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Review

Methods for the determination of sulphonamides in meat

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ABSTRACT

Sulphonamides, due to their important antibacterial effects, are widely used in veterinary practice and animal husbandry. Residues arising from administration without observing withdrawal time sufficiently are normally the parent compounds and the N^4-acetyl derivatives, the latter being hydrolyzed to the parent compounds only during extraction under acidic conditions. It is therefore quite conceivable that many authors concentrate on determining these metabolites. In the past decade, we have witnessed a considerable increase in new analytical techniques dealing with the determination of sulphonamides. Among these procedures, especially the so-called multimethods using high-performance liquid chromatography —though sometimes including toilsome clean-up steps— can be mentioned. However, current approaches also utilize gas chromatography, gas chromatography–mass spectrometry, liquid chromatography–mass spectrometry, supercritical fluid chromatography–mass spectrometry, thin-layer chromatography and immunological methods. For most of these techniques, a strong trend towards lowering the level of detectability (down to the sub-ppb range) and improving accuracy and reproducibility can be established.

CONTENTS

1. Introduction . . . 426
2. Sample preparation and extraction procedures . . . 426
3. High-performance liquid chromatography . . . 426
   3.1. Procedures without derivatization . . . 426
      3.1.1. Simple routine methods to determine a few sulphonamides . . . 427
      3.1.2. Multi-methods to determine many sulphonamides . . . 428
      3.1.3. Multi-methods to determine many sulphonamides and other chemotherapeutics . . . 428
   3.2. Procedures requiring derivatization . . . 429
      3.2.1. Precolumn derivatization . . . 429
      3.2.2. Postcolumn derivatization . . . 429
         3.2.2.1. Derivatization with DMBA . . . 429
         3.2.2.2. Derivatization with Fluram . . . 429
4. Gas chromatography . . . 429
5. Coupled techniques . . . 432
   5.1. Liquid chromatography–mass spectrometry . . . 432
   5.2. Supercritical fluid chromatography–mass spectrometry . . . 433
6. Thin-layer chromatography . . . 433
7. Non-chromatographic methods . . . 433
8. Conclusions . . . 433
9. Abbreviations . . . 435
References . . . 435

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1. INTRODUCTION

Sulphonamides (SA) are a very important class of antibacterial compounds widely used in veterinary practice. Residues of these drugs and in many instances also their metabolites may occur in foods of animal origin and present a potential danger to consumers' health. Many of the parent compounds are approved for use in species under limitations which require withdrawal long enough to allow elimination of the residues from edible tissues.

This review deals with the analytical aspects of SA in meat. In Switzerland, as in many other countries, the legal tolerance is 0.1 mg/kg. This level includes the parent compounds and their possible N^4-acetylated metabolites. Many analytical methods have been applied to investigate concentrations of SA in meat. In the following, an attempt is made to summarize the different procedures developed since about 1982.

2. SAMPLE PREPARATION AND EXTRACTION PROCEDURES

The critical step in all SA residue methods is the clean-up, which is normally expected to remove constituents showing similar behaviour and/or reactions to the SA. Classical methodologies for the isolation of SA residues involve processes such as repeated homogenization of samples in an extraction or several extraction solvents, centrifugation of the extracts, back-extraction, pH adjustments, re-extraction and evaporation of the solvent. All these methods are more or less laborious and time-consuming and do not allow the analysis of large numbers of samples per day.

Long *et al.* [3] reported a new approach to the isolation of SA in catfish muscle tissue. With this procedure, called MSPD, the samples were blended into C_{18} by means of a glass pestle until the mixture appeared homogeneous. The semi-dry blend was transferred into a plastic syringe column and compressed. The column was then eluted with solvent(s) so as to isolate the SA. The authors proposed that the disruption of the tissue architecture that occurred was due both to mechanical shearing forces from blending and to hydrophobic forces of the covalently bound C_{18}. They stated that MSPD greatly speeded the process of SA residue screening.

As for the analytical methods, a considerable number of new techniques have been introduced to complement the already known methods in the last decade, such as EIA, ELISA, HPTLC, SFC and GC–tandem MS.

3. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

3.1. *Procedures without derivatization*

In practice, SA screening for qualitative and quantitative residue control in meat is performed by RP-HPLC. The great advantage of HPLC is that SA may be analysed without derivatization and can be quantified by UV absorption at 254 or 270 nm. With more effort it is possible to analyse SA residues from meat with pre- or postcolumn derivatization to obtain results without matrix interferences. Some published methods are concerned with a single SA, others manage to extract the most important ones (up to twenty) from meat. Even multi-methods are known that permit the detection of up to 60 chemotherapeutics, antiparasites and growth promoters in one procedure [4].

Because of the inductive effect of the SO_2 group, SA are amphoteric (Fig. 1) and have different pK_a values, and consequently a work-up and clean-up procedure is aggravated. Several liquid–liquid and solid-phase sample clean-ups have been reported. Between pH 5.0 and 5.2 the commonly used SA are uncharged compounds [5].

In general, SA are poorly soluble in water, diethyl ether and chloroform, but readily soluble in polar organic solvents such as acetone. SA that are acetylated at the N^4-position, can be hydrolysed in boiling acidified acetone [5]. However, some workers have reported the determination of SA and their metabolites in one procedure without hydrolysation.

Usually, SA are fairly stable compounds, only a few being sensitive to UV radiation. Mainly, there is

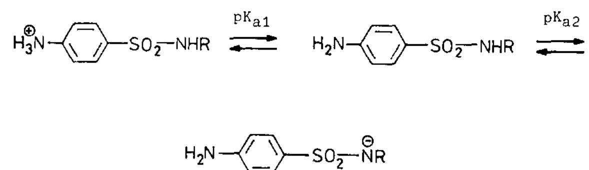

Fig. 1. Dissociation of sulphonamides.

an absorbance band at 270 nm, commonly used for UV detection, whereas fluorescence detection has been reported for SAA and SGU [6] and electrochemical detection is rarely used for SA [7].

Severe matrix interferences and low recoveries were the reason for developing highly sophisticated methods to determine different SA in one procedure. The isolation of SA residues from a complex mixture such as muscle, liver or kidney usually requires several time-consuming steps: homogenization of the sample with the extraction solvent; centrifugation; purification by solid-phase extraction or liquid–liquid partition by a series of washes, re-extractions and pH adjustments; and determination by RP-HPLC.

3.1.1. Simple routine methods to determine a few sulphonamides

Paulson and co-workers [8,9] reported the analysis of SMZ by HPLC in connection with pharmacological studies. Animal tissue was extracted with methanol, degreased with hexane, purified on an XAD-2 column and separated on a Radial-Pak C_{18} column.

Another study of SMZ and the N^4-metabolite in swine liver during frozen storage was described by Parks [10]. Meat was extracted with water, filtered and passed through a column of Duolite ES-863, a column of neutral alumina and a second column of Duolite ES-863 and chromatographed on Supelco LC-18.

Haagsma *et al.* [11] reported a method for the determination of SMZ in swine tissue. Extraction was performed with CH_2Cl_2 in an ultrasonic bath for 10 min. Clean-up was carried out by passing the extract through a Sep-Pak C_{18} cartridge. The purified extract was analysed on a Hypersil ODS column. Recovery for pig muscle tissue was reported to be 89.5%. Other determinations of SMZ were reported by Haagsma and co-workers [12,13].

A modified method by Haagsma and VanDeWater [14] can be used to determine five SA simultaneously (SAA, SMZ, SQX, SDA and SDO). After the extraction of meat with chloroform–acetone (1:1) and ultrasonic treatment, the acidified extract was passed through a 3-ml Baker cation-exchange column. A CP Spher C_8 column was used for HPLC analysis. Recoveries were reported to be high for muscle tissue (80–90%). However, low recoveries were sometimes found for kidney tissue, for unknown reasons.

Other pharmacokinetic studies have been published for SMZ, the N^4-acetyl metabolite and hydroxy metabolites in food-producing animals by Nouws and co-workers [15–19], but sample preparation and HPLC analysis were reported in only one of the papers [15].

Further pharmacokinetic studies have been described for SMZ in plasma, milk and uterine fluid of different animals [20,21], for SPR [22] and for SDA in the rat [23]. Good reviews on the pharmacokinetics of several differently used chemotherapeutics in animal husbandry were given by Lutz [24] and by Vree and Hekster [25].

Endoh *et al.* [26] reported the determination of SD in swine tissue, muscle, liver, kidney and fat. SD, widely used in Japan, was extracted with acetonitrile saturated with hexane. Further purification was achieved on an alumina phase. The purified drug was then analysed on a Nucleosil 5 C_{18} column. The reported recovery was 81–95%.

For SDM and OMP in tissue of cattle, chicken and catfish, a method was described by Weiss *et al.* [27]. Tissue was extracted at pH 10 with CH_2Cl_2 and tetrabutylammonium hydroxide, an ion-pair reagent, and both compounds were separated on a Porasil column. Tetrabutylammonium hydroxide is a reagent for ion-pair extractions, that is, anions from weak acids can be extracted with CH_2Cl_2 or $CHCl_3$ [28]. A similar strategy for the simultaneous extraction of STA and SMZ was used by Parks [29,30].

The same ion-pair extraction method was proposed by Rona *et al.* [31] for the determination of SPR in human serum. Further purification was obtained with an Extrelut column and on an RP-8 MOS-Hypersil column. They subsequently reported [32] a modified method for SPR in human serum and urine. The crude extract was purified with a Sep-Pak C_{18} cartridge. The analytical column used for separation was Hypersil ODS.

Petz [6] mentioned a liquid–liquid partition method for the determination of SAA and SGU. Muscle and liver tissue was extracted with acetonitrile at pH 8.5. Water was separated by adding sodium chloride, the evaporated residue was partitioned between aqueous methanol and hexane and the aqueous layer was used for chromatography at pH 2

on a Nucleosil 5 SA column. The native fluorescence was used for detection with excitation at 275 nm and emission at 340 nm.

Horii *et al.* [33] determined SMZ, SDM and SMM in animal tissue by extraction with acetonitrile and liquid–liquid partitioning. The residue was purified on a Bond Elut ODS cartridge at a relatively low pH and the three SA were separated on a Nucleosil 100 ODS column. The recovery in meat was 81–93%.

Another method for SMZ and its metabolites in body fluids was reported by Van 't Klooster *et al.* [34]. Samples were extracted and purified by liquid–liquid partitioning and separated on a Hypersil ODS column. This method can be applied to plasma, urine and cell culture media.

A new method for the determination of SDM and SMM in fish was published by Ueno *et al.* [35]. This method included the extraction of the two SA together with the N^4-acetylated metabolites with acetone and re-extraction with chloroform. The concentrated solution was cleaned up on a Sep-Pak alumina B cartridge and analysed on a YMC-Pack C_{18} column. An average recovery of 85% was measured at a level of 2 mg/kg.

3.1.2. Multi-methods to determine many sulphonamides

A simple and rapid method for thirteen SA residues (SAA, STA, SPR, SMZ, SCP, SDM, SGU, SAC, SDA, SME, SMP, SQX, SMX) in meat, liver and kidney, published by Rychener *et al.* [5], is also applicable to N^4-metabolites. A 10-g homogenized sample was extracted with acetone and partitioned between water and hexane. After neutralization, the SA were re-extracted with ethyl acetate and purified on a small silica gel column. The determination was effected by analysis on a Superspher 100 phase and on a cation-exchange phase (Nucleosil 5 SA) column. The SA were determined by UV detection at 270 nm. The two different HPLC analyses were necessary for confirmation purposes. It was not possible to separate all thirteen SA in one run without a gradient programme. The recovery was between 50 and 80%, depending on the type of SA.

Long and co-workers [36,37] published a simple multi-method for eight SA residues in milk which they called matrix solid-phase dispersion (MSPD). The same time-saving method was practicable with pork tissue [38] for SAA, STA, SDA, SME, SMZ, SMX, SDM and SIA. A 0.5-g sample of meat was mixed with 2 g of C_{18}-derivatized silica. The mixture was used to prepare a column. After washing the column with hexane, the SA were eluted with CH_2Cl_2. Without further purification the extract was chromatographed on an ODS column and no problems with interfering peaks were observed. Recoveries were between 70 and 95%.

Ikai *et al.* [39] reported a multi-method for ten SA residues in meat and fish (STA, SMX, SDA, SME, SMZ, SDM, SMP, SQX, SIZ and SMM). Ethyl acetate was used for extraction of 5 g of meat. Clean-up was performed with a Baker 10 amino cartridge. All ten SA were retained with ethyl acetate and eluted with 5 ml of acetonitrile. The separation was performed on Wakosil $5C_{18}$. The recoveries were 74–99%. This method is simple and rapid.

3.1.3. Multi-methods to determine many sulphonamides and other chemotherapeutics

For extensive residue monitoring control it is necessary to have multi-methods and to determine many different chemotherapeutics with one procedure. Single-residue determination is only efficient in case of suspicion.

Petz [40] proposed a multi-method for the determination of chloramphenicol, furazolidone and five SA (SDA, SME, SMZ, SMX and SQX) in meat, milk and eggs. A 25-g sample was extracted with acetonitrile, separated from co-extracted water and purified by liquid–liquid partitioning with hexane. For the analysis an MOS-Hypersil column was used and the detector was set at 275 nm. Recoveries were stated to be between 70 and 90%.

A new and extensive field was opened up by Malisch [4,41] with a multi-method for about 60 chemotherapeutics, antiparasitics and growth promoters in one procedure by combination of HPLC–UV detection and GC–ECD. After a complex and time-consuming sample preparation and clean-up, up to 28 SA were analysed on a Spherisorb ODS column with a gradient programme, a variable-wavelength detector and a photodiode-array detector. Recoveries for eleven SA were between 70 and 90%, but only 35% for SGU.

A method for the simultaneous determination of eleven synthetic antibacterial agents, including four SA (SME, SDM, SMM and SIZ), in cultured fish was described by Nose *et al.* [42]. A 10-g sample of

fish meat was extracted twice with acetone, purified by liquid–liquid partitioning and chromatographed on neutral alumina with different solvents. The drugs were analysed on a Nucleosil C_{18} column and with UV detection at 260 nm. Recoveries for the four SA were 70–91%.

Parks [7] described a screening procedure for six nitro-containing drugs, including one SA (SN), in chicken tissue. A 2.5-g amount of tissue was extracted with 20 ml of chloroform–DMSO–ethyl acetate (50:0.8:50) and purified on a small neutral alumina column. The drugs were eluted with phosphate buffer (pH 6)–methanol solution (1:1) and chromatographed on Supelcosil LC-18. Detection was effected with an amperometric detector with a glassy carbon electrode at −0.8 V *versus* Ag/AgCl. The recovery of SN was 91–97%.

A method for the simultaneous determination of eight antibacterial drugs, including three SA (SMZ, SMM and SIZ), used in cultured fish production was developed by Horie *et al.* [43]. The method is closely related to an earlier method to determine SMZ in meat [44]. The drugs were extracted with acidic methanol, followed by a Bond Elut clean-up procedure. Analysis of the drugs was carried out on an Intersil ODS column with detection at 265 nm. The recoveries for each drug added to the fish were 65–89%.

3.2. Procedures requiring derivatization

Fluorescamine {4-phenylspiro[furan-2-(3*H*),1′-(3′*H*)-isobenzofuran]-3,3′-dione; Fluram} and *p*-dimethylaminobenzaldehyde (DMBA) are widely used as derivatization reagents for SA in various TLC and HPTLC methods.

3.2.1. Precolumn derivatization

Takeda and Akiyama [45] derivatized specifically eight SA (SDA, SME, SMZ, SMX, SDM, SQX, SMM and SIM) with Fluram at pH 3 to give highly fluorescent compounds. The derivatized drugs were analysed on a Chemosorb 5-ODS-H column and detected with a fluorescence detector at an excitation wavelength of 405 nm and an emission wavelength of 495 nm.

3.2.2. Postcolumn derivatization

3.2.2.1. Derivatization with DMBA. SMZ and STA were easily extracted from feed and analysed using a LiChrosorb RP-18 column with detection at 450 nm after postcolumn derivatization with DMBA. This method was published by Smallidge *et al.* [46] as an extension of their earlier work [47].

A similar derivatization method and a combination of automated clean-up and concentration of milk, eggs and meat samples together with an HPLC separation was described by Aerts and co-workers [1,48]. Thirteen SA residues (STA, SQX, SAA, SMX, SME, SMZ, SGU, SDO, SDM, SDA, SAC, STR and SCP) and a few other drugs could be easily analysed in meat by automated on-line dialysis of the aqueous extract through a flat cellulose acetate membrane. The aqueous dialysate was concentrated on a small column (XAD-2 or XAD-4). After this concentration, the drugs were back-flushed with the HPLC eluent and analysed on a LiChrosorb RP-8 column. Because of many matrix interferences, a specific detection was necessary. For this reason, postcolumn derivatization with DMBA and sensitive detection of the SA residues at 450 nm were chosen.

3.2.2.2. Derivatization with Fluram. Sista *et al.* [49] determined SPR in human saliva by HPLC using postcolumn derivatization with Fluram and detection of the generated fluorophore with a fluorimetric detector at 395 nm (emission) and 470 nm (excitation). Fluram is expensive and only stable in a cooled solution for about 48 h. A similar method for the determination of twelve SA (SAA, SGU, SPR, SDA, SME, STA, SMZ, SMP, SCP, SDO, SDM and SMT) in meat and kidney was recently reported by Pacciarelli *et al.* [50]. The drugs were extracted with CH_2Cl_2–acetone (1:1). The extract was purified by solid-phase extraction on a cation-exchange cartridge (Chromabond SA 500) and chromatographed on LiChrospher 100 RP-18, followed by Fluram derivatization and fluorescence detection. Relative fluorescences and recoveries were reported.

4. GAS CHROMATOGRAPHY

GC methods for screening, quantification and confirmation could have the advantage of being more sensitive than LC. Sensitive detectors such as the electron-capture detector, in conjunction with

appropriate derivatizations, may improve the detectability, or a strong confirmation tool like MS could provide good identifications. The low vapour pressure and the high polarity of the SA necessitate derivatization. However, a few workers have described some technically advanced methods for the introduction of the underivatized SA into a mass spectrometer. Although these methods did not apply the GC technique, they are listed because of their mass spectrometric aspects.

Screening, confirmation and quantification of SDA, SDO, SMP, SMZ and SQX without derivatization were accomplished by Finlay *et al.* [51] in crude ethyl acetate extracts of pig kidney, introducing them by a solid probe or a moving belt interface into a hybrid tandem MS system and recording the collisionally activated (CAD) spectra. The mass spectrometer consisted of an electric and magnetic sector part, a quadrupole collision cell and a quadrupole mass analyser. Chemical ionization (CI) employing ammonia as the reagent gas and argon as the collision gas yielded daughter ion spectra suitable for confirmation by means of an intense protonated molecular ion and a set of fragments of the sulphanil moiety common to all the substances under study. Screening was accomplished by scanning the CI spectrum in the magnetic sector part and detecting the sulphanil fragment with the quadrupole mass analyser; detection limits of 100 μg/kg were achieved. A subsequent confirmatory experiment was performed by scanning the daughter ion spectra of the protonated molecular ions. The multiple ion detection mode was used for quantitative analysis by monitoring some intense daughter ions. The method was described as being very fast, and up to 400 crude extracts could be analysed before cleaning of the ion source became necessary.

Brumley *et al.* [52] applied collision-induced dissociation–mass analysed ion kinetic energy spectrometry (CID–MIKES) to the identification of SA in tissues. The CID–MIKE spectra of eighteen SA were presented. After a routine tissue clean-up, spiked liver samples were directly introduced with a solid probe and the spectra were obtained by isobutane chemical ionization and with helium as the collision gas. SDM, SMZ and SQX were determined in the range 100–200 μg/kg by scanning the electric sector in a magnetic sector instrument and recording the full-scan CID–MIKE spectra.

Manuel and Steller [53] reported a GC method for determining SBM, SCP, SDM, SMZ, SQX and STA in cattle and swine tissues. After an extraction by the Tishler method, the SA were methylated at the N^1-position by diazomethane, then separated on a packed column and detected with an electron-capture detector. The hydrolysis of the N^4-acetyl metabolite of SMZ in fortified tissue was studied. The methylation of SMZ by diazomethane was found to be approximately 90%. Recovery studies were performed in the range 100–1000 μg/kg.

Suhre *et al.* [54] developed an assay for SMZ using a packed column and determining the N^1-methyl derivative by MS with electron impact ionization. Quantification was accomplished after a modified Tishler clean-up and by comparing the ratio of two fragments of the derivative in the multiple-ion detection mode with the corresponding fragments of previously added ^{13}C-labelled SMZ. The method was tested on swine liver and muscle tissues, fortified with SMZ in the range 50–200 μg/kg.

In the investigation of incurred residues of SMZ, Matusik *et al.* [55] synthesized desaminosulphamethazine, N^4-acetylsulphamethazine, N^4-D-glucosylsulphamethazine and N^4-(1-deoxy-D-glucuronyl)sulphamethazine. In order to compare the Tishler spectrophotometric method and the GC methods of Manuel and Steller [53] and Suhre *et al.* [54], swine liver and muscle tissues were fortified with SMZ and the metabolites mentioned above in the range 100–200 μg/kg. The N^4-derived metabolites yielded poor chromatographic and recovery properties. With the addition of a hydrolysis step using dilute hydrochloric acid, all metabolites were determined as N^1-methylsulphamethazine or N^1-methyldesaminosulphamethazine, respectively, by GC. Recovery studies were reported for the different metabolites and assays.

Paulson *et al.* [56] described another procedure for the identification and determination of ^{14}C-labelled SMZ, N^4-acetylsulphamethazine, N^4-glycosylsulphamethazine and desaminosulphamethazine in swine tissue after oral administration of laballed SMZ. After clean-up of the tissue, the substances in question were separated by RP-LC and determined by measuring the carbon-14 activity by liquid scintillation counting. The fractions of these substances were collected separately and methylated with di-

azomethane; N^4-glycosylsulphamethazine had to be hydrolysed before derivatization and GC. The derivatives were subsequently analysed by on-column injection capillary GC and full-scan MS with electron-impact ionization. Determinations were carried out routinely in the range 50–100 μg/kg, but also a few μg/kg could be detected.

Stout *et al.* [57] developed a confirmatory method for SMZ in cattle and swine tissue. After a clean-up, a derivatization step in accordance with Manuel and Steller [53] provided that N^1-methylsulphamethazine, which was determined by packed-column GC and by CI-MS, using methane as the reagent gas. The advantage of the CI mode was that the spectrum generated an intense protonated molecular ion and a fragment indicative of the methylated amine functionality of the molecule in the positive-ion CI mode and an intense fragment of the sulphanil moiety in the negative-ion CI mode. In this way and with an accessory for pulsed positive-ion–negative-ion CI, it was possible to detect a few nanograms of SMZ. Satisfactory recoveries were obtained in cattle and swine tissues in the 100 μg/kg range, and even residues of less than 10 μg/kg were confirmed.

Matusik *et al.* [58] modified the method of Manuel and Steller [53] using GC–ECD in order to separate and determine SMZ and two of its metabolites, N^4-acetylsulphamethazine and desaminosulphamethazine, and applied it to incurred residues in cattle and swine tissues. This method, performed on a packed column, provided the contents of tissues of dosed animals after a 1-week withdrawal time. These derivatives were also separated on a short, non-polar capillary column and identified by positive-ion CI-MS, using methane as reagent gas. Full-scan spectra were provided and residues of fortified and incurred tissues were confirmed in the multiple-ion detection mode.

In a later approach, Matusik *et al.* [59] extended the method by ECD to SMZ, SDM, SCP and STA, all of which have the highest violation rate in the USA, and to a confirmation method using tandem MS. As in the procedure mentioned above, the extraction was accomplished by a modified Manuel–Steller or Tishler method and quantification was performed with ECD of the N^1-methylated substances. Recovery studies on fortified and incurred cattle and swine tissues were presented. The confirmation method by quadrupole tandem MS was performed by using a short capillary column for the separation and GC introduction of the N^1-methylated SA, ammonia as the reagent gas for positive-ion CI (this providing the most abundant protonated molecular ions) and argon as the collision gas for the daughter ion experiments. The daughter ion spectra derived from the protonated molecular ions contained the latter and ions corresponding to the sulphanil moiety, the methylated amino moiety and cleavage products of the sulphanil part. The procedure had sufficient sensitivity to provide full-scan daughter ions spectra in tissue residues at the 100 μg/kg level. This method provides better information than the multiple-ion detection mode.

Takatsuki and Kikuchi [60] described a method based on the N^1-methylated derivatives for SMZ, SDM, SME, SMX and SQX using capillary GC and MS with EI ionization in the multiple-ion detection mode. They focused on faster eluting by-products with similar fragmentation modes but mass spectra different from those of het isomers reported by Feil *et al.* [61]. The formation of these by-products seemed to be favoured during methylation by diazomethane by light or heating to evaporation.

Carignan and Carrier [62] desorbed a determination and confirmation procedure based on a clean-up by extraction and LC and subsequent N^1-methylation, followed by determination by GC and MS with EI ionization in the multiple-ion detection mode. SA in fortified swine tissues were measured in the range 1–100 μg/kg.

The formation of isomeric by-products in the N^1-methylation step of derivatizations was first studied by Gilbert *et al.* [63]. An isomer of N^1-methylsulphapyridine was isolated and characterized by ^{1}H NMR and MS. It was shown that to some extent the methylation took place at the pyridine-nitrogen. Similar effects could be observed with SMZ and SDA, the greatest amount of the by-product occurring with SPR at levels of a few percent. A similar by-product was obtained in a large-scale methylation of SMZ by Feil *et al.* [61] and was characterized by means of ^{1}H NMR spectroscopy and fast atom bombardment (FAB) MS as a tautomeric methylation product, the methylation taking place at a pyrimidine-nitrogen. Fortified tissues showed different yields of the by-product in the range 8–30%.

Although N^1-methyl derivatives of SA provided

good GC properties and the possibility of applying sensitive detection techniques such as ECD or MS in different ionization modes, several attempts were made to improve these properties by acylating the sulphanil amino group.

Gyllenhaal *et al.* [64] described the extractive alkylation with pentafluorobenzyl halides and a subsequent acylation with heptafluorobutyric or trifluoroacetic anhydride in order to detect the derivatized SA by ECD.

Garland and Miwa [65] proposed a method to determine SDM in cattle and swine. After clean-up, the extract was treated with diazomethane and pentafluoropropionic anhydride. The derivative was detected at the residue level by positive-ion CI using isobutane as the reagent gas by multiple ion detection. For quantification, deuterated SDM was used, the synthesis of which was described. The spectra showed a protonated molecular ion and a fragment corresponding to the amine part. Data for the spectra of derivatized SMZ, SCP, SDA, SDM, SDO, SMP, SPY, SQX and STA were listed. The method was tested on spiked tissue samples in the range 50–200 μg/kg. The identification of SDM or SDO was difficult because of the almost identical mass spectra of these isomeric substances and of the restricted resolution of packed-column chromatography.

Roach *et al.* [66] listed the EI and positive- and negative-ion CI mass spectra, using methane as reagent gas, of seventeen SA and of the N^1-methyl- and N^1-methyl-N^4-pentafluoropropionyl derivatives of SMZ. Extensive interpretations were given for these spectra. Positive-ion CI yields intense fragments such as the protonated molecular ion and the amine part of the substance or the derivatives, respectively. Negative-ion CI showed an intense ion corresponding to the sulphanil moiety. The authors suggested applying these fragmentation properties in a pulsed positive-ion–negative-ion CI procedure for detection at the residue level.

The good GC properties of the N^1-methyl-N^4-heptafluorobutyryl derivatives were described by Holtmannspötter and Thier [67]. Capillary GC was used in conjunction with flame ionization detection. The clean-up of the tissue extracts involved a gel chromatographic step and recovery data for SAA, SDA, SME, SMZ, STA and SQX were listed at concentrations of 100 μg/kg in tissue, eggs and milk, together with data for chloramphenicol and furazolidone. The detection limit was 10 μg/kg.

Simpson *et al.* [68] determined SMZ, SBM, SDM, SQX and STA after an extraction according to Tishler. The substances were methylated with diazomethane and acylated with pentafluoropropionic anhydride. The method was elaborated with fortified tissues using packed-column GC and MS with EI ionization in the single-ion monitoring mode. Quantification was performed by using the ^{13}C-labelled substances as internal standards. Recovery studies were presented in the range 50–200 μg/kg.

Mooser and Koch [69] proposed a confirmation method following quantification by LC. SMZ, SCP, SDA, SDM, SME, SMP, SMX, SPR, SQX and STA were determined as the N^1-methyl-N^4-trifluoroacetyl derivatives by capillary GC–MS. Extracts were methylated with diazomethane and trifluoroacetylated with N-methylbistrifluoroacetamide. The positive-ion CI mass spectra using methane as the reagent gas yielded intense ions corresponding to the protonated molecular ion and to the amine moiety of the derivatives, and in the negative-ion mode the sulphanil part yielded very intense ions. Prior to the derivatization, SGU was cyclizized with hexafluoroacetylacetone to give the fluorinated analogue of SMZ. SAC was methylated with iodomethane and showed similar fragmentations. Detection was performed at the residue level using pulsed positive-ion–negative-ion CI. SAA was detected as the methyl derivative by EI ionization in the multiple-ion detection mode. The method was routinely tested on violation samples of cattle, swine and rabbit.

Kmostak and Dvorak [70] described a capillary GC-ECD method in order to determine SDM with an external standard. The clean-up involved a sorption step with extractive alkylation using iodomethane and subsequent acylation with trifluoroacetic anhydride. The method was applied to incurred residues in swine tissue with a detection limit of 10 μg/kg.

5. COUPLED TECHNIQUES

5.1. Liquid chromatography–mass spectrometry

Henion *et al.* [71] applied atmospheric-pressure CI in conjunction with LC to the MS determination

of SMZ, SDA and SDM in racehorse urine. The mass spectra were very simple and the authors described a tandem MS system producing more characteristic daughter ion spectra.

Horie *et al.* [72] described the detection of SMZ, SCP, SDA, SDO, SDM, SME, SMX, SQX and STA in meat by means of LC and thermospray MS. The mass spectra yielded mainly the protonated molecular ion. These ions were used in the single-ion monitoring mode for determination by means of external standards. Chromatographic analyses of incurred swine tissue at the level of 1 mg/kg were shown.

Pleasance *et al.* [73] reported the separation and identification of 21 SA by RP-LC and ion-spray MS. In accordance with the results of Henion *et al.* [71], positive-ion mass spectra yielded only abundant protonated molecular ions. Further information was provided by tandem MS, giving more structural information by daughter ion spectra. Detection of SDM in incurred salmon tissue at the level of 25 μg/kg using a diode-array UV spectrometer as compared with LC–MS by single-ion monitoring using the protonated molecular ion for confirmation.

5.2. Supercritical fluid chromatography–mass spectrometry

Perkins *et al.* [74] reported a packed-column SFC separation on silica or amino-bonded silica, using carbon dioxide with methanol modifier as the mobile phase. The effects of column pressure and modifier concentration were studied in the separation of SMZ, SCP, SDA, SDO, SME, SMP, SPY, SQX and STA. Detection was accomplished by UV spectrophotometry or MS using moving-belt or thermospray interfaces. EI and ammonia CI mass spectra were presented and showed simple spectra, mainly with the protonated molecular ion as the base peak. The possibility of detecting residues in spiked tissue was tested on SMZ at the level of 3 mg/kg, comparing UV absorption with MS by single-ion monitoring and a moving-belt interface.

6. THIN-LAYER CHROMATOGRAPHY

LC and GC are capable of detecting SA at the low-μg/kg level. TLC was described by Horwitz [75] as lacking sensitivity and precision for quantification. Meanwhile, several approaches have been published (see Table 1) in order to detect or even determine SA in nanogram amounts in edible tissues by means of TLC. In fact, the technique has several distinct advantages over other chromatographic methods: the possibility of analysing many samples simultaneously rather than serially and, in conjunction with newer HPTLC materials giving shorter run times, results in significant time savings. The use of selective detection reagents such as the Bratton–Marshall reagent or fluoresamine can provide sensitive and, with a scanner, rapid quantitative assays. Bratton *et al.* [86] introduced N-(1-naphthyl)ethylenediamine as a diazotization reagent, whose application was extensively discussed by Horwitz [75]. Parks [80] proposed a modified version for the rapid development of thin-layer plates. Fluorescamine (Fluram) reacts very rapidly with primary amines, forming intensely fluorescent derivatives.

7. NON-CHROMATOGRAPHIC METHODS

Dixon-Holland and Katz [87] described a direct competitive ELISA for the detection of SMZ in swine urine and muscle tissue. Urine without any clean-up or an extract of muscle were analysed by absorbance measurement, using 2,2′-azino(3-ethylbenzothiazoline)sulphonic acid as a chromogen. SMZ was detected in concentrations as low as 20 μg/kg in muscle and 10 μg/kg in urine.

A similar competitive solid-phase EIA for the detection of SMZ in swine plasma was developed by Singh *et al.* [88] for the concentration range 10–1000 μg/kg. Validation values and comparison with TLC were reported for plasma, obtained without any clean-up step. Among the 36 SA studied, only SME showed a cross-reaction in the assay.

Ram *et al.* [89] presented an EIA in order to determine SMZ in swine plasma or serum with larger handling volumes of sample solutions and an automated technique. Comparison with TLC showed a good correlation in the range 1–5 μg/kg. The assay was tested on SMZ-fed pigs and their plasma and serum.

8. CONCLUSIONS

There has been an enormous increase in new analytical procedures on the one hand and consider-

TABLE 1

SUMMARY OF TLC METHODS

Ref.	Sulphonamides	Purpose	Type of plate	Type of extraction	Type of detection	Detection limits, remarks
76	SMZ, SAA, SCP, SDA, SDM, SDO, SGU, SME, SMP, SMX, SPR, SQX, STZ	TLC:screening HPLC:quantification, except for SGU, SAA	HPTLC, silica gel, 3 eluents HPLC:RP-18, acetate buffer (pH 4.6)–methanol	10 g tissue, solid–liquid extraction, CH_2Cl_2	HPTLC:fluorescamine HPLC:UV (266 nm)	HPTLC:20 µg/kg HPLC:5 µg/kg
77	SAA, SDA, SMZ, SQX, SDO	Screening	Silica gel, $CHCl_3$–C_4H_9OH (4:1)	10 g tissue, solid–liquid extraction, CH_2Cl_2	Fluorescamine	50 µg/kg
78	SAA, SDA, SMZ, SQX, SDO	Screening	Silica gel, $CHCl_3$–C_4H_9OH (4:1)	10 g tissue, solid–liquid extraction, CH_2Cl_2	Fluorescamine	50 µg/kg Collaborative study
79	20 SA	Screening	HPTLC, silica gel, 4 eluents	5 g tissue, solvent extraction, C_2H_5OAc	Fluorescamine	100 µg/kg
80	SMZ, STZ	Screening	Silica gel, EtOAc–CH_3OH (4:1)	2.5 g tissue, solvent extraction, $CHCl_3$–C_2H_5OAc	Bratton–Marshall	20 µg/kg
81	SDM, SQX	Screening	Silica gel, $CHCl_3$–EtOAc–CH_3OH (5:5:1)	5 g tissue, solvent extraction, $CHCl_3$–C_2H_5OAc (1:1)	Bratton–Marshall	<100 µg/kg Tested: 100–400 µg/kg Also coccidiostaticss and their metabolites
82	23 SA	Screening, quantification	HPTLC, silica gel, 6 eluents, two-dimensional	2 g tissue, solvent extraction, (1) buffer, acetone–CH_3OH or (2) C_4H_9OAc	Fluorescamine	50 µg/kg (serum: 5 µg/kg)
83 84	SMZ, SDM, SQX	Screening, quantification	Silica gel, CH_3OH, $CHCl_3$–C_4H_9OH (4:1)	2.5 g tissue, solvent extraction, C_2H_5OAc	Fluorescamine, densitometry	Tested: 50–200 µg/kg Collaborative study (comparison with Tishler and Manuel–Steller methods)
85	16 SA	Screening	HPTLC, silica gel, 3 eluents, two-dimensional	10 g tissue, solid–liquid extraction, $CHCl_3$–acetone (1:1)	Fluorescamine	10 µg/kg (10 ng)

able progress in lowering detection limits of SA on the other in the past decade. There is no doubt that reducing the level of detectability is still continuing despite the fact that many methods reach the sub-ng/g range. In this context the question might arise of whether this trend is reasonable from a practical point of view. It can be stated that a great many methods for SA in meat and meat products exist and that there is, although perhaps sounding rather presumptuous, no or little actual need for additional ones.

Various workers are paying great attention to problems arising from clean-up and possible metabolites of originally administered SA. A new and promising approach seems to be the matrix solid-phase dispersion isolation of substances, although the limits of this procedure are not yet clear.

9. ABBREVIATIONS

The pK_a values (SO_2-NH) are taken from refs. 1 and 2.

DMSO	Dimethyl sulphoxide
ECD	Electron-capture detection
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
GC	Gas chromatography
HPLC	High-performance liquid chromatography
HPTLC	High-performance thin-layer chromatography
LC	Liquid chromatography
MS	Mass spectrometry
MSPD	Matrix solid-phase dispersion
RP	Reversed-phase
SFC	Supercritical fluid chromatography
TLC	Thin-layer chromatography
DMBA	*p*-Dimethylaminobenzaldehyde
SA	Sulphonamide(s)
OMP	Ormetoprim
SAA	Sulphanilamide (Sigma S-9251; pK_a 10.43)
SAC	Sulphacetamide (Serva 35630; pK_a 5.38)
SBM	Sulphabrommethazine
SCL	Sulphaclozine
SCP	Sulphachlorpyridazine (Sigma S-9892; pK_a 5.1)
SD	Sulphamoyldapsone
SDA	Sulphadiazine = sulphapyrimidine (Sigma S-8626; pK_a 6.4)
SDM	Sulphadimethoxine (Sigma S-7007; pK_a 6.2)
SDO	Sulphadoxine
SFU	Sulphafurazole
SGU	Sulphaguanidine (Sigma S-8751; pK_a 11.25)
SIA	Sulphisoxazole
SIM	Sulphisomidine
SIZ	Sulphisozole
SME	Sulphamerazine (Sigma S-8876; pK_a 7.0)
SMM	Sulphamonomethoxine
SMP	Sulphamethoxypyridazine (Sigma S-7257; pK_a 6.7)
SMT	Sulphamethizole
SMX	Sulphamethoxazole (Sigma S-7507; pK_a 5.6)
SMZ	Sulphamethazine = sulphadimidine (Serva 35635; pK_a 7.4)
SN	Sulphanitran
SPE	Sulphaperine
SPH	Sulphaphenazole
SPR	Sulphapyridine (Serva 35860)
SPY	Sulphapyrazole
SQX	Sulphaquinoxaline (Sigma S-7382; pK_a 5.5)
STA	Sulphathiazole (Serva 35690; pK_a 7.2)
STO	Sulphatolumide
STR	Sulphatroxazole (pK_a 5.8)

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