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Determination of sulfonamides by packed column supercritical fluid chromatography with atmospheric pressure chemical ionisation mass spectrometric detection

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Sulfonamide antibiotics are widely used to prevent bacterial infections in livestock, and residues are commonly found in milk and meat. Packed column supercritical fluid chromatography (pSFC) with detection using ultra violet (UV) and atmospheric pressure chemical ionisation (APCI) mass spectrometry (MS) provides a versatile method for the detection and quantification of six major sulfonamides. The APCI mass spectra for all the sulfonamides consisted of protonated molecules at low cone voltages. Increasing the cone voltage led to informative fragmentation patterns, which provided structural information for identification purposes. The pSFC-APCI-MS technique was shown to be linear ($r^2 \geq 0.999$) over the concentration range 0.1–50 μg ml^{-1} using total ion current. The precision and the accuracy of the system and validation of sample preparation are acceptable, with RSD $< 2\%$ and relative error 8%. Selected ion monitoring gave detection limits as follows: sulfadiazine 41, sulfamethoxazole 45, sulfamerazine 47, sulfamethizole 59, sulfamethazine 181 and sulfadimethoxine 96 μg l^{-1}, which are lower than the amounts permitted in milk products. The APCI pSFC-MS system was shown to have a high degree of reproducibility. The technique was then applied to determine the above sulfonamides in milk. The results obtained show that there are no matrix effects from the milk and that the detection limits remained as stated for the standard solutions.

Introduction

Sulfonamides are antibacterial agents widely used in veterinary practice to prevent infections in livestock.[1] They have also been used widely in animal feeds to promote growth and to treat disease.[2] Residues are often found in meat[3] and milk products where they enter the human food chain. The presence of sulfonamide residues in food is of concern because some of the compounds are carcinogenic[4] and they enhance the risk of developing bacterial resistance, which makes the therapeutic use of this medicine inefficient.[5] Sulfamethazine, for instance, produces thyroid tumours in rodents.[6]

Numerous methods have been employed to determine sulfonamide drugs. Thin-layer chromatography is prone to interferences and is inadequate for quantitative analysis.[3,7] Gas chromatography (GC) coupled to electron ionisation (EI) mass spectrometry (MS) is both sensitive and selective for the determination of sulfonamides but derivatisation of non-volatile and thermally labile sulfonamides is required prior to analysis.[8,9] This increases the overall analysis time and is an extra source of error for the analytical technique. GC with atomic emission detection (AED) is one of the recent techniques applied for the determination of sulfonamide antibiotics. Quantitative mass calculations of the sulfonamides are based on the proportion of the peak areas to the number of the atoms in the compounds, C, S and N. Derivatisation is, of course, required for the GC, and AED can only be used to identify already known species.[10] Capillary zone electrophoresis (CZE) coupled with nano-electrospray MS-MS-MS has been applied successfully to the determination of sulfonamide in milk samples. It was found, however, that the separation suffers from interference from salt and fat in milk, so a clean-up procedure was required prior to the analysis.[11] Liquid chromatography interfaced with fluorescence detection has also been reported to have a low limit of detection but the technique certainly requires derivatisation to improve the fluorescence properties for detection; in addition, the resolution is often poor and the detection is non-specific.[12,13] However, LC-MS using APCI,[6,14] thermospray,[15,16] thermospray tandem MS[17,18] and also UV[17,19,20] interfaces has been successfully applied to the determination of some of the sulfonamides in milk, chicken liver, swine muscle tissue, porcine muscle and swim wastewater. Packed column supercritical fluid chromatography (pSFC) using UV detection has recently been applied to the determination of eight sulfonamides and good resolution was achieved by coupling two columns in-line.[21] pSFC-MS using a moving belt and SFC-MS with EI interfaces are useful for the determination of sulfonamides in kidney, biological matrices and an extract from *Claviceps purpurea*.[22,23] The moving belt interface has also been used without chromatography to analyse extracts of pig's kidneys for these drugs.[24] pSFC interfaced to FT-IR spectrometry has also been applied to determine eight sulfonamides[25] and satisfactory resolution was obtained with the exception of sulfamerazine, sulfadimethoxine and sulfapyridine, but FT-IR detection is non-specific for such compounds. Capillary SFC has also been applied to these compounds, but was unable to achieve complete separation of the test analytes.[26]

Although, as mentioned above, the determination of sulfonamides by LC-MS or UV has been investigated,[6,15–17,19,20] the analysis technique requires time for sample preparation and clean-up and can give low peak resolution. The technique does, however, provide satisfactory results for routine screening of sulfonamides in milk, meat, *etc*.

It would be preferable to have, in addition, a more selective and sensitive technique. Also, from the environmental point of view, it is important to minimise the use of undesirable solvents in the determination of sulfonamides. SFC-atmospheric pressure chemical ionisation (APCI) MS is such a technique and considerable attention has been paid to it.[27–31] Addition of an

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organic modifier to the mobile phase is very common in SFC. However, the amount of organic solvent used is very much less than for other analytical techniques, such as HPLC.

In this work, pSFC with on-line UV and APCI- MS detection using environmentally friendly supercritical (sc) CO_2 as the mobile phase has been developed and used for the determination of six major sulfonamides (Fig. 1). On-line UV was used for detection in the optimisation stage and both UV and APCI-MS gave identical chromatograms; therefore, no UV chromatograms will be presented. The technique was found to be a useful alternative to the more conventional analytical techniques currently used in terms of time consumption, sample preparation, selectivity and sensitivity. It gave a shorter overall analysis time, provided good chromatographic resolution for all six sulfonamides,[12,17] especially sulfamethazine and sulfamethizole,[15] and produced excellent linearity over a wide concentration range.[16]

Experimental

Analysis for the sulfonamides was performed using a Gilson SF3 pSFC system (Gilson Medical Electronics, Middleton, WI, USA). Separation of the standard mixture was achieved using a 250 × 4.6 mm id column with a cyanopropyl stationary phase (5 μm particle size), maintained at an oven temperature of 50 °C. Samples were introduced using a Model 7125 10 μl injection loop (Rheodyne, Cotati, CA, USA) and the components were eluted using a $scCO_2$ mobile phase, modified with methanol. The modifier concentration started at 10% for the first 5 min and increased linearly to 15% over the next 10 min (15 min total run time). The flow rate was 2 ml min^{-1}, producing a pressure of ~180 bar at the column inlet. UV detection was performed using an on-line Jasco (Tokyo, Japan) Model 875-CE UV detector operated at 230 nm.

The pSFC system was coupled to a single quadrupole VG Trio-2000 mass spectrometer equipped with an APCI ionisation source (Micromass, Manchester, UK). Control of the system was effected with a Lab-Base data system (version 2.12). The interface comprised a tapered 75 μm id restrictor, connected to the outlet of the UV detector and inserted into the APCI probe. The total SFC column effluent was delivered into the atmospheric pressure ion source through the heated probe (300 °C) using nitrogen boil-off gas from a liquid nitrogen Dewar vessel as the bath gas at a flow rate of 200 l h^{-1}. The source temperature was maintained at 120 °C. Ionisation was achieved *via* a 3kV discharge at the corona pin, generating proton donor methanol reagent ions, $[MeOH_2]^+$, and/or methanol clusters, $[(MeOH)_nH]^+$, which transfer protons to the analytes. The mass spectrometer was operated in the positive ion mode and the cone voltage was set at 20 V to give measurements of protonated molecules ($[M + H]^+$) without any fragmentation. To obtain mass spectra containing several fragment ions, the cone voltage was varied between 20 and 70 V and MS data were collected in the full-scan mode from 100 to 400 u in 1 s. Selected ion monitoring (SIM) was used for quantification of the sulfonamides.

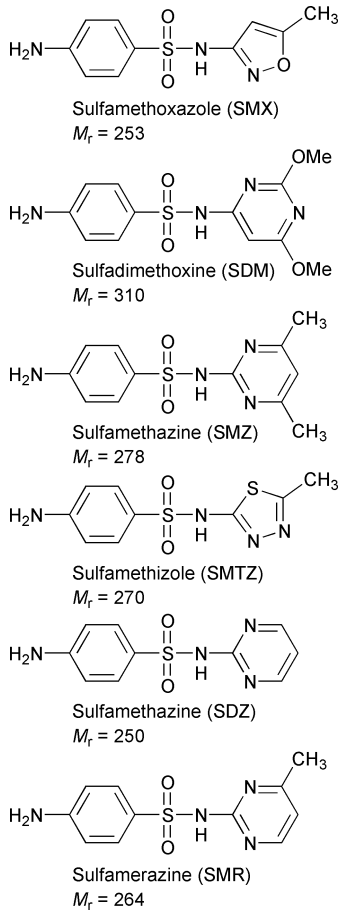

Fig. 1 Structures of the sulfonamides.

Chemicals

In all analyses, SFC grade carbon dioxide (BOC, Guildford, Surrey, UK) was used. Methanol (HPLC grade) was obtained from Fisher Scientific (Loughborough, UK). Standards for sulfamethoxazole, sulfamethazine, sulfadiazine, sulfamerazine, sulfamethizole and sulfadimethoxine were obtained from Sigma (Poole, Dorset, UK).

Sample preparation

Laboratory samples were prepared to determine the six sulfonamides, sulfamethoxazole (SMX), sulfadimethoxine (SDM), sulfamethazine (SMZ), sulfamerazine (SMR), sulfadiazine (SDZ) and sulfamethizole (SMTZ), in milk with SFC-APCI-MS. First, the milk was filtered using a Whatman (Maidstone, Kent, UK) filter-paper to remove solid particles. At the same time the six sulfonamides were dissolved in 10 ml of water to give a concentration of 50 μg ml^{-1} and the solution was used to spike the milk samples.

For the determination of sulfonamides in milk, two spiked samples at concentrations of 10 and 25 μg ml^{-1} were used. Milk samples spiked with the standard solution containing the six sulfonamides were prepared by transferring milk (1 ml) into two different 10 ml calibrated flasks, then adding the spiked solution to the flasks to give the desired concentrations and diluting with MeOH to 10 ml, resulting in mixtures of sulfonamides at concentrations of 10 and 25 μg ml^{-1} each (100 and 250 ng on-column). Another 1 ml milk sample was prepared in the same way, but the sample was then treated with 10 ml of dichloromethane (DCM) to extract all the sulfonamides. The main purpose of preparing the samples in both MeOH and DCM was to study possible effects arising from water present in milk and also the effect of the solvent on the recovery. The two resulting solutions were injected on-column for the determination of the sulfonamides. A blank was also prepared in exactly the same manner, but without the addition of sulfonamides.

A standard mixture of sulfonamides in MeOH was prepared with a concentration of 50 μg ml^{-1} of each component. The mixture was used to optimise the system and to prepare solutions at concentrations of 0.1, 1, 5, 10, 30 μg ml^{-1} to plot a calibration graph. All standard solutions and the samples were analysed by pSFC-APCl-MS with on-line UV detection.

Results and discussion

A standard solution containing the six sulfonamides was injected into the mobile phase for separation on a cyanopropyl column under the optimum conditions in the full-scan mode and the total ion current (TIC) chromatogram was obtained. The separation is achieved in 14 min without derivatisation and the peaks are both sharp and well resolved. The APCI mass spectra of the sulfonamides at the optimum cone voltage (20 V) all give only the protonated molecules expected in each case: sulfamethoxazole (*m*/*z* = 254), sulfadimethoxine (*m*/*z* 311), sulfamethazine (*m*/*z* 279), sulfamerazine (*m*/*z* 265), sulfadiazine (*m*/*z* 251) and sulfamethizole (*m*/*z* = 271). These APCI mass spectra obtained at 20 V confirm that the technique can be used for the trace determination of sulfonamides in samples, especially when SIM is performed, and can be used for the quantification of known species, rather than providing structural information.

To obtain informative structural information, the cone voltage was increased to 50 V to give rise to collision-induced dissociation (CID) reactions in the intermediate pressure region of the APCI-MS, leading to progressive fragmentation, thereby providing information for structural elucidation and hence further confirmation of the target compounds. Fig. 2 (a–f) shows the APCI mass spectra acquired at 50 V. All of the mass spectra show the protonated molecules for the corresponding sulfonamides. The fragment ion at *m*/*z* 156 is a characteristic ion for the sulfonamides, produced by cleavage of the S–N bond in the structure. The other fragment ions are all specific for each compound. The scan range was limited down to *m*/*z* 100 to prevent interference from reagent ion species. The main reagent ion was the protonated dimer of methanol (*m*/*z* 65) at the optimum cone voltage (20 V). At the same time, the protonated methanol molecule and trimeric ions were also present, at *m*/*z* 33 and 97, respectively. Therefore, the mass acquisition was carried out above *m*/*z* 100, and so another common ion, as described elsewhere[17] for the sulfonamides, at *m*/*z* = 92 was not observed.

Fig. 3 shows the proposed structures for the SMR-derived fragments at a cone voltage of 50 V. At 20 V, the mass spectrum consists entirely of the protonated molecule at *m*/*z* 265. As the cone voltage is increased, loss of the common aromatic amine group ($C_6H_5NH_2$) produces the fragment ion at *m*/*z* 172. Cleavage of the bond between the amine and the methylpyrimidine could produce the same ion, but this is less likely since the single bond energy for N–C is higher than that for S–C.[32] The ions at *m*/*z* 156 and 110 are produced by splitting the molecule between S and N, producing aromatic amine, sulfur dioxide and methylpyrimidine amine groups, respectively. Similar fragmentation schemes can be derived for the other analytes. All of the sulfonamides studied, with the exception of SMTZ, produce a rearrangement ion by losing H_2SO_2 from the protonated molecules, *m*/*z* 188 (SMX), 245 (SDM), 218 (SMZ),

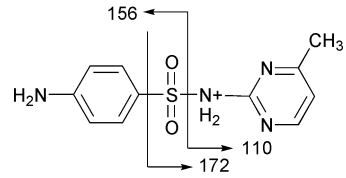

Fig. 3 Proposed fragmentation reactions of sulfamerazine.

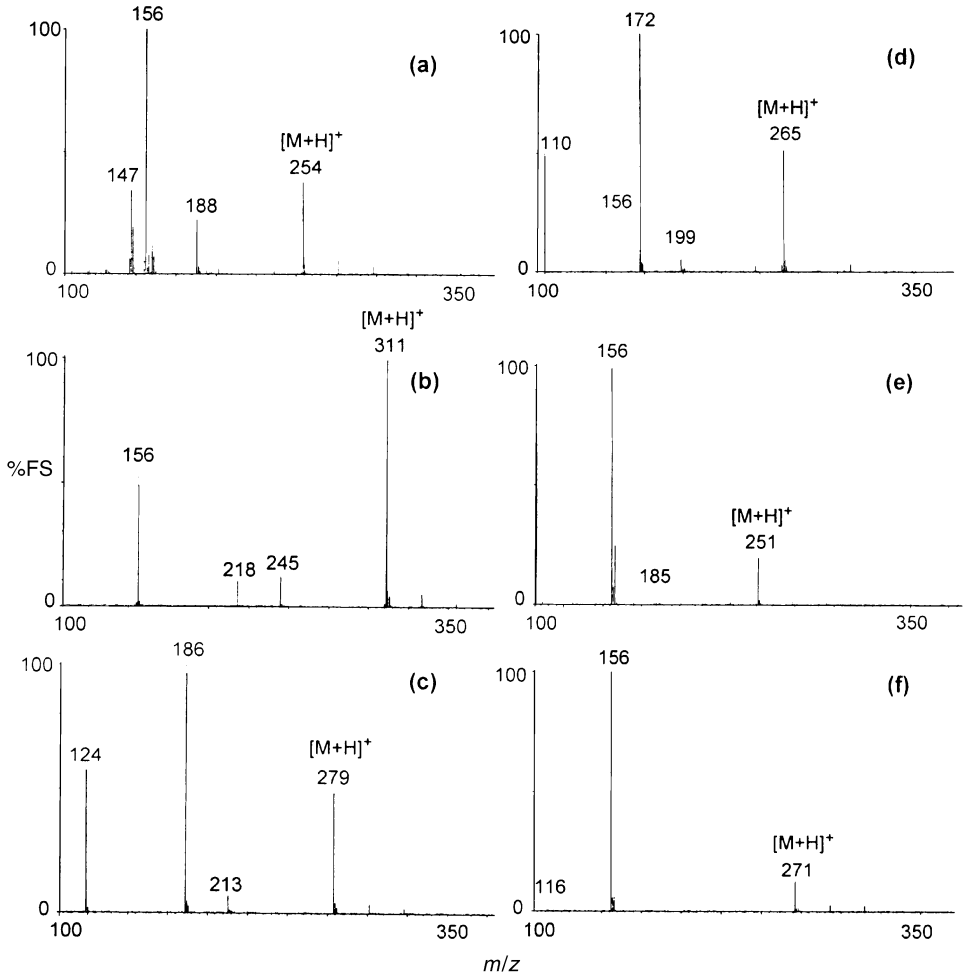

Fig. 2 Mass spectra at 50 V for (a) sulfamethoxazole (SMX), (b) sulfadimethoxine (SDM), (c) sulfamethazine (SMZ), (d) sulfamerazine (SMR), (e) sulfadiazine (SDZ) and (f) sulfamethizole (SMTZ).

199 (SMR) and 185 (SDZ). All of the sulfonamides produce very similar fragmentation patterns under the conditions applied by cleaving the bond at the same point, and they produce the specific fragment ion corresponding to each specific structure.

The linearity of the system was determined by injecting the standard solution containing the six sulfonamides. A high degree of linearity was observed for all the analytes over the concentration range 0.1–50 μg ml^{-1}, with values of r^2 = 0.999 in each case.

The detection limits were determined for a signal-to-noise ratio of 3:1 and were in the picogram range on-column for all analytes in the SIM mode (with the exception of sulfamethazine): sulfadiazine 41, sulfamethoxazole 45, sulfamerazine 47, sulfamethizole 59, sulfamethazine 181 and sulfadimethoxine 96 μg l^{-1}.

The on-line UV pSFC-MS system was also tested for reproducibility. The results are summarised in Table 1 and the excellent reproducibility is reflected by the RSD, which is < 1% in each case. The mean RRT (average relative retention time), *s* (standard deviation) and RSD were calculated from eight injections of the standard solution of the six sulfonamides in the full-scan mode.

Determination of sulfonamides in milk

The milk used in this experiment was normal pasteurised milk obtained from a local supermarket. All of the milk samples to be analysed were diluted with methanol to prevent any blockage at the end of the heated restrictor by fat, proteins or other material in the milk. When the milk was diluted in MeOH a colloidal solution appeared, and therefore to remove any precipitate the solution was filtered through a 0.2 μm Gelman Nylon Acrodisk (Fisher Scientific) to obtain a clear solution.

A portion of the blank sample was injected into the mobile phase to obtain a TIC chromatogram, using the experimental conditions optimised for the sulfonamides. The TIC chromatogram shows a few peaks at short retention times, a weak broad peak at around 8 min and finally a very broad peak at approximately 17 min (Fig. 4). Most importantly, no peaks appear in the range of the retention times for sulfonamides. The earlier peaks are believed to arise from the material present in the Acrodisk, as the TIC chromatogram of MeOH filtered through the Acrodisk shows the same peaks at the same retention times and also the same mass spectra as those of the blank milk sample. Thus, the blank chromatogram shows no interference arising from milk. In addition, no problems were encountered due to blockage of restrictors or other instrumental difficulties caused by the milk.

The milk samples spiked with the six sulfonamides in MeOH without any further extraction were injected on-column for separation in the full-scan mode, again using the experimental conditions described above. Fig, 5 shows the TIC chromatogram in the full-scan mode for the spiked milk sample containing the six sulfonamides. All of the six sulfonamide peaks can be seen very clearly on the chromatogram, with no interference from any milk components.

Quantification of the sulfonamides in milk was studied with the samples spiked at two concentrations, 10 and 25 μg ml^{-1}. Therefore, MS was switched to the SIM mode, in which the protonated molecules were detected. The aim of using two spiked milk samples was to investigate the effect of the concentration of sulfonamides on the validation of the sample preparation. The results obtained from analysis of the three injections for each concentration of the two spiked samples show very good precision in terms of the *s* and RSD calculated: 0.2 μg ml^{-1} and 2% for 10 μg ml^{-1} and 0.5 μg ml^{-1} and 2% for 25 μg ml^{-1}, respectively (Table 2). The accuracy of the system was estimated by calculating the absolute and relative errors for both spiked milk samples. The results show an absolute error of < 2 μg ml^{-1} for both spiked samples, with the exception of SDM in the spiked sample at 25 μg ml^{-1} (4.88 μg ml^{-1}). The relative error, which is a measure of the accuracy, was < 8% at both concentrations for all sulfonamides, except for SDM, which gave high values.

Quantification of sulfonamide residues in the material removed from the samples by filtration was also performed. Samples were prepared by washing the precipitate with a certain amount of MeOH (10 ml) and subjected to the same procedure as above. The residue concentration of the sulfonamides in the samples differed according to the original spiked concentration but, most importantly, the percentages retained in the precipitate were the same in both cases (< 2%).

SIM, under the same conditions as described earlier, showed that the limit of detection for the sulfonamides under these conditions is lower than approximately 50 μg l^{-1} in each case, except for sulfamethazine (~ 200 μg l^{-1}), all of which are lower than the maximum residue limit permitted in food.[25]

Fig. 6 shows the TIC chromatogram of the six sulfonamides extracted from the spiked milk solution into DCM. A good response is found for five sulfonamides but the sixth (SMTZ) gives only a weak response. This suggests that the solubility of SMTZ is lower in DCM than in water. Thus, as the water present in milk gives no interference in the determination of sulfonamides in milk, direct injection from milk solution gave better analyte recovery and a more rapid analysis while requiring no additional toxic chemicals.

Hence the presence of other components in samples approximating those likely to be found in actual analytical case studies

Table 1 Summary of the relative retention times (RRTs) for the sulfonamide standards

Sulfonamides	Mean RRT	*s*	RSD (%)
SMX	0.92	0.0035	0.38
SDM	0.96	0	0
SMZ	1	0	0
SMR	1.04	0.0035	0.34
SDZ	1.09	0	0
SMTZ	1.16	0.0052	0.45

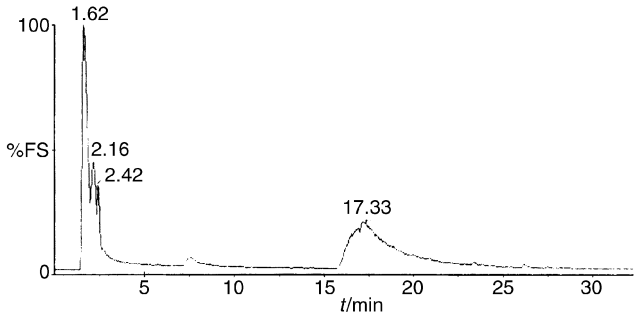

Fig. 4 TIC chromatogram of the blank solution (non-spiked milk sample).

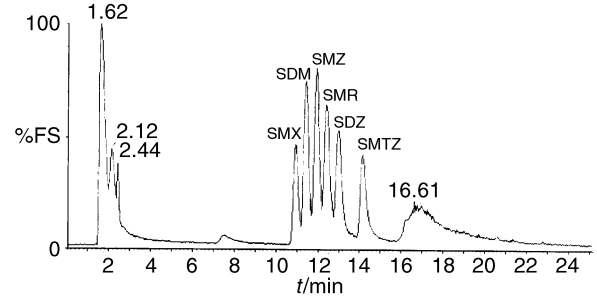

Fig. 5 TIC chromatogram of the sample of milk spiked with a mixture of the six sulfonamides in MeOH.

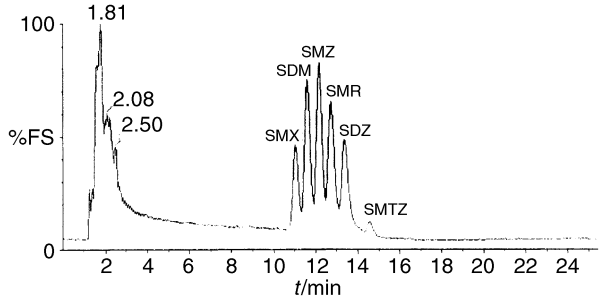

Fig. 6 TIC chromatogram of the milk sample spiked with a mixture of the six sulfonamides, then extracted from milk into DCM.

Table 2 Summary of the results of the determination of sulfonamides in two spiked milk samples at concentrations of 10 and 20 μg ml^{-1} and residues removed by filtration

Compound	Average/ μg ml^{-1}	*s*/μg ml^{-1}	RSD (%)	Absolute error/ μg ml^{-1}	Relative error (%)
Spiked with 10 μg ml^{-1}—					
SMX	10.61	0.04	0.38	0.61	6.11
SDM	11.55	0.13	1.10	1.55	15.45
SMZ	10.54	0.13	1.26	0.54	5.42
SMR	10.41	0.14	1.31	0.41	4.14
SDZ	10.15	0.13	1.31	0.15	1.45
SMTZ	10.43	0.20	1.90	0.43	4.27
Spiked with 25 μg ml^{-1}					
SMX	26.35	0.16	0.62	1.35	5.39
SDM	29.88	0.12	0.40	4.88	19.54
SMZ	26.98	0.25	0.93	1.98	7.93
SMR	26.61	0.22	0.82	1.61	6.44
SDZ	25.91	0.32	1.22	0.91	3.63
SMTZ	24.96	0.43	1.73	0.04	0.16

Residue analysis—

	From 10 μg mg^{-1}		From 25 μg mg^{-1}	
	μg mg^{-1}	%	μg mg^{-1}	%
SMX	0.18	1.75	0.28	1.13
SDM	0.18	1.77	0.33	1.30
SMZ	0.15	1.52	0.26	1.05
SMR	0.16	1.64	0.28	1.11
SDZ	0.16	1.61	0.26	1.02
SMTZ	0.17	1.72	0.26	1.02

cause no difficulties or interference in the proposed method for sulfonamides. In samples containing lower levels of sulfonamide contamination, as may be encountered in practice, the same technique could be employed, but in such cases it would be necessary to concentrate the samples by an appropriate factor before injection on to the SFC column.

Conclusions

The six sulfonamide drugs tested in this work can be separated and characterised using packed column SFC-UV-APCI-MS. The mass spectra of all the sulfonamides consisted exclusively of protonated molecules at low cone voltage and diagnostic fragment ions were formed at higher cone voltages through CID. This system can clearly be used to identify other sulfonamide species in terms of their characteristic mass spectra and fragmentation patterns. The detector response for the mass spectrometer was found to be linear over a large concentration range (0.1–50 μg ml^{-1}) and the detection limits (using SIM) were estimated to be approximately 50 μg l^{-1} (S/N = 3 : 1). The system was also found to be reproducible, precise and accurate for the quantification of the sulfonamides in milk (Tables 1 and 2). One area where additional work may be necessary is in the detection of sulfonamide metabolites in appropriate matrices.

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