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SEPARATION AND QUANTIFICATION OF CAPSAICINOIDS USING COMPLEXATION CHROMATOGRAPHY

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ABSTRACT.—A reliable and reproducible method to separate and quantitate capsaicin and its analogues using argentation hplc, which permits the base-line separation of norcapsaicin, zucapsaicin (civamide), capsaicin, nordihydrocapsaicin, nonivamide, homocapsaicin, dihydrocapsaicin, and homodihydrocapsaicin-I, is described.

Fruits of plants in the genus *Capsicum* have been used for centuries as spices and also in the practice of medicine. For these reasons a quick and easy method to completely separate and quantitate the alkaloids responsible for the biological activity in Capsicum was thought desirable. Capsicum is defined as the dried ripe fruits of *C. frutescens* L., *C. annuum* L. var. *conoides* Irish, or *C. annuum* var. *longum* Sendt. in the *National Formulary XI* and the *British Pharmaceutical Codex*. However, the British Standards Institution states that Capsicum includes certain varieties of *C. annuum* L. For a review of the complex botany of *Capsicum*, one is directed to Govindarajan (1).

Capsicum Oleoresin is the solvent-free extract of Capsicum. The alkaloid capsaicin is one of the active constituents of Capsicum Oleoresin, together with at least 100 other compounds, ranging from capsaicinoids (capsaicin and analogues) to terpenes and miscellaneous chemicals (2). Both the natural trans-isomer capsaicin [**1**] and the synthetic cis-isomer, zucapsaicin (civamide) [**2**], but not the crude Capsicum Oleoresin, have been demonstrated to be effective in managing the pain of post-herpetic neuralgia, rheumatoid arthritis, diabetic neuropathy, osteoarthritis, and cluster headache (3–5). The disadvantage of using Capsicum Oleoresin as a topical analgesic is that it has not been proven reliable as a neuropeptide-active agent. This may be due to the wide variability in the content of **1** and the potential that some of the phenolic compounds in the oleoresin may have neuropeptide-agonist activity (6).

Peripheral neuropathic pain disorders have a common denominator in that the pain is mediated by substance P. Substance P is an undecapeptide and appears to be a principal neurotransmitter in pain. Substance P also contributes to inflammation by stimulating cells to produce various cytokines, thus increasing capillary permeability. Application of **1** blocks substance P production and storage in peripheral nerves and blocks C-fiber conduction, thereby reducing neurogenic inflammation and pain response (7). Other major capsaicinoids found in Capsicum Oleoresin include dihydrocapsaicin [**3**] and nordihydrocapsaicin [**4**], but they are less abundant than **1**. Minor capsaicinoids that have been detected in Capsicum Oleoresin include norcapsaicin [**5**], homocapsaicins I [**6**] and II [**7**], homodihydrocapsaicins I [**8**] and II [**9**], nonivamide [**10**], caprylic acid vanillylamide [**11**], and decyclic acid

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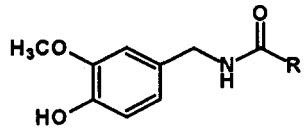

$R=(CH_2)_nCH=CH\text{-}CH\text{-}(CH_3)_2$	
$n=4$, *trans*	capsaicin (CAP) [**1**]
$n=4$, *cis*	zucapsaicin (CIV) [**2**]-synthetic
$n=3$	norcapsaicin (NC) [**5**]
$n=5$	homocapsaicin (SHC)-synthetic
$R=(CH_2)_4CH=CH\text{-}CH_2CH\text{-}(CH_3)_2$	homocapsaicin-I (HCAP-I) [**6**]
$R=(CH_2)_4CH=CH\text{-}CH\text{-}(CH_3)\text{-}CH_2\text{-}CH_3$	homocapsaicin-II (HCAP-II) [**7**]
$R=(CH_2)_nCH_2CH\text{-}(CH_3)_2$	
$n=4$	dihydrocapsaicin (DHC) [**3**]
$n=3$	nordihydrocapsaicin (NDHC) [**4**]
$n=5$	homodihydrocapsaicin-I (HDHC-I) [**8**]
$R=(CH_2)_5CH_2CH_2CH(CH_3)\text{-}CH_2\text{-}CH_3$	homodihydrocapsaicin-II (HCAP-II) [**9**]
$R=(CH_2)_nCH_3$	
$n=7$	nonivamide (NVA) [**10**]
$n=6$	caprylic acid vanillylamide (CVA) [**11**]
$n=8$	decylic acid vanillylamide (DVA) [**12**]

FIGURE 1. Structures of natural and synthetic capsaicinoids.

vanillylamide [**12**] (8). Figure 1 presents a structure summary of natural and synthetic capsaicinoids. Since capsaicinoids are important for the spice and pharmaceutical industries, there have been numerous methods developed to analyze these alkaloids, ranging from simple colorimetric methods to complex methods using gc-ms or hplc-ms. A review by Suzuki and Iwai summarizes a number of analytical methods used in the history of Capsicum research (9).

Inexpensive adulterants have been added to Capsicum Oleoresin for food use and to over-the-counter topical analgesic creams and gels (10). One adulterant, misleadingly named "synthetic capsaicin," is in fact nonivamide [**10**]. Adding to the confusion, **10** has a retention time similar to **1** in most hplc systems. In a study to analyze the content of **1** in currently available over-the-counter products, over 25% of the creams and gels that were analyzed using the present method had this adulterant present (11). Jurenitsch and Kampelmuhler, using argentation-chromatography, described a method in which **1** and **10** were separated (12). Their method included high flow rates and elevated column temperatures, and the time for the complete elution of all capsaicinoids was greater than 80 minutes on a C_8 column. No data were presented for **2**. The present paper discusses a simple hplc method to separate both natural and synthetic capsaicinoids, including *cis*- and *trans*- **1**.

In the present study, the initial conditions included a μBondapak C_{18} column with a mobile phase of 53% MeOH/47% H_2O (13). Using this method, the elution time of the last major capsaicinoid (dihydrocapsaicin) was 45 min, there was no baseline separation of **1** from **2** ($R_s<0.8$), and **1** and **2** were not resolved. It was therefore decided to examine a 5-μm packing material to increase efficiency. With a mobile phase of 60% MeOH/40% H_2O, **1** and **2** were almost resolved ($R_s\approx1$), but **1** and **10** were not resolved.

Complexation chromatography (addition of Ag^+ ions to the mobile phase) increases the selectivity of this system by complexing preferentially with unsaturated capsaicinoids, thus increasing their polarity and decreasing their R_fs. Concentrations of $AgNO_3$ ranging from 15 mM to 30 mM were explored, and 20 mM was found to be appropriate. Using

$AgNO_3$, baseline resolution was achieved for **1** and **10**. A substantial increase in resolution between **1** and **2** was also achieved due to their steric differences at the site of complexation.

Figure 2 shows a chromatogram of some capsaicinoid standards. The retention times observed were 15, 16, 22, 25, 29, 40, 46, and 90 min for **5**, **2**, **1**, **4**, **10**, homocapsaicin, **3**, and **8**, respectively. For quantitative work, an internal standard of propiophenone was used having a R_t of about 9 min.

To ascertain if this method separated the capsaicinoids in Capsicum Oleoresin from one another, the oleoresin was injected into the chromatographic system after reducing the viscosity by adding a small amount of MeOH. Figure 3 is a chromatogram of a typical sample of Capsicum Oleoresin. Capsaicinoids are well separated from each other and from other non-capsaicinoid compounds found in the oleoresin. The peak labeled NVA is tentatively identified as nonivamide based on retention time data and on mass spectrometry; definitive structural proof is forthcoming presently. Using a diluted sample of the oleoresin, the peak at about 10 min did not interfere with the internal standard (propiophenone) when used for quantification studies.

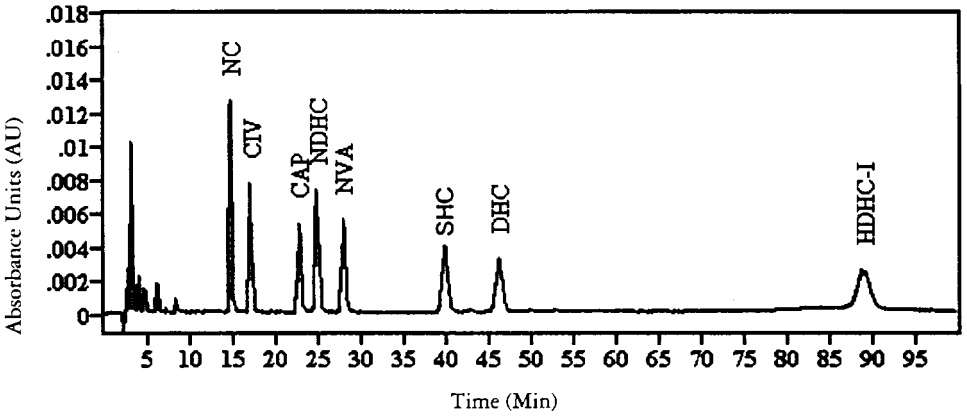

FIGURE. 2. Chromatogram trace of capsaicinoid standards (see Figure 1 for explanation of abbreviations). Conditions: Mobile phase 60% MeOH/40% H_2O with 20 mM $AgNO_3$. Flow rate 1.25 ml/min.

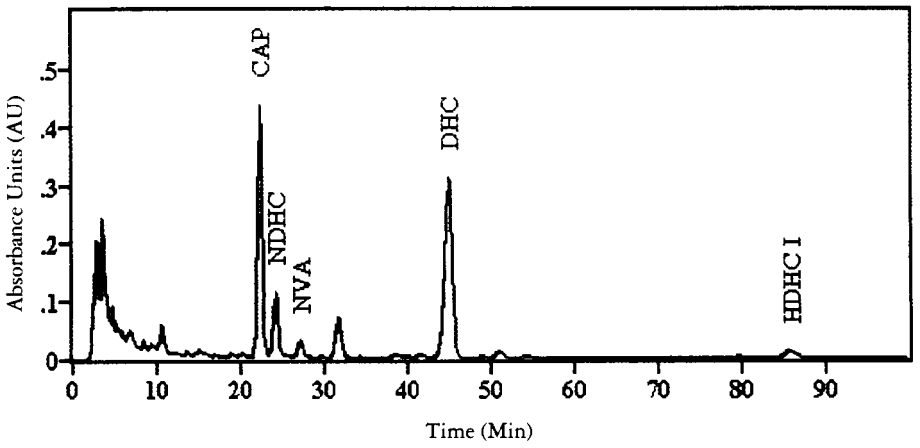

FIGURE 3. Chromatogram trace of Capsicum Oleoresin (see Figure 1 for explanations of abbreviations) Conditions: Mobile phase 60% MeOH/40% H_2O with 20 mM $AgNO_3$. Flow rate 1.25 ml/min.

Due to the importance of **1** as a therapeutic agent, a method to separate and quantitate it from other natural homologues and related adulterants is essential. The hplc technique described here is a reliable and reproducible method which for the first time rapidly resolves all of the known homologues without the need for elevated temperatures. The use of propiophenone as an internal standard allows accurate quantification.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Separations were accomplished using two different Waters hplc systems (Milford, MA). The first system consisted of two pumps (model 510), an autoinjector (model 712), and a model 991 photodiode array detector set at 280 nm. The second system was a LCModule I hplc system (600 model pump, autoinjector, and 486 model detector set at 280 nm) with a 996 photodiode array detector add-on. The column was purchased from YMC (Wilmington, NC) and the column specifications were 4.6×250 mm with 5 μm C_{18} packing material. The mobile phase was comprised of a 60% MeOH/40% H_2O mixture with a $AgNO_3$ concentration of 2.0×10^{-2} M at a flow rate of 1.25 ml/min. It was found that columns from other manufacturers may be used, but in such cases 1.2 ml of glacial HOAc per 1 liter of mobile phase were added. A Waters (Milford, MA) guard column containing C_{18} packing or a guard column packed with pelicular C_{18} material (Upchurch Scientific, Oak Harbor, WA) was used.

Samples of natural capsaicin and dihydrocapsaicin were purchased from Sigma (St. Louis, MO). Nonivamide was purchased from Fluka (Ronkonkoma, NY), zucapsaicin (*cis*-isomer), and capsaicin (*trans*-isomer) were obtained from Macfarlan Smith Ltd. (Edinburgh, UK). Nordihydrocapsaicin, norcapsaicin, homocapsaicin, and homodihydrocapsaicin-I were generously provided by Dr. Kazuhiko Orito (Sapporo, Japan). All standard compounds were used without further purification.

MeOH (hplc grade) was purchased from Fisher Scientific (Pittsburgh, PA) and water was drawn from a Barnstead NANOpure system (Dubuque, IA). The $AgNO_3$ was purchased from Baker (Phillipsburg, NJ). All solvents were filtered with a 0.45-μm filter and degassed before use.

CONDITIONS OF CHROMATOGRAPHY.—Capsaicin was dissolved in a 60% MeOH/40% H_2O solution containing a known amount of propiophenone as the internal standard. A detailed description of the quantification of capsaicin using this mobile phase system is the one outlined in the *United States Pharmacopeial Forum* (14).

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