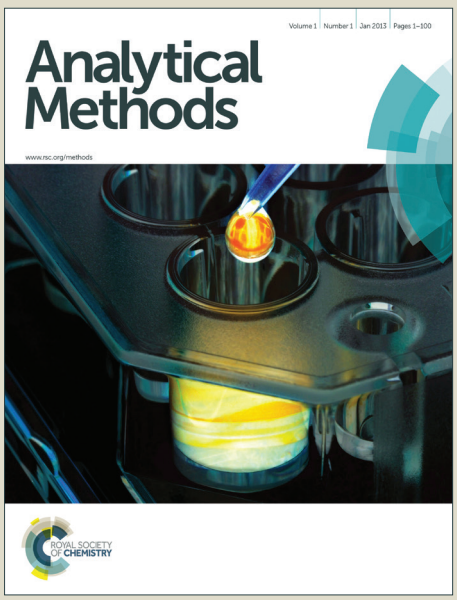
Analytical Methods

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/methods

ANALYSIS OF DANOFLOXACIN, DIFLOXACIN, CIPROFLOXACIN AND SARAFLOXACIN IN HONEY USING MICELLAR LIQUID CHROMATOGRAPHY AND VALIDATION ACCORDING TO THE 2002/657/EC DECISION

K. Tayeb Cherif, J. Peris-Vicente*, S. Carda-Broch, J. Esteve-Romero

Química Bioanalítica, QFA, ESTCE, Universitat Jaume I, 12071, Castelló, Spain

**Corresponding author. E-mail: vicentej@uji.es; Tel.: +34964728099*

Abstract

A reliable and a 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 interferences 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, respectively), calibration range (10-200 μg/kg), linearity (r^2 > 0.9990), decision limit (4 μg/kg), detection capability (4.7-6.2 μg/kg), 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.

Keywords: Honey; Micellar; Optimization; Fluoroquinolone; Validation

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 growing of mutated pathogens resistant to these quinolones, which can lately jump to humans. Besides, drug residues may persist in the edible products of the 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 the quinolones, and would be unaffected by future antibiotic treatments[2]. In the European Union (EU), the presence of these drugs in foodstuff 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 Po/w = 0.14; pKa = 6.22/9.43)[5], difloxacin (log Po/w = 0.77; pKa = 5.66/7.24)[6,7], ciprofloxacin (log Po/w = 0.77; pKa = 6.09/8.09)[7,8] and sarafloxacin (log Po/w = 0.86; pKa = 4.12/6.78)[7], which structures are shown in Figure 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 is these compounds are detected, and then the corresponding batch would not be allowed to be distributed within the EU.

Honey is world-wide consumed, 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 are produced worldwide and 400000 tons are traded internationally[9]. In the last 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 were found in honey originating from China, demonstrating that such broad spectrum antibiotics are used by some beekeepers[10]. Therefore, the 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 comply with the EU regulation and to detect a possible threat to the consumers.

Many methods based on separative 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 works 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 are tedious and time-consuming, because of the viscosity and the presence of a large amount of sugars. The most usual 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 are required[16,18,20] or previous screening by microbiological methods[28]. The enlargement of the experimental procedure increases the probability of loss of analyte, thus reducing the quality of the experimental results. Several authors have proposed the analysis of FQs in honey by an automated on-line sample purification, using turbulent flow chromatography coupled to LC - MS[15].

Micellar liquid chromatography (MLC), using mobile phases containing an aqueous solution of sodium dodecyl sulfate as 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 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, 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 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 %) come from Merck (Darmstadt, Germany). Hydrochloric acid (reagent grade, 37 %), triethylamine (>99.5 %) and ethanol (HPLC grade) were bought to 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 solving 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 solutions. 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 with the aid of a vacuum pump through a 0.45 μm nylon membrane.

Individual stock solutions of the studied FQs were prepared as follows: the adequate quantity of the solid standard was weighed and solved in few mLs of ethanol, and then filled up with a micellar solution of 0.05 M SDS at pH 3 (fixed with phosphate buffer), to reach a final concentration of nearly 100 mg L^{-1}. The solution was ultrasonicated to assure the complete solubilization. These solutions were stored at 4ºC 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 4ºC 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 x 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. The software Agilent Chemstation (Rev. B.03.01) software was used to control the HPLC instrumentation and to acquire the 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 (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[29].

2.4 Sample collection and processing

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

- "Granja San Francisco" (Nutrexpa, Barcelona, Spain): multi flower, eucalyptus-lime, forest, orange blossom.
- "Consum" (Reina Apícola Levantina, Alzira, Spain): multi flower, rosemary, orange blossom, eucalyptus.
- "El Brezal" (Mielso, Almazora, Spain): orange blossom, rosemary, multi flower, thyme, black eucalyptus, white eucalyptus, mountain (several mountain flowers), forest (honeydew), acacia, Yucatan (Nahonal and Dzidzilche flowers).
- "El Quexigal" (El Quexigal, Cebreros, Spain): heather, 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 the 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 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 amount was between the minimum and 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 concentration of SDS, indicating that the FQ bind to the micelles. On the other hand, at higher concentrations of 1-butanol, the retention times diminish and the efficiency increase.

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 which 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. The equation (1) is used to describe the retention factor of the analyte, depending on the concentration of SDS ([M]) and 1-butanol (φ):

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

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} depends of the analyte, the surfactant and the alcohol.

The peak shape is modelled by the equation (2) and can be used to calculate N and B/A. It considers that the distribution of signal *h(t) v.s.* 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 and depends on 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 the equations (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 *h(t) v.s.* 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 concentration 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 times. 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 needing 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 solvent. 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 the 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 then spectral data from other mobile phases and matrices cannot be taken.

The excitation and emission spectra of the four drugs were by analyzing a honey sample spiked with 40 μg/kg of each antibiotic, using the optimized chromatographic conditions. The maximum excitation and emission wavelengths (nm) were found 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: λexc = 280 nm and λem = 455 nm. Under these conditions, the four analytes are quantified close to their maximum signal-to-noise ratio and no changing of detection wavelength during the run was needed.

3.2 Sample preparation

Honey sample was mixed with a micellar solution of 0.05 M SDS at pH 3[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 needing 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 a multi flower honey (trademark "Consum" and manufactured in Spain), free of FQs was analyzed using the optimized method (Figure 2A). Several peaks were observed, but they elute before 10 min and do not interfere with the analytes.

The higher advantage of this experimental procedure is the absence of extraction and clean-up steps, expediting it to a dilution and filtration. Thus, the sample is quantitatively introduced in the chromatographic system. This simplified operating procedure reduces the probability of operator error and strongly shorten the analysis time. As consequence, the possible sources of variability and the risk of loss of analyte are minimized, thus improving the reproducibility. Besides, the analysis can be achieved using a few 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 for 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, cross over, 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 multi flower honey (same as in 3.2), initially free

of analytes. The concentrations refer to the w/w amount of FQ in the honey sample, not in injected aliquot.

3.3.1 Specificity

The specificity was studied by analyzing the twenty samples of honey described in 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 of each FQ, and analyzed. The resulting chromatograms show similar profile than the blanks, the only difference was the occurrence of the peaks from the analytes. No overlapping was observed between the analytes and endogenous compounds. Therefore, the method is enough specific to unequivocally distinguish the analytes in a wide range of honey varieties.

As an example, chromatograms obtained before and after spiking a sample of a multi flower honey (same as in 3.2) can be seen in Figure 2A and 2B, respectively. Smaller peaks appear from the dead time to ≈ 10 min, enough 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 range. The equation relating the peak area of each analyte and the concentration was adjusted using the least-square 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 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 the Table 1. An excellent linearity (r^2 > 0.9990) was obtained for danofloxacin, difloxacin, ciprofloxacin and sarafloxacin in the considered range.

The limit 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 the Table 1. A chromatogram obtained by the analysis of a honey sample spiked at the LOQ for each analyte is shown in Figure 2C. The low values prove that the method has enough

sensitivity to detect low amount of these FQs in honey. These values are similar to those obtained using other HPLC-FLD-based methods: 4.4 μg/kg[27] and 7 μg/kg[28], using an easier sample preparation.

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 to 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. 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 the 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 inside 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 the 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 of each analyte. The relative standard deviation 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 enough robust 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* 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.

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 a 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 interferences form 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 levels of FQ, due to the use of fluorescence detection. The method uses innocuous inorganic reagents and low concentration of organic solvent, and then meets the requirements of the "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.

5. Conflict of interest

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

6. Acknowledgements

This work was supported by Project P1.1B2012-36 del Pla de Promoció de la Investigació de la Universitat Jaume I.

7. References

1. A. Fàbrega, S. Madurga, E. Giralt, J. Vila, *Microb. Biotechnol.*, 2009, **2**, 40-61
2. *Emerging and other communicable diseases, surveillance and control. Use of quinolones in food animals and potential impact on human health*, Report of a WHO meeting, World Health Organization, Geneva, Switzerland, 1998. Available at: http:// whqlibdoc.who.int/hq/1998/WHO_EMC_ZDI_98.10.pdf (Accessed: 14/06/2015)
3. W. Reybroeck, E. Daeseleire, H.F. De Brabander, L. Herman, *Vet. Microbiol.*, 2012, **158**, 1–11
4. European Council, Commission Regulation (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin, *OJEU* 2010, **L15**, 1-72. Available at: http://ec.europa.eu/health/files/eudralex/vol-5/reg_2010_37/reg_2010_37_en.pdf (Accessed: 14/06/2015)
5. *Danofloxacin, Environmental Assessment*, Food and Drug Administration, Rockville, MD, USA, 2000. Available at: http://www.fda.gov/downloads/AnimalVeterinary/Development

ApprovalProcess/EnvironmentalAssessments/UCM072389.pdf (Accessed: 14/06/2015)

6. M.H. Abraham, K. Takács-Novák, R.C. Mitchell, *J. Pharm. Sci.*, 1997, **86**, 310-315
7. A.I. Caço, L.C. Tomé, R. Dohrn, I.M. Marrucho, *J. Chem. Eng. Data*, 2010, **55**, 3160-3163
8. V. Law, C. Knox, Y. Djoumbou, T. Jewison, A.C. Guo, Y. Liu, A. Maciejewski, D. Arndt, M. Wilson, V. Neveu, A. Tang, G. Gabriel, C. Ly, S. Adamjee, Z.T. Dame, B. Han, Y. Zhou, D.S. Wishart, DrugBank 4.0: shedding new light on drug metabolism, *Nucleic Acids Res.*, 2014, **42**, D1091–S1097, Available at: http://www.drugbank.ca/ (Accessed: 14/06/2015)
9. V. Michaud, *Apiacta* 2005, **40**, 52–54. Available at: http:// www.apimondia.com/apiacta/articles/2005/michaud_1.pdf (Accessed: 14/055/2015)
10. *Import Alert IA3604*, Food and Drug Administration, Rockville, MD, USA, 2006. Available at: http://www.accessdata.fda.gov/cms_ia/importalert_111.html (Accessed: 14/06/2015)
11. D. Barrón, E. Jiménez-Lozano, S. Bailac, J. Barbosa, *J. Chromatogr. B*, 2002, **767**, 313-319
12. J.A. Hernández-Arteseros, J. Barbosa, R. Compañó, M.D. Prat, Analysis of quinolone residues in edible animal products, *J. Chromatogr. A*, 2002, **945**, 1–24
13. M.P. Hermo, E. Nemutlu, S. Kir, D. Barrón, J. Barbosa, *Anal. Chim. Acta*, 2008, **613**, 98–107
14. J.A. Paschoal, F.G. Reyes, S. Rath, *Food Addit. Contam. Part A-Chem.*, 2009, **26**, 1331-1340
15. P. Mottier, Y.A. Hammel, E. Gremaud, P.A. Guy, *J. Agric. Food Chem.*, 2008, **56**, 35-43
16. R. Galarini, G. Saluti, D. Giusepponi, R. Rossi, S. Moretti, *Food Control*, 2015, **48**, 12-24
17. J. Wang, D. Leung, *Drug Test. Anal.*, 2012, **4**, 103-111
18. M. Lombardo-Agüí, A.M. García-Campaña, L. Gámiz-Gracia, C. Cruces-Blanco, *Talanta*, 2012, **93**, 193– 199
19. M.L. Gómez-Pérez, P. Plaza-Bolaño, R. Romero-González, J.L. Martínez-Vidal, A. Garrido-Frenich, *J. Chromatogr. A*, 2012, **1248**, 130– 138
20. M.I. Lopez, J.S. Pettis, I.B. Smith, P.S. Chu, *J. Agric. Food Chem.*, 2008, **56**, 1553-1559
21. E.A. Christodoulou, V.F. Samanidou, I.N. Papadoyannis, *J. Chromatogr. B*, 2007, **859**, 246-255
22. E.A. Christodoulou, V.F. Samanidou, I.N. Papadoyannis, *J. Sep. Sci.*, 2007, **30**, 2676-2686
23. J.F. Huang, B. Lin, Q.W. Yu, Y.Q. Feng, *Anal. Bioanal. Chem.*, 2006, **384**, 1228-1235
24. I. Pecorelli, R. Galarini, R. Bibi, A Floridi, E. Casciarri, A. Floridi, *Anal. Chim. Acta*, 2003, **483**, 81-89
25. V. Andreu, C. Blasco, Y. Picó, *TrAC - Trends Anal. Chem.*, 2007, **26**, 534-556
26. J. Zhou, X. Xue, F. Chen, J. Zhang, Y. Li, L. Wu, L. Chen, J. Zhao, *J. Sep. Sci.*, 2009, **32**, 955-964
27. Y.I. Yatsukawa, H. Ito, T. Matsuda, M. Nakamura, M. Watai, K. Fujita, Determination of residual fluoroquinolones in honey by liquid chromatography using metal chelate affinity chromatography, *J. AOAC Int.*, 2001, **94**, 1319-1327

28. M. Kanda, T. Kusano, S. Kanai, H. Hayashi, Y. Matushima, T. Nakajima, K Takeba, T. Sasamoto, T. Nagayma, *J. AOAC Int.*, 2010, **93**, 1331-1339

29. M. Rambla-Alegre, J. Peris-Vicente, S. Marco-Peiró, B. Beltrán-Martinavarro, J. Esteve-Romero, *Talanta*, 2010, **81**, 894–900

30. J. Peris-Vicente, I. Casas-Breva, P. Roca-Genovés, J. Esteve-Romero, *Bioanalysis*, 2014, **6**, 1975–1988

31. J. Peris-Vicente, M. Villarreal-Traver, I. Casas-Breva, S. Carda-Broch, J. Esteve-Romero, *J. Sep. Sci.*, 2014, **37**, 2825–2832

32. M. Rambla-Alegre, J. Peris-Vicente, J. Esteve-Romero, S. Carda-Broch, *Food Chem.*, 2010, **123**, 1294–1302

33. M. Rambla-Alegre, M.A. Collado-Sánchez, J. Esteve-Romero, S. Carda-Broch, *Anal. Bioanal. Chem.*, 2011, **400**, 1303-1313

34. European Council, Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results, *OJEC*, 2002, **L221**, 8-36. Available at: http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32002D0657&from=EN (Accessed: 14/06/2015).

35. ICH Expert Working Group, *ICH Harmonized Tripartite Guideline: Validation of Analytical procedures, text and methodologies Q2(R1)*, ICH, Geneva, Switzerland, 2005. Available at: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf (Accessed: 14/06/2015)

36. J.R. Torres-Lapasió, *Michrom Software*, Marcel-Dekker, New York, USA, 2000

37. I. Casas-Breva, J. Peris-Vicente, M. Rambla-Alegre, S. Carda-Broch, J. Esteve-Romero, *Analyst*, 2012, **137**, 4327-4334

38. J. Peris-Vicente, K. Cherif-Tayeb, P. Roca-Genovés, J. Esteve-Romero, *Madhya Bharti Research Journal*, 2014, LVIII, 1-12

FIGURE CAPTIONS

Figure 1. Structure of the studied fluoroquinolones.

Figure 2. Chromatogram obtained by the analysis of a multi flower honey sample (trademark "Consum", manufactured in Spain): A) blank; B) spiked with a mixture of DAN, DIF, CIP and SAR B) 40 μg/kg, and c) at their corresponding LOQ.

Table 1. Calibration parameters for the analytes (Linear range = 10 - 200 μg/kg)

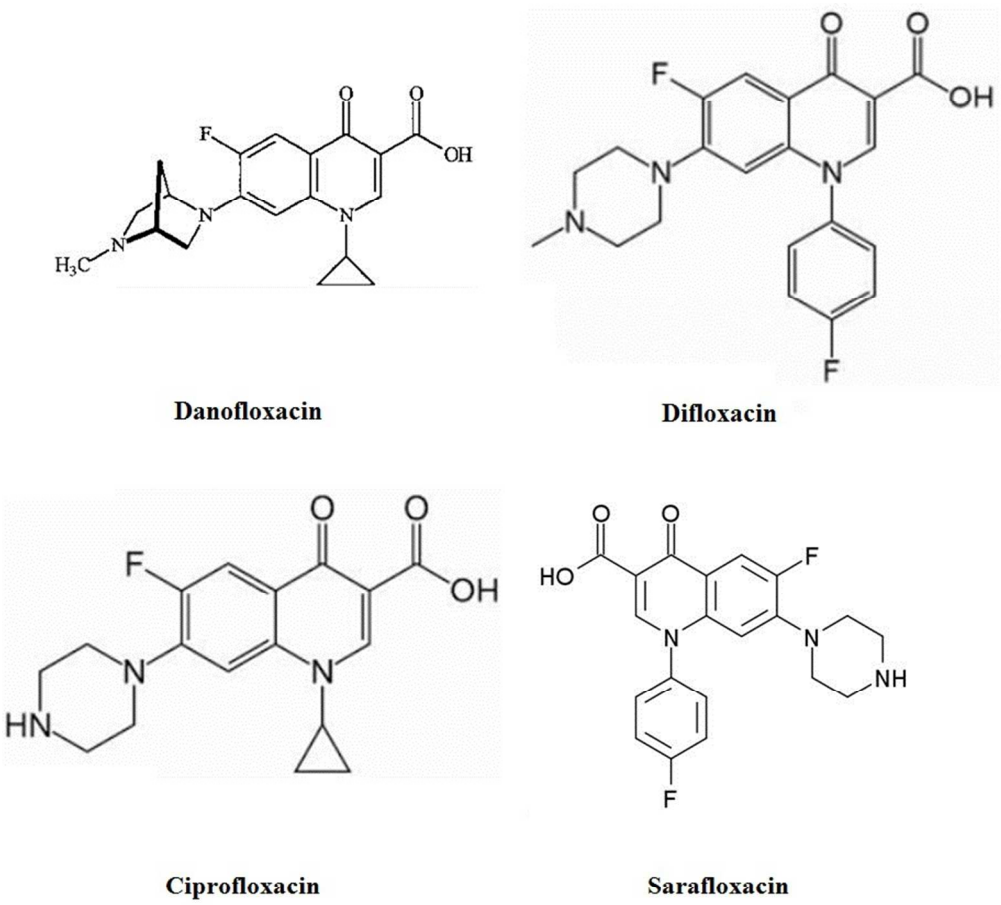
Compound	Slope	Intercept	r^2	LOD/CCα	LOQ	CCβ
Danofloxacin	2.000 ± 0.004	24.0 ± 0.9	0.9991	4	10	5.5
Difloxacin	3.31 ± 0.03	- 15 ± 8	0.9990	4	10	5.2
Ciprofloxacin	2.64 ± 0.04	-11 ± 9	0.9995	4	10	6.2
Sarafloxacin	1.424 ± 0.008	-2.5 ± 1.7	0.9993	4	10	4.7

n = 5; all concentrations in μg/kg

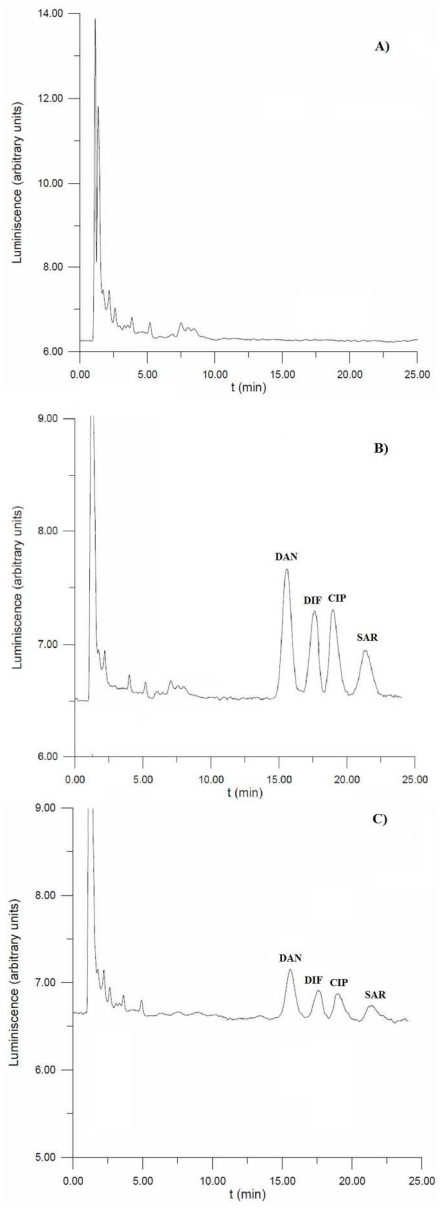
Table 2. Intra- and inter-day accuracy and precision for the studied fluoroquinolones.

Fluoroquinolone	Concentration (μg/kg)	Intra-day[a] Accuracy (%)	Intra-day[a] Precision (RSD, %)	Inter-day[b] Accuracy (%)	Inter-day[b] 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



Structure of the studied fluoroquinolones
154x151mm (150 x 150 DPI)



Chromatogram obtained by the analysis of a multi flower honey sample (trademark "Consum", manufactured in Spain): A) blank; B) spiked with a mixture of DAN, DIF, CIP and SAR B) 40 ng/g, and c) at their corresponding LOQ
165x457mm (150 x 150 DPI)