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Liquid chromatographic–tandem mass spectrometric determination of selected sulphonamides in milk

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Abstract

Liquid chromatography–tandem mass spectrometry is used for the quantitative analysis of selected sulphonamides in milk. Ultrafiltration is the only sample pre-treatment technique which is required. Consequently, sample throughput is much higher than with conventional procedures, and analyte recoveries are high. As for quantification, both external standard and isotope dilution calibration yield satisfactory results. The method is fully validated for five sulphonamides with a maximum residue limit of 100 μg/kg, and which are included in the Dutch control programme on residues. Furthermore, results are presented on the applicability of the method to detect compounds at a much lower concentration level exemplified by a banned sulphonamide, dapsone, which has a provisional action limit of 5 μg/kg. The main conclusion is that the present, novel approach to the trace-level determination of veterinary drugs is simple and straightforward and has a wide-ranging application potential which is briefly exemplified by the analysis of selected benzimidazoles in milk by essentially the same procedure.

Keywords: Milk; Ultrafiltration; Tandem mass spectrometry; Sulphonamides; Benzimidazoles; Dapsone

1. Introduction

Many sulphonamides, which all exhibit antimicrobial activity, possess the *p*-aminobenzenesulphone moiety. Sulphonamides have been in use as antibiotic agents in veterinary practice for several decades. They exhibit antimicrobial synergy with trimethoprim and are frequently co-administered with this compound. Although many sulphonamides have been described, only few are registered for veterinary use, the most important being sulphadiazine, sulphadimidine, sulphamethoxazole, sulphadoxine and sulphadimethoxine. These compounds have been registered for use in medicated feeds and for therapeutic use [1]. Within the EU, the maximum residue limit (MRL) in milk has been set at 100 μg of total sulphonamides/kg [2]. There is therefore a need for methods of analysis that can detect individual substances at lower concentration levels. Some countries require the absence of sulphonamides in food products for human consumption, control of which also requires methods of analysis with low limits of detection. Generally, screening of sulphonamides in milk is based on microbiological methods [3–5], while more conclusive results are obtained by means of instrumental methods of analysis, which are frequently based on LC with UV or fluorescence detection [6–8]. However, these methods require

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extensive sample preparation to avoid interference of matrix components and, hence, are characterised by a limited sample throughput. There is, therefore, a need for sensitive and conclusive analytical methods that provide reliable identification of sulphonamide drugs and their quantitation but, at the same time, should be relatively simple and enable high sample throughput.

We have studied the possibility of developing such methods by applying modern mass spectrometric techniques in combination with non-discriminative techniques for sample preparation. In this paper we report on the use of ultrafiltration as the sole sample-preparation step for LC–MS–MS of veterinary drugs at trace levels in milk. The emphasis has been on the demonstration of the analytical potential offered by this approach to residue analysis, using sulphonamides as a highly relevant group of compounds. In a further attempt to demonstrate this potential, essentially the same technique was applied to the analysis of a sulphonamide that has been banned for veterinary use and, consequently, should be detected at a much lower concentration level. Brief application to some benzimidazoles was regarded a suitable final test case because these drugs have lower MRLs [2] than sulphonamides and have less favourable mass spectrometric properties.

2. Experimental

2.1. Materials

All reagents were of analytical-reagent grade unless stated otherwise. Ammonium acetate, glacial acetic acid and acetonitrile were obtained from Merck (Darmstadt, Germany).

Sulphadiazine (SDZ), sulphadimidine (SMZ), sulphamethoxazole (SMX), sulphadoxine (SDX), sulphadimethoxine (SDM) and dapsone (DDS) were from Sigma (St. Louis, MO, USA). d_7-Sulphadimidine (CEC-MAT 41) was obtained from the Institute for Public Health and the Environment (dr L. van Ginkel, RIVM, Bilthoven, NL). Microcon 0.5-ml ultrafilters with a molecular weight cut-off (MWCO) of 30 kDa were from Millipore (Bedford, MA, USA).

2.2. Sample preparation

Raw milk was fortified with the analytes of interest by spiking with a standard solution in water. The spiked milk samples contained 200 μg/kg d_7-sulphadimidine and from 25 to 500 μg/kg of the analytes of interest. For dapsone, which is a banned substance, the spiking levels were 20-fold lower and ranged from 1.25 to 25 μg/kg.

After spiking and mixing, the fortified milk was allowed to stand for at least 30 min at room temperature to allow equilibration. The milk samples (1.0 ml) were subsequently mixed with an equal volume of acetonitrile to effect precipitation of proteins and simultaneously to solubilize the analytes of interest. The mixture was centrifuged for 15 min at room temperature at 3500 *g* and the supernatant was isolated. A 20-μl aliquot of the supernatant was diluted to 1.0 ml with demineralized water and 0.5 ml of the diluted sample was applied to a Microcon ultrafilter and centrifuged at 5000 *g* for 30 min at room temperature to remove any residual proteins. The resulting ultrafiltrate was used for analysis without any further clean-up.

2.3. LC–MS–MS

A Waters (Milford, MA, USA) Alliance chromatography Module 2690 was used which consisted of a gradient LC system, an autosampler with a cooled sample tray and a column oven set at 40 °C. LC separation of the sulphonamides was performed on a 15 cm×3 mm I.D. Waters Symmetry C_{18} column (d_f=5 μm). An acetonitrile–10 m*M* ammonium acetate (pH 3.5) eluent was used with a 15-min linear gradient from 0 to 90 vol.% acetonitrile, starting 5 min after injection; the flow-rate was 400 μl/min. In all instances, the injection volume was 100 μl.

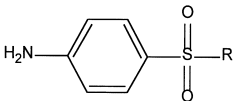
The LC system was coupled to a MicroMass (Manchester, UK) Quattro Ultima mass spectrometer equipped with an electrospray (ESI) interface. The column effluent was split 1:2 immediately before the ESI interface. The mass spectrometer was operated with ion source settings optimised for maximum sensitivity. Data acquisition was done in the MRM mode by monitoring the major fragmentation re-

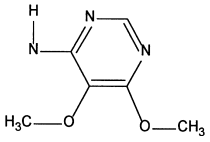
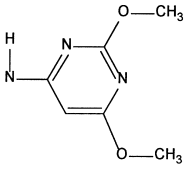
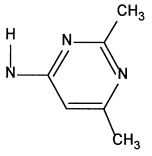
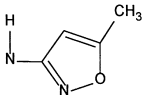
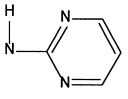
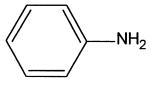
actions characteristic of the sulphonamides (Table 1).

2.4. Samples

The present method was primarily applied to raw milk samples collected randomly from the Dutch National Surveillance Scheme. In addition it was used to analyse preserved milk such as sterilised, pasteurised and ultra-high temperature (UHT) treated milk. These samples were obtained from local retailers. For each of these types of milk, two different batches were used to prepare spiked samples at the same concentration levels as for raw milk. During

Table 1
Molecular structures and LC–MS–MS characteristics of the studied sulphonamide compounds. The most abundant product ion is underlined



Name	Abbreviation	R	RT (min)	Molecular weight	$[M+H]^+$	Product ions (m/z)
Sulphadoxine	SDX		15.2	310.3	311.1	245 <u>156</u> 140 108 92
Sulphadimethoxine	SDM		13.9	310.3	311.1	245 218 <u>156</u> 108 92
Sulphadimidine	SMZ		12.5	278.3	279.2	204 <u>186</u> 156 124 108 92
Sulphamethoxazole	SMX		13.6	253.3	254.1	188 <u>156</u> 108 92
Sulphadiazine	SDZ		10.0	250.3	251.1	<u>156</u> 108 92
Dapsone	DDS		12.7	248.3	249.0	<u>156</u> 108 92

this study, milk replacers used as calf feed which contained sulphadimidine became available. These samples were used to further demonstrate the applicability of the present concept.

3. Results and discussion

3.1. Analytical conditions

For the separation of the six test analytes, a conventional reversed-phase LC separation on C_{18}-bonded silica was used, with a buffer–acetonitrile gradient. By using an ammonium acetate/acetic acid buffer, compatibility with MS detection was ensured. As regards mass spectrometry, experience has shown that, from the several modes of atmospheric pressure ionisation available, ESI in the positive ion mode provides the best results for sulphonamides [9,10]. For MS–MS detection, the protonated molecules are selected as precursor ions and the most abundant fragment ions obtained at collision energy of 15–20 eV are monitored as the product ions. Most sulphonamides yield the *p*-aminobenzenesulphone moiety at m/z 156 as the most abundant fragment ion upon collision-induced dissociation (CID) and this ion was indeed chosen as the fragment ion to monitor all test analytes except sulphadimidine. Table 1 presents details concerning the LC separation and MS detection of the analytes. From the retention times it is obvious that a satisfactory separation is obtained. The CID fragment ions can be used for confirmatory analysis; in this study only the underlined product ions were used for MRM measurements.

As indicated in the Introduction, the main aim of the present project was to study whether the high selectivity, and sensitivity, of MS–MS detection will allow a rapid one-step sample preparation even when veterinary drugs have to be determined at the μg/kg level in samples as complicated as milk. Essentially, the 1.0-ml milk samples were diluted two-fold with acetonitrile to effect protein precipitation. Since acetonitrile readily dissolves all sulphonamides, adsorption to proteins which is reported [11] to occur for several of the test compounds in aqueous solutions, was expected to be very limited. Experimental data on analyte recoveries for the total procedure ranged from 69 to 87% which seems to indicate that an appreciable amount of the analytes is lost during the clean-up procedure either by adsorption on precipitated proteins or by adsorption to the ultrafiltration (UF) membrane. Although the overall recovery data are in compliance with the requirements for residue analysis, the cause of the loss of analytes was further investigated. After protein precipitation, sample aliquots were subjected to ultrafiltration after a further 50-fold dilution with water. Adsorption of the sulphonamides to the UF membranes in the resulting, largely aqueous solution that was applied to the UF device, was studied by analysing aqueous standard solutions before and after ultrafiltration. For all analytes, the same peak areas were obtained to within ±5%, which indicates that adsorption to the UF membrane is essentially absent. Consequently, incomplete recovery of the analytes is most likely caused by adsorption on proteins or inclusion in the precipitate, even in the presence of 50% of acetonitrile. Repeatability data however, demonstrate that RSDs are acceptable (cf. Table 2 below) and do allow accurate quantitative determination of the analytes. One final remark should be added: since 100 μl of the ultrafiltrate finally obtained, were injected on the LC column, effectively the equivalent of only 1 μl of milk was analysed.

Fig. 1 presents typical chromatograms of a non-spiked milk sample and a milk sample spiked at the MRL level of 100 μg/kg as obtained with the present UF-LC–MS–MS method.

The mass chromatograms of Fig. 1A were scaled to the same vertical scale as the corresponding traces in the spiked sample (Fig. 1B) in order to facilitate comparison. Both samples contained the internal standard (I.S.) at a concentration of 200 μg/kg. The non-spiked milk sample shows a small peak at the retention time of sulphadimidine corresponding to ~10 μg/kg. This peak originates from an unknown compound and not from a residue of sulphadimidine as was demonstrated by the absence of other product ions characteristic for sulphadimidine. The other mass chromatograms clearly illustrate the selectivity introduced by applying LC–MS–MS. Despite the low mass of analyte injected on the column at the MRL level, which is 100 pg only, the signal-to-noise (S/N) ratios typically are on the order of 100–300. Because the excellent analyte detectability, in its

Table 2
Validation data for the determination of sulphonamides in milk by external calibration ($n=6$)[a]

Analyte	Level (MRL)	Day 1		Day 2		Day 3	
		Accuracy (%)	RSD	Accuracy (%)	RSD	Accuracy (%)	RSD
Sulphadoxine	0.5	102	3.6	105	2.9	95	4.8
	1.0	100	2.4	102	3.3	107	2.1
	1.5	103	2.2	98	3.0	113	4.4
Sulphadimethoxine	0.5	101	5.8	105	1.7	94	3.3
	1.0	102	1.1	102	2.2	106	2.6
	1.5	110	2.1	97	2.9	110	3.1
Sulphadimidine	0.5	95	7.9	104	3.5	90	5.0
	1.0	91	7.9	104	3.7	101	2.7
	1.5	101	4.4	106	3.2	104	4.6
Sulphamethoxazole	0.5	101	4.9	102	2.3		
	1.0	104	5.2	96	4.8		
	1.5	107	2.9	92	2.5		
Sulphadiazine	0.5	105	3.3	103	2.3	93	7.0
	1.0	104	2.6	105	3.0	108	3.4
	1.5	104	3.9	103	2.4	125	4.9

[a] SMX was not included on day 3; for UF-LC–MS–MS conditions, see text.

turn, allows the sample introduction to be limited to the 1-μl milk equivalent mentioned above, there is very little danger that the present approach will adversely affect the performance of the MS–MS measurements. Indeed, in the course of our 4-month study, no such effects were observed. Furthermore, the consistency of the quantitative data obtained by external standard calibration over a period of several months from extended series of analyses, provides strong evidence for the absence of matrix-related adverse influences on MS–MS performance.

3.2. Quantitative analysis

An isotope-labelled analogue was available only for sulphadimidine. Therefore, comparison of isotope-dilution and external-standard calibration was done for this analyte. For the other sulphonamides, quantification was performed by external-standard calibration using blank milk samples spiked at increasing concentrations as calibrants. Fig. 2 shows calibration plots for sulphadimidine constructed by using both approaches. In both instances milk samples were used that had been spiked with analyte concentrations ranging from 25 to 500 μg/kg. Although the slopes of the two calibration plots are different, they are both characterised by high regression coefficients and acceptable intercepts. For a further comparison, samples spiked at 0.5, 1.0 and 1.5 times the MRL were analysed in six-fold on 3 consecutive days. Somewhat surprisingly, the accuracy and precision were essentially the same for isotope-dilution and external-standard calibration, with mean accuracies of 103 and 100%, respectively. RSD values typically ranged from 2 to 10% ($n=6$) with a high RSD (17%) for isotope-diluted quantification at the lowest spiking level on 1 day.

MS-based quantification is frequently adversely influenced by ionisation suppression caused by matrix co-extractants. Isotope dilution is generally used to eliminate such problems. A further advantage of this approach is that an intrinsic recovery correction is achieved. It should be mentioned that, by using spiked milk samples as calibrants, the recovery correction is made also when using external-standard calibration. In our opinion, the main reason why there is no distinct benefit from the application of isotope dilution, is that the sensitivity of the MS–MS procedure allows a considerable, i.e. 50–100-fold, dilution of the samples (cf. above). In other words,

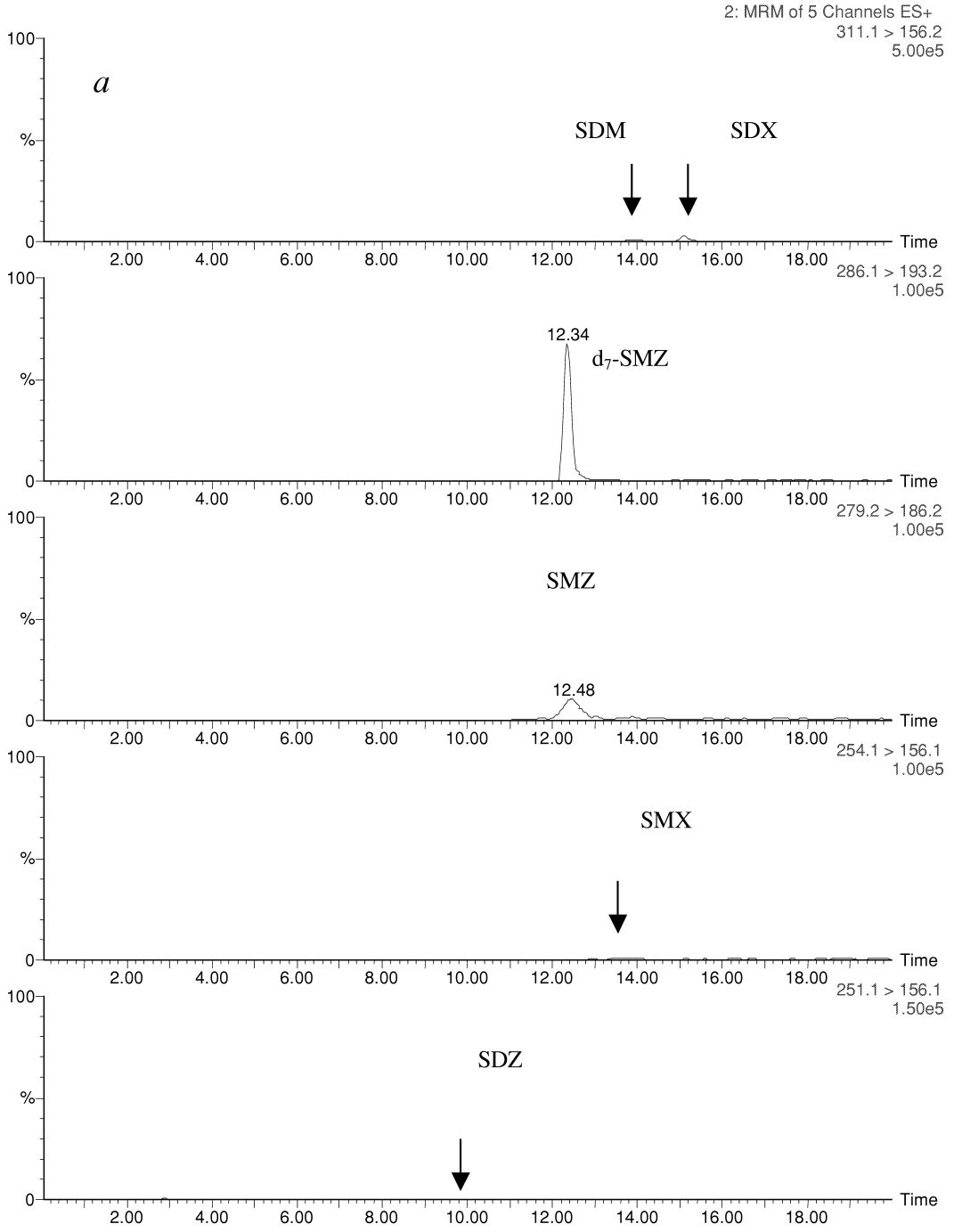


Fig. 1. MRM mass chromatograms of (a) blank milk and (b) milk spiked at 100 μg/kg with sulphadoxine, sulphadimethoxine, sulphadimidine, sulphamethoxazole and sulphadiazine, obtained by UF-LC–MS–MS. Concentration of d_7-sulphadimidine (I.S.), 200 μg/kg.

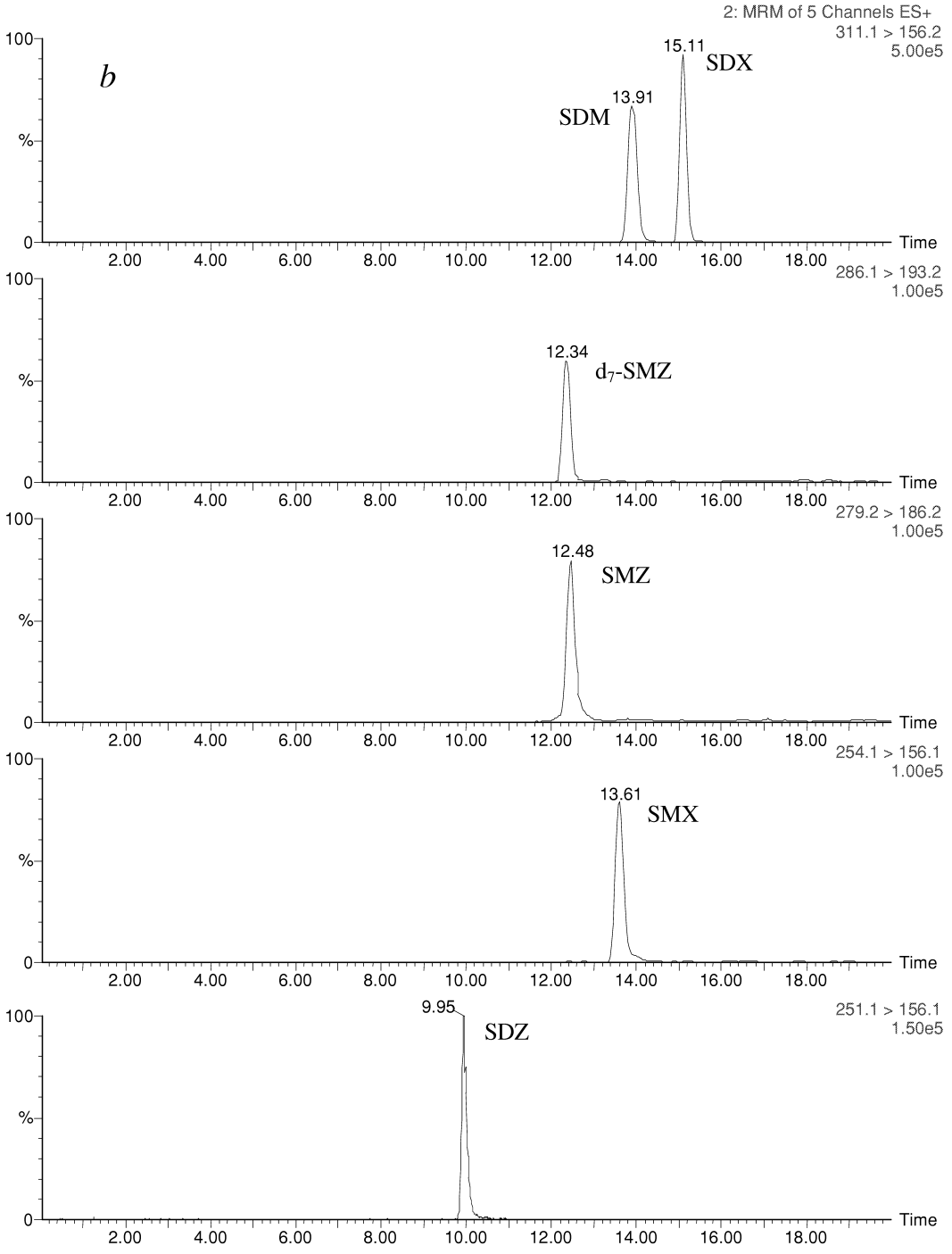


Fig. 1. (*continued*)

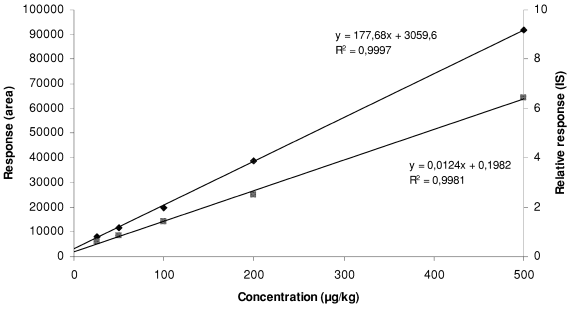


Fig. 2. Calibration curves obtained for sulphadimidine spiked in blank milk at concentrations of 25–500 μg/kg, obtained by plotting (♦) absolute peak areas and (■) the response factor using d_7-sulphadimidine at 200 μg/kg as isotope-labelled analogue.

the extract actually analysed closely resembles an aqueous standard solution and, hence, the factors that cause the sometimes poor quantitative performance of mass spectrometers, such as ionisation suppression, are largely eliminated.

3.3. Method validation

To demonstrate the feasibility of the present approach for residue analysis and to test its robustness and practicability, the method was fully validated.

Linearity was demonstrated on 3 different days using spiked raw milk samples. For all analytes linear calibration plots were obtained with coefficients of correlation of 0.99 or higher (five data points). These plots were reproducible from day to day and, hence, allow accurate quantitative analysis. Table 2 presents the data obtained at spiking levels of (0.5–1.5)×MRL. On each of the 3 days another batch of raw milk was used for the preparation of spiked samples. Also on each of the 3 days, spiking solutions as well as standards were freshly prepared from the stock solutions in methanol. All data were calculated by external-standard calibration.

The mean accuracies are seen to be close to 100% in all instances but one (sulphadiazine, day 3; 1.5× MRL level) and are closely similar to the accuracies that can be obtained by isotope dilution, as was shown above for sulphadimidine. The precision was fully satisfactory with RSDs ranging from 1 to 8%. Considering the limited sample purification and the fact that MS data in general suffer from a slightly higher variability than LC–UV data, this is remarkably good.

Comparison of the data obtained on different days reveals that within-lab reproducibility is fully acceptable. Unifactorial analysis of variance which was applied using these data, yielded RSDs for the within-lab reproducibility ranging from 2.7 to 12% which is fully acceptable.

The limit of quantification (LOQ) which was determined by extrapolating to an S/N of 10, was conveniently low, that is, all analytes except sulphamethoxazole (10 μg/kg) could be detected and quantified at levels as low as 5 μg/kg.

For residue analysis of veterinary drugs, the EU has drafted criteria for method performance [12]. The performance characteristics included in the most recent draft include the decision limit, CC_α, and the detection capability, CC_β [13]. The decision limit is the limit from which on it can be decided that a sample is truly violative with an error probability of α. The detection capability is the smallest content of the analyte that may be detected, identified and/or quantified in a sample with an error probability of β. The validation experiments were designed to permit the calculation of these characteristics; the results are presented in Table 3. It is obvious that both CC_α and CC_β are highly reproducible. A statistically significant ($\alpha = 0.05$) violation of the MRL can be concluded when a concentration of 103–107 μg/kg (compound-dependent) is found. This is very near to the MRL and indicates the capability of the present method, despite its simplicity, to determine accu-

Table 3
Values for CC_α and CC_β obtained from the quantitative analysis of sulphonamides in raw milk on 3 consecutive days

Analyte	CC_α		CC_β	
	Mean	SD	Mean	SD
Sulphadoxine	104.4	1.4	109.9	1.7
Sulphadimethoxine	102.8	0.9	107.7	1.7
Sulphadimidine	107.1	3.2	114.8	4.9
Sulphamethoxazole	105.1	1.2	110.3	2.0
Sulphadiazine	104.6	0.4	110.6	0.6

rately the concentration of sulphonamides at MRL levels.

3.4. Application to preserved milk and milk replacers

The present procedure was developed and optimised for raw milk samples but its applicability to preserved milk also is an important aspect. UF-LC–MS–MS was therefore applied, without any modification, to pasteurised, sterilised and ultra-high temperature (UHT) treated milk spiked with the sulphonamides at the same concentration levels as the raw milk samples; the MRL level was analysed in six-fold. In all instances, essentially the same results were obtained as for raw milk for parameters such as linearity, repeatability and recovery. No interfering peaks showed up in any of the LC–MS–MS traces, but with UHT milk, the noise level was some two-fold higher. As a consequence, the LOQs were the same as for raw milk, i.e. 5–10 μg/kg, except for UHT milk (10–20 μg/kg). With the MRLs for sulphonamides at 100 μg/kg, this is amply sufficient for routine quantification.

The method was also applied to the analysis of artificial milk used as calf feed. Compared to raw cows' milk this is a quite different matrix. The sample was reconstituted in water in a 1:10 ratio;

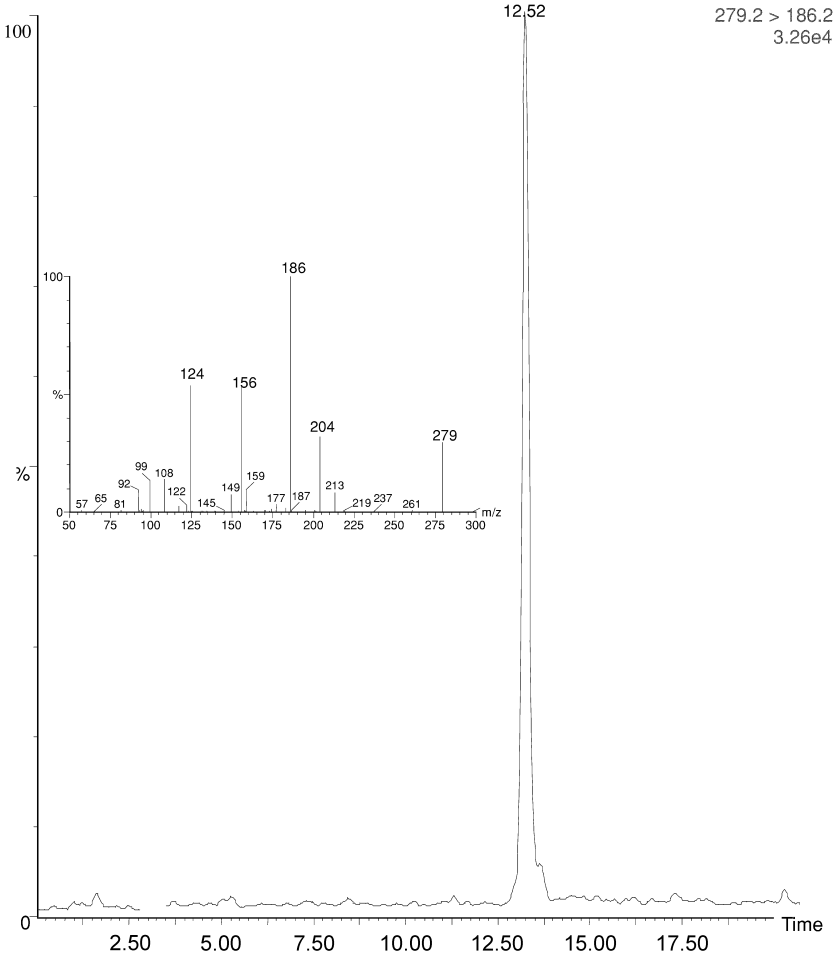


Fig. 3. MRM mass chromatogram of an artificial milk sample containing sulphadimidine and (insert) its CID spectrum.

next, the UF-LC–MS–MS procedure was applied. Fig. 3 shows the mass chromatogram for an artificial milk sample suspected to contain sulphadimidine, presenting the most abundant transition of sulphadimidine and its CID spectrum. It is evident that detection of the sulphonamide presents no problems. Its identity could readily be confirmed according to EU requirements based on the ratio of two product ions (viz. m/z 186 and m/z 204). Comparison with the corresponding ion ratio in a standard revealed a high degree of similarity, viz. 10%. The concentration of sulphadimidine was found to be ~250 μg/kg in the reconstituted milk. The analytical performance data were, again, similar to those found for raw milk.

3.5. Dapsone

The analytes discussed above have MRLs of 100 μg/kg. Another sulphonamide however, dapsone, is a banned substance. This means that there is a zero tolerance: it is not allowed for veterinary use and, hence, should be absent from food products. Designing proper analytical methods for such compounds is rapidly becoming more important in view of EU regulations. Today, for dapsone the pursued detection limit is set at 5 μg/kg.

To illustrate the usefulness of UF-LC–MS–MS at the required ultra-trace level, raw milk was spiked with 1.25–25 μg/kg of dapsone. Fig. 4 presents mass chromatograms obtained for a blank milk sample and a milk sample spiked at 5 μg/kg. Obviously, detection and quantification do not pose any problems at this low concentration, earlier in this paper quoted as the LOQs for the majority of test analytes. In other words, the target value for this particular banned compound is readily achieved. The mass chromatogram of the blank milk sample indicates the presence of a minute amount of less than 1 μg/kg of dapsone that was found to be due to carry-over from an earlier injected standard solution. Apparently, at analyte levels of 1–2 μg/kg—which, obviously, can be analysed—it will become necessary to take extra measures to avoid cross-contamination.

Once again, one should note that this analysis was performed using 100-fold diluted milk samples. In other words, in order to avoid clogging and/or

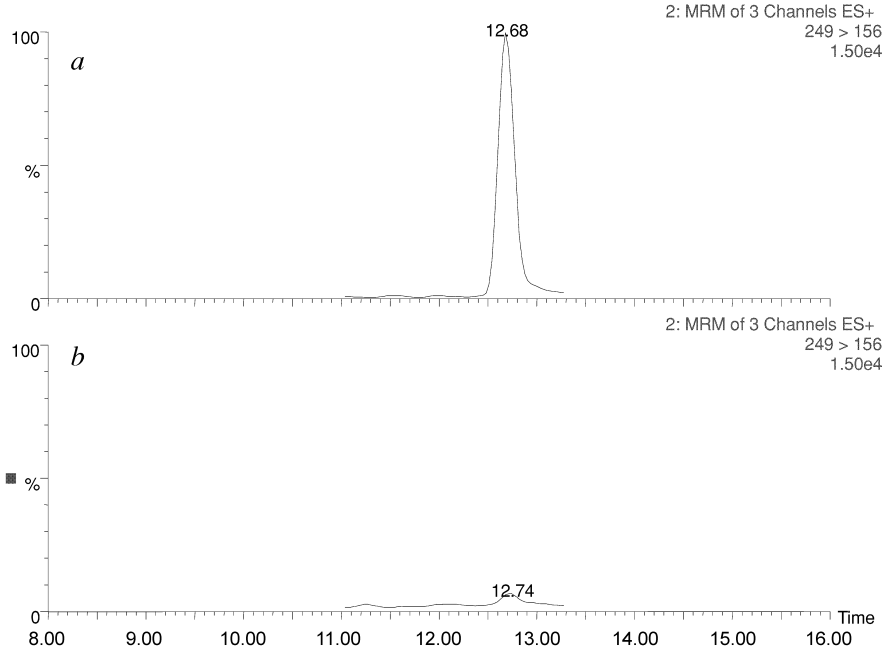


Fig. 4. MRM mass chromatograms of (a) milk spiked at 5 μg/kg with dapsone and (b) blank milk. For UF-LC–MS–MS conditions, see text.

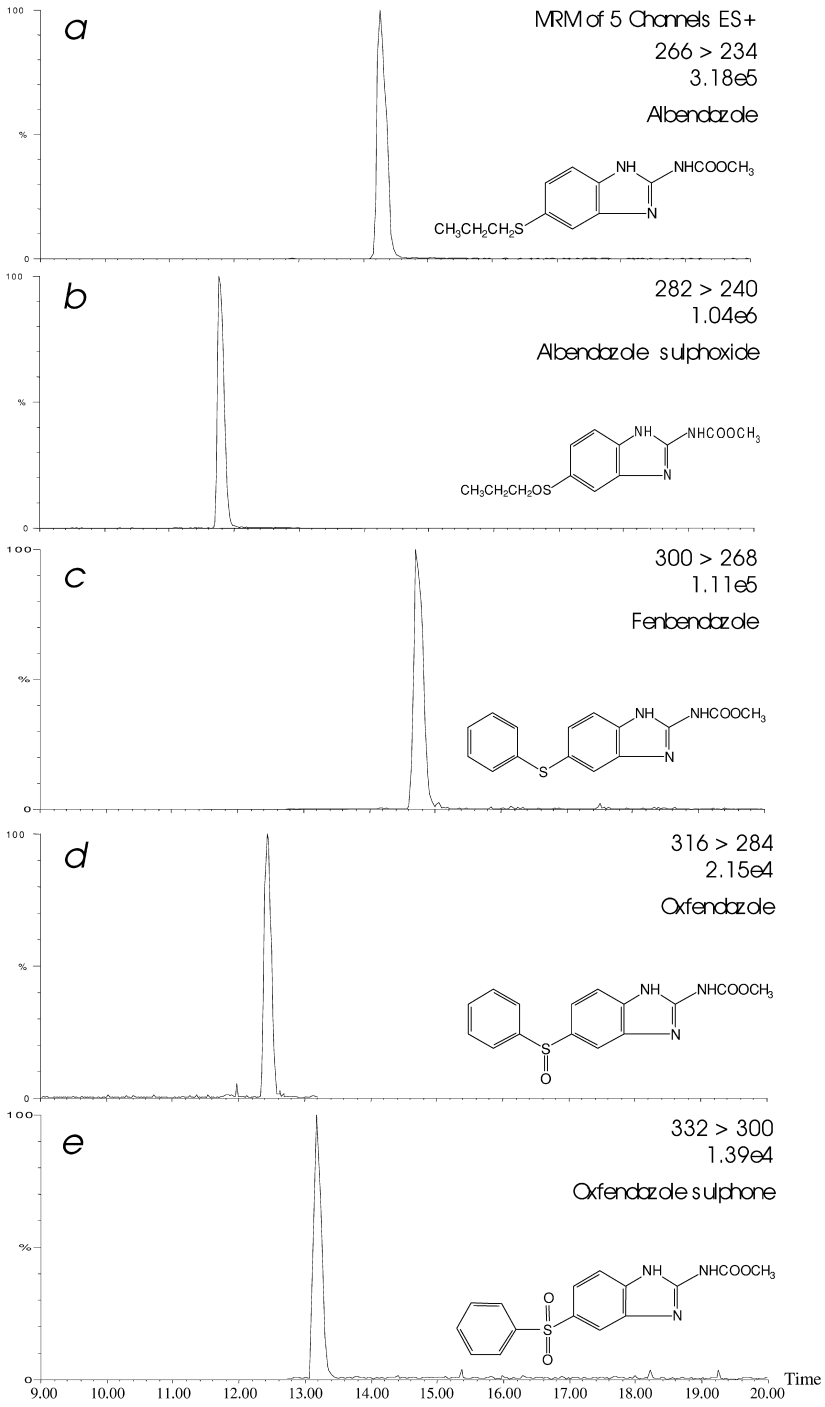


Fig. 5. Mass chromatograms of blank milk spiked with (a) albendazole (50 μg/kg), (b) albendazole sulphoxide (50 μg/kg), (c) fenbendazole (2.5 μg/kg), (d) oxfendazole (2.5 μg/kg) and (e) oxfendazole sulphone (2.5 μg/kg). MRM transitions are indicated in the figure. For UF-LC–MS–MS conditions, see text.

interferences due to matrix completely, part of the inherent detection potential was sacrificed.

3.6. Extending the application range

Because of its simplicity and the absence of a selectivity-inducing step in the sample preparation procedure, the present approach may be expected to be applicable to a wide range of compounds from several classes. Preliminary experiments with the determination of benzimidazoles in milk are presented here to verify this suggestion. Benzimidazoles are anthelmintic compounds widely used in veterinary practice. Their MRLs differ from 100 μg/kg for albendazole including its metabolites to 10 μg/kg for fenbendazole and metabolites. Because of the different demands and the, in our experience, somewhat poor ionisation efficiency of the benzimidazoles, the sample preparation was slightly modified.

Ultrafiltration was carried out with the 50% acetonitrile mixture to avoid adsorption of the analytes to the UF membrane. Of the ultrafiltrate obtained, a volume corresponding to 5 μl of milk was subjected to LC–MS–MS. Fig. 5 presents mass chromatograms obtained from a milk sample spiked at the MRL level for albendazole, albendazole sulphoxide, fenbendazole, oxfendazole and oxfendazolesulphone. The MRM transitions used are included in Fig. 5.

Albendazole and its sulphoxide yield very intense peaks with S/N ratio of several hundreds, which clearly indicates that detection well below their MRL is easily possible. Actually for these compounds a sample dilution as applied for the sulphonamides can readily be used and, consequently, incorporation in a single assay is feasible.

Fenbendazole and its metabolites are also readily detected at the MRL level and a five-fold lower concentration should not create problems. However, the distinctly lower signal intensities recorded, indicate that, in this instance, further dilution of the sample (analogous to that for sulphonamides) had best be avoided.

The added example, with estimated LOQs of 0.5–2.5 μg/kg, does demonstrate the general potential of the present approach, which is especially useful because for compounds such as the benzimidazoles, inexpensive and fast microbiological screening methods such as exist for the sulphonamides, are lacking.

4. Conclusions

The off-line combination of ultrafiltration and LC–MS–MS has been shown to be a promising technique with good analytical performance for the trace-level determination, i.e. identification and quantification, of veterinary drugs in raw and preserved milk and milk replacers. Although UF-based sample preparation is performed off-line, the possibility of treating 24 samples simultaneously ensures that there is no barrier here to high sample throughput.

With sulphonamides as test analytes, it is demonstrated that no problems are encountered at or below the MRL levels of such analytes, nor at the much lower concentrations typically required in the case of a banned compound. Brief study of another class of compounds, the benzimidazoles, indicates that the present approach can, with minor modification, also be applied there. Therefore, future work of our group will be directed at further exploring this generic approach for more classes of veterinary drugs and other matrices. Proper consideration of analyte characteristics, MS–MS detectability and required sensitivity (MRLs, banned substances) will be a main aspect of such studies aiming at wide-ranging multi-residue methods. Preliminary results for the analysis of nitroimidazoles in porcine muscle reveal a promising perspective for this approach [14].

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