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Trace Analysis of Cinnamaldehyde in Animal Feed, Human Urine, and Wastewater by Electron Capture Gas Chromatography

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Cinnamaldehyde is a popular food flavoring agent that is under toxicological evaluation at the National Center for Toxicological Research. Methods that ensure purity, concentration, homogeneity, and stability of the chemical in feed and the safety of personnel and the environment were prerequisites for the toxicological tests. The chemical is extracted from feed with a solution of 40% methanol, 5% acetic acid, and 55% deionized H_2O. For levels >10 ppm, the feed extract is partitioned against benzene and assayed directly by FID-GC. For feed levels <10 ppm, the benzene extract is derivatized with *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine (PFBH) for analysis by EC-GC. Wastewater and human urine are extracted with benzene and derivatized. Wastewater is assayed by EC-GC while extracts of human urine require additional cleanup on a silica gel column prior to EC-GC analysis. Theoretical minimum detactable levels of cinnamaldehyde in feed, human urine, and wastewater were 1 ppm, 5 ppb, and 1 ppb, respectively.

The principal ingredient of cinnamon oil is cinnamaldehyde (CNMA) (formula shown in eq 1), which is also present in cassia oil. It is a popular food flavoring and is used as a component of cosmetics, soaps, and detergents. Its use as a flavor and fragrance ingredient was estimated to be 5.00×10^8 g/year, based upon sales in the United States as reported by the U.S. International Trade Commission (1978). Its use as a fragrance ingredient was estimated by Opdyke (1979) to be 4.5×10^7 g/year. CNMA is used as a flavoring ingredient in fruits and juices at levels as high as 6400 ppm, 3500 ppm in baked goods, 2200 ppm in breakfast cereals, 2000 ppm in baby food and desserts, and 1100 ppm in chewing gum.

Jenner et al. (1964) reported an oral LD_{50} for CNMA in rats of 2.22 g/kg, while Sporn et al. (1965) reported 3.35 g/kg and Zaitsev and Rakhmanina (1974) reported 3.4 g/kg. Jenner et al. (1964) explained that rats were characterized by depression, diarrhea, and scrawny appearance and deaths occurred within 2–3 h after administration of cinnamaldehyde. Zaitsev and Rakhmanina reported oral LD_{50}s of 3.4 g/kg for guinea pigs and mice. They noted no differences in LD_{50}s for different sexes of rats, guinea pigs, or mice.

Current human toxicity data for CNMA is limited to its irritant and sensitizing properties. It is a primary irritant that has been implicated as a frequent cause of allergic reactions to perfumes by Schorr (1975). Epidemiological studies or case reports have not been found in the literature that link CNMA exposure to human cancer. However, there are at least two related compounds that have been reported to induce tumors in experimental animals. Schoental and Gibbard (1972) reported that 3,4,5-trimethoxycinnamaldehyde was found to induce tumors in rats after intraperitoneal injection. A report from the National Cancer Institute (NCI) (NCI/NTP, 1980), for a study performed under the National Toxicology Program (NTP), states that cinnamyl anthranilate induced tumors in both rats and mice when administered in the diet at 15 000 or 30 000 ppm.

CNMA is oxidized to cinnamic acid and excreted in the urine as benzoic or hippuric acid. Teuchy et al. (1971) reported that as much as 25% of a dose may be excreted in the urine as hippuric acid within 24 h after an intraperitoneal injection of the compound.

While there is currently no evidence for the carcinogenicity of CNMA, this compound is a potential alkylating agent that could react with cellular macromolecules. This potential, together with human exposure to the compound at relatively high concentrations, and the demonstration of tumorigenic activity of two related compounds, makes the chemical a prime candidate for carcinogenic bioassay. These studies were proposed to be conducted at the National Center for Toxicological Research (NCTR). Prerequisites for these studies were development of chemical methodology to ensure that accurate doses of CNMA were administered to the test animals via spiked feed and that the chemical was uniformly distributed and stable during preparation of the feed as well as during the feeding period of the animal study. Preparation of a stable homogeneous dosed diet required selection of proper medium by which this volatile compound could be added to the feed. Methods are required for the trace analysis of all compounds tested at the NCTR, in the urine of laboratory personnel to monitor their exposure to the compounds and in wastewater to ensure that none of the compounds are discharged into the environment.

Heikki et al. (1972) reported preparation of 2,4-dinitrophenylhydrazone derivatives of carbonyl compounds for subsequent analysis by electron capture gas chromatography (EC-GC). Koshy et al. (1975), and Nambara et al. (1975) described methods for derivatizing carbonyl groups with *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine (PFBH) in pyridine for EC-GC analysis. Levine et al. (1981) described *O*-alkyloxime derivatization of aldehydes for gas chromatographic–mass spectrometric analysis.

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Evans and Truesdale (1973) reported the cyanosilation of aldehydes and ketones as a route to cyanohydrin derivatives. None of these methods offered the sensitivity and specificity necessary for the trace analysis of cinnamaldehyde in the various substrates required for the proposed toxicological studies.

This paper describes modified procedures employing EC-GC for the analysis of CNMA in animal feed at levels as low as 1 ppm after derivatization of the compound with PFBH (formula of derivative shown in eq 1). EC-GC procedures are also described for analyses of CNMA in human urine and wastewater at levels as low as 5 and 1 ppb, respectively. Ancillary data are presented concerning the stability of the encapsulated chemical in animal feed and analysis of residues in feed by flame ionization gas chromatography (FID-GC) at levels ≥10 ppm.

EXPERIMENTAL SECTION

Apparatus. The gas chromatograph was a Hewlett-Packard (Avondale, PA) Model 5710A equipped with dual injection ports, temperature programmer, flame ionization detector, linearized electron capture detector, and a Model 7127A strip chart recorder.

Reagents. Cinnamaldehyde was purchased from (Chem-Fluer, Inc., Newark, NJ) and the purity was determined to be 99.4% by FID-GC. The structure was confirmed as cinnamaldehyde by mass and ^{13}C NMR spectrometry.

The silica gel (No. 3405, J. T. Baker Chemical Co., Phillipsburg, NJ) was heated overnight in an oven at 130 °C and placed in a desiccator to cool. The silica gel was then partially deactivated to contain 3% water by wetting the inner surface of a 1-L glass-stoppered bottle with 3 mL of deionized water and adding 100 g of the activated silica gel. The contents were thorughly mixed and allowed to stand overnight prior to use.

The PFBH hydrochloride (Thiokol-Ventron Division, Danvers, MA) solution used to derivatize CNMA in sample extracts was prepared by adding 10 mg of PFBH hydrochloride to 1 mL of glacial acetic acid in an 8-mL culture tube with screw cap and Teflon seal and heating in a block heater at 65 °C for 15 min to ensure dissolution.

The microencapsulated CNMA (Eurand America, Inc., Dayton, OH) was prepared with a complex coasceravate of gelatin and gum arabic cross-linked with glutaraldehyde as described by Scarpelli (1982). This material was extracted and analyzed as described for feed by FID-GC and was found to contain 81.5% CNMA.

The extraction solution used in the analysis of feed samples was prepared to contain by volume 40% methanol, 5% acetic acid, and 55% deionized water. All solvents were UV grade and all additional reagents were CP grade.

Preparation of Cleanup Columns. The silica gel columns (12 mm i.d., No. 420000, Kontes Glass Co., Vineland, NJ) equipped with a 50-mL reservoir were prepared immediately prior to use by successively adding a plug of glass wool, 2 g of anhydrous sodium sulfate, 1 g of silica gel (3% water), and 2 g of anhydrous sodium sulfate and were washed with 10 mL of hexane that was discarded. (Note: The silica gel must be evaluated prior to use to ascertain that derivatized CNMA will elute as indicated in the analytical procedure.)

FID-GC. The column, injection port, and detector temperatures were 160, 200, and 300 °C, respectively. Gas flows were 60 mL/min of helium carrier, 60 mL/min of hydrogen, and 240 mL/min of air. The glass column used was 4 mm i.d. × 183 cm long packed with 10% OV-101 on Gas-Chrom Q (80–100 mesh). Under these conditions the retention time (t_R) of CNMA was 2.2 min. All injections were in 5 μL of benzene and the flame was extinguished for 1 min after injection to allow the benzene to elute before reigniting.

EC-GC. The column, injection port, and detector temperatures were 210, 240, and 300 °C, respectively. The carrier gas (5% CH_4 in argon) flow was 100 mL/min. The glass column was 4 mm i.d. × 91.4 cm long packed with 5% OV-17 on Anachrom Q (80–100 mesh). Under these conditions the retention time (t_R) for PFBH–CNMA was 2.1 min. All injections were in 5 μL of benzene. The benzene extract used as the positive control standard described under FID-GC, after PFBH derivatization, served as the positive control standard for quantitation of feed samples by EC-GC. A solution of CNMA in benzene subjected only to PFBH derivatization was used as the standard for quantitation of wastewater and human urine samples by EC-GC.

Extraction of Animal Feed. A sample (20 g or less) of animal feed was weighed into a 250-mL Erlenmeyer flask fitted with a ground glass stopper and mechanically extracted for 1 h with 100 mL of the extraction solution on a reciprocating shaker (No. 6000, Eberbach Corp., Ann Arbor, MI) at a rate of 200 excursions/min. Ten milliliters of the extract was placed in a 12-mL culture tube and centrifuged at 500 rpm for 3 min. (Note: All culture tubes were borosilicate glass equipped with Teflon-lined screw caps.) Four milliliters of the extract, or a suitable dilution with extraction solution, was transferred to an 8-mL culture tube containing exactly 2 mL of benzene. The contents were mixed with moderate shaking and centrifuged at 500 rpm for 3 min. The benzene layer, for samples containing 10 ppm of CNMA or more, was injected into the gas chromatograph for analysis by FID-GC. For samples containing less than 10 ppm of CNMA, 6 mL of the extract was added to a 12-mL culture tube containing 3 mL of benzene, and then the solution was mixed and centrifuged as described above. Two milliliters of the benzene layer was reserved for derivatization. Standard solutions of CNMA in the feed extract were prepared and extracted with benzene as described above for quantitation of feed samples.

Derivatization of CNMA for EC-GC Assays. Two milliliters of the benzene extract of the sample was placed in an 8-mL culture tube, followed by 0.25 g of anhydrous sodium sulfate and 20 μL of PFBH solution in acetic acid. The tube was shaken gently to mix the contents and placed in a heating block at 65 °C for 1 h. The reaction was terminated by the addition of 2 mL of 3 N HCl, and the contents were mixed with moderate shaking for 2 min and centrifuged at 500 rpm for 2 min. The aqueous layer (bottom) was withdrawn and discarded. The benzene phase was either subjected to a cleanup procedure (for extracts of human urine) or dried with 0.25 g of anhydrous sodium sulfate and injected into the gas chromatograph after appropriate dilution with benzene.

Extraction and Analysis of Wastewater. One milliliter of sodium acetate solution (10% $NaC_2H_3O_2$ dissolved in 0.1 N NaOH), 3 g of NaCl, and 2 mL of benzene were successively added to a 10-mL sample of wastewater in a 16-mL culture tube. The contents were mixed with moderate shaking for 2 min and centrifuged at 500 rpm for 3 min. The benzene layer was carefully withdrawn and placed in an 8-mL culture tube containing 0.25 g of anhydrous sodium sulfate for derivatization and analysis by EC-GC.

Extraction, Derivatization, Cleanup, and Analysis of Human Urine. One milliliter of sodium acetate solution (10% $NaC_2H_3O_2$ dissolved in 0.1 N NaOH), 3 g of

NaCl, and 3 mL of benzene were added to a 16-mL culture tube containing a 10-mL sample of urine. The contents were shaken for 2 min and centrifuged at 500 rpm for 3 min. Two milliliters of the benzene layer was carefully pipetted into an 8-mL culture tube containing 0.25 g of anhydrous sodium sulfate for derivatization as previously described except that when the 3 N HCl was added, the benzene layer was carefully withdrawn, percolated through a plug of anhydrous sodium sulfate (ca. 2 g in a 25 mm diameter funnel), and collected in a 50-mL round-bottom flask. The aqueous phase was extracted with two additional 2-mL portions of benzene and collected in a like manner. Keeper solution (0.5 mL of benzene, containing 10 mg of Nujol) was added to the combined benzene extracts, which were then evaporated to dryness with a 60 °C water bath and a water pump vacuum.

The residue was transferred to a silica gel cleanup column prepared as previously described by using three successive 3-mL portions of hexane, allowing each portion to percolate into the column bed. The column was then eluted with 10 mL of hexane followed by 10 mL of hexane–3% benzene; each portion was allowed to percolate through the adsorbent, and the combined eluates were discarded. The derivatized CNMA was then eluted from the column with 70 mL of hexane–5% benzene and the eluate collected in a 100-mL round-bottom flask. Keeper solution (0.5 mL) was added, and the eluate was evaporated to dryness with a 60 °C water bath with a water pump vacuum. The residue was dissolved in at least 2 mL of benzene for analysis by EC-GC.

Preparation of Dosed Feed for Stability Experiments. Batches of feed (2.0 kg) (Laboratory Chow, Type 5010M, Ralston Purina Co., St. Louis, MO) containing dose levels of 0, 1000, and 20,000 ppm of CNMA were prepared by mixing the appropriate amounts of microencapsulated CNMA and animal feed. The feed was mixed in a Model LV twin-shell laboratory blender (Patterson-Kelly Co., East Stroudsburg, PA) with the shell of the blender rotating at 20 rpm during the 30-min mixing process. The intensifier bar was not operated. At the end of the blending process each batch was transferred to a stainless steel pan, divided into 500- and 1500-g portions, and reserved for the following stability experiments.

Stability Experiments. The 500-g portion of each batch of dosed feed was placed in a crystallizing dish (ca. 19-cm diameter by 10 cm deep) and allowed to stand in the open vessel in a fume hood under incandescent lighting at ambient temperature for 16 days. This portion was used for short-term stability tests to simulate animal test conditions. Duplicate 5-g samples were taken from each dish immediately and 1, 2, 5, 8, and 16 days later for analysis of CNMA. The feed was mixed throughly prior to the removal of each sample. The 1500-g portion of each batch was sealed in an amber bottle, stored in a light-free cabinet at ambient temperatue, and used for long-term stability tests under simulated storage conditions. Duplicate 5-g samples were taken from each bottle immediately and 1, 2, 4, 8, and 16 weeks later for analysis of CNMA. All samples from the stability experiments were analyzed by FID-GC.

Recovery Experiments. Triplicate 20-g samples of animal feed were spiked with 0, 1, 10, and 100 ppm of CNMA. The spiking solutions were prepared by diluting a stock solution of CNMA (20 mg/mL in corn oil) to the appropriate level with hexane so that each spike was added in a 1-mL volume. Triplicate 5-g samples of animal feed were spiked with 1000 and 20 000 ppm of CNMA by accurately weighing the proper amount of microencapsulated compound on a microbalance (Cahn Model 4700, Cahn Instruments, Cerritos, CA) and adding the weighed amount to each sample of feed.

Table I. Results of Stability Study of Encapsulated Cinnamaldehyde in Animal Feed Spiked at Two Levels

	CNMA recovered at target level indicated,[a] ppm	
sample interval	1000	20 000
Short-Term Study[b]		
0 days	950	18 600
1 day	895	20 000
2 days	905	18 800
5 days	866	18 200
8 days	910	19 500
16 days	955	19 800
Long-Term Study[c]		
0 weeks	950	18 600
1 week	915	19 200
2 weeks	980	18 400
4 weeks	930	19 800
8 weeks	915	19 400
16 weeks	960	19 900

[a] Mean of duplicate assays corrected for background of control samples and recovery. [b] Open container, incandescent light, and ambient temperature. [c] Sealed container, light-free cabinet, and ambient temperature.

Triplicate 10-mL samples of human urine and wastewater were spiked with 0, 5, 10, and 50 ppb of CNMA by adding the appropriate amount of compound in 1 mL of methanol. All feed, urine, and wastewater samples were then allowed to stand overnight at ambient temperature under fluorescent lighting prior to analysis by the previously described methods to determine the accuracy and precision of the procedures.

RESULTS AND DISCUSSION

Several experiments were performed in determining a means of preparing CNMA-dosed feed whereby an adequately stable concentration of the volatile chemical could be maintained for a sufficient length of time to allow animal feeding experiments to be performed. A premixture of Syloid 244 FP, CNMA, and corn starch was prepared and mixed with feed. After 5 days at 1000 ppm about 24% of the compound was lost and the mixture was noticeably less homogeneous. A feed mixture containing 3% corn oil and 0.1% (1000 ppm) CNMA was prepared and tested for stability. After 3 days, about 26% of the CNMA was lost and again a loss in homogeneity was observed. Feed was then dosed with 1250 ppm of CNMA encapsulated in gum arabic and evaluated for stability. After 16 days the mixture showed a loss of CNMA of approximately 25%.

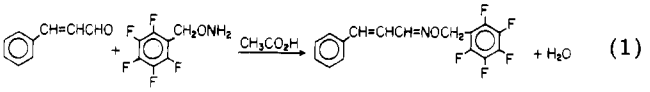
Since the previous attempts to prepare a stable mixture were unsuccessful, capsules with a different composition were evaluated. A sample of CNMA encapsulated with gelatin and gum arabic cross-linked with glutaraldehyde was obtained for evaluation. Feed mixtures containing 1000 and 20 000 ppm of the encapsulated chemical were prepared and evaluated for stability. The results of these studies are shown in Table I. There was no apparent loss in concentration for the duration of the tests; however, there was an obvious lack of homogeneity. This was attributed to the particle size distribution of the sample material. The particle size distribution was as folows: 500–600 μm, 27%, for 350–500 μm, 45.4%, for 297–350 μm, 11.8%, for 212–297 μm, 9.7%, and, for 105–212 μm, 6.1% as reported by the supplier. We recommended that the size of capsules be below 350 μm, to assure homogeneity of the dosed feeds that were to be used in the animal feeding experiments.

Initial extractions of CNMA from feed with methanol and with acetonitrile resulted in poor recoveries even at the 1000-ppm level. The addition of as little as 0.5% acetic acid gave near 100% recovery of CNMA from this substrate. Water is needed in the extraction of encapsulated CNMA from feed to break the cross-linking in the encapsulation material to free the compound for dissolution into the extraction solution. After evaluating several solvents for extracting CNMA from encapsulated CNMA dosed feed, the volume/volume solution of 40% methanol, 5% glacial acetic acid, and 55% deionized water was selected to give optimum recoveries. Procedures for the analysis of CNMA in feed were not required for initial animal feeding experiments at levels below 1000 ppm. However, results of initial feeding experiments often dictate changes in feed concentrations necessary for further experimentation. Recoveries were therefore determined for CNMA spiked feed at levels of 1,10, and 100 ppm to ensure analytical capability at these levels in the event that they are needed. It was not feasible to attempt to spike these feed samples at levels below the 1000-ppm level with the encapsulated compound because of the problems previously discussed concerning particle size distribution that are magnified when smaller amounts of encapsulated CNMA are added to achieve lower levels. The use of microcapsules of less than 350-μm diameter and containing less CNMA than those used in this experiment is a means of overcoming this problem since more of the capsules would have to be added to feed to achieve the desired concentration. The 1-, 10-, and 100-ppm levels were achieved by spiking feed with hexane dilutions of CNMA stock solution (20 mg/mL in corn oil). Control standards were run with each analysis of feed samples by making standards in the extraction solution and extracting into benzene for analysis exactly as the sample extracts were analyzed. This corrected for the p value of 0.905 for CNMA between benzene and the extraction solution.

The volatility of CNMA dictated the procedures used for extraction and cleanup of samples. Very careful evaporations of solutions of CNMA in benzene, in a 60 °C water bath with a water pump vacuum, in the presence of a keeper (0.5 mL of benzene containing 10 mg of Nujol), resulted in losses as high as 60%, whereas the PFBH–CNMA derivative could be evaporated to dryness without loss. The derivatives were prepared from a single extraction of the substrate before any cleanup could be performed. Cleanup procedures were not required for the wastewater samples, so a single 2-mL extraction in benzene was derivatized for analysis. Human urine samples required cleanup on a silica gel column, evaporation to dryness, and dissolution into a specific volume of solvent. Therefore, the sample was extracted with 3 mL of benzene and exactly 2 mL of this extract was derivatized. This same logic was followed in the derivatization of feed extracts. Benzene was selected as the solvent for extractions from aqueous solutions because of the p value of 1.0 between the organic and aqueous portions even at the 1:5 v/v ratio used for trace assays. Hexane, for example, has a p value of 0.64 under indentical conditions. However, benzene is a suspected carcinogen and should be handled under a hood whenever possible. Only limited quantities of benzene should be used in an open laboratory.

Several approaches were apparent for derivatization of the carbonyl group for trace analysis: cyanosilation as a route to the cyanohydrin, formation of hydrazones, and the nucleophilic addition of a hydroxylamine to form the corresponding oxime. The synthesis of cyanohydrin from a carbonyl compound requires two steps: (a) the addition of trimethylsilyl cyanide and (b) hydrolysis of the silyl group to form the corresponding cyanohydrin. This compound requires an additional derivatization with a halogenated acetyl compound for EC-GC analysis. The reaction of a fluorinated phenylhydrazine to form the corresponding phenylhydrazone apparently did not go to completion as we were unable to detect trace levels of derivatized CNMA in this manner. The methods referenced for making PFBH derivatives from carbonyl compounds recommend pyridine as the solvent, which produces a high background when using EC-GC, that eclipses the response from the PFBH–CNMA derivative. These methods reported in the literature require sample extracts to be evaporated to dryness, so the PFBH in pyridine is added to the dry sample. This is not applicable to a volatile compound such as CNMA.

Nucleophilic addition reactions, typical of the carbonyl group, are catalyzed by bases, by acids, and sometimes even by weak Lewis acids. Thus, it was possible in our experiment to react PFBH with CNMA by dissolving the PFBH in acetic acid before adding it to a benzene solution of CNMA. This reaction produces the desired derivative and a molecule of water as shown in eq 1. The anhydrous



(1)

CNMA PFBH-CNMA

sodium sulfate that has been added in the PFBH derivatization step of the analytical procedure absorbs the water and allows the reaction to go to completion. The structure of the product shown in eq 1 was confirmed by gas chromatography–mass spectrometry, and the mass spectrum showed no CNMA present, which confirmed that the reaction was essentially complete.

Derivatization of CNMA with PFBH produces syn and anti isomers of the PFBH–CNMA that can be at least partially separated by using a 4 mm i.d. × 274.3 cm glass column packed with 10% OV-101 on Gas-Chrom Q (80–100 mesh). Enough resolution was achieved to quantitate the isomers separately. However, by using the 4 mm i.d. glass column packed with 5% OV-17 on Anachrom Q (80–100 mesh), the isomers eluted in one peak, giving greater sensitivity and very good precision.

Recovery experiments were initiated to determine the accuracy and precision of the analytical procedures for the analysis of CNMA residues in animal feed. The results from triplicate assays of each level of CNMA spiked into animal feed are presented in Table II. The recoveries ranged from 71.0% at 1 ppm to 99.2% at 20 000 ppm. The volatility of CNMA certainly had an effect upon the recoveries at the lower levels since the compound in hexane and corn oil was added to a dry sample of feed and allowed to reach equilibrium in the 250-mL flask overnight. When the flask was opened to add the extraction solvent, the CNMA in the vapor phase escaped.

Typical FID-GC chromatograms of the CNMA positive control standard, its associated background, and animal feed unspiked and spiked with 1000 ppm of the chemical are presented in Figure 1. Also presented in Figure 1 are typical EC-GC chromatograms of the PFBH–CNMA positive control standard, its associated background, and animal feed unspiked and spiked with 1 ppm of CNMA and then carried through the analytical procedure.

Recovery experiments were also conducted to evaluate the accuracy and precision of the procedures for analyzing CNMA residues in human urine and wastewater. The results from triplicate assays of each level of CNMA spiked

Table II. Analysis of Animal Feed, Human Urine, and Wastewater Spiked at Various Levels with Cinnamaldehyde

CNMA added		method of analysis	mg equiv of sample injected	CNMA recovered, $\bar{x}$ ± SD[a]		
μg	ppm			μg	ppm	%
				Animal Feed[b]		
0	0	EC-GC	0.20	1.94 ± 0.12	0.097 ± 0.06	
20	1	EC-GC	0.20	14.2 ± 0.39	0.710 ± 0.19	71.0 ± 1.9
0	0	FID-GC	2.00			
200	10	FID-GC	2.00	153 ± 11.0	7.63 ± 0.55	76.3 ± 0.6
2 000	100	FID-GC	2.00	1 750 ± 59.0	87.6 ± 2.90	87.6 ± 2.9
20 000	1 000	FID-GC	0.5	19 100 ± 243	957 ± 12.0	95.7 ± 1.2
400 000	20 000	FID-GC	0.05	397 000 ± 10 200	19 800 ± 508	99.2 ± 2.5
				Human Urine[c]		
0	0	EC-GC	16.67	0.036 ± 0.008	0.00036 ± 0.00008	
0.05	0.005	EC-GC	16.67	0.052 ± 0.006	0.0052 ± 0.0006	104.0 ± 1.2
0.100	0.010	EC-GC	16.67	0.104 ± 0.001	0.0104 ± 0.0010	104.0 ± 1.0
0.500	0.050	EC-GC	3.33	0.491 ± 0.028	0.0491 ± 0.0028	98.2 ± 2.8
				Wastewater[c]		
0	0	EC-GC	25.00	0.0029 ± 0.0003	0.00029 ± 0.00003	
0.05	0.005	EC-GC	25.00	0.048 ± 0.002	0.0048 ± 0.0002	96.0 ± 1.7
0.10	0.010	EC-GC	25.00	0.967 ± 0.005	0.00967 ± 0.00005	96.7 ± 0.5
0.50	0.050	EC-GC	25.00	0.509 ± 0.005	0.0509 ± 0.0005	102.8 ± 0.5

[a] Mean and standard deviation from triplicate assays; spiked samples are corrected for background of control samples. [b] Per 20 g of animal feed, except for the 1000- and 20 000-ppm samples, 5 g of feed was spiked with encapsulated CNMA. [c] Per 10 g of sample.

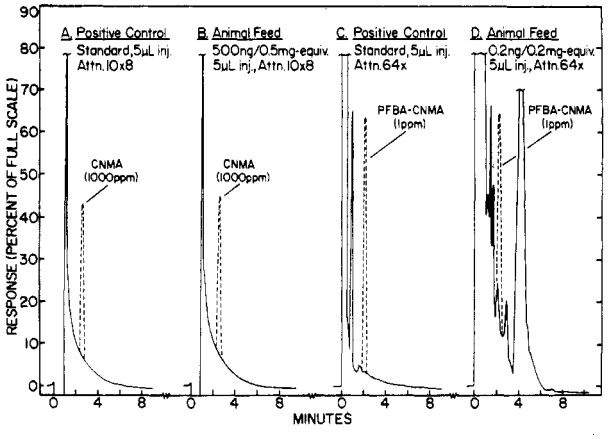


Figure 1. Gas chromatograms: (A and B) Solid lines represent FID-GC responses of the background associated with the CNMA positive control standard and unspiked animal feed; broken lines (superimposed) illustrate responses of the CNMA positive control standard and animal feed spiked at the 1000-ppm level. (C and D) Solid lines represent EC-GC responses of the background associated with the PFBH–CNMA positive control standard and unspiked animal feed; broken lines (superimposed) depict responses of the derivatized CNMA positive control standard and animal feed spiked with 1 ppm of CNMA and carried through the analytical procedure for EC-GC analysis.

into human urine and wastewater are also presented in Table II. Recoveries ranged from 98.2% at 50 ppb to 104% at 5 ppb for human urine and 96% to 102.8% for the same levels in wastewater. Theoretical minimum detectable levels of CNMA in human urine and wastewater were 5 and 1 ppb, respectively, based on twice background.

Figure 2 depicts EC-GC chromatograms of the derivatized CNMA standard, its associated background, and wastewater and human urine unspiked and spiked with 10 ppb of CNMA and then taken through the analytical procedures.

The methods presented provide specific and sensitive procedures for the analysis of CNMA in animal feed, human urine, wastewater, and microcapsules. These methods were not previously available and are necessary to conduct toxicological studies that are proposed for this chemical.

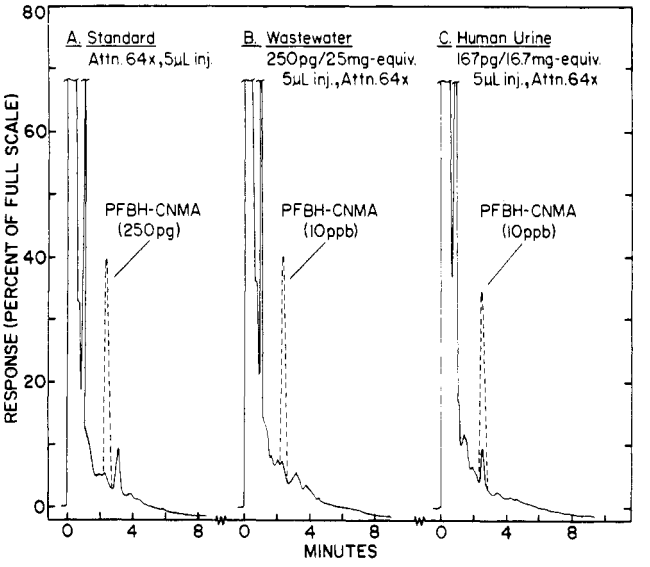


Figure 2. EC-GC chromatograms: In (A), the solid line represents the background associated with the derivatized CNMA standard; the broken line (superimposed) represents the response of the derivatized CNMA standard. (B and C) Solid lines illustrate the responses from unspiked wastewater and human urine taken through the analytical procedure; broken lines (superimposed) represent responses of the same substrates spiked with 10 ppb of CNMA and carried through the analytical procedure.

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Identification of Sheep Liver Volatiles

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The volatile constitutents of sheep liver were investigated to find new coyote (*Canis latrans*) attractants that could be useful for reducing predation of sheep. Steam disstillation–solvent extraction of the liver yielded a concentrate which showed good coyote attractancy. In order to identify the most active substances from the many compounds present, silica gel and acid/base fractionations were carried out. Capillary column GLC/MS of these fractions showed over 200 peaks of which 108 compounds were identified. They included a series of aldehydes, hydrocarbons, esters, and aromatic compounds, 15 thiazoles, 9 pyrazines, and 5 pyridines.

Coyotes (*Canis latrans*) cause considerable damage to livestock, mainly sheep. Efforts at using lures for coyote trapping suffer from attractance of nontarget animals; therefore, development of new lures is aimed at improving specificity, as well as attractancy, solely for coyotes.

A cooperative project between the University of California, Davis, and the Western Regional Research Center, U.S. Department of Agriculture, Albany, CA has resulted in the identification of a number of substances that are promising as specific coyote lures (Teranishi et al., 1980; Fagre et al., 1981). Such lures must satisfy several requirements. They must be at least as effective as the empirically developed commercial lures used so far. They should of known composition and consist of a minimum number of components for simple and inexpensive production. From the ecological point of view, lures should be specific for coyotes.

Sheep liver volatiles were investigated as a possible source for attractants (Fagre, 1982). A study of cooked pork liver volatiles was published by Mussinan and Walradt (1974); however, there are no publications on sheep liver volatiles. Testing of cooked sheep, pork, and beef liver volatiles on penned coyotes has been carried out by cooperating scientists from the University of California, Davis. This paper presents the analytical portion of the project: the identification of volatiles from cooked sheep liver.

EXPERIMENTAL SECTION

Extraction of Volatiles. A total of 35 kg of lamb liver provided by the Animal Science Department, University of California, Davis, was extracted in 2–3-kg batches. The liver was blended with an equal amount of water (2–3 L) in a Waring Blendor, transferred to a 12-L round-bottomed flask, and atmospherically steam distilled and extracted with diethyl ether (125 mL) by using a Likens-Nickerson head, as described by Schultz et al. (1977). A dry ice–2-propanol reflux condenser was attached at the extraction head exit port. The ether extracts were combined, dried and concentrated by using a Vigreux column. The extraction yielded 1.27 g of sheep liver volatiles. Material which codistilled with ether during initial concentration was recovered by distilling off the ether with more efficient glass helix distillation system. This low-boiling fraction totalled 10 mg, which is 0.8% of the total extract.

Silica Gel Fractionation. A total of 400 mg of the sheep liver extract was placed on a silica gel column (45 cm × 1.5 cm i.d.; silica gel 100–200 mesh, deactivated with 10% H_2O) and eluted successively with hexane, hexane/diethyl ether (5%), hexane/ether (50%), ether, and methanol. The eluents were divided into nine fractions, as determined by TLC analysis, by using phosphomolybdic acid for visualization. Each fraction was concentrated and weighed. Total recovery was 32%.

Acid/Base Fractionation. A 240-mg portion of the sheep liver extract was dissolved in 50 mL of ether and extracted with 3 N HCl as described by Buttery et al. (1977) to yield the basic fraction. The remaining ether solution was treated successively with 5% $NaHCO_3$ and 1 N NaOH to obtain acidic fractions I and II. Yields were basics 12.5%, acidic I 5%, and Acidic II 38.8%. A portion of the acidic fractions was esterified with dimethylformamide dimethyl acetal (Pierce No. 49355) in order to identify the methyl esters.

Capillary GC/Mass Spectral Analyses. The nine fractions obtained from the silica column were analyzed by gas chromatography/mass spectrometry using a 42 m × 0.6 mm OV-101 glass capillary column which was temperature programmed from 60 to 200 °C at 3 °C/min and

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