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Direct stereochemical resolution of aspartame stereoisomers and their degradation products by high-performance liquid chromatography on a chiral crown ether based stationary phase

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ABSTRACT

The direct stereochemical resolution of the four stereoisomers of aspartame (N-DL-α-aspartyl-DL-phenylalanine methyl ester) and their degradation products was achieved on a high-performance liquid chromatography chiral stationary phase based upon a chiral crown ether. The chromatographic conditions included a mobile phase composed of aqueous perchloric acid adjusted to a pH of 2.8 and modified with 1.5% of 2-propanol and a temperature gradient. The active L,L-isomer (sold under the brand name NutraSweet) was measured in a diet cola and coffee sweetened with NutraSweet. Degradation products of NutraSweet were also detected but no racemization of stereochemical contamination was observed.

INTRODUCTION

Aspartame (N-DL-α-aspartyl-DL-phenylalanine methyl ester, or APM, Fig. 1) is a dipeptide derivative which can exist in four possible stereoisomeric forms: L,L-APM, D,D-APM, D,L-APM and L,D-APM. The L,L-isomer (NutraSweet) is a low-calorie sweetener widely used as a sugar substitute in diet soft drinks and other food products.

Various high-performance liquid chromatographic (HPLC) methods have been developed for the analysis of L,L-APM in food products including isocratic[1–3] and gradient[4] approaches. These assays were able to separate L,L-APM from other ingredients such as caffeine, benzoic acid, colorants and flavors. However, these methods did not measure L,L-APM degradation products which may also be present.

L,L-APM is unstable over time and the degradation of the compound is accelerated by elevated temperature and pH[5–9]. For example, in diet soft drinks, only 30 to 40% of the sweetener remained after 6 months of storage at ambient temperature[6]. The major degradation pathway in acidic media involves initial cyclization to a diketopiperazine (DKP) followed by hydrolysis which yields the

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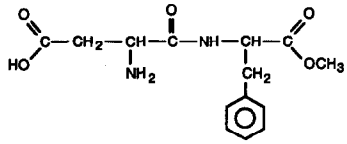
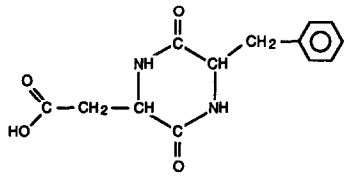
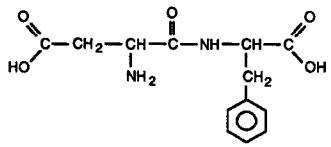


Aspartame (APM)



Diketopiperazine (DKP)



Aspartyl-phenylalanine (Asp-Phe)

Fig. 1. APM and its breakdown products.

aspartyl-phenylalanine dipeptide, Asp-Phe, which is eventually cleaved to the free amino acids, aspartic acid (Asp) and phenylalanine (Phe)[9]. A second pathway in basic media involves the production of the Asp-Phe through direct hydrolysis of the APM[9]. The structures of the DKP and dipeptide are presented in Fig. 1. The breakdown of APM also results in the release of methanol.

While a number of methods have been developed to measure the decomposition products of L,L-APM[5–7], they do not take into account any possible racemization during the synthesis or degradation processes of L,L-APM which could generate one or more of the three other stereoisomers. To our knowledge, only three articles deal with this problem[8–10].

Gaines and Bada[9] observed racemization at high temperature of L,L-APM degradation products, specifically L,L-DKP. However, the analytical method utilized an achiral reversed-phase HPLC system and only diastereomeric pairs could be separated; an accurate measurement of each stereoisomer could not be obtained[8].

Jadaud and Wainer[10] were able to resolve the enantiomeric pairs L,L-/D,D-APM and L,D-/D,L-APM using a column based upon α-chymotrypsin. However, the resolutions of the diastereomeric pairs L,D-/D,D-APM and D,L-/L,L-APM were poor, which led to overlapping when the four stereoisomers were chromatographed together.

In this paper, we report the chromatographic resolution of the four stereoisomers of APM on an HPLC chiral stationary phase based upon a chiral crown ether (CR-CSP). The system was also able to resolve the diastereomeric pairs of DKP and the four corresponding dipeptides. The method was used to analyze the composition of a diet cola and coffee sweetened with NutraSweet. Degradation products of

NutraSweet were detected but no racemization or stereochemical contamination was observed.

EXPERIMENTAL

Chemical

The four stereoisomers of APM were kindly provided by NutraSweet (Mt. Prospect, IL, U.S.A.). L-Asp-L-Phe, β-L,L-APM, DL-Asp and DL-Phe were purchased from Sigma (St. Louis, MO, U.S.A.). The perchloric acid was from Aldrich (Milwaukee, WI, U.S.A.) and the 2-propanol was from Burdick & Jackson (Muskegon, MI, U.S.A.). The diet cola and Equal (the commercially available packet form of NutraSweet) were purchased at the local market.

Apparatus

The chromatographic system consisted of a Beckman 110B solvent delivery module (Beckman, Houston, TX, U.S.A.), a Rheodyne 7125 injector equipped with a 20-μl loop (Alltech Assoc., Deerfield, IL, U.S.A.), a Spectra-Physics SP8773XR UV detector set at 210 nm (Spectra-Physics, San Jose, CA, U.S.A.) and a Shimadzu C-R6A integrator (Shimadzu, Columbia, MD, U.S.A.). The column temperature was controlled by a Forma Scientific module 2006 circulating water bath (Forma Scientific, Marietta, OH, U.S.A.) equipped with an Alltech HPLC column jacket. The column used for this study consisted of a 150 × 4 mm I.D. stainless-steel column packed with a chiral stationary phase composed of a chiral crown ether coated on a polymeric support (Crownpack CR(+), Daicel, Los Angeles, CA, U.S.A.).

Chromatographic conditions

The mobile phase was prepared by addition of perchloric acid to HPLC grade water until a pH of 2.8 was obtained. The aqueous perchloric acid was modified with 1.5% of 2-propanol. The flow-rate was 0.6 ml/min. To prevent corrosion and decomposition of the stationary phase, the column was washed every night with HPLC grade water.

Temperature gradient

The chromatography was carried out using the following temperature gradient: 0–26 min, T = 10°C; 26–70 min, T = 10–40°C (linear gradient); 70–80 min, T = 40°C.

Samples

The chromatographic standards were prepared in distilled water (100 μ*M*) and directly injected onto the column. The diet cola was degassed in an ultrasonic bath, diluted to 1/5 with distilled water and filtered through a 0.45-μm Millipore filter (Millipore, Bedford, MA, U.S.A.) before injection. One packet of Equal (1 g NutraSweet) was added to 150 ml of warm coffee. The solution was heated to a temperature of 70°C on a hotplate and held at that temperature for 10 min, diluted while hot to 1/40 with distilled water, filtered through a 0.45-μm Millipore filter and immediately injected onto the column.

RESULTS AND DISCUSSION

The results from the chromatography of the stereoisomers of APM and the major degradation products are presented in Table I and Fig. 2. For APM, the D,L- and D,D-isomers eluted first with capacity factors of 9.94 and 11.03, respectively, and were separated from each other with a resolution factor (R_s) of 1.20. The two other isomers (L,D-APM and L,L-APM) were only slowly eluted from the column under the isocratic and isothermal conditions used for the separation of D,L-APM and D,D-APM, *i.e.*, at a constant temperature of 10°C.

TABLE I

RESULTS FROM THE CHROMATOGRAPHY OF THE STEREOISOMERS OF APM AND THEIR MAJOR DEGRADATION PRODUCTS ON A CR-CSP

See Fig. 2 for experimental conditions.

Peak	*Compound*	*k'*	α^a	R_s^b
1	L,L-APM	33.52		
2	L,D-APM	23.62	1.42	7.32
3	D,D-APM	11.03	2.14	11.08
4	D,L-APM	9.94	1.11	1.20
5	L-Asp-D-Phe	8.92		
6	D,D + L,L-DKP	7.24		
7	D,L + L,D-DKP	5.12		
8	D-Asp-D-Phe	4.74		
9	D-Asp-L-Phe	3.67		
10	L-Asp-L-Phe	24.34		

[a] Selectivity between two adjacent peaks.
[b] Resolution between two adjacent peaks.

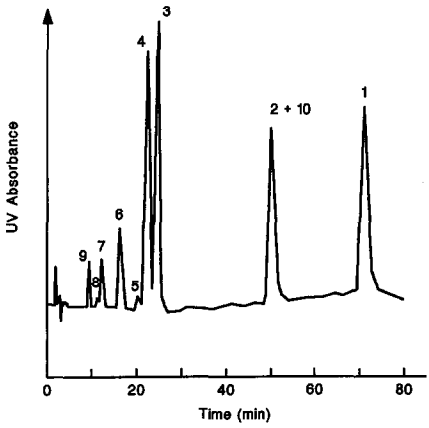


Fig. 2. Chromatogram of a 1-day-old synthetic mixture of the four stereoisomers of APM (0.1 m*M* of each isomer). Peaks: **1** = L,L-APM; **2** = L,D-APM; **3** = D,D-APM; **4** = D,L-APM; **5** = L-Asp-D-Phe; **6** = D,D-DKP + L,L-DKP; **7** = D,L-DKP + L,D-DKP; **8** = D-Asp-D-Phe; **9** = D-Asp-L-Phe; **10** = L-Asp-L-Phe. Chromatographic conditions: column: Crownpack CR(+), Daicel, 150 × 4 mm I.D. Injection loop: 20 µl. Mobile phase: aqueous $HClO_4$ (pH = 2.8)–2-PrOH (98.5:1.5, v/v). Flow-rate: 0.6 ml/min. Temperature gradient: 0–26 min: T = 10°C; 26–70 min: linear gradient from T = 10–40°C. Detector: 210 nm, sensitivity = 0.02 a.u.f.s., attenuation = 2^5.

The use of larger concentrations of organic modifiers to improve the chromatography of the late eluting peaks was not attempted due to the characteristics of the stationary phase and the recommendations of the manufacturer. Therefore, standard solvent gradient techniques were not explored and, instead, a temperature gradient was used. In this approach, the temperature was raised from 10–40°C, starting after the elution of the first two isomers. Under these conditions, both L,D- and L,L-APM were eluted within 80 min, $k' = 23.62$ and 33.52, respectively, with significant peak compression. The L,D- and L,L-isomers were separated from each other with a resolution factor (R_s) of 7.32. The enantiomeric pairs were resolved with stereoselectivities (α) of 2.38 (D,L/L,D) and 3.04 (D,D/L,L).

These data indicate that the elution order of the APM isomers was determined by the configuration of the aspartyl moiety. The results are consistent with the proposed chiral recognition mechanism in which inclusion complexes are formed between a protonated primary amino group in the vicinity of the chiral center of the solute and the polyether rings of the chiral stationary phase (CSP). The difference in the stabilities of the diastereomeric solute–CSP complexes is due to additional π–π and steric interactions[11,12]. The APM and dipeptide solutes chromatographed in this study contain a primary amino function in the aspartyl moiety of the molecule and it is the configuration of this moiety which should, and in fact does, control the enantiomeric elution order, *i.e.*, the compounds which contain D-Asp, D,L- and D,D-APM, elute before those which contain L-Asp, L,D- and L,L-APM. It is of interest to note that the diastereomeric elution order is also affected by the configuration of the Asp moiety. When Asp is in the D-configuration, the APM containing L-Phe elutes before the one containing D-Phe. On the contrary, when Asp is in the L-configuration, the APM containing D-Phe elutes before the one containing L-Phe.

According to the scheme proposed by Gaines and Bada[9], under slightly acidic conditions, *e.g.*, distilled water, the primary amino group of APM rapidly reacts with the methylester moiety to give a cyclised compound, the 5-benzyl-3,6-dioxo-2-piperazine acetic acid (DKP, Fig. 1). The ring reopens to give the corresponding dipeptide, aspartyl-phenylalanine (Asp-Phe), which can thereafter by hydrolysed to the free amino acids, Asp and Phe. At acidic pH values, DKP is the major decomposition product.

Although authentic samples of the four possible DKPs were not available, they were synthesized following the procedure described by Gaines and Bada[8,9]. Each isomer of APM was dissolved in distilled water and stored at ambient temperature. The composition of the solution was followed during a 14-day period. The results of this study are illustrated by the experiment involving L,L-APM which is presented in Fig. 3. The predominant component of the fresh solution was L,L-APM; a second minor peak, **6**, was also present, Fig. 3a. After 2 days, **6** had significantly increased, and a third peak, **10**, had appeared, Fig. 3b. By day 14, both L,L-APM and **10** had disappeared from the chromatogram leaving only **6**, Fig. 3c. Peak **10** was identified as L-Asp-L-Phe by injection of a standard solution of the dipeptide. Under the chromatographic conditions used in this study, the free amino acids DL-Asp and DL-Phe were eluted at the solvent front and could not be detected. It is assumed that the disappearance of **10** was due to hydrolytic cleavage of the dipeptide[9].

Similar results were obtained with D,D-APM. After 14 days, peak **6** was also the predominant peak in the chromatogram of the D,D-APM solution. These results

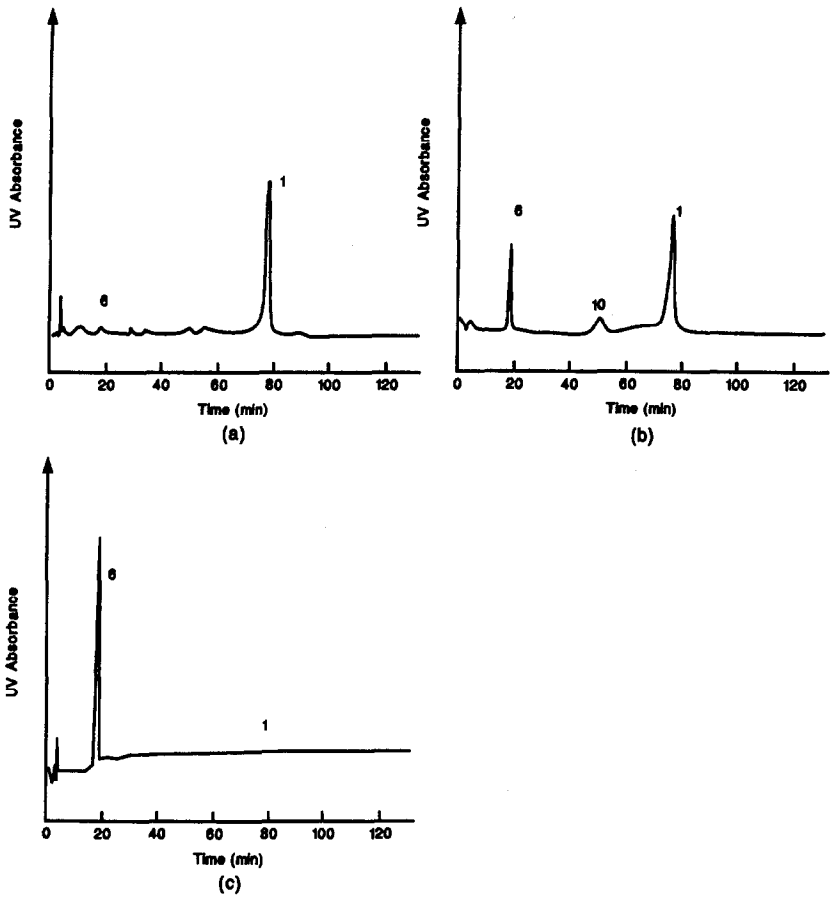


Fig. 3. Degradation of L,L-APM in distilled water as a function of time, where (a) fresh solution; (b) 2-day-old solution; (c) 14-day-old solution and **1** = L,L-APM; **6** = L,L-DKP; **10** = L-Asp-L-Phe. Chromatographic conditions: see Fig. 2.

support the identification of **6** as the DKP arising from D,D- and L,L-APM. Since DKP does not contain a primary amino group, it is unable to form an inclusion complex with the chiral stationary phase and enantioselective separations cannot occur. Thus, the pair of enantiomers, D,D- and L,L-DKP, gives rise to only one peak.

When D,L- and L,D-APM were studied, a common predominant peak, **7**, was found in the chromatograms of the two solutions at day 14. Using the same approach employed for the identification of peak **6**, these results allowed us to identify **7** as the enantiomeric D,L-/L,D-DKP. In addition, since **7** was not detected in any of the chromatograms run during the degradation studies of L,L- and D,D-APM and since **6** was not found in similar studies carried out with L,D- and D,L-APM, it appears that racemization did not occur. It is of interest to note that although the CR-CSP was unable to resolve the enantiomeric DKPs, the separation of the diastereomeric D,D-/L,L-DKP and L,D-/D,L-DKP was achieved under these conditions.

A third peak was also detected in each of the chromatograms from the aqueous

solutions of D,L-, D,D- and L,D-APM with capacity factors of $k' = 3.67$ (**9**), 4.74 (**8**) and 8.92 (**5**), respectively. The magnitudes of the peaks and the time courses of their appearances were similar to the appearance of L-Asp-L-Phe in the aqueous solution of L,L-APM (Fig. 3). The three peaks were, accordingly, assumed to be D-Asp-L-Phe (**9**), D-Asp-D-Phe (**8**) and L-Asp-D-Phe (**5**). The chromatograms of the aqueous solutions of each of the four APM stereoisomers contained only a single peak which could be assumed to be Asp-Phe. This is further evidence that racemization did not occur under the experimental conditions used in this study.

This method was applied to the analysis of L,L-APM in a diet cola and in coffee sweetened with the commercially available packet form of L,L-APM (Equal). The results of the assay of the diet cola are presented in Fig. 4. The chromatogram demonstrates that L,L-APM (**1**) is the predominant form of the sweetener and that L,L-DKP (**6**) is only a minor contamination.

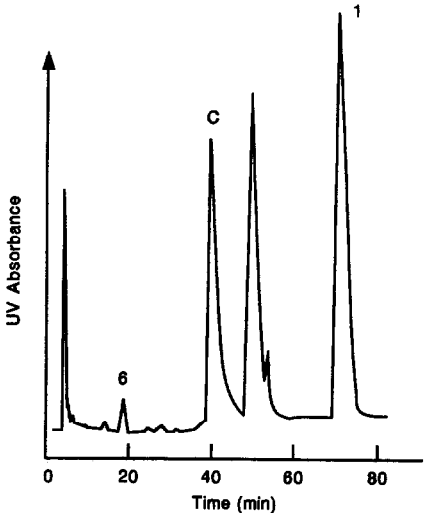


Fig. 4. Determination of L,L-APM and its decomposition products in a diet cola, where **1** = L,L-APM; **6** = L,L-DKP; C = caffeine. Chromatographic conditions: see Fig. 2.

Peak C was identified as caffeine by injection of a standard solution of this compound. Peak C was absent from a caffeine-free form of the diet cola. The peak next to peak C in the chromatogram was not present in a sugar-sweetened form of the cola and was present in both the caffeinated and decaffeinated diet colas. The peak does not co-elute with L-Asp-L-Phe, which was added to the analytical samples, and therefore does not represent this dipeptide. The compound was not identified. The small peak at 26 min is eluted at the retention time of the β form of L,L-APM and could, therefore, indicate that this compound is a minor contaminant. Trace amounts of β-L,L-APM have been found previously in a diet cola by Lawrence and Iyengar[13]. In this study, further identification of this component was also not pursued.

If we consider L,L-DKP as the only degradation compound, the degree of degradation can be estimated using the ratio between the areas of the L,L-DKP and

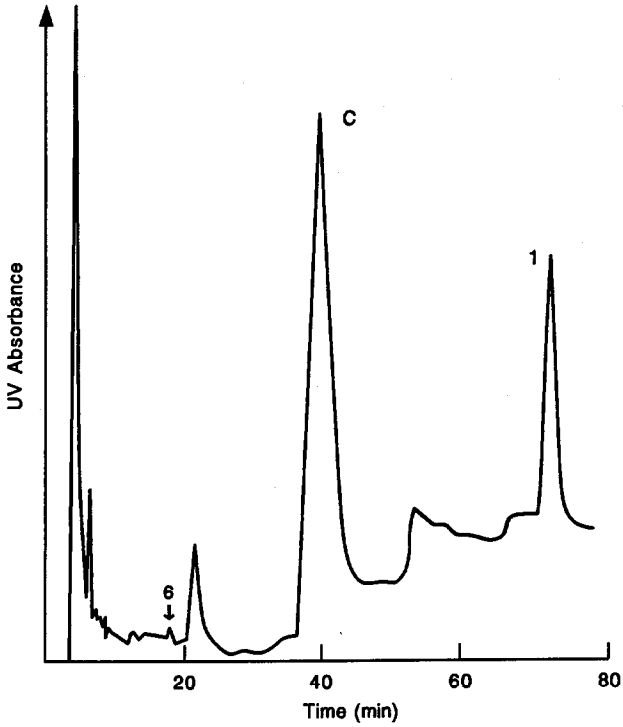


Fig. 5. Determination of L,L-APM and its decomposition products in coffee sweetened with Equal, where **1** = L,L-APM; **6** = L,L-DKP; C = caffeine. Chromatographic conditions: see Fig. 2.

L,L-APM peaks. For this sample of diet cola, the calculated degree of degradation of L,L-APM was 1.3%.

The results of the experiment involving the addition of Equal to hot coffee followed by heating at 70°C for 10 min are presented in Fig. 5. Peaks corresponding to L,L-APM (**1**), L,L-DKP (**6**) and caffeine (C) were identified. The other peaks in the chromatogram and caffeine were present in a sample of unsweetened coffee. In this experiment, the degradation of L,L-APM to L,L-DKP was less than 1%. The same experiment, carried out with a heating time of 20 min instead of 10 min, gave similar results. In contrast, when water is used instead of coffee, after 10 min of heating at 70°C, 21% of the L,L-APM was degraded to L,L-DKP. These observations indicate that L,L-APM is more thermally stable in coffee than in water.

Racemization of the L,L-APM was not observed in either the diet cola or the sweetened coffee.

CONCLUSION

The results of this study indicate that the isomers of aspartame and their degradation products can be chromatographically separated on the CR-CSP. The analytical method can be used to rapidly determine the isomeric composition of

aspartame and the degree of its degradation in food products. The CR-CSP appears to be quite stable under the conditions used in this study. The column has been in constant use for over two months without significant deterioration in its chromatographic performance.

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