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Note

High-performance liquid chromatographic analysis of Aspartame

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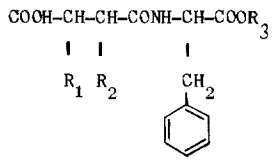
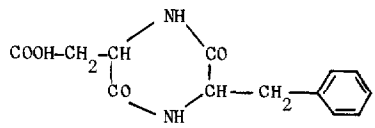
Aspartame[1] (L-α-aspartyl-L-phenylalanine methyl ester, I) is a sweetener discovered 20 years ago, but which has been marketed only since 1981 in the U.S.A. after final approval[2] by the Food and Drugs Administration. It has been used as a table-top sweetener mainly in European countries, and as a food additive and a sugar substitute in carbonated soft drinks in the U.S.A.

Its stability is strongly dependent on the pH in aqueous solution[3] and on temperature[4] during drying and storage; the products of degradation are the dipeptide L-α-aspartyl-L-phenylalanine (II) and chiefly 5-benzyl-3,6-dioxo-2-piperazine-acetic acid (III), hereafter diketopiperazine, which are non-toxic[5] but devoid of sweetness[1].





III

I : $R_1 = H$; $R_2 = NH_2$; $R_3 = CH_3$

II : $R_1 = H$; $R_2 = NH_2$; $R_3 = H$

IV : $R_1 = H$; $R_2 = NH\text{-}CHO$; $R_3 = CH_3$

V : $R_1 = NH\text{-}CHO$; $R_2 = H$; $R_3 = CH_3$

VI : $R_1 = NH_2$; $R_2 = H$; $R_3 = CH_3$

The official method[6] of analysis of Aspartame requires a titration with lithium methoxide, but chromatographic methods, using an amino acid analyzer[7,8] or liquid chromatograph[8–12], are also available. We now report a method designed to obtain a very short analysis time and to separate Aspartame at a higher retention time with respect to the above degradation products, and intermediates in its synthesis.

EXPERIMENTAL

Materials and reagents

Reference standards of Aspartame (I), the dipeptide (II), diketopiperazine (III),

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N-formyl-α-aspartame (IV), N-formyl-β-Aspartame (V), β-Aspartame (L-β-aspartyl-L-phenylalanine methyl ester, VI), were prepared in Pierrel's Chemical Synthesis Laboratories. Their purity was tested by high-performance liquid chromatography (HPLC), IR, ^{1}H NMR and C,H,N analysis and found to be not less than 99% on a dry basis. Aspartame samples were from Pierrel. Potassium dihydrogen phosphate and 85% phosphoric acid were reagent grade from E. Merck (Darmstadt, F.R.G.), while acetonitrile was HPLC grade from the same supplier.

HPLC

An Hewlett-Packard Model 1090 A chromatograph equipped with a Model 1040 variable-wavelength UV detector (diode array) operating at 215 nm and a Model 3390 A recording integrator was employed. The column (120 × 4 mm) was packed with 5-μm Hypersil MOS (Hewlett-Packard, Cernusco sul Naviglio, Italy).

The mobile phase was prepared as follows. Potassium dihydrogen phosphate (1.36 g) was dissolved in distilled water (800 ml) and a 5% (w/v) solution of phosphoric acid was used to adjust the pH to 2.5. Finally the mixture was brought to volume (1 l) and filtered through a 0.45-μm membrane. This solution (770 parts) was diluted in acetonitrile (230 parts) and degassed in an ultrasonic bath by applying a moderate vacuum.

The temperature of the column was maintained at 25°C and the flow-rate of the mobile phase was 2 ml/min.

Sample and standard preparations

Aspartame (*ca.* 50 mg) was weighed in a volumetric flask (100 ml) and dissolved in acetonitrile (25 ml) and a 0.01 *M* phosphate buffer, pH 4.5. Solutions of compounds II–VI at concentrations 1/50 of that of Aspartame were prepared similarly. Aliquots (10 μl) of these solutions were injected into the chromatograph. Quantitation was performed by use of an appropriate external standard in similar concentration, in triplicate experiments.

RESULTS AND DISCUSSION

As Aspartame had been produced by Pierrel since 1981, we needed a procedure not only for the quantitation of Aspartame in the final product, but also for the identification of the impurities present. Apart from the amino acid analyzer[7,8], HPLC is usually more sensitive to small quantities of impurities. An excellent method for the separation of impurities in Aspartame bulk products was presented by Signoretti *et al.*[10], requiring about 14–16 min.

In our synthesis[13] of Aspartame there is the need to separate α- from β-Aspartame, derived respectively from the hydrolysis of N-formyl-α- or β-Aspartame, which result from the coupling between L-phenylalanine methyl ester and N-formyl-aspartic anhydride. In fact this acylation step affords a mixture of the α- and β-isomers. In the final product, mainly during the later steps of the synthesis and in the recovery of the dried products, dipeptide (II) and diketopiperazine (III) have to be monitored accurately, as stated before. Finally a very short analysis time is the key rapid monitoring of the process.

Fig. 1 shows the separation of an artificial mixture of Aspartame and related

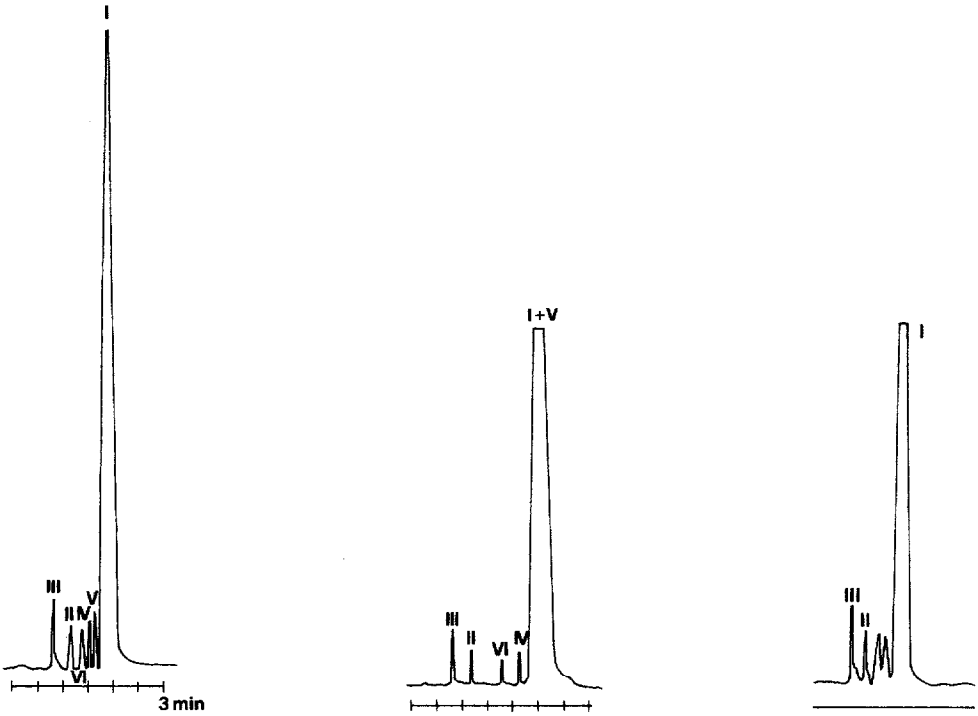

Fig. 1. Chromatogram of an artificial mixture of aspartame and related products.

Fig. 2. Chromatogram obtained by lowering the acetonitrile content in the mobile phase to 18%.

Fig. 3. Chromatogram obtained by increasing the acetonitrile content in the mobile phase to 25%. Compounds IV–VI are eluted in the two unlabelled peaks.

impurities II–VI, each at 1% of the level of I. The resolution of the six-component mixture is achieved in less than 2.5 min. The quantity of acetonitrile is the critical parameter in the separation of the substances: small changes in its concentration, lower or higher than the value of 23%, make the separation impossible. Lowering the acetonitrile concentration to 18% (fig. 2) results in better resolution of some products but causes compound V to overlap Aspartame; increasing its content to 25% (Fig. 3) does not allow a complete resolution, as compounds V and VI have the same retention time. Another important parameter is the pH. Fig. 4 shows a chromatogram of an artificial mixture in which Aspartame is approximately at the same concentration as those of the other products: it is seen that the retention times are completely different to those presented in Fig. 1; Aspartame is less retained than its isomer (VI) and intermediate (V). Thus it is practically impossible to determine compounds V and VI in a trace analysis (Fig. 5).

Table I collects the data corresponding to the calibration curve for Aspartame in Fig. 6, obtained under the same conditions as Fig. 1. The correlation coefficient[14], $r = 0.9998$, is excellent in the range 0.4–0.6 mg/ml Aspartame, and the coefficient of variation is lower than 1% at each concentration tested. Table II shows data pertaining to the quantitation of compounds II–VI at a concentration relative to that

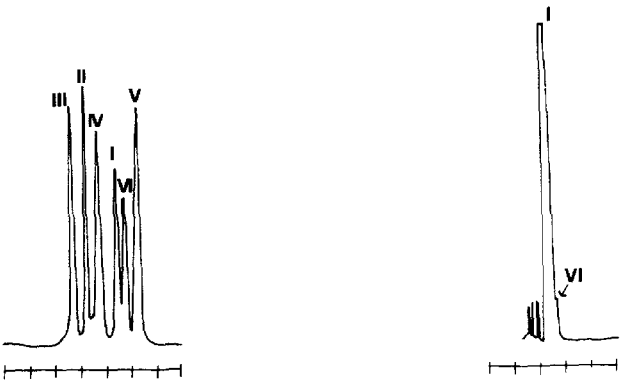

Fig. 4. Separation obtained at pH 4.5 of an artificial mixture of components I–VI each at 1% concentration.

Fig. 5. Chromatogram obtained under the same conditions as in Fig. 4, but with β-Aspartame (VI) at 1% of the concentration of Aspartame (I).

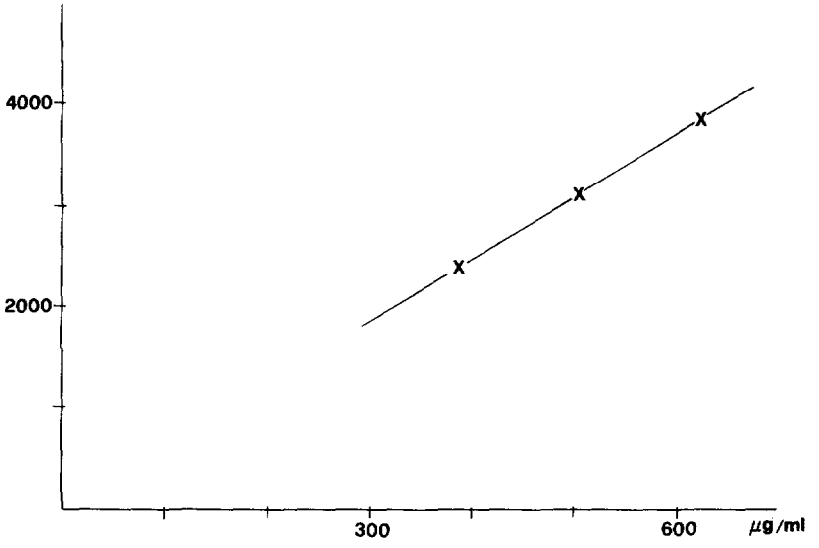

Fig. 6. Calibration curve of Aspartame (I) showing area counts *vs.* concentration (μg/ml).

of Aspartame of about 0.4% for diketopiperazine(III), the dipeptide II and β-Aspartame (VI) and 0.2% each for N-formyl-α- and β-Aspartame (IV) and (V), the last two compounds normally being absent in Aspartame batches.

The HPLC method described may be applied using different commercial columns and liquid chromatographs; Fig. 7 shows the chromatogram of the same artificial mixture as in Fig. 1 with the same mobile phase and chromatographic conditions, but using a 5-μm reversed-phase C_8 column of different size (25 × 0.4 cm; Brownlee Labs., Italab, Florence) and a Perkin-Elmer Model 3B chromatograph with

TABLE I

VALIDATION OF ASPARTAME ANALYSIS

Experiment	*Aspartame concentration (mg/ml)*		
	0.387	*0.505*	*0.625*
1	2413.9*	3095.0	3896.3
2	2371.0	3132.0	3895.2
3	2390.0	3130.0	3871.0
4	2415.0	3078.0	3811.0
5	2409.0	3085.0	3849.0
Average	2399.2	3104.0	3864.4
Standard deviation	18.5	25.4	35.9
Coefficient of variation (%)	0.77	0.81	0.92

* Area counts.

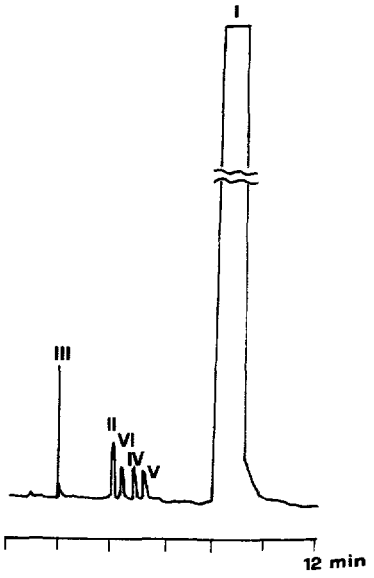

Fig. 7. Chromatogram of the same artificial mixture as in Fig. 1 but obtained on a Perkin-Elmer 3B chromatograph.

LC 75 detection and integrator Σ 10. The separation of impurities is excellent, but the analysis time is about four times that obtained with the previous columns; however, it is still less than needed by other methods.

Further work is in progress to find other methods for separating intermediates of the chemical synthesis.

TABLE II

STATISTICAL ANALYSIS OF THE IMPURITIES II–VI IN ASPARTAME

	Diketopiperazine (III)	*Dipeptide (II)*	*β-Aspartame (VI)*	*Formyl-α-Aspartame (IV)*	*Formyl-β-Aspartame (V)*
Concentration (μg/ml)	2.01	2.07	1.98	0.84	1.04
1	24.92*	15.51	9.99	4.78	4.63
2	22.5	14.65	10.40	5.15	4.91
3	23.56	14.47	9.94	5.69	5.34
4	24.23	16.45	9.14	4.98	4.89
5	22.10	15.02	10.56	5.33	4.64
Average	23.462	15.22	10.006	5.186	4.882
Standard deviation	1.17	0.79	0.56	0.35	0.288
Coefficient of variation (%)	4.98	5.2	5.6	6.7	5.9

* Area counts.

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