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Dispersive solid-phase extraction for the determination of sulfonamides in chicken muscle by liquid chromatography

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Abstract

A new, fast and low-cost sample preparation for the determination of sulfonamide (SA) residues in chicken muscle by LC technique has been developed. The procedure involves single extraction of sample with acetonitrile, followed by a rapid clean-up and was called "dispersive solid-phase extraction" (dispersive SPE). Using dispersive SPE 25 mg of octadecyl sorbent was added to 1 ml of acetonitrile extract, mixed and centrifuged. The acetonitrile layer was evaporated and residue was dissolved in acetate buffer (pH 3.5). Analysed compounds were detected by fluorescence detector after pre-column derivatization with fluorescamine. The separation of analytes was performed with gradient elution with mobile phase methanol: 2% acetic acid and RP-LC analytical column. The whole procedure was evaluated for six sulfonamides (sulfadiazine, sulfamerazine, sulfamethazine, sulfametoxypirydazine, sulfametoxazole and sulfadimetoxine) according to the European Commission Decision 2002/657/EC. Specificity, decision limit (CCα), detection capacity (CCβ), trueness and precision were determined during validation process. The dispersive SPE with octadecyl sorbent was found suitable for sample preparation before sulfonamide determination in chicken muscle. As it was found the most of endogenous matrix components were removed and the analytes were isolated from spiked samples with recoveries above 90%. The used analytical conditions allow to successively separate all the tested sulfonamides with the limit of detection at the level of 1–5 μg/kg. The method is simple, rapid and more effective than conventional methods.

Keywords: Sulfonamides; Residues; Muscle; Dispersive SPE; LC

1. Introduction

Sulfonamides (SAs) are a very important class of antibacterial compounds widely used in veterinary practice for therapeutic, prophylactic, or growth-promoting purposes. Residues of SAs may occur in animal tissues if the adequate withdrawal time have not been observed or if SAs have been improperly administered. The maximum residue limit (MRL) in the European Union countries for SAs in animal muscle tissue was established at the level of 100 μg/kg (the residue of interest is the sum of parent compounds) [1].

Several liquid chromatographic (LC) methods have been developed for the determination of SA compounds in animal tissues and body fluids [2–10]. Extraction of SAs from tissues traditionally employ liquid–liquid partitioning, the solid-phase extraction (SPE) technique or matrix solid-phase extraction (MSPD), however, the recoveries of analytes from spiked samples were sometimes low and variable.

Dispersive SPE was previously used by Anastadiades et al. [11] for the determination of pesticide residues in fruit and vegetable samples. Dispersive SPE is similar in same respects to MSPD, but the sorbent is added to an aliquot of the extract rather than to the original sample as in MSPD. The high cost of the sorbent limits the sample size that can be used in MSPD. This leads to concern about sample representation and homogeneity, but dispersive SPE relies on the extraction process to provide a homogenous aliquot from an original sample of any size and only a small amount of sorbent is used.

The aim of this work was to develop a simple and low-cost procedure, which involves the dispersive SPE instead of classical SPE or MSPD methods, and has a large dynamic concentration range in order for the determination of SA levels

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in chicken muscle tissue. We have validated the use of dispersive SPE and show that such sample taking can provide the proper results.

2. Experimental

2.1. Reagents and materials

Acetonitrile, methanol, acetone and acetic acid (HPLC grade) were from Baker (Deventer, The Netherlands). Also Baker supplied a non-polar sorbent Bakerbond octadecyl (C_{18}) 40 μm (Catalog No. 7025-00). The sorbent was prewashed twice with hexane and dried at 50 °C. Sodium acetate was from P.O.Ch. (Poland). Water was purified trough a Milli-Q plus system from Millipore (France). Fluorescamine reagent (0.02%) was prepared by dissolving 10 mg of FLuram (F. Hoffman-La Roche, Switzerland) in 5 ml of acetone. Analytical standards of sulfadiazine (SDA), sulfamerazine (SMR), sulfamethazine (SMZ), sulfametoxypirydazine (SMD), sulfametoxazole (SMX) and sulfadimetoxine (SDM) were obtained from Sigma (Poole, UK). Chemical structures of the SAs included in this study are shown in Fig. 1.

2.2. Buffer and standard solutions

A buffer solution (pH 3.5) was prepared by dissolving 0.82 g of sodium acetate in 500 ml of water and completing it to 1000 ml. This solution was filtered through a 0.45 μm PTFE filter from Milipore (France).

Individual stock solutions of SAs (1 mg/ml) were prepared in methanol and stored at −20 °C (stable for at least 12 months). Six sulfonamide combined stock solution (100 μg/ml) was prepared by diluting individual stock solutions with methanol and stored in dark at 4 °C (stable for at least 6 months). Working standard aqueous solutions of the analytes were prepared by serial dilution of the combined stock solution with acetate buffer at pH 3.5 (stable for at least 3 months).

Working standard solutions of SAs were also added to chicken tissues at appropriate microliter aliquots of the standard aqueous solutions (quality control samples).

2.3. Sample preparation equipment

A refrigerated centrifuge type Verifuge 3.0R was purchased from (Heraus, Germany) and a stirrer type IKA was from Laboratory Equipment (USA). The pH of the buffer solution was measured with a 780 pH Meter (Metrohm, Switzerland).

2.4. Sample preparation

The breast and thigh muscles were obtained from the healthy adult broiler chickens that were not treated with any veterinary drugs. The samples were minced and deep-frozen until analysis.

An accurately weighed 5 g amount of sample was placed into 40 ml centrifuge tube and vortexed for 1 min with 5 ml of acetonitrile, then the mixture was shaken for 10 min and centrifuged at 4000 rpm for 10 min.

An aliquot of 1 ml of acetonitrile phase was transferred to 1.5 ml microcentrifuge vial containing 25 mg of octadecyl sorbent. The vial was tightly capped and vortexed for 30 s.

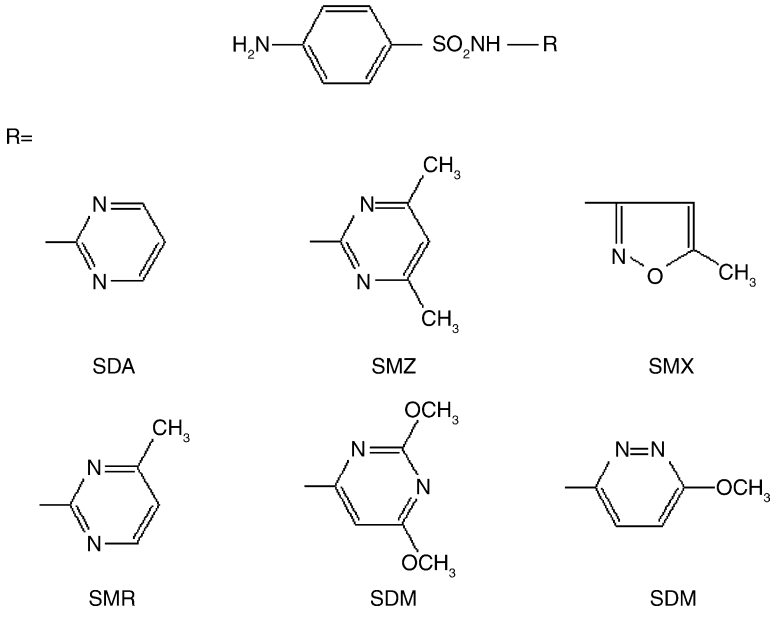

Fig. 1. Structures of the compounds studied. SDA: sulfadiazine; SMR: sulfamerazine; SMZ: sulfamethazine; SMD: sulfametoxypirydazine; SMX: sulfametoxazole; and SDX: sulfadimetoxine.

After centrifugation for 5 min at 5000 rpm the solution was separated from the solids and transferred to test tube. The solution was accurately evaporated to dryness under nitrogen stream at 50 °C, the residue was dissolved in 400 μl of acetate buffer (pH 3.5) and was ready for derivatization (Section 2.5).

2.5. Derivatization with fluorescamine

For LC analyses 400 μl of working standards or 400 μl of muscle extracts dissolved with acetate buffer (pH 3.5) were taken, then 100 μl of the fluorescamine reagent was added and whole solution was mixed with a vortex mixer. The sample was filtered through a 0.45 μm nylon filter and after standing for 30 min at ambient temperature was ready for determination. Aliquots of 10 μl were injected into the analytical column.

2.6. Liquid chromatography

A Shimadzu VP Series liquid chromatograph (Duisburg, Germany) was equipped with a degasser and a mixer of mobile phase. A fluorescence detector FR-10AXL with excitation wavelength of 405 nm and emission wavelength of 495 nm was used to analyse the tested solutions. LC control, data acquisition and peak integration was performed by system controller SCL-10A utilizing the RS-232C interface for communication with the CLASS-VP chromatography workstation.

The chromatographic separation was performed with gradient elution on a Luna RP-18 (150 mm × 4.6 mm, 3 μm) analytical column (Phenomenex, Germany). A C_{18} guard cartridge (40 mm × 2 mm, Phenomenex) was used prior to the analytical one. The mobile phase for LC analyses consisted of methanol and a 2% solution of acetic acid. The gradient mobile phase was pumped through the analytical column with the program presented in Table 1. Separation of the analytes was accomplished with flow of 0.5 ml/min at ambient temperature.

2.7. Validation study

The evaluation of the suitability of the whole procedure for the determination of six SAs residues in the breast and thigh chicken muscles was carried out according to the European Commission Decision 2002/657/EC [13]. The linearity of the assay was checked by calculation of the regression line by the method of least squares and expressed by the coefficient of determination (R^2). Five-point matrix-matched calibration curves were constructed by spiking of blank muscle samples with increasing amounts of each of the six SAs. The calibration curves were obtained for each component by plotting the recorded peak area (% F) versus the corresponding concentrations of the analytes injected (ng/10 μl).

Repeatability (precision) and recovery (trueness) of SAs were measured in blank chicken muscle that were spiked at MRL level (100 μg/kg), at half the MRL level (50 μg/kg) and one and half the MRL level (150 μg/kg). The spiked samples were analysed and the recoveries were calculated by comparing the measured concentration to the spiked concentrations.

To verify the specificity, the extracts from 20 blank chicken (breast and thigh) muscles were analysed. The absence of endogenous compounds at the retentions times of the analytes was investigated.

Decision limit (CCα) and detection capability (CCβ) were determined by using the calibration curve procedure. CCα was calculated with a statistical certainty of $1-\alpha$ ($\alpha = 5\%$) from the MRL value, CCβ was calculated with a statistical certainty of $1-\beta$ ($\beta = 5\%$) to detect MRL concentration.

The following elements of stability of SAs were determined: (i) in solvent (stock solutions), (ii) in matrix (spiked samples at 100 μg/kg), and (iii) derivatized solution stored prior the LC–FLD analysis.

3. Results and discussion

3.1. Dispersive SPE

Traditional sample preparation strategies for SAs in animal tissues involve isolation with organic solvent (e.g. acetonitrile, methanol, ethyl acetate) followed by SPE with polar, non-polar or ion-exchange sorbent materials [2–10].

In our preliminary studies, the extraction steps were performed with different organic solvents (ethyl acetate, methyl chloride, acetonitrile or methanol), and co-extraction of endogenous compounds from chicken muscles was evaluated. In this experiment, the samples were treated with different solvents and centrifuged. After that organic layers were separated, evaporated to dryness in pre-weighed test tubes, and the amount of co-extracted matrix was determined by differences in weight. As it was shown in Table 2 the most amounts of co-extractive compounds was isolated after sample treatment

Table 1
Program of gradient mobile phase

Time (min)	Methanol (%)	2% Acetic acid (%)
0	50	50
4	50	50
10	60	40
18	50	50
30	Stop	Stop

Table 2
Co-extracted matrix (mg/g) with different solvents

Solvent	Chicken muscle	
	Breast	Thigh
Methyl chloride	3.5	4.6
Ethyl acetate	3.2	3.7
Acetonitrile	1.2	1.5
Methanol	1.8	1.9

with methyl chloride as well as with ethyl acetate. In difference to non-polar solvent, the smallest ones were isolated after the application of acetonitrile, so we decided to use acetonitrile as extraction solvent.

In the next step, different amounts (from 10 to 100 mg) of octadecyl sorbent were used for the isolation of co-extracted compounds from acetonitrile extracts. The results of the experiments showed that 25 mg octadecyl sorbent was enough to remove the matrix compounds tested for a 1 ml aliquot of 1 g sample.

3.2. Fluorescamine derivatization

As it was previously reported by Takeda and Akiyama [12], optimal fluorescence detection for SAs was obtained after pre-column derivatization with fluorescamine in acetate buffer at pH 3.5. The optimal incubation period at above buffer was found to be 20–60 min. In our experiments the reaction was performed for 30 min, since much longer incubation lowered the intensity. In practice, a reproducible reaction period was achieved as follows: the stop time of integration was set at 30 min (LC separation was completed within the time) and the sample solution for the next injection was prepared immediately after the previous sample had been injected. In our opinion, pre-column derivatization with fluorescamine is useful for SAs determination by LC, it seems to be more laborious than post-column one but it is cheep and does not need expensive equipment or additional apparatus, and does not involve setting up and optimizing system.

3.3. LC separation

Usually, separation of SAs is performed by LC using reversed-phase C_{18} or C_8 silica columns with mixture of low-pH environment—acetonitrile or/and methanol as mobile phase [2–10]. In the presented paper, the separation of the mixture of six SAs was developed on a Luna C_{18} analytical column and the mobile phase that contained methanol and 2% acetic acid. In preliminary experiments, when isocratic elution was used, SDZ and SMR or SMZ and SMT could not be fully separated, and the separation time needs more than 45 min since SDX is highly retained. Thus, it was necessary to apply the gradient elution. The details of optimized stepwise linear gradient elution are presented in Table 1. Typical chromatogram corresponding to a separation under developed conditions is shown in Fig. 2. The obtained peaks are symmetrical and fully separated with the retention times as follows Table 3.

3.4. Validation method

The results of the linearity are reported in Table 3. The working range of the curves were from 0 to 500 μg/kg. The correlation coefficients of the calibration curves were above 0.999.

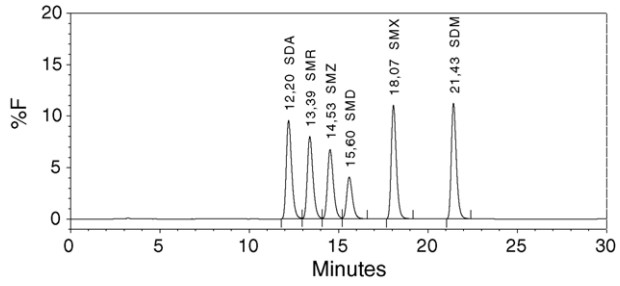

Fig. 2. Chromatogram of six SAs standard at 100 ng/ml.

Table 3
Retention time (t_R) and linearity in solution of six SAs

Sulfonamide	t_R (min)	b[a]	a[b]
SDA	12.20	20,273	−17,479
SMR	13.39	17,738	−6563
SMZ	14.53	15,520	−4149
SMD	15.60	9263	−13,647
SMX	18.07	22,252	−43,189
SDM	21.43	22,231	−22,543

Linear range investigated.
[a] b: Slope.
[b] a: Intercept.

The specificity was evaluated by the analysis of 20 blank samples taken from the chicken breast and thigh muscles. The chromatograms obtained from the analysis of the blank muscle extract and the spiked muscle extract are shown in Figs. 3 and 4, respectively. No interfering peaks from endogenous compounds as well as from other antibacterial agents were found in the retention time of the target SAs.

The recovery (trueness) and precision of the method are summarised in Table 4. The results show good accuracy

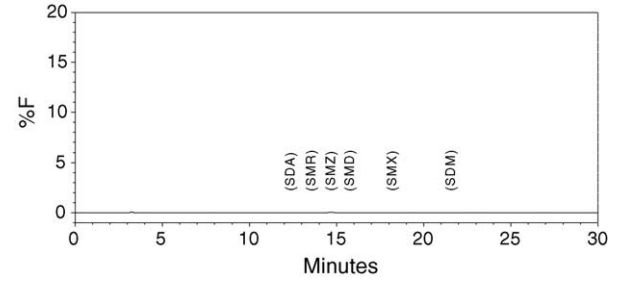

Fig. 3. Chromatogram of blank chicken muscle.

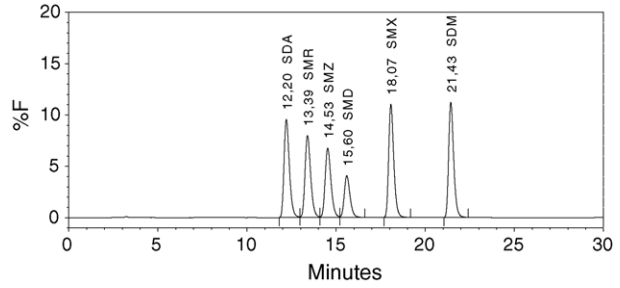

Fig. 4. Chromatogram of spiked chicken muscle at the level of 50 μg/kg.

Table 4
Repeatability and recovery for six SAs determined in spiked breast and thigh chicken muscles ($n = 6$)

Sulfonamide	Parameter	Level of spiking (μg/kg)					
		Breast chicken muscle			Thigh chicken muscle		
		50	100	150	50	100	150
SDA	Average (μg/kg)	46	94	138	46	92	139
	SD (μg/kg)	1.8	5.8	9.9	1.1	1.0	2.5
	RSD (%)	3.8	6.2	7.2	2.3	1.1	1.8
	Recovery (%)	92	94	92	92	92	93
SMR	Average (μg/kg)	46	95	142	46	95	140
	SD (μg/kg)	1.1	4.5	8.4	0.5	0.8	1.8
	RSD (%)	2.4	4.8	5.9	1.0	0.8	1.3
	Recovery (%)	92	95	94	93	95	93
SMZ	Average (μg/kg)	47	95	143	47	94	140
	SD (μg/kg)	2.4	7.8	6.6	4.0	5.5	2.6
	RSD (%)	5.1	8.2	4.6	8.5	5.8	1.9
	Recovery (%)	94	95	95	93	94	94
SMD	Average (μg/kg)	46	94	142	46	93	141
	SD (μg/kg)	8.2	12.3	14.3	3.1	5.4	6.3
	RSD (%)	17.9	13.0	10.0	6.8	5.8	4.5
	Recovery (%)	92	94	95	92	93	94
SMX	Average (μg/kg)	45	90	138	46	91	137
	SD (μg/kg)	3.7	2.1	10.2	3.4	1.3	2.3
	RSD (%)	8.1	2.3	7.4	7.4	1.4	1.7
	Recovery (%)	91	90	92	93	91	92
SDM	Average (μg/kg)	46	93	140	45	91	139
	SD (μg/kg)	5.7	5.3	5.5	3.8	2.6	1.4
	RSD (%)	12.4	5.8	3.9	8.4	2.9	1.0
	Recovery (%)	92	93	93	90	91	93

ranged between 90 and 95% with a good repeatability, less than 20%.

According to the concept of the European Commission Decision 2002/657/EC, the CCα (decision limit) and CCβ (detection capability) have been estimated. The values of the CCα and CCβ are presented in Table 5. Additionally limit of detections (LODs), traditional analytical parameter, have been established for all the tested sulfonamides. As it was found, the used analytical conditions allow to detect SAs at the level of 1–5 μg/kg, depending on the analyte.

The stability of the stock standard solutions was examined every month. The individual stock solution prepared in methanol and stored at −20 °C were stable for 1 year. The stability of the combined stock solutions stored at 4 °C was 6 month and working aqueous solution was stable for 3 months. The stability of derivatized compounds was at least 30 min. The spiked muscles at level of 100 μg/kg were examined every week. The stability of the analysed sample was estimated for at least 6 weeks.

Table 5
CCα and CCβ (μg/kg) obtained for six SAs in breast and thigh chicken muscles

Sulfonamide	Breast chicken muscle		Thigh chicken muscle	
	CCα	CCβ	CCα	CCβ
SDA	112.0	132.2	111.1	129.8
SMR	108.7	120.6	107.4	120.8
SMZ	106.3	118.8	104.8	116.7
SMD	111.9	125.1	121.9	137.4
SMX	103.8	119.3	107.8	115.0
SDM	125.1	153.1	127.6	157.2

4. Conclusions

A simple, rapid and inexpensive extraction and clean-up procedure for the detection and determination of six SAs in chicken muscles has been developed and can be used for residue control purposes. The obtained validation results indicate accordance the method performance with the European Commission Decision 2002/657/EC.

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