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PENTAFLUOROBENZYLATION OF CAPSAICINOIDS FOR GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION

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SUMMARY

The alkylation of capsaicinoids with pentafluorobenzyl (PFB) bromide for gas chromatography with electron-capture detection was studied. The PFB derivatives were formed within 30 min at 60°C and did not show any signs of decomposition. The alkylated capsaicinoids had a hydrophobic character making them suitable for gas chromatography on a non-polar column. The electron-capture response for the PFB derivatives of capsaicinoids was very sensitive and amounts of capsaicin down to 10 pg were easily detected. The method was succesfully applied to measurement of capsaicinoids in *Capsicum* products using vanillylamide of octanoic acid as an internal standard.

INTRODUCTION

Due to growing interest in the pharmacological actions of capsaicin and its analogues called capsaicinoids[1–3], there is a need for sensitive and accurate analytical methods for their detection. Colorimetric[4], direct and differential spectrophotometric[5,6], thin-layer and paper chromatographic[7,8] methods can be used only for the analysis of total capsaicinoids. Gas chromatography (GC)[9,10], high-performance liquid chromatography (HPLC)[11–13] as well as high-performance thin-layer chromatography (HPTLC)[4] have been succesfully used for identification and quantitation of individual capsaicinoids with varying degrees of sensitivity. Recently Kawada *et al.*[13] reported an HPLC method with electrochemical detection (ED) capable of detecting capsaicin down to the picogram level. However, this highly sensitive method does not appear to be very practical because it requires an expensive detector that is not commonly found in research laboratories.

GC with electron-capture detection (GC–ECD) has been demonstrated to be a very sensitive tool for quantitative determinations of low concentrations of a variety of compounds. Compounds with low or no electron-capture response can usually be made electron-capture sensitive by means of derivatization. Capsaicinoids, due to the presence of a polar hydroxyl group in the molecules (see Fig. 1) require derivatization to improve their chromatographic performance. Silylation[9] and methylation[10] of a hydroxyl group have been used to modify the chromatographic properties of capsai-

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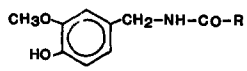

naturally occurring:

R = $(CH_2)_5-CH-(CH_3)_2$ nordihydrocapsaicin

R = $(CH_2)_4-CH=CH-(CH_3)_2$ capsaicin

R = $(CH_2)_6-CH-(CH_3)_2$ dihydrocapsaicin

synthetic analogues:

R = $(CH_2)_7-CH_3$ vanillylamide of nonanoic acid

R = $(CH_2)_6-CH_3$ vanillylamide of octanoic acid

Fig. 1. Structures of capsaicinoids.

cinoids by rendering them less polar and thus reducing undesirable column interactions. Derivatives containing a pentafluorobenzyl (PFB) group have been reported to have a high electron-capture response[15,16]. Pentafluorobenzylation has been applied to a broad spectrum of organic compounds containing an active hydrogen by reacting the compounds with PFB bromide in acetone in the presence of potassium carbonate[17–20].

The goal of this work was to study conditions for the pentafluorobenzylation of capsaicinoids and to evaluate the GC and ECD properties of the PFB derivatives.

EXPERIMENTAL

Gas chromatography

Electron-capture detection. The GC analysis of PFB derivatives of capsaicinoids was performed on a Varian 3400 gas chromatograph (Varian, Palo Alto, CA, U.S.A.) fitted with a ^{63}Ni-electron-capture detector. The column was a 30 m × 0.326 mm I.D. fused-silica capillary column with a chemically bonded DB-5 stationary phase at a film thickness of 0.25 μm (J&W Scientific, Rancho Cordova, CA, U.S.A.). Nitrogen was used as a carrier gas at a flow-rate of 1.5 ml/min as well as a make-up gas at a flow-rate of 20 ml/min. The oven temperature program for optimal peak separation was as follows: initial temperature = 200°C, initial time = 0 min, program rate = 8°C/min, final temperature = 265°C. The injector and detector temperatures were 250 and 350°C, respectively. A 1-μl volume of a sample was injected at an attenuation of 32 in a splitless mode with an open insert fitted in a 1075 split/splitless capillary injector. Peaks were recorded and integrated by a 3390A Hewlett-Packard integrator.

Flame ionization detection (FID). A Varian Model 3700 gas chromatograph equipped with a FID system was used for a model study of the pentafluorobenzylation reaction of capsaicinoids. A glass column (2.1 m × 2 mm I.D.) packed with 5% SE-52 on Chromosorb W AW DMCS 100–120 mesh (Supelco, Bellefonte, PA, U.S.A.) was prepared. The injector and detector temperatures were 250 and 270°C, respectively. The temperature program was the same as in GC–ECD. A 2-μl volume of a sample was injected into the gas chromatograph at a sensitivity setting of $8 \cdot 10^{-10}$ A f.s. Peaks were recorded using a Varian Model 9176 recorder and areas integrated by Varian CDS 111 data system.

Materials

PFB bromide was obtained from Pierce (Rockford, IL, U.S.A.). Vanillylamide of nonanoic acid, a commercially available synthetic capsaicin analogue, used in the study of the derivatization reaction, was from Pfaltz and Bauer (Waterbury, CT, U.S.A.). Pure nordihydrocapsaicin, capsaicin and dihydrocapsaicin used for GC standards were isolated from natural capsaicin (Pfaltz and Bauer), by liquid chromatographic (LC) methods[10,21].

Vanillylamide of octanoic acid used as an internal standard (I.S.) for determination of capsaicinoids in *Capsicum* products was prepared from vanillylamine and octanoyl chloride[22]. The concentration of vanillylamide of octanoic acid was adjusted to yield a GC peak area comparable to the areas of the capsaicinoid peaks. Solution of I.S. was added to the samples before the extraction.

Tetracosane and 1,2-dibromododecane used as I.S. for GC–FID and GC–ECD injections, respectively, were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.) and from Fluka Chemical (Ronkonkoma, NY, U.S.A.).

All solvents used for the extraction, purification and derivatization of samples for GC analysis were "Baker Analyzed" reagents (J. T. Baker, Phillipsburg, NJ, U.S.A.).

Synthesis and characterization of PFB capsaicinoids

The PFB derivatives of two representative capsaicinoids, namely vanillylamide of nonanoic acid and capsaicin, were synthesized as follows: 40 mg capsaicinoid (about 0.13 mmol) were dissolved in 2 ml acetone in a micro round-bottom flask; 100 μl PFB bromide (0.71 mmol) and 300 mg of anhydrous potassium carbonate were added. The mixture was heated under reflux for 5 h. The acetone was evaporated under a gentle stream of nitrogen, the residue dissolved in 10 ml distilled water and the derivative was extracted into diethyl ether. After washing with 0.1 *M* sodium hydroxide, 0.1 *M* hydrochloric acid and water, the ether was evaporated and the derivative crystallized from the acetone–water mixture.

The purity and identity of the synthesized derivatives was established by IR spectrometry, mass spectrometry (MS) and melting point determination. IR and MS demonstrated that only the phenolic hydroxyl group underwent pentafluorobenzylation. No alkylation occurred on an amide group although pentafluorobenzylation[23,24] as well as other derivatizations[25–28] involving an active hydrogen of an –NH group have been reported. Characteristic bands of a hydroxyl group, i.e. at 3400–3200 cm^{-1} and at 1200 cm^{-1} were absent in the IR spectra of both derivatized compounds. This allowed the detection of a characteristic band of an "associated" –NH group of a secondary amide at 3300 cm^{-1}, indicating no change in the amide structure and alkylation of only a hydroxyl group. The mass spectra confirmed the introduction of one PFB group into the molecule. Both derivatives showed molecular ion peaks increased by 180 a.m.u. (*m/e* 473 and *m/e* 485 for PFB derivative of vanillylamide of nonanoic acid and capsaicin, respectively). Fragment ions, also increased by 180 a.m.u., showed patterns similar to the parent compounds with additional peaks present in the spectra corresponding to cleavage of the PFB group from the derivatized molecule. PFB capsaicin melted at 87–88.5°C while PFB vanillylamide of nonanoic acid softened at 104.5°C and melted at 106.5–108°C.

These synthesized analogues were used for verification of the identity of the

products of the derivatization reaction by co-chromatography and MS. Injection of a reaction mixture containing either derivatized vanillylamide of nonanoic acid or capsaicin yielded single peaks with identical retention times as those of synthesized analogues using both FID and ECD. Also, mass spectra of both derivatized capsaicinoids were identical with their respective synthesized analogues.

Model study of capsaicinoid pentafluorobenzylation

The optimal conditions for the pentafluorobenzylation of capsaicinoids were determined using vanillylamide of nonanoic acid and tetracosane as an I.S. Tetracosane did not undergo derivatization and therefore served as a constant reference. A 7.126 m*M* solution of vanillylamide of nonanoic acid was prepared in acetone containing tetracosane (0.25 g/1000 ml); 1 ml of the above solution was transferred to the screw-cap vial. The reaction was carried out varying the following conditions: the reaction time at different temperatures, the amount of anhydrous potassium carbonate, the volume of acetone in the reaction mixture at different temperatures, and the amount of PFB bromide.

After stopping the reaction, samples were evaporated to dryness under a stream of nitrogen at 40°C; 1 ml benzene and 2 ml water were added and the vials shaken vigorously. After the layers separated, 2 μl of the benzene layer were injected into the GC–FID system. Changes of both the PFB derivative as well as underivatized vanillylamide of nonanoic acid were plotted against the I.S. peak.

Sample preparation

Samples were prepared by a procedure described elsewhere[10] involving extraction of capsaicinoids with acetone in a Soxhlet apparatus, purification with petroleum ether and reextraction of capsaicinoids from the aqueous phase with diethyl ether. The diethyl ether was evaporated, the residue dissolved in 5 ml acetone and 1 ml of this solution transferred into a screw-cap vial. The volume was reduced to 0.3 ml with a gentle stream of nitrogen at 40°C. Next, 500 mg anhydrous potassium carbonate and 20 μl PFB bromide were added, the vial was tightly capped and the reaction mixture heated for 0.5 h in a water bath maintained at 60°C. The vials were carefuly agitated every 10 min. After completion of the reaction, the acetone and the derivatizing agent were evaporated under a stream of nitrogen at 40°C and 1 ml benzene and 2 ml distilled water were added. The mixture was vigorously shaken, the layers were allowed to separate and 15 μl of the organic layer diluted further with 1 ml acetone. A 1-μl volume of this solution was injected into the GC–ECD system. The concentration of the alkylated capsaicinoids was determined from the ratio of the peak areas to that of the I.S. by reference to calibration curves for nordihydrocapsaicin, capsaicin and dihydrocapsaicin.

RESULTS AND DISCUSSION

Optimal conditions for capsaicinoid pentafluorobenzylation

The pentafluorobenzylation was considered complete when no underivatized vanillylamide of nonanoic acid was detected by GC–FID, and the amount of derivative formed was constant. As can be seen in Fig. 2, large differences were found in the extent of pentafluorobenzylation of vanillylamide of nonanoic acid with varying

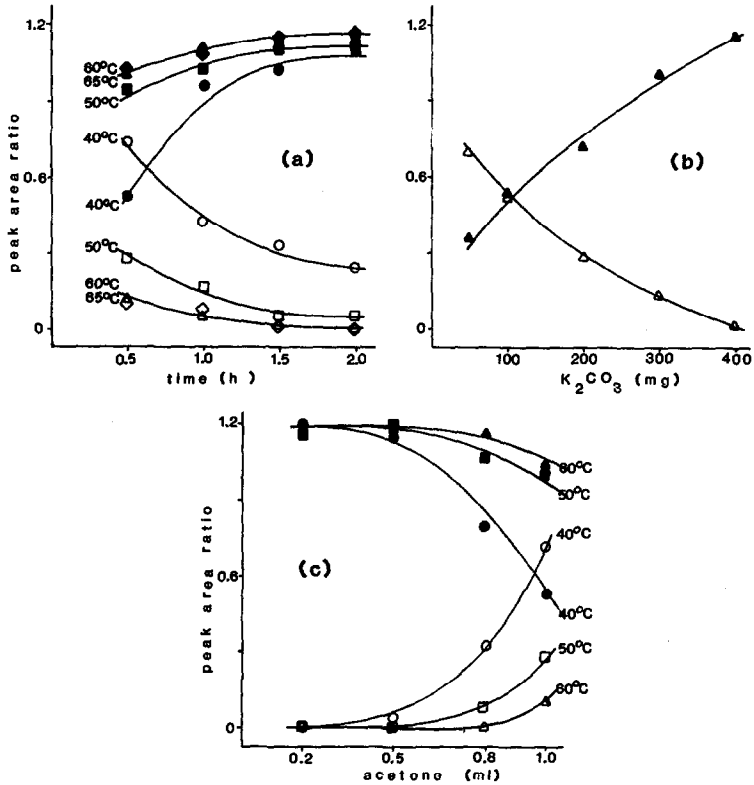

Fig. 2. Yield of pentafluorobenzylated vanillylamide of nonanoic acid as determined by GC–FID; 7.126 μmol vanillylamide of nonanoic acid reacted with 4 μl PFB bromide; underivatized vanillylamide of nonanoic acid at 40°C (○), 50°C (□), 60°C (△), 65°C (◇); PFB derivative at 40°C (●), 50°C (■), 60 °C (▲), 65°C (◆). (a) Effect of reaction time at different temperatures with 400 mg K_2CO_3 used in 1 ml acetone; (b) effect of amount of K_2CO_3 in the reaction mixture; reaction carried out for 1.5 h at 60°C in 1 ml acetone; (c) effect of volume of acetone at different temperatures; reaction carried out for 0.5 h in the presence of 400 mg K_2CO_3.

time, temperature, the amount of anhydrous potassium carbonate and the volume of acetone in the reaction mixture. The reaction was practically complete after heating for 1.5 h at 60°C (see Fig. 2a). As shown in Fig. 2b, 400 mg potassium carbonate were adequate, but any smaller amount resulted in incomplete derivatization. As seen in Fig. 2c, when the volume of acetone was reduced to 0.2 ml, 0.5 h at 40°C was sufficient to complete pentafluorobenzylation. The reaction was found to be equimolar and 1 μl PFB bromide (7.126 μmol) was enough for the complete derivatization of 1 ml of the vanillylamide of nonanoic acid solution.

Thus, it was concluded that the pentafluorobenzylation reaction of vanillylamide of nonanoic acid was complete and reproducible when the reaction mixture was heated for 0.5 h in the presence of 400 mg anhydrous potassium carbonate in 0.3 ml acetone and when an adequate volume of PFB bromide was added.

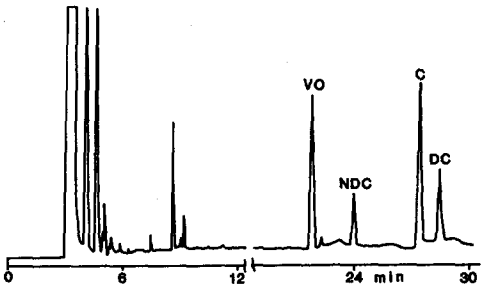

Fig. 3. GC–ECD of pentafluorobenzylated standard mixture of capsaicinoids; VO = vanillylamide of octanoic acid (I.S.), NDC = nordihydrocapsaicin, C = capsaicin, DC = dihydrocapsaicin; 1 μl injected at attenuation × 32 representing 3.0, 10.5 and 6.0 ng of NDC, C and DC, respectively; GC conditions: capillary column (30 m × 0.326 mm I.D.) with chemically bonded DB-5 stationary phase; nitrogen flow-rate = 1.5 ml/min, initial temperature = 200 °C, initial time = 0 min, program rate = 8 °C/min, final temperature = 265 °C.

Electron-capture detection of PFB derivatives of capsaicinoids

Using the conditions determined for pentafluorobenzylation of vanillylamide of nonanoic acid, a series of capsaicin solutions in the range of 4.3–192.4 μM was derivatized and injected with 1,12-dibromododecane (0.14 g/1000 ml) as an I.S., into the GC–ECD system. A linear relationship (r^2 = 0.99) was obtained within the range tested. The PFB capsaicin was stable in solution for several weeks at room temperature. A series of dilutions of PFB capsaicin was injected to determine the electron-capture detection limit. As little as 10 pg of capsaicin was easily detected. This value is comparable to the sensitivity of HPLC–electrochemical detection (ED), *i.e.*, 12 pg reported by Kawada *et al.*[13]. Sensitivities of other chromatographic systems for capsaicin are much lower: 100 ng by HPLC with UV (279 nm) detection[11], 3 ng by HPLC with fluorescence (270/330 nm) detection[12] and 12.5 ng by GC–FID of methyl derivatives[29].

The applicability of the method was examined using the extracts of two kinds of peppers and one oleoresin. To assure completeness of the pentafluorobenzylation of the extracts, more PFB bromide and anhydrous potassium carbonate than needed for a pure capsaicinoid was used in case other substances present in the extracts underwent derivatization. Vanillylamide of octanoic acid was used as an I.S. in the extracts and standard solutions. Standard solutions for the determination of the three major

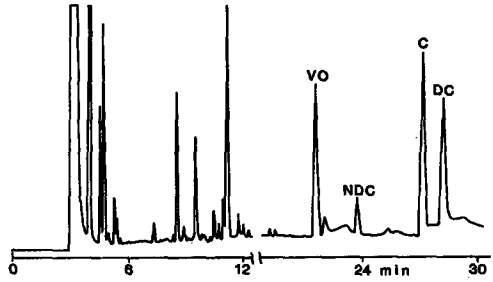

Fig. 4. GC–ECD of pentafluorobenzylated extract of jalapeno peppers; codes and GC conditions as in Fig. 3.

TABLE I

CAPSAICINOID CONTENT IN SELECTED *CAPSICUM* PRODUCTS AS DETERMINED BY GC–ECD OF PFB DERIVATIVES

Product	*Capsaicinoid content* (mg/100 g original wt.)*					
	*NDC***	*C.V. (%)*	*C***	*C.V. (%)*	*DC***	*C.V. (%)*
Jalapeno peppers	3.36	2.7	20.81	2.3	17.06	3.5
Red finger hot peppers	4.41	4.0	28.08	2.7	10.04	3.9
Oleoresin (African chillies)	763.90	3.0	1154.62	1.3	1182.21	3.7

* Average of three determinations.
** NDC = Nordihydrocapsaicin, C = capsaicin, DC = dihydrocapsaicin.

capsaicinoids were in the ranges 1.5–6.0, 5.25–21.0, and 3.0–12.0 μg/ml I.S. solution for nordihydrocapsaicin, capsaicin and dihydrocapsaicin, respectively. The relative concentrations of each capsaicinoid in the standard mixtures were such that they reflected the relative content of capsaicinoids in *Capsicum* extracts (see Figs. 3 and 4).

The results of the determination of capsaicinoid content in selected *Capsicum* products are presented in Table I. Coefficient of variation (C.V.) in the range of 1.3–4.0% based on three replications indicated excellent reproducibility of the method. This was mainly due to use of vanillylamide of octanoic acid, a synthetic analogue of capsaicinoids, as an I.S. Its addition before sample preparation decreased the error in the extraction and purification and its behaviour during pentafluorobenzylation identical with capsaicinoids reduced the error of derivatization. The extraction and purification procedure of capsaicinoids from *Capsium* products used in this study was examined earlier and the recovery established[10].

As seen in Fig. 3 and 4, excellent GC separation of PFB derivatives of capsaicinoids was obtained using the GC conditions described. Peaks were very well resolved and therefore easy to quantitate.

CONCLUSIONS

This study demonstrated that pentafluorobenzylation is a useful method of derivatization for capsaicinoids. This derivatization results in formation of stable analogues suitable for GC with increased sensitivity to ECD. Use of vanillylamide of octanoic acid or other closely related compound as an I.S. is strongly recommended for reproducible results.

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