ELSEVIER

Journal of Chromatography A, 871 (2000) 37–42

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Quantitative determination of sulfonamide residues in foods of animal origin by high-performance liquid chromatography with fluorescence detection

G. Stoev*, Al Michailova

Central Laboratory for Veterinary Control and Ecology, Iskarsko Shosse 5, Sofia 1528, Bulgaria

Abstract

An HPLC method with fluorescence detection is proposed for the quantitative determination of residues of ten of the most used sulfonamides as their derivatives. Sulfonamides were isolated from meat, mix meat and kidney with ethyl acetate (first extraction) and acetone (second extraction) and further purified by partitioning three times with water–methylene chloride. The recovery for mix meat spiked with 1, 5 and 10 μg/kg of sulfonamides averaged 64%, 68% and 75%, respectively. Limits of quantitation were 1 μg/kg for sulfaquinoxaline and 0.5 μg/kg for the remaining sulfonamides.

Keywords: Residue determination; Fluorescence detection; Sulfonamide

1. Introduction

Sulfonamides (SAs) are extensively used in veterinary practice for the treatment of various bacterial infections. Because of their use in food producing animals, the risk of occurrence of unwanted residues in edible products exists. The European Community has adopted for SAs a maximum residue level (MRL) of 100 μg/kg in foodstuffs of animal origin [1]. Most commonly, control of residues of SAs in meat and milk is performed microbiologically [2]. With this simple test qualitative non-specific information is obtained which requires further confirmation. GC and GC–MS methods are very sensitive and specific [3,4] but routine application of these methods for a large number of samples is not easy because of the many purification and derivatization steps required. HPLC methods with UV-detection have also been reported using liquid–liquid, solid-phase sample clean-up [5–7] or on-line dialysis with column-switching [8]. Because SAs are polar compounds severe matrix influences have occurred resulting in rather complicated clean-up procedures, low recoveries, and difficulty distinguishing the SAs among tissue constituents. For these reasons, assessing the MRL has been difficult. HPLC–APCI-MS with selected ion monitoring overcomes these problems and sulfadiazine was confirmed in muscle tissue from fish at a concentration of 20 μg/kg [9].

TLC screening methods using derivatization of SAs with fluorescamine have also been developed [10–12]. These methods are rather selective and sensitive but are not reproducible enough.

Post-column derivatization with fluorescamine has been applied for the HPLC determination of sulfapyridine in human saliva [13], and for sulfonamides in salmon [14,15]. In another approach SA residues were previously derivatized with fluores-

*Corresponding author.

0021-9673/00/$ – see front matter
PII: S0021-9673(99)00904-8

camine and subsequently analyzed by HPLC with fluorescence detection [16].

When we applied these methods on different meat matrices we met some difficulties at the sample purification step because these objects consist of many substances of variable concentration. We then modified these methods and obtained easier clean-up and lower detection limits – below 1 μg/kg.

2. Experimental

2.1. Chemicals and materials

Acetonitrile and methanol (HPLC-grade) were purchased from LabScan, Dublin, Ireland. Methanol, ethyl acetate, methylene chloride, orthophosphoric acid (H_3PO_4), *n*-hexane (*n*-C_6), dipotassiumphosphate (K_2HPO_4) and acetone, purchased from Fluka, were of analytical-grade quality.

Fluorescamine and some of the most used sulfonamides as: Sulfanilamide (SA), Sulfadiazine (SD), Sulfamerazine (SM), Sulfadimidine (Sdm), Sulfamethoxypyridazine (SMP), Sulfachloropyridazine (SCP), Sulfadoxine (SDX), Sulfamethoxazole (SMT), Sulfadimethoxine (SDM), Sulfaquinoxaline (SQ), were purchased from Sigma.

Bond Elut cartridges (250 mg, 40 μm): Silica gel, Florisil, Strong Cation Exchanger (SCX) and C_{18} (16% carbon loaded), were supplied by Varian.

The water was purified and deionized by a Milli-Q system (Millipore). The solvents for HPLC were filtered through 0.45 μm filters (Millipore) and degassed in an ultrasonic bath.

2.2. Standard solution, sample preparation and fortification

A stock solution of SAs (100 mg/l) was prepared by dissolving 1 mg in 10 ml methanol and was stored at −4°C. The desired concentrations of 1, 5, 10 and 100 μg/l were adjusted by diluting the stock solution with methanol. A sample of 25 μl of each concentration was analyzed five times for defining the calibration curve (peak area/concentration).

Samples – meat, mix meat and kidney, were purchased at a local market. Before analysis they were assayed by microbiological tests for inhibitors. Ground tissue samples were fortified with the desired quantities of SAs by mixing the SAs into the sample and leaving it in contact with the tissue for 30 min.

2.3. Apparatus

The HPLC system consisted of a Waters 501 HPLC pump; Rheodyne injector with 100 μl loop; fluorescence detector Waters, mod. 470 (λ_{ex}=405 nm and λ_{em}=490 nm) with Shimadzu C-1RB integrator.

A Chrompack analytical column (250×4 mm, 5 μm 100 RP_{18} ODS-2) was used in this study. An acetonitrile–water (35/65, v/v) mobile phase of pH 3.0 containing 0.01 *M* K_2HPO_4 was pumped at a rate of 1.5 ml/min. The pH of the mobile phase was adjusted with H_3PO_4 and measured with a Chemcadet pH-meter (Cole Parmer).

2.4. Sample preparation

A 5 g homogenized sample was extracted two times with 15 ml ethyl acetate by vortexing for 2 min and filtered (Whatman). The sample was then extracted with 10 ml acetone and was filtered again. The extracts were combined and the solvent was evaporated in a vacuum evaporator at 50°C to dryness. The residue was dissolved in 15 ml water by ulrasonication for 1 min and the solution was filtered. The solution was further extracted three times in a separatory funnel with 5 ml methylene chloride portions. The lower layers were combined in a graduated tube and evaporated to dryness in a 60°C water bath under a stream of nitrogen. 0.25 ml of 0.1% fluorescamine in acetone and 0.25 ml of 1 *M* K_2HPO_4 were added to the tube for the derivatization which was performed at 60°C for 10 min. Aliquots of 25 μl were analyzed 20 min following the end of the derivatization procedure.

3. Results and discussion

3.1. Derivatization of sulfonamides

Fluorescamine is a fluorogenic reagent specific for primary aliphatic and aromatic amines [10] which produces fluorophors of a high fluorescence yield and potential selectivity having an essentially similar excitation–emission spectral characteristic (λ_{ex}=

395–410 nm and λ_{em}=490–510 nm). Fluorescamine and its hydrolysis products are nonfluorescent. This property eliminates extensive clean-up and chromatographic separation of the fluorescent derivatives from the excess reagent. Edder et al. [16] reported that at ambient temperature the optimal concentration of fluorescamine and reaction time for the derivatization of SAs were 0.1% and 20 min, respectively. Gehring et al. [15] speeded up the reaction by heating at 70°C for post-column derivatization. We found, however, a difference between the fluorescence yield of the derivatized SAs analyzed immediately after heating at 70°C for 2 min as compared to the yield obtained for the same derivatives 15 min following the heating step. This clearly suggests that the reaction time is among the important factors to be considered. Accordingly, we carried out the derivatization by heating at 60°C for 10 min and waiting for 20 min before analyzing the derivatives.

By examining the influence of pH on the fluorescence yield we concluded that the optimum pH range for the analyzed SAs is 2.5–3.5. We presume that at these pH-values hydrogen bonding exists (Fig. 1) and the derivatives acquire fluorescence properties. For this reason the pH value of the mobile phase was fixed at 3.0 with phosphate buffer. The fluorescence yields of the SA derivatives were approximately equal with the exception of the yield of sulfaquinoxaline which was half the average value.

3.2. Sample preparation

We devoted a significant amount of effort in the attempt to reproduce numerous published methods with respect to the extraction and the purification of SAs from meat matrices. Some of these procedures failed to yield the described recovery, while others employed time consuming and tedious sample cleanup. In some cases the recovery and the detection limits for part of the SAs were achieved but for others these parameters were very low, regardless of the application of solid-phase extraction with C_{18}, Florisil, Silica, weak or strong cation-exchange cartridges. These circumstances clearly limit the utility of these methods for the screening of SAs.

By studying every step of the sample preparation it was revealed that the binding of the SAs to the meat matrix, mainly to the proteins, and purification of SAs, especially from primary amines, are predominant. These two factors determine the recovery, the sensitivity and the selectivity. Solvents such as methanol, acetonitrile, water, ethyl acetate, and methylene chloride have been used for the extraction of the SAs. The highest recovery rate – about 40% at a 5 μg/kg SA concentration was achieved with acetonitrile and ethyl acetate. We selected the latter because its extracts contain a lower level of contaminants. By repeating the extraction with ethyl acetate we increased the recovery to 55%. The recovery was considerably increased, above 80%, by repeating the extraction with acetone. We assume that acetone attacks the hydrogen bonds between the amide groups of the meat proteins and the amine moiety of the SAs more efficiently than does ethyl acetate. Interestingly, the extract was markedly cleaner as compared to the one obtained with acetone only.

3.3. Validation of the method

3.3.1. Recovery, precision, accuracy, and sensitivity

The extraction recovery was determined by comparing the peak areas of the SAs extracted from

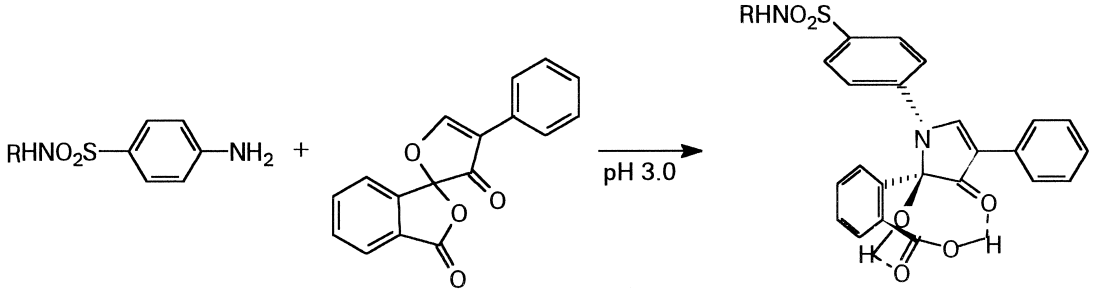
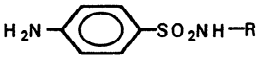
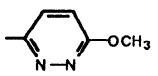
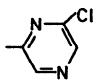
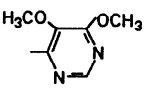
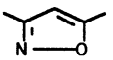
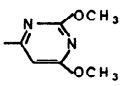
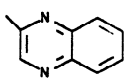


Fig. 1. Structural formula of derivatized sulfonamides.

Table 1
Recovery of sulfonamides from fortified mixed meat samples

Sulfonamides		Recovery, %, indicated fortification								
	R	1 μg/kg			5 μg/kg			10 μg/kg		
		Mean	SD	RSD %	Mean	SD	RSD %	Mean	SD	RSD %
Sulfanilamide	H	54	14.0	26	60	9.9	17	63	10.1	16
Sulfadiazine		63	13.2	21	64	7.7	12	73	6.6	9
Sulfamerazine		69	9.7	14	65	3.9	6	73	4.4	6
Sulfadimidine		67	6.7	10	71	2.8	4	73	5.8	8
Sulfamethoxypyridazine		63	14.5	23	72	5.8	8	87	12.2	14
Sulfachloropyridazine		64	8.3	13	66	5.9	9	85	9.3	11
Sulfadoxine		68	8.2	12	72	5.8	8	83	7.4	9
Sulfamethoxazole		61	5.5	9	69	3.5	5	74	7.4	10
Sulfadimethoxine		66	7.3	11	70	7.0	10	68	6.1	9
Sulfaquinoxaline		65	14.3	22	66	9.2	14	75	11.2	15

tissue samples with that of the unextracted standards containing the same amount of SAa. The mean recovery ranged from 54% to 87% in the concentration range of 1–100 μg/kg, and that of SAs at 5 μg/kg was 60–72% (Table 1). The precision of the method is sufficient because the RSD values are lower than the maximum permissible relative standard deviation at a fraction of the analyte 10^{-8} according to Decisions 93/257/EEC. The peak areas were linear between 1 and 100 μg/l concentration range of SAs. The correlation coefficients, *r*, of the standard curves (peak's area/concentration) were 0.993–0.997. The lowest value was for Sulfanilamide and Sulfaquinoxaline. It was estimated that the limit of quantification of SAs in meat was 0.2 μg/kg. Only for sulfaquinoxaline it was 1 μg/kg. These values are lower than the recently reported limits of quantitation 1–5 μg/kg [15,16] and are results of the simplified clean-up procedure. They are many times lower than the adopted by the European Community MRL value of 100 μg/kg. The detection limit, determined as a concentration and resulting in a signal-to-noise ratio of 5, was 0.05 μg/kg.

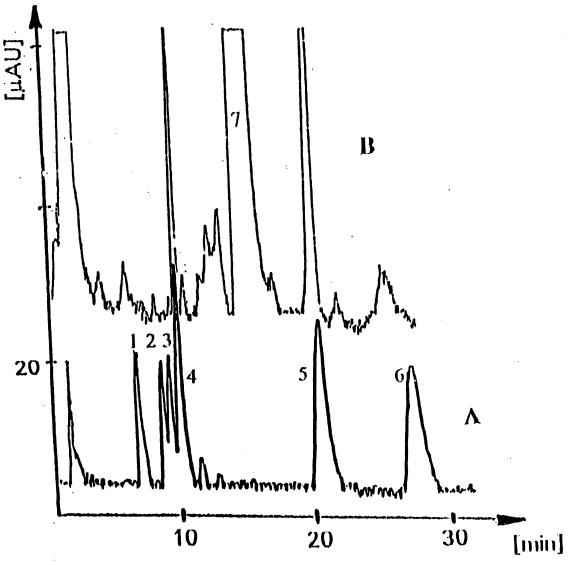


Fig. 2. (A) Chromatogram of a mixed meat sample, spiked with 2 μg/kg of the following sulfonamides: 1: Sulfanylamide, 2: Sulfadiazine, 3: Sulfamerazine, 4: Sulfadimidine, 5: Sulfadoxine, 6: Sulfadimethoxine. (B) Chromatogram of real sample with content of 86 μg/kg [7] and trace quantities of Sulfadimidine (4 μg/kg) and Sulfadimethoxine (5 μg/kg).

3.3.2. Selectivity

The absence of interference from tissue samples in the area of SAs in the chromatogram and the fluorescence detection of SAs at λ_{ex}=405 nm and λ_{em}=490 nm guaranteed high selectivity of the method. The probability of other compounds to acquire fluorescence properties at this excitation and emission at pH=3.0 is very small.

3.3.3. Ruggedness

The ruggedness was evaluated by six replicate injections of one sample containing 1, 5 or 10 μg/kg of SAs. Analyses were performed during ten days with different standard solutions, different mobile phases batches, and by two independent analysts. This procedure was repeated after a month without any considerable deviations (Table 2).

Over 60 mix meat samples were analyzed with the same column that was used for the development of the method resulting in no significant decrease in column efficiency (Fig. 2B).

Chromatogram of a sample consisting of 2 μg/kg is presented in Fig. 2A. The sample has been analyzed in different days. The results show that the system and the method precision, as well as the

Table 2
Extraction efficiency within-day and between-day reference standard deviation (RSD) as determined with 1–50 μg/kg concentration of sulfonamides[a] in mix meat samples

Concentration (μg/kg)	Extraction recovery (%)	Within-day[b] RSD (%)	Between-day[b] RSD (%)
1	58–68	8–18	6–18
10	63–83	4–12	7–14
50	65–84	2–9	3–8

[a] Sulfonamides are listed in Table 1.
[b] Six replicate analysis for each sample.

ruggedness, were within the acceptable range (RSD<18%) suggesting that the method could be transferable between different laboratories.

Method accuracy results, expressed as percent recoveries, are summarized in Table 2. Acceptable mean recoveries and RSD values are shown, although the recovery parameter displayed values slightly lower than desirable at the lowest concentration tested (1 μg/kg). However this fact has little, if any, practical relevance because even these low concentrations can be quantified with acceptable accuracy.

4. Conclusion

An HPLC method with fluorescence detection characterized by a good reliability for quantitative determination of residues of ten sulfonamides in meat has been developed. The proposed simplified clean-up procedure, including extraction with ethyl acetate and acetone and liquid–liquid partition with methylene chloride, enables quantitative determination of ten of the most used sulfonamides at concentrations below 0.5 μg/kg. This level is considerably lower than the level of MRL 100 μg/kg adopted by the European Community.

References

[1] Establishment of Maximum Residue Levels of Veterinary Medical Products in foodstuffs of animal origin, Council Regulation No. 2377/90 of EEC.
[2] J.F.M. Nouws, Arch. Lebensmittelhyg. 32 (1981) 97.
[3] S.J. Stout, W.A. Steller, A.J. Manuel, M.O. Poeppel, A.R. da Cunha, J. Assoc. Off. Anal. Chem. 67 (1984) 142.
[4] G.D. Paulson, A.D. Mitchell, R.G. Zaylskie, J. Assoc. Off. Anal. Chem. 68 (1985) 1000.
[5] Y. Ikai, H. Oka, N. Kawamura, J. Hayakawa, M. Yamada, K.-I. Harada, M. Suzuki, H. Nakazawa, J. Chromatogr. 541 (1991) 393.
[6] J.-M. Diserens, Nestec Ltd., 1993, private communication
[7] A.R. Long, C.R. Short, S.A. Barker, J. Chromatogr. 502 (1990) 87.
[8] M.M.L. Aerts, PhD Thesis, Vrije Universiteit, Amsterdam, 1990.
[9] Th.A. Gehring, L.G. Rushing, M.I. Churchwell, D.R. Doerge, K.M. McErlane, H.C. Thompson, J. Agric. Food Chem. 44 (1996) 3164.
[10] J.A.F. de Silva, N. Strojny, Anal. Chem. 47 (1975) 714.
[11] J.P. Abjean, J. Planar Chromatogr. 6 (1993) 147.
[12] J.-P. Abjean, J. AOAC Int. 4 (1997) 737.
[13] H.S. Sista, D.M. Dye, L. Leonardo, J. Chromatogr. 273 (1983) 464.
[14] Th.A. Gehring, L.G. Rushing, H.C. Thompson Jr., J. AOAC Int. 78 (1995) 1161.
[15] Th.A. Gehring, L.G. Rushing, H.C. Thompson Jr., J. AOAC Int. 80 (1997) 751.
[16] P. Edder, A. Cominoli, C. Corvi, Trav. Chim. Aliment. Hyg. 88 (1997) 554.