Journal of Chromatography, 367 (1986) 267–270
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 887

Note

Isolation of naturally occurring capsaicinoids by reversed-phase low-pressure liquid chromatography

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(First received January 14th, 1986; revised manuscript received June 24th, 1986)

Capsaicin and its analogues, called capsaicinoids, are the pungent compounds of the *Capsicum* fruit. Many chromatographic methods have been employed for analytical separation, quantitation and identification of naturally occurring capsaicinoids by gas chromatography[1–4], high-performance liquid chromatography (HPLC)[5–8] and thin-layer chromatography[9–11]. There has been no report on a separation of capsaicinoids on a preparative scale yielding high-purity compounds for investigation of their structurally dependent effects such as sensory, pharmacological or toxicological.

This paper describes a reversed-phase low-pressure liquid chromatographic (LPLC) method which resulted in successful isolation of major capasicinoids, namely capsaicin, dihydrocapsaicin, nordihydrocapsaicin and homodihydrocapsaicin. To separate nordihydrocapsaicin and capsaicin, an intermediate bromination step was required. This method is simple and less costly than preparative-scale HPLC.

EXPERIMENTAL

Reagents

Natural capsaicin (a mixture of naturally occurring capsaicinoids) was obtained from Pfaltz and Bauer (Stanford, CT, U.S.A.). Methanol and water used for HPLC and column separations were HPLC grade. Gibb's reagent used as 0.1% solution in methanol and pyridinium bromide perbromide (PBPB) used as 0.005 *M* solution in glacial acetic acid were from Aldrich (Milwaukee, WI, U.S.A.).

Instrumentation

A heavy-wall glass column (40 cm × 2 cm I.D.) was packed with 40-μm octadecyl-silica (J. T. Baker, Phillipsburgh, NJ, U.S.A.) to a bed height of 19 cm as described by Still *et al.*[12] and Crane *et al.*[13]. Nitrogen from a cylinder served as a driving force for the mobile phase.

The HPLC system (Waters Assoc., Milford, MA, U.S.A.) equipped with 10-μm μBondapak C_{18} analytical column (30 cm × 3.9 mm I.D.), a 440 dual beam UV–visible absorbance detector set at 280 nm, a U6K injector and a 6000 A solvent delivery system was used for monitoring the composition of fractions eluted from the low-pressure column. A mobile phase consisting of methanol–water (60:40) at a flow-rate of 1.8 ml/min was used.

0021-9673/86/$03.50

TABLE I

CONDITIONS FOR LOW-PRESSURE CHROMATOGRAPHIC SEPARATION OF CAPSAICINOIDS

Column: 40 × 2 cm I.D.; bed height: 19 cm; packing: 40-μm octadecyl-silica.

Methanol–water	*Solvent volume (ml)*	*Flow-rate (ml/min)*	*Fraction size (s)*	*Number of fractions*
50:50	800	7.2	–*	–
55:45	300	7.5	30	85
60:40	150	7.8	30	40
70:30	150	8.6	45	25
80:20	200	10.2	90	10

* Not collected.

Procedure

A 10% solution of natural capsaicin in methanol (0.5 ml) was applied to the top of the adsorbent bed. A stepwise elution with methanol–water in the range of 50:50 to 80:20 was used with nitrogen pressure regulated to about 13.2 p.s.i. The conditions for the elution are summarized in Table I. Since the elution was very fast no fraction collector was used, and the fraction size was measured by time rather than by volume (Table I). To determine which fractions contained eluted capsaicinoids, a few microliters of each fraction were spotted on a filter paper, sprayed with Gibb's reagent and exposed to ammonia vapors. Fractions which developed a blue color with Gibb's reagent were dried in a freeze drier, dissolved in 0.5 ml methanol and every third or fourth fraction was analyzed by HPLC. Fig. 1 represents the HPLC separation of mixture of capsaicinoids. Fractions containing the pure capsaicinoids were combined. Fractions containing a mixture of unseparated nordihydrocapsaicin and capsaicin were subjected to bromination with PBPB. The solution of PBPB was added gradually to the mixture of nordihydrocapsaicin and capsaicin dissolved in chloroform at room temperature. Next, the reaction mixture was washed three times with 1 *M* hydrochloric acid, then with a saturated solution of sodium hydrogen carbonate till no carbon dioxide was produced, and finally rinsed three times with water and dried with anhydrous sodium sulphate. After evaporation of the chloroform, the sample was dissolved in methanol and run through the low pressure column to separate nordihydrocapsaicin from dibromocapsaicin.

The purity and identity of the isolated capsaicinolds was determined by HPLC, mass spectrometry and infrared spectrometry[14].

RESULTS AND DISCUSSION

Scaling up from analytical HPLC to less efficient preparative LPLC separation can be difficult especially with compounds whose retention times are similar. In reversed-phase separations, compounds containing long saturated aliphatic side-chains are co-eluted with their homologues containing one double bond but one carbon atom more[15]. This is the case with the pairs nordihydrocapsaicin, capsaicin and

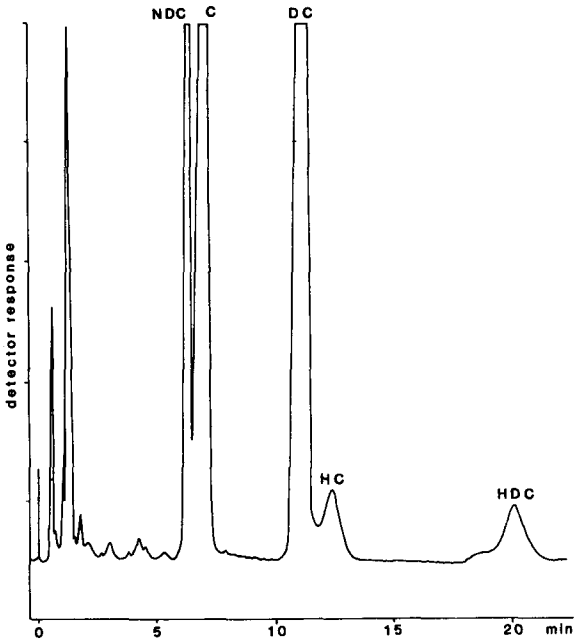

Fig. 1. HPLC separation of natural capsaicinoids on a 10 μm μBondapak C_{18} column (30 cm × 3.9 mm I.D.) with methanol–water (60:40); flow-rate 1.8 ml/min; UV detection at 280 nm; 58.5 μg injected at 0.05 a.u.f.s. NDC = Nordihydrocapsaicin; C = capsaicin; DC = dihydrocapsaicin; HC = homocapsaicin; HDC = homodihydrocapsaicin.

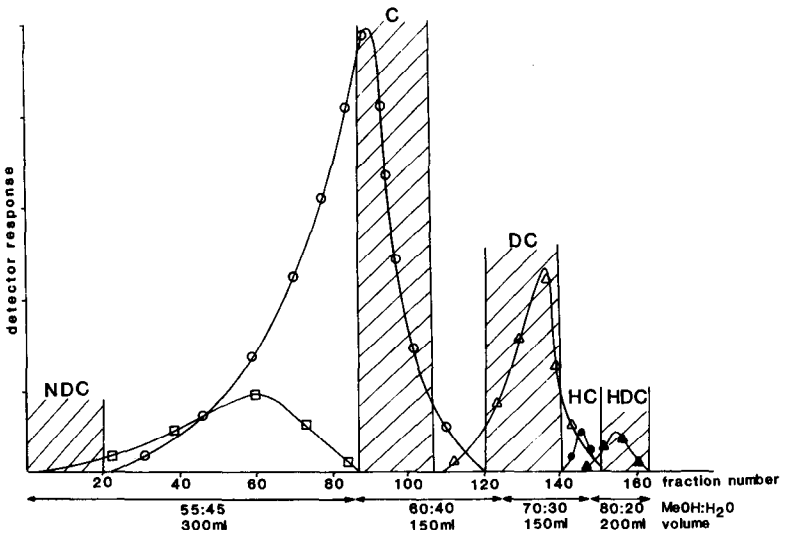

Fig. 2. Elution pattern of natural capsaicinoids from reversed-phase low-pressure chromatographic column as determined by off-line analytical HPLC fraction control. A 50-mg sample of a capsaicinoid mixture was applied. Compounds: □ NDC = nordihydrocapsaicin; ○ C = capsaicin; △ DC = dihydrocapsaicin; ● HC = homocapsaicin; ▲ HDC = homodihydrocapsaicin. Shaded areas indicate ranges of fractions containing pure capsaicinoids. Conditions as in Table I.

dihydrocapsaicin, homocapsaicin (See Fig. 1). Under optimal LPLC conditions neither of the pairs could be separated.

As can be seen in Fig. 2, a stepwise elution with methanol–water was found to be optimal for preparative separation of capsaicinoids by low-pressure chromatography. Pure capsaicin, dihydrocapsaicin and homodihydrocapsaicin were obtained by combining corresponding fractions. Pure nordihydrocapsaicin was isolated from the mixture of nordihydrocapsaicin and capsaicin by brominating capsaicin and repeating the separation on the low-pressure column. Since the structure of dibromocapsaicin resembles that of dihydrocapsaicin, bromination of capsaicin changed its retention time to that of dihydrocapsaicin, making the separation of nordihydrocapsaicin from capsaicin possible. Because nordihydrocapsaicin contains a saturated acid side-chain, its structure and elution properties were not changed by bromination. Due to the very low initial concentration of homocapsaicin and its co-elution with dihydrocapsaicin, isolation of homocapsaicin was not attempted.

The final yields of nordihydrocapsaicin, dihydrocapsaicin and homodihydrocapsaicin were in the range of 80 to 90% while yield of capsaicin was lower because of the loss due to the bromination reaction.

Fraction control by off-line analytical HPLC has the advantage over on-line UV detection in that the content of each compound in effluent can be determined as demonstrated in Fig. 2.

It is therefore possible, as described in this paper, to obtain major capsaicinoids from natural sources in a highly purified state by an inexpensive and simple reversed-phase low-pressure column chromatography.

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