HPLC Method for the Simultaneous Analysis of Sulfadimethoxine and Ormetoprim in Tissues and Blood of Cattle, Chickens, and Catfish

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A method has been developed to simultaneously quantitate sulfadimethoxine (SDM) and ormetoprim (OMP) levels in cow, chicken, and catfish tissues and cow blood. SDM and OMP were extracted from the tissues with methylene chloride at pH 10 after the addition of tetrabutylammonium hydroxide to form an ion pair with SDM. Both compounds were separated by HPLC on a Waters μ-Porasil column and detected at 288 nm. The method exhibits good recovery and reproducibility for both compounds at levels between 0.05 and 40.0 ppm in tissues and 0.05 and 100 ppm in cow blood.

Sulfadimethoxine (SDM, Figure 1) is a relatively potent and long-acting sulfonamide that is effective when used alone or in combination with the potentiator ormetoprim (OMP, Figure 1) for the treatment of bacterial (Mitrovic et al., 1969, 1971b) or coccidial (Mitrovic et al., 1971a) infections. A 5:1 combination of SDM and OMP is approved for use in catfish and salmon.

Current federal regulations set the allowable residues for each drug in edible tissues at 0.1 ppm. Current assay methodologies for SDM in animal tissues and fluids are based on the following: (1) the Bratton–Marshall colorimetric assay (Tischler, 1968; Fellig and Westheimer, 1968); (2) paper or thin-layer chromatography using either a Bratton–Marshall (Parks, 1985) or fluorescamine (Thomas et al., 1983a,b) spray for quantitation; (3) gas–liquid chromatography following derivatization with diazomethane and/or pentafluoropropionic anhydride (PFPA) with detection either by electron capture (Goodspeed et al., 1978) or selected ion mass spectrometry either in the positive chemical ionization mode using deuterium-labeled internal standard (Garland et al., 1980) or in the electron impact mode using ^{13}C-labeled internal standard (Simpson et al., 1985).

In addition to the published methods, an unpublished HPLC method (Chen and Palko, 1979a) has been developed for the assay of SDM in horse blood at the 1–100 ppm level. The method employs a chloroform extraction at pH 6.0–6.5, ammonium sulfate wash, concentration, and HPLC analysis on a Whatman Partisil PXS 10-μm silica column. The mobile phase consists of chloroform, methanol, water, and concentrated ammonium hydroxide.

Except for the latter method, all procedures employ derivatization steps, either for detection (Bratton–Marshall, fluorescamine, PFPA) or for facilitation of the gas chromatography (diazomethane, PFPA). All the methods also involve several cleanup steps, mostly based on the method of Tischler (1968) or Fellig and Westheimer (1968) which take advantage of the amphoteric nature of SDM to facilitate removal of natural tissue constitutents. In addition, all the chromatographic methods employ one or more concentration steps. These concentrations have to be carried out just to dryness to avoid volatilization and loss of some SDM.

Only one method has been published for OMP in animal tissues or fluids (Fellig et al., 1971). This method involves the permanganate oxidation of OMP to a fluorescent product, 4,5-dimethoxy-*o*-toluic acid, and measurement of the fluorescence at 345 nm while excitating at 305 nm. An unpublished HPLC method (Chen and Palko, 1979b) has been developed for the assay of OMP in dog blood that uses the same cleanup steps as the published fluorimetric procedure. The HPLC system is similar to that described by Chen and Palko (1979a) for SDM in horse blood.

All of the SDM and OMP methods require time-consuming extraction/back-extraction and concentration steps. Additionally, the colorimetric assays suffer from high blank values and lack of specificity.

To minimize the work required to assay for the SDM and OMP combination products, a method was sought that would (1) assay for both compounds simultaneously, (2) require a minimum number of cleanup steps, (3) require no reaction steps, and (4) require no concentration steps.

Sulfadimethoxine is amphoteric and extracts well only between pH 6.0 and 6.5. Ormetoprim is basic and extracts optimally at pH 10. Thus, to extract both compounds simultaneously, the tetrabutylammonium ion pair of SDM was formed and both compounds were extracted at pH 10.0 into methylene chloride. The methylene chloride extract was dried with sodium sulfate and analyzed by HPLC using a modification of the system described above (Chen and Palko, 1979b). Parks (1985) used a similar strategy for the simultaneous extraction of two sulfa compounds along with two basic dinitrobenzamide coccidiostats at pH 11.0 into dichloromethane.

EXPERIMENTAL SECTION

I. Reagents and Apparatus. Chloroform, methanol, and methylene chloride were from Burdick & Jackson (Muskegon, MI). The chloroform was preserved with amylene and 1% ethanol. The water was distilled and deionized by a Hydro service (Research Triangle Park, NC) system. The sulfuric acid and ammonium hydroxide were concentrated reagent grade from J. T. Baker (Phillipsburg, NJ). The tetrabutylammonium hydroxide (TBAH) was 1 M from Fisher (Springfield, NJ) and was diluted to 0.5 M before use. The pH 10 buffer was 0.05 M potassium carbonate/potassium borate from Fisher. The sodium sulfate was anhydrous from J. T. Baker. The SDM and OMP were Hoffmann-La Roche standards. For the preparation of external standards, a combined stock solution of 100 μg/mL SDM and OMP was prepared by dissolving 10 mg of each compound in 100 mL of chloroform. Subsequent dilutions were prepared in chloroform. The external standard solutions were kept refrigerated and were found to be stable for up to 3 months.

The fortification stock solutions were prepared independently for the two compounds. A 100-mg portion of SDM was dissolved in ca. 20 mL of water with the aid of a few drops of concentrated NH_4OH. The sample was made up to 100 mL with water, and subsequent dilutions were made with water. The OMP stock solution was prepared as 100 mg/mL in 0.01 N sulfuric acid. Subse-

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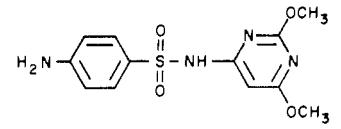
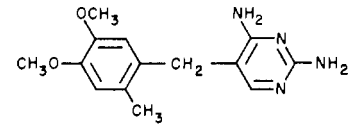


SULFADIMETHOXINE



ORMETOPRIM

Figure 1. Structures of sulfadimethoxine and ormetoprim.

quent dilutions were made with water. All the solutions used for sample fortifications were kept refrigerated and were found to be stable for 1 month.

The HPLC system consisted of the following components: (1) pump, Model 590 or M6000 (Waters Associates, Milford, MA); (2) injector, Model 710B autoinjector using 4-mL screw-cap vials (Waters Associates); (3) precolumn filter, Model 7302 with a 2-μm filter element (Rheodyne, Cotati, CA); (4) column, 300 mm × 3.9 mm (i.d.) μ-Porasil, stainless steel (Waters Associates, Milford, MA); (5) detector, Model 773 UV–visible, set at 288 nm (Kratos Analytical, Ramsey, NJ); (6) system control and data system, Model 840 (Waters Associates); (7) two mobile phases, A (used for analysis of chicken tissues) chloroform/methanol/water/concentrated ammonium hydroxide (1000:28:2:0.5, v/v/v/v) and B (used for bovine and catfish tissues and blood) chloroform/methanol/water/concentrated ammonium hydroxide (1000:28:2:0.6, v/v/v/v).

II. Sample Preparation. *a. Tissues.* Tissue samples were frozen shortly after harvesting. They were ground in a semifrozen state into plastic bags, mixed well, and stored frozen for assay.

Semifrozen tissue (2.5 g) was weighed into a 50-mL polypropylene centrifuge tube. To this was added the SDM and OMP spiking solutions (100 μL of each), 200 μL of 0.5 M TBAH, 1 mL of 0.05 M pH 10 buffer, and 0.5 mL of 1 N sodium hydroxide (final pH ≈10). After brief manual mixing, the sample was extracted with 10 mL of methylene chloride on a vortex mixer at high speed for 30 s. The phases were separated by centrifuging for 10 min at 3300*g* at 10 °C. The upper layer was suctioned off, the solid tissue plug was pushed to one side, and an aliquot of the methylene chloride layer was removed and filtered through sodium sulfate into 20-mL glass liquid scintillation counting vials. Samples can be stored overnight at room temperature prior to HPLC analysis.

b. Bovine Blood. Blood samples were collected over an oxalate solution (25 mL of blood to 1 mL of oxalate solution prepared from 6 g of ammonium and 4 g of potassium oxalate diluted to 100 mL with water). They were frozen until assayed. To one milliliter of oxalated blood in a 15- or 50-mL polypropylene centrifuge tube (Corning, Corning, NY) were added the SDM and OMP spiking solutions (100 μL of each), 100 μL of 0.5 M TBAH, and 1 mL of pH 10 buffer. After brief mixing, this was extracted with 4 mL of glass-distilled methylene chloride on a reciprocating shaker (New Brunswick Scientific, New Brunswick, NJ) at high speed for 2 min. The layers were separated by centrifugation (10 min at 3300*g* at 10 °C). The upper layer was suctioned off, the solid blood plug on top of the organic phase was pushed to one side, and a portion of the bottom layer was decanted into a 4-mL HPLC vial. Samples can

Table I. Recoveries of SDM and OMP from Fortified Bovine Tissues

fortificn level, ppm	average recovery,[a] % ± SD									
	liver		kidney		muscle		fat		blood	
	SDM	OMP	SDM	OMP	SDM	OMP	SDM	OMP	SDM	OMP
0.05	90.3 ± 21.9 (8)	112.3 ± 8.1 (8)	114.7 ± 11.4 (9)	113.1 ± 9.2 (9)	100.8 ± 6.8 (6)	101.3 ± 6.0 (6)	106.0 ± 15.7 (9)	121.3 ± 14.1 (9)	87.8 ± 7.7 (5)	102.8 ± 4.8 (5)
0.10	93.0 ± 14.4 (3)	114.7 ± 0.6 (3)	103.8 ± 14.3 (4)	103.5 ± 14.4 (4)	94.3 ± 15.4 (3)	96.0 ± 0.0 (3)	94.8 ± 8.38 (5)	88.0 ± 20.2 (5)	105.0 ± 5.8 (4)	97.8 ± 7.9 (5)
1.00	94.7 ± 3.2 (3)	96.7 ± 2.1 (3)	95.75 ± 11.8 (4)	104.8 ± 12.0 (4)	102.7 ± 9.5 (4)	102.0 ± 9.8 (4)	87.5 ± 12.2 (4)	94.8 ± 12.6 (4)	97.3 ± 4.9 (6)	93.5 ± 1.6 (6)
10.00	100.5 ± 0.7 (2)	96.5 ± 0.7 (2)	74.0 ± 0 (2)	76.0 ± 0 (2)	81.5 ± 0.7 (2)	72.5 ± 0.7 (2)	70.0 ± 0 (3)	85.3 ± 0.57 (3)	95.2 ± 0.8 (5)	93.3 ± 0.4 (5)
40.00	87.0 ± 14.4 (3)	91.3 ± 0.6 (3)	88.0 ± 0 (3)	95.0 ± 0 (3)	94.0 ± 1.0 (3)	94.3 ± 1.5 (3)	83.0 ± 0 (3)	84.7 ± 0.07 (3)		
50.00									92.5 ± 0.5 (5)	97.5 ± 1.0 (5)
100.00									94.0 ± 2.8 (5)	95.4 ± 1.4 (5)
overall mean ± SD	93.1 ± 5.1	102.3 ± 10.5	95.3 ± 15.5	98.5 ± 14.1	94.7 ± 8.3	93.2 ± 12.1	88.3 ± 13.4	94.8 ± 15.3	95.3 ± 5.7	96.7 ± 4.0

[a] Numbers in parentheses are the number of replicates determined.

Table II. Recoveries of SDM and OMP from Fortified Chicken Tissues

fortifcn level, ppm	average recovery,[a] % ± SD					
	liver		muscle		skin/fat	
	SDM	OMP	SDM	OMP	SDM	OMP
0.05	90.2 ± 16.4 (10)	90.2 ± 8.9 (9)	81.5 ± 11.0 (8)	82.7 ± 11.7 (8)	83.2 ± 13.4 (8)	100.1 ± 4.1 (8)
0.10	89.8 ± 8.0 (8)	87.4 ± 11.8 (8)	80.3 ± 4.5 (5)	75.0 ± 8.0 (5)	86.4 ± 8.0 (5)	90.8 ± 1.1 (5)
0.50	77.1 ± 2.2 (3)	71.7 ± 5.2 (3)	79.2 ± 10.3 (4)	84.4 ± 16.0 (4)	77.4 ± 1.8 (4)	96.7 ± 4.0 (4)
1.00	83.7 ± 4.8 (6)	79.6 ± 2.0 (6)	84.4 ± 2.3 (6)	85.2 ± 3.2 (6)	81.0 ± 2.6 (7)	89.3 ± 2.1 (7)
10.00	82.0 ± 7.7 (6)	72.1 ± 7.1 (6)	83.7 ± 5.4 (3)	77.0 ± 3.5 (3)	81.0 ± 0.8 (3)	80.2 ± 9.3 (3)
40.00	87.3 ± 2.8 (4)	80.6 ± 3.0	83.0 ± 1.3 (6)	76.3 ± 1.8 (6)	80.0 ± 4.1 (6)	80.2 ± 2.5 (6)
overall mean ± SD	86.3 ± 10.5	83.7 ± 13.9	82.0 ± 6.7	79.9 ± 9.1	81.7 ± 7.7	90.6 ± 8.4

[a] Numbers in parentheses are the number of replicates determined.

Table III. Recoveries of SDM and OMP from Fortified Catfish Tissues

fortifcn level, ppm	average recovery,[a] % ± SD					
	muscle		liver		kidney	
	SDM	OMP	SDM	OMP	SDM	OMP
0.05	101.3 ± 6.5 (6)	107.7 ± 11.7 (6)	117.8 ± 14.7 (5)	128.0 ± 17.6 (5)	114.0 ± 25.8 (5)	115.0 ± 12.7 (4)
0.10	92.5 ± 10.4 (6)	103.7 ± 10.0 (6)	101.3 ± 21.4 (11)	97.15 ± 21.9 (13)	85.0 ± 12.7 (5)	104.8 ± 16.8 (6)
40.00	96.3 ± 2.3 (6)	97.5 ± 3.5 (6)	99.0 ± 0 (3)	97.7 ± 0.6 (3)	98.4 ± 1.9 (7)	99.1 ± 2.1 (7)
overall mean ± SD	96.7 ± 7.7	102.9 ± 9.6	102.3 ± 20.3	112.3 ± 16.5	98.9 ± 18.3	104.9 ± 12.6

[a] Numbers in parentheses are the number of replicates determined.

be stored overnight at room temperature for HPLC analysis.

III. Analysis. Columns were equilibrated with mobile phase until reproducible retention times and areas were obtained for standards. This usually required overnight conditioning at 2 mL/min flow. Fresh mobile phase was prepared daily. The retention times using mobile phase A were ca. 4.9 and 6.0 min for OMP and SDM, respectively, and the retention times for mobile phase B were ca. 4.8 and 6.4 min for OMP and SDM, respectively.

Samples (400 μL) of both the external standards and samples were chromatographed with each set of samples. At least a three-point external standard curve bracketing the expected sample concentrations, a control sample, and a fortified sample were run with each set of samples. External standards are interspersed among the samples to check chromatographic consistency.

The flow rate for analysis of all tissues was 2 mL/min, and data were collected for 10 min. However, it was found that endogenous components eluted past this time, and conditions were used to ensure the elution of these components prior to subsequent injections. For bovine and fish tissues, the flow rate was increased to 4 mL/min at 10 min and the next injection cycle was started at 20 min. For bovine blood the flow rate was kept at 2 mL/min, but an extra 5 min was allowed before the next sample injection cycle was initiated. For chicken tissues and extra 10 min at 2 mL/min was allowed.

The peak areas obtained for the external standards were analyzed by the method of least squares or weighted least-squares analysis to obtain linear regression equations. Where peaks were detected for control tissues at the retention times for SDM or OMP, their areas were subtracted from those obtained for the samples before they were converted to concentrations by the linear regression equation.

RESULTS AND DISCUSSION

Both compounds exhibited good linear response from 0.005 to 40 μg/mL. Typical chromatograms of SDM and OMP using mobile phase A as well as control and 0.05 ppm fortified chicken liver are shown in Figure 2. The elution profile is very sensitive to the volume of ammonia used.

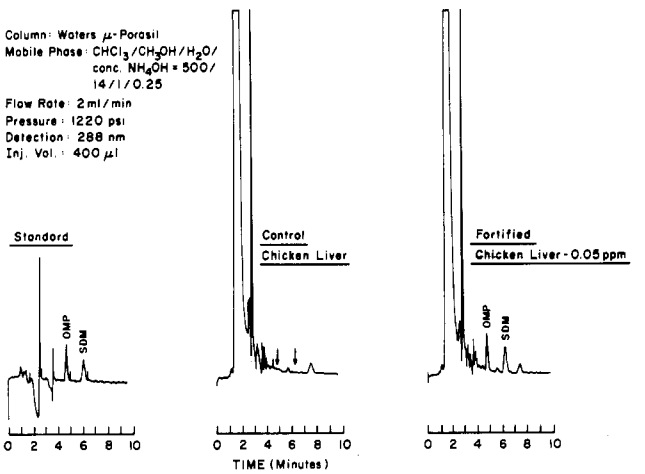


Figure 2. Typical chromatograms obtained for standards, control liver extract, and extract of control chicken liver fortified with 0.05 ppm each of sulfadimethoxine (SDM) and ormetoprim (OMP).

Table IV. SDM and OMP Levels (ppm) Obtained from 10 Aliquots of an Incurred Liver Sample Assayed on a Single Day

aliquot	SDM	OMP	aliquot	SDM	OMP
1	8.69	0.21	7	7.81	0.18
2	8.44	0.19	8	8.10	0.18
3	8.10	0.19	9	8.39	0.20
4	7.77	0.19	10	8.41	0.19
5	7.76	0.18	mean	8.146	0.19
6	7.99	0.19	RSD	3.974	4.96

Decreasing the ammonia concentration brings the SDM and OMP peaks closer together and eventually reverses the elution order while increasing the ammonia concentration pulls the peaks further apart. Thus, adjusting the ammonia concentration is a convenient method for creating a window to separate the compounds from endogeneous tissue components.

Recoveries from fortified controls are presented in Tables I–III for bovine, chicken, and catfish tissues and bovine blood. Satisfactory recoveries were obtained from all

Table V. SDM and OMP Levels (ppm) Obtained from Five Incurred Bovine Kidney Samples Assayed in Duplicate on Six Days

sample	assay day 1	2	3	4	5	6	mean	RSD
				SDM				
1	0.132	0.135	0.150	0.143	0.152	0.116	0.137	7.9
	0.146	0.127	0.124	0.141	0.141	0.136		
2	0.424	0.393	0.530	0.548	0.445	0.462	0.474	12.5
	0.425	0.443	0.519	0.589	0.445	0.460		
3		0.465	0.639	0.618	0.703	0.711	0.639	16.9
	0.749	0.451	0.541	0.715	0.747	0.687		
4	10.897	9.010	11.421	10.694	10.721	11.121	10.909	12.3
	11.948	8.829	13.922	11.415	11.037	9.895		
5	14.945	12.786	18.023	13.851	16.321	14.767	15.061	10.9
	14.159	12.346	16.744	15.352	15.307	16.134		
				OMP				
1	nd	nd	nd	nd	nd	nd		
2	nd	nd	nd	nd	nd	nd		
3	nd	nd	nd	nd	nd	nd		
4	0.486	0.331	0.475	0.362	0.465	0.459	0.452	17.1
	0.521	0.342	0.597	0.499	0.460	0.427		
5	0.718	0.555	0.805	0.643	0.666	0.701	0.677	13.2
	0.754	0.549	0.802	0.660	0.563	0.713		

Table VI. Compounds Tested for Interference with Analysis of SDM and OMP in Bovine Tissues

monensin sodium	sulfamethazine	lincomycin hydrochloride
sulfaethoxypyridazine	erythromycin	streptomycin sulfate
amprolium	neomycin sulfate	oxytetracycline hydrochloride
decoquinate	zinc bacitracin	coumaphos
thiobendazole	famphur	bacitracin
tylosin	levamisole hydrochloride	rabon
bacitracin MD	melengestrol acetate	ronnel
zeranol	chlortetracycline hydrochloride	
phenothiazine		
procaine penicillin G		

Table VII. Compounds Tested for Interference with Analysis of SDM and OMP in Chicken Tissues at 1.0 ppm

clopidol	virginiamycin	hygromycin B
ethylenediamine dihydriodide (EDDI)	nicarbazin	4-nitrobenzene arsenic acid
nitrofurazone	salinomycin sodium	sodium arsenilate
3,5-dinitro-*O*-toluamide	piperazine anhydrous	nystatin
furazolidone	buquinolate	bacitracin MD
ethopabate	butynorate	halofunginone hydrobromide
	narasin	

tissues at all fortification levels.

Two experiments were performed to test the reproducibility of the method with tissues from drug-treated animals. In one, 10 aliquots of a bovine liver sample were assayed as a single set. Data presented in Table IV show good, single-day reproducibility for both compounds.

In the next experiment, five bovine kidney samples containing various levels of SDM and OMP were assayed in duplicate on six successive days. The data obtained are presented in Table V. The average coefficient of variation (CV) for duplicate analysis of all five tissues for the 6 days was 5.5% for SDM. For OMP, only two of the tissues showed measurable levels. For these tissues, the average CV for duplicate analysis was 6.7%. The CV for each tissue over the 6 days was higher than for the duplicate analysis. These values are given in Table V. The overall CV for SDM was 12.1% and for OMP 15.2%.

To test for possible interference, 25 chemical entities commonly used in bovine husbandry were chromatographed with use of the HPLC system employed for bovine tissues. These compounds are given in Table VI. Of the 26, only sulfamethazine eluted close to either SDM or OMP, eluting just 0.26 min prior to OMP. However, it is a simple matter to differentiate between the two compounds by using wavelength ratioing. A convenient second wavelength would be 254 nm. The intensity of a peak due to sulfamethazine would increase on going from 288 to 254 nm while that due to OMP would show a marked decrease.

Similarly, 19 compounds used in raising chickens were chromatographed in the HPLC system used to assay the chicken tissues. These compounds are listed in Table VII. Again, only sulfamethazine showed a peak in the area of SDM or OMP. However, in this system, the separation is good, sulfamethazine eluting ca. 0.7 min after OMP.

We have found the durability of the column to be adequate with most of the tissues tested. However, with increasing number of samples, the retention of OMP decreases and eventually separation from matrix peaks is no longer attained. We have not found any means to regenerate column performance.

The method seems to be applicable to many tissues. The retention times for OMP and SDM can be conveniently varied by adjusting the ammonia concentration, and usually this provides a convenient method to separate the compounds from tissue matrix components.

CONCLUSION

A rapid and convenient method has been presented for the simultaneous assay of SDM and OMP in various animal and fish tissues. The method employs only a single extraction of both compounds followed by HPLC in which both compounds are detected at a single wavelength. Good recoveries and reproducibilities are attained for both compounds from bovine, chicken, and catfish tissues and bovine blood. The general approach used should be applicable to the assay of the sulfa/pyrimidine combinations in many biological matrices.

ABBREVIATIONS USED

SDM, sulfadimethoxine; OMP, ormetoprim; ppm, parts per million; PFPA, pentafluoropropionic anhydride; TBAH, tetrabutylammonium hydroxide; CV, coefficient of variation; nd, not detectable.

Registry No. Sulfadimethoxine, 122-11-2; ormetoprim, 6981-18-6.

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Analysis of Acrolein from Heated Cooking Oils and Beef Fat

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Acrolein formed from heated cooking oils and from beef fat was quantified as the morpholine derivative. Headspace volatiles formed from cooking oils or beef fat heated at various temperatures were purged into an aqueous morpholine solution with either a nitrogen or an air stream. 3-Morpholinopropanal produced from acrolein and morpholine was extracted with dichloromethane and subsequently analyzed by a gas chromatograph equipped with a thermionic detector and a fused silica capillary column. Five cooking oils and beef fat were separately heated at 300 °C for 2 h, and the quantities of acrolein formed were determined. The amount of acrolein formed from 120-g samples ranged from 30 mg (soybean oil) to 72 mg (olive oil).

Acrolein is the simplest α,β-unsaturated aldehyde. It has been known as a lachrymator, and the vapor causes eye, nose, and throat irritation. Acrolein is used for many purposes including that of a biocide for aquatic weed control and that of an intermediate in the synthesis of many organic chemicals. Thus, it is often present in commerical products as a trace impurity. Acrolein has been found at very low levels in ambient air in urban and suburban areas (Brodzinsky and Singh, 1982), in emission from plants manufacturing acrylic acid (Serth et al., 1978), and in exhaust gas from a cornstarch manufacturing works (Hoshika et al., 1981).

Acrolein has been found in various foods such as sugarcane molasses (Hrdlicka and Janicek, 1968), souring salted pork (Cantoni et al., 1969), cooked horse mackerel (Shinomura et al., 1971), and white bread (Mulders and Dhont, 1972). Kishi (1975) detected acrolein at levels between 2.5 and 30 mg/m^3 in the air 15 cm above the surface of a heated oil. Acrolein was proposed to form from the dehydration of glycerol when animal or vegetable fats were heated to high temperatures (Izard and Libermann, 1978). In the present study, the amounts of acrolein formed from various heated cooking oils and from beef fat were determined as the morpholine derivative, 3-morpholinopropanal.

EXPERIMENTAL SECTION

Materials. Morpholine, acrolein, and tributylamine were purchased from Aldrich Chemical Co., Milwaukee, WI. The extraction solvent, dichloromethane, was obtained from J. T. Baker Chemical Co., Philipsburg, NJ. Corn oil was from Sigma Chemical Co., St. Louis, MO. Soybean oil, sunflower oil, olive oil, and sesame oil were purchased from a local market. Frozen fatty tissue, which was obtained from the renal periphery of beef carcasses, was ground to a powder in a blender with a small amount of dry ice and then melted in a flask in a hot water bath at 70–80 °C. All of the nonfatty tissue, including blood, muscle, and connective tissue, was removed from the liquid fat by filtration. The pure beef fat was then stored in a freezer for future experiments. The standard stock solution of tributylamine for gas chromatographic (GC) analysis was prepared by adding 50 mg of tributylamine to 1 mL of dichloromethane and was stored at 5 °C. The standard stock solution of acrolein for the gas chromatographic calibration curve was prepared by adding 1 g of

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