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Determination of sulfonamides in animal tissues using cation exchange reversed phase sorbent for sample cleanup and HPLC–DAD for detection

W. Hela*, M. Brandtner, R. Widek, R. Schuh

Bundesanstalt für veterinärmedizinische Untersuchungen, Robert Kochgasse 17, A-2340 Mödling, Austria

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Abstract

A new extraction method for 12 sulfonamides (sulfadiazine, sulfathiazole, sulfapyridine, sulfamerazine, sulfamethizole, sulfadimidine, sulfisoxazole, sulfamethoxazole, sulfatroxazole, sulfachlorpyrazine, sulfaphenazole and dapsone) in muscle, liver and kidney was developed. Fortified tissues of muscle, liver and kidney from bovine, pig and chicken were studied. A 10 g tissue and 10 ml acetonitrile were homogenised with an Ultra-Turrax. Next, the tissue was defatted with 5 ml *n*-hexane, centrifuged and filtered into flasks. Then the extracts were acidified with 10 ml hydrochloric acid and diluted with deionised water. Next, the samples were loaded on OASIS® MCX columns, which were conditioned with 5 ml methanol and 5 ml water. The columns were washed with 5 ml hydrochloric acid and 5 ml methanol. Then the samples were eluted with 5 ml ammonia solution/acetonitrile (v/v 1/19), allowed to dry under nitrogen and reconstituted in 200 µl acetonitrile/water-mixture (v/v 1/4). The analyses were carried out on HPLC–DAD. Mobile phase was 0.01 M ammonium acetate pH 4.6 (A) and acetonitrile (B). Chromatographic separation was obtained by gradient elution (5% B to 40% within 16 min, back to 5% in 1 min, equilibration for 3 min). The sulfonamides were detected at 260 nm and dapsone at 294 nm. The detection limits of the HPLC method were 1 ppb for all analytes.

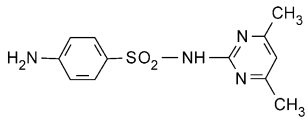
Keywords: Sulfonamides; Dapsone; HPLC

1. Introduction

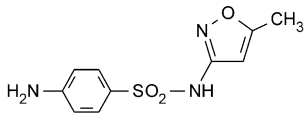
The sulfonamides are synthetic antibiotics with a wide spectrum against most gram-positive and many gram-negative organisms. Sulfonamides inhibit multiplication of bacteria by acting as competitive inhibitors of *p*-aminobenzoic acid in the folic acid metabolism cycle (Forth, Henscheler, & Rummel, 1987).

They are widely used as growth promoters and antimicrobial agents in animal production. But, many strains of an individual species may be resistant.

To monitor the sulfonamide residues a reliable and sensitive method is needed. Within the EU the maximum residue limit is 100 µg/kg in muscle, kidney and liver in any animal, which is used for food production. Dapsone is generally forbidden (Commission of the European Communities). In the following the structure of the two most common used sulfonamides and dapsone is given:



Sulfadimidine (Panazin®)



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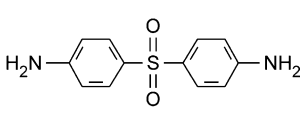

Dapsone

* Corresponding author. Tel.: +43-2236-46640; fax: +43-2236-46640-368.

E-mail address: wolfgang_hela@yahoo.de (W. Hela).

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There is a variety of methods to analyse sulfonamides:

- Capillary electrophoresis for separation and UV for detection (Lin, 1997)
- Derivatization and gas chromatography (Cannavan, 1996)
- High pressure liquid chromatography and UV (Malisch, 1986) or mass spectrometry (Kaufmann, Roth, Ryser, Widmer, & Guggisberg, 2002) for detection
- Supercritical fluid extraction (Pensabene, Fiddler, & Parks, 1997)

The method which was used before (Malisch, 1986) had some disadvantages: poor yield, poor purity of the sample extracts especially in liver and kidney and low sample throughput. The method consisted of a liquid–liquid extraction with acetonitrile and ethyl acetate. This extract was evaporated in a rotary evaporator, reconstituted in methanol/water (v/v 80/20) and defatted with *n*-hexane. The extract was dried under nitrogen and reconstituted in a acetonitrile/water-mixture (v/v 1/4). The analyses were carried out by HPLC–DAD. The aim of the present work was to develop a reliable method with a higher sample throughput and better recoveries especially for dapsone.

2. Experimental

2.1. Chemicals

All chemicals were analytical or LC grade and were purchased from Merck (Darmstadt, Germany). The sulfonamides, except sulfaphenazole which was purchased from Pfizer, were purchased from Sigma (Deisenhofen, Germany). The stock solutions (c = 1 mg/ml) were prepared in methanol. The spiking solution (c = 5 μg/ml) was prepared in deionised water.

2.2. Sample extraction

A 10 g homogenised tissue was weighed into a 50-ml polypropylene tube and spiked with 1 μg sulfonamides and 0.3 μg dapsone. The sample was allowed to stand for 10 min. Then 10 ml acetonitrile were added and the sample was homogenised with an Ultra-Turrax. Next, 5 ml

Table 1
Decision limit (CCα)[a] and detection capability (CCβ)[b] of the analytes

Analytes	Muscle		Kidney		Liver	
	CCα [ppb]	CCβ [ppb]	CCα [ppb]	CCβ [ppb]	CCα [ppb]	CCβ [ppb]
Sulfadiazine	109	118	117	134	115	130
Sulfathiazole	112	124	112	124	126	151
Sulfapyridine	113	126	127	154	128	157
Sulfamerazine	112	125	108	116	117	133
Sulfadimidine	113	126	107	114	128	157
Dapsone	17	19	17	20	20	24
Sulfisoxazole	112	123	104	107	124	147
Sulfamethoxazole	112	124	104	109		
Sulfatroxazole	113	125	116	132	127	154
Sulfachlorpyridazine	113	126	105	110	125	150
Sulfaphenazole	115	130	105	110	127	154

[a] CCα is the concentration of the analyte is—95% possibility—above the residue limit, max. 5% false-positive results. CCα = 100 + 1.64×RSD×100.

[b] CCβ is the concentration value where—if spiked samples are analysed—not more than 5% false-negative results according to the decision limit (CCα) are obtained. CCβ = 100 + 2 × 1.64 × RSD × 100.

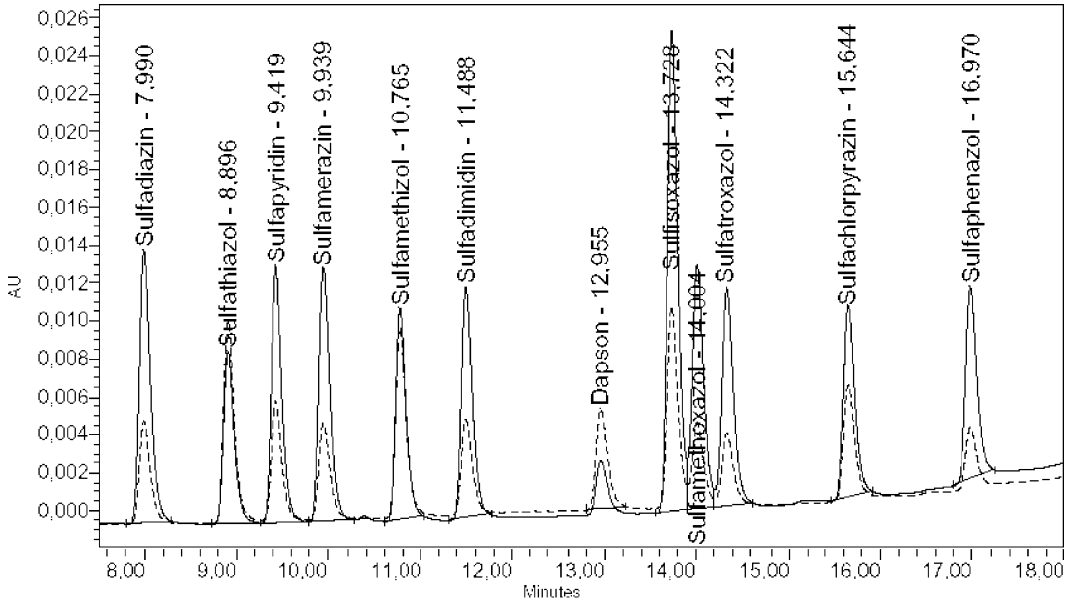

Fig. 1. A 50 μl injection of a standard containing 2 ppb sulfonamides (solid line) and 0.6 ppb dapsone (dotted line).

n-hexane was added, the sample was mixed in a vortex stirrer and was treated in an ultrasonic-bath. Then the sample was centrifuged at 3600 min^{-1} (=3000 g) for 10 min. The upper layer (*n*-hexane) was discarded. The layer in the middle (acetonitrile) was filtered into a 50-ml flask. The filter was washed with 10 ml 1 M hydrochloric acid and then the flask was filled up with deionised water.

2.3. Solid phase extraction

An OASIS® MCX (6 ml, 500 mg) (Milford, Massachusetts) was conditioned with 5 ml methanol and 5 ml deionised water. After sample loading, the column was washed with 5 ml 0.1 N hydrochloric acid and 5 ml methanol. Then the sulfonamides were eluted with 5 ml ammonia solution/acetonitrile (v/v 1/19), allowed to dry under nitrogen at 40 °C and reconstituted in 200 µl acetonitrile/deionised water (v/v 1/4).

Table 2
Recoveries and RSD of spiked muscle tissue[a]

Analyte	Spiking level 50 µg/kg		Spiking level 100 µg/kg	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Porcine				
Sulfadiazine	27.4	6.4	22.7	4.6
Sulfathiazole	58.0	7.4	49.0	6.7
Sulfapyridine	49.0	6.5	44.6	8.0
Sulfamerazine	39.5	4.4	34.7	3.4
Sulfadimidine	48.0	5.6	43.9	6.8
Dapsone	52.8	4.3	41.8	7.7
Sulfisoxazole	42.8	5.5	39.1	7.0
Sulfamethoxazole	51.3	5.9	44.5	7.3
Sulfatroxazole	47.3	4.8	43.1	7.6
Sulfachlorpyrazine	43.6	5.4	39.0	7.8
Sulfaphenazole	44.7	4.5	40.3	9.3
Chicken				
Sulfadiazine	23.1	5.4	17.4	5.4
Sulfathiazole	66.6	3.0	57.1	7.2
Sulfapyridine	44.5	7.9	50.6	6.1
Sulfamerazine	28.8	2.7	29.4	7.6
Sulfadimidine	44.3	8.5	47.5	8.0
Dapsone	53.4	7.1	49.0	6.9
Sulfisoxazole	43.0	5.8	44.5	4.9
Sulfamethoxazole	50.1	7.9	50.6	5.3
Sulfatroxazole	44.7	9.2	49.8	6.1
Sulfachlorpyrazine	43.8	7.9	45.4	5.6
Sulfaphenazole	45.5	9.4	49.4	6.3
Bovine				
Sulfadiazine	25.7	8.9	23.7	4.4
Sulfathiazole	60.7	8.8	53.0	4.7
Sulfapyridine	47.3	5.5	45.5	6.5
Sulfamerazine	41.3	5.6	38.5	4.4
Sulfadimidine	47.8	4.1	45.9	5.5
Dapsone	52.8	3.2	43.5	4.9
Sulfisoxazole	41.4	5.4	40.7	6.9
Sulfamethoxazole	49.3	5.6	46.5	6.2
Sulfatroxazole	45.3	4.4	45.0	6.6
Sulfachlorpyrazine	42.9	5.5	41.1	6.6
Sulfaphenazole	43.4	4.2	42.1	6.1

[a] Precision within 1 day at three concentration levels ($n=6$ for each level). Extraction is done by one technician. RSD should be below 23% (Horwitz term: $RSD=2^{(1-0.5\log C)}$).

2.4. HPLC–DAD

The analyses were carried out on a Waters 2690 (Milford, Massachusetts) separations module coupled with a Waters 996 photodiode array detector. Mobile phase was 0.01 M ammonium acetate pH 4.6 (A) and acetonitrile (B). Chromatographic separation was obtained by gradient elution (5% B at the beginning linear descend to 40% within 16 min, back to 5% in 1 min, equilibration for 3 min) using a Phenomenex Luna C_{18} (250×2 mm; 5µm) (Torrance, California) at 40 °C at a flow of 0.35 ml/min. 25 µl of the sample was injected. These conditions are also suitable for LC–MS. The sulfonamides were detected at 260 nm and dapsone at 294 nm. The detection limits were 1 ppb for all analytes (Fig. 1).

2.5. Calibration and quantitation

The calibration curves were based on an external standard mixture. The curves ranged for all sulfonamides from 10 to 100 ppb, for dapsone from 3 to 30 ppb. The

Table 3
Recoveries and RSD of spiked kidney tissue[a]

Analyte	Spiking level 50 µg/kg		Spiking level 100 µg/kg		Spiking level 150 µg/kg	
	Recovery	RSD	Recovery	RSD	Recovery	RSD
Porcine						
Sulfadiazine	17.0	6.1	14.0	10.3	13.9	2.4
Sulfathiazole	60.6	4.1	46.2	7.4	43.0	3.4
Sulfapyridine	53.1	3.7	59.1	16.5	61.9	11.1
Sulfamerazine	31.9	3.0	27.4	3.9	26.0	2.0
Sulfadimidine	60.8	0.8	52.5	3.0	50.4	1.8
Dapsone	61.2	1.8	51.4	2.6	50.9	2.3
Sulfisoxazole	52.7	1.4	48.7	2.2	48.6	1.0
Sulfamethoxazole	77.9	3.4	61.3	2.5	56.8	2.3
Sulfatroxazole	86.1	2.2	65.7	2.1	60.4	1.1
Sulfachlorpyrazine	59.6	2.6	53.2	2.5	53.7	1.8
Sulfaphenazole	55.8	1.8	52.0	3.2	52.5	1.3
Bovine						
Sulfadiazine	15.9	8.4	13.1	7.0	13.3	8.0
Sulfathiazole	69.3	7.9	52.6	6.9	47.2	8.2
Sulfapyridine	57.9	3.2	55.1	4.5	51.4	4.4
Sulfamerazine	33.6	6.9	29.3	4.9	27.8	4.4
Sulfadimidine	50.8	4.5	48.4	4.3	46.7	4.4
Dapsone	62.1	7.7	50.3	9.6	47.2	10.6
Sulfisoxazole	50.9	2.9	47.7	2.1	46.5	2.8
Sulfamethoxazole	67.5	4.6	57.3	2.7	53.0	3.3
Sulfatroxazole	67.4	14.9	60.9	9.6	61.0	8.1
Sulfachlorpyrazine	58.1	2.6	52.0	2.9	48.5	2.3
Sulfaphenazole	52.3	3.3	47.7	1.6	45.7	3.6

[a] Precision within 1 day at three concentration levels ($n=6$ for each level). Extraction is done by one technician. RSD should be below 23% (Horwitz term: $RSD=2^{(1-0.5\log C)}$).

correlation coefficients of the linear standard curves were greater than 0.99.

The efficiency of extraction of the sulfonamides was determined by comparing the concentration of the spiked samples with those of the corresponding standards.

Table 4
Recoveries and RSD of spiked liver tissue[a]

Analyte	Spiking level 50 μg/kg		Spiking level 100 μg/kg		Spiking level 150 μg/kg	
	Recovery	RSD	Recovery	RSD	Recovery	RSD
Porcine						
Sulfadiazine	13.1	18.0	13.6	9.3	11.0	9.1
Sulfathiazole	47.7	18.9	39.1	10.2	35.2	8.5
Sulfapyridine	28.8	6.4	36.7	14.9	39.7	12.0
Sulfamerazine	27.3	5.6	30.1	10.2	25.6	6.0
Sulfadimidine	37.5	5.2	32.6	14.3	34.3	8.9
Dapsone	39.0	8.3	32.2	16.2	32.2	10.7
Sulfisoxazole	25.0	15.7	27.9	13.2	26.8	14.2
Sulfamethoxazole						
Sulfatroxazole	47.0	8.4	41.4	11.4	40.1	8.6
Sulfachlorpyrazine	28.7	20.4	30.4	10.4	32.4	8.1
Sulfaphenazole	31.3	15.2	33.2	11.7	34.7	7.8
Bovine						
Sulfadiazine	18.1	5.3	15.2	7.6	16.2	5.9
Sulfathiazole	39.0	11.3	37.4	15.7	34.6	5.7
Sulfapyridine	34.8	6.1	36.3	17.4	39.8	7.3
Sulfamerazine	33.5	7.6	30.0	7.0	30.1	6.3
Sulfadimidine	35.8	6.5	36.6	17.3	38.2	3.2
Dapsone	38.8	8.9	32.8	19.2	38.8	11.8
Sulfisoxazole	28.3	6.8	27.6	14.4	30.3	4.4
Sulfamethoxazole						
Sulfatroxazole	50.4	4.9	41.3	16.4	45.4	3.3
Sulfachlorpyrazine	32.9	4.1	30.2	15.3	32.8	5.0
Sulfaphenazole	35.8	6.5	33.9	16.5	37.9	4.2

[a] Precision within 1 day at three concentration levels ($n = 6$ for each level). Extraction is done by one technician. RSD should be below 23% (Horwitz term: $RSD = 2^{(1-0.5\log C)}$).

3. Validation procedure

This method was validated for muscle (pig, chicken, bovine), liver (pig, bovine) and kidney (pig, bovine). Sulfamethizole was not validated because it was not available at the beginning of the validation. The decision limit (CCα), detection capability (CCβ), trueness, precision, selectivity and ruggedness were determined according to lit. (Kaufmann et al., 2002).

4. Results and discussion

4.1. HPLC method development

In the HPLC method (Malisch, 1986) which was used before, sodium acetate buffered water (pH 4.8) and acetonitrile were used to separate the sulfonamides on a Spherisorb ODS column.

It was planned to analyse the samples on a LC–MS system. So a volatile buffer was needed, and sodium acetate was substituted with ammonium acetate. To get better peak shape the Spherisorb column was changed to a Luna C_{18} column. At pH 4.6 baseline separation was achieved for nine analytes. For sulfisoxazole, sulfamethoxazole and sulfatroxazole no baseline separation was obtained. The retention time was stable and the peak shape was quite good because the pH of the buffer was about two pH-units below the pKa of the analytes (Eger, Troschütz, & Roth, 1999).

To improve the detection limit of dapsone 50 μl instead of 25 μl were injected into the HPLC column (Fig. 1). To get better results from library search the UV-spectra measurement started at 240 instead of 220 nm.

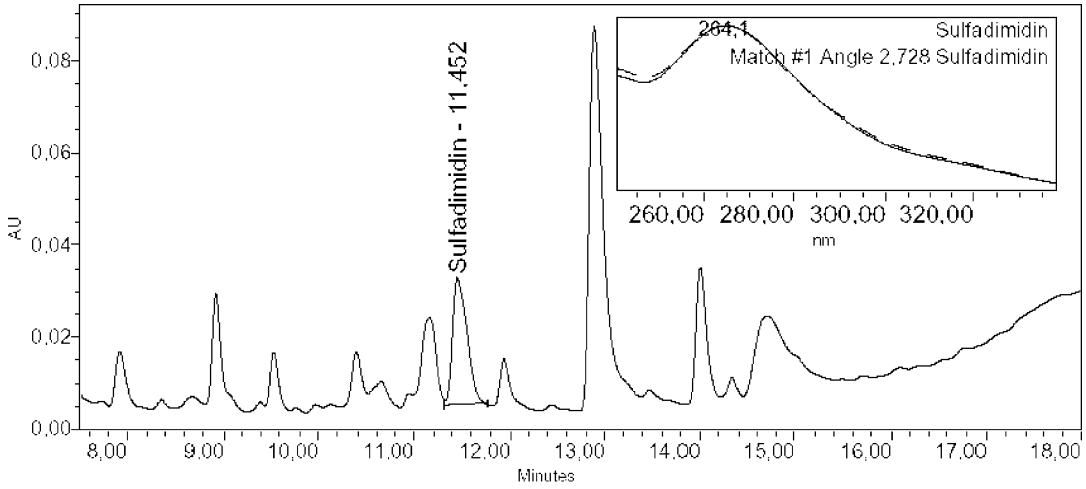

Fig. 2. Positive sample containing 11 ppb sulfadimidine.

4.2. Extraction method development

First OASIS® HLB cartridges were used as reported in Waters Corp (2001a), but a lot of interference's made it impossible to find all analytes. Experiments with some endcapped C_{18} cartridges of different manufacturers also did not lead to satisfying results.

Next, OASIS® MCX cartridges were chosen. First the SPE procedure was optimised as described in Waters Corp (2001b). Then the extraction step with acetonitrile and the defatting were optimised.

The validation experiment showed that in the muscle extracts, less interference's were found and that the recovery in kidney was higher than in muscle or liver. The results are shown in Tables 1–4.

Ruggedness was determined by comparing the percentage recoveries of the samples spiked with 100 ppb. The difference was not significant, if the value was smaller than the critical *F*-value (data not shown). Ruggedness showed that the kind of matrix influenced the recovery more than the type of animal. That means that for every different matrices, except pig and bovine liver, a separate spiked sample has to be analysed.

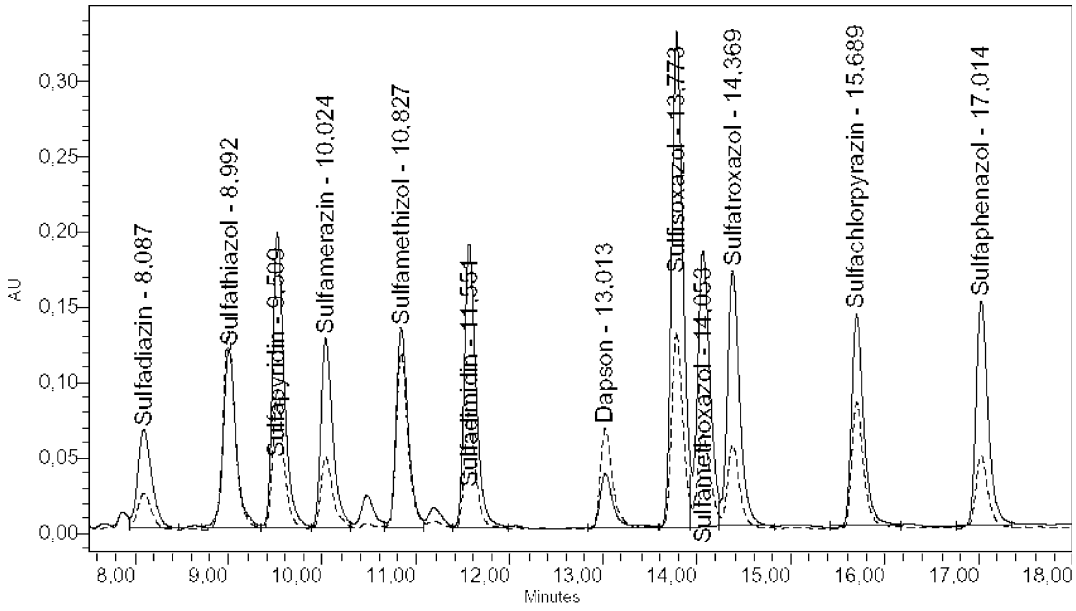

Fig. 3. Porcine muscle extract (new method) spiked with 100 ppb sulfonamides (solid line) and 30 ppb dapsone (dotted line).

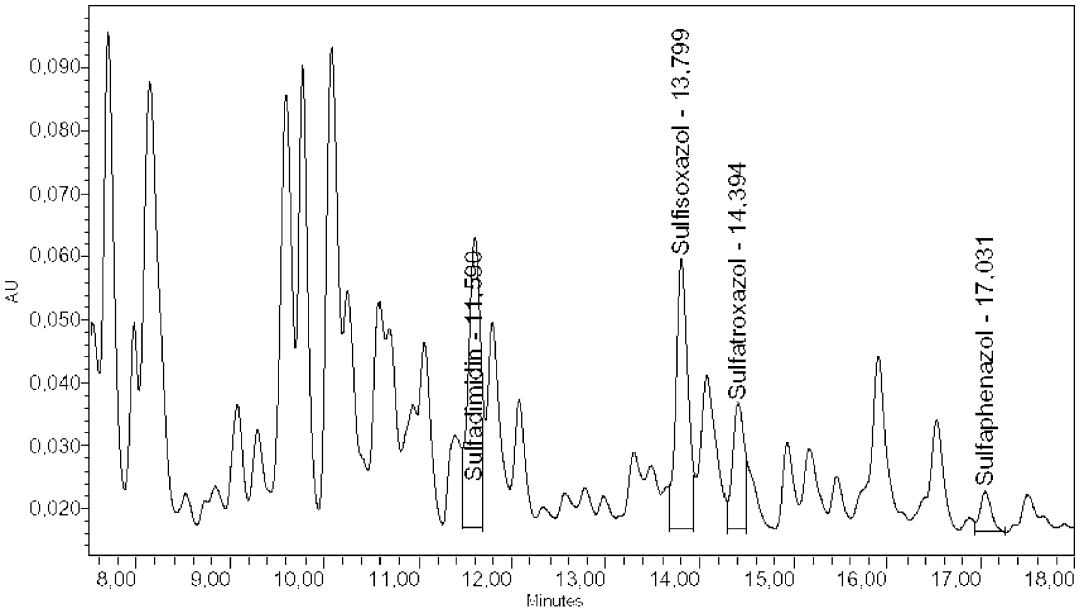

Fig. 4. Porcine muscle extract (old method) spiked with 100 ppb sulfonamides (solid line) and 30 ppb dapsone (not found). Seven sulfonamides were not found because of interferencés recovery was also quite poor.

In Figs. 3–8 it can be seen, that the extracts obtained with the new extraction method show less interference's than the extracts which were done with the old one. Further can be seen that dapson and some sulfonamides were not found in the extracts done with the old extraction method (Figs. 4, 6, 8). The new extraction method is also suitable for kidney (Fig. 5) and liver (Fig. 7). Interference's made it impossible for the computer to match sulfathiazole, sulfamethizole, sulfamethoxazole and sulfatroxazole in the kidney extracts at half MRL with the UV-spectra library. At the MRL a correct library match for all analytes was obtained except sulfamethoxazole. In the liver extract sulfamethoxazole (Fig. 7) was not found, because an interference overlaid the sulfamethoxazole peak.

Until now 580 samples were analysed with this method. The samples were taken by federal veterinary surgeons according to the national monitoring plan. This plan prescribes that a certain percentage of the in Austria slaughtered animals, which were used for food production has to be analysed.

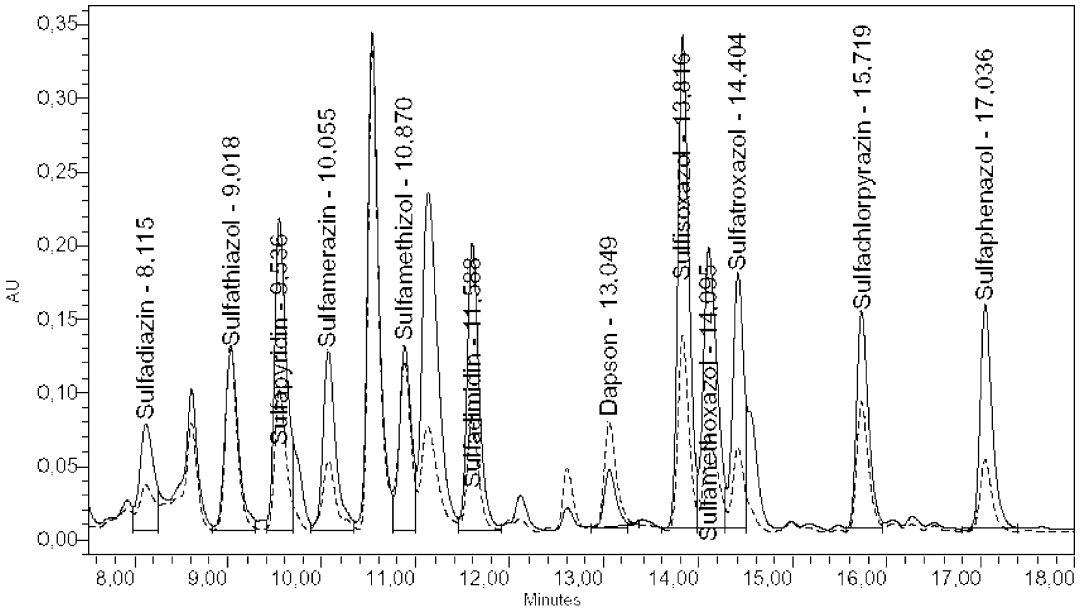

Fig. 5. Porcine kidney extract (new method) spiked with 100 ppb sulfonamides (solid line) and 30 ppb dapsone (dotted line).

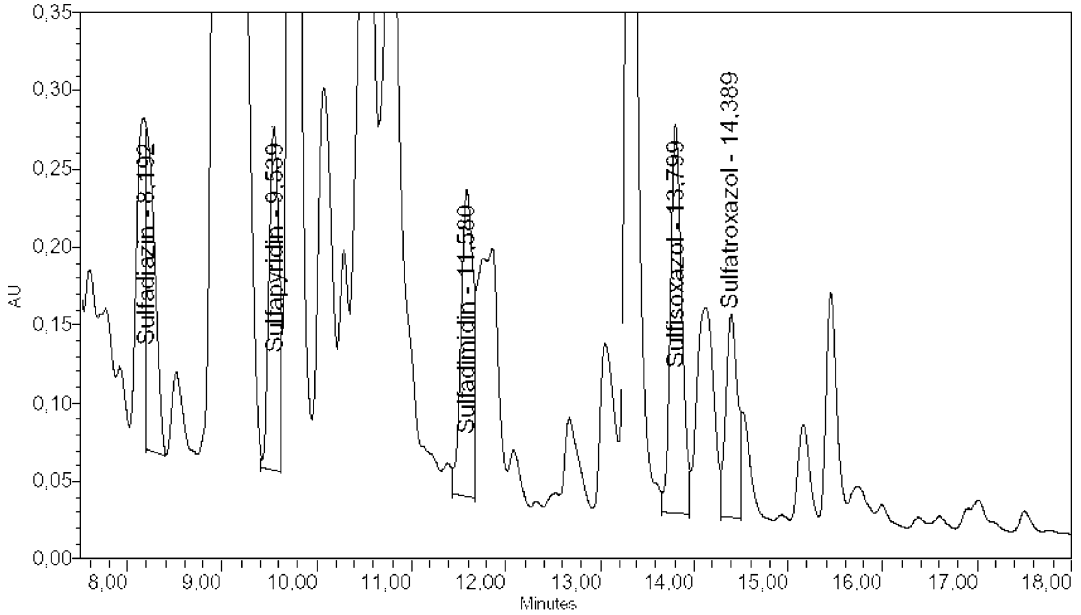

Fig. 6. Porcine kidney extract (old method) spiked with 100 ppb sulfonamides (solid line) and 30 ppb dapsone (not found). Six sulfonamides were not found because of interferences.

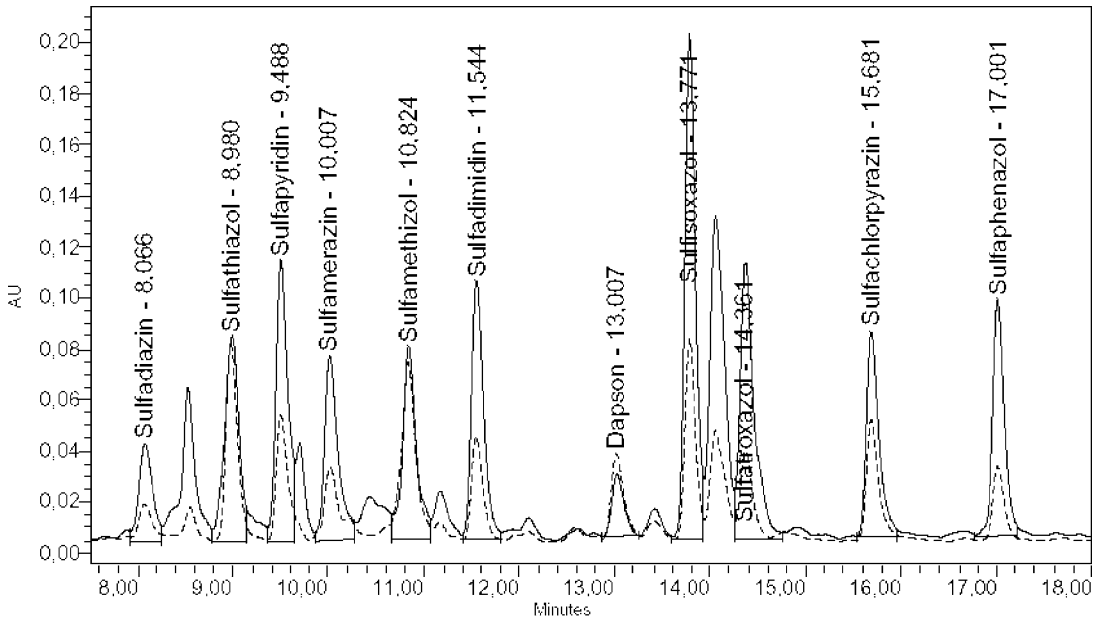

Fig. 7. Bovine liver extract (new method) spiked with 100 ppb sulfonamides (solid line) and 30 ppb dapsone (dotted line).

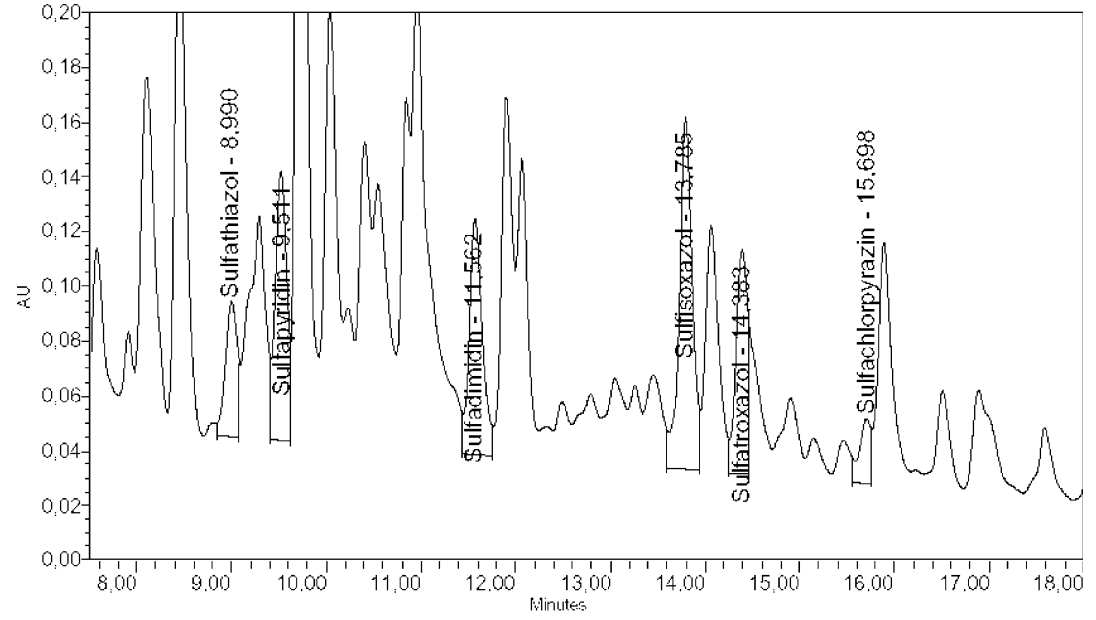

Fig. 8. Bovine liver extract (old method) spiked with 100 ppb sulfonamides and 30 ppb dapsone (not found). Five sulfonamides were not found because of interferences.

Twelve samples were positive, sulfadimidin was found in them. The sulfadimidin content of the positive samples ranged from 7 to 1232 ppb. As shown in Fig. 2, sulfadimidin could be detected in low concentration and a library match was obtained.

In conclusion, the extraction method developed for this 12 compounds is more reliable and more sensitive then the method (Malisch, 1986) used before. Further a higher sample throughput is achieved and the consumption of the chemicals could be lowered to 25%.

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