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# Multiresidue Method for the Determination of Sulfonamides in Pork Tissue

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A multiresidue technique for the isolation and liquid chromatographic determination of eight sulfonamides from pork muscle tissue is described. Sulfanilamide, sulfathiazole, sulfadiazine, sulfamerazine, sulfamethazine, sulfamethoxazole, sulfisoxazole, and sulfadimethoxine standards were fortified into pork tissue (0.5 g) and blended with $C_{18}$ (octadecylsilyl-derivatized silica) packing material (2 g). The blended $C_{18}$/muscle tissue matrix was used to prepare a column that was washed with hexane (8 mL). Sulfonamides were eluted with methylene chloride (8 mL). Sample extracts contained sulfonamide analytes (62.5–2000 ng/g) that were free from interfering compounds when examined by HPLC utilizing photodiode array detection at 270 nm. Correlation coefficients of standard curves of sulfonamides isolated from fortified pork tissue ranged from 0.994 (±0.006) to 0.999 (±0.001). Percentage recoveries (70.4–95.8%) and intra- (3.46–6.17%) and interassay variabilities (4.04–14.05%) for the eight sulfonamides were indicative of a suitable quantitative method for the analysis of these compounds in a muscle tissue matrix.

The use of antibiotics as chemotherapeutic agents in animal production has increased in the last decade. Antibiotics such as sulfonamides have become an integral part of the livestock production industry and function to prevent disease and/or increase feed efficiency. However, residues of these drugs in foods derived from treated animals could pose a health threat to consumers, and the constant exposure of some microorganisms to these drugs may manifest itself in the development of drug-resistant strains. Recent evidence has implicated sulfamethazine as a possible carcinogen (Littlefield, 1988), which has magnified risk assessment concerns. These concerns have prompted the U.S. Department of Agriculture/Food Safety Inspection Service to include sulfamethazine, sulfathiazole, and five other sulfonamides in the Compound Evaluation and Analytical Capability National Residue Program Plan (USDA, 1988). Regulatory agencies have established withdrawal periods for such drugs for animals treated prior to slaughter, as well as maximal residue levels allowable in foods (USDA, 1988), to minimize their impact.

The widespread use of sulfonamides in animal production necessitates the development of multiresidue techniques by which residue levels can be monitored. Techniques used for sulfonamide determinations include, but are not limited to, microbiological, high-performance liquid chromatographic, gas chromatographic, and/or mass

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**Table I. Compounds Studied (Concentrations 62.5, 125, 250, 500, 1000, and 2000 ng/g; 200 ng/g Sulfamerazine Internal Standard), Correlation Coefficients (±SD), Percentage Recoveries, and Inter- and Intraassay Variabilities of the Eight Sulfonamides Isolated from Fortified Pork Muscle Tissue**

| compound | correln coeff ($r \pm$ SD, $n = 5$) | % recovery ($\bar{x} \pm$ SD, $n = 30$) | interassay variability, % ($n = 30$) | intraassay variability, % ($n = 5$) |
|---|---|---|---|---|
| sulfanilamide | 0.997 ± 0.002 | 70.4 ± 12.7 | 11.2 ± 3.7 | 5.5 |
| sulfathiazole | 0.994 ± 0.006 | 80.3 ± 11.1 | 10.8 ± 3.5 | 3.5 |
| sulfadiazine | 0.998 ± 0.002 | 95.1 ± 15.1 | 7.2 ± 5.5 | 3.8 |
| sulfamerazine | | 78.1 ± 4.1 | 9.1 ± 4.8 | 4.8 |
| sulfamethazine | 0.999 ± 0.001 | 84.7 ± 8.2 | 7.4 ± 4.3 | 4.0 |
| sulfamethazole | 0.999 ± 0.001 | 95.7 ± 14.8 | 9.5 ± 5.4 | 3.9 |
| sulfisoxazole | 0.998 ± 0.001 | 92.8 ± 11.8 | 10.0 ± 8.0 | 5.3 |
| sulfadimethoxine | 0.997 ± 0.003 | 95.8 ± 12.4 | 13.3 ± 8.1 | 6.2 |

spectrometric methods. A review of analytical methods for sulfonamide determinations has been published (Horwitz, 1981). There are inherent problems associated with each of these techniques when dealing with extracts obtained from a complex matrix such as animal tissue. A major difficulty associated with detecting targeted compounds isolated from complex matrices is that of interfering coextractants, which can limit the choice of detection methods and complicate the analysis. Thus, the sample extraction and preparation steps become of paramount importance when trying to extract residues from tissues.

Because of the number of different sulfonamide drugs and metabolites that may be present, the need for multiresidue methods of analysis becomes obvious. Recently our laboratory reported (Barker et al., 1988, 1989; Long et al., 1989a,b) a simple multiresidue/multidrug-class/solid-phase extraction (SPE) method for the isolation of drug residues from tissues. The extracts obtained from this method, which we have named matrix solid-phase dispersion (MSPD), contained targeted residues that were free from interfering coextractants. We report here the first use of the MSPD methodology for the isolation and liquid chromatographic determination of eight sulfonamides as residues in pork tissue.

## EXPERIMENTAL SECTION

**Chemicals and Expendable Materials.** All standard compounds and solvents were obtained from commercial sources and were of the highest purity available. Water for HPLC analyses was triple-distilled water polished by a Modulab Polisher I (Continental Water Systems Corp., San Antonio, TX) water purification system. Bulk $C_{18}$ (22 g, 40 μm, 18% load, endcapped, octadecylsilyl-derivatized silica; Analytichem Int., Harbor City, CA) was placed in a column (50-mL syringe barrel) and sequentially washed with two column volumes each of hexane, methylene chloride (DCM), and methanol by vacuum aspiration until dry to remove contaminants inherent in manufacture.

Stock sulfonamide solutions (1 mg/mL) were prepared by dissolving standard compounds in HPLC-grade methanol and serially diluting to the desired microgram per milliliter level (3.13, 6.25, 12.5, 25.0, 50.0, 100.0 μg/mL) with methanol. Syringe barrels (10 mL), which were used to prepare elution columns for samples, were thoroughly washed and dried prior to use.

**Extraction Procedure.** Pork muscle tissue was obtained from a local market. A 2-g portion of $C_{18}$ packing was placed in a glass mortar, and fortified pork tissue (0.5 g) was placed onto the $C_{18}$. Sulfonamide standards (10 μL, 3.13–100 μg/mL) and internal standard sulfamerazine (10 μL, 10 μg/mL) were injected randomly into the tissue and were allowed to stand for 2 min. Blank tissues were prepared similarly, except that 20 μL of methanol containing no sulfonamides was added. The tissues were then blended into the $C_{18}$ material with a glass pestle until a homogeneous mixture was observed (30 s). The resultant $C_{18}$/tissue matrix was transferred to a 10-mL syringe barrel containing two filter paper disks (Whatman No. 1). Two filter paper disks were placed on the column head, and the column was compressed to a final volume of 4.5 mL with use of a syringe plunger that had the rubber end and pointed plastic portion removed. A plastic pipet tip (100 μL) was placed on the column outlet to increase the residence time of the eluting solvents on the column.

The column was first washed with HPLC-grade hexane (8 mL) by gravity flow under a hood. When flow had ceased, excess hexane was removed by applying positive pressure (pipet bulb) to the column head until any remaining hexane was eluted. The hexane was discarded appropriately. Sulfonamides were then eluted with methylene chloride (8 mL) as described above. The DCM extract was dried under a steady flow of dry nitrogen gas under a hood. To the dry residue were added 0.1 mL of methanol and 0.4 mL of 0.017 M $H_3PO_4$. The sample was sonicated (5–10 min) to disperse the residue, which resulted in a suspension. The resultant suspension was transferred to a microcentrifuge tube and centrifuged at 17000*g* for 10 min (IEC Centra M, International Equipment Co., Needham Heights, MA). The supernatant was filtered through a 0.45-μm filter (Micro Prep-Disc; Bio-Rad Laboratories, Richmond, CA), and a portion (20 μL) of this solution was analyzed by HPLC.

**HPLC Analysis.** Analyses of standard and extracted sulfonamides were conducted utilizing a Hewlett-Packard HP1090 (HP 79994A HPLC Chemstation) liquid chromatograph equipped with a photodiode array detector (UV, 270 nm, 20-nm bandwidth, 0.1 mAUFS, reference spectrum range 200–350 nm). The solvent system was a 70:30 (v/v) ratio of 0.017 M $H_3PO_4$ to acetonitrile at an isocratic flow rate of 1 mL/min. A reversed-phase (octadecylsilyl-derivatized silica, ODS) column (30 cm × 4 mm, Varian MCH-10), maintained at 40 °C, was used for all determinations.

Peak area ratio (PAR) curves of standards and samples were obtained by plotting areas of integrated peaks as a ratio to the area of the internal standard (sulfamerazine). A comparison of spiked sample PAR's to PAR's of pure standards run under identical conditions gave percent recoveries (mean of 30 samples ± standard deviation (SD)). The interassay variability was determined as follows: The mean of the PAR's for five replicates of each concentration (62.5, 125, 250, 500, 1000, 2000 ng/g) was calculated. The standard deviation corresponding to each mean was divided by its respective mean, which resulted in the coefficient of variation (CV) for each concentration. The mean of these CV's was calculated along with its SD, multiplied by 100, and defined as the interassay variability plus or minus the SD. Interassay variability was determined as the coefficient of variation (standard deviation of the mean divided by the mean) of the mean PAR's of five replicates of an identical sample.

## RESULTS

Table I lists the compounds examined, concentrations analyzed, standard curve correlation coefficients (±SD), percentage recoveries, and inter- and intraassay variabilities of the eight sulfonamides isolated from spiked pork tissue. Representative chromatograms of extracted tissue blanks and fortified tissue samples are shown in Figure 1, parts A and B, respectively.

## DISCUSSION

A critical aspect of drug residue analyses is the sample extraction and preparation steps required to isolate the residue from a complex biological matrix. The tech-

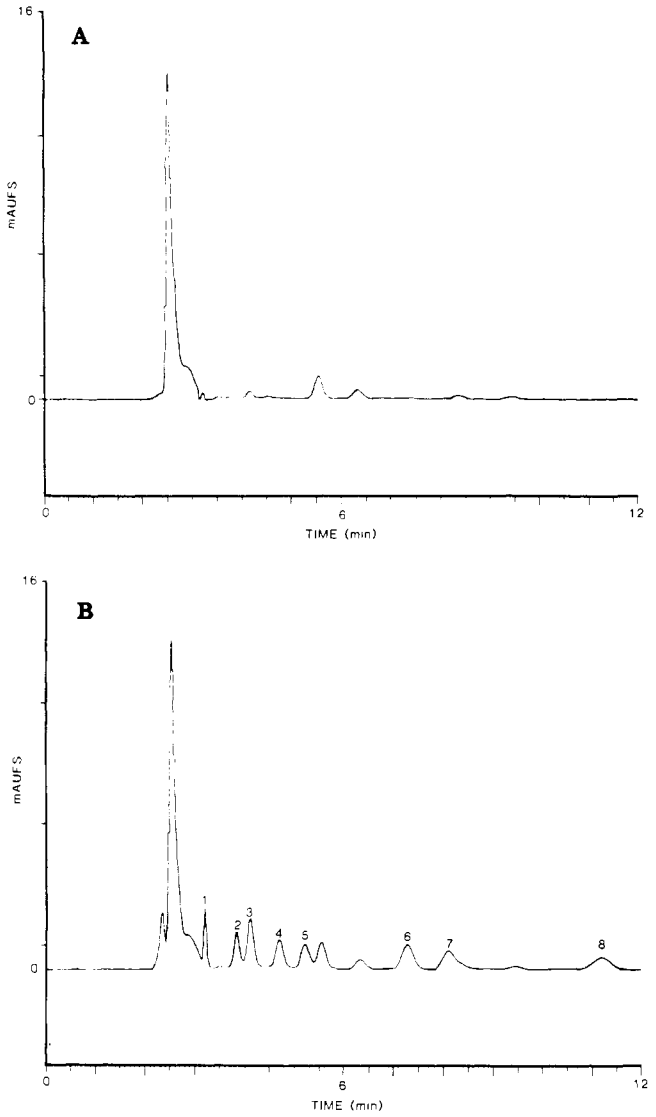


**Figure 1.** Representative chromatograms obtained from the HPLC/photodiode array (270-nm) analysis of the methylene chloride extract of (A) blank pork tissue and (B) sulfonamide-fortified (250 ng/g) pork tissue. Order of elution is sulfanilamide (1), sulfathiazole (2), sulfadiazine (3), sulfamerazine (4), sulfamethazine (5), sulfamethoxazole (6), sulfisoxazole (7), and sulfadimethoxine (8).

niques utilized should be such that they can be completed in a short period of time while simultaneously limiting expendable materials, especially solvents. Traditionally, the isolation of sulfonamide residues (USDA, 1979; Tishler et al., 1968) from a complex matrix such as tissue requires the homogenization of the sample in an extracting solvent(s). The homogenized sample extract must then be centrifuged to pellet cellular debris, which can lead to residue losses due to entrainment or in the electrostatic binding of the target residue on the pellet. Multiple extractions of the pellet are generally required to increase residue recoveries. The extract is further purified by a series of washes, pH adjustments, and reextractions to minimize or remove interfering coextractants, which then requires the evaporation or disposal of large volumes of organic solvents. Emulsion formation during these isolation procedures hinders the recovery of target compounds and complicates the analysis. Thus, the time-limiting steps involved are in the sample-extracting procedures especially when the residues are being isolated from a complex matrix such as muscle tissue. Additionally, these classical extraction methods are generally applicable for only a few residues (one to three compounds) within a compound class that may contain greater than ten different residues.

In contrast, the dispersion of pork muscle tissue onto the $C_{18}$ according to the MSPD procedure and the subsequent elution of eight sulfonamides with DCM is simple and results in extracts free from interferences, as can be seen in HPLC chromatograms of a blank tissue (Figure 1A) and sulfonamide-fortified tissue (Figure 1B) extracts.

The dispersion of tissue onto the $C_{18}$ packing material apparently involves a mechanism whereby membranes are disrupted by mechanical and hydrophobic forces, thereby allowing the external and internal membrane lipids to associate with the lipophilic $C_{18}$ to essentially unfold the structural components of the tissue. The theoretical aspects of this procedure have been published (Barker et al., 1988, 1989; Long, et al., 1989a,b). Scanning and transmission electron micrographs of uncoated and tissue-coated $C_{18}$ packing show that this process totally disrupts the organelle structure, thereby supporting this hypothesis (unpublished observations). This would allow the more hydrophilic regions of proteins to extend outward away from the nonpolar inner $C_{18}$/lipid region. Water and more polar constituents would preferentially associate with these hydrophilic ends. The distribution of cellular components is apparently uniform, which allows the nonpolar lipid constituents to be selectively eluted with hexane prior to eluting the sulfonamides with DCM.

In the MSPD procedure the sample is dispersed over a large surface area (1000 $m^2$/2 g of $C_{18}$) and exposes the entire sample to the extraction process. The extraction is an exhaustive extraction process whereby a large volume of solvent is passed over an extremely thin layer of sample. The hexane wash functions to remove lipid material and neutral chromophores that might interfere with the UV detection of sulfonamides. Other more polar compounds that are less soluble in DCM remain on the column. By utilizing a sequential elution protocol, one obtains extracts containing sulfonamides free of interferences.

The resulting extracts show minimal interferences when monitored by photodiode array detection at 270 nm. The linearity of standards extracted from spiked tissue samples, as well as recovery percentages and inter- and intraassay variabilities, is given in Table I. A small peak at 4.1 min was inherent to the method blank and had a similar retention time as that of sulfamerazine. However, this did not affect quantitative determinations, as reflected in good standard curve correlation coefficients and percentage recoveries.

The minimal detectable limit utilizing photodiode array detection was between 31.25 and 62.5 ng/g, utilizing a 20-$\mu$L injection volume from a 0.5-mL sample (approximately 1.25 ng on column), which reflects the sensitivity characteristics of the detection system utilized in this study. The analytical capability for the violative level of concern for sulfamethazine (100 ng/g) and sulfathiazole (100 ng/g) in the edible tissue of swine (USDA, 1988) is met by the MSPD method. The analytical capability for the other sulfonamides examined here for edible tissues has not been determined by regulatory agencies. Thus, this method may provide a framework for their isolation from muscle tissue. Additionally, the utility of the MSPD technique is exemplified in the fact that eight sulfonamides can be isolated simultaneously from a complex biological matrix. This is extremely difficult to accomplish by classical isolation techniques. Furthermore, the sample

extract has a minimal number of interfering compounds. Therefore, an increase in sensitivity may be achieved by increasing the injection volume and/or dissolving the extract residue in a smaller final volume. The cleanliness of the extracts may facilitate the use of more sensitive detection methods, including immunoassay, and allow for the detection of sulfonamide residue levels in the ppt range.

The results presented here are based on spiked samples, such as would be required and obtained for the preparation of standard curves or for conducting recovery studies for the quantitative analysis of drug residues in tissue incurred from the administration of a drug. The purpose of the present study was to examine the application of matrix solid-phase dispersion for the isolation of the eight sulfonamides from a single tissue sample, demonstrating the prospect that such methodology may be used to screen for a wide range of drugs in a single sample. While an examination of tissues from animals actually administered these eight sulfonamides would be ideal, such samples were not available to us and is outside the scope and limits of practicality of the present research. Such studies are currently under way, examining incurred residues of individual sulfonamides in tissues obtained from animals used in drug depletion studies, with the assistance of the U.S. Food and Drug Administration.

The method as outlined here eliminates many of the problems associated with classical techniques for the isolation of sulfonamides from tissue. The method uses small sample sizes, has a minimal number of steps and no chemical manipulations (such as pH adjustments), and requires a minimal amount of solvent. Conversely, classical sulfonamide isolation schemes based on the method of Tishler et al. (1968) require 50 g of tissue, a minimum of 300 mL of extracting solvents, pH adjustments, and repetitive back-washing in order to obtain an extract suitable for analysis. Thus, the savings in terms of time and solvent requirements make this procedure an attractive alternative to classical isolations. In addition, this method may be suitable for the isolation of different residues from other tissues or matrices.

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**Registry No.** Sulfanilamide, 63-74-1; sulfathiazole, 72-14-0; sulfadiazine, 68-35-9; sulfamerazine, 127-79-7; sulfamethazine, 57-68-1; sulfamethoxazole, 723-46-6; sulfisoxazole, 127-69-5; sulfadimethoxine, 122-11-2.

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