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Analysis of Sulfonamides in Animal Feeds by Liquid Chromatography with Fluorescence Detection

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ABSTRACT: Two analytical methodologies for the simultaneous analysis of eight sulfonamide antibiotics in animal feeds were developed. Analytes were extracted in a simple and rapid procedure by manual shaking with an ethyl acetate/ultrapure water mixture (99:1, v/v) without further sample cleanup. Mean recoveries ranging from 72.7% to 99.4% with relative standard deviations below 9% were achieved from spiked animal feed samples. Determination was carried out by high-performance liquid chromatography using fluorometric detection with precolumn derivatization. The separation of the derivatized compounds was performed using two different chromatographic columns: a conventional C_{18} column and a recently available core−shell particle Kinetex C_{18} column. Both methods were validated in-house in six different feed matrices, and the two approaches were compared. The experiments showed that the method using the Kinetex column was superior with regard to speed of analysis and precision, both under repeatability and intermediate reproducibility conditions. The limits of detection and quantification were also greatly improved, below 0.10 and 0.34 μg/g, respectively. Finally, this novel approach was successfully applied to the analysis of real feed samples.

KEYWORDS: sulfonamides, feed analysis, liquid chromatography, fluorescence detection, Kinetex

INTRODUCTION

The widespread usage of antibiotics in veterinary practices can lead to the presence of residues in foodstuffs of animal origin.[1] Residues of these compounds can have a harmful effect on human health, such as allergic reactions in some hypersensitive individuals as well as generation of drug resistant bacterial strains in humans,[2−4] and for these reasons their use in animal husbandry must be subjected to strict control.[5] Consequently, authorities around the world have laid down a large number of regulations to ensure food safety and reduce human exposure. In the European Union, maximum residue limits (MRLs) have been established for antibacterials in animal-derived foods, and in the case of feeds no antibiotics other than coccidiostats and histomonostats can be marketed and used as feed additives.[6] Moreover, medicated feeds, which contain active principles at therapeutic levels, must be prepared from authorized medical premixes and used under veterinary prescription.[7] However, the fraudulent use of medicated feeds to illegally promote animal growth must be considered.

Cross-contamination from one medicated feed batch to the next nonmedicated one, usually in the 2−10 μg/g concentration range,[8] can occur either during manufacturing or transport or even at the farm. The critical control points for this chain of events, in which adequate precautions must be taken, are both in the feed mill and on the farm.[9] Thus, the development of sensitive and reliable analytical methodology is required to ensure the compliance of animal feeds with the current legislation.

Sulfonamides (SAs) are among the most widely administered groups of antimicrobials in animal husbandry within the European countries,[10] and, as a consequence, they have been commonly identified as contaminants in animal feeds.[11]

Several methods have been developed for the analysis of SAs in different matrices, especially in edible animal tissues[12−16] and other food products such as milk,[3,17−20] eggs[21,22] or honey.[23,24] However, methods concerning animal feeds are still scarce.[11,25−32]

Analytes are usually extracted with polar solvents or hydroorganic mixtures, mainly based on acetonitrile or methanol, using techniques such as mechanical and manual shaking, sometimes combined with sonication, as well as microwave assisted extraction (MAE) or pressurized liquid extraction (PLE). Some of the current methods also involve a cleanup of the extracts to remove matrix interferences. Methods like liquid−liquid extraction (LLE) or solid-phase extraction (SPE), frequently with C_{18} or polymeric cartridges, are the most common approaches. In some cases, however, a dilution of the extract is enough to avoid matrix interferences. Finally, separation and determination of SAs is normally performed by liquid chromatography with ultraviolet (LC−UV), fluorescence (LC−FL) or mass spectrometry detection (LC−MS). In recent years, some multiresidue methods using LC−MS/MS[31,32] have been published, but they often deal with a few analytes from the same group of antibacterials. Alternative assays based on immunochemical techniques, such as enzyme-linked immunosorbent assay (ELISA),[26,28] have also been reported for rapid screening of SA residues, but, in spite of their low cost and simplicity of analysis, these methods only provide semiquantitative measurements.

In this paper, two methods for the simultaneous determination of eight sulfonamides at low carry-over levels in animal feeds are described. Both methods involve a simple extraction, without further cleanup, followed by a precolumn derivatization with fluorescamine prior to the analytical determination by high-performance liquid chromatography with fluorescence detection using a conventional C_{18} column and a core−shell particle

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Kinetex C_{18} column. The efficiency of the recently available Kinetex columns[33,34] for conventional LC instruments was investigated in order to reduce time of analysis and increase productivity, which is of great interest for laboratories today, especially for those performing routine analysis. The two methods developed were validated and compared. Due to the variety and complexity of the feed matrices, the validation included assays with six different kinds of feeds, to ensure that the proposed methods were reliable with a wide variety of matrices. The concentration level of the target analytes in the feeds used for the validation experiments was set at 2 μg/g, which roughly corresponds to a drug-free feed cross-contaminated during the production process. Finally, the applicability of the Kinetex method, which improved the speed and efficiency of the analysis, was also assessed by analyzing real feed samples.

MATERIALS AND METHODS

Chemicals and Reagents. Sulfadiazine (SDZ), sulfadimidine (SDD), sulfamethoxypyridazine (SMP), sulfachloropyridazine (SCP), sulfadoxine (SDX), sulfamethoxazole (SMX), sulfadimethoxine (SDM), sulfaquinoxaline (SQX) and the internal standard (IS) sulfamethoxydiazine (SME), Vetranal grade, were purchased from Riedel-de Haën (Buchs, Switzerland). Fluorescamine (≥99.0%) was supplied by Fluka (Buchs, Switzerland).

HPLC grade ethyl acetate (AcEt), acetonitrile (ACN) and methanol (MeOH) were obtained from Merck (Darmstadt, Germany), and doubly deionized water (Milli-Q, Millipore, Molsheim, France) of 18.2 mΩ/cm resistivity was used. All other chemicals were of analytical reagent grade.

A mixed stock standard solution containing 50 mg/L of each sulfonamide and another containing the same concentration of the internal standard (SME) were prepared monthly in methanol from the solid compounds and stored in dark glass bottles at 4 °C. Working standard solutions (from 40 to 800 μg/L) were freshly prepared by dilution of the stock solution with acetonitrile. Internal standard (200 μg/L) and fluorescamine solutions (0.2%, w/v) in acetonitrile were prepared daily.

Mobile phases of several compositions were prepared by mixing ACN with the appropriate volume of aqueous formic acid/sodium formate buffer solutions, previously filtered through a 0.22 μm nylon filter.

Feed Samples. Samples of six different feeds for pigs, piglets, cows, chickens, laying hens and rabbits were provided by the Associació Catalana de Fabricants de Pinsos (ASFAC). On arrival, they were stored at 4 °C in poly(vinyl chloride) (PVC) flasks, and pelleted samples were ground with a domestic chopper (Moulinex, France) before being used. Blank samples were tested to be free of sulfonamides by LC−MS/MS.[14]

For the validation studies, samples spiked at 2 μg/g were prepared "in-house" by mixing blank feed with the corresponding amount of a quality control material consisting of a pig feed spiked at 50 μg/g with the eight sulfonamides, which was prepared as described elsewhere.[35] The mixtures were placed in PVC flasks containing ceramic balls and rolled on a rolling table for at least 90 h. After homogenization, spiked feeds were stored at 4 °C. Prior to analysis, the samples were allowed to reach room temperature.

Real feed samples were obtained from production lines, where medicated feeds containing SDZ had been recently prepared.

Apparatus. Chromatographic analysis was performed with a Shimadzu system (Kyoto, Japan), consisting of a LC-10AD VP quaternary pump, a SIL-10AD VP automatic injector and a RF-10A XL fluorescence detector with a 150 W xenon lamp. LC Solution for Shimadzu LC-Workstations was used for instrument control and data processing. Two analytical columns were used, a LiChrospher 100 RP-18 (Merck, 250 × 4 mm, 5 μm), equipped with a LiChrospher 100 RP-18 (Merck, 4 × 4 mm, 5 μm) guard column, and a Kinetex C_{18} (150 × 4.6 mm, 2.6 μm), equipped with a Guard Cartridge System KJ0-4282 (4 × 2 mm), both from Phenomenex (Torrance, CA, USA). Both columns were thermostated at 25 °C.

A Milestone ETHOS E system (Sorisole, Italy), designed for extraction using organic solvents and able to hold twelve 100 mL extraction vessels with magnetic stirring, was used for microwave assisted extraction (MAE).

Mechanical shaking was carried out with a Rotary Mixer 34526 (Breda Scientific, Breda, Netherlands). The pH was measured using a Crison GLP21 pH-meter (Alella, Spain), equipped with a Crison 52−02 Ag/AgCl combined glass electrode.

Other instruments used in sample preparation were a Heraeus Christ Labofuge 400 centrifuge (Osterode am Harz, Germany), a TurboVap LV Caliper evaporator (Hopkinton, USA), a Stuart vortex mixer SA8 (Staffordshire, U.K.) and a RollerMix D RM120-DE Ovan (Badalona, Spain) for the homogenization of spiked feed.

Feed Extraction Procedure. Feed samples (1 g) were placed in 25 mL centrifuge tubes and extracted by hand-shaking for one minute with 10 mL of a mixture of ethyl acetate and deionized water (99:1, v/v). The resulting extracts were centrifuged at 1370*g* for ten minutes.

Precolumn Derivatization. Either 1 mL of feed extract or 1 mL of a standard solution in acetonitrile was transferred to a glass vial, evaporated to dryness under a nitrogen stream (about 5 min, 50 °C, 10 psi) and reconstituted with 1 mL of the internal standard solution (200 μg/L). Subsequently, 2 mL of aqueous formic acid/sodium formate buffer solution at pH 3.4 and 1 mL of 0.2% fluorescamine solution were added. The mixture was left to stand in the dark for at least 2 h at room temperature and filtered through a 0.45 μm nylon membrane before injection (50 μL) into the chromatographic system. Fluorescence of the derivatized sulfonamides remained constant for about 8 h.

LC−FL Analysis. The chromatographic separation was carried out at 25 °C using a binary mobile phase in gradient mode. Mobile phase A consisted of a 0.01 M formic acid/sodium formate buffer at pH 3.4, and mobile phase B was acetonitrile. The following gradient program, at a flow rate of 1.5 mL/min, was applied for the LiChrospher column: 0−15 min, 33% B; 15−25 min, linear increase to 40% B; 25−28 min, decrease to 33% B; and finally 28−35 min, 33% B. The gradient program for the Kinetex column, at a flow rate of 1.6 mL/min, was as follows: 0−6 min, 33% B; 6−10 min, linear increase to 38% B; 10−12 min, decrease to 33% B; and finally 12−17 min, 33% B. Excitation/emission wavelengths were 405 and 485 nm, respectively. Quantification was performed by external calibration curves.

RESULTS AND DISCUSSION

Feed Extraction Procedure. Preliminary tests for the extraction of sulfonamides from animal feed were carried out with a quality control material consisting of pig feed spiked with SDZ and SDD at 5 μg/g.[35] At this initial stage of method development, a LC−UV method free of matrix interferences developed in the same study[35] was used to evaluate the recovery of the target antibiotics.

In order to select a suitable solvent composition, the following solvent combinations were tested: ACN/H_2O (95:5, v/v), AcEt, and mixtures of AcEt/H_2O (95:5 and 90:10, v/v), AcEt/HAc (99:1 and 98:2, v/v) and AcEt/MeOH (99:1 and 98:2, v/v). For these experiments, 1 g of the quality control material was extracted by manual shaking for 1 min using 10 mL of the extracting solvent. The results, shown in Figure 1, indicated that ACN/H_2O (95:5, v/v) and AcEt/H_2O mixtures gave the highest recoveries for both SAs, about 80−85% for SDZ and 75−80% for

SDD. However, the analysis of blank feeds of different kinds showed that dirtier extracts were obtained when ACN was used, making them less suitable for the subsequent quantification of the selected antibiotics by LC−FL. When AcEt/H_2O mixtures were used, only one interfering peak corresponding to matrix components overlapped with those corresponding to SDD. Figure 2 shows the chromatograms obtained for blank feed extracts. Therefore, 95:5 and 90:10 (v/v) AcEt/H_2O mixtures were chosen for further work.

Different extraction techniques, including manual shaking (1 min), mechanical shaking (30 min) and microwave assisted extraction (20 min at 80 °C), were also assessed. Five extractions were carried out to evaluate the performance of each technique. The results obtained are shown in Figure 3. Best extraction efficiencies were obtained with manual and mechanical shaking, whereas MAE provided lower recoveries and also poorer reproducibility. Manual shaking for 1 min was selected as the most appropriate for the solid−liquid extraction, as it offered the best compromise between extraction time and efficiency.

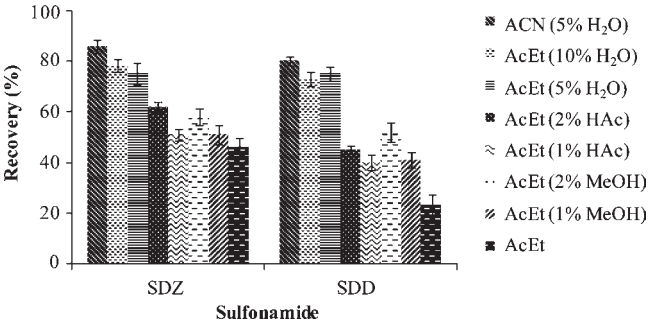

Figure 1. Recoveries obtained for SDZ and SDD using different extraction solvent compositions (mean values of 5 experiments).

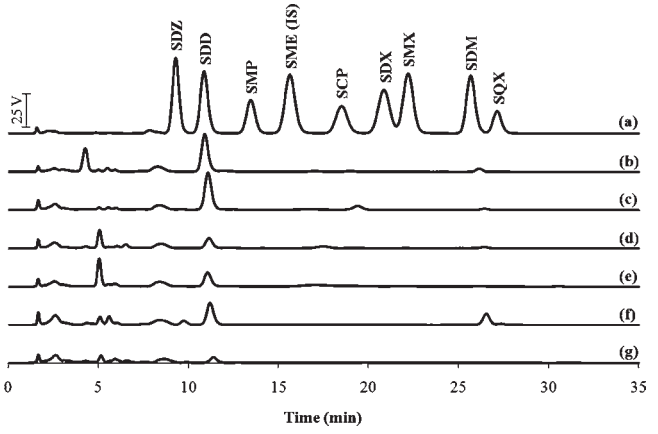

Figure 2. LC−FL chromatograms of a 200 μg/L standard mixture (a) and of blanks of different animal feeds extracted with AcEt/H_2O (95:5, v/v): pig (b), piglet (c), cow (d), chicken (e), laying hens (f) and rabbit (g).

These extraction conditions were tested in pig feed spiked at 2 μg/g with the 8 SAs, and the effect of lower water percentages, 1 and 2%, was also assessed. As shown in Figure 4, decreasing the water proportion in the extraction solvent from 5% to 1% had no significant effect on the recovery of the analytes, but the interference from the feed matrix coeluting with SDD was notably reduced.

Therefore, the conditions finally adopted consisted of manually shaking 1 g of feed with 10 mL of 99:1 (v/v) AcEt/H_2O for 1 min in a 25 mL centrifuge tube. Recoveries in these conditions ranged from 60 to 114%.

LC−FL Optimization. Sulfonamides themselves are not fluorescent, and derivatization with fluorescamine to form highly fluorescent pyrrolidine-type derivatives has often been used for fluorometric detection of SAs during LC, either in precolumn[10,35] or postcolumn[24,36] mode. In the present paper, a method previously applied to the analysis of sulfonamides in surface water and soils[10] was adapted for their determination in feed samples. No changes were made to the already optimized parameters of the derivatization step with fluorescamine (reaction time, temperature, reagent concentration and solvent composition), but the elution gradient and flow rate were suitably modified to attain a good chromatographic separation using two different columns: one packed with 5 μm LiChrospher C_{18} totally porous particles and another with 2.6 μm Kinetex C_{18} core−shell particles.

First attempts were done using the conventional reversed-phase C_{18} column. Several experiments were performed to test a wide range of gradient profiles, with acetonitrile percentages varying between 25% and 42% and flow rates ranging from 1 to 1.5 mL/min. An acceptable baseline chromatographic separation was achieved in 35 min for the majority of the target analytes using the elution program described in the LC−FL Analysis section, except for a slight overlap of the SDX and SMX peaks. Other parameters, such as column temperature and injection volume, were also studied to get a reliable separation, obtaining the best results when the column was held at 25 °C and an injection volume of 50 μL was used.

Subsequently, this method was adapted for the Kinetex column using the Kinetex Scaling Calculator[37] available online on the Phenomenex Web site. Only minor adjustments were needed to

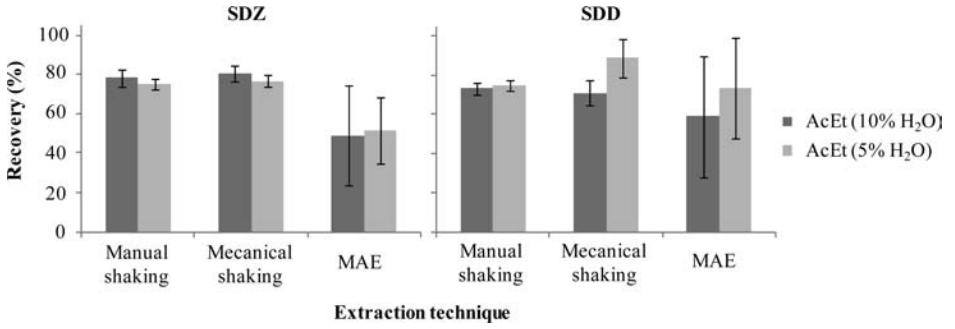

Figure 3. Recoveries obtained for SDZ and SDD using different extraction techniques (mean values of 5 experiments).

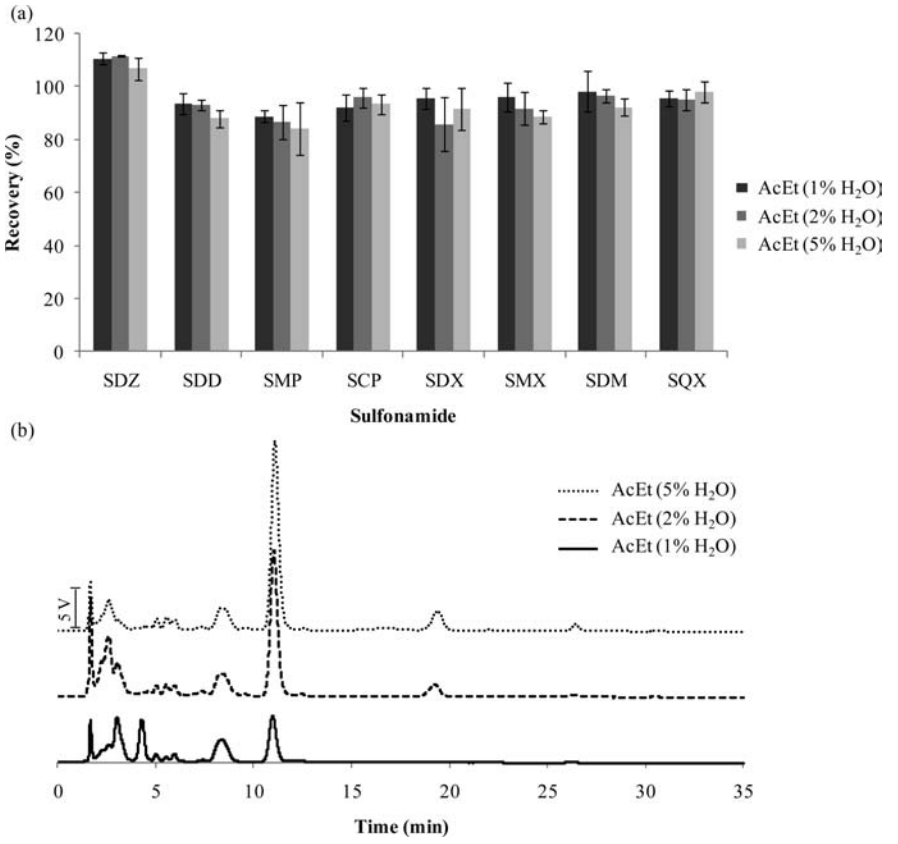

Figure 4. Recoveries obtained for SAs (a) and LC−FL chromatograms of blank piglet feed extracts (b) decreasing the water proportion in the extraction solvent (mean values of 5 experiments).

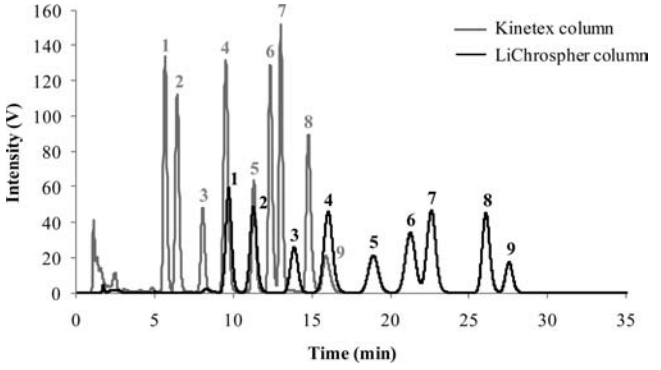

Figure 5. LC−FL chromatograms of a 200 μg/L standard mixture of the eight SAs and the internal standard (SME) using the two different columns: SDZ (1), SDD (2), SMP (3), SME (4), SCP (5), SDX (6), SMX (7), SDM (8) and SQX (9).

reach adequate analyte separation in about half the time (17 min). Moreover, an improvement in the resolution for SDX and SMX was achieved with this column, though separation was still not complete. Figure 5 shows a representative chromatogram obtained from a standard mixture (200 μg/L) of the antibiotics with both columns.

Once optimized, both methods were compared in terms of the quality parameters obtained injecting standard solutions and solvent-based blanks.

Method linearity was assessed by means of five-point calibration curves obtained with derivatized standard solutions at concentrations ranging from 40 to 800 μg/L for each compound.

Table 1. Typical Retention Times and Calibration Parameters for the Two Columns

	LiChrospher column				Kinetex column			
compd	t_R (min)	slope	intercept	r	t_R (min)	slope	intercept	r
SDZ	9.2	0.9281	−0.0443	0.9998	5.1	0.7993	−0.0010	0.9998
SDD	10.6	0.8822	−0.0440	0.9998	7.2	0.6161	−0.0002	0.9998
SMP	13.2	0.5294	−0.0286	0.9997	8.7	0.3563	−0.0008	0.9999
SCP	15.3	0.5761	−0.0297	0.9997	10.0	0.3773	−0.0006	0.9999
SDX	18.0	0.9256	−0.0481	0.9997	11.4	0.7505	−0.0023	0.9998
SMX	20.3	1.1401	−0.0565	0.9998	12.0	1.0240	−0.0039	0.9999
SDM	21.8	0.9327	−0.0459	0.9998	13.7	0.8207	−0.0040	0.9999
SQX	25.3	0.3499	−0.0204	0.9997	14.3	0.2648	−0.0059	1.0000

Linear regression analysis was carried out by plotting the ratio between the peak area of the analyte and of the internal standard versus their concentration ratio. Typical retention times (t_R) and calibration parameters for the two columns are presented in Table 1. The response obtained was linear in the tested range with correlation coefficients (r) higher than 0.9997 in all cases.

Precision was checked under repeatability conditions, where the experiments were carried out on the same day, and under intermediate reproducibility conditions, where the experiments were distributed over three different days. All experiments were conducted by the same technician using the same instrumentation. Five derivatized standard solutions at two concentration

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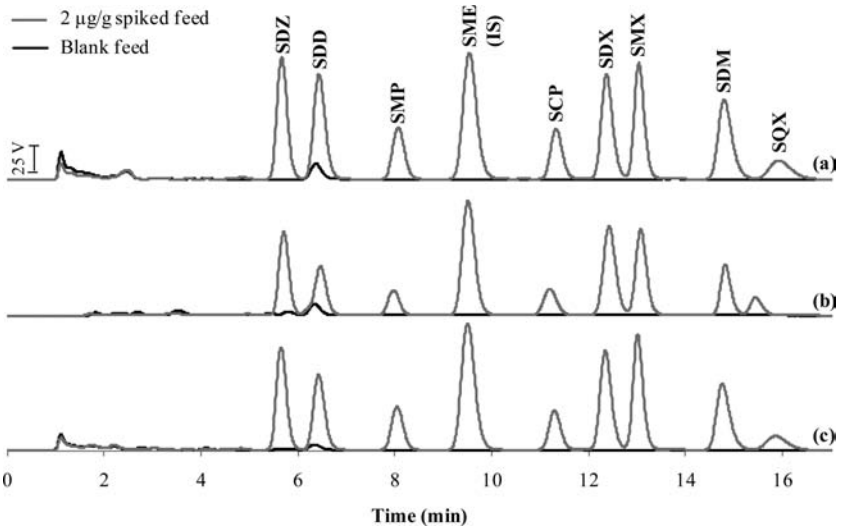

Figure 6. LC−FL chromatograms of blank and 2 μg/g spiked animal feed extracts: pig (a), laying hens (b) and rabbit (c).

Table 2. Recovery and Precision Studies with Feed Matrices

	LiChrospher column			Kinetex column		
compound	recovery[a] (%)	RSD_r[b] (%)	RSD_R[b] (%)	recovery[a] (%)	RSD_r[b] (%)	RSD_R[b,c] (%)
SDZ	99.3 (90.8−111.4)	3.1	4.1	99.4 (91.7−109.9)	3.2	3.2
SDD	77.7(65.7−98.6)	4.3	5.2	87.3 (67.7−97.0)	4.1	4.8
SMP	72.7 (64.0−89.2)	5.1	6.5	81.1 (66.3−90.9)	5.4	5.9
SCP	73.2 (59.5−87.2)	5.5	6.6	78.6 (61.2−86.6)	5.6	6.3
SDX	96.1 (83.5−113.6)	6.5	7.4	98.0 (72.3−111.4)	5.9	6.5
SMX	82.0 (70.4−101.1)	5.7	8.4	85.8 (73.9−90.0)	5.8	7.3
SDM	79.2 (67.0−98.5)	4.0	5.0	88.5 (72.0−95.7)	4.3	4.5
SQX	84.8 (64.9−102.4)	3.3	4.9	93.1 (72.7−103.3)	3.4	3.3
	LiChrospher column			Kinetex column		
feed	recovery[d] (%)	RSD_r[b] (%)	RSD_R[b] (%)	recovery[d] (%)	RSD_r[b] (%)	RSD_R[b,c] (%)
pig	92.6 (81.7−111.4)	4.6	5.6	96.6 (86.6−109.9)	4.6	5.5
piglet	81.3 (66.3−101.9)	5.3	6.9	80.2 (66.4−100.8)	4.9	
cow	75.3 (59.5−95.9)	4.8	5.7	75.7 (67.7−91.7)	5.9	
chicken	81.4 (64.0−113.6)	5.4	6.3	78.2 (61.2−103.8)	7.9	
laying hens	81.6 (65.9−107.5)	4.8	6.1	85.6 (70.7−111.4)	6.7	
rabbit	82.7 (68.6−103.5)	4.9	6.7	82.3 (70.7−96.9)	5.2	5.2

[a] Mean value for each compound in all the feeds. [b] Pooled relative standard deviations. [c] For piglet, cow, chicken and laying hen feeds only a single-day experiment setup was applied with the Kinetex column. [d] Mean value for all the compounds in each feed.

levels (200 and 600 μg/L) were injected for this purpose. A one-way (day) analysis of variance (ANOVA) for a 95% confidence level was applied to obtain intersession (s_L), intrasession (s_r) and intralaboratory total standard deviations (s_R). The Kinetex method yielded better precision than the LiChrospher method, with repeatability (RSD_r) and intermediate reproducibility (RSD_R) values, expressed as relative standard deviation, about 1−2% vs 6−7% and 5−14% vs 11−14%, respectively, for all the sulfonamides.

Limits of detection (LODs) and limits of quantification (LOQs) for the sulfonamides were determined on the basis of three and ten times, respectively, the standard deviation of the baseline of 10 derivatized acetonitrile blanks using low-concentration standard solutions (from 0.2 to 4 μg/L). LODs for the Kinetex column were similar to those obtained with the conventional column (0.1 to 0.5 μg/L vs 0.1 to 0.3 μg/L), but LOQs were lower (0.2 to 0.7 μg/L vs 0.6 to 1.2 μg/L).

The increase in resolution, the shorter analysis time, resulting in reduced solvent consumption, and the better precision and sensitivity obtained demonstrate the advantages of the core-shell technology versus the conventional particle technology.

Method Validation. The in-house validation of the developed analytical methods requires the estimation of the parameters explained in detail in the paragraphs below.

Table 3. LOD and LOQ Values Determinated in Feed Matrices[a]

	LiChrospher column		Kinetex column		LiChrospher column		Kinetex column	
	LOD (μg/L)	LOQ (μg/L)	LOD (μg/L)	LOQ (μg/L)	LOD (μg/g)	LOQ (μg/g)	LOD (μg/g)	LOQ (μg/g)
SDZ								
pig	0.3	1.1	0.5	1.8	0.01	0.05	0.02	0.07
piglet	0.6	2.0	0.4	1.4	0.02	0.08	0.02	0.06
cow	0.8	2.7	0.5	2.2	0.03	0.11	0.02	0.09
chicken	0.2	0.6	0.2	0.5	0.01	0.02	0.01	0.02
laying hens	0.4	1.5	0.3	0.9	0.02	0.06	0.01	0.04
rabbit	0.4	1.2	0.2	0.8	0.01	0.05	0.01	0.03
SMP								
pig	1.4	4.5	1.0	3.3	0.05	0.18	0.04	0.13
piglet	2.5	8.2	1.6	5.9	0.10	0.33	0.06	0.24
cow	0.8	2.6	0.5	1.7	0.03	0.10	0.02	0.07
chicken	3.0	10.1	2.2	7.3	0.12	0.40	0.09	0.29
laying hens	0.9	3.2	0.6	2.1	0.04	0.13	0.02	0.08
rabbit	1.8	6.1	1.3	4.5	0.07	0.24	0.05	0.18
SCP								
pig	1.2	4.1	0.2	0.7	0.05	0.16	0.01	0.03
piglet	5.7	18.9	2.6	8.5	0.23	0.76	0.10	0.34
cow	3.9	13.1	1.4	5.8	0.16	0.52	0.06	0.23
chicken	3.5	11.6	1.1	4.6	0.14	0.47	0.04	0.18
laying hens	1.6	5.3	0.4	3.2	0.06	0.21	0.02	0.13
rabbit	5.0	16.8	2.3	7.6	0.20	0.67	0.09	0.31
SDX								
pig	0.2	0.7	0.4	1.5	0.01	0.03	0.02	0.06
piglet	2.0	6.6	0.7	3.4	0.08	0.26	0.03	0.14
cow	0.7	2.4	0.2	1.1	0.03	0.10	0.01	0.04
chicken	1.0	3.4	0.6	1.9	0.04	0.14	0.02	0.08
laying hens	1.0	3.2	0.9	2.2	0.04	0.13	0.04	0.09
rabbit	0.6	2.1	0.2	0.7	0.03	0.08	0.01	0.03
SMX								
pig	0.4	1.4	0.1	0.3	0.02	0.06	0.01	0.03
piglet	2.3	7.8	0.9	4.2	0.09	0.31	0.04	0.17
cow	0.6	1.8	0.2	0.8	0.02	0.07	0.01	0.03
chicken	1.6	5.4	0.9	3.9	0.06	0.22	0.04	0.16
laying hens	0.9	2.9	0.6	1.2	0.04	0.12	0.02	0.05
rabbit	0.3	1.0	0.1	0.2	0.01	0.04	0.01	0.04
SDM								
pig	0.4	1.4	0.1	0.2	0.02	0.06	0.01	0.04
piglet	0.7	2.5	0.3	1.3	0.03	0.10	0.01	0.05
cow	0.7	2.3	0.2	0.9	0.03	0.09	0.01	0.04
chicken	1.2	4.1	0.7	1.9	0.05	0.16	0.03	0.08
laying hens	0.7	2.2	0.4	1.6	0.03	0.09	0.02	0.06
rabbit	0.3	1.1	0.3	1.0	0.01	0.04	0.01	0.04
SQX								
pig	4.3	14.5	1.7	5.5	0.17	0.58	0.07	0.22
piglet	3.0	10.2	0.9	4.4	0.12	0.41	0.04	0.18
cow	2.0	6.7	0.6	2.1	0.08	0.27	0.02	0.08
chicken	4.4	14.8	1.2	5.9	0.18	0.59	0.05	0.24
laying hens	2.6	8.6	0.7	3.5	0.10	0.34	0.03	0.14
rabbit	4.5	15.0	1.1	3.7	0.04	0.15	0.04	0.15

[a] LOD is given by $(3S_{y/x})/b$ and LOQ by $(10S_{y/x})/b$ for each analyte, where $S_{y/x}$ corresponds to the standard deviation of the residuals and b is the slope of the calibration curve.

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Linearity. Since external calibration using standard solutions prepared in pure solvent is proposed, the results reported for method linearity in the LC−FL Optimization section were also applicable here. Although matrix-matched calibration curves would have further compensated for discrepancies between different matrices, the variability of feed matrices and the difficulty of finding representative blank samples often make this practice unfeasible in the case of feed analysis, unless blank feed samples are available.

Specificity. Specificity was checked by testing samples of blank feeds produced for six different animal species. Only one peak, coeluting with SDD, was found in all feeds. Another peak, coeluting with SDZ, was also observed in the case of laying hen feed. Bearing in mind the magnitude of the interferences, SDZ and SDD could be quantified with an error ≤10% in sample feeds containing concentrations of the target analytes over 0.5 and 1.5 μg/g, respectively. The absence of background peaks at the retention time of the remaining compounds showed that the method was free of endogenous matrix interferences for them. The chromatograms of blank and 2 μg/g spiked pig, laying hen and rabbit feed samples are shown in Figure 6.

Trueness and Precision. Feed samples spiked at a carryover level of 2 μg/g were analyzed during three different days. Repetitive application of the whole procedure to five independent spiked samples of each kind of feed was carried out each day. Data from this batch of experiments was used to evaluate the trueness and the precision of both methods in terms of repeatability (intraday precision) and intermediate reproducibility (interday precision). Results obtained are summarized in Table 2. In order to avoid the consignment of an excessive amount of data, the range and the average of the recoveries obtained for each compound in all feeds are given. Similarly, for each feed, the range and the average of the recoveries corresponding to all sulfonamides are also given. Concerning precision, the pooled standard deviations for compounds and for feeds are consigned. As can be observed, good precisions and recoveries, ranging from 59.5% to 113.6%, were obtained with both methods for all compound/matrix combinations. Intermediate reproducibility results were, as expected, higher than repeatability values. The lowest RSD_r and RSD_R corresponded to SDZ and SQX, and the highest to SDX and SMX, a consequence of the slight overlapping of their peaks in the chromatographic separation.

Sensitivity. Limits of detection (LODs) and limits of quantification (LOQs) were assessed using matrix-matched calibration curves established at appropriate low concentration levels of the target analytes in feed. Extracts of blank samples of each type of feed were individually spiked prior to analysis with the sulfonamides at four increasing levels of concentrations, 1, 2, 5, and 10 μg/L, equivalent to 10, 20, 50, and 100 times the lowest instrumental LOD previously calculated using standard solutions. Based on these measurements, calibration curves for each compound were then used to calculate the LODs and LOQs. As shown in Table 3, limits in feed extracts were in all cases higher than the corresponding values in solvent because of matrix influence. Moreover, despite the extract dilution during the derivatization step, satisfactory LODs and LOQs were obtained in all cases, below 0.23 μg/g and 0.76 μg/g in feed respectively, which indicates that the developed methods are sensitive enough for their intended purpose. However, results again revealed a better performance of the new generation column against the conventional one.

Application to Real Feed Samples. Analysis of real feed samples was carried out in triplicate with the newly developed Kinetex method. The method was applied to four samples, one for piglets and three for pigs, which were obtained from production lines where medicated feeds with SDZ had been recently prepared. Results show that all samples tested contained SDZ concentrations in the 0.7−6.5 μg/g range, demonstrating that the feed samples were affected by cross-contamination after the production of medicated formulations.

In conclusion, the two procedures developed provide precise, sensitive and accurate methods for multiresidue analysis of 8 sulfonamides in animal feeds. The simple and rapid extraction procedure by manual shaking with an ethyl acetate/ultrapure water mixture (99:1, v/v) gives high recoveries for all the target analytes, and no further cleanup step is necessary. The excellent sensitivity of the fluorescence detection allows SAs to be quantified, with a previous derivatization, at the required concentration levels and makes it an inexpensive and applicable alternative to MS based methods for routine laboratories. Moreover, using the recently available Kinetex C_{18} column instead of a conventional C_{18} column reduces analysis time and improves resolution and precision. The limits of detection and quantification are also greatly improved. Based on the method performance characteristics, this new validated analytical methodology is considered to be suitable for the determination of SAs in drug-free animal feeds that have been contaminated during the fabrication process. Finally, the application of the proposed method to test samples demonstrates its usefulness to evaluate possible cross-contaminations in the feed mill laboratories and, thus, ensure safety in the first stage of the food chain.

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