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# Determination and quantification of sulfadiazine and trimethoprim in swine tissues using liquid chromatography with ultraviolet and mass spectrometric detection

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High-performance liquid chromatographic procedures with ultraviolet detection were developed for the quantitative determination of sulfadiazine (SDA) and trimethoprim (TMP) in swine tissues (kidney, liver, muscle, fat and fat+skin). In addition, high-performance liquid chromatography with atmospheric pressure chemical ionization mass spectrometry was used for the confirmation of the identity of the analytes of interest. Chromatographic separation was achieved on a Spherisorb ODS-2 column (250 × 4.6 mm id, dp 5 μm). The mobile phase for SDA analysis consisted of 1% acetic acid in water–acetonitrile (85 + 15, v/v). For TMP analysis a 80 + 15 + 5 (v/v/v) mixture of 0.25% triethylammonium acetate in water, acetonitrile and methanol was used as the eluent. Sulfamerazine and ormethoprim were used as the internal standards for SDA and TMP analysis, respectively. For the isolation of the compounds of interest from biological samples, a liquid–liquid extraction with acetone and ethyl acetate, followed by a clean-up using a solid-phase extraction column (aminopropyl and benzenesulfonic acid for SDA, benzenesulfonic acid for TMP) was performed. Calibration graphs were prepared for all tissues and linearity was achieved over the concentration ranges tested (50–1000 ng $g^{-1}$ for SDA, $r \geq 0.9979$; 25–500 ng $g^{-1}$ for TMP, $r \geq 0.9994$). The method was validated at the maximum residue level (MRL, 100 ng $g^{-1}$ for SDA and 50 ng $g^{-1}$ for TMP), at half the MRL and at double the MRL for both SDA and TMP. The accuracy and precision (expressed as the within-day repeatability) were found to be within the required ranges for each specific concentration. The quantification limits were 50 ng $g^{-1}$ for SDA and 25 ng $g^{-1}$ for TMP. The limits of detection were below one half the MRLs. Both methods were selective for the determination of SDA and TMP. Biological samples (kidney, liver, muscle, fat and fat + skin) from pigs that received a commercial SDA–TMP preparation with the feed for five consecutive days (dose rate: 25 mg SDA and 5 mg TMP kg body $weight^{-1}$ $day^{-1}$) were analyzed using the described methods. The quantitative results were used to calculate a withdrawal time (12 days) to reach residue levels below the respective MRLs. This calculation was performed according to the recommendations of the European Agency for the Evaluation of Medicinal Products (EMEA/CVMP/036/95).

## 1. Introduction

Combinations of sulfonamides and trimethoprim (TMP) are widely used as antibacterials in veterinary practice for the prevention or treatment of respiratory or gastro-intestinal tract infections. However, the large scale application of these drugs raises the risk of the occurrence of residues in food products due to improper observance of withdrawal times. Such residues particularly are of concern because the possibility exists that resistance against these antibiotics is developed. Therefore, regulatory agencies within the European Union (EU) have defined maximum residue limits (MRLs) of 100 ng $g^{-1}$ for sulfonamides in muscle, fat, liver and kidney tissues of all food-producing animals and 50 ng $g^{-1}$ for TMP in muscle, fat + skin, liver and kidney tissues of pigs.[1]

A number of chromatographic methods for the determination of sulfonamides in tissues have been published. These include gas chromatography (GC) with electron capture detection[2] and GC with mass spectrometric detection (MS) after methylation.[3,4] Most analytical methods, however, use reversed-phase high-performance liquid chromatography (RP-HPLC) in combination with ultraviolet (UV) detection ($\lambda$ = 254, 270 or 275 nm).[5–11] In addition, mass spectrometry was suggested or used for both quantitative and confirmatory analysis.[12,13] TMP analyses are performed by using RP-HPLC. The compound is detected by measuring UV absorbance at wavelengths of 229,[11] 240,[10] 270[8,9] or 288 nm.[7] For the chromatography of both compounds mixtures of phosphate buffer and acetonitrile are usually applied as mobile phase.[7–12] Only a few methods for the determination of sulfonamides use volatile mobile phases (*e.g.* ammonium acetate–acetonitrile) which can be used for LC-MS applications.[6,12,13] For sample pre-treatment both liquid–liquid extraction (LLE) procedures[8,9] as well as procedures which combine LLE with solid-phase extraction (SPE)[10–12] have been described. Quantification limits ranging from 50 to 100 ng $g^{-1}$ for sulfonamide analysis[8,12] and from 50 to 160 ng $g^{-1}$ for TMP analysis[8,9,11] have been reported. In the Commission Decision No. 93/256/EEC it is defined that methods for the determination of residues in tissues have to be validated at the MRL, at half the MRL and at double the MRL.[14] As the EU has set an MRL of 50 ng $g^{-1}$ for the determination of TMP in all tissues, the quantification limits which were reported in the literature are not low enough to fulfil the EU requirements for residue analysis.

This work is focused on the quantitative LC-UV determination of low concentrations (≤1/2 MRLs) of sulfadiazine (SDA)

and TMP in swine tissues. Special care has been devoted to the choice of the HPLC mobile phase with the aim to allow a simple methodological transfer of the HPLC-UV methods to an LC system with atmospheric pressure chemical ionization mass spectrometric detection (APCI-MS/MS), which is used for qualitative purposes. The principle of internal standardization was applied. Extraction and clean-up procedures for the determination of both compounds are described. The methods were validated by a set of parameters which are in compliance with the requirements as defined in the Rules Governing Medicinal Products in the European Community.[15]

Moreover, since the methods developed were able to quantify residues as low as half the MRLs, they were tested on real biological samples (kidney, liver, muscle, fat and fat + skin). These samples were taken during a residue study in pigs after oral administration of a commercial SDA–TMP formulation with the feed for five consecutive days. The results obtained were used for withdrawal time calculation.[16]

## 2. Experimental

### 2.1. Chemicals

SDA and TMP were Chemical Reference Substances (CRS) of the European Pharmacopoeia (Strasbourg, France). Sulfamerazine (SMZ) was obtained from Sigma Aldrich Chemie (Steinheim, Germany). Methyltrimethoprim and ormethoprim (OMP) were a gift from Roche (Basle, Switzerland). Stock solutions of 1000 μg mL $^{-1}$ of each compound in methanol were prepared and stored in the dark at ≤ −15 °C for 1 month. By diluting each stock solution with water, appropriate working solutions were obtained (SDA concentration: 20, 10, 4, 2 and 1 μg mL $^{-1}$, TMP concentration: 10, 4, 2, 1 and 0.5 μg mL $^{-1}$, SMZ concentration: 5 μg mL $^{-1}$, OMP concentration: 10 and 2 μg mL $^{-1}$). The working solutions were stored in the dark at 4 °C and were replaced every 14 d.

All products (acetic acid, concentrated ammonia solution, potassium hydroxide and triethylammonium acetate) and solvents used for the extraction procedures (acetone, ethyl acetate, hexane and methanol) were of analytical-reagent grade. All solvents used for the mobile phases (acetonitrile, methanol and water) were of LC grade.

Isolute aminopropyl ($NH_2$) and benzenesulfonic acid (SCX) solid-phase extraction columns (both 500 mg per 10 mL) were purchased from Sopachem (Brussels, Belgium).

### 2.2. Apparatus

**HPLC-UV system.** The HPLC-UV system consisted of a quaternary gradient pump P4000, an autosampler AS3000 with cooling device, and a UV-DAD detector type UV 6000LP, all from ThermoQuest (San José, CA, USA). Chromatographic separations were achieved using a RP $C_{18}$ Spherisorb ODS-2 column (250 × 4.6 mm id, dp 5 μm, kept at room temperature) in combination with a guard column (Chrompack Belgium, Antwerp, Belgium). The mobile phases were prepared with LC grade solvents and contained 1% acetic acid in water–acetonitrile (85 + 15, v/v) for the analysis of SDA. For TMP analysis, a mobile phase consisting of a 0.25% aqueous solution of triethylammonium acetate–acetonitrile–methanol (80 + 15 + 5, v/v/v) was used. Both analytes of interest and their respective internal standards (IS) were eluted isocraticaly with a flow rate of 1.0 and 1.5 mL min $^{-1}$ for SDA and TMP, respectively. Respective injection volumes were 50 and 100 μL. UV absorption of SDA and TMP was measured at a wavelength of 270 and 240 nm, respectively.

**LC-MS/MS system.** The LC-MS/MS system consisted of a quaternary gradient pump P4000, an autosampler AS3000 with cooling device, and a UV detector type UV1000, all from ThermoQuest. The LC conditions were the same as described above for the HPLC-UV system. The MS detector used was a Finnigan LCQ® instrument (ThermoQuest). The samples were analyzed in the positive APCI–MS/MS mode. The instrument was first calibrated and tuned in the APCI mode using a solution of caffeine, L-methionyl-arginyl-phenylalanyl-alanine acetate·$H_2O$ (MRFA) and Ultramark® 1621. Thereafter, aqueous solutions of SDA and SMZ (IS), and of TMP and OMP (IS) were introduced *via* loop injections into the APCI source in combination with the LC eluent in order to tune and optimize the MS detector settings. The instrument was tuned in the full scan MS/MS mode and the relative collision energy (RCE) was set at a level at which the parent ions were fragmented for (nearly) 100% into their product ions (RCE = 20% for both SDA and TMP and their respective IS). Data concerning the fragmentation of the analytes of interest and the relative abundance of the product ions are presented in Table 1.

### 2.3. Biological samples

The samples (kidney, liver, muscle, fat and fat + skin) were from 18 healthy pigs of a Belgian breed (Belgian Landrace Pietrain × Seghers), of a weight ranging between 16.8 and 31.2 kg, of the same age (11–12 weeks) and of the two sexes (females and castrates, equally divided). The pigs received TRIMAZIN 30% Kela (Kela Laboratoria, Hoogstraten, Belgium) twice daily with the feed for five consecutive days at a dose of 1 g powder per 10 kg body weight (BW) per day (equivalent to 25 mg SDA and 5 mg TMP kg BW$^{-1}$ day$^{-1}$). The animals were slaughtered at 1 ($n$ = 4), 3 ($n$ = 4), 5 ($n$ = 5) and 8 ($n$ = 5) days after cessation of medicated feed. The samples (±100 g) were collected immediately after slaughtering and transported to the laboratory. They were cut into small pieces and homogenized by using a Moulinette blender (Moulinex, Paris, France). The samples were placed in identified plastic bags and kept frozen at ≤ −15 °C until analysis.

Known drug-free samples of kidney, liver, muscle, fat and fat + skin tissues were collected from animals which did not receive any medication.

### 2.4. Tissue extraction

**SDA analysis.** Five grams of kidney, liver and muscle tissue (or 2.5 g for fat tissue) were weighed into a plastic centrifuge tube and 250 μL of the IS working solution (125 μL for fat tissue) and 3 mL of 0.1 M acetic acid in water were added. The sample was vortex-mixed for 15 s after each addition of standards or reagents. Three millilitres of acetone and 12 mL of ethyl acetate were added, followed by an extraction for 10 min by rotation. After centrifugation for 10 min at 3500 rpm, the organic phase was transferred into another centrifuge tube. The

**Table 1** Parent ions and product ions of SDA, SMZ, TMP and OMP

| Compound | Parent ion *(m/z)* | Product ions *(m/z)* |
|---|---|---|
| SDA | 251.3 | 174.0; 156.1 (80%)[a]; 108.2 (12%)[a]; 92.1 |
| SMZ | 265.3 | 189.9; 174.0 (56%)[a]; 156.1 (22%)[a]; 110.3; 108.2; 92.2 |
| TMP | 291.3 | 275.2; 258.1 (35%)[a]; 230.2; 181.2; 123.2 (28%)[a] |
| OMP | 275.3 | 260.3 (86%)[a]; 231.1 (8%)[a]; 123.2 |

[a] Ions used for ion ratio measurements; the relative abundance is shown in parentheses.

same amount of organic phase was added to the remainder of the tissue sample and the extraction was repeated. The combined organic phases were kept at ≤ −15 °C for at least 1 h.

**TMP analysis.** Five grams of kidney, liver and muscle tissue (or 2.5 g for fat + skin tissue) were weighed into a plastic centrifuge tube and 250 μL of the 2 μg mL$^{-1}$ IS working solution (125 μL for fat + skin) and 3 mL of 0.1 M acetic acid in water were added. The sample was vortex-mixed for 15 s after the addition of each standard solution or reagent. To the sample was added 1 mL of 2 M KOH and the pH was measured using pH indicator paper. If the pH fell out of the range 10–14, another 1 mL of the 2 M KOH solution was added and the pH of the sample was checked again. After the sample had been vortex-mixed, it was further analysed as described for SDA analysis.

### 2.5. Solid-phase clean-up

**SDA analysis.** *$NH_2$ procedure*. The $NH_2$ cartridge was preconditioned with 3 mL of hexane, followed by the application of the organic tissue extracts obtained during the tissue extraction step. After passage through the cartridge, the ethyl acetate extract was collected into a small Pyrex centrifuge tube. The $NH_2$ column was further washed with 6 mL of methanol, which was also collected in a centrifuge tube.

*SCX procedure*. After the preconditioning of the SCX cartridge with 3 mL of hexane and 6 mL of a 5% solution of acetic acid in ethyl acetate, the collected tissue extract and the methanol washings from the $NH_2$ cartridge were applied onto the SCX column. The column was washed with 6 mL of water and 12 mL of methanol, followed by the elution of SDA and the IS with 3 mL of a 25% solution of concentrated ammonia in methanol. The eluate was concentrated to dryness under a gentle stream of nitrogen at a temperature of 40–45 °C. The dry residue was dissolved in 200 μL of the aqueous part of the mobile phase and the sample was vortex-mixed for 15 s. The dissolved residue was centrifuged for 1 min at 10 800 rpm. The clear supernatant was transferred into an autosampler vial and a 50 μL aliquot was injected onto the HPLC column.

*TMP analysis*. Prior to solid-phase extraction, 2 mL of glacial acetic acid were added to the organic tissue extract, which was obtained during the liquid–liquid extraction procedure.

The SCX column was preconditioned with 3 mL of hexane and 6 mL of a 5% acetic acid solution in ethyl acetate. The organic tissue extract was subsequently applied onto the extraction column, followed by a washing step with 6 mL of water and 12 mL of methanol. TMP and the IS were finally eluted using 3 mL of a 25% solution of concentrated ammonia in methanol.

The organic eluate was evaporated to dryness at 60 °C under a gentle stream of nitrogen. The dry residue was dissolved in 250 μL of the aqueous part of the mobile phase and centrifuged for 1 min at 10 800 rpm. The clear supernatant was transferred into an autosampler vial and a 100 μL aliquot was injected onto the HPLC column.

### 2.6. Method validation

The linearity of the method was evaluated by analyzing calibration graph samples. Samples for the calibration graphs were prepared by spiking blank tissues from pigs which did not receive any medication with SDA and TMP. The addition of 250 μL (or 125 μL for fat or fat + skin tissue) of each of the above mentioned standard working solutions resulted in SDA concentrations of 50, 100, 200, 500 and 1000 ng g$^{-1}$ and in TMP concentrations of 25, 50, 100, 200 and 500 ng g$^{-1}$. The calibration graph samples were treated in a similar way to the unknown samples. Peak area ratios between SDA or TMP and their respective IS were plotted against the corresponding concentration ratios (SDA : IS and TMP : IS).

Within-day precision was evaluated by analyzing six blank tissue samples, which were spiked with SDA and TMP at three different concentrations (MRL, half the MRL and double the MRL), on the same day.

The limit of quantification (LOQ) was defined as the lowest concentration of SDA and TMP for which the method is validated with an accuracy and precision that fall within the recommended ranges (accuracy: −20 to +10%, precision: relative standard deviation (RSD) < $RSD_{max}$ with $RSD_{max} = 2^{(1-0.5\log Conc)} \times 2/3$).[14,17] The LOQ was also established as the lowest point of the calibration graph.

The limit of detection (LOD) was defined as the lowest concentration of SDA and TMP that could be recognized by the detector with a signal-to-noise ratio ≥ 3. The noise was determined by analyzing blank tissue samples. The LOD values were calculated, based on a signal-to-noise ratio of 3, for spiked tissue samples.

The selectivity of the method was demonstrated by analyzing blank tissues from two pigs (negative samples) and injecting solutions of several sulfonamides and TMP analogues onto the HPLC column at a concentration of 10 μg mL$^{-1}$ (cross-contamination). The chromatographic conditions were the same as those used for the analysis of SDA and/or TMP.

## 3. Results and discussion

### 3.1. Internal standardization

In order to enhance the precision and accuracy of the analysis, internal standardization was performed. During preliminary experiments, several sulfonamides were evaluated as candidate IS for the analysis of SDA (Fig. 1A). SMZ (Fig. 1B) was

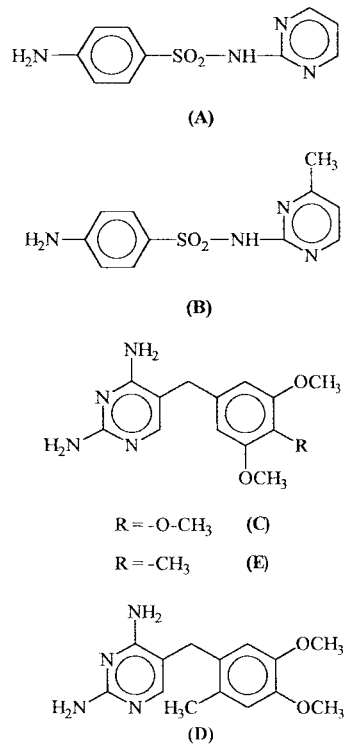


**Fig. 1** Chemical structures of sulfadiazine (A), sulfamerazine (B), trimethoprim (C), methyltrimethoprim (D) and ormethoprim (E).

retained because it elutes closely to the analyte of interest, behaves similarly during the sample preparation procedure and is detectable under the same conditions as the analyte.[18]

For TMP (Fig. 1C) analysis, two compounds which were not commercially available, *viz*., 2,4-diamino-5-(3,5-dimethoxy-4-methylbenzyl)pyrimidine or methyltrimethoprim and 2,4-diamino-5-(4,5-dimethoxy-2-methylbenzyl)pyrimidine or ormethoprim, were evaluated as candidate IS (Fig. 1D and E). To elute methyltrimethoprim (MTMP) within a reasonable time (< 30 min) gradient elution was necessary. Ormethoprim (OMP), which was more structurally related to TMP than MTMP, eluted within 2 min of the elution position of TMP under isocratic conditions. Therefore, OMP was chosen as the IS for TMP analysis.

### 3.2. Tissue extraction and clean-up

The critical step in all sulfonamide residue methods is the clean-up procedure, especially since sulfonamides have amphoteric properties (Fig. 2). Hence, for the extraction of SDA and SMZ in an organic solvent, the pH should be kept between 5.0 and 5.2, where both drugs are uncharged compounds.[5] This condition is fulfilled since a pH of 5 is obtained after the addition of 0.1 M acetic acid to the tissue samples.

TMP, which has a p$K_a$ value of 7.2, is best extracted into an organic solvent in alkaline medium. A pH value of > 10 is obtained by adding ≥ 1 mL of 2 M KOH to the initially acidified tissue extract.

In an effort to eliminate residues of interfering compounds, the organic tissue extracts were further purified by performing a solid-phase extraction. In a preliminary phase of our work, a single-stage solid-phase clean-up was performed, using strong cation-exchange (SCX) columns for both SDA and TMP analysis. Interference-free extracts were obtained for the analysis of TMP. The SDA tissue extracts, however, were not clean enough to allow a proper quantification of this compound. Moreover, injection of many dirty extracts would accelerate the deterioration of the HPLC column. This problem was resolved by performing an additional solid-phase extraction step, using an $NH_2$ column prior to the SCX extraction step.

### 3.3 Chromatography

**HPLC-UV analysis.** In Fig. 3 the chromatograms of the HPLC-UV analysis of a blank kidney sample (A: SDA analysis, B: TMP analysis) and of a blank kidney sample spiked with SDA (Fig. 3C) and TMP (Fig. 3D) and their respective IS are depicted. Fig. 3E and F shows the results of the HPLC-UV analysis of an incurred kidney sample from a pig that received TRIMAZIN 30% Kela (dose rate 25 mg SDA kg $BW^{-1}$ $day^{-1}$ and 5 mg TMP kg $BW^{-1}$ $day^{-1}$ for five consecutive days) and that was slaughtered at 1 day after cessation of medication.

As can be seen, SDA and the IS SMZ are well separated from each other and from endogenous compounds present in the extract. TMP and OMP elute well separated from each other too, but the OMP peak is not completely baseline-separated from an endogenous compound peak present in the kidney extract. The same phenomenon, although less pronounced, was also observed for the other tissue extracts. However, this small interference does not influence the reliability of the quantitative results in a negative way as can be seen from the validation results in Table 2.

In order to allow the elution of late eluting peaks the chromatographic run for the analysis of SDA was elongated to 30 min, which is still an acceptable run time.

Special care was devoted to the choice of the mobile phases. We preferred to use mobile phases containing volatile buffers (1% acetic acid in water for SDA analysis and 0.25% triethylammonium acetate in water for TMP analysis) to allow a simple transfer of the HPLC-UV methods to the LC-MS system.

**LC-MS/MS analysis.** The EU has recommended some type of mass spectrometry for confirmatory purposes to increase specificity. Hence, a LC-APCI-MS/MS system was used for the identification of the compounds of interest in some preselected incurred tissue samples.

The methodological cross-over between HPLC-UV and LC-APCI-MS/MS was simple since no modifications were necessary. The same column was used and the samples could be injected on both systems after the same extraction procedure. Initially, the LC-MS was operated in the full scan MS mode. The full scan APCI mass spectra, however, were devoid of

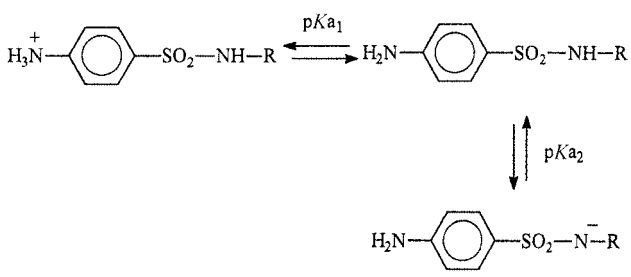


**Fig. 2** Amphoteric properties of sulfonamides.

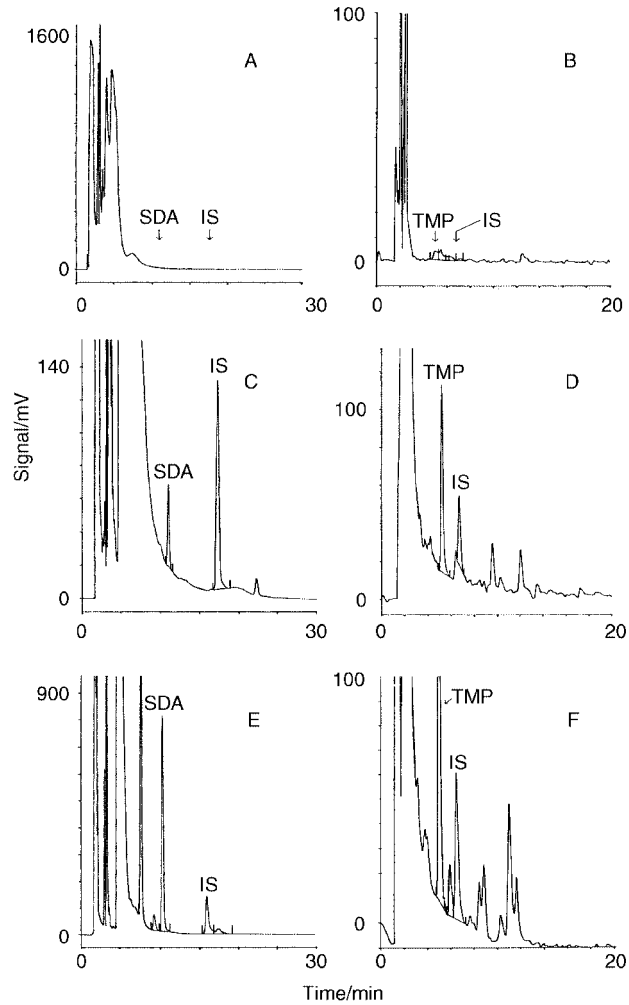


**Fig. 3** LC-UV traces of the analysis of blank kidney (panel A: SDA analysis, panel B: TMP analysis), blank kidney spiked with SDA (100 ng $g^{-1}$, panel C) and TMP (50 ng $g^{-1}$, panel D) and an incurred kidney sample (panel E: SDA, concentration 1100 ng $g^{-1}$, panel F: TMP, concentration 254 ng $g^{-1}$).

much structural information, since they generally contained a strong molecular ion signal ($[M - H]^+$ at a $m/z$ value of 251.3 for SDA, 265.3 for SMZ, 291.3 for TMP and 275.3 for OMP) and very small signals from fragment ions. By operating the LC-MS in the MS/MS scanning mode, product ion spectra were afforded which contained at least three product ions. Some of these product ions were used for ion ratio measurements.

In comparison to the LC-UV chromatograms, the total ion chromatograms (TIC) were much cleaner and totally free from interferences from endogenous compounds.

The presence of SDA and TMP in the preselected incurred pig samples was confirmed since the LC-MS/MS data agreed with the following criteria, which are based on the EU Commission Decision No. 93/256[14] and on the criteria applied in our laboratory: the retention times of the SDA and TMP peaks in the incurred samples were within a ±3% range of those in standard samples (Fig. 3C–F), the same product ions were present in the MS/MS full scan mass spectra of SDA and TMP in the incurred samples and the standard samples (Fig. 4A–D), and the relative abundance of the selected product ions in the mass spectra of SDA and TMP in incurred samples fell within a ±20% range of those in standard samples (Table 1).

### 3.3. Method validation

**Linearity.** The calibration graphs for SDA and TMP were linear over the concentration ranges tested (50–1000 ng $g^{-1}$ and 25–500 ng $g^{-1}$, respectively) in all tissues (correlation coefficients $\geq 0.9994$) as shown in Fig. 5. The bias for the recalculated concentration of all calibrators of the calibration graphs in kidney, liver, muscle, fat and fat + skin was within the required range of –20 to +10%.[17]

**Precision and accuracy.** The within-day precision and accuracy results are presented in Table 2. As can be seen, the RSD values for SDA and TMP were below the $RSD_{max}$ values in all tissues. Moreover, the accuracy fell within the required range of –20 to +10%.[17]

**Limit of quantification.** The LOQ was determined by analyzing spiked samples ($n = 6$) at a level of 50 ng $g^{-1}$ of SDA and 25 ng $g^{-1}$ of TMP. As the data for within-day precision and accuracy fell within the ranges specified, the LOQ for both compounds was set at these concentration levels.

The LOQ levels obtained are low enough to meet the EU requirements which impose the quantification of residues of veterinary medicinal products in foodstuffs (*i.e.* tissues) at levels which are as low as half the MRLs. Moreover, a LOQ of 25 ng $g^{-1}$ for the determination of TMP in tissues is lower than the LOQ values reported in the literature.[9,11]

**Limit of detection.** LOD values of 12, 38, 15 and 17 ng $g^{-1}$ were obtained for the determination of SDA in kidney, liver, muscle and fat, respectively. For the analysis of TMP, the LOD values were as low as 22 ng $g^{-1}$ in kidney, 18 ng $g^{-1}$ in liver, 20 ng $g^{-1}$ in muscle and 11 ng $g^{-1}$ in fat + skin tissue.

**Selectivity and specificity.** The methods proved to be selective for SDA, SMZ and TMP since the chromatograms of blank tissues showed no interfering peaks from endogenous compounds eluting at the retention time of these drugs (Fig. 3 A and B). In the eluting zone of OMP an endogenous compound was observed in all tissues. However, as mentioned above, this interference did not influence the reliability of the quantitative results. The methods were also selective for SDA and TMP with respect to the interference from analogous compounds: the sulfonamides tested (sulfathiazole, sulfamerazine, sulfapyridine, sulfamethazine, sulfadoxine, sulfamethoxazole, sulfaquinoxaline and sulfadimethoxine) and TMP did not interfere with SDA. In addition, the TMP analogues (OMP and

**Table 2** Validation results for the determination of SDA and TMP in various swine tissues using LC-UV

| | | SDA | | | | TMP | | |
|---|---|---|---|---|---|---|---|---|
| Matrix | | Conc./ ng $g^{-1}$ | Accuracy (%) | Precision[a] (RSD, %) | | Conc./ ng $g^{-1}$ | Accuracy (%) | Precision[a] (RSD, %) |
| *Kidney*— | | | | | *Kidney*— | | | |
| Calibration graph | $r = 0.9998$ | 0 → 1000 | within −20 to 10% | | $r = 0.9978$ | 0 → 500 | within −20 to 10% | |
| Accuracy and precision | | 50 | 4.0 | 7.5 | | 25 | −8.5 | 12.3 |
| (within-run) | | 100 | 3.0 | 9.2 | | 50 | −7.7 | 6.5 |
| | | 200 | −8.5 | 10.3 | | 100 | 3.9 | 8.0 |
| LOQ | | 50 | −15.0 | 7.4 | | 25 | −9.0 | 10.7 |
| LOD | | 12.5 | | | | 21.6 | | |
| *Liver*— | | | | | *Liver*— | | | |
| Calibration graph | $r = 0.9999$ | 0 → 1000 | within −20 to 10% | | $r = 0.9995$ | 0 → 500 | within −20 to 10% | |
| Accuracy and precision | | 50 | −15.7 | 6.9 | | 25 | 6.0 | 9.2 |
| (within-run) | | 100 | 1.7 | 5.7 | | 50 | 8.0 | 7.5 |
| | | 200 | 6.3 | 6.9 | | 100 | −2.2 | 11.9 |
| LOQ | | 50 | −17.0 | 6.9 | | 25 | 8.5 | 12.4 |
| LOD | | 36.7 | | | | 18.1 | | |
| *Fat*— | | | | | *Fat + skin*— | | | |
| Calibration graph | $r = 0.9994$ | 0 → 1000 | within −20 to 10% | | $r = 0.9996$ | 0 → 500 | within −20 to 10% | |
| Accuracy and precision | | 50 | 8.3 | 10.1 | | 25 | −3.5 | 12.3 |
| (within-run) | | 100 | −5.7 | 11.6 | | 50 | −4.8 | 9.1 |
| | | 200 | 8.1 | 11.7 | | 100 | 1.4 | 4.6 |
| LOQ | | 50 | −8.7 | 12.6 | | 25 | −11.6 | 8.6 |
| LOD | | 16.7 | | | | 11.4 | | |
| *Muscle*— | | | | | *Muscle*— | | | |
| Calibration graph | $r = 0.9998$ | 0 → 1000 | within −20 to 10% | | $r = 0.9997$ | 0 → 500 | within −20 to 10% | |
| Accuracy and precision | | 50 | −17.0 | 5.2 | | 25 | 10.0 | 12.7 |
| (within-run) | | 100 | −1.8 | 6.0 | | 50 | −10.0 | 6.8 |
| | | 200 | 7.1 | 4.3 | | 100 | −8.5 | 2.6 |
| LOQ | | 50 | −17.0 | 5.2 | | 25 | 10.0 | 7.3 |
| LOD | | 15.0 | | | | 20.0 | | |

[a] RSD max: 18.6, 16.7, 15.1 and 13.6% for a concentration of 25, 50, 100 and 200 ng $g^{-1}$, respectively.

MTMP) and SDA did not co-elute with TMP. The interference from metabolites of SDA and TMP analogues was not tested. However, if the presence of chemotherapeutics other than SDA and TMP is expected in an incurred tissue sample, the identity of the 'SDA' or 'TMP' peak can be unequivocally confirmed by performing an LC-MS analysis.

### 3.4. Analysis of biological samples

The applicability of the methods described was proved by the analysis of incurred tissue samples, which were obtained during a residue study in pigs after oral administration of SDA and TMP with the feed. One day after cessation of medication the following mean ($n$ = 4) quantitative results were obtained: for SDA: 430 ng $g^{-1}$ in kidney, < 50 ng $g^{-1}$ in liver, 180 ng $g^{-1}$ in muscle and 180 ng $g^{-1}$ in fat tissue; for TMP: 322 ng $g^{-1}$ in kidney, 65.6 ng $g^{-1}$ in liver, 29.2 ng $g^{-1}$ in muscle and 141 ng $g^{-1}$ in skin and fat tissue. The concentrations of SDA and TMP in all matrices were below the MRL at 3 days after cessation of medication, indicating a rapid elimination of both drugs. These results correspond with those found by other workers. Söli *et al.*[19] reported that, after oral administration of commercial SDA–TMP formulations, no unacceptable or antibacterial residues of SDA and TMP were found in the kidneys of pigs slaughtered at 5, 7 and 10 days after a single dose administration. Garwacki *et al.*[7] proposed a withdrawal period of at least 5 days for an oral SDA–TMP formulation which was administered for three consecutive days (dose rate 6 mg $kg^{-1}$ BW of TMP and 30 mg $kg^{-1}$ BW of SDA). This withdrawal period of at least 5 days was based on the moment that the concentrations of SDA and TMP were below the MRL in all tissues.

Using the quantitative results obtained after sample analysis a withdrawal time of 6 days could be calculated for SDA. For TMP a longer withdrawal time (12 days) was obtained. This can be explained by the different chemical properties of both

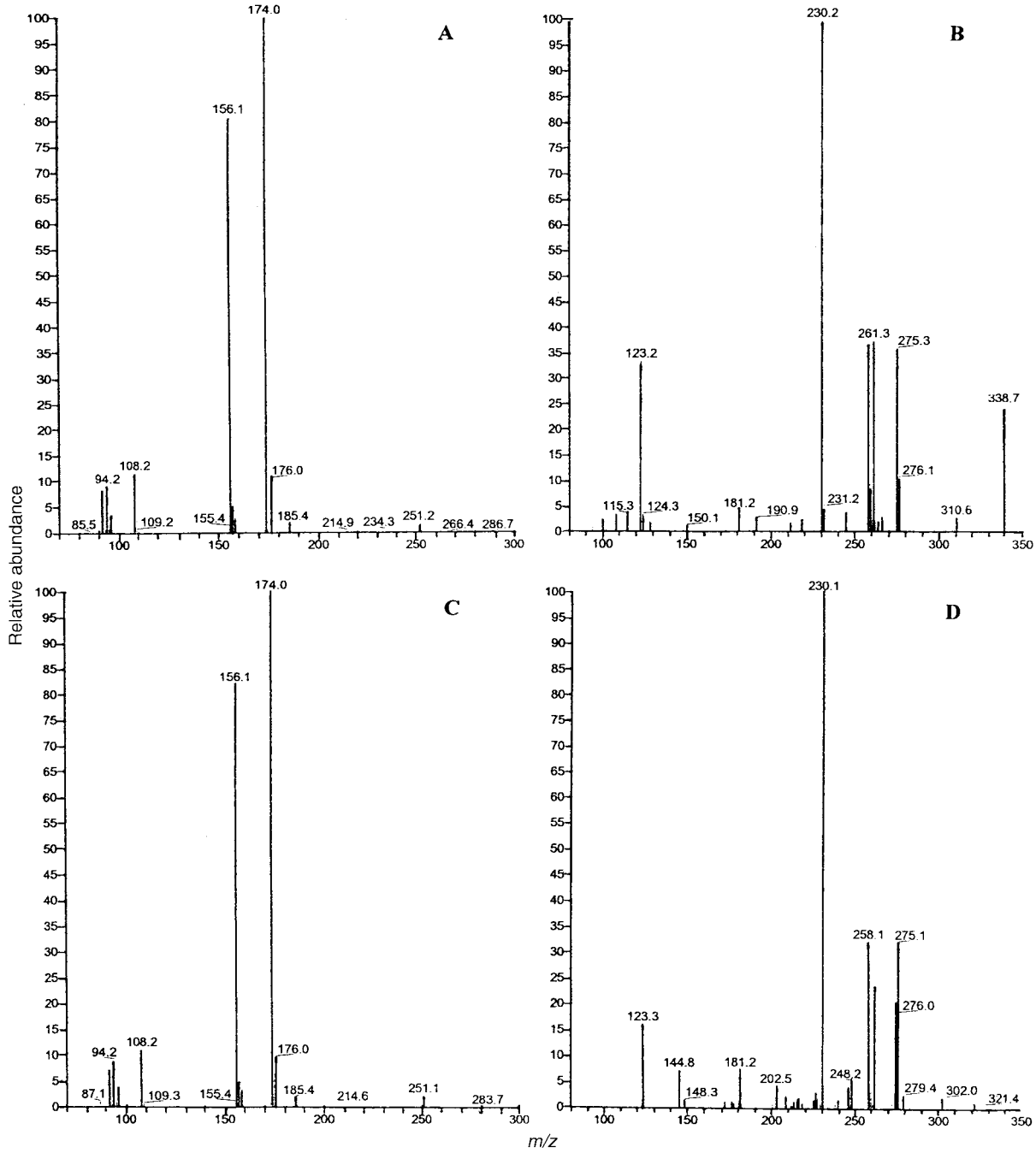


**Fig. 4** LC-APCI-MS/MS scans of SDA (panel A) and TMP (panel B) in a spiked kidney sample and of SDA (panel C) and TMP (panel D) in an incurred kidney sample.

compounds: TMP is a lipid-soluble organic base, whereas the more hydrophilic sulfonamides are weak organic acids. Therefore, sulfonamides are well distributed in the interstitial aqueous space of most tissues, whereas TMP rapidly passes from the blood into tissues, in which it tends to concentrate.[7] The same conclusions could be drawn from the pharmacokinetic parameters for SDA and TMP, which were obtained during a pharmacokinetic study with TRIMAZIN 30% Kela at our laboratory.[20] These results will be presented in a forthcoming paper.

From the results obtained it can be concluded that after the administration of 1 g per 10 kg BW per day of TRIMAZIN 30% Kela for five consecutive days, a withdrawal time of 12 days is recommended to reach concentrations below the MRL for both SDA and TMP in all tissues. This withdrawal time is longer than the withdrawal time of at least 5 days proposed by Garwacki *et al*. However, this can be explained by the different calculation methods used in both studies. As mentioned above, Garwacki *et al*. proposed the moment that the concentrations of SDA and TMP were below the MRL in all tissues as the withdrawal time. In our study, however, the withdrawal time was calculated according to the EU regulations, which take a safety margin into account.[16] It was determined at the time when the one-sided 95% upper tolerance limit with a 95% confidence level was below the MRL.

## 4. Conclusion

The methods described were successfully used for the quantitative HPLC-UV determination of SDA and TMP in several swine tissues (kidney, liver, muscle, fat and fat + skin). The combination of a structurally related IS, a specific extraction procedure and good chromatographic properties resulted in reliable quantitative data. In addition, the methods were suitable for LC-MS/MS application, which gave the opportunity for a complementary identification of the analytes of interest on the basis of their unique MS/MS profiles. The methods were validated (linearity, within-day precision, accuracy, LOD, LOQ and selectivity) and the results were within the specified ranges. The LOQ values of 50 ng $g^{-1}$ for SDA and 25 ng $g^{-1}$ for TMP analysis were sufficiently low to allow the quantification of residues of both compounds at levels which were as low as half the MRLs.

Finally, the validated methods were used to analyze incurred swine tissue samples in a tissue residue depletion study in pigs after oral administration of a commercial formulation of SDA–TMP with the feed for five consecutive days. Using the results obtained, a withdrawal time of 12 days was calculated.

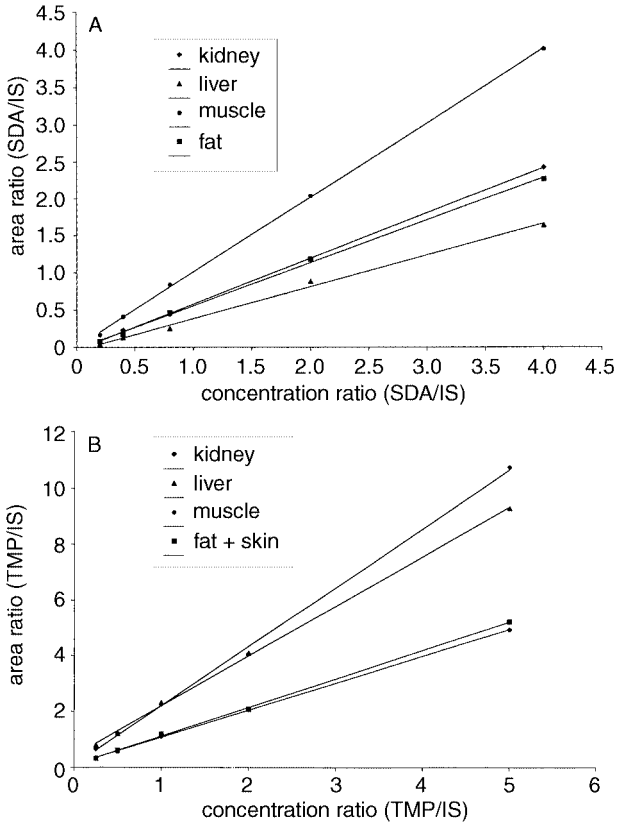


**Fig. 5** Calibration graphs for: (A) SDA in kidney ($y = 0.6143x - 0.03787$), liver ($y = 0.4259x - 0.04302$), muscle ($y = 1.005x + 0.00462$) and fat ($y = 0.5777x - 0.02278$); (B) TMP in kidney ($y = 2.1194x + 0.08599$), liver ($y = 1.7880x + 0.4157$), muscle ($y = 0.9663x + 0.1161$) and fat + skin ($y = 1.02279x + 0.09617$).


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