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Review

High-performance liquid chromatographic methods for the determination of sulfonamides in tissue, milk and eggs

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ABSTRACT

In the last decade, significant research has been done to improve the existing high-performance liquid chromatographic (HPLC) methods and also towards developing simple, reliable and sensitive HPLC methods for sulfonamides in meat, milk and eggs. The replacement of solvent extraction with solid-phase extraction or matrix solid-phase dispersion techniques is a step forward. Significant improvements in sensitivity have been achieved. This review concentrates on HPLC methods for the determination of sulfonamides in foods of animal origin published after 1980. The existing methods are critically evaluated and suggestions for future research are made.

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1. INTRODUCTION

Within the last three decades, the use of veterinary drugs in animal husbandry programs has increased substantially. Although the use of veterinary drugs has helped to increase the food supply, negative consequences, such as the presence of drug residues in foods, cannot be ignored. As the use of veterinary drugs has increased, the possibility of consumers being exposed to these drugs has also increased. The United States Food and Drug Administration (FDA) sets the tolerance levels of approved drugs in foods. As long as the residues are below the allowed tolerance limits, the food is considered safe for human consumption. Some of the food supply which reaches the consumer, however, does contain drug residues over tolerance limits.

There are two main reasons for the presence of

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TABLE 1
STRUCTURES OF SULFONAMIDES

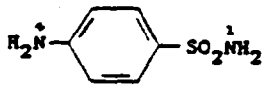
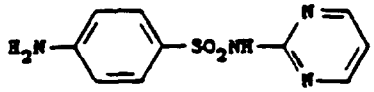
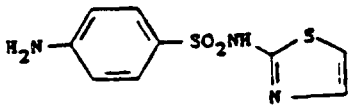
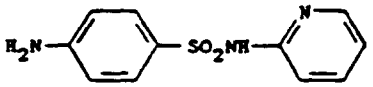
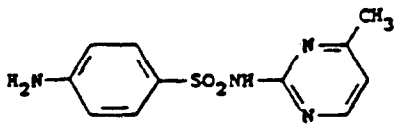
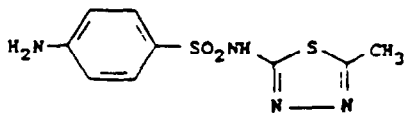
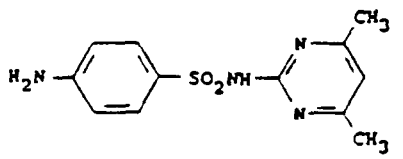
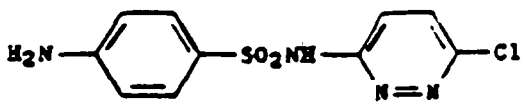
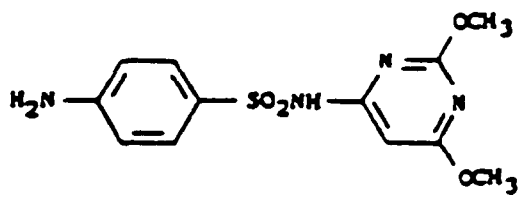
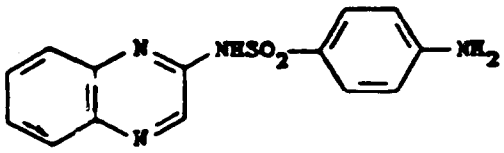
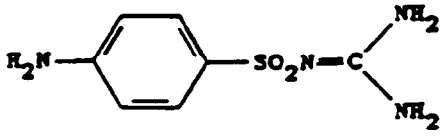
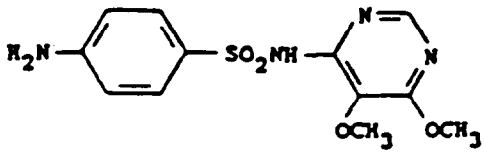
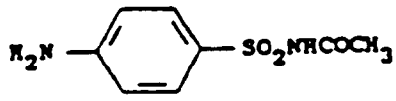
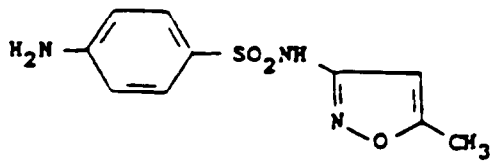
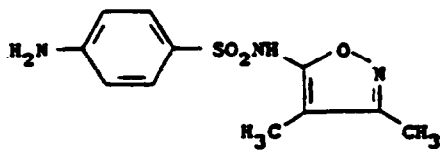
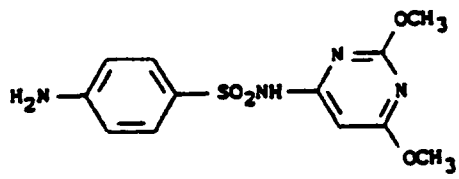
Compound	Structure	pK_a
Sulfanilamide		10.4
Sulfadiazine		6.4
Sulfathiazole		7.2
Sulfapyridine		8.56
Sulfamerazine		7.0
Sulfamethiazole		5.45
Sulfamethazine		7.5
Sulfachloropyridazine		5.1
Sulfadimethoxine		6.2

TABLE 1 (*continued*)

Compound	Structure	pK_a
Sulfaquinoxaline		5.5
Sulfaguanidine		11.3
Sulfadoxine		5.9
Sulfaacetamide		6.1
Sulfamethoxazole		5.4
Sulfisoxazole		4.79
Sulfamonomethoxine		6.5

violative residues in foods. First, improper, illegal or extra-label use of drugs can result in violative residues. Second, an insufficient withdrawal period can also cause violative residues. Every drug has a set withdrawal period before the residue levels in the animal body drops below the tolerance level. If this withdrawal period is not maintained before slaughter, higher residue level may be present in food.

The presence of drug residues in foods can be a health hazard to consumers. First, carcinogenicity of some drugs may be a serious concern. Second, continuous exposure of certain microorganisms to these drugs may result in the development of drug-resistant strains.

Sulfonamides are a class of antibacterial drugs which are used in farm animals for the treatment of a variety of bacterial infections. In food-producing animals, sulfonamides are used not only for therapeutic but also for prophylactic purposes. Chemically, sulfonamides are substituted aromatic amines substituted at the N-1 position. Table 1 shows the structures of important sulfonamides which are discussed here.

In the past, residues of sulfa drugs have been found in milk offered for sale. A nationwide survey by the FDA in 1988 reported that 45% of milk samples contained detectable amounts of sulfamethazine [1]. Another survey of 30 samples, done in ten cities across Canada, found sulfamethazine residues in two samples at levels of 11.40 and 5.24 ppb [2].

After a report from the National Center for Toxicological Research (NCTR) in 1988 indicating that sulfamethazine may be a carcinogen, concern over the presence of residue of sulfamethazine and other sulfonamides in milk and other foods has grown [3]. The FDA has, therefore, set tolerance limits of sulfonamides in meat, milk and poultry (Table 2). Currently, sulfadimethoxine is the only sulfa drug allowed for use in lactating animals, and the residue may not exceed 10 ppb in milk [4]. In meat and poultry, residues of sulfonamides may not exceed 100 ppb [4].

This review concentrates on high-performance liquid chromatographic (HPLC) methods for the determination of sulfonamides in foods of animal origin. The existing methods are critically evaluated and suggestions for future research are made.

TABLE 2

FDA TOLERANCES FOR SULFONAMIDES IN FOODS OF ANIMAL ORIGIN

Other sulfonamides not included in the table have a zero tolerance.

Drug	Tolerance (ppb)			
	Cattle	Swine	Poultry	Milk
Sulfamethazine	100	100	100	–
Sulfathiazole	–	100	–	–
Sulfadimethoxine	100	–	100	10
Sulfachloropyridazine	100	100	–	–

2. METHODS OF ANALYSIS

Owing to the concern over residues of sulfonamides in food products of animal origin, a number of techniques have been proposed for their detection, including, microbiological [5,6], immunoassay [7], thin-layer chromatography (TLC) [8–14], gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS) [15–24] and HPLC [25–38].

2.1. Microbiological and immunoassay

Microbiological and immunoassay methods [5–7] are not considered very specific and may give false-positive results. These methods can be applied to liquid samples directly, but for tissue samples the tissue must be extracted to isolate drug residues before applying these tests.

2.2. Thin-layer chromatography

TLC [8–14] has been used for the detection of sulfa drug residues in foods, but these methods have limited application and are generally used only for screening or qualitative analysis. The method of Clark *et al.* [14] can screen for eight sulfonamides in milk at a low level of 10 ppb. This method is being used by the FDA as a rapid screening method, which must be followed by an HPLC method for quantitative analysis.

2.3. Gas chromatography

GC methods [15–24] have not gained wide acceptance in spite of being very sensitive and specific. In general, GC methods require solvent extraction

followed by a sample clean-up step. It is essential to derivatize sulfonamides to prepare a volatile derivative before GC analysis. The derivatization generally involves either N-methylation or N-methylation followed by acylation of the N^4-primary amino function with pentafluoroalkane carboxylic anhydride [23].

2.4. High-performance liquid chromatography

HPLC has become the most widely used technique for the determination of sulfa drug residues in meat, milk and poultry, and a number of methods have been published. Table 3 summarizes the HPLC methods which are reviewed here.

3. HPLC METHODOLOGY

A major problem in developing methodology for drug residue analysis is the detection of these drugs in biological matrices such as meat, milk and eggs at low ppb levels in the presence of potentially interfering compounds. A general approach for the determination of sulfonamide residues in foods of animal origin involves extraction, sample clean-up and HPLC analysis steps. A critical and extensive review of the methodologies developed up to 1980 was published by Horowitz [39,40]. Therefore, only methods reported after 1980 are reviewed here.

3.1. Extraction

Traditionally, the extraction of sulfonamide from meat, milk and eggs has been done with organic solvents. The use of large amounts of organic solvents makes these methods very laborious and time consuming. Sulfonamides are not very soluble in non-polar solvents, but have good solubility in polar solvents. Therefore, the extraction is generally done with chloroform, methylene chloride, acetone, acetonitrile or ethyl acetate. Some organic solvents also denature the sample protein, which results in a cleaner extract and also helps in extracting the drug residues bound to proteins. Some of the newer methods have replaced the traditional liquid extraction step with solid-phase extraction (SPE), which eliminate the use of large amounts of solvent for extraction.

3.2. Sample clean-up

Sample clean-up step is performed by back-extraction of sulfonamides into an aqueous medium. When extracting from an organic into an aqueous phase, the adjustment of the pH of the aqueous phase is critical to obtain complete recoveries. SPE has also been used for sample clean-up in newer methods. Depending on the sample matrix, sometimes a few more clean-up steps are needed before HPLC analysis. The purpose of these additional clean-up steps is to extract sulfonamides selectively while leaving other interfering compounds behind.

3.3. HPLC analysis

After the sample has been cleaned up, the HPLC analysis is performed to identify and determine sulfonamides. In general, a reversed-phase HPLC column is used with a mobile phase consisting of buffer and organic solvent (methanol or acetonitrile). For detection, either variable-wavelength UV or UV–VIS photodiode-array detectors are used. In some instances sulfonamides are derivatized and a fluorescence detector is used.

4. METHOD DEVELOPMENT AND EVALUATION CRITERIA

An analytical method must meet the criteria of evaluation, which is primarily based on its precision, specificity, accuracy and practicability. Precision is a measure of repeatability and reproducibility of the method. Repeatability indicates the variability within the laboratory or the variability of the analyst. Reproducibility indicates the variability between laboratories. The specificity of the method means that under the conditions used, no other compound (from matrix of potential external source) should show a response that may interfere with the analysis. The specificity of the method may be proven by spiking the sample with potential interferents and analyzing by the proposed method. Accuracy of the method is determined by spiking the blank sample with known amounts of standards at various concentrations and recovering the parent compound and their metabolites, if any. It is emphasized that the method development should also include incurred samples as the method may perform differently on an incurred sample than in a spiked sample. Finally, the method should be practical, which is judged on the basis of total operational requirements of the method such as chem-

TABLE 3

HPLC METHODS FOR THE DETERMINATION OF SULFONAMIDES IN MEAT, MILK AND EGGS

Abbreviations: SNL = sulfanilamide, SDZ = sulfadizine, STZ = sulfathiazole, SPD = sulfapyridine, SMR = sulfamerazine, SMTZ = sulfamethiazole, SMZ = sulfamethazine, SCP = sulfachloropyridazine, SDM = sulfadimethoxine, SQX = sulfaquinoxaline, SG = sulfaguanidine, SMX = sulfamethoxazole, SDX = sulfadoxine, SAA = sulfaacetamide, STX = sulfatroxazole, SFZ sulfisoxazole, SMM = sulfamonomethoxine.

Sulfonamide	Sample matrix	Sample preparation	Mobile phase	Column	Detection	Detection limit (ppb)	Ref.
SDZ, SMZ	Milk	Extraction with chloroform	Water–methanol (75:25)	RP-C_2, 10 μm, 250 × 4.6 mm I.D.	UV, 263 nm, electrochemical	10	Alawi and Rüssel [25]
27 Sulfonamides	Milk, meats eggs	Deproteination with acetonitrile. Extraction with hexane. Extraction with methylene chloride. Partition between hexane and aq. methanol. Extraction with ethyl acetate	Sodium acetate–CH_3CN	Spherisorb ODS, 5 μm, 250 × 4.6 mm I.D.	UV, 290, 310 nm	20–50	Malisch [26]
SDZ, SMR, SDM, SQX, SMX, SDX, SMZ, SCP, SAA, STX, STZ, SNL, SG	Milk, meat eggs	Dialysis with cellulose membrane. Enrichment on Bondapak C_{18}–Corasil, XAD-2 or XAD-4 column	Sodium acetate–CH_3CN	LiChrosorb RP-8, 10 μm' 250 × 4.6 mm I.D. CpTM-Sphere C_{18}, 7 μm, 250 × 4.6 mm I.D. μBondapak C_{18}, 10 μm, 300 × 3.9 mm I.D.	UV, 280 nm or fluorescence, 450 nm, after postcolumn deriv. with DMAB	10	Aerts *et al.* [27]
SDZ, SMR, SMZ, SMX, SQX	Milk, meats eggs	Extraction with acetonitrile. Water removal with methylene chloride. Dissolution in methanol–buffer. Extraction with hexane	Sodium acetate–CH_3CN	MOS Hypersil, 3 μm, 120 × 4.6 mm I.D.	UV, 275 nm	100	Petz [28]
SMZ	Milk	SPE extraction with C_{18}, acidic alumina and ion exchange	Potassium phosphate–methanol	LC-18, 5 μm, 250 × 4.6 mm I.D.	UV, 270 nm	0.5	Unruh *et al.* [29]

STZ, SDZ, SMR, SMZ, SMX, SDM, SFZ	Infant formula	MSPD extraction with C_{18} material. Elution with methylene chloride	Phosphoric acid–CH_3CN	MCH-10, 300 × 4 mm I.D.	Photodiode, 270 nm	62.5	Long *et al.* [30]
SNL, STZ, SDZ, SMR, SMZ, SMX, SFZ, SDM	Milk	MSPD extraction with C_{18} material. Elution with methylene chloride	Phosporic acid–CH_3CN	Supelcosil LC-18, 3 μm, 75 × 4 mm I.D.	Photodiode, 270 nm	31.2	Long *et al.* [31]
SNL, STZ, SDZ, SMR, SMZ, SMX, SFZ, SDM	Pork tissue	MSPD extraction with C_{18} material. Elution with methylene chloride	Phosporic acid–CH_3CN	MCH-10, 300 × 4 mm I.D.	Photodiode, 270 nm	31.2	Long *et al.* [32]
SNL, SMZ, SD, SQX, SDX	Swine tissue	Extraction with chloroform–acetone. Cation exchange with aromatic sulfonic acid column. Treatment with ammonia. Elution with methanol	Ammonium acetate–CH_3CN	Spherisorb C_8, 250 × 4.6 mm I.D.	UV, 254 nm	50	Haagsma and De Water [33]
SMZ, SDM, SMM	Tissue, eggs	Extraction with acetonitrile. Extraction with hexane to remove lipids. SPE with Bond-Elut C_{18}. Elution with 0.1% TEA–CH_3CN	Potassium phosphate–CH_3CN	Nucleosil 100 C_{18}, 5 μm, 250 × 4.6 mm I.D.	UV, 268 nm	20–40	Horie *et al.* [34]
SMZ	Milk	Extraction with chloroform. Partition between potassium phosphate buffer and hexane	Potassium phosphate–methanol	LC-18-DB, 5 μm, 250 × 4.6 mm I.D.	UV, 265 nm	5	Weber and Smedley [35]
SNL, SDZ, STZ, SPD, SMR, SMZ, SMTZ, SCP, SDM, SQX	Milk	Extraction with chloroform–acetone. Partition between potassium phosphate buffer and hexane	Potassium phosphate–methanol	LC-18-DB, 5 μm, 250 × 4.6 mm I.D.	UV, 265 nm	5	Smedley and Weber [36]

(Continued on p. 418)

TABLE 3 (*continued*)

Sulfonamide	Sample matrix	Sample preparation	Mobile phase	Column	Detection	Detection limit (ppb)	Ref.
SMZ		Extraction with chloroform. Partition between potassium phosphate buffer and hexane. SPE extraction with Cyclobond-I column	Ammonium acetate–methanol	LC-18-DB, 5 μm, 250 × 4.6 mm I.D.	UV, 265 nm	5	Agarwal [37]
SDZ, STZ, SPD, SMR, SMZ, SCP, SMTZ, SDM, SQX		Extraction with chloroform. Partition between potassium phosphate buffer and hexane. SPE extraction with Cyclobond-I column	Ammonium acetate–methanol	LC-18-DB, 5 μm, 250 × 2 mm I.D.	UV, 265 nm	10	Agarwal [38]

icals, instrumentation and time required for completion of the analysis. A good method may not meet al the criteria, however, as discussed above.

As the number of drugs being used on animals is increasing, the methods developed for individual drugs are becoming unattractive, and efforts are being concentrated towards developing multi-residue methods. In the case of sulfonamides, several multi-residue methods have been developed which can determine a whole range of sulfonamides simultaneously.

The detection limit of the method is another important aspect. The method should be able to determine the residue below, or at, the allowed tolerance levels. For sulfonamides, as indicated earlier, the tolerance limit is 10 ppb in milk and 100 ppb in meat and poultry. Unless a method can achieve such detection limits, it is not considered useful for regulatory purposes for a given sample type.

5. HPLC METHODS FOR THE DETERMINATION OF SULFONAMIDES

The method reported by Alawi and Rüssel [25] is applicable to milk. This method requires a simple chloroform extraction followed by HPLC analysis. Electrochemical detection was found to be more selective and sensitive than UV detection. When a UV detector was used at 263 nm the peaks co-eluting with sulfonamides interfered with the analysis. Electrochemical detection was more specific for sulfonamides and the co-eluting peak did not show any response to the electrochemical detector. An RP-C2 column was used for HPLC analysis. The mobile phase was methanol–water (25:75) containing 0.01 *M* $LiClO_4$ as an electrolyte. This method, however, is applicable only to sulfadiazine and sulfamethazine down to a low level of 10 ppb.

A method reported by Malisch [26], which requires very extensive clean-up steps, was applied to 27 sulfonamides in meat, milk and eggs. Milk was deproteinated with acetonitrile and the aqueous acetonitrile phase was extracted with hexane to remove lipid material. The aqueous acetonitrile phase was saturated with sodium chloride and extracted with methylene chloride. After evaporating the methylene chloride extract to dryness on a rotary evaporator, the residue was further purified by partitioning between hexane and aqueous methanol. The hexane phase was discarded and the aqueous methanol extract was evaporated on a rotary evaporator to remove methanol. The concentrated aqueous extract was diluted with water and extracted twice with ethyl acetate. The combined ethyl acetate extract was evaporated to dryness and the residue dissolved in an aqueous acetonitrile–methanol for HPLC analysis. HPLC analysis was done using a reversed-phase Spherisorb ODS column with sodium acetate–acetonitrile as the mobile phase and UV detection at 290 and 310 nm. The detection limits of sulfonamides varied from 20 to 50 ppb. This method is certainly advantageous as it can determine several sulfonamides simultaneously. The lengthy extraction procedure, however, makes it difficult to use this method as a routine method and to obtain consistent recoveries.

Aerts *et al.* [27] developed an HPLC method for the detection of thirteen sulfonamides in meat, milk and eggs at a low level of 10 ppb. They explored a number of options for the clean-up and HPLC analysis and a detailed comparison was made. The fat was removed from the milk and then diluted with saline solution. Eggs were directly diluted with saline solution and meats were homogenized with saline solution. Various concentrations of sodium azide solution were added to these saline solutions as an antioxidant.

Samples were dialyzed using a flat cellulose membrane to separate drugs from larger proteins or lipid molecules. The aqueous dialysate containing sulfa drugs was loaded on a short stainless-steel column which served as a concentrator. A variety of packing materials were tested, including Bondapak C_{18}-Corasil, Perisorb KAT, Perisorb RP-2, XAD-2, XAD-4 and Baker-C_8. With respect to retention and elution of sulfonamides, XAD-2 and XAD-4 were excellent but the retention was not very specific to sulfonamides as numerous UV-absorbing compounds present in the sample matrix were also retained on these phases and were eluted with sulfonamides. Bondapak C_{18}-Corasil gave very good results with milk samples but meat and egg samples were not cleaned up enough on this packing material. After concentration, the sample was backflushed to an analytical reversed-phase HPLC column and chromatographed with an appropriate mobile phase. Three different HPLC columns were examined; LiChrosorb RP-8, CpTM-Sphere C_{18} and

μBondapak C_{18}. The capacity factors (k') on CpTM-Sphere were highest, LiChrosorb RP-8 showed intermediate and μBondapak C_{18} the lowest k' values. The mobile phases, which contained sodium acetate–acetonitrile, were able to separate all sulfonamides on LiChrospher and CP TM-Sphere columns. The retention behaviour of sulfonamides was dependent not only on the polarity, but also on the ionization of sulfonamides. Therefore, the pH of the mobile phase also played an important role in the chromatographic separation. Direct UV detection at 280 nm could detect sulfonamide in milk but the chromatograms of meat and egg samples were not clean. Further clean-up of these samples was necessary before HPLC analysis. Aerts *et al.* [27] used a more specific detection approach to eliminate interferences instead of further clean-up. Postcolumn derivatization with *p*-dimethylaminobenzaldehyde (DMAB) and fluorescence detection at 450 nm were specific for sulfonamides and eliminated the interferences. Postcolumn derivatization not only improved the specificity but also the overall response by approximately 1.5 times. Based on the sample matrix and the level of sulfonamide residues present, the specific enrichment column, the HPLC column and the detection system can all be selected to determine all the sulfonamides in a single chromatographic run.

The method developed by Petz [28] for the detection of five sulfonamides in meat, milk and eggs has a detection limit of only 100 ppb. Milk was extracted with acetonitrile and co-extracted water was removed by the addition of sodium chloride and dichloromethane. The organic phase, which contained sulfonamides, was evaporated to dryness and the residue dissolved in a mixture of methanol and mobile phase. The extract in the methanol–mobile phase was washed with hexane to remove lipids, and then analyzed by reversed-phase HPLC with UV detection at 275 nm. A reversed-phase MOS-Hypersil column and with sodium acetate buffer (0.01 *M*, pH 4.6)–acetonitrile (75:25) as the mobile phase were used. This method is a simple approach for the determination of five sulfonamides without extensive clean-up. This method, however, is not suitable for milk samples owing to the detection limit of only 100 ppb.

A method developed by Unruh *et al.* [29] for the determination of sulfamethazine in milk at a low level of 0.5 ppb uses an SPE technique followed by either TLC or HPLC. Milk was diluted with phosphate buffer (pH 5.7) and passed through a C_{18} SPE column. Sulfamethazine, which was retained on the SPE column, was eluted with methanol. The eluent was passed through a small acidic alumina column and the eluent from this column was loaded directly on an ion-exchange column (AGMP-1). Sulfamethazine, retained on the AGMP-1 column, was then eluted with methanol–acetic acid–acetone (1:5:94) and analyzed by TLC with fluorescamine derivatization. For confirmation, HPLC analysis was done using a Supelco LC-18 column with a mobile phase consisting of 0.05 *M* K_2HPO_4 (pH 6.0)–methanol (65:35) and UV detection at 270 nm. Before loading the milk on the C_{18} SPE column, it was necessary to adjust the pH to 5.9. This serves two purposes: first, better retention of sulfamethazine was achieved at pH 5.9 than at pH 7, and second, milk was diluted and a better flow through the C_{18} SPE column was achieved. The eluate obtained from the C_{18} SPE column contained not only sulfamethazine but also other components, *e.g.*, lipids and riboflavin from the sample matrix which interfered with chromatographic analysis. These interferences were removed by using alumina and AGMP-1 resin columns. This method can determine sulfamethazine at a low level of 0.5 ppb. The use of HPLC was optional if further confirmation was required. This method is the first method that has such a low detection limit of 0.5 ppb. Introduction of SPE replaced the traditional solvent extraction step and thus made this method very convenient and attractive. This method has great potential for application to multi-residue determination of sulfonamides in milk and other foods of animal origin.

Long and co-workers [30–32] developed three methods which are applicable to the determination of eight sulfonamides in milk, infant formula and pork tissue, using a new technique, known as matrix solid-phase dispersion (MSPD), which was developed by Barker *et al.* [41]. This technique is based on the principle of surfactant or detergent use for disrupting the cell membrane of biological matrices by solubilizing the membrane components.

In the MSPD technique, milk, tissue or infant formula was blended with C_{18} packing material using gentle mechanical force. The C_{18} packing mate-

rial, which contains a C_{18} polymer phase bound to a silica support, works as a lipid-solubilizing material. This results in the disruption of the cell membrane of the sample, making the cell contents accessible for extraction. The polymer–sample matrix prepared in this manner was loaded on a column and washed with hexane to remove lipid materials. Sulfonamides which were more polar remained on the column and were eluted with methylene chloride. The eluate was analyzed by HPLC using a photodiode-array detector at 270 nm. The extracts from pork tissue and infant formula were analyzed by HPLC using an MCH-10 ODS HPLC column and phosphoric acid–acetonitrile (70:30) as the mobile phase. For milk, a Supelcosil LC-18 HPLC column with phosphoric acid–acetonitrile as mobile phase was used. The detection limits of these methods were 31.2 ppb for tissue and milk samples and 62.5 ppb for infant formula. Eight sulfomamides were determined in tissue and milk. Only seven sulfonamides were determined in infant formula. The mobile phase for infant formula contained a higher concentration of acetonitrile and sulfanilamide therefore could not be determined, as it eluted very early and was not separated from earlier eluting sample matrix peaks.

The MSPD technique simplifies the overall methodology and removes most of the interfering components from milk, tissue and infant formula without using solvent extraction [30–32]. The problem of the detection limits in milk and infant formula remains. A large sample volume with the same amount of C_{18} material could not be used as the polymer–sample matrix obtained was not dry enough for further analysis. Therefore, larger amounts of C_{18} material were required when larger volumes of milk were used. Further work is needed in this area to improve the detection limits. The MSPD technique, however, is a significant improvement over other existing methods and opens up a new direction in drug residue analysis. The only drawback of this method is the sensitivity for liquid samples, which needs to be improved.

Haagsma and De Water [33] developed a multiresidue method for five sulfonamides in tissue. The tissue sample was extracted with chloroform–acetone. It was necessary to adjust the pH of the sample to 5.5 before extraction, otherwise the recoveries were low. The recoveries from kidney tissue were still not very reproducible by this method. Clean-up was done by using an aromatic sulfonic acid cation-exchange column. All the sulfonamides were retained on the ion-exchange column when the acidified extract of tissue was passed through the column. The column was then treated with ammonia vapor and sulfonamides were eluted with methanol. Sulfonamides could also be eluted from the column with an alkaline buffer, but the eluate was not suitable for HPLC. HPLC analysis was then carried out on a Chrompack Spherisorb C_8 HPLC column with ammonium acetate (pH 4.6)–acetonitrile as mobile phase and UV detection at 254 nm. This method has a detection limit of 50 ppb and is applicable only to swine tissue.

Horie *et al.* [34] developed a method for determination of three sulfonamides in animal tissue and eggs. Extraction was done with acetonitrile, and the acetonitrile extract was further extracted with hexane to remove lipid materials. Further clean-up was done using a Bond-Elut C_{18} SPE column. It was necessary to adjust the pH of the extract to a low pH of 1–2 before loading on to a C_{18} SPE column. Excellent retention of all three sulfonamides was achieved. Elution of sulfonamides from C_{18} SPE column could be done with acetonitrile, but a larger volume was required. Use of 0.1% TEA in acetonitrile eluted sulfonamides very efficiently because the amine modifier competes with unbound silanols on the silica substrate. The eluate was evaporated to dryness and the residue dissolved in in 10 m*M* potassium dihydrogenphosphate solution. HPLC analysis was done on a Nucleosil 100 C_{18} column with potassium phosphate–acetonitrile as the mobile phase and UV detection at 268 nm. A column temperature of 40°C rather than ambient temperature resulted in better peak shapes. The detection limit of this method was 20 ppb for sulfamethazine and sulfamonomethoxine and 40 ppb for sulfadimethoxine.

A method developed by Weber and Smedley [35] for the detection of sulfamethazine in milk at a low level of 5 ppb, involving a simple chloroform extraction followed by partitioning between potassium phosphate buffer and hexane to remove lipids. The aqueous buffer phase containing sulfonamides was then analyzed by HPLC with UV detection at 265 nm. A Supelco LC-18-DB HPLC column with potassium phosphate–methanol was used as the

mobile phase. An extension of this work by Smedley and Weber [36] has been applied to the detection of ten sulfonamides in milk at a low level of 10 ppb and is currently being used by the FDA for testing milk. This method involves a chloroform–acetone extraction followed by partitioning between hexane and potassium phosphate buffer. Sulfonamides, dissolved in buffer, were then determined by HPLC. A Supelco LC-18-DB HPLC column with potassium phosphate–methanol as mobile phase was used. This method, although a simple approach for the determination of ten sulfonamides in milk, does have certain drawbacks. The method requires two different sets of HPLC conditions to determine ten sulfonamides. For this, either two HPLC systems are required, or all the samples are first analyzed for seven sulfonamides, and then the HPLC conditions are changed to analyze for the remaining three sulfonamides. It was also necessary to clean up the column after a few runs in order to avoid interference with any later eluting peaks. Additionally, a number of extraneous peaks were present in the LC trace which could make quantification difficult for earlier eluting sulfonamides.

A method developed by Agarwal [37], for the detection of sulfamethazine in milk involves SPE. Milk was extracted with chloroform, the extract evaporated to dryness and the residue dissolved in potassium phosphate buffer (pH 5.0). The extract in buffer was then passed through a Cyclobond I SPE column, which was washed with potassium phosphate buffer and then sulfamethazine was eluted with aqueous methanol and determined by HPLC with UV detection at 265 nm. A Supelco LC-18-DB HPLC column with ammonium acetate–methanol as mobile phase was used. This method has a detection limit of 5 ppb for sulfamethazine in milk.

The same technique was also used for the determination of nine sulfonamides in milk [38]. Milk was extracted with chloroform–acetone and the extract evaporated to dryness. The concentrated extract was then partitioned between hexane and 1 *M* potassium phosphate buffer (pH 4.4). The aqueous buffer, containing sulfonamides, was passed through a Cyclobond-I SPE cartridge. The sulfonamides, retained on the cartridge, were eluted with aqueous acetonitrile. Acetonitrile was removed from the eluate and the latter diluted with ammonium acetate buffer. HPLC analysis was done using a Supelco LC-18-DB microbore column and a gradient mobile phase. Use of a microbore HPLC column enhanced the sensitivity for the detection of sulfonamides. The mobile phase consisted of (A) 25 m*M* ammonium acetate buffer (pH 4.6)–methanol (850:150) and (B) 25 m*M* ammonium acetate buffer (pH 8.0)–methanol (700:300), with a gradient from 0 to 100%B in 30 minutes. All nine sulfonamides were eluted in 30 min and the column could be equilibrated to the initial conditions within the next 20 min. Use of a gradient also increased the peak resolution.

In this method a Cyclobond-I column was selected for SPE owing to the selective retention of sulfonamides on these columns. Cyclobond-I SPE columns contain β-cyclodextrin bonded to silica. Cyclodextrins are D-(+)-glucopyranose units connected by α-(1,4)-bonds to form cyclic oligosaccharides [42]. The glucose units are arranged in a fashion to form a truncated cone shape. The orientation of glucose units is such that there are no hydroxyl groups on the interior of the cavity, making it hydrophobic [43]. Formation of an inclusion complex between sulfonamides and β-cyclodextrin has been reported [44]. A detailed study to optimize the conditions for the retention of sulfonamides on the Cyclobond-I SPE column was done [45]. As sulfonamides are ionic in nature, their retention on Cyclobond-I SPE columns is pH dependent. The first ten sulfonamides listed in Table 1, which were used in this study, have a pK_a range from 5.5 to 10.4. With the exception of sulfanilamide, all nine sulfonamides were retained within the pH range 4.0–5.5. The effect of pH was most pronounced with sulfamethazine and sulfamethiazole. Sulfamethazine gave maximum retention towards higher pH (5.5) whereas sulfamethiazole gave the maximum retention at lower pH (4.0). The optimum pH of the buffer was chosen was 4.4, at which all sulfonamides except sulfanilamide gave excellent retention. This method is a significant improvement over the method of Smedley and Weber [36] and has overcome the shortcomings of that method. The drawback of this method is that it requires solvent extraction. Research is in progress in our laboratory to eliminate the solvent extraction step.

6. CONCLUSION

In the last decade, research has been carried out to improve the existing HPLC methods and also towards developing simple, more reproducible, reliable and sensitive HPLC methods for the determination of sulfonamides in meat, milk and eggs. The replacement of solvent extraction with SPE or MSPD techniques is a step forward. A significant improvement in the sensitivity of the methods has been achieved. None of these methods, which appear to be simplified and precise, have been subjected to collaborative studies. Therefore, the ruggedness and practicability of these methods have not yet been proven.

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