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Multiresidue analysis of sulfonamides in meat by supramolecular solvent microextraction, liquid chromatography and fluorescence detection and method validation according to the 2002/657/EC decision

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ABSTRACT

A multiresidue method was described for determining eight sulfonamides, SAs (sulfadiazine, sulfamerazine, sulfamethoxypyridazine, sulfachloropyridazine, sulfadoxine, sulfamethoxazole, sulfadimethoxine and sulfaquinoxaline) in animal muscle tissues (pork, chicken, turkey, lamb and beef) at concentrations below the maximum residue limit (100 $\mu g\,kg^{-1}$) set by the European Commission. The method was based on the microextraction of SAs in 300-mg muscle samples with 1 mL of a supramolecular solvent made up of reverse micelles of decanoic acid (DeA) and posterior determination of SAs in the extract by LC/fluorescence detection, after in situ derivatization with fluorescamine. Recoveries were quantitative (98–109%) and matrix-independent, no concentration of the extracts was required, the microextraction took about 30 min and several samples could be simultaneously treated. Formation of multiple hydrogen bonds between the carboxylic groups of the solvent and the target SAs (hydrogen donor and acceptor sum between 9 and 11) were considered as the major forces driving microextraction. The method was validated according to the European Union regulation 2002/657/EC. Analytical performance in terms of linearity, selectivity, trueness, precision, stability of SAs, decision limit and detection capability were determined. Quantitation limits for the different SAs ranged between 12 $\mu g\,kg^{-1}$ and 44 $\mu g\,kg^{-1}$, they being nearly independent of matrix composition. Repeatability and reproducibility, expressed as relative standard deviation, were in the ranges 1.8–3.6% and 3.3–6.1%. The results of the validation process proved that the method is suitable for determining sulfonamide residues in surveillance programs.

1. Introduction

Sulfonamides (SAs) are among the most used veterinary drugs owing to their broad-spectrum antimicrobial activity, effectiveness as growth promoters of livestock and low price [1]. The possible presence of residues of these drugs in edible animal products is of major concern because they can cause serious health problems in humans, such as allergic or toxic reactions [2,3]. They are frequently detected in a variety of food-producing animals [4–6] at concentrations above the maximum residue limit (MRL) set by different countries (100 $\mu g\,kg^{-1}$ for total SAs [7–9]).

Quantification of these wide polarity range compounds in edible animal products is a challenging task. The technique most extensively used for this purpose is liquid chromatography (LC) coupled to mass spectrometry (MS) [1,4,10–12] or fluorescence (FL) detection [13–16]. LC/MS using matrix-matched calibration is strongly recommended to determine veterinary residues because the high sensitivity and specificity it provides [17,18]. Derivatization is required to determine SAs using fluorescence detection. Although both LC/MS and LC/FL are considered as reference for many matrices, a quick, widely applicable sample treatment is lacking.

Most sample handling procedures reported for SAs involve the use of repetitive extractions followed by clean up and concentration steps. Because of the absence of suitable solvents to quantitatively extract such wide polarity range analytes, extraction with large solvent volumes (typically 15–140 mL per sample [4,5,10,13–15,19,20]) assisted by auxiliary energies (i.e. ultrasounds [5,2], microwaves [21] or pressure [6]) is a common strategy. The most frequently used solvent is acetonitrile, alone or in the presence of salts. Alternative solvents such as supercritical fluids have been also reported [22]. Clean up is usually carried out by liquid–liquid (LLE) [4,5,10,13–15] and/or solid phase (SPE) [6,10,19] extraction, while preconcentration is generally achieved by solvent evaporation. Simplified sample treatments such as matrix solid-phase dispersion extraction (MSPDE) [23], dispersive SPE [16] or liquid phase microextraction (LPME) [22] permit to reduce analysis time and solvent consumption (5–7 mL). How-

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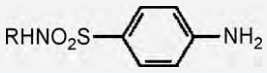
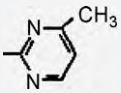
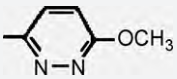
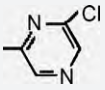
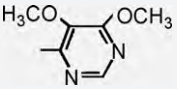
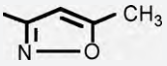
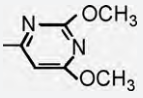
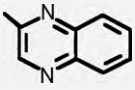
Table 1
Chemical structure, octanol–water partition coefficients ($\log K_{ow}$), ionization constants ($pK_{a,1}$ and $pK_{a,2}$) and number of donor and acceptor groups for sulfonamides.

General structure	Sulfonamide antibiotic	R	[a]Log K_{ow}	[b]p$K_{a,1}$	[b]p$K_{a,2}$	[b]Hydrogen donor and acceptor sum
	Sulfadiazine (SD)		−0.09	1.64	6.50	9
	Sulfamerazine (SM)		0.14	1.64	6.98	9
	Sulfametoxypyridazine (SMP)		0.32	2.18	7.19	10
	Sulfachloropyridazine (SCP)		0.31	1.88	5.90	9
	Sulfadoxine (SDX)		0.70	1.59	6.16	11
	Sulfamethoxazole (SMT)		0.89	1.39	5.81	9
	Sulfadimethoxine (SDM)		1.63	1.30	6.21	11
	Sulfaquinoxaline (SQ)		1.68	1.77	5.65	9

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

ever, many of the above approaches have been optimized and validated for a unique food matrix (i.e. chicken or pork muscle [6,16,2,23,24]) and others provide low and/or matrix-dependent recoveries [5,10,14,20].

This article deals with the assessment of supramolecular solvents (SUPRAS) for the extraction of sulfonamide residues in edible animal tissues in order to develop a quick, simple and efficient sample treatment procedure that along with a simple technique, LC–FL, provides a low-cost method able to fulfil the requirements of the 2002/657/EC decision.

SUPRAS are nano-structured liquids generated from amphiphiles through a sequential, self-assembly process occurring on two scales, molecular and nano [25]. Basic components of SUPRAS are water and amphiphiles arranged in ordered structures (e.g. aqueous or reverse micelles and vesicles). These solvents are well-known to the analytical community and have been used for long years in extraction processes under different names (e.g. cloud point technique [26–28] or coacervates [29,30]). The outstanding properties of SUPRAS for extraction processes derive from the special structure of the ordered aggregates that constitute them and the large concentration of amphiphiles in the solvent (0.1–1 mg μL^{-1}). Both features determine the high capability of solubilisation of SUPRAS for a variety of solutes, which renders them ideal for multiresidue analysis in the environmental and agrifood fields, an outstanding property hardly explored so far.

Application of SUPRAS made up of non-ionic micelles to the extraction of hydrophobic organic compounds and metals, mainly from environmental and biological liquid samples, is a consolidated area and excellent published reviews compile this information [31–34]. Effective extraction of polar compounds by SUPRAS depends on the nature of surfactant polar groups and the type of interactions they can establish with analytes (viz. ionic, hydrogen bonding, π-cation, π–π, etc.) [31]. A great deal of research has been conducted in the last decade related to the extraction of polar compounds by SUPRAS; the major advances being obtained with the development of SUPRAS based on ionic surfactants and other aggregates than aqueous micelles (viz. reverse micelles and vesicles) [35,36]. Applications include the extraction of pesticides [37], surfactants [38], bioactive compounds [29], dyes [39], endocrine disruptors [40] and phenols [41], among others. Only a few applications have been devoted to the extraction of residues in food (viz. pesticides in fruits [42] and cereals [43], endocrine disruptors in canned foods [44], drug residues in seafood [45], contaminants in drinks [23]) and most of them involve the extraction of 1–2 analytes.

SUPRAS have been used for the first time in this work for the microextraction of multiresidues of SAs in animal muscle tissues Table 1 shows the molecular structures and physico-chemical properties of the SAs assayed. The SUPRAS used for extraction was made up of reverse micelles of decanoico acid (DeA) [36]. The solvent was expected to provide high extraction efficiencies for SAs on the basis of: (1) the strong hydrogen bonds that can be established between sulfonamides and carboxylic groups and (2) the high concentration of DeA in the SUPRAS (0.76 mg μL^{-1}). The study included the optimization of the variables affecting the efficiency of the extraction and the yield of the derivatization reaction. The proposed method was validated following the guidelines established in the 2002/657/EC Commission Decision [46], and SAs were determined in different edible animal tissues (muscle of pork, chicken,

turkey, lamb and beef). The main results obtained are presented and discussed below.

2. Experimental

2.1. Chemicals

All the chemicals were of analytical reagent-grade and were used as supplied. Phosphoric acid and LC-grade acetonitrile, methanol and tetrahydrofuran (THF) were obtained from Panreac (Barcelona, Spain) and decanoic acid (DeA) from Sigma (St. Louis, MO). Ultra-high-quality water was obtained from a Mili-Q water purification system (Millipore, Madrid, Spain). A fluorescamine solution (5%, w/v) was prepared by dissolving the reagent [Fluka (Buchs, Switzerland)] in acetone. This solution was stable for at least 3 months. Sulfonamides [sulfadiazine (SD), sulfamerazine (SM), sulfamethoxypyridazine (SMP), sulfachloropyridazine (SCP) sulfadoxine (SDX), sulfamethoxazole (SMT), sulfadimethoxine (SDM) and sulfaquinoxaline (SQ)] were supplied by Riedel-de Haën (Seelze, Germany). Stock solutions of 100 mg L^{-1} individual SAs were prepared in methanol and stored at −20 °C. A 2-mL working solution containing 5 mg L^{-1} of each SA was daily prepared by appropriate dilution of the stock solutions with the supramolecular solvent. Six calibration solutions, containing amounts of SAs in the range 2–360 ng were prepared in 0.5-mL volumetric flasks from the working solution by dilution with the supramolecular solvent.

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_8 column (5 μm, 250 mm × 4.0 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 × 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 8 meat 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 closed glass vial to avoid THF losses and stored at 4 °C. The volume of solvent obtained can be adjusted at will by choosing an appropriate, constant DeA/THF/water proportion.

2.4. Determination of SAs in meat samples

2.4.1. Sample preparation

Meat samples (pork, chicken, turkey, lamb and beef) were bought in local supermarkets and stored at −20 °C until analysis. After thawing, about 300 g of sample was chopped and homogenized using a homogenizer-disperser. Then, portions of about 300 mg were taken for analysis and recovery experiments, which were performed in triplicate. Spiking of chopped samples (300 mg) was done by adding volumes in the range 4–230 μL of a solution containing SAs (3 mg L^{-1} each) in methanol. The spiked samples were allowed to stand at room temperature for 15 min before analysis. Spiking for sample representativity studies was made using 4 g aliquot samples.

2.4.2. Microextraction of SAs

A portion (about 300 mg) of chopped sample and 1 mL of supramolecular solvent were mixed in a 2-mL microtube Safe-Lock from Eppendorf Ibérica (Madrid, Spain). A microPTFE-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 30 min. Then, the mixture, thermostated at 15 °C, was centrifuged at 15,000 rpm (16,720 × *g*) for 10 min. The supramolecular extract was withdrawn using a micropipette and transferred to an auto-sampler vial.

2.4.3. Derivatization

Calibration solutions or supramolecular extracts (140 μL) containing amounts of SAs in the interval 0.6–100 ng and 60 μL of fluorescamine solution (5%, w/v) were placed in auto-sampler vials. Then, they were vortex-shaken at 2500 rpm for 10 s, placed in the auto-sampler and allowed to stand at room temperature for 70 min before injecting an aliquot (30 μL) of the resulting solution into the liquid chromatograph.

2.4.4. Liquid chromatography–fluorescence detection

Derivatized SAs were separated by LC and quantified by fluorescence measurements performed at 405/490 nm excitation/emission wavelengths. The mobile phase consisted of acetonitrile/concentrated H_3PO_3 (99.91:0.09, v/v) (A) and 15 mM of phosphate buffer (pH = 3) (B). Two sequential isocratic elution steps were used: (1) 0–32 min: 25% A and 75% B for elution of matrix components and (2) 32–65 min: 35% A and 65% B for separation of SA derivatives. After each run, initial elution conditions were restored for 5 min before the next injection. The flow-rate of the mobile phase and the temperature of the stationary-phase column were kept constant at 1.1 mL min^{-1} and 40 °C, respectively. Calibration curves were run from six calibration solutions.

3. Results and discussion

3.1. Supramolecular solvent-based microextraction of SAs

Reverse micelles of DeA in THF self-assemble in the presence of water undergoing phase separation from the bulk homogeneous solution as an immiscible liquid made up of large reverse micelles, THF and water. The SUPRAS is produced within a wide range of THF/water ratios (e.g. from 5/95 to 80/20, v/v) and DeA concentrations below 8% (w/v). Reverse micelles are only formed from decanoic acid ($pk_a = 4.8 \pm 0.2$), so maximal production of this solvent occurs at pH below 4.

SUPRAS composition mainly depends on the THF/water ratio (v/v) in the bulk solution. It keeps constant at a given THF/water ratio, independently of the amount of DeA in the mixture. The volume of solvent produced is linearly and exponentially dependent on the amount of decanoic acid and percentage of THF, respectively.

So, the amount of DeA in the solvent decreases as the concentration of THF increases (e.g. 0.76 mg μL^{-1} and 0.21 mg μL^{-1} for 5% and 30% of THF, respectively [45]).

3.1.1. Optimization

The extraction of SAs from meat samples involves the breaking down of hydrogen bonds between the amide groups of matrix proteins and the amine moiety of analytes and the solubilisation of free SAs in the SUPRAS based on hydrogen bond and hydrophobic interactions.

Optimization of SUPRAS extraction was carried out using pork tissue (100–300 mg), which was used as a sample model, spiked with the eight SAs at a concentration of 200 $\mu g\,kg^{-1}$ each. Experiments were made in triplicate. The percentages of protein in the samples analysed were within the interval 16.6–21.8% (w/w), 16.9% for pork [47], and their fact content mainly depended on the part of the animal being analysed (viz. it varies from 2.9% to 23.4%). Selection of the optimal conditions was based on the recoveries and method quantitation limits (MQLs) obtained. The level of interferences extracted in the supramolecular solvent was also taken into account. MQLs were calculated from the instrumental quantitation limits (80 pg for SD and SMT, 90 pg for SM and SDX, and 100 pg, 150 pg, 190 pg and 250 pg for SDM, SCP, SMP and SQ, respectively), the volume of supramolecular solvent used for extraction, the recoveries obtained and the weight of sample used for analysis. The variables investigated were: composition and volume of extractant, pH, 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 meat analysis, after applying the sample treatment recommended in Section 2.4.1, was also evaluated.

SUPRAS of different compositions were prepared from a constant concentration of DeA [8% (w/v)] and variable THF/water ratios (5/95 to 30–70, v/v)] and a volume of 1 mL was used to extract SAs from 300-mg pork samples. In parallel, non-fortified samples were analyzed to assess the influence of the SUPRA composition on the cleanliness of extracts. The concentration of DeA in these solvents ranged between 0.21 mg μL^{-1} and 0.76 mg μL^{-1}. SUPRAS composition was found not to influence SA recoveries however, extracts became dirtier as the THF in the SUPRAS increased which resulted in interferences in the chromatographic determination of SAs. The solvent providing the cleanest extracts was that produced from a 5/95 (v/v) THF/water ratio, and therefore, it was selected for further studies.

Table 2 shows the recoveries and MQLs obtained for SAs as a function of the volume of SUPRAS used for extraction. Recoveries were nearly 100% for all the SAs and MQLs ranged between 13 $\mu g\,kg^{-1}$ and 40 $\mu g\,kg^{-1}$ (i.e. lower than the MRLs legislated for meat, 100 $\mu g\,kg^{-1}$ [7–9]) as 1 mL of SUPRAS was used. So, this volume was selected as optimal.

The pH had not influence on SA extraction. This parameter was investigated by producing the supramolecular solvent from water solutions in which the pH was adjusted between 1 and 4 with hydrochloric acid. Then, 1 mL aliquots of these solvents were used for extraction. Both recoveries and MQLs kept constant in the interval investigated.

Table 3 shows the influence of the time of extraction and centrifugation on SA recoveries. About 30 min of extraction were required to achieve recoveries nearly 100% for all the sulfonamides. Effective separation of particles from the SUPRAS was reached after centrifugation for 10 min.

To evaluate the representativity of the amount of pork sample used for analysis, the variances obtained for the measurement of SAs in 300-mg pork subsamples fortified with 100 $\mu g\,kg^{-1}$ of each SA were compared with those obtained from the measurement of 300-mg aliquots taken from a 4 g pork sample spiked at the same concentration level. No statistically significant differences between both variances were observed by applying a Fisher test [48]. The experimental F-values were in the interval 1.09–1.59 and were below the critical F-value (2.98, $n_1 = n_2 = 11$, significant level = 0.05).

3.2. Derivatization of SAs in the SUPRAS

Sulfonamides were derivatized in the SUPRAS extract using fluorescamine [13]. Because of both the concentration of reagent and time necessary to obtain maximal product yield have been known to highly depend on the composition of the reaction medium [13,14,49], we studied how these variables behave in the SUPRAS. Experiments performed to select the optimal concentration of fluorescamine were as follows: variable volumes of fluorescamine solution (5%, w/v) in acetone were added to standard solutions containing 60 ng of each SA dissolved in the supramolecular solvent (total fluorescamine + standard solution volume = 200 μL). After 70 min of reaction, the mixture was analysed by LC–FL. Peak areas increased as the concentration of fluorescamine did up to 1.5% (w/v) and they slightly decreased at higher reagent amounts probably due to quenching effects produced by fluorescamine hydrolysis products [49]. A reagent concentration of 1.5% (w/v) was selected as optimum because it provides the maximum sensitivity for the determination of all the analytes tested.

Kinetic curves obtained for the reaction of derivatization of the different SAs investigated were similar to those previously reported in hydro-organic solutions [13]. Maximal signals were obtained at reactions times between 50 min and 100 min. In practice, it is convenient to match the time for derivatization and chromatographic run in order to automate the first one in the auto-sampler [15], so a reaction time of 70 min was selected.

No influence of the matrix components extracted from samples of pork, chicken, turkey, lamb and beef on the yield of the reaction of derivatization was observed. Signals obtained for SA-fortified pure and sample SUPRAS extracts were similar.

3.3. Validation

Validation of the proposed approach was carried out according to the guidelines established by the 2002/657/EC Commission Decision [46] for quantitative methods of analysis. This decision provides rules for the analytical methods to be used for determining veterinary drug residues in products of animal origin.

3.3.1. Sensitivity and linearity

Calibration parameters and detection (MDLs) and quantitation (MQLs) limits for the determination of SAs by the proposed method are shown in Table 4. Calibration curves were run using six standard solutions prepared in the supramolecular solvent. The linear range was confirmed by the visual inspection of the plot residuals versus analyte amount [50]; the residuals were randomly scattered within a horizontal band and a random sequence of positive and negative residuals was obtained. The MDLs were calculated from six independent complete analysis (experimental procedure in Section 2.4) of pork, chicken, turkey, lamb and beef samples, containing no detectable levels of SAs, by using a signal-to-noise ratio of 3 (the ratio between the peak areas for each target analyte and peak area of noise). The MQLs were calculated in a similar way by using a signal-to-noise ratio of 10. Very small differences were observed between the MDLs and MQLs achieved for the determination of a given SAs in the different meat matrices assayed and MQLs lower than 100 $\mu g\,kg^{-1}$ (the current MRL established for total SAs in meat [7–9]) were obtained for all the analytes determined.

Table 2
Mean recoveries and method quantification limits obtained for sulfonamides as a function of the volume of supramolecular solvent used for extraction.

Sulfonamide	Volume of supramolecular solvent (mL)							
	0.4		0.6		0.8		1.0	
	Recovery[a] ± s[b] (%)	MQL (μg kg^{-1})	Recovery[a] ± s[b] (%)	MQL (μg kg^{-1})	Recovery[a] ± s[b] (%)	MQL (μg kg^{-1})	Recovery[a] ± s[b] (%)	MQL (μg kg^{-1})
SD	44 ± 1	11	50 ± 1	15	81 ± 1	12	104 ± 4	13
SM	64 ± 2	9	76 ± 2	11	104 ± 8	11	102 ± 2	14
SMP	60 ± 1	20	75 ± 3	24	103 ± 9	24	101 ± 2	30
SCP	54 ± 1	17	69 ± 6	21	91 ± 12	21	104 ± 3	24
SDX	77 ± 1	7	87 ± 6	10	100 ± 1	11	101 ± 1	14
SMT	65 ± 1	8	74 ± 4	10	105 ± 1	10	101 ± 3	13
SDM	73 ± 1	9	88 ± 1	11	105 ± 4	13	103 ± 4	16
SQ	63 ± 1	25	76 ± 7	31	93 ± 8	34	98 ± 1	40

[a] 300 mg of pork spiked with 200 μg kg^{-1} of each SA.
[b] Standard deviation, $n = 3$.

Table 3
Recovery (mean value ± standard deviation)[a] obtained for sulfonamides using different operational conditions.

Sulfonamide	Extraction time (min)[b]				Centrifugation time (min)[c]		
	5	15	30	45	5	10	15
SD	82 ± 2	88 ± 1	104 ± 4	103 ± 2	83 ± 1	104 ± 4	104 ± 3
SM	78 ± 1	87 ± 4	102 ± 2	104 ± 2	95 ± 1	102 ± 2	103 ± 2
SMP	69 ± 4	92 ± 1	101 ± 2	100 ± 1	94 ± 1	101 ± 2	101 ± 2
SCP	77 ± 2	100 ± 3	104 ± 3	100 ± 2	95 ± 1	104 ± 3	104 ± 5
SDX	77 ± 2	94 ± 1	101 ± 1	100 ± 2	92 ± 1	101 ± 1	106 ± 4
SMT	76 ± 1	93 ± 2	101 ± 3	102 ± 1	95 ± 1	101 ± 3	102 ± 3
SDM	78 ± 1	96 ± 2	103 ± 4	103 ± 2	98 ± 1	103 ± 4	99 ± 2
SQ	84 ± 3	96 ± 2	98 ± 1	99 ± 1	88 ± 2	98 ± 1	98 ± 3

[a] 300 mg of pork spiked with 200 μg kg^{-1} of each SA; volume of supramolecular solvent = 1.0 mL; vibration motion = 2500 rpm, and centrifugation rate = 15,000 rpm; $n = 3$.
[b] Centrifugation time = 10 min.
[c] Extraction time = 30 min.

3.3.2. Selectivity

To assess the possible interference from matrix components, two approaches were implemented. The first one consisted in analysing six blank muscle samples from pork, chicken, turkey, lamb and beef, and checking the chromatograms obtained for any peaks in the regions of interest where the target analytes were expected to elute. The second approach involved the comparison of the slopes of the calibration curves obtained from standards in supramolecular solvent with those run from meat fortified with known amounts of the target analytes (50–2300 μg kg^{-1}) analyzed using the whole recommended procedure (see Section 2.4). Chromatograms obtained from most blank samples analysed showed a peak near the retention time of SD but it did not affect to the accuracy achieved for the determination of the analyte as it could be inferred from the results obtained from the analysis of spiked samples. The slopes of the calibration curves obtained for SD ($n = 6$) from pork, chicken, turkey, lamb and beef samples were 6.83 ± 0.03 pg^{-1}, 7.03 ± 0.27 pg^{-1}, 6.76 ± 0.04 pg^{-1}, 6.92 ± 0.05 pg^{-1}, 7.06 ± 0.04 pg^{-1}, 6.75 ± 0.09 pg^{-1} and that obtained for this analyte from standards in supramolecular solvent 6.83 ± 0.03 pg^{-1}. No statistically significant differences between these slopes were observed by applying a Student's t test [51]. The experimental t-values were in the interval 0.01–0.15 and were far below the critical t-value (3.36, significant level = 0.01). Similarly, slopes of the calibration curves obtained for the rest of SAs from standard and from meat samples did not shown statistically significant differences (experimental t-values = 0.01–2.01, critical t-value = 3.36, significant level = 0.01).

3.3.3. Trueness

As no certified reference materials for SAs in meat were available, the trueness of the method was assessed by repetitive analysis ($n = 11$) of blank pork samples spiked at two concentration levels; i.e. 100 μg kg^{-1} and 200 μg kg^{-1}. The concentrations

Table 4
Analytical figures of merit of the proposed method.

Sulfonamide antibiotic	Calibration parameters					MDL[d] (μg kg^{-1})	MQL[e] (μg kg^{-1})
	Linear range[a] (ng)	Slope ± s (pg^{-1})	Intercept ± s × 10^3	r[b]	$S_{x/y}$[c] × 10^2		
SD	0.08–15	6.83 ± 0.03	0.4 ± 0.2	0.99997	3.7	3.4–4.0	12.4–13.3
SM	0.09–15	5.61 ± 0.06	0.6 ± 0.5	0.9998	8.6	4.5–4.8	15.1–16.2
SMP	0.19–15	2.83 ± 0.01	0.5 ± 0.1	0.99997	1.5	9.0–9.7	29.9–32.1
SCP	0.15–15	3.48 ± 0.05	0.1 ± 0.4	0.9995	7.0	7.3–7.8	24.3–26.1
SDX	0.09–15	5.93 ± 0.06	−0.1 ± 0.4	0.9998	7.4	4.3–4.6	14.3–15.3
SMT	0.08–15	6.31 ± 0.07	−0.1 ± 0.6	0.9998	9.3	4.0–4.3	13.4–14.4
SDM	0.10–15	5.13 ± 0.05	0.7 ± 0.4	0.9998	6.4	4.9–5.3	16.5–17.7
SQ	0.25–15	2.09 ± 0.02	0.5 ± 0.2	0.9998	2.9	12.1–13.1	40.5–43.5

[a] Instrumental quantitation limit calculated by using a signal-to-noise ratio of 10.
[b] Correlation coefficient.
[c] Standard error of the estimate.
[d] Range of detection limits obtained for the determination of SAs in muscle of pork, chicken, turkey, lamb and beef.
[e] Range of quantitation limits obtained for the determination of SAs in muscle of pork, chicken, turkey, lamb and beef.

found, expressed as mean values ($n=6$) ± the expanded uncertainly, estimated as *ks* [52] (coverage factor $k=2$ for a significant level of 0.05), were $100 \pm 7\ \mu g\ kg^{-1}$ and $199 \pm 4\ \mu g\ kg^{-1}$ for SD, $102 \pm 6\ \mu g\ kg^{-1}$ and $199 \pm 2\ \mu g\ kg^{-1}$ for SM, $102 \pm 8\ \mu g\ kg^{-1}$ and $200 \pm 4\ \mu g\ kg^{-1}$ for SMP, $100 \pm 5\ \mu g\ kg^{-1}$ and $200 \pm 3\ \mu g\ kg^{-1}$ for SCP, $100 \pm 5\ \mu g\ kg^{-1}$ and $201 \pm 2\ \mu g\ kg^{-1}$ for SDX, $100 \pm 6\ \mu g\ kg^{-1}$ and $201 \pm 3\ \mu g\ kg^{-1}$ for SMT, $101 \pm 6\ \mu g\ kg^{-1}$ and $201 \pm 4\ \mu g\ kg^{-1}$ for SDM, and $101 \pm 6\ \mu g\ kg^{-1}$ and $199 \pm 5\ \mu g\ kg^{-1}$ for SQ. Recoveries ranged between 99% and 102% (according to the 2002/657/EC Commission Decision [46] recovery data are acceptable when they are within the interval 80–110% for analyte concentrations higher than $10\ \mu g\ kg^{-1}$).

3.3.4. Precision

The precision was evaluated in terms of repeatability and reproducibility by analysing thirty aliquots of a blank pork sample spiked with $100\ \mu g\ kg^{-1}$ of SAs. Analyses were performed in 5 days (six aliquots each) using different supramolecular solvents, standard solutions and mobile phases. The within-assay precision, expressed as standard deviation, was calculated as the square root of the average value of the intra-day variances obtained and, the between-assay one as the square root of the mean intra-day variance plus the inter-day variance. The relative standard deviations under repeatability and reproducibility conditions varied within the intervals 1.8–3.6% and 3.3–6.1%, respectively (according to the 2002/657/EC Commission Decision [46] the relative standard deviation under within-laboratory reproducibility conditions should not exceed 15% for analyte concentrations comprised between $100\ \mu g\ kg^{-1}$ and $1000\ \mu g\ kg^{-1}$).

3.3.5. Stability

The stability of SAs was assessed in both stock solutions and sample extracts. Stock solutions of each SA at a concentration of $100\ mg\ L^{-1}$ were stable for at least 1 month as they were stored at −20 °C and working solutions containing $1.5\ mg\ L^{-1}$ of each analyte were stable for 12 h at room temperature. To evaluate the stability of SAs in meat extracts, samples of pork, chicken, turkey, lamb and beef containing no SAs were fortified with the analytes at two concentration levels ($100\ \mu g\ kg^{-1}$ and $500\ \mu g\ kg^{-1}$) and extracted using the proposed microextraction procedure (Section 2.4.2). Each of the extract obtained was divided in two aliquots. SAs were determined in one aliquot immediately after extraction and the other one was stored at room temperature for 12 h before analysis. No differences were obtained between the results obtained from both aliquots.

With regard to the stability of SAs in samples, previous reported studies [16,19] have demonstrated that these drug residues are stable in edible animal tissues (e.g. muscle of chicken, beef, pork, etc.) for several weeks as they were stored at −20 °C.

3.3.6. 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 pork samples fortified with SAs at the permitted limit (i.e. $100\ \mu g\ kg^{-1}$), and it was calculated from the concentration at the permitted limit plus 1.64 times the standard deviation of the blank samples measurements ($\alpha=5\%$). The decision limits obtained for SD, SM, SMP, SCP, SDX, SMT, SDM and SQ were $106\ \mu g\ kg^{-1}$, $105\ \mu g\ kg^{-1}$, $107\ \mu g\ kg^{-1}$, $104\ \mu g\ kg^{-1}$, $106\ \mu g\ kg^{-1}$, $105\ \mu g\ kg^{-1}$, $105\ \mu g\ kg^{-1}$ and $105\ \mu g\ kg^{-1}$, 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 pork samples fortified at the CCα level ($\beta=5\%$). The obtained CCβ values were $111\ \mu g\ kg^{-1}$, $110\ \mu g\ kg^{-1}$, $113\ \mu g\ kg^{-1}$, $109\ \mu g\ kg^{-1}$, $111\ \mu g\ kg^{-1}$, $110\ \mu g\ kg^{-1}$, $109\ \mu g\ kg^{-1}$ and $110\ \mu g\ kg^{-1}$, respectively.

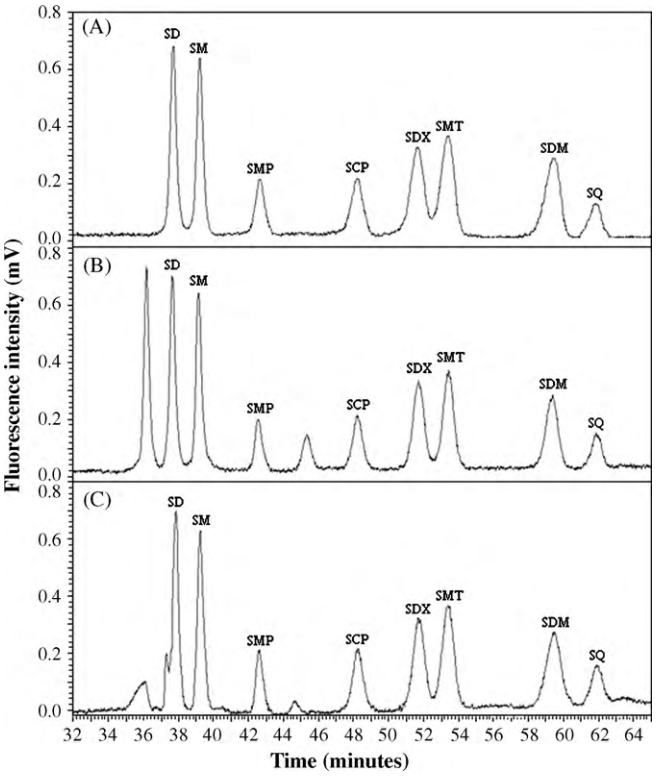


Fig. 1. Chromatograms obtained from (A) a standard solution containing $70\ \mu g\ L^{-1}$ of SAs, and (B and C) 300 mg of meat (B: lamb, C: chicken) sample spiked with $500\ \mu g\ kg^{-1}$ of SAs. Chromatographic conditions as specified in Section 2.4.4.

3.4. Analysis of meat samples

The developed method was used to determine SAs in non-fortified and fortified muscle samples of pork, chicken, turkey, lamb and beef, bought in local supermarkets in Córdoba (Spain). The spiking of samples was performed at two concentration levels, i.e. $100\ \mu g\ kg^{-1}$ and $500\ \mu g\ kg^{-1}$, and experiments were made in triplicate. No SAs were detected in the non-spiked samples tested. The mean concentrations found and the recoveries obtained from the analysis of fortified samples are listed in Table 5. The recoveries ranged from 99% to 98% with relative standard deviations from 1% to 5%.

Fig. 1 compares the chromatogram obtained from a standard solution containing $70\ \mu g\ L^{-1}$ of SAs (A) with those obtained from the analysis of a lamb (B) and chicken (C) sample fortified with $500\ \mu g\ kg^{-1}$ of SAs. Identification of SAs was based on their retention times. Differences between the retention times measured for the analytes from standards and fortified samples were lower than 0.5% in all the cases, and the repeatability obtained for consecutive measurements of this parameter from samples, expressed as relative standard deviations ($n=6$) were 0.34%, 0.28%, 0.35%, 0.19%, 0.15%, 0.19%, 0,12% and 0.10% for SD, SM, SMP, SCP, SDX, SMT, SDM and SQ, respectively. The identification of the target analytes in meat samples containing SAs at detectable concentration levels should be performed by co-chromatography [46] comparing the retention time and the peak width at half-maximum height obtained for each analyte from non-spiked and spiked samples.

Table 5
Mean concentrations and recoveries, along with their respective standard deviations, obtained from the determination of sulfonamide antibiotics (SAs) in fortified meat samples.

Sulfonamide antibiotic	Pork		Chiken		Turkey		Lamb		Beef	
	Concentration found[a] ± s^b ($\mu g\,kg^{-1}$)	Recovery ± s^b (%)	Concentration found[a] ± s^b ($\mu g\,kg^{-1}$)	Recovery ± s^b (%)	Concentration found[a] ± s^b ($\mu g\,kg^{-1}$)	Recovery ± s^b (%)	Concentration found[a] ± s^b ($\mu g\,kg^{-1}$)	Recovery ± s^b (%)	Concentration found[a] ± s^b ($\mu g\,kg^{-1}$)	Recovery ± s^b (%)
SD	101 ± 1[c]	101 ± 1[c]	100 ± 3[c]	100 ± 3[c]	99 ± 3[c]	99 ± 3[c]	99 ± 2[c]	99 ± 2[c]	102 ± 3[c]	102 ± 3[c]
	502 ± 27[d]	100 ± 5[d]	499 ± 2[d]	100 ± 1[d]	501 ± 3[d]	100 ± 1[d]	500 ± 3[d]	100 ± 1[d]	496 ± 7[d]	99 ± 1[d]
SM	102 ± 1[c]	102 ± 1[c]	104 ± 2[c]	104 ± 2[c]	101 ± 2[c]	101 ± 2[c]	102 ± 2[c]	102 ± 2[c]	102 ± 3[c]	102 ± 3[c]
	529 ± 21[d]	106 ± 4[d]	503 ± 5[d]	100 ± 1[d]	496 ± 4[d]	99 ± 1[d]	499 ± 6[d]	100 ± 1[d]	506 ± 6[d]	101 ± 1[d]
SMP	102 ± 2[c]	102 ± 2[c]	104 ± 4[c]	104 ± 4[c]	107 ± 3[c]	107 ± 3[c]	102 ± 2[c]	102 ± 2[c]	104 ± 2[c]	104 ± 2[c]
	495 ± 6[d]	99 ± 1[d]	488 ± 6[d]	98 ± 2[d]	492 ± 14[d]	98 ± 3[d]	502 ± 2[d]	100 ± 1[d]	497 ± 13[d]	99 ± 3[d]
SCP	102 ± 1[c]	102 ± 1[c]	103 ± 2[c]	103 ± 2[c]	107 ± 5[c]	107 ± 5[c]	100 ± 1[c]	100 ± 1[c]	103 ± 4[c]	103 ± 4[c]
	513 ± 13[d]	103 ± 3[d]	545 ± 16[d]	109 ± 3[d]	540 ± 2[d]	107 ± 3[d]	498 ± 5[d]	100 ± 1[d]	503 ± 3[d]	101 ± 1[d]
SDX	102 ± 2[c]	102 ± 2[c]	101 ± 1[c]	101 ± 1[c]	101 ± 1[c]	101 ± 1[c]	101 ± 1[c]	101 ± 1[c]	103 ± 5[c]	103 ± 5[c]
	499 ± 11[d]	100 ± 2[d]	522 ± 7[d]	104 ± 2[d]	511 ± 2[d]	102 ± 1[d]	504 ± 1[d]	101 ± 1[d]	501 ± 2[d]	100 ± 1[d]
SMT	100 ± 1[c]	100 ± 1[c]	104 ± 1[c]	104 ± 1[c]	103 ± 2[c]	103 ± 2[c]	103 ± 1[c]	103 ± 1[c]	104 ± 1[c]	104 ± 1[c]
	515 ± 4[d]	103 ± 1[d]	529 ± 9[d]	106 ± 2[d]	526 ± 3[d]	105 ± 2[d]	495 ± 1[d]	99 ± 1[d]	502 ± 4[d]	100 ± 1[d]
SDM	101 ± 1[c]	101 ± 1[c]	101 ± 1[c]	101 ± 1[c]	106 ± 5[c]	106 ± 5[c]	104 ± 3[c]	104 ± 3[c]	101 ± 1[c]	101 ± 1[c]
	512 ± 3[d]	102 ± 1[d]	539 ± 2[d]	107 ± 4[d]	533 ± 22[d]	106 ± 4[d]	509 ± 2[d]	102 ± 1[d]	511 ± 4[d]	102 ± 1[d]
SQ	102 ± 1[c]	102 ± 1[c]	101 ± 4[c]	101 ± 4[c]	102 ± 5[c]	102 ± 5[c]	104 ± 4[c]	104 ± 4[c]	103 ± 3[c]	103 ± 3[c]
	499 ± 11[d]	100 ± 2[d]	505 ± 6[d]	101 ± 1[d]	501 ± 6[d]	100 ± 1[d]	498 ± 8[d]	100 ± 2[d]	500 ± 3[d]	100 ± 1[d]

[a] Mean of three independent determinations.
[b] Standard deviation.
[c] Sample spiked with 100 $\mu g\,kg^{-1}$.
[d] Sample spiked with 500 $\mu g\,kg^{-1}$.

4. Conclusions

Based on the use of a supramolecular solvent made up of reverse micelles of DeA, a microextraction was developed for the fast and reliable extraction of SAs from a variety of edible animal tissues. In contrast with previously reported extraction methods [4–6,13–15,19,20], recoveries obtained were quantitative and independent of the matrix composition and no concentration steps were needed. The volume of organic solvent used for sample handling was drastically reduced (0.5 mL per sample) and, because of the simplicity of the microextraction, several samples could be simultaneously treated. These characteristics, combined with LC–FL determination, make the proposed method a valuable alternative to those based on the use of organic solvents for the routine monitoring of SA residues in meat. Although the method has been demonstrated to fulfil accuracy requirements established in the Commission Decision of the European Union 2002/657/EC, the use of a suitable recovery internal standard (e.g. sulfapyridine) could result in more accurate measurements. Finally, even though a long time is required for analyte derivatization, the total time spends per analysis is not so high taking into account the complexity of samples and the multiresidual character of the proposed method.

Because of the ability of SUPRAS to extract analytes in a wide polarity range and the high extraction efficiency they provide, these solvents have a great potential for developing generic sample handing approaches for the determination of organic contaminants in foods.

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