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Validation of a confirmatory method for the determination of sulphonamides in muscle according to the European Union regulation 2002/657/EC

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Abstract

A simple multiresidue method is described for assaying 10 sulphonamides (SAs) (sulfadiazine, sulfathiazole, sulfapyridine, sulfamerazine, sulfamethazine, sulfamonomethoxine, sulfachlorpyridazine, sulfamethoxazole, sulfaquinoxaline and sulfadimethoxine) in muscle samples. Samples were prepared by homogenizing the tissue, extracting with ethyl acetate and cleaning up with a cation-exchange solid-phase extraction (SPE) column. The detection of analytes was achieved by HPLC–diode array detection (DAD) at 270 nm. The procedure was validated according to the European Union regulation 2002/657/EC determining specificity, decision limit, detection capability, trueness and precision. The results of validation process demonstrate that the method is suitable for application in European Union statutory veterinary drug residue surveillance programmes.

Keywords: Validation; Sulphonamides

1. Introduction

Sulphonamides (SAs) are an important group of synthetic antimicrobials which have been used in veterinary and human therapy over 60 years. More recently these drugs are being extensively introduced in food production and their residues are a great concern, due to the possibility of risk to human health, such as resistance development and toxicity. It has been reported that sulfamethazine produces tumours in rodent bioassay [1] and some evidence on the toxicity of sulphonamides on the thyroid gland has been presented [2,3]. To protect consumers' health the European Union has adopted for SAs a maximum residue level (MRL) of 100 μg/kg in foodstuffs of animal origin [4]. The substances with MRLs (permitted) are contained in group B of Annex I of Council Directive 96/23/EC [5].

Recently, the European Union (EU) has issued a specific regulation decision (2002/657/EC) concerning the performance of methods and the interpretation of results in the official control of residues in products of animal origin [6]. Some new parameters must be calculated as limit of decision (CCα) and detection capability (CCβ).

The application of this law is not very clear and the authors themselves admit some difficulties in its interpretation [7]. However the methods currently applied for the analysis of official samples of the substances in group B of Annex I of Council Directive 96/23/EC will have to comply with the decision 2002/657/EC by 1 September 2007.

Several analytical procedures are currently available for the determination of sulphonamide residues in animal tissues and at present the LC is the instrumental technique most adopted [8–16]. Because SAs are polar compounds severe matrix influences have occurred; therefore the analytical procedures must utilise either a very selective but expensive detector (i.e. MS) or a good matrix clean-up. This work describes the simultaneous determination of 10 molecules of SAs (sulfadiazine, sulfathiazole, sulfapyridine, sulfamerazine, sulfamethazine, sulfamonomethoxine, sulfachlorpyridazine, sulfamethoxazole, sulfaquinoxaline and sulfadimethoxine) in muscle samples. The purification step uses a strong cation-exchange solid-phase extraction (SPE). Determination of analytes is then performed by HPLC–diode array detection (DAD). Ionic exchange clean up, proposed for the first time by Haagsma and Van De Water, [17] assures

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an adequate selectivity without the use of MS detection considering also the relatively high levels of validation.

None of the methods published meet new European criteria for the confirmation of substances, such as SAs that are contained in group B. The validation proposed here represents a possible compromise between the requirements of decision 2002/657/EC and the resources of an official laboratory with high samples throughput.

2. Experimental

2.1. Materials and reagents

The chemical and chromatographic reagents used were of LC or analytical grade. Ammonia (30%), ethyl acetate, *n*-hexane, methanol, methanol LC grade, acetic acid (99.5%) and anhydrous sodium sulphate were purchased from Panreac (Barcelona, Spain).

Sodium acetate trihydrate (99% Merck, Darmstadt, Germany) was used as buffer for HPLC mobile phase.

HPLC mobile phase acetate buffer pH 4.5 (0.05 M) was prepared dissolving 6.80 g of sodium acetate trihydrate in a 1000 ml volumetric flask with about 700 ml of high-purity water. The pH was adjusted to 4.5 with some drops of 10% (v/v) acetic acid and the buffer diluted to 1000 ml volume.

Acetonitrile (LC grade) was obtained from Carlo Erba (Milan, Italy).

Eluents for HPLC and standard solutions were prepared with high-purity water obtained from a Milli-Q system (Millipore, Bedford, MA, USA). LC solvents were filtered with 0.45 μm Durapore membrane (Millipore) and purified samples were filtered through (17 mm ∅) 0.45 μm nylon filters Stepbio (Bologna, Italy).

Aromatic sulfonic acid Speedisk SPE columns (200 mg/6 ml) were supplied by J.T. Baker (Deventer, The Netherlands).

2.2. Standards

Ten SAs standards [sulfadiazine (SDA), sulfathiazole (STZ), sulfapyridine (SP), sulfamerazine (SM), sulfamethazine (SMZ), sulfamonomethoxine (SMM), sulfachlorpyridazine (SCP), sulfamethoxazole (SMX), sulfaquinoxaline (SQ) and sulfadimethoxine (SDM)] were obtained from Sigma (St. Louis, MO, USA).

Standard stock solutions were prepared by accurately dissolving approximately 10 mg of SAs in 10 ml of methanol LC grade and stored at 4 °C. Working standards were prepared daily by appropriate dilution in acetate buffer at pH 4.5.

2.3. HPLC–DAD equipment and conditions

The chromatographic apparatus was a ThermoFinnigan Spectrasystem (Milano, Italy) composed of a P4000 quaternary pump, an AS3000 autosampler equipped with a 7010 Rheodyne valve (Bensheim, Germany) interfaced with a diode array (UV6000LP) detector.

Table 1
Gradient timetable

Time (min)	A (acetonitrile) (%)	B (acetate buffer, pH 4.5) (%)
0	15	85
22	41	59
24	15	85
30	15	85

The chromatographic separation was accomplished in 30 min with gradient elution on a C_8 (250 mm × 3 mm, 5 μm) analytical column (Phenomenex, Torrance, CA, USA). A C_8 guard cartridge (4 mm × 2 mm, Phenomenex) was used prior to the analytical one. The gradient mobile phase (Table 1) was pumped at a flow rate of 0.4 ml/min. The detector was set at 270 nm.

The blender was a 1 l Waring Commercial (New Hartford, CT, USA). Rotary evaporating system was a Büchi RE-111 (Milan, Italy).

2.4. Procedure

The muscle sample was cut into pieces and blended. An accurately weighed 10 g amount of the tissue was placed in 50 ml centrifuge tube. Extraction of SAs was achieved by introducing 20 ml of ethyl acetate and 10 g of anhydrous Na_2SO_4, shaking for about 15 min. The tube was then centrifuged at 3000 rpm for about 10 min. The supernatant (organic phase) was transferred in a 250 ml conical flask and the same extraction procedure was repeated twice. The extracts (60 ml) were combined and evaporated under vacuum. The residue was suspended in 40 ml of ethyl acetate. For the purification step, the Speedisk column was conditioned with 2 × 3 ml of *n*-hexane and 2 × 4 ml of ethyl acetate. After the application of the sample, the column was washed with 5 ml of water and 5 ml of methanol.

Table 2
Retention time (t_R), linearity and detection limit in solution of 10 SAs

Analyte	t_R (min)	b[a]	a[b]	DL[c] (μg/ml)
SDA	8.98	977002 ± 1471	2919 ± 5987	0.04
STZ	9.64	731909 ± 763	−2854 ± 3105	0.03
SP	10.46	852569 ± 2277	18045 ± 9270	0.07
SM	11.39	973141 ± 1308	4053 ± 5324	0.04
SMZ	13.32	869553 ± 1497	2019 ± 6088	0.05
SMM	15.71	908545 ± 1053	−2827 ± 4288	0.03
SCP	16.58	879430 ± 1082	−8898 ± 4403	0.03
SMX	18.87	934301 ± 2272	−2703 ± 9251	0.07
SQ	22.50	711413 ± 860	−5841 ± 3503	0.03
SDM	22.74	781760 ± 1388	−9045 ± 5649	0.05

Linear range investigated: 0.1, 0.25, 0.5, 1.0, 1.5, 2.0, 5.0 and 10.0 μg/ml.
[a] b = slope (±S.D. of slope).
[b] a = intercept (±S.D. of intercept).
[c] DL is the instrumental limit of detection.

The SAs were eluted with 20 ml of a methanol–ammonia (97.5:2.5, v/v) mixture. The eluate was accurately evaporated to dryness in a rotary evaporator and the residue was dissolved in 1 ml of acetate buffer (pH 4.5) with 0.1% of methanol. The resulting solution was filtered through a 0.45 μm disposable syringe filter and 20 μl of the filtrate were injected into the HPLC system.

2.5. Specificity

To verify the absence of interfering substances around the retention time of analytes (specificity), 20 muscle samples of various species, among those present in the monitoring programmes implemented by the European Union, were analysed.

2.6. Decision limit and detection capability

The critical concentrations for MRL compliance (CCα, where $\alpha = 0.05$) were calculated from the MRL value plus 1.64 times the standard deviation of the fortified samples at the MRL. The CCβ is obtained adding to CCα 1.64 times the same standard deviation.

2.7. Precision and trueness

Repeatability and recovery were assessed by performing tests on 54 spiked samples at 0.5, 1 and 1.5 times the MRL (50, 100 and 150 μg/kg, respectively) according to the criteria of decision.

2.8. Stability

The SAs stability was determined in three different ways: (i) in solvent (stock solutions), (ii) in matrix (spiked muscles at 50 μg/kg), and (iii) in sample purified extracts stored prior the HPLC–DAD analysis.

The procedure to determine stability is reported in Section 3.

3. Results and discussion

The calibration graphs obtained by plotting peak area versus drug concentration in 0.1–10 μg/ml range are reported in Table 2. The linear correlation coefficients (r^2) are all above 0.9999. The instrumental detection limit (DL) of

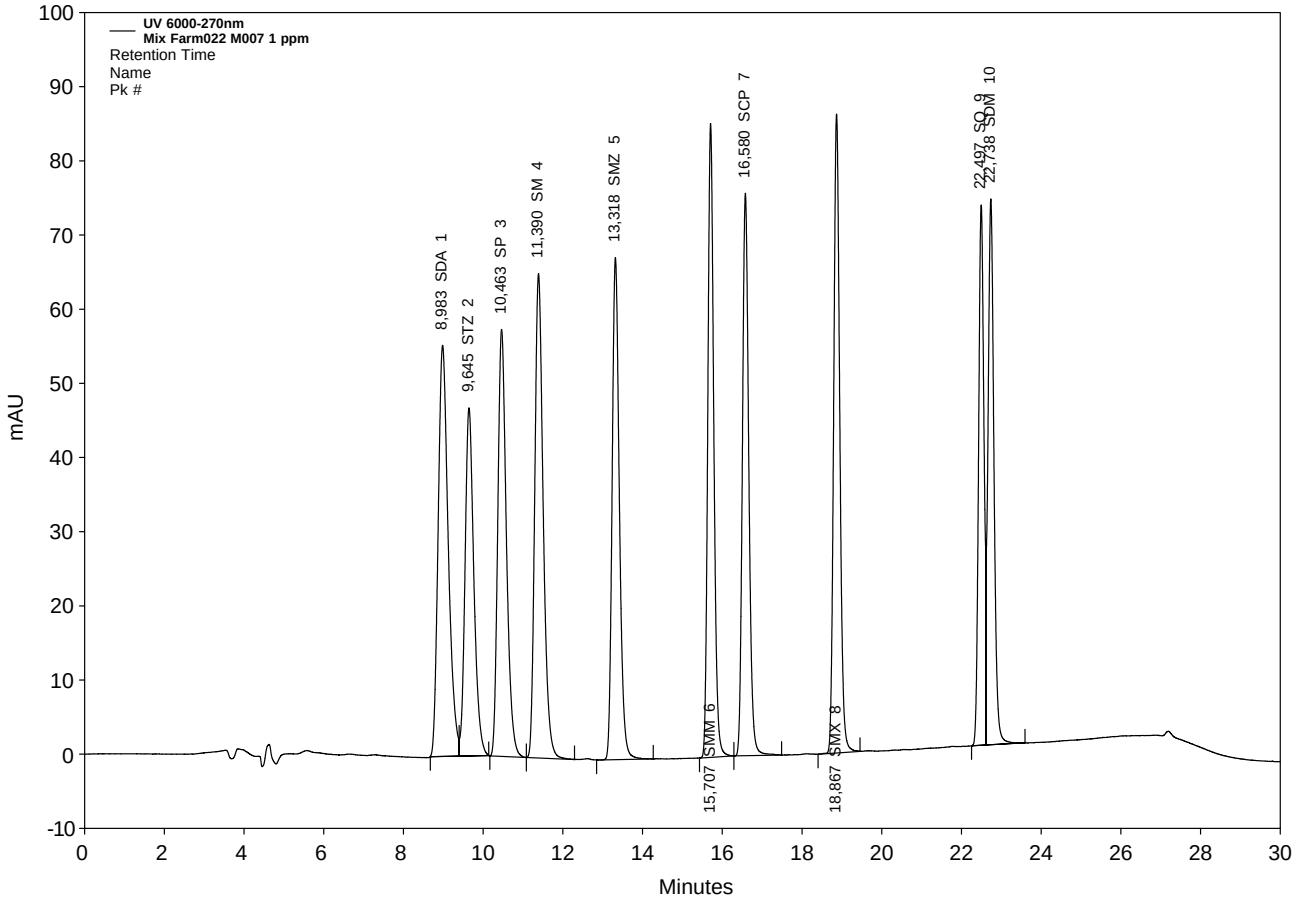

Fig. 1. The chromatogram of 10 sulphonamides standard at 1 μg/ml. Peaks: 1: sulfadiazine, 2: sulfathiazole, 3: sulfapyridine, 4: sulfamerazine, 5: sulfamethazine, 6: sulfamonomethoxine, 7: sulfachlorpyridazine, 8: sulfamethoxazole, 9: sulfaquinoxaline, and 10: sulfadimethoxine.

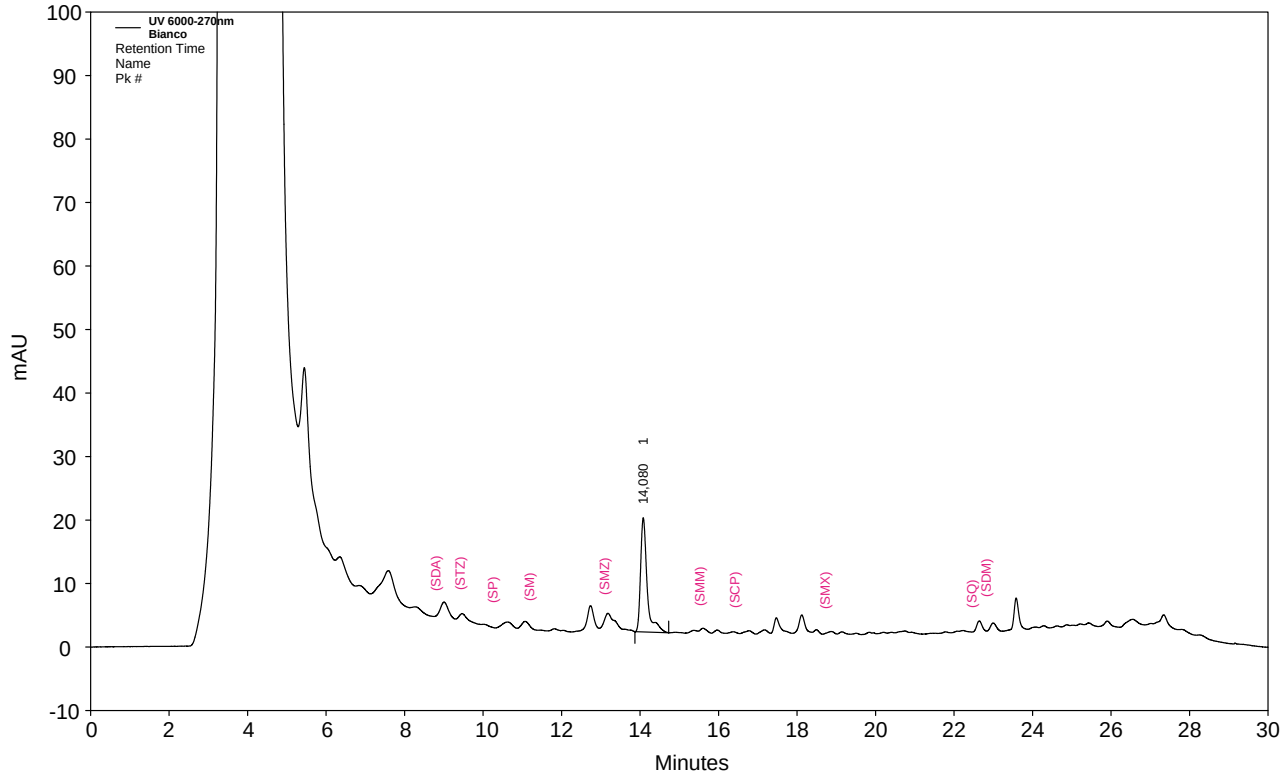

Fig. 2. The chromatogram of blank swine muscle.

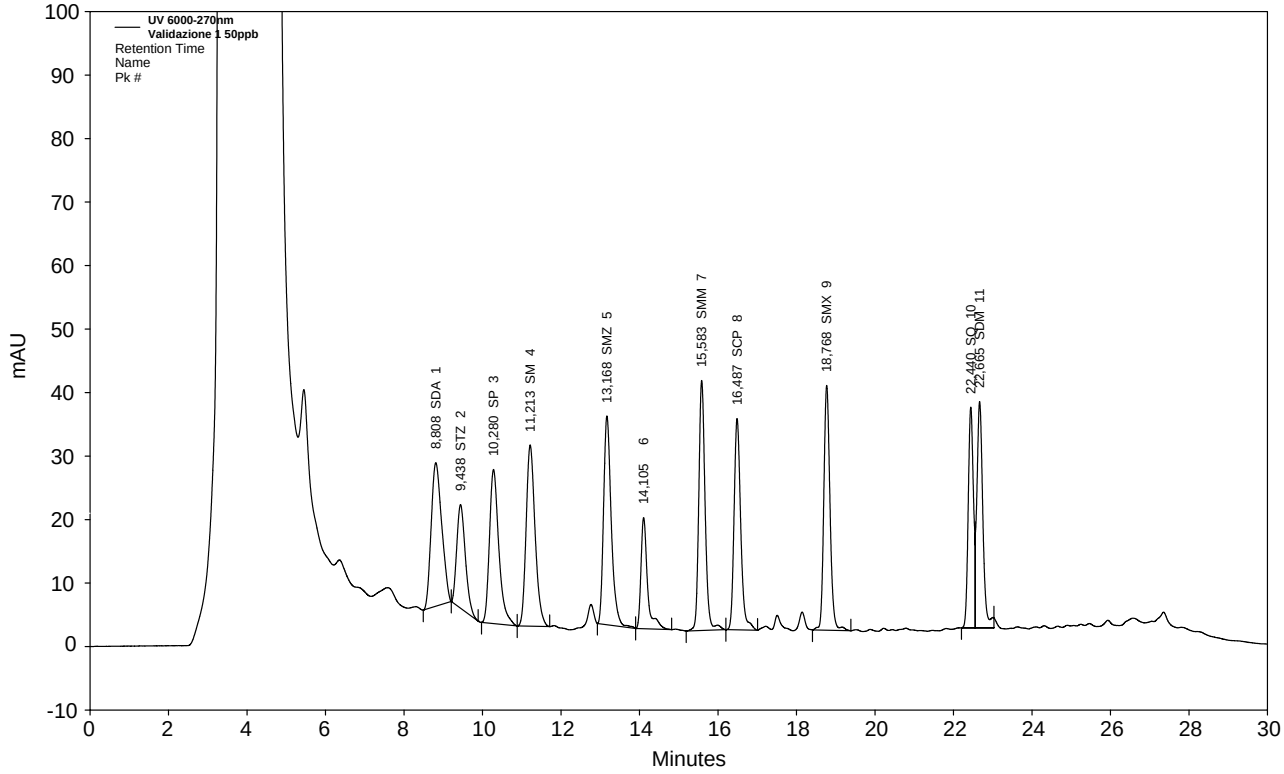

Fig. 3. The chromatogram of the spiked sample with 10 sulphonamides at 50 μg/kg. Peaks: 1: sulfadiazine, 2: sulfathiazole, 3: sulfapyridine, 4: sulfamerazine, 5: sulfamethazine, 7: sulfamonomethoxine, 8: sulfachlorpyridazine, 9: sulfamethoxazole, 10: sulfaquinoxaline, and 11: sulfadimethoxine (peak 6: interference).

each analyte is calculated following the equation proposed by Miller and Miller [18]:

$$y = a + 3s_{y/x}$$

where a is the intercept and $s_{y/x}$ is the standard error. DL is obtained by calculating y and using this value in the regression equation. All DL values are lower than first point of calibration curve (0.1 μg/ml).

The 95% confidence interval for the intercept a, calculated considering its standard deviation, includes the zero value in all cases, thus indicating the absence of systematical instrumental bias.

Chromatograms of a standard mixture, of a blank sample and of a spiked sample of swine muscle are shown in Figs. 1–3, respectively.

The application of the whole procedure to 20 blank samples from different species (bovine, swine and poultry) in order to verify the method specificity demonstrates that no interference around the retention time of the 10 analytes was detected in any of the muscles analysed.

The recovery and repeatability of the method were measured by the analysis of six blank mixed muscles of bovine, swine and poultry fortified with 10 SAs at each of three concentrations (50, 100 and 150 μg/kg) on three separate occasions (Tables 3–5). The identification criteria of decision 2002/657/EC for full scan UV–Vis detection were accomplished for all samples.

All R.S.D.s were lower or equal to 15% according to the values calculated using the Horwitz equation. Only sulfathiazole and sulfachlorpyridazine at 50 μg/kg level have an R.S.D. value of 19 and 16%, respectively. On the other hand, for mass fractions lower than 100 μg/kg the "classical" Horwitz equation is not applicable.

The mean recoveries were in the range of 72–92% for all analytes except for sulfathiazole with values of 63% at 100 μg/kg and 55% at 150 μg/kg.

The revised criteria also introduce the CCα (decision limit) and CCβ (detection capability) to replace the limit of detection and quantification, respectively. These new parameters are based on the critical value of the net state variable (CCα) and the minimum detectable value of the net state variable (CCβ). In fact, these concepts had already been introduced in the ISO Guide 11843-1 [19].

In the 2002/657/EC decision, the CCα was defined as "the limit at and above which it can be concluded, with an error probability of α, that a sample is non-compliant" (greater than the MRL for group B substances) while CCβ represents "the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of β". For group B substances, α and β errors must be $\leq$5%.

Unfortunately, the decision itself proposes two different options for determining CCα and CCβ, inducing possible confusion [20]. A practical choice is the elaboration of the data obtained during validation of the method reported in Tables 3–5 [21,22]. The CCα and CCβ values obtained for each sulphonamide are shown in Table 6.

Table 3
Repeatability and recovery for the determination of 10 SAs in muscle at 50 μg/kg

SAs	Day 1	Day 2	Day 3	Overall
SDA measured (μg/kg)	47	39	41	42
S.D. (μg/kg)	2	2	6	5
R.S.D. (%)	4	5	14	12
Recovery (%)	94	77	81	84
STZ measured (μg/kg)	39	28	40	36
S.D. (μg/kg)	2	2	6	7
R.S.D. (%)	6	9	15	19
Recovery (%)	78	56	81	72
SP measured (μg/kg)	45	37	40	41
S.D. (μg/kg)	2	5	3	5
R.S.D. (%)	4	13	7	11
Recovery (%)	90	74	80	81
SM measured (μg/kg)	46	39	41	42
S.D. (μg/kg)	2	1	4	4
R.S.D. (%)	4	3	10	9
Recovery (%)	91	78	82	83
SMZ measured (μg/kg)	47	40	43	43
S.D. (μg/kg)	3	1	6	5
R.S.D. (%)	6	2	14	11
Recovery (%)	93	79	85	86
SMM measured (μg/kg)	45	41	42	43
S.D. (μg/kg)	2	1	3	3
R.S.D. (%)	4	3	8	7
Recovery (%)	91	81	83	85
SCP measured (μg/kg)	49	36	38	41
S.D. (μg/kg)	2	2	4	7
R.S.D. (%)	4	6	11	16
Recovery (%)	99	71	77	82
SMX measured (μg/kg)	43	38	39	40
S.D. (μg/kg)	2	2	4	3
R.S.D. (%)	4	4	9	8
Recovery (%)	85	77	79	80
SQ measured (μg/kg)	44	35	37	39
S.D. (μg/kg)	2	2	4	5
R.S.D. (%)	4	6	11	12
Recovery (%)	88	70	74	77
SDM measured (μg/kg)	42	36	35	38
S.D. (μg/kg)	1	2	4	4
R.S.D. (%)	4	5	10	10
Recovery (%)	83	72	70	75

Six sample were analysed on each of 3 days ($n = 18$).

The CCα and CCβ were elaborated using the 18 curves obtained at three levels (50, 100 and 150 μg/kg). As shown in Table 6 the higher CCα and CCβ are those of sulfathiazole according to the high R.S.D.s observed.

The stability of the stock standard solutions in methanol was 1 year at 4 °C. The stock solutions were analysed every month and the instrumental responses were compared with the peak areas obtained at the moment of solution preparation ($t = 0$). The acceptance criterion was a response comprised between 95 and 105% of the initial one [23].

Table 4
Repeatability and recovery for the determination of 10 SAs in muscle at 100 μg/kg

Sas	Day 1	Day 2	Day 3	Overall
SDA measured (μg/kg)	80	79	78	79
S.D. (μg/kg)	3	2	5	3
R.S.D. (%)	4	3	7	4
Recovery (%)	80	79	78	79
STZ measured (μg/kg)	65	61	61	63
S.D. (μg/kg)	2	3	4	4
R.S.D. (%)	3	5	7	6
Recovery (%)	65	61	61	63
SP measured (μg/kg)	84	107	84	92
S.D. (μg/kg)	3	15	5	14
R.S.D. (%)	3	14	7	15
Recovery (%)	84	107	84	92
SM measured (μg/kg)	83	85	81	83
S.D. (μg/kg)	3	3	6	4
R.S.D. (%)	4	4	7	5
Recovery (%)	83	85	81	83
SMZ measured (μg/kg)	86	87	81	85
S.D. (μg/kg)	3	4	7	5
R.S.D. (%)	3	4	9	6
Recovery (%)	86	87	81	85
SMM measured (μg/kg)	84	93	84	87
S.D. (μg/kg)	2	10	6	8
R.S.D. (%)	2	11	7	9
Recovery (%)	84	93	84	87
SCP measured (μg/kg)	80	85	78	81
S.D. (μg/kg)	2	11	7	7
R.S.D. (%)	3	13	8	9
Recovery (%)	80	85	78	81
SMX measured (μg/kg)	78	92	79	83
S.D. (μg/kg)	2	13	6	10
R.S.D. (%)	2	14	7	12
Recovery (%)	78	92	79	83
SQ measured (μg/kg)	84	79	76	80
S.D. (μg/kg)	2	6	7	6
R.S.D. (%)	2	7	9	7
Recovery (%)	84	79	76	80
SDM measured (μg/kg)	79	80	74	78
S.D. (μg/kg)	1	3	5	4
R.S.D. (%)	2	4	7	5
Recovery (%)	79	80	74	78

Six sample were analysed on each of 3 days ($n = 18$).

Table 5
Repeatability and recovery for the determination of 10 SAs in muscle at 150 μg/kg

Sas	Day 1	Day 2	Day 3	Overall
SDA measured (μg/kg)	111	108	110	110
S.D. (μg/kg)	13	11	6	10
R.S.D. (%)	11	10	6	9
Recovery (%)	74	72	73	73
STZ measured (μg/kg)	81	83	84	83
S.D. (μg/kg)	11	13	5	10
R.S.D. (%)	13	15	6	12
Recovery (%)	54	55	56	55
SP measured (μg/kg)	116	125	119	120
S.D. (μg/kg)	9	4	4	7
R.S.D. (%)	7	3	4	6
Recovery (%)	77	84	79	80
SM measured (μg/kg)	118	120	115	118
S.D. (μg/kg)	10	4	4	6
R.S.D. (%)	8	3	4	5
Recovery (%)	79	80	77	79
SMZ measured (μg/kg)	118	124	119	120
S.D. (μg/kg)	12	3	5	8
R.S.D. (%)	11	3	4	7
Recovery (%)	78	83	79	80
SMM measured (μg/kg)	119	121	123	121
S.D. (μg/kg)	10	4	5	7
R.S.D. (%)	9	3	4	6
Recovery (%)	80	80	82	81
SCP measured (μg/kg)	109	103	112	108
S.D. (μg/kg)	14	8	5	10
R.S.D. (%)	13	8	4	9
Recovery (%)	73	69	75	72
SMX measured (μg/kg)	113	112	116	114
S.D. (μg/kg)	11	5	5	7
R.S.D. (%)	9	4	4	6
Recovery (%)	75	75	77	76
SQ measured (μg/kg)	116	112	118	115
S.D. (μg/kg)	14	5	5	9
R.S.D. (%)	12	4	4	8
Recovery (%)	78	75	79	77
SDM measured (μg/kg)	114	111	114	113
S.D. (μg/kg)	12	5	4	7
R.S.D. (%)	10	5	4	6
Recovery (%)	76	74	76	75

Six sample were analysed on each of 3 days ($n = 18$).

Spiked muscles at 50 μg/kg stored at −20 °C were analysed after 1, 2 and 4 weeks and stability was demonstrated for at least 4 weeks.

Finally, in order to check the stability of purified extracts of spiked samples at 50 μg/kg, an aliquot of extracts of a validative round ($n = 6$), stored at 4°C, was reanalysed by HPLC–DAD after 2 and 7 days respectively (Table 7). *t*-Test and *F*-test were performed. No significant differences were detected with the exception of four sulphonamides (SMM, SMX, SQ and SDM) for which the *F*-test shows a significant difference after a week of storage ($P = 0.05$).

Table 6
CCα and CCβ (μg/kg) obtained for 10 SAs

Sulphonamide	CCα	CCβ
SDA	109.3	120.0
STZ	116.2	134.9
SP	110.2	121.7
SM	105.2	110.9
SMZ	106.6	113.9
SMM	106.6	114.1
SCP	108.8	119.1
SMX	107.1	115.0
SQ	107.8	116.4
SDM	107.0	114.4

Table 7
Stability tests at 4 °C of purified extracts of samples spiked at 50 μg/kg

Sas	Time 0	After 2 days	Time 0	After 7 days
SDA measured (μg/kg)	47	48	39	37
S.D. (μg/kg)	2	2	2	2
R.S.D. (%)	4	5	5	5
Recovery (%)	94	95	77	74
STZ measured (μg/kg)	39	38	28	26
S.D. (μg/kg)	2	3	2	3
R.S.D. (%)	6	7	9	11
Recovery (%)	78	77	56	51
SP measured (μg/kg)	45	43	37	34
S.D. (μg/kg)	2	1	5	11
R.S.D. (%)	4	3	13	33
Recovery (%)	90	86	74	68
SM measured (μg/kg)	46	44	39	36
S.D. (μg/kg)	2	2	1	2
R.S.D. (%)	4	4	3	5
Recovery (%)	91	88	78	72
SMZ measured (μg/kg)	47	50	40	35
S.D. (μg/kg)	3	3	1	2
R.S.D. (%)	6	5	2	7
Recovery (%)	93	101	79	70
SMM measured (μg/kg)	45	44	41[a]	41[a]
S.D. (μg/kg)	2	1	1	5
R.S.D. (%)	4	3	3	13
Recovery (%)	91	88	81	82
SCP measured (μg/kg)	49	43	36	33
S.D. (μg/kg)	2	2	2	5
R.S.D. (%)	4	4	6	14
Recovery (%)	99	85	71	65
SMX measured (μg/kg)	43	41	38[a]	41[a]
S.D. (μg/kg)	2	2	2	6
R.S.D. (%)	4	4	4	15
Recovery (%)	85	83	77	82
SQ measured (μg/kg)	44	43	35[a]	37[a]
S.D. (μg/kg)	2	2	2	15
R.S.D. (%)	4	4	6	40
Recovery (%)	88	86	70	75
SDM measured (μg/kg)	42	41	36[a]	34[a]
S.D. (μg/kg)	1	1	2	10
R.S.D. (%)	4	4	5	31
Recovery (%)	83	81	72	68

An aliquot of each six samples stored at 4 °C were reanalysed by HPLC–DAD after 2 and 7 days.
[a] The *F*-test shows a significant difference at 95% level.

Consequently, the purified extracts can be stored prior to instrumental determination for 2 days only.

4. Conclusions

This quantitative confirmatory method for 10 SAs has been validated according to the revised EU criteria in bovine, swine and poultry muscle. Our approach utilises a traditional detection by HPLC–DAD without the use of a very sophisticated system such as LC–MS which can be better dedicated to banned substances (group A of Annex I of directive 96/23/EC). The described procedure has now been applied for the quantitative confirmation of sulphonamides in samples collected as part of residue control EU programmes.

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