Improved High-Performance Liquid Chromatography Method for the Determination of Major Capsaicinoids in Capsicum Oleoresins

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The major capsaicinoids responsible for pungency in capsicum oleoresins were separated and quantified in an oleoresin model system and in a pharmaceutical model system using HPLC. An analogue of capsaicin, dimethoxybenzylmethyloctamide (DMBMO), was synthesized for use as an internal standard and used for quantification of capsaicin, nordihydrocapsaicin, and dihydrocapsaicin. This novel internal standard has a retention time between that of capsaicin and dihydrocapsaicin and is nonpungent. A precollaborative test was used to check the robustness of the procedure for variations in instrumentation and sample preparation. Changes in sample media were not shown to affect the results.

INTRODUCTION

Capsaicinoids are responsible for the pungent flavor in many "hot" foods. Since the effect they produce is actually an irritation to the nerve endings responsible for heat and pain sensation in the mouth, standardization of the level of heat is essential. Methods of their analysis include organoleptic (Govindarajan et al., 1977) and spectrophotometric methods (Ramos, 1979).

Historically, the organoleptic method has been preferred by the food industry since it is a direct measure of levels of heat. The disadvantages of this technique are the extensive training of panelists and the monitoring of their sensitivity to environmental factors to get reliable results (Meilgaard et al., 1987).

Spectrophotometric methods involve reactions of capsaicinoids with either vanadium oxytrichloride or phosphomolybdic acid to produce a colored species. Although not specific for capsaicinoids, the assay will give a result that is proportional to the amount of heat (Ramos, 1979; Bajaj, 1980; Rymal et al., 1984).

A variety of GC and HPLC methods have been proposed. The GC methods require some form of derivatization to form compounds that are sufficiently volatile for the analysis (Todd et al., 1977; Krajewska and Powers, 1987). HPLC analysis can be done by both normal and reverse-phase chromatography (Iwai et al., 1979; Hoffman et al., 1983; Law, 1983; Weaver et al., 1984; Chiang, 1986; Weaver and Awde, 1986). Reverse-phase chromatography is preferred because separation of the individual capsaicinoids can be accomplished. This is not possible with normal-phase chromatography because the capsaicinoids differ only in their fatty acid side chains (Table I).

The aim of this research was to develop a general procedure for the determination of the three major capsaicinoids in a variety of media using an internal standard. An internal standard was developed to improve accuracy and to correct for losses that may result from sample preparation. Recommendations for assaying oleoresin samples by HPLC in a collaborative study are given as well.

The major capsaicinoids measured in this work are capsaicin (C), dihydrocapsaicin (DC), and nordihydrocapsaicin (NDC). The minor capsaicinoids and the synthetic analogue of capsaicin, *N*-vanillyl-*n*-nonamide, are listed in Table I. The capsaicin analogue (4,5-dimethoxybenzyl)-4-methyloctamide (DMBMO) is used as the internal standard. Its structure is shown in Table I along with those of the three major capsaicinoids for comparison.

EXPERIMENTAL PROCEDURES

Preparation of 4-Methyloctanoyl Chloride Used in the Preparation of DMBMO. Thionyl chloride (20 mL; Aldrich) was added to 4-methyloctanoic acid (5 g; Aldrich) and heated on a steam bath (30 min). After cooling, methylene chloride (100 mL) was added and removed under vacuum twice to remove excess thionyl chloride.

Preparation of Dimethoxybenzyl-4-methyloctamide (DMBMO). 4-Methyloctanoyl chloride, prepared above, was added to dimethoxybenzylamine (1.6 g; Aldrich) which had been dispersed in 10% aqueous NaOH (10 mL). The two layers were well mixed, and the pH adjusted with 10% aqueous NaOH to 8.5–9.0. The white precipitate was filtered and washed with cold distilled water. The product was recrystallized, first from hexane and then from pentane (mp = 62–63 °C). It was found to be >99% pure by HPLC at 280 nm. The molar absorptivity, ϵ, of the product in methanol was 2970 at 278 nm and 8240 at 230 nm. A stock solution was prepared by dissolving 100 mg in 1 L of methanol; 50-mL aliquots of this stock solution were diluted to 100 mL with methanol for standards and sample solutions.

Preparation of Model Capsicum Systems. Oleoresin capsicum (Kalsec) was vacuum distilled (200–250 mTorr, 150–220 °C), and the distillate was recrystallized from hexane. the result was a red waxy solid without vegetable oils. This material was weighed into 100 mL of acetone; 10 mL aliquots were added to 100 mL of ethanol or soy oil to simulate a pharmaceutical tincture (*United States Pharmacopeia*, 1974) of capsicum or an oleoresin, respectively.

Capsaicin and Dihydrocapsaicin Standards. Capsaicin was purchased from Sigma and recrystallized from hexane or used as is. By HPLC, recrystallization did not improve purity. Dihydrocapsaicin was prepared by hydrogenation of capsaicin (1 g) over 5% platinum on carbon (0.25 g; Engelhard) in ethanol (17 mL), followed by extraction into hexane/ethyl acetate and recrystallization. It was identified by retention time on the HPLC and found to be at least 99% pure. Stock solutions were prepared by adding 10 mg of the capsaicinoid to 100 mL of methanol and then diluting to 20, 40, 60, 80, and 100 ppm to generate calibration curves.

HPLC Conditions. Separations were accomplished using either a Hitachi 655A-11 liquid chromatograph equipped with an L-5000 LC controller or a Waters 600E gradient pump with system controller. Detection was by either a Hitachi F-1000 fluorescence detector or a Waters 990 photodiode array detector. The column was either a Supelco C_{18}, 25 × 0.46 cm i.d., with 5-μm particle size or the equivalent Baxter column. A Valco guard column with removable cartridge was placed ahead of the analytical column. The output from the detector was recorded on a Waters 990 photodiode array workstation or a Waters

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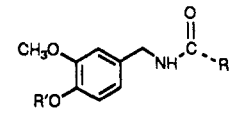
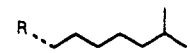
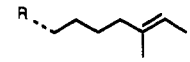
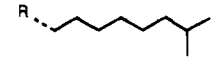
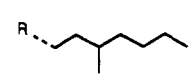
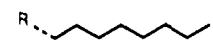
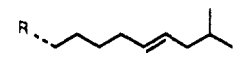
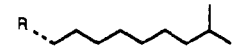
Table I. Structures of Major and Minor Capsaicinoids and Internal Standard Labeled in Figure 1 and Vanillylnonamide (Coelutes with Capsaicin)



major capsaicinoids		
nordihydrocapsaicin (NDC)		R′ = H
capsaicin (C)		R′ = H
dihydrocapsaicin (DHC)		R′ = H
internal standard		
dimethoxybenzylmethyloctamide (DMBMO)		R′ = CH_3
minor capsaicinoids		
N-vanillyl-*n*-nonamide		R′ = H
homocapsaicin (HC)		R′ = H
homodihydrocapsaicin (HDC)		R′ = H

Maxima 820 chromatography workstation. A mobile phase of 60:40 (v/v) methanol/water at a flow rate of 1.5 mL/min was found good for baseline separation of the major capsaicinoids. Acetonitrile was added up to 10%, to adjust for differences in columns or when decreased analysis time was called for. Citric acid at 1% was used to buffer the aqueous portion of the mobile phase at pH 3.00 for ion suppression. The fluorescence detector was set at 229-nm excitation and 320-nm emission with a 3-s time constant. *N*-Vanillyl-*n*-nonamide was placed in the detector cell to align the cell and the xenon lamp for maximum fluorescence. UV detection was at 280 nm with sensitivity set at 0.1 AUFS. Either 10- or 20-μL sample loop injections were used. A methanol/water gradient was used to wash out mobile phases containing citric acid buffers, and then methanol was used to wash out the column at the end of each day or after 20–30 injections.

Sample Preparation. Two methods of sample preparation were investigated. In the first, an oleoresin sample is weighed into a centrifuge tube to which is added 10 mL of 50 ppm internal standard solution in methanol, and the tube is shaken to mix the two layers. Centrifugation separated the two layers, and the top layer was injected onto the HPLC after filtering through a 0.45-μm filter. For the tincture of capsicum, a 1-mL aliquot of sample plus a 10-mL aliquot of 500 ppm stock solution of internal standard was diluted to 100 mL with methanol.

In the second method, a silica gel solid-phase extraction cartridge (500 mg; Burdick and Jackson) was utilized. First, the silica gel bed was activated with 1 mL of hexane. Second, a sample of oleoresin capsicum or tincture of capsicum (100 mg) was weighed directly onto the cartridge. This was then eluted with 10 mL of hexane followed by elution with methanol containing 50 ppm of the internal standard. Before oleoresin capsicum is sampled, the container is first heated in a water bath and shaken to ensure homogeneity.

RESULTS AND DISCUSSION

Previously, quantitation used an external standard method with capsaicin (obtained synthetically or isolated) or a synthetic analogue of capsaicin, *N*-vanillyl-*n*-nonamide (Hoffman et al., 1983). Both of these have very high pungencies and, as such, are difficult to handle. In our work, an analogue of capsaicin is used as the internal standard. It is obtained synthetically in high purity, is chromatographically resolved from the capsaicinoids of interest, and responds similarly in the detection systems. DMBMO is also nonpungent; it is well-known that the phenolic group is necessary for pungency (Susuki and Iwai, 1984; Szolcsanyi, 1982). In addition, it will correct for any sample preparation errors if added early in the extraction of ground chili peppers and finished food products such as hot sauces.

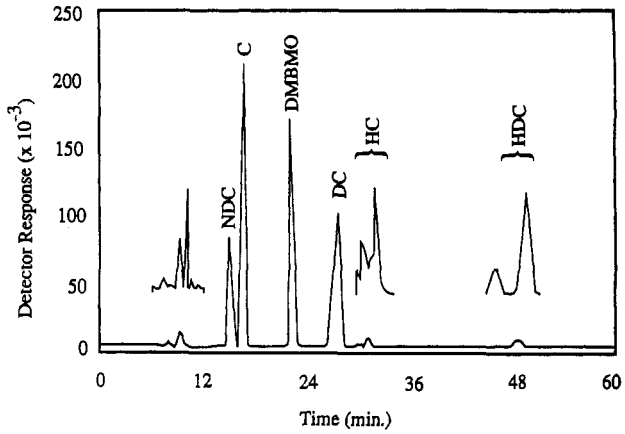


Figure 1. HPLC separation of crystalline capsaicinoids with DMBMO internal standard. Mobile phase: 60% methanol/40% water, pH 3.00, 1% citric acid at 1.5 mL/min. Stationary phase: Supelcosil C_{18} 25 cm × 0.46 mm, 5-μm particle size. See Table I for key to abbreviations.

The chromatogram of crystalline capsaicinoids shown in Figure 1 illustrates the separation that can be obtained on a C_{18} column. Baseline resolution is obtained for the internal standard as well as the three major capsaicinoids. Identification of the different capsaicinoids has been obtained by mass spectrometry (Heresch and Jurensitsch, 1979; van der Greef, 1985). The capsaicinoids elute from the C_{18} column in order of molecular weight. An external standard used previously, *N*-vanillyl-*n*-nonamide, coelutes with capsaicin and therefore cannot be used as an internal standard. It is also a minor naturally occurring capsaicinoid (Susuki and Iwai, 1984).

It can be seen from the chromatogram (expanded scale) that there are at least two minor components that elute before nordihydrocapsaicin as well as two that elute directly after dihydrocapsaicin and two that elute long after dihydrocapsaicin. Two of these minor components, for which pungency data are known, are identified in Table I by comparison to previous work (Heresch and Juren-

Table II. Relative Response Factors and Retention Times for the Major Capsaicinoids Referenced to DMBMO

capsaicinoid	rel response factor[a]	rel retention time
nordihydrocapsaicin	0.90[b]	0.71
capsaicin	0.94	0.79
DMBMO	1.00	1.00
dihydrocapsaicin	0.96	1.19

[a] Fluorescence detection used. [b] Calculated from molecular weight.

itsch, 1979). These minor components do not contribute significantly to the pungency (less than 2%) and are not included in this assay.

Both methanol and acetonitrile were investigated as mobile-phase organic modifiers. It was found that methanol provided better resolution of the internal standard from the capsaicinoids. In addition, methanol is cheaper and less toxic, which makes it more desirable for routine analysis.

To determine relative response factors of capsaicin and dihydrocapsaicin to the internal standard, calibration curves were constructed by plotting concentration ratios vs the ratios of area counts. A straight line was obtained through the points with a correlation coefficient greater than 0.99. The slopes of these lines give the relative response factors. The ratio of the slopes of the two analyte calibration curves gives the relative response for the two capsaicinoids. These ratios are very close to the ratios of the molecular weights of dihydrocapsaicin and capsaicin (Table II). On this basis, the relative response of nordihydrocapsaicin was also assumed to be proportional to molecular weight.

Although the determination of capsaicin and its major naturally occurring analogues has been performed in a wide array of matrices (Iwai et al., 1979; Hoffman et al., 1983; Law, 1983; Weaver et al., 1984; Chiang, 1986; Weaver and Awde, 1986), to our knowledge, no one has validated a method for the determination of capsaicinoids in such diverse matrices as vegetable oils (oil-soluble oleoresins) and ethanol (tincture of capsicum). To validate the method for the major capsaicinoids in these media, a simple factor analysis based on Youden's description of precollaborative testing of analytical methods was employed (Youden, 1963). The paired factors used in this study were A,a, lot of capsaicin external standard; B,b, lot of internal standard; C,c, lot of column used; D,d, detector used; E,e, sample medium; and F,f, sample preparation step used.

These six factors under two conditions were compared for their influence on the assay results. A six by eight matrix was set up for the six factors and labeled alphabetically; capital letters designate one condition, and lower case letters designate a second condition. The factors were organized in such a way that the average of four results under one set of conditions compared to the average of results under a second set of conditions will give predominantly the effect of a single factor. These combinations and the results of the analysis are given in Table III. At the left are shown combinations of capital and lower case letters representing the conditions used to get the result at the right.

By averaging the results that have a given parameter set at a primary condition and then averaging those set at the secondary condition and obtaining the difference of the resulting means, it is possible to rank the effects of those parameters on the basis of this difference. The factors ranked in order of importance are E,B < F,A < C < D.

Factor C, the column, and factor D, the two instruments, represent the largest sources of variability by this analysis.

Table III. Factors Investigated in This Analysis for Precollaborative Validation of HPLC Method

A,a, lot of capsaicin external standard
B,b, lot of DMBMO internal standard
C,c, lot of C_{18} column used
D, UV detector
d, fluorescence detector
E, sample in soy oil
e, sample in ethanol
F, liquid/liquid extraction[a]
f, solid-phase extraction[a]

	result,[b] %
combination 1, A B C D E F	1.07
combination 2, a b C D e f	1.07
combination 3, a B C d e F	1.10
combination 4, A b C d E f	1.07
combination 5, a B c d E f	1.09
combination 6, A b c d e F	1.09
combination 7, A B c D e f	1.01
combination 8, a b c D E F	1.03

[a] Explained in text. [b] Total major capsaicinoids defined as the sum of nordihydrocapsaicin, dihydrocapsaicin, and capsaicin.

It was apparent that relative retention times did vary by 4–5% when the column was changed. Using Youden's method, the standard deviation was estimated at about 3%. Thus, this is the level of error that should reasonably be expected among several laboratories in a collaborative study. Detection (D,d) was found to be possible by either fluorescence or UV detection with equivalent precision but with different linear ranges. The response from the fluorescence detector was found to be nonlinear at higher concentrations of capsaicin.

Capsaicin standards did not vary significantly from lot to lot. The internal standard was significantly less pure from one lot to the other without significantly influencing assay results *when calibrated using a reliable standard.*

Direct injection of oleoresin solution is possible, but a stronger solvent (less polar) than the mobile phase must be used as a carrier onto the column. Tetrahydrofuran works well as a carrier solvent. However, this results in mobile-phase insolubles such as waxes and fatty acids being injected into the system. Solid-phase extraction using an octadecylsilane column has been used with much success in the sample cleanup of solvent extracts of capsicum fruit (Attuquayefio and Buckle, 1987). Although these very polar interferants are present in extracts of capsicum fruits using a polar solvent, they are usually removed in the manufacture of oleoresins.

Two different sample preparation techniques (described under Experimental Procedures) were investigated for both model systems dissolved in soy oil and ethanol. Sample preparation (F,f) was not found to have a large influence on assay results, although it will presumably affect column life. The solid-phase sample cleanup technique is advantageous for extending column life because when the silica gel column is washed with hexane, methanol insolubles are removed and irreversibly adsorbed components, which will degrade the analytical column, remain on the strongly adsorbant silica gel surface. It is apparent that some carotenoids and vegetable oils are removed with the hexane while some remain on the silica gel column. However, all of the vegetable oil is not removed by this method. For the model system, it was determined that about 50% of the soy oil was removed by this sample cleanup procedure.

Factor E, which represents the model system in either soy oil or ethanol, has no detectable effect on variability.

The results obtained here were compared to those obtained spectrophotometrically using vanadium oxytrichloride ($VOCl_3$). Some discrepancies are normal since this colorimetric reaction is not selective for capsaicinoids and the absorbance is time dependent. Protic solvents such as water will interfere with the colorimetric reaction. The $VOCl_3$ method on these model systems showed a higher value for capsaicinoids dissolved in ethanol compared to vegetable oil. For these two samples, a relative error of ±8% was obtained, while the HPLC method was about ±3% for the lowest and highest values from Table III in soy oil and ethanol.

This analysis does not take into account differences in methods of integration or environmental variations. It indicates that when assay results from two laboratories for the same sample are compared, variables outside these controllable factors must be considered.

Previous oleoresin capsicum HPLC assays submitted for interlaboratory collaborative testing have failed, and there is currently no standard assay listed in the *Official Methods of Analysis* of the Association of Official Analytical Chemists (AOAC, 1990). It is hoped that the introduction of an internal standard which is structurally similar to the capsaicinoids will increase accuracy and give better recovery during sample preparation. This will also result in a more favorable collaborative outcome.

CONCLUSIONS

This proposed method of capsaicinoid analysis by HPLC has the following unique features: (1) the use of a novel internal standard that has a detector response equivalent to that of capsaicinoids in both UV and fluorescence detection modes and is nonpungent; (2) a sample preparation procedure employing a silica gel solid-phase extraction column; and (3) applicability to both oil-soluble and water-dispersible oleoresins.

For an interlaboratory collaborative effort, samples of the internal standard, the calibration standard, and model oleoresin samples as well as commercial oleoresin samples should be provided. It is recommended that an initial calibration curve be constructed by each laboratory and standards be run periodically to check for drift in the equipment. A standard containing capsaicin, dihydrocapsaicin, and the internal standard at about equal concentrations should be run after at least every three samples. This helps greatly to inspire confidence in the results.

Although tested predominantly in oleoresins, the measurement of major capsaicinoids in commercial hot sauces has been found to work well in our laboratory. This method can be extended to materials with lower concentrations of capsaicinoids.

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Received for review August 5, 1991. Accepted October 11, 1991.

Registry No. C, 404-86-4; DC, 19408-84-5; NDC, 28789-35-7; DMBMO, 137175-12-3; thionyl chloride, 7719-09-7; 4-methyloctanoic acid, 54947-74-9; 4-methyloctanoyl chloride, 137175-11-2; dimethoxybenzylamine, 5763-61-1.