Trace Analysis of Natural Capsaicinoids in Animal Feed, Human Urine, and Wastewater by High-Pressure Liquid Chromatography

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Capsicum oleoresin, a natural food flavoring additive, contains a mixture of closely related amides called capsaicinoids. Because of its widespread use and a deficiency of toxicological data, Capsicum oleoresin was proposed for toxicological evaluation. Analytical chemical procedures that ensure proper concentration, homogeneity, and stability of the capsaicinoids in dosed feed as well as the safety of personnel and the environment during animal studies are prerequisites for such toxicological tests. Therefore, high-pressure liquid chromatographic methods using both UV absorbance and fluorescence detection were developed for analysis of capsaicinoids in animal feed, human urine, and wastewater at levels as low as 500, 10, and 10 ppb, respectively. These procedures provide specific and sensitive techniques not previously available for the separation and determination of natural capsaicinoids.

The red pepper fruits of *Capsicum annum* and *Capsicum frutescens* contain a complex mixture of closely related amides which are called capsaicinoids. These amides, the pungent principals of red peppers, are capsaicin (C), dihydrocapsaicin (DC), nordihydrocapsaicin (NDC), homocapsaicin (HC), and homodihydrocapsaicin (HDC), whose formulas are shown in Figure 1. The oily residue of the extracted red peppers is normally referred to as Capsicum oleoresin and is widely used as a flavoring agent for such foods as curries, sauces, and both Chinese and Mexican dishes. Other uses include incorporation into topical ointments and cough medicines. In addition to capsaicinoids, the oleoresin is generally composed of fixed oils of the polyunsaturated vegetable oil type (Boersma, 1980).

Only limited information is available relative to the toxicology of capsaicinoids. However, Todd et al. (1977) showed that each capsaicinoid elicits a pungent effect when in contact with mucosa in humans, although with differing intensities. In a summary on the pharmacological effects of capsaicin, Molnar (1965) reported that ingestion of capsaicin in dogs produced hypothermia, somnolence, bradypnea, and bradycardia and that intravenous administration of capsaicin produced desquamation of the convoluted tubules of the kidneys as well as petechial intertubular hemorrhages.

According to Molnar, the first data on the toxic effects of capsaicin were reported in 1931 where ingestion of capsaicin in rabbits produced histologically degenerative changes in the heart, liver, and kidneys. Further, toxic doses of capsaicin by parenteral injection, in guinea pigs (450–500 μg/kg), induced a reaction similar to that of histamine, resulting in anaphylactic shock. The animals lost died of asphyxia.

Molnar also reported that the urine from humans having ingested food containing capsaicin showed a positive capsaicin colorimetric reaction. Thus, at least part of the capsaicin ingested by humans appeared to be eliminated intact in the urine; however, the colorimetric test is not specific.

Since only minimal toxicological data are available concerning Capsicum oleoresin and in view of its use in foods, initial toxicity studies were proposed to be conducted at the National Center for Toxicological Research (NCTR). Before such studies could be initiated, analytical chemical methodology was required to provide assurance that accurate doses of capsaicinoids were administered to the test animals via spiked feed and that the test agents were uniformly distributed and stable during preparation of the feed and during the feeding period of the animal study. Also, trace-level assays were required for urine of laboratory personnel to signal any accidental exposure to the capsaicinoids and for wastewater to provide assurance that the compounds were not discharged into the environment.

Various gas chromatography–mass spectrometry (GC–MS) (Masada et al., 1971; Iwai et al., 1979), gas chromatography (GC) (Grushka and Kapral, 1977; Jurenitsch and Leinmüller, 1980), high-pressure liquid chromatography (HPLC) (Johnson et al., 1979; Jurenitsch and Kampelnuhler, 1980), and HPLC–MS (Heresch and Jurenitsch, 1979) methods have been reported for the analysis of capsaicinoids. None of these procedures provide the sensitivities or analytical capabilities required for assays of the capsaicinoids in the various substrates for the proposed toxicological studies.

This paper describes procedures for the analysis of capsaicinoids in animal feed at levels as low as 500 ppb and at 10 ppb in both human urine and wastewater as determined by HPLC. Data concerning the stability of capsaicinoids in animal feed, partition values with various solvents, and ancillary analytical information pertaining to the simultaneous determination of individual capsaicinoids in animal feed and Capsicum oleoresin at levels as low as 10 ppm are also presented.

EXPERIMENTAL SECTION

Apparatus. The system for HPLC consisted of a Waters Associates (Milford, MA) Model M6000A solvent delivery system, a Rheodyne (Berkley, CA) Model 7120 septumless injector, an Altex (Berkley, CA) guard column, 40 mm × 3.2 mm i.d. packed with 37–40 μm of Corasil C_{18} (Waters Associates), an Altex 5-μm ODS Ultrasphere analytical column, No. 256-05, 25 cm × 4.6 mm i.d., a Tracor, Inc. (Austin, TX), Model 970 variable-wavelength UV–visible absorbance detector, a Farrand Optical Co. (Valhalla, NY) Cat. No. 15170 fluorescence HPLC monitor, and a Hewlett-Packard (Palo Alto, CA) Model 7127A strip chart recorder. The detectors were run in tandem with the absorbance of the UV detector set at 280 nm, followed by the fluorescence detector with λ_{ex} = 280 nm and λ_{em} = 316 nm (7–39 filter) employing a 10-nm slit program.

Reagents. Preparations of natural capsaicin were purchased from Pfaltz and Bauer, Inc. (Stamford, CT), Fluka, Tridom Chemical Co. (Hauppauge, NY), and Sigma

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Table I. HPLC Assays of Natural Capsaicin Preparations and Capsicum Oleoresin

sample	source	total capsaicinoid content, %	% composition of capsaicinoids present				
			C	DHC	NDC	HDC	HC
natural capsaicin	Pfaltz & Bauer, Inc., Stamford, CT	100	55.0	29.1	13.2	2.0	0.8
natural capsaicin	Fluka, Tridom Chemical Inc., Hauppauge, NY	100	54.9	30.0	12.6	1.9	0.5
natural capsaicin	Sigma Chemical Co., St. Louis, MO	100	88.2	10.4	1.4	0.0	0.0
Capsicum oleoresin	Kalsec, Inc., Kalamazoo, MI	2.27	63.4	31.8	3.1	1.5	0.3

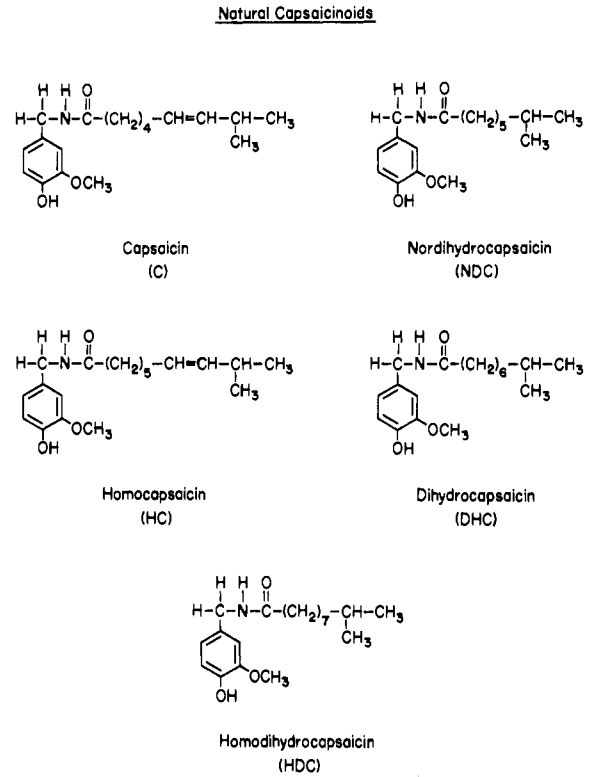

Figure 1. Formulas of natural capsaicinoids and their abbreviations.

Chemical Co. (St. Louis, MO); all were examined by HPLC to determine if they could be used as an analytical standard. All preparations were found to be mixtures of capsaicinoids with capsaicin being the major constituent of each (Table I). No additional extraneous peaks were detected in our analyses, and the capsaicinoids in the preparations were positively identified by mass spectrometry (MS). Since no pure standards of the individual capsaicinoids were commercially available, the Sigma preparation was chosen for separation of capsaicin from other capsaicinoid constituents by HPLC to obtain the standard sought. This preparation was chosen because of its high content of capsaicin (88.2%). Pure capsaicin, prepared as described in a later section, was positively identified by MS.

Capsicum oleoresin (No. 01-100-03-01, Kalsec, Inc., Kalamazoo, MI) contained 2.27% capsaicinoids as determined by HPLC. The relative percentage of each component in the oleoresin is also presented in Table I.

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 25% water by wetting the inner surface of a 1-L glass-stoppered bottle with 50 mL of deionized water and adding 150 g of the activated silica gel. The contents were throughly mixed and allowed to stand overnight prior to use.

The silver nitrate (Mallinckrodt, Inc., Paris, KY) was certified ACS grade and was used as received. All solvents were UV grade and all additional reagents were CP grade.

Preparation of Cleanup Columns. The silica gel columns (14.5-mm i.d., No. 420250, Kontes Glass Co., Vineland, NJ) equipped with 250-mL reservoirs used for the cleanup of extracts from animal feed, human urine, and wastewater were prepared just prior to use by adding successively a plug of glass wool and 5 g of deactivated silica gel (25% water). Twenty-five milliliters (25 mL) of benzene was then used to wash the column and the eluate was discarded. (Note: The silica gel must be evaluated prior to use to provide assurance that the capsaicinoids elute as indicated in the analytical procedure.)

High-Pressure Liquid Chromatography. The mobile phase used for analysis of total capsaicinoid residues in dosed feed stability experiments and recovery experiments for feed, human urine, and wastewater was 80% methanol–20% water (pH adjusted to 2.5 with glacial acetic acid). This mobile phase which flowed at a rate of 1 mL/min at 1300 psi is hereafter referred to as the "total capsaicinoid" mobile phase. Under these conditions C, NDC, and HC elute at 4.3 min; DHC and HDC elute at 5.0 min.

The mobile phase used for the simultaneous determination of individual capsaicinoids in Capsicum oleoresin or feed samples containing 10 ppm or more of each congener was 60% methanol–40% water (pH adjusted to 2.5 with glacial acetic acid) with both constituents made 0.05 M with $AgNO_3$. This mobile phase, hereafter referred to as "silver ion modified", flowed at a rate of 1 mL/min at 2100 psi. By use of this mobile phase, the retention times (t_R's) for C, NDC, HC, DHC, and HDC were 5.6, 6.9, 7.6, 10.7, and 11.8 min, respectively.

Total capsaicinoid residues from all three substrates were quantitated on the basis of peak heights. Feed spiking solutions (described in a later section) cleaned up as described for the analysis of Capsicum oleoresin and then added to cleaned up control extracts served as standards for the quantitation of feed samples. Spiking solutions that were prepared as described under Recovery Experiments for human urine and wastewater and added as prepared to cleaned up control extracts served as standards for the quantitation of human urine and wastewater samples.

Separation of Constituents of Commercial Capsaicin. The sample of commercial capsaicin from Sigma, which contained 88.2% C, 10.4% DHC, and 1.4% NDC, was separated into its constituents to provide a standard of pure capsaicin. The HPLC system previously described was used with a mobile phase of 65% methanol–35% water. Methanol (100 μL) containing 20 μg of commercial capsaicin was injected into the chromatograph, and the separated constituents were collected as they eluted. This procedure was repeated 120 times, and HPLC assays of the separated fractions indicated that each constituent was

free of congeners; the individual capsaicinoid in each fraction was positively identified by MS. The capsaicin fraction was evaporated to dryness by using a 30 °C water bath and water pump vacuum; then 2 mg was analytically weighed and dissolved in 2 mL of methanol. This pure standard was used for quantitation of capsaicinoids in the Capsicum oleoresin.

Analysis of Capsicum Oleoresin. A 5 g sample of Capsicum oleoresin was dissolved in 50 mL of hexane and extracted 3 times with 50-mL portions of acetonitrile in a 165-mL culture tube. The combined extracts, removed by syringe and cannula, were evaporated to dryness as previously described. The residue was dissolved in 5 mL of methanol and analyzed by HPLC using the silver ion modified mobile phase.

The oleoresin capsaicin was quantitated on the basis of peak height (H) as compared to the pure capsaicin standard. Each oleoresin capsaicin homologue was also quantitated by peak height based on the response normalized to that of capsaicin. For each homologue of capsaicin, the normalization was performed by simply dividing the retention time (t_R) of the homologue by that of capsaicin and then multiplying by the peak height of the homologue [i.e., $H_{\text{homologue}}(t_{R,\text{homologue}}/t_{R,\text{capsaicin}})$ = normalized H of homologue].

Extraction and Cleanup of Animal Feed. A 20-g sample of animal feed was weighed into a 250-mL Erlenmeyer flask fitted with a glass stopper and mechanically extracted for 1 h with 100 mL of 80% methanol–20% 0.1 N HCl on a reciprocating shaker (No. 6000, Eberbach Corp., Ann Arbor, MI) at a rate of 200 excursions/min. The extract was filtered through a plug of glass wool, and a 5-mL aliquot (1 g equiv of animal feed) was transferred to a 30-mL culture tube containing 5 mL of deionized water. (Note: All culture tubes were borosilicate glass equipped with Teflon-lined screw caps.) After the solution was briefly mixed, 10 mL of dichloromethane was added to the culture tube; the contents were throughly shaken and then centrifuged for 2 min at 1000 rpm. The dichloromethane layer was withdrawn with a syringe and cannula, percolated through a plug of anhydrous sodium sulfate (ca. 18 × 20 mm thick), and collected in a 50-mL round-bottom flask. The aqueous layer was extracted in the same manner with two additional 10-mL portions of dichloromethane which were also collected in the same flask. The combined extracts were evaporated to dryness by using a rotary evaporator, a water pump vacuum, and a 30 °C water bath.

The residue was transferred to a silica gel cleanup column by using 1 mL of benzene, followed by two successive 2-mL portions of benzene, each portion was allowed to percolate into the adsorbent. The column was then sequentially washed with 15 mL of benzene, 10 mL of 90% benzene–10% acetone, and 3 mL of 75% benzene–25% acetone; each portion was allowed to percolate through the adsorbent, and the combined eluates were discarded. The capsaicinoids were then eluted with 15 mL of 75% benzene–25% acetone and collected in a 50-mL round-bottom flask. The eluate containing the capsaicinoids was evaporated to dryness, and the residue was dissolved in 5 mL of methanol for analysis by HPLC as described.

Preparation of Feed Spiking Solutions and Dosed Feed for Stability Experiments. *Spiking Solutions.* Three separate acetone solutions (200 mL) containing Capsicum oleoresin, Sigma natural capsaicin, and/or corn oil were prepared with total capsaicinoid concentrations of 0, 100, and 20000 ppm for subsequent incorporation into animal feed to provide mixtures spiked with 0, 5, and 1000 ppm of total capsaicinoids. The solution for spiking control feed (0 ppm) contained 80 g of corn oil, and the one for spiking feed at the 5 ppm total capsaicinoid level contained 79.12 g of corn oil plus 0.88 g of Capsicum oleoresin. The solution for spiking feed at the 1000-ppm total capsaicinoid level contained 80 g of Capsicum oleoresin plus 2.182 g of Sigma natural capsaicin. A portion of each solution (100 mL) was reserved for spiking the appropriate feed sample; the remaining portions were reserved for use as standards to quantitate capsaicinoids in the respective spiked feed samples from stability experiments.

Dosed Feed. Batches of animal feed (Laboratory Chow, Type 5010M, Ralston Purina Co., St. Louis, MO) spiked to yield 0, 5, and 1000 ppm of total capsaicinoids were prepared by adding 100 mL of the appropriate spiking solution previously described to 1.96 kg of animal feed through the intensifier bar of the blender with an additional 100 mL of acetone added through the bar as a rinse. The feed was mixed in a Model LV Twin-Shell Lab Blender (Patterson-Kelly Co., East Stroudsburg, PA) with the shell of the blender operated at 20 rpm during the 45-min mixing process. The intensifier bar was operated at 3300 rpm during the mixing. At the end of the blending process, each batch was transferred to a stainless steel pan and dried at ambient temperature with frequent stirring for 1 h. Each batch was then divided into a 500- and 1500-g portion 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 × 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 20-g samples were taken from each dish immediately and 1, 2, 4, 8, and 16 days later for analysis of the capsaicinoids. The feed was thoroughly mixed just 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 temperature, and used for long-term stability tests under simulated storage conditions. Duplicate 20-g samples were taken from each bottle immediately and 1, 2, 4, 8, and 12 weeks later for analysis of capsaicinoids by HPLC.

Extraction of Human Urine and Wastewater. A 50-mL sample of human urine or wastewater was added to a 165-mL culture tube containing 20 mL of dichloromethane. The tube was gently shaken by hand for 2 min with care taken to prevent emulsification of the contents. The tube was then centrifuged for 2 min at 750 rpm, and the dichloromethane layer was removed by syringe and cannula, percolated through a plug of anhydrous sodium sulfate (ca. 18 × 20 mm thick), and collected in a 100-mL round-bottom flask. The extraction process was repeated with two additional 20-mL portions of dichloromethane, and the combined extracts were evaporated to dryness, cleaned up on silica gel, and assayed by HPLC as described.

Recovery Experiments. Triplicate 20-g samples of animal feed were spiked with 0, 0.5, 1, 5, 10, 100, and 1000 ppm of total capsaicinoids. The feed spikes were prepared at the appropriate level in the same manner as dosed feed spiking solutions previously described. Each of the spikes was added to the feed sample in 5 mL of acetone and thoroughly mixed.

Triplicate 50-mL samples of human urine and wastewater in 165-mL culture tubes were spiked with 0, 10, and

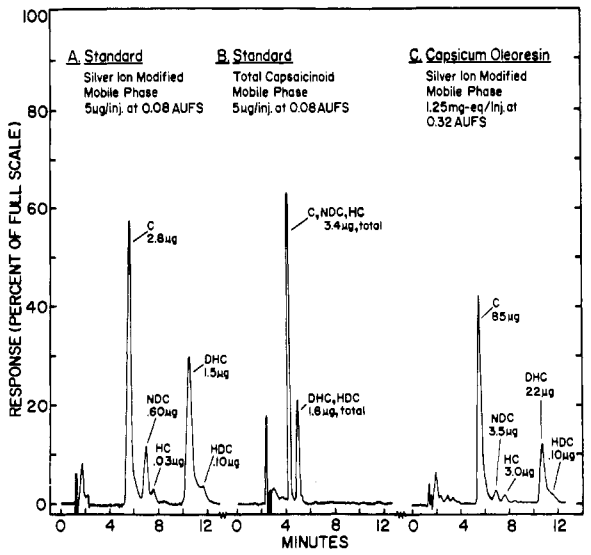

Figure 2. High-pressure liquid chromatograms. (A) illustrates the response from a natural capsaicinoid standard using the silver ion modified mobile phase; (B) represents the response from the same standard using the total capsaicinoid mobile phase; (C) illustrates the response of Capsicum oleoresin after liquid–liquid partitioning cleanup. All injections were in 5 μL of methanol.

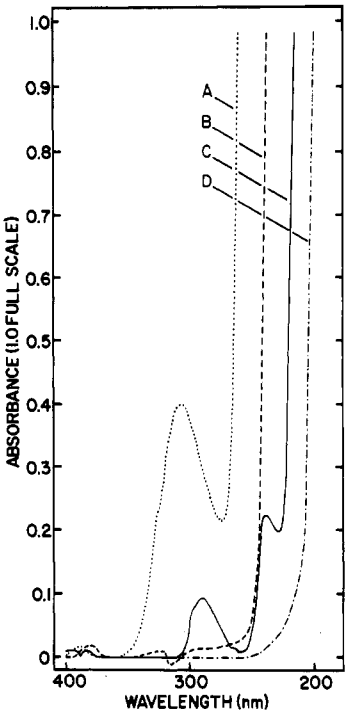

Figure 3. UV absorption spectra of (A) 65% methanol–35% deionized water (pH adjusted to 2.5 with glacial acetic acid) with both constituents made 0.05 M with $AgNO_3$, (B) 65% methanol–35% deionized water (pH adjusted to 2.5 with glacial acetic acid), (C) admixture of natural capsaicinoids (1 mg/mL in methanol), and (D) 65% methanol–35% deionized water.

100 ppb of total capsaicinoids by adding an appropriate amount of Sigma natural capsaicin in 1 mL of methanol. The tubes were sealed and the contents were thoroughly mixed. All feed, urine, and wastewater samples were then allowed to stand overnight at ambient temperature under fluorescent light prior to extraction, cleanup, and analysis by HPLC as previously described to determine the accuracy and precision of the procedures.

RESULTS AND DISCUSSION

Since the capsaicinoids exhibited substandard gas chromatographic properties, high-pressure liquid chromatography appeared to be the choice for analysis. Recent advances in HPLC (Johnson et al., 1979; Jurenitsch and Kampelnuhler, 1980) for the analysis of capsaicinoids include development of their optimum separation by the addition of $AgNO_3$ to the mobile phase which forms silver chelates with the olefinic portion of the capsaicin and homocapsaicin molecules. However, this technique required a relatively long analysis time (ca. 24 min) which resulted in low sensitivity. On the other hand, our experiments have demonstrated that the addition of acid to act as an ionic suppressor significantly reduces the analysis time to about 12 min and increases the sensitivity to as low as 10 ppm for the substrates tested. Chromatograms which illustrate the improved system are shown in Figure 2. Nevertheless, the improved sensitivity was inadequate for the proposed toxicological tests. The ultraviolet absorption spectra of the mobile phase with and without $AgNO_3$ were then determined, and it was found that the $AgNO_3$ produced significant absorption from 350 to 270 nm with a maximum at 305 nm (Figure 3). Consequently, further experiments employing the silver-ion modified mobile phase for trace level assays were abandoned. Therefore, subsequent attempts to meet the trace-level requirements of the proposed toxicological tests were focused on an HPLC mobile phase without $AgNO_3$ (total capsaicinoid mobile phase) which eluted all five capsaicinoids under two peaks that were easily quantitated as total capsaicinoids (Figure 2). Quantitation of all five capsaicinoids under two peaks was possible since the absorption maxima and response of each of the five compounds normalized to that of capsaicin appeared to be essentially the same as determined by both HPLC systems. The removal of $AgNO_3$ from the mobile phase failed to yield adequate trace-level sensitivity for assays of capsaicinoids via UV-absorbance detection. Fluorescence detection, which was subsequently investigated and found to give a 3-fold enhancement of sensitivity for capsaicinoids, was adopted for use in assays of feed, human urine, and wastewater.

Once HPLC conditions were established for the analysis of capsaicinoids, experiments were initiated to develop an effective means to extract the test agents from feed. A variety of solvents including benzene and methanol were investigated. After the capsaicinoids had been in contact with the feed for 14 days, only 59% of the total capsaicinoids could be recovered at the 5-ppm level by using benzene as the extraction solvent. Additional experiments using 0.1 N HCl to deactivate the feed and extraction with methanol significantly improved the recovery (91%) of the total capsaicinoids from 14-day spiked feed samples at the 5-ppm level. Methanol–0.1 N HCl was therefore selected as the extraction solvent because of the excellent recoveries obtained and its compatibility with subsequent cleanup procedures.

Since the feed, urine, and wastewater extractives contained a broad spectrum of materials that interfered with both UV and fluorescence detection for HPLC assays at levels below about 10 ppm, cleanup procedures were sought to enhance the sensitivity of the analyses. The partition values (p values) of the capsaicinoids in various solvent systems were therefore investigated for possible use in a liquid–liquid partitioning cleanup system. The p values of the capsaicinoids in dichloromethane or benzene vs. aqueous solutions at pH 2, 4, 6, 8, 10, and 12 or aqueous methanol (1:1 v/v) were all found to be 1.0. The p value of capsaicinoids in hexane–acetonitrile was 0.00, and this property is useful for separating capsaicinoids from the oily components of the oleoresin. Dichloromethane was the solvent of choice for the liquid–liquid partitioning cleanup

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

capsaicinoids added		g equiv of	capsaicinoids recovered ($\bar{x}$ ± SD)[a]		
μg	ppm	sample injected	μg	ppm	%
			Animal Feed[b]		
0	0.0	0.0125	0.340 ± 0.02	0.017 ± 0.001	
10	0.5	0.0125	9.10 ± 0.00	0.459 ± 0.000	91.8 ± 0.0
20	1.0	0.0125	18.6 ± 0.12	0.931 ± 0.006	93.1 ± 0.6
100	5.0	0.005	93.2 ± 0.1	4.66 ± 0.00	93.2 ± 1.2
200	10.0	0.005	191 ± 1.4	9.55 ± 0.07	95.5 ± 0.7
2000	100	0.001	1930 ± 10.0	96.8 ± 0.5	96.8 ± 0.5
20000	1000	0.001	19300 ± 48.0	967 ± 24.0	96.7 ± 2.4
			Human Urine[c]		
0	0.0	1.25	0.015 ± 0.000	0.00030 ± 0.00001	
0.5	0.01	1.25	0.451 ± 0.013	0.00902 ± 0.00025	90.2 ± 2.5
5.0	0.1	0.250	4.58 ± 0.040	0.0916 ± 0.0008	91.6 ± 0.8
			Wastewater[c]		
0	0.0	1.25	0.010 ± 0.001	0.00022 ± 0.00002	
0.5	0.01	1.25	0.420 ± 0.011	0.00840 ± 0.00021	84.0 ± 2.1
5.0	0.1	0.250	4.58 ± 0.070	0.0916 ± 0.0014	91.6 ± 1.4

[a] Mean and standard deviation from triplicate assays; spiked samples are corrected for background of control samples. [b] Per 20 g of animal feed. [c] Per 50 g of sample.

based on these data as well as its compatibility with the oils present in the oleoresin-spiked feed and the fact that it minimizes formation of emulsions during the cleanup.

High-pressure liquid chromatographic assays of total capsaicinoids (5 ppm) in feed extracts subjected to the dichloromethane partitioning procedure indicated that an additional cleanup would be required to further remove feed coextractives in order to perform assays below the 5-ppm level. Experiments with gravity-flow columns of silica gel and Sephadex LH-20 indicated that the most efficient column was silica gel deactivated to contain 25% water and eluted with mixtures of benzene–acetone of increasing polarity. This column eluted essentially all of the total capsaicinoids applied at all levels (0.01–1000 ppm) in the three substrates while retaining sufficient amounts of the coextractives to allow assays of feed, urine, and wastewater at levels as low as 500, 10, and 10 ppb, respectively. The use of silica gel containing less than 25% water resulted in significantly lower recoveries of capsaicinoids from the column. The cleanup system adopted for all three substrates consisted of the liquid–liquid partitioning procedure with dichloromethane, followed by the column cleanup on silica gel (25% water).

Recovery experiments were initiated to determine the accuracy and precision of the analytical procedure for the analysis of capsaicinoid residues in animal feed. Recoveries from triplicate assays of capsaicinoids in animal feed by HPLC are presented in Table II. The recoveries ranged from 93.2 to 96.7% for the higher levels (5 to 1000 ppm) while recoveries for the lower levels (0.5 and 1 ppm) were 91.8 and 93.1%, respectively. Although total capsaicinoids may be detected in feed at levels as low as 100 ppb, precise quantitation was not attempted at levels below 500 ppb because of the steep slope of the chromatogram. Typical HPLC chromatograms of the Capsicum oleoresin standard and background of the cleaned up animal feed extract, unspiked and spiked with 0.5 and 5 ppm of total capsaicinoids, are presented in Figure 4.

An explanation is necessary to clarify the reasons for the unusual composition of the solutions containing Capsicum oleoresin, Sigma natural capsaicin, and/or corn oil used for spiking the samples of feed. During animal bioassay tests at the NCTR, the animal feed (Ralston-Purina Chow, Type 5010M) is made available to the test animals (rats or mice) through a gravity-type feeder box. It has previously been determined that the feed will not flow

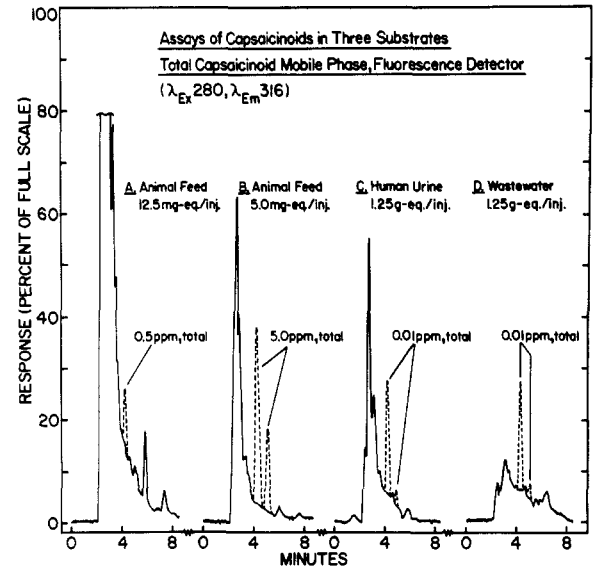

Figure 4. High-pressure liquid chromatograms. (A) and (B) solid lines represent responses from cleaned up animal feed extracts and (C) and (D) solid lines represent responses from cleaned up human urine and wastewater extracts. Dashed lines (superimposed) for each illustrate responses of capsaicinoid residues spiked into the extracts. All injections were in 25 μL of methanol.

downward in the box as it is consumed by the animals if it contains oil in excess of 2% (weight/weight basis) of that normally present in the feed. For example, the feed normally contains no more than 6.7% fats and oils, and if that content exceeds about 8.7%, the feeder will not operate properly. For this reason, the total amounts of corn oil and/or oleoresin could not exceed the 2% limitation. All feed samples used in this study contained a total of 2% oil in excess of the amount normally present in untreated feed. Capsicum oleoresin without the capsaicinoids was needed for the preparation of control feed. Since the p value of capsaicinoids in hexane–acetonitrile was shown to be 0.00, we attempted to use this system to separate the capsaicinoids from the oleoresin to provide a source of control oil for preparing the control feed. However, the procedure was abandoned since the acetonitrile also selectively extracted some of the oils from the oleoresin. Consequently, corn oil was selected to spike control feed at the 2% level (capsaicinoids absent). Corn

Table III. Results of Stability Study with Five Capsaicinoids in Animal Feed Spiked at Two Levels

sampling interval	capsaicinoids recovered (ppm) at target level indicated[a] (ppm)		
	control	5	1000
	Short-Term Study[b]		
0 day	0.017 ± 0.000	4.96 ± 0.12	965 ± 30
1 day	0.016 ± 0.000	4.68 ± 0.08	930 ± 18
2 days	0.020 ± 0.004	4.63 ± 0.00	938 ± 25
4 days	0.008 ± 0.000	4.53 ± 0.06	990 ± 25
8 days	0.018 ± 0.002	4.60 ± 0.04	1030 ± 10
16 days	0.019 ± 0.000	3.88 ± 0.02	953 ± 14
	Long-Term Study[c]		
0 week	0.017 ± 0.000	4.96 ± 0.12	965 ± 30
1 week	0.054 ± 0.009	4.49 ± 0.13	1010 ± 25
2 weeks	0.021 ± 0.001	4.51 ± 0.00	991 ± 30
4 weeks	0.060 ± 0.000	3.80 ± 0.05	943 ± 3
8 weeks	0.028 ± 0.000	3.74 ± 0.13	988 ± 7
12 weeks	0.036 ± 0.003	3.81 ± 0.08	982 ± 13

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

oil was also used in the 5-ppm feed preparation to make up the difference between the weight of oleoresin and the additional 2% of oil required for the spiked feed. In preparing the feed spiked with 1000 ppm of capsaicinoids (no corn oil added), we used Sigma natural capsaicin to fortify the oleoresin since the addition of 2% of oleoresin to the feed was deficient in the amount of capsaicinoids required to yield feed that contained 1000 ppm of the homologues.

Recovery experiments were also conducted to establish the accuracy and precision of the analytical procedure for determining capsaicinoid residues in human urine and wastewater. Results of recoveries from triplicate assays of human urine and wastewater unspiked and spiked with 10 and 100 ppb of capsaicinoids are presented in Table II. Typical HPLC chromatograms using fluorescence detection of the unspiked human urine and wastewater carried through the entire analytical procedure and 10-ppb levels of the capsaicinoids spiked into each matrix are shown in Figure 4.

After the analytical procedure was developed and thoroughly evaluated for the analysis of capsaicinoids in the three substrates, it was necessary to conduct additional experiments to determine the stability of the capsaicinoids in animal feed. Results from the short- and long-term stability studies of capsaicinoids in animal feed are presented in Table III. These data indicate that after 16 days in the short-term study (open dish exposed to light) about 22% of the total capsaicinoids at the 5-ppm level were not recovered with our procedure. After 2 weeks in the long-term study (closed container in the dark), only about 9% could not be recovered at the 5-ppm level. However, after 4 weeks in the long-term study, 23% of the total capsaicinoids were not recovered. The percentages of unrecovered capsaicinoid residues at the 1000-ppm level in both the short- and long-term studies were insignificant during the test period. On the basis of these data, spiked animal feed must be prepared approximately every 2 weeks to ensure that requisite dosages are administered to the test animals.

The procedures reported in this paper provide the analyst and/or toxicologist with specific and sensitive techniques not previously available for the separation and determination of natural capsaicinoids in four different substrates. Toxicological tests with capsaicinoids would not be possible without the successful development of these analytical procedures.

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