical micellar concentration (CMC) and, eventually, a low amount of a short-chain alcohol, has been applied for the analysis of organic compounds in food.[29] Micellar solutions solubilize both polar and hydrophobic compounds. Thus, samples can be directly injected without the risk of precipitation into the column, thus shortening the experimental protocol. As a result, the analysis time, cost and environmental impact are lower than hydroorganic HPLC.[30] Besides, the chromatographic behavior of the analytes in micellar mobile phases is highly stable and reproducible, and can be related to the concentration of SDS and alcohol using several equations. Therefore, the composition of the mobile phase can be easily optimized by testing a few mobile phases.[31] MLC has been successfully used to analyze the quinolones in fish from fisheries,[32] in eggs and milk.[33]

The aim of this work was to develop an MLC procedure for the screening of DAN, DIF, CIP and SAR in honey. The analytical procedure must be reliable, simple, inexpensive and non-polluting, and useful for the routine analysis of honey samples. The method must be validated following the requirements of the EU Commission Decision 2002/657/EC regulation in terms of selectivity, linearity, decision limit, detection capability, accuracy, precision, and robustness.[34] The sensitivity was evaluated through the ICH Harmonized Tripartite Guideline.[35] The procedure developed would be applied to the analysis of the studied antibiotics in commercial honey samples.

2. Experimental

2.1 Standards and chemicals

The solid standards of danofloxacin (purity >99.9%), difloxacin (>99.8%) and sarafloxacin (>97.2%) were supplied by Fluka (Buchs, SG, Switzerland), whereas ciprofloxacin (>99.9%) was purchased from Sigma (St. Louis, MO, USA).

Sodium dodecyl sulfate (>99.9%) and sodium hydroxide (>99.0%) were purchased from Merck (Darmstadt, Germany). Hydrochloric acid (reagent grade, 37%), triethylamine (>99.5%) and ethanol (HPLC grade) were bought from J. T. Baker (Deventer, the Netherlands). Sodium dihydrogen phosphate 1-hydrate (99%), 1-propanol, 1-butanol and 1-pentanol (HPLC grade) were obtained from Scharlab (Barcelona, Spain). Ultrapure water was in-lab generated from distilled water using an ultrapure water device (Millipore S.A.S., Molsheim, France).

2.2 Preparation of solutions and mobile phases

The mobile phases were prepared by weighing the adequate amount of SDS and sodium dihydrogen phosphate, and dissolving them in ultrapure water by shaking. The appropriate volume of triethylamine (TEA) was added to obtain a final

concentration of 0.5% (v/v) and the pH was fixed to 3 by adding drops of HCl solution. Furthermore, the organic solvent was added to reach the desired proportion (%, v/v), and then ultrapure water was added up to the mark of the volumetric flask. Finally, the solution was ultrasonicated and filtered through a 0.45 μm nylon membrane with the aid of a vacuum pump.

Individual stock solutions of the studied FQs were prepared as follows: the adequate quantity of the solid standard was weighed and dissolved in few mLs of ethanol, and then filled up with a micellar solution of 0.05 M SDS at pH 3 (fixed with a phosphate buffer), to reach a final concentration of nearly 100 mg L^{-1}. The solution was ultrasonicated to assure complete solubilization. These solutions were stored at 4 °C in darkness for 1 month. Working solutions were prepared by successive dilutions with the solution of 0.05 M SDS at pH 3. Working solutions containing the four fluoroquinolones were prepared by mixing the stock solutions. These solutions were kept at 4 °C in darkness for 1 week.

2.3 Chromatographic instrumentation and conditions

The chromatographic system used for this study was a Series HP1100 supplied by Agilent Technologies (Palo Alto, CA, USA), equipped with an isocratic pump, an autosampler tray and a fluorescence detector. The stationary phase was in a reverse-phase C18 Kromasil column (150 × 4.6 mm; 5 μm particle size; 10 nm pore size) supplied by Scharlab. The mobile phase was an aqueous solution of 0.05 M SDS–1% (v/v) 1-butanol–0.5% (v/v) TEA at pH 3 running under isocratic mode at room temperature at 1 mL min^{-1}. The injection volume was 20 μL. The excitation and emission wavelengths were set at 280 and 455 nm, respectively. The Agilent Chemstation (Rev. B.03.01) software was used to control the HPLC instrumentation and to acquire chromatographic data. The obtained chromatograms were processed by the Michrom software[36] to measure the main chromatographic parameters: peak area (A), dead time (t_0, min), retention time (t_R, min), retention factor (k), efficiency (a number of theoretical plates, N) and asymmetry (B/A).[37] The special care required for the chromatographic system when dealing with micellar mobile phases is described in ref. 29.

2.4 Sample collection and processing

Twenty commercial honey samples were purchased from local supermarkets and kept in a fridge. The trademark, supplier and variety are indicated below:

- "Granja San Francisco" (Nutrexpa, Barcelona, Spain): multiflower, eucalyptus-lime, forest, and orange blossom.
- "Consum" (Reina Apícola Levantina, Alzira, Spain): multiflower, rosemary, orange blossom, and eucalyptus.
- "El Brezal" (Mielso, Almazora, Spain): orange blossom, rosemary, multiflower, thyme, black eucalyptus, white eucalyptus, mountain (several mountain flowers), forest (honeydew), acacia, and Yucatan (Nahonal and Dzidzilche flowers).
- "El Quexigal" (El Quexigal, Cebreros, Spain): heather and lavender.

All the honey samples were manufactured in Spain except acacia honey and Yucatan honey, which were elaborated in Central Europe and Mexico, respectively.

The samples were taken out 30 min before analysis to warm up to room temperature. Then, 5 g were introduced into a 10 mL-volumetric flask, and filled up with a micellar solution of 0.05 M SDS at pH 3. The diluted solution was filtered through a 0.45 μm nylon membrane, placed into the vials and injected into the chromatographic system. The remaining solutions were not stored.

For spiked samples, the appropriate amount was injected into the honey, immediately before mixing with the micellar solution.

3. Results and discussion

3.1 Optimization of the chromatographic conditions

The main chromatographic conditions (injection volume, 20 μL; flow-rate, 1 mL min^{-1}; surfactant, SDS; pH, 3; buffer, 0.01 M phosphate and addition of 0.5% of TEA) were taken from previously published papers about the analysis of difloxacin and sarafloxacin in fish[32] and danofloxacin and difloxacin in eggs and milk.[33] These papers also recommend the use of hybrid mobile phases with a short-chained alcohol to obtain adequate retention times and peak shapes.

The composition of the mobile phase (concentration of SDS and the organic solvent) and the detection conditions were optimized. In all the optimization tests, a standard solution of DAN, DIF, CIP and SAR at 20 ng L^{-1} was used.

3.1.1 Selection of the alcohol for the mobile phase. Hybrid mobile phases containing 1-propanol, 1-butanol and 1-pentanol were tested. Using mobile phases with SDS/1-pentanol, the analytes were barely retained on the C18 column, and then they overlapped and were eluted too close to the dead time. Therefore, 1-butanol was selected, as mobile phases using SDS/1-butanol provides better peak shapes and less retention times than using SDS/1-propanol.

The studied range of SDS and 1-butanol amounts was between the minimum and the maximum concentration recommended for MLC, 0.05–0.15 M, and 1–7%, respectively. In order to evaluate the chromatographic behavior of each analyte, five mobile phases were tested, at the following SDS (M)/1-butanol (% v/v): 0.05–1; 0.05–7; 0.10–4; 0.15–1 and 0.15–7.[30]

The chromatographic parameters (t_0; t_R; k; N and B/A) were taken for each FQ and mobile phase, using the Michrom software.[36] The retention time and the efficiency decrease at higher concentrations of SDS, indicating that the FQ binds to the micelles. On the other hand, at higher concentrations of 1-butanol, the retention times diminish and the efficiency increases.

3.1.2 Optimization of the composition of the mobile phase. The concentration of SDS and 1-butanol were simultaneously optimized following an interpretative strategy, using a chemometrical approach. This mathematical model is based on equations that relate the chromatographic behaviour of the analytes with the composition of the mobile phase.[31] This

approach would be more effective and rapid than a sequential (one by one) optimization. Eqn (1) is used to describe the retention factor of the analyte, depending on the concentration of SDS ([M]) and 1-butanol (φ):

$$k = \frac{K_{AS}\dfrac{1}{1+K_{AD}\varphi}}{1+\dfrac{K_{AM}[M](1+K_{MD}\varphi)}{1+K_{AD}\varphi}} \quad (1)$$

K_{AS} and K_{AM} are the partition coefficients of the analyte between the bulk water and stationary phase and the micelle, respectively. K_{AD} and K_{MD} measure the relative variation of the analyte in the mobile phase and inside the micelles, because of the presence of the alcohol. K_{AM} and K_{AS} depend on the analyte and surfactant, whereas K_{AD} and K_{MD} depend on the analyte, the surfactant and the alcohol.

The peak shape is modelled by eqn (2) and can be used to calculate N and B/A. It considers that the distribution of the signal $h(t)$ *vs.* elution time follows a modified normal (Gaussian) model, which maximum is at the retention time. The standard deviation is substituted by a linear equation:

$$h(t) = H_0 e^{-0.5\left(\frac{t-t_R}{s_0+s_1(t-t_R)}\right)^2} \quad (2)$$

H_0 represents the height at the retention time, and depends on the concentration and the fluorescence emission of the analyte. The constant s_0 is a measure of the peak width and s_1 constants quantify the distortion of the peak. The s_i constants depend on N and B/A, as well as the FQ and the mobile phase.

The chromatographic data obtained by the five mobile phases containing 1-butanol (see Section 3.1.1) were processed by the Michrom software[36] as "calibration levels" to fit eqns (1) and (2). Thus, the obtained equations are able to predict k; N; B/A and $h(t)$ for the four FQs in the range 0.05–0.15 M (SDS) and 1–7% (1-butanol) by interpolation. Combining these values, the software calculates the resolution (r_{ij}) of consecutive peaks following the valley-peak criterion, and the global resolution (Z) as the r_{ij} of the least-resolved peak pair.[38] Besides, theoretical chromatograms can be drawn by the simultaneous plotting of the $h(t)$ *vs.* time for the four analytes. Thus, the changes in the chromatograms and chromatographic behaviour for each analyte, when the amount of SDS/butanol progressively varies, can be easily visualized.

The concentrations of SDS and 1-butanol were selected to obtain the maximum resolution between the studied FQs at the minimum analysis time. The optimal mobile phase was an aqueous solution of 0.05 M sodium dodecyl sulfate, 1% 1-butanol and 0.5% triethylamine buffered at pH = 3. Under these conditions, the analytes were completely resolved (Z = 0.998) in 25 min, and the peaks were nearly Gaussian. The chromatographic parameters (t_R; N; B/A) were: danofloxacin (15.5; 4201; 1.085), difloxacin (17.6; 1652; 1.012), ciprofloxacin (19.1; 1750; 0.985) and sarafloxacin (21.4; 3100; 1.047). As required by the 2002/657/EC regulation,[34] the less retained compound was eluted more than two times the dead time. The errors in the predicted values for retention factors were <5%.

The use of a chemometric tool has allowed the optimization of the two parameters testing only five mobile phases, thus reducing time and effort. The optimized mobile phase has attractive advantages to apply the method for routine analysis. The use of isocratic mode removes the need of stabilization time between two injections, thus reducing the total time of analysis. As a result, the successive analysis of a large amount of samples is expedited and the analysis can be sold at a lower price. Besides, the optimized mobile phase contains harmless inorganic reagents and a minimal amount of organic solvents. This reduces the risk of the laboratory staff to handle toxic volatile solvents and the waste of toxic compounds to the environment.

3.1.3 Optimization of detection conditions. The studied FQs show an intense fluorescence in micellar media.[32,33] However, the fluorescence properties can strongly vary depending on the chemical environment, and the spectral data from other mobile phases and matrices cannot be taken.

The excitation and emission spectra of the four drugs were obtained by analyzing a honey sample spiked with 40 μg kg^{-1} of each antibiotic, using the optimized chromatographic conditions. The maximum excitation and emission wavelengths (nm) were found to be similar for the studied analytes: danofloxacin, 280 and 450; difloxacin, 280 and 455; ciprofloxacin, 285 nm and 465, and sarafloxacin, 280 nm and 455 nm, respectively. As the spectral data were similar for the studied fluoroquinolones, the detection conditions were set at intermediate values: λ_{exc} = 280 nm and λ_{em} = 455 nm. Under these conditions, the four analytes are quantified close to their maximum signal-to-noise ratio and no changing of the detection wavelength during the run was needed.

3.2 Sample preparation

A honey sample was mixed with a micellar solution of 0.05 M SDS at pH 3 (ref. 32) in order to solubilize the saccharides and then obtain a liquid sample with low viscosity. Furthermore, the diluted sample must be filtered to avoid the injection of high particles and remaining aggregates. There is no risk of precipitation after the injection, because the compounds would remain in a micellar medium. The dilution ratio was optimized considering the need of avoiding an early obstruction of the filter before obtaining a volume sufficiently representative of the whole sample, but without excessively diminishing the sensitivity. Several dilution ratios were tested, by varying the amount of honey: 50 : 1; 20 : 1; 10 : 1; 5 : 1; 1 : 1. In all cases, an aliquot of 2 mL was easily obtained without obstruction of the filter. Thus, 1 : 1 was selected to maximize the sensitivity.

A sample of multiflower honey (trademark "Consum" and manufactured in Spain), free of FQs was analyzed using the optimized method (Fig. 2A). Several peaks were observed, but they elute before 10 min and do not interfere with the analytes.

The greatest advantage of this experimental procedure is the absence of extraction and clean-up steps, expediting it to dilution and filtration. Thus, the sample is quantitatively introduced in the chromatographic system. This simplified operating procedure reduces the probability of operator errors and strongly shortens the analysis time. As a consequence, the possible sources of variability and the risk of the loss of analytes are minimized, thus improving the reproducibility. Besides, analysis can be achieved using a small amount of innocuous reagents, without requiring specific instrumentation and large volumes of toxic organic solvents. This would improve the productivity of the laboratory, the safety of the laboratory staff and lessen the environmental impact of the analysis.

3.3 Method validation

The method was validated following the directives of the EU Commission Decision 2002/657/EC.[34] The studied validation parameters were: selectivity, linearity, calibration range, crossover, intra- and interday accuracy and precision, decision limit, detection capability and robustness. The limits of detection and quantification were determined by the ICH Harmonized Tripartite Guideline,[35] as the EU Commission Decision does not mention them. The whole validation was performed using spiked samples of multiflower honey (same as in Section 3.2), initially free of analytes. The concentrations refer to the w/w amount of FQ in the honey sample, not in the injected aliquot.

3.3.1 Specificity. The specificity was studied by analyzing the twenty samples of honey described in Section 2.4. In all cases, several peaks were detected from the dead time to nearly 5 min, corresponding to the matrix endogenous compounds. No peaks were observed near the retention times of the analytes, and the baseline was quite stable at >10 min. Furthermore, the studied samples were spiked with 40 μg kg^{-1} of each FQ, and analyzed. The resulting chromatograms show similar profiles to the blanks, the only difference being the occurrence of the peaks from the analytes. No overlapping was observed between the analytes and the endogenous compounds. Therefore, the method is specific enough to unequivocally distinguish the analytes in a wide range of honey varieties.

As an example, chromatograms obtained before and after spiking a sample of multiflower honey (same as in Section 3.2) can be seen in Fig. 2A and B, respectively. Smaller peaks appear from the dead time to ≈10 min, sufficiently far from the elution times of the analytes. The difference between the retention time of the analytes in standard solution and in spiked samples was <2.0%, and the peak shape was similar.

3.3.2 Linearity and sensitivity. For calibration purposes, five solutions containing increasing concentrations (three replicates) of the four studied FQs were analyzed in the 10–200 μg kg^{-1} range. The equation relating the peak area of each analyte and the concentration was adjusted using the least-squares linear regression, in order to calculate the slope and *y*-intercept. The goodness-of-fit of the data to the curve was evaluated through the determination coefficient. In order to

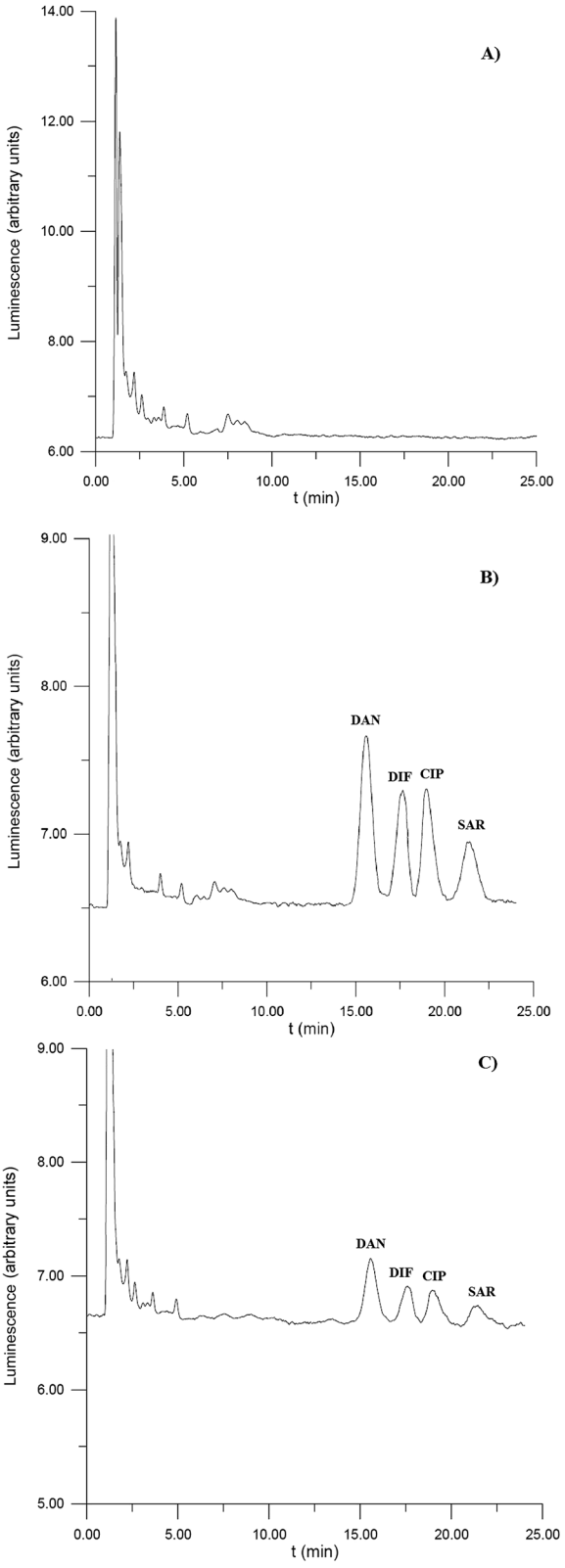

Fig. 2 Chromatograms obtained by the analysis of a multiflower honey sample (trademark "Consum", manufactured in Spain): (A) blank; (B) spiked with a mixture of DAN, DIF, CIP and SAR (B) 40 μg kg^{-1}, and (C) at their corresponding LOQs.

consider the interday variability, five calibration curves were constructed in different days over a 3 months period, using new solutions each time. The average values can be seen in Table 1. An excellent linearity (r^2 > 0.9990) was obtained for danofloxacin, difloxacin, ciprofloxacin and sarafloxacin in the considered range.

The limits of detection (LOD) and quantification (LOQ) were calculated as the minimal concentration providing a chromatographic peak 3 or 10 times higher than the baseline noise, respectively.[35] The LOQ was taken as the minimal level of the calibration curve. The values are shown in Table 1. A chromatogram obtained by the analysis of a honey sample spiked at the LOQ for each analyte is shown in Fig. 2C. The low values prove that the method has enough sensitivity to detect low amounts of these FQs in honey. These values are similar to those obtained using other HPLC-FLD-based methods: 4.4 μg kg^{-1} (ref. 27) and 7 μg kg^{-1},[28] using an easier sample preparation method.

3.3.3 Accuracy and precision. The intraday accuracy was calculated as the average value of the concentration measured by the method (6 successive analyses) and the true value, whereas the intraday precision was calculated as the relative standard deviation between the obtained peak areas by six successive injections of the same solution. The same solutions were used for accuracy and precision and, different from those used in calibration studies. The accuracy and precision of the method were determined for the four studied FQs at 10, 20 and 40 μg kg^{-1}. The interday values were calculated as the average of five intraday measurements taken at several days during a three-months period. The solutions were remade each day. The results are shown in Table 2.

The method was found quite accurate (81.0–103.4%) and precise (<12.3%). These values are in accordance with the EC Decision 2002/657/EC regulation, which accepts values within 80–110% for accuracy and <15% for precision.[34]

3.3.4 Decision limit and detection capability. The EU Commission Decision 2002/657/EC has introduced the determination of two validation parameters, the decision limit (CCα) and the detection capability (CCβ), which assess the critical concentrations (detected and really present) above which the method is able to distinguish a non-compliant sample, considering the method variability and the statistical risk of making a wrong decision. As no MRLs have been stated for the studied FQs, the samples are non-compliant if the analytes are detected.

The CCα refers to the detected concentration above which it can be concluded that the sample is not compliant, with a probability of α to have a false positive. For compounds without MRL, α = 1%, and the CCα is taken as the limit of detection.

The detection capability (CCβ) is the smallest concentration of FQ in honey samples that can produce a non-compliant result with a maximal probability of β to make a false negative. Considering β = 5%, this value was calculated as the decision limit plus 1.64 times the standard deviation of a honey sample spiked at the CCα.

CCα and CCβ values are shown in Table 1. According to the results, the method is able to notice non-compliant samples in honey batches even containing low concentrations of the studied FQs.

3.3.5 Robustness. The robustness was examined by measuring the changes in the retention time and peak area of each FQ, at small, but deliberate variations of the composition of the mobile phase (pH, SDS, 1-butanol, and TEA) and flow rate. These studies were performed using a processed honey sample spiked with 40 μg kg^{-1} of each analyte. The relative standard deviations of the retention time and peak area values, taken at: the optimal value, slightly over and slightly under (each one by three replicates), were calculated. Each parameter was separately studied, maintaining the other constant.

The retention time (<8.5%) and the peak area (<6.5%) are not significantly affected, when the above-mentioned parameters were modified. The concentration of TEA has the strongest influence on the retention of the analytes, compared to the other parameters. This coincides with that found in a previous paper.[32] Anyway, the method is robust enough to provide consistent results, when the experimental parameters oscillate within a realistic range.

3.4 Analysis of real samples

According to the results of the study, the method has been successfully validated following the EU Commission Decision 2002/657/EC, and then could be implemented in laboratories approved for the official residue control of these FQs in honey, or used as a test *prior* to sending honey batches to the EU market. Finally, the method was applied to the commercial honey samples described in Section 2.4. No significant differences were found in the chromatograms, and the studied fluoroquinolones were not detected.

Table 1 Calibration parameters for the analytes (linear range = 10–200 μg kg^{-1})[a]

Compound	Slope	Intercept	R^2	LOD/CCα	LOQ	CCβ
Danofloxacin	2.000 ± 0.004	24.0 ± 0.9	0.9991	4	10	5.5
Difloxacin	3.31 ± 0.03	−15 ± 8	0.9990	4	10	5.2
Ciprofloxacin	2.64 ± 0.04	−11 ± 9	0.9995	4	10	6.2
Sarafloxacin	1.424 ± 0.008	−2.5 ± 1.7	0.9993	4	10	4.7

[a] n = 5; all concentrations in μg kg^{-1}.

Table 2 Intra- and inter-day accuracy and precision for the studied fluoroquinolones

		Intra-day[a]		Inter-day[b]	
Fluoroquinolone	Concentration (μg kg^{-1})	Accuracy (%)	Precision (RSD, %)	Accuracy (%)	Precision (RSD, %)
Danofloxacin	10	92.7	2.2	95.6	3.4
	20	100.2	0.9	101.2	2.1
	40	100.0	1.9	98.5	1.8
Difloxacin	10	82.3	6.1	87.5	4.5
	20	102.3	6.9	98.5	6.4
	40	99.9	2.8	100.8	4.1
Ciprofloxacin	10	82.1	12.3	86.5	10.2
	20	85.2	3.4	90.5	5.3
	40	99.7	2.2	97.5	2.0
Sarafloxacin	10	81.0	4.9	83.8	5.2
	20	103.4	5.9	101.2	4.6
	40	99.9	3.8	98.6	2.5

[a] $n = 6$. [b] $n = 5$.

4. Conclusions

The obtained results indicate that micellar liquid chromatography is an interesting alternative to analyze danofloxacin, difloxacin, ciprofloxacin and sarafloxacin in honey. Despite the viscosity of the sample, it can be directly injected after simple dilution and filtration, thus avoiding tedious and time-consuming extraction procedures, reducing the global analysis time. The studied antibiotics have been eluted using an isocratic mobile phase, without interference from endogenous compounds of honey. The method was successfully validated following the requirements of the EU Commission Decision 2002/657/EC in terms of selectivity, calibration range, linearity, accuracy, precision, decision limit, detection capability and robustness. Besides, the method ensures that a honey sample declared as compliant has only up to μg kg^{-1} levels of FQ, due to the use of fluorescence detection. The method uses innocuous inorganic reagents and a low concentration of organic solvents, and then meets the requirements of "green chemistry". Besides, it facilitates the successive analysis of a high amount of samples, and it is relatively inexpensive, thus making it more advantageous. Therefore, the method is applicable to be used for routine analysis of residues of danofloxacin, difloxacin, ciprofloxacin and sarafloxacin in honey, in order to evaluate the suitability of the samples to be distributed with the European Union.

Conflict of interest

The authors state that there is no financial/commercial conflict of interest.

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