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Supramolecular solvents in solid sample microextractions: Application to the determination of residues of oxolinic acid and flumequine in fish and shellfish

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ABSTRACT

Supramolecular solvents are here proposed firstly as extractants in solid sample microextractions. The approach was evaluated by extracting flumequine (FLU) and oxolinic acid (OXO), two widely used veterinary medicines, from fish and shellfish muscle using a supramolecular solvent made up of decanoic acid (DeA) reverse micelles. The antibiotics were extracted in a single step (~15 min), at room temperature, using 400 μL of solvent. After centrifugation, an aliquot of the extract was directly analyzed by liquid chromatography and fluorescence, without the need of clean-up or solvent evaporation. Contrary to the previously reported methods, both OXO and FLU were quantitatively extracted from fish and shellfish, independently of sample composition. The high extraction efficiencies observed for these antibiotics were a consequence of their amphiphilic character which resulted in the formation of DeA-OXO and DeA-FLU mixed aggregates. The quality parameters of this quantitative method including sensitivity, linearity, selectivity, repeatability, trueness, ruggedness, stability, decision limit and detection capability were evaluated according to the 2002/657/EC Commission Decision. Quantitation limits in the different samples analyzed (salmon, sea trout, sea bass, gilt-head bream, megrim and prawns) ranged between 6.5 and 22 $\mu g\,kg^{-1}$ for OXO and, 5 and 15 $\mu g\,kg^{-1}$ for FLU. These limits were far below the current maximum residue limits (MRLs) set by the European Union (EU) (i.e. 100 and 600 $\mu g\,kg^{-1}$, for OXO and FLU, respectively). The trueness of the method was determined by analyzing a Certified Reference Material (CMR, BCR®-725) consisting of a lyophilised salmon tissue material. Recoveries for fortified samples (50–100 $\mu g\,kg^{-1}$ of OXO and 50–600 $\mu g\,kg^{-1}$ of FLU) and their relative standard deviations were in the intervals 99–102% and 0.2–5%, respectively. The repeatability, expressed as relative standard deviation, was 3.6% for OXO and 2.3% for FLU ([OXO] = [FLU] = 200 $\mu g\,kg^{-1}$ and $n = 11$).

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1. Introduction

Legislation intended to meet the worldwide demand for safe food supplies has become increasingly restrictive. As a result, food control laboratories have to deal with a huge number of samples for which they must ensure unequivocal identification and exact quantitation of the prohibited/legislated substances. In this context, analysis of residues/contaminants in solid food samples (e.g. edible tissues from animals) is a challenge for lab scientists, specially the sample treatment step, for which breakthrough methodologies are lacking [1–3].

Most of the sample preparation approaches routinely used in solid food analysis involve repetitive extractions with organic solvents, filtration or centrifugation, interference removal by liquid–liquid (LLE) and/or solid-phase (SPE) extraction and solvent evaporation [4–9]. These procedures often take up most of the total analysis time, contribute highly to the analysis error and cost and are the main sources of waste.

Different techniques have been accepted to some extent by labs in order to increase extraction efficiency (e.g. microwave-assisted extraction, MAE, and pressurized liquid extraction, PLE [10]) and reduce solvent consumption (e.g. supercritical fluid extraction, SFE [11]) or complexity (e.g. matrix solid-phase dispersion, MSPD [12]). However, there is a gap in the development of microextraction techniques for the treatment of solid food samples, mainly because of the low extraction efficiency of organic solvents in such complex matrices (e.g. edible tissues from animals contain collagen, muscle proteins, elastin, fat, other lipids and mineral constituents).

In this work, the capability of supramolecular solvents for the microextraction of residues from solid food samples is firstly investigated. Supramolecular solvents are water-immiscible liquids made up of large surfactant aggregates dispersed in a continuous phase (usually water) [13]. They spontaneously form in micellar or vesicular aqueous or hydro-organic solutions by the action of an external stimulus (e.g. temperature, electrolyte, pH, solvent), which induces the formation of larger aggregates, often keeping the mor-

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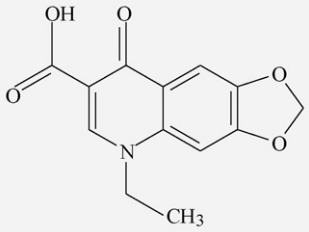
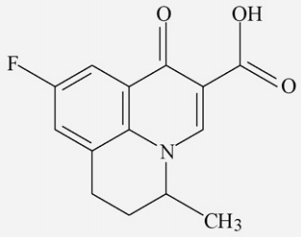
Table 1
Chemical structures, ionization constants (pK_a), octanol-water partition coefficients ($\log K_{ow}$) and numbers of donor and acceptor groups for oxolinic acid and flumequine.

Quinolone antibiotic	Chemical structure	[a]pK_a	[b]Log K_{ow}	[c]Hydrogen donor and acceptor sum
Oxolinic acid (OXO)		6.78	0.94	7
Flumequine (FLU)		6.65	1.6	5

[a] Obtained from Ref. [28].
[b] Obtained from the ChemIDplus Lite database, National Institutes of Health (USA). Available from: http://toxnet.nlm.nih.gov.
[c] Calculated using the Advanced Chemistry Development (ACD/Labs) Software V8.14 for Solaris.

phology, and causes their separation from the bulk solution by a phenomenon named coacervation [14].

Supramolecular solvents have two outstanding properties that should make them suitable for microextractions. The first one derives from the special structure of the ordered aggregates that constitute them. Thus, they have regions of different polarities that provide a variety of interactions for analytes. The type of interaction may be tuned varying the hydrophobic or the polar group of the surfactant and in theory one may design the most appropriate extractant for a specific application because amphiphiles are ubiquitous in nature and synthetic chemistry. A second major feature of supramolecular solvents is the high concentration of amphiphiles, and therefore of binding sites, they contain (typically 0.1–1 mg μL^{-1}). This characteristic permits to achieve high extraction efficiencies using low extractant volumes, which is requisite in microextractions. Additional interesting properties for extractions include non-volatility and non-flammability, which permits the implementation of safer processes, and the use of self-assembly based synthetic procedures that are within everyoneĭs reach.

Application of non-ionic micelle-based supramolecular solvents to the analytical extraction of contaminants from environmental waters has been known for a long time and the corresponding extraction approach has been named *cloud point technique* in the analytical literature [15–17]. In the last decade, developments in this area have focused on the use of supramolecular solvents made up of zwitterionic [18], cationic [19] or anionic [20] micelles, which have avoided the problems of coelution caused by non-ionic surfactants in LC and have made the extract compatible with MS. Recently, supramolecular solvents consisting of vesicles [21] and reverse micelles [22] of biosurfactants, such as alkylcarboxylic acids, have been reported and have marked a turning point with regard to the type of aggregates that constitute them, the variety of interactions they can establish with analytes and the high concentration of amphiphiles they contain.

Despite the high potential of supramolecular solvents for the extraction of contaminants in solid samples, only a few applications involving sludge [23,24], soils [24], ashes [25] and foods [26,27] have been reported. Extractions in these matrices invariably involve sample amounts below 1 g and aqueous solutions (10–40 mL) containing the dispersed supramolecular solvent (typically 100–500 μL), which is separated from the bulk solution after extraction by centrifugation. This approach is not convenient for the extraction of highly polar analytes, which may distribute between the water and surfactant-rich phase, and makes extraction miniaturization difficult. To our knowledge, no applications involving the direct addition of the supramolecular solvent to the solid sample have been developed.

In this work, the potential of supramolecular solvents to be used in solid food microextractions was investigated using oxolinic acid (OXO) and flumequine (FLU) (Table 1) as model analytes. These compounds are two highly polar acidic quinolone antibiotics extensively used as antimicrobial agents in aquaculture. The food matrices selected were the muscles from a variety of fishes and shellfishes. Maximum residue limits (MRLs) set by the EU for OXO and FLU in seafood are 100 and 600 μg kg^{-1}, respectively [29]. Analytical strategies to determine quinolone residues in food and the environment have been critically reviewed [30,31]. Methods for the determination of quinolone residues in seafood are based on liquid chromatography with fluorescence [32–34] or mass spectrometric detection using matrix-matched calibration [34–36]. Reported sample treatment procedures consume considerable volumes of toxic organic solvents (typically 7–24 mL per sample [32,34–36]) and include repetitive extractions, solvent evaporation and clean-up steps, which makes them slow and tedious. The supramolecular solvent made up of decanoic acid reverse micelles [22] was used as extractant on the basis of its capacity to bind analytes through hydrophobic and hydrogen bonding interactions and the high concentration of aggregates (0.76 mg μL^{-1}) making it up. Microextractions were carried out in 2 mL microtubes involving minute sample amounts and supramolecular solvent volumes.

The influence of experimental variables on the efficiency of the extraction of the target analytes was investigated, the quality parameters of the method including sensibility, linearity, selectivity, repeatability, trueness, ruggedness, stability, decision limit

(CCα) and detection capability (CCβ)] were evaluated according to the 2002/657/EC Commission Decision [37], and OXO and FLU were quantified in the muscle of aquaculture seafood. Below, the main results obtained are presented and discussed.

2. Experimental

2.1. Reagents and standards

All chemicals were of analytical reagent-grade and were used as supplied. Oxalic acid and LC-grade acetonitrile, methanol and tetrahydrofuran (THF) were supplied by Panreac (Barcelona, Spain) and decanoic acid (DeA) by Fluka (Buchs, Switzerland). Ultra-high-quality water was obtained from a Mili-Q water purification system (Millipore, Madrid, Spain). High-purity (>98%) oxolinic acid (OXO) and flumequine (FLU) standards were obtained from Sigma (Saint Louis, MI, USA). Stock solutions, 100 mg L^{-1} of each antibiotic, were prepared in acetonitrile and stored at 4 °C. A 2 mL-working solution containing 1.5 mg L^{-1} of OXO and FLU was daily prepared from the stock solution by dilution with the supramolecular solvent. Seven calibration solutions, containing amounts of OXO and FLU in the ranges 2.5–500 and 1.5–500 ng, respectively, were prepared in 0.5 mL-volumetric flasks from the working solution by dilution with the supramolecular solvent. The Certified Reference Material (CRM) used for method validation (BCR®-725) consisted of lyophilised salmon tissue (skin included) and was supplied by LGC Standards GmbH (Wesel, Germany). It was stored under dark at −70 °C. The certified contents of OXO and FLU were 600 ± 100 μg kg^{-1} and 1170 ± 210 μg kg^{-1}, respectively.

2.2. Apparatus

The liquid chromatographic system used consisted of a TermoQuest Spectra System (San Jose, CA, USA) furnished with a P4000 quaternary pump, a SCM 1000 vacuum membrane degasser, an AS3000 auto-sampler and a FL3000 fluorescence detector. The stationary-phase column was a Kromasil C_{18} column (5 μm, 150 mm × 4.6 mm) from Analisis Vinicos (Tomelloso, Spain). A homogenizer-disperser Ultra-Turrax T25 Basic from Ika (Werke, Germany), a vortex-shaker REAX Top equipped with an attachment (ref. 549-01000-00) for 10 microtubes from Heidolph (Schwabach, Germany) and a high speed brushless centrifuge MPW-350R equipped with an angle rotor 36 × 2.2/1.5 mL (ref. 11462) from MPW Med-Instruments (Warschaw, Poland) were used for sample preparation. A magnetic stirrer Basicmagmix from Ovan (Barcelona, Spain) and a digitally regulated centrifuge Mixtasel equipped with an angle rotor 4 mL × 100 mL (ref. 7001326) from JP-Selecta (Abrera, Spain) were used for supramolecular solvent production.

2.3. Supramolecular solvent production

The following procedure, which permits to obtain a supramolecular solvent volume (~8.5 mL) able to treat 20 fish samples, was routinely followed. Decanoic acid (6.5 g) was dissolved in THF (4.2 mL) in a 100 mL-glass centrifuge tube. Then, 80 mL of a 10 mM hydrochloric acid aqueous solution was added. The mixture was magnetically stirred for 5 min, time in which the supramolecular solvent spontaneously formed into the bulk solution. Then, the suspension was centrifuged at 3500 rpm for 10 min to speed solvent separation up, which is less dense than water. Next, it was withdrawn using a 10 mL-syringe, transferred to a hermetically close storage glass vial to avoid THF losses and stored at 4 °C. Under these conditions, the solvent produced was stable for at least one month. The volume of solvent obtained can be adjusted at will by choosing an appropriate, constant DeA/THF/water proportion.

2.4. Determination of OXO and FLU in aquaculture products

2.4.1. Sample preparation

Aquaculture fishes (salmon, sea trout, sea bass, megrim and gilt-head bream) and shellfishes (prawns) were bought in supermarkets in Córdoba (Spain). Their head, fishbone and backbones were removed and the muscle, including the skin, filleted. Headless prawns were peeled and the tail removed. Cleaned fishes and prawns were stored at −20 °C until analysis. After thawing, about 200 g of sample was chopped and homogenized using a homogenizer-disperser. Then, portions of about 200 mg were taken for analysis and recovery experiments, which were performed in triplicate. Spiking of chopped samples (200 mg) was done by adding volumes in the range 2.5–200 μL of a solution containing OXO and FLU (2 mg L^{-1} each) in acetonitrile. Spiked samples were allowed to stand at room temperature for 15 h before analysis. Both analytes were stable in the samples during the holding time.

2.4.2. Microextraction of OXO and FLU

About 200 mg of chopped sample and 400 μL of supramolecular solvent were mixed in a 2 mL-microtube Safe-Lock from Eppendorf Ibérica (Madrid, Spain). A micro PTFE-coated bar (3 mm × 10 mm, Pobel, Madrid, Spain) was introduced in the microtube to favour sample dispersion during extraction, which was made by sample vortex-shaken at 2500 rpm for 15 min. Then, the mixture, thermostated at 15 °C, was centrifuged at 15,000 rpm (16,720 × *g*) for 15 min. The supramolecular extract was withdrawn using a microsyringe, transferred to an auto-sampler vial and injected (20 μL) into the liquid chromatographic system.

2.4.3. Liquid chromatography-fluorescence detection

Separation of OXO and FLU was carried out by liquid chromatography using isocratic elution and their quantitation was performed by fluorescence detection. The mobile phase consisted of 55% oxalic acid (0.01 M) and 45% acetonitrile/methanol (75:25, v/v) at a flow rate of 1 mL min^{-1} [34]. Fluorescence measurements were performed at 325 nm (excitation wavelength) and 360 nm (emission wavelength). Calibrations curves ($n = 7$) were run from standards dissolved in supramolecular solvent by injecting amounts of OXO and FLU in the intervals 0.1–20 ng and 0.06–20 ng, respectively, and quantitation was performed by measuring peak areas. Daily, cleaning of the column was performed by flushing methanol through it.

3. Results and discussion

3.1. Supramolecular solvent-based microextraction of OXO and FLU

3.1.1. Solvent composition

The reverse micelle-based supramolecular solvent used in this research spontaneously forms in ternary mixtures of DeA, water and THF at well-defined proportions (Fig. 1). Its formation occurs through two sequential self-assembly processes. First, DeA molecules aggregate as reverse micelles in THF and then, under the addition of water, they rearrange in larger reverse micelles that separate from the bulk solution, as an immiscible liquid, through a mechanism that remains elusive. The immiscible liquid is made up of reverse micelles, THF and minute amounts of water. Because of reverse micelles are only formed from protonated DeA molecules ($pK_a = 4.8 \pm 0.2$), the pH of the ternary mixture should be kept below 4 to ensure maximal solvent production.

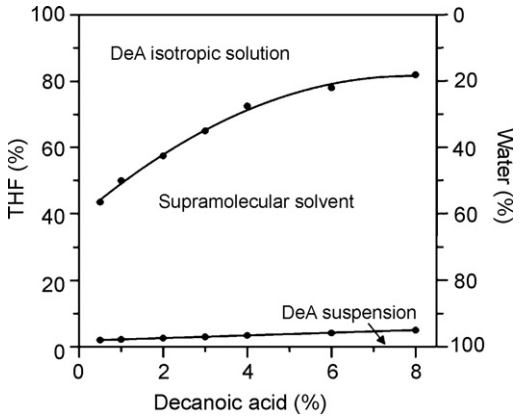


Fig. 1. Diagram of phase boundaries for THF-decanoic acid-water mixtures.

Both solvent volume and composition depend on the absolute amount of DeA and/or the THF/water proportion in the bulk solution. The volume of solvent formed (V_S, in mL) can be estimated from the following equation [38]:

$$V_S = 1.035\, A_{DeA} e^{0.04731\,[THF]}$$

where A_{DeA} is the amount of DeA in g and [THF] the percentage (v/v) of this solvent in the water:THF solution. The linear relationship between V_S and A_{DeA} indicates that the composition of the supramolecular solvent keeps constant as the percentage of THF in the bulk solution remains unchanged. On the other hand, the exponential relationship between V_S and [THF] reveals that the volume of THF incorporated into the supramolecular solvent increases as the percentage of THF used to produce it does, which results in decreased biosurfactant concentration in the supramolecular solvent. The concentration of DeA in supramolecular solvents produced from solutions containing different THF percentages was found by LC-UV and the results obtained are shown in Table 2. The concentration of biosurfactant in the solvent decreased about 3.6-fold by increasing the concentration of THF in the bulk solution from 5 to 30%.

3.1.2. Solvent binding capability

A good knowledge of the interactions between the solvent and analytes is important for setting up an efficient extraction scheme. Reverse micelles of decanoic acid may solubilise solutes based on both hydrophobic interactions in the hydrocarbons tails and hydrogen bonds in the carboxylic acid polar groups. On the other hand, the planarity of the quinolone ring and the nature of the functional groups present in OXO and FLU molecules (Table 1) suggest the possibility of several types of intra- and inter-molecular interactions. The self-association of OXO in aqueous solutions through intramolecular interactions has been proved [39]. Intermolecular interactions between OXO/FLU and the solvent can be of two types; hydrophobic and hydrogen bonding, and according to analyte structure, multiple solvent–analyte intermolecular interactions can occur. These strong solvent:analyte associations should permit the development of robust and efficient microextraction methods.

3.1.3. Factors affecting the extraction efficiency of the solvent

The effect of experimental variables on the efficiency of the extraction of AQAs from aquaculture seafood was investigated by extracting salmon muscle (skin included, ~200 mg) spiked with OXO and FLU at a concentration of 200 μg kg^{-1} each. Experiments were made in triplicate. The protein content of salmon (19.9 g per 100 g of sample) was representative of the content of this ingredient in 100 g of the fishes/shellfishes analyzed (it ranged between 15.8 and 23.8 g), while its fat content (10.8 g) was far above that of the other ones (1.3–3.6 g) [40,41]. Selection of the optimal conditions was based on the recoveries and the method quantitation limits (MQLs) obtained. MQLs were calculated from the instrumental quantitation limits (0.11 ng for OXO and 0.06 ng for FLU), the volume of supramolecular solvent used for extraction, the recoveries obtained and the sample weight used for analysis. The variables investigated were: composition and volume of extractant, pH, sample amount, time required to reach equilibrium conditions and time of centrifugation necessary to obtain free-particle extracts. The representativity of the amount of sample selected for seafood analysis, after applying the sample treatment recommended in Section 2.4.1, was also investigated.

Supramolecular solvents containing different amounts of DeA per unit volume were produced from solutions containing 6.5 g of decanoic acid and variable percentages of THF (5–30%; total water + THF volume = 85 mL) according to the procedure specified in the Section 2.3. In this way, supramolecular solvents containing DeA concentrations between 210 and 763 mg μL^{-1} were obtained (Table 2) and 400 μL aliquots were used for extraction of OXO and FLU from salmon. The recoveries and MQLs obtained from these experiments are shown in Table 2. The extraction capability of the supramolecular solvents decreased as its biosurfactant content did owing to the reduction of micellar solubilisation sites. The solvent produced using a 5% of THF provided quantitative recoveries and the lowest MQLs for both OXO and FLU, so it was selected for further studies. Lower THF percentages could not be used for solvent production because decanoic acid (6.5 g) was insoluble in the resulting water:THF solutions.

The volume of supramolecular solvent used to extract the target antibiotics from salmon influenced both extraction efficiency and reproducibility (Table 3). Both recoveries and precision increased as the volume of the supramolecular solvent did while MQLs progressively degraded. Because of the MRLs set for both OXO and FLU are far above the MQLs found in this study, recoveries and precision were the parameters used for selection. In consequence, a volume of 400 μL aliquots was chosen as optimal.

The pH had no influence on OXO and FLU extraction. This parameter was investigated by producing the supramolecular solvent

Table 2
Concentrations of decanoic acid in the supramolecular solvent, mean recoveries and method quantitation limits obtained for oxolinic acid and flumequine as a function of the percentage of THF used to produce the solvent.

%THF	[DeA] (mg μL^{-1})	Recovery[a] ± s[b] (%)		MQL (μg kg^{-1})	
		OXO	FLU	OXO	FLU
5	0.76	98 ± 1	99 ± 1	11.5	6.5
10	0.54	91 ± 7	94 ± 3	12.4	6.8
15	0.43	86 ± 1	88 ± 1	13.1	7.3
20	0.34	80 ± 5	83 ± 1	14.1	7.7
30	0.21	76 ± 1	78 ± 1	14.9	8.2

[a] 200 mg of salmon sample spiked with 200 μg kg^{-1} of OXO and FLU; volume of supramolecular solvent = 400 μL.
[b] n = 3.

Table 3
Mean recoveries and method quantitation limits obtained for oxolinic acid and flumequine as a function of the volume of supramolecular solvent used for extraction.

Volume of supramolecular solvent (μL)	Recovery[a] ± s[b] (%)		MQL (μg kg^{-1})	
	OXO	FLU	OXO	FLU
100	35 ± 8	34 ± 10	8.1	4.7
200	77 ± 3	74 ± 1	7.3	4.3
300	89 ± 5	99 ± 1	9.5	4.8
400	98 ± 1	100 ± 1	11.5	6.4
500	99 ± 1	99 ± 2	14.3	8.0

Table 4
Mean recoveries[a] and standard deviations ($n = 3$) obtained for oxolinic acid and flumequine using different operational conditions.

Extraction time (min)	[b]Recovery ± s (%)		Centrifugation time (min)	[c]Recovery ± s (%)	
	OXO	FLU		OXO	FLU
5	69 ± 1	71 ± 2	5	68 ± 1	61 ± 1
10	89 ± 3	91 ± 1	10	89 ± 2	92 ± 4
15	100 ± 2	100 ± 5	15	100 ± 2	100 ± 5
30	99 ± 1	99 ± 1	30	100 ± 1	98 ± 3

[a] 200 mg of salmon spiked with 200 μg kg^{-1} of OXO and FLU; volume of supramolecular solvent = 400 μL; vibration motion = 2500 rpm, and centrifugation rate = 15,000 rpm.
[b] Centrifugation time = 15 min.
[c] Extraction time = 15 min.

from water solutions in which the pH was adjusted between 1 and 4 with hydrochloric acid. Then, 400 μL aliquots of these solvents was used for extraction. Both recoveries and reproducibility remained constant in the interval investigated.

The time required to reach equilibrium conditions for the extraction, under the defined operation conditions (vibration motion = 2500 rpm), was 15 min. Recoveries decreased at lower extractions times (e.g. they were 69 ± 1 and 71 ± 2 for OXO and FLU, respectively, after 5 min of extraction). Once the extraction was completed, samples were centrifuged at different rotation rates and times to separate the material suspended in the supramolecular extract. Centrifugation of the sample at 15,000 rpm for 15 min is recommended.

Time used for extraction and centrifugation of samples influenced the recoveries obtained for AQAs (Table 4). Equilibrium conditions were reached after 15 min of vortex shaking-assisted extraction (vibration motion = 2500 rpm). Recoveries kept about 100% at higher extraction times and decreased at lower ones. The minimum centrifugation time required to reach quantitative recoveries was 15 min. At shorter rotation times, no effective separation of the supramolecular extract from the sample particles was obtained which resulted in decreased recoveries for both OXO and FLU. Recoveries were not affected by the rotation rate in the range studied (13,000–17,000 rpm).

The influence of matrix components on recoveries was investigated by extracting different amounts of fortified salmon samples. Table 5 shows the results obtained in this study. Recoveries were quantitative up to around 250 mg and then progressively decreased at higher sample amounts as a result of the deficient solvation of the sample at solvent volume/sample amount ratios below around 1.5. So, 200 mg of muscle samples is recommended for the extraction of the target antibiotics.

To evaluate the representativity of such low amount of salmon, the variances obtained for the measurement of OXO and FLU in 200 mg-subsamples ($n = 11$) of a certified reference material (CRM BCR®-725) were compared with those reported by the different laboratories (variances: 52–548 μg^2 kg^{-2} for OXO and 207–1918 μg^2 kg^{-2} for FLU; 500–1000 mg-subsamples) [42]. These laboratories used a wide range of sample treatment procedures including extraction with methanol, dichloromethane, ethyl acetate, or basic solutions (alone or mixed with organic solvents); clean-up with SPE with C_{18} materials, protein precipitation or several back extractions, and solvent evaporation. The variances obtained using the developed method (262 μg^2 kg^{-2} for OXO and 918 μg^2 kg^{-2} for FLU) were within the interval reported for the CRM material thus indicating that no problems of homogeneity were derived from the amount of sample treated by the recommended procedure in this manuscript. Additionally, salmon samples were fortified with OXO and FLU at the same concentration level than that certified for the CRM (i.e. 600 and 1170 μg kg^{-1} for OXO and FLU, respectively) and analyzed. No statistically significant differences between the variances obtained from 200 mg of sample and CRM were observed by applying a Fisher test [43]. The experimental F-values were 2.27 and 1.02 for OXO and FLU, respectively, and were below the critical F-value (2.98; $n_1 = n_2 = 11$; significant level = 0.05).

3.2. Analytical performance

Analytical performance of the developed method was assessed according to the guidelines established in the 2002/657/EC Commission Decision [37]. This Decision provides rules for the analytical methods to be used in the determination of veterinary drug residues in animal products.

3.2.1. Sensitivity and linearity

Calibration curves for the target analytes were run using standard solutions prepared in the supramolecular solvent. Retention times for analytes, expressed in min, were 3.5 for OXO and 7.5 for FLU. No appreciable changes in these retention times were observed after at least 32 consecutive injections; their relative standard deviations ($n = 32$) being 1.7% and 1.1% for OXO and FLU, respectively. The sensitivities, expressed as the slope of the calibration curves, were 1.40 ± 0.03 pg^{-1} for OXO and 1.61 ± 0.01 pg^{-1} for FLU, and linearity was obtained in the intervals 0.1–20 and 0.06–20 ng (correlation coefficient = 0.9993 and 0.99990), respectively. The linear range was confirmed by the visual inspection of the plot residuals versus analyte amount [44]; the residuals were randomly scattered within a horizontal band and a random sequence of positive and negative residuals was obtained. The detection limits of the method, MDL [45], were calculated from six independent complete analyses (experimental procedure in Section 2.4) of salmon, sea trout, sea bass, gilt-head bream, megrim and prawn samples, containing no detectable levels of OXO or FLU, by

Table 5
Mean concentrations of oxolinic acid and flumequine found in fortified salmon samples, and recoveries obtained, as a function of the amount of sample analyzed.

Sample amount (mg)	Solvent volume/sample amount (μL mg^{-1})	OXO		FLU	
		Concentration found[a] ± s[b] (μg kg^{-1})	Recovery[a] ± s[b]	Concentration found[a] ± s[b] (μg kg^{-1})	Recovery[a] ± s[b]
100	4.0	202 ± 3	101 ± 1	200 ± 1	100.3 ± 0.6
200	2.0	196 ± 1	98.0 ± 0.5	200 ± 2	100 ± 1
250	1.6	198 ± 5	99 ± 2	199 ± 2	99 ± 1
300	1.3	162 ± 4	81 ± 2	160 ± 10	80 ± 5
400	1.0	133 ± 2	66 ± 1	156 ± 1	78.2 ± 0.6

using a signal-to-noise ratio of 3 (the ratio between the peak areas for each target analyte and peak area of noise). The quantitation limits of the method (MQL) were calculated in a similar way by using a signal-to-noise ratio of 10. The MDLs found for OXO were in the interval 2–3.6 $\mu g\,kg^{-1}$, except for salmon (6.6 $\mu g\,kg^{-1}$), while those found for FLU ranged from 1.5 to 4.5 $\mu g\,kg^{-1}$. The MQLs for OXO and FLU ranged between 6.5 and 12 (22 for salmon) $\mu g\,kg^{-1}$ and 5 and 15 $\mu g\,kg^{-1}$, respectively. Consequently, the method permitted the quantitation of OXO and FLU at concentrations far below the current MRLs established by the EU for these antibiotics in seafood; i.e. 100 $\mu g\,kg^{-1}$ for OXO and 600 $\mu g\,kg^{-1}$ for FLU (Council Directives 1356/2005 and 1181/2002, respectively).

3.2.2. Selectivity

The possible interference from matrix components was evaluated by two approaches. Firstly, six blank samples of each seafood tested, i.e. salmon, sea trout, sea bass, gilt-head bream, megrim and prawn, were analyzed and the chromatograms obtained were checked for any peaks in the regions of interest where the target analytes were expected to elute. Secondly, calibration curves for OXO and FLU were run from 200 mg samples fortified with known amounts of the target analytes (25–2000 $\mu g\,kg^{-1}$), extracted using 400 μL of the supramolecular solvent and their slopes compared with those obtained from standards in supramolecular solvent. No peaks at the retention times of OXO and FLU appeared in the chromatograms obtained from the blank samples analyzed and no statistically significant differences between the slopes obtained from standards and those obtained from the samples were observed by applying a Student t test [46]. For example, the slopes and correlation coefficients ($n = 10$) obtained from salmon samples for OXO and FLU were 1.36 ± 0.03 and 1.56 ± 0.05, respectively and those obtained for standards in supramolecular solvent 1.40 ± 0.03 for OXO and 1.61 ± 0.01 for FLU. The experimental t-values were in the interval 0.03–1.42 and were below the critical t-value (3.36 significant level = 0.01).

Veterinary drug residues that can be encountered in seafood with the target analytes were also investigated as potentially interfering substances. Drugs tested include chemically related compounds, namely acidic (nalidixic acid) and piperazinyl (sarafloxacin and enrofloxacin) quinolones and other fluorescent substances used as antimicrobial agents in aquaculture, i.e. tetracyclines (chlortetracycline and oxytetracycline) and fenicols (florfenicol). The possible interference of these substances in the identification and quantification of OXO and FLU was assessed by analyzing blank salmon samples fortified with 1600 $\mu g\,kg^{-1}$ of foreign specie. Sarafloxacin, enrofloxacin, chlortetracycline, oxytetracycline and florfenicol did not provide any signal at the excitation and emission wavelengths used for the fluorescence detection of OXO and FLU (i.e. 325 and 360 nm, respectively) whereas the peak maximum corresponding to nalidixic acid separated 1.8 and 2.3 min from those corresponding to OXO and FLU, respectively. So, no interference from this acidic quinolone, both in the identification and quantitation of the target analytes, is expected to occur.

3.2.3. Precision

The precision of the method was assessed by applying the whole analytical process to eleven salmon samples spiked with 200 $\mu g\,kg^{-1}$ of AQAs. The repeatability, expressed as relative standard deviation was 3.6% for OXO and 2.3% for FLU.

3.2.4. Trueness

This parameter was evaluated by analyzing a Certified Reference Material (CMR, BCR®-725), consisting of lyophilised salmon tissue material, skin included, with a certified content of OXO and FLU of 600 $\mu g\,kg^{-1}$ (uncertainty = 100 $\mu g\,kg^{-1}$) and 1170 $\mu g\,kg^{-1}$ (uncertainty = 210 $\mu g\,kg^{-1}$), respectively. The results obtained, expressed as mean values ($n = 11$) ± the expanded uncertainty, estimated as $k{\cdot}s$ [47] (coverage factor $k = 2$ for a significant level of 0.05), were 580 ± 32 $\mu g\,kg^{-1}$ for OXO and 1167 ± 60 $\mu g\,kg^{-1}$ for FLU. The trueness calculated was −3.3% for OXO and −0.3% for FLU (according to the 2002/657/EC Commission Decision [37], the trueness of quantitative methods should be comprised between −20% and 10% for analyte concentrations equal or higher than 10 $\mu g\,kg^{-1}$).

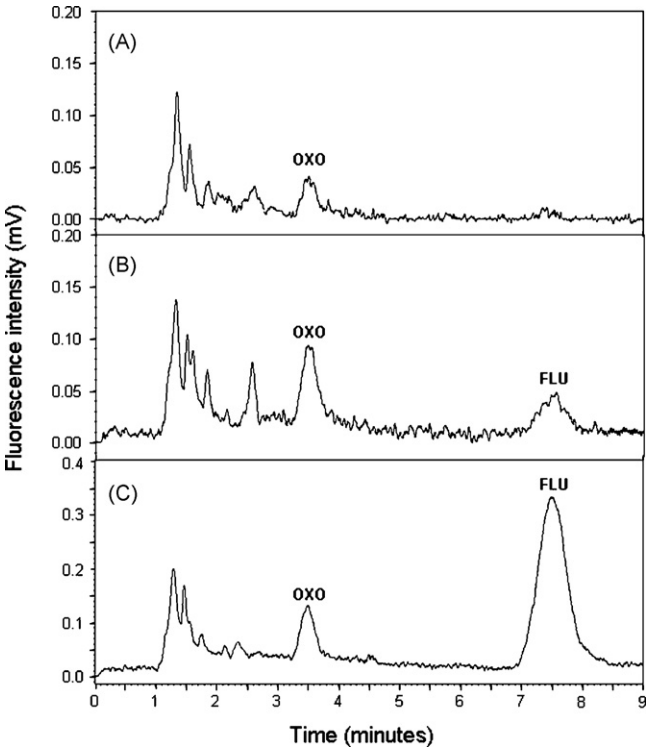


Fig. 2. Chromatograms obtained from 200 mg of megrim sample (A) non-spiked and (B and C) spiked with the target analytes at two concentration levels: (B) 50 $\mu g\,kg^{-1}$ OXO and 50 $\mu g\,kg^{-1}$ FLU and (C) 100 $\mu g\,kg^{-1}$ of OXO and 600 $\mu g\,kg^{-1}$ FLU. Chromatographic conditions as specified in Section 2.4.3.

3.2.5. Ruggedness

Blank salmon samples fortified with 200 $\mu g\,kg^{-1}$ OXO and FLU were analyzed introducing deliberate small variations in the extraction method and their effect on the accuracy of the results was evaluated using the Youden approach [37]. Experimental conditions were varied in an order of magnitude that matched the deviations usually encountered among laboratories. The following conditions were tested: (a) THF used for preparation of the supramolecular solvent: 5% and 5.8%; (b) volume of solvent used for extraction: 340 and 460 μL; (c) vibration motion: 13 and 17 min at a rate of 2100 and 2500 rpm; (d) centrifugation for 13 and 17 min at a rate of 13,000 and 17,000 rpm. The averages obtained at the two nominal values of each variable were compared and the standard deviation of the differences for all the variables, S_{Di}, was calculated for OXO ($S_{Di} = 7.2$ ng g^{-1}) and FLU ($S_{Di} = 4.5$ ng g^{-1}). The differences between these standard deviations and those of the method carried out under repeatability conditions were found to be not statistically significant by applying a Fisher test [43]. The calculated F-values were 3.78 and 2.11 and were below the critical t-value (4.24), being significance established at 0.05 levels. Therefore the method is considered robust against the chosen modifications.

Table 6
Mean concentrations and recoveries, along with their respective standard deviations, obtained from the analysis of OXO and FLU in fortified seafood samples.

Sample	Concentration found[a] ± s[b] (μg kg^{-1})		Recovery ± s[b] (%)	
	OXO	FLU	OXO	FLU
Fishes				
Salmon	49.8 ± 0.4[c]	50.4 ± 0.9[c]	99.6 ± 0.8[c]	101 ± 2[c]
	100 ± 1[d]	594 ± 4[d]	100 ± 1[d]	99.1 ± 0.7[d]
Sea trout	50 ± 2[c]	50.5 ± 0.7[c]	100 ± 3[c]	101 ± 1[c]
	100 ± 1[d]	597 ± 4[d]	100 ± 1[d]	99.5 ± 0.6[d]
Sea bass	49 ± 2[c]	49.9 ± 0.7[c]	99 ± 3[d]	100 ± 1[d]
	100 ± 3[d]	609 ± 4[d]	100 ± 3[d]	101.4 ± 0.7[d]
Gilt-head bream	50.8 ± 0.5[c]	50 ± 1[c]	102 ± 1[c]	100 ± 2[c]
	102 ± 2[d]	605 ± 1[d]	102 ± 2[d]	100.8 ± 0.2[d]
Megrim	87 ± 3[c]	50.6 ± 0.7[c]	99 ± 2[c]	101 ± 1[c]
	140 ± 7[d]	609 ± 3[d]	103 ± 5[d]	101.5 ± 0.6[d]
Seafood				
Prawn	51 ± 3[c]	50 ± 2[c]	102 ± 5[c]	100 ± 3[c]
	100 ± 2[d]	608 ± 1[d]	100 ± 2[d]	101.4 ± 0.2[d]

[a] Mean of three independent determinations.
[b] Standard deviation.
[c] About 200 mg of sample spiked with 50 μg kg^{-1} OXO and 50 μg kg^{-1} FLU.
[d] About 200 mg of sample spiked with 100 μg kg^{-1} OXO and 600 μg kg^{-1} FLU.

3.2.6. Stability

The stability of the analytes was tested both in standard solutions and samples. Stock solutions containing 100 mg L^{-1} of OXO or FLU were stable for one month as they were stored at 4 °C and working solutions containing 1.5 mg L^{-1} of both analytes were stable for 12 h at room temperature. To assess the stability of OXO and FLU in the samples, a blank salmon sample was divided into aliquots and each one was fortified with 200 μg kg^{-1} OXO and FLU. Then, the aliquots were stored at −20 °C and analyzed after one, two and three weeks. Under these conditions, the analytes were stable for two weeks. After three weeks of storage, the concentration of both OXO and FLU in the sample decreased to about 150 μg kg^{-1}.

3.2.7. Decision limit and detection capability

The decision limit (CCα) means the limit at and above which it can conclude with an error probability of α that a sample is not compliant. It was established by analyzing 20 blank salmon samples fortified with OXO and FLU at the permitted limit (i.e. 100 and 600 μg kg^{-1}, respectively), and it was calculated from the concentration at the permitted limit plus 1.64 times the standard deviation of the blank salmon sample measurements (α = 5%). The decision limits obtained for OXO and FLU were 104 and 611 μg kg^{-1}, respectively. The detection capability (CCβ) is the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of β. It was calculated as the value of the decision limit plus 1.64 times the standard deviation of the measurements obtained from the analysis of 20 blank salmon samples fortified with 104 μg kg^{-1} OXO and 611 μg kg^{-1} (β = 5%). The obtained CCβ values were 109 μg kg^{-1} for OXO and 622 μg kg^{-1} for FLU.

3.3. Analysis of aquaculture seafood

A variety of aquaculture fishes (salmon, sea trout, sea bass, gilt-head bream and megrim) and shellfishes (prawn) were bought in supermarkets in Córdoba (Spain) and their muscles spiked at two concentration levels: (1) 100 μg kg^{-1} OXO and 600 μg kg^{-1} FLU, the current European MRLs for these antibiotics in seafood, and (2) 50 μg kg^{-1} OXO and FLU, concentrations below these MRLs. In parallel, non-fortified muscle samples were also analyzed. No detectable concentrations of FLU were found in any of the non-fortified samples. OXO was only detected in the megrim sample at a concentration of 37 ± 2 μg kg^{-1}, which is below the current European MRL for this antibiotic and above the MQL of the developed method for this matrix (8 μg kg^{-1}). The identification of the analyte was confirmed by co-chromatography comparing the retention time and the peak width at half-maximum height obtained for OXO from non-spiked and spiked samples. Fig. 2 shows the chromatograms obtained from the megrim sample (A) non-fortified and fortified with (B) 50 μg kg^{-1} OXO and FLU or (C) 100 μg kg^{-1} OXO and 600 μg kg^{-1} FLU. The retention times obtained for OXO from the non-fortified and the two fortified samples were within a margin of 2% (2002/657/EC Commission Decision establishes a margin of 5% for the identification of analytes by co-chromatography [37]), and the widths at half-maximum height of the peaks recorded from the spiked samples were between 95% and 106% of the corresponding widths for the non-spiked sample (2002/657/EC Commission Decision establishes a range of 90–110% [37]). No detectable concentrations of OXO were found in the further non-fortified samples analyzed.

The results obtained for the fortified seafood samples are shown in Table 6. Both the concentrations of analytes and recoveries were expressed as the mean value of three independent determinations, besides their corresponding standard deviations. Recoveries and their relative standard deviations were in the intervals 99–102% and 0.2–5%, respectively.

Fig. 2 compares the chromatograms obtained from a standard solution containing 50 μg L^{-1} of OXO and 300 μg L^{-1} of FLU (A) with those obtained from the analysis of a salmon (B) and prawn (C) sample fortified with 100 μg kg^{-1} OXO and 600 μg kg^{-1} FLU. No interference from matrix components was detected for any of the samples analyzed.

4. Conclusions

Supramolecular solvents consist of amphiphilic nanostructures that provide multiple binding sites and regions of different polarity. These outstanding properties make them suitable to extract a variety of analytes with high efficiency and render them ideal for microextractions. In this research, the suitability of supramolecular solvents to be used in solid sample microextractions has been firstly proved from a practical point of view through the development of a reliable, rapid and low-cost method for the determination of two widely used quinolone antibiotics in fishes and shellfishes. The proposed sample treatment highly surpasses to the previously reported ones in: (1) extraction efficiency; recoveries are quantitative and independent of the composition of sample matrix, (2) simplicity; extractions are performed in a single step and nei-

ther clean-up nor solvent evaporation is required and (3) sample throughput; because of the short time spent in the sample treatment (about 30 min) and the possibility of treating several samples simultaneously. The high capability of DeA reverse micelle-based solvents to extract OXO and FLU residues from aquaculture products can be explained on the basis of the formation of DeA-OXO and DeA-FLU mixed aggregates through hydrophobic interactions and hydrogen bonding. Most veterinary drugs are amphiphilic and contain hydrogen bond donors (OH) and/or acceptors in their molecular structures, therefore the use of DeA reverse micelle-based solvents is expected to be a suitable general strategy for the extraction of drug residues (e.g. antibiotics belonging different structural groups, anti-inflammatories, steroid hormones, etc.) from products of animal origin.

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