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Determination of aspartame and its degradation and epimerization products by capillary electrophoresis[1,2]

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Abstract

Two capillary zone electrophoretic assays using run buffers of pH 9.35 and pH 2.70 have been developed for the determination of aspartame (α-L-Asp-L-PheOMe) and its potential degradation products including Phe, PheOMe, 5-benzyl-3,6-dioxo-2-piperazineacetic acid (DKP), the dipeptides Asp-Phe and Phe-Asp, as well as the isomeric β-aspartame (β-L-Asp-L-PheOMe). As an uncharged species at pH 2.7 DKP could not be determined. Between pH 2.0 and 3.5 the resolution of the diastereomers of aspartame and β-aspartame was investigated. While the resolution of the epimeric β-isomers exhibited a plateau between pH 2.3 and 2.7, resolution of the aspartame diastereomers peaked at pH 3.0. Using salicylic acid and Phe-Gly as internal standards at pH 9.35 and 2.70, respectively, linear calibration curves were obtained for a concentration range between 5 μg ml^{-1} and 1 mg ml^{-1}. The R.S.D. for intraday and interday analysis ranged from 1.0 to 3.6% and 1.5% to 9.1%, respectively. The capillary electrophoresis assays were applied to analyze aspartame solutions heated to 70°C. In agreement with the literature data aspartame was found to be less stable at pH 7 compared to pH 3. In contrast to aspartame itself, an approximate 20% epimerization of β-aspartame was observed in the incubation mixtures.

Keywords: Aspartame; β-aspartame; Epimerization; Degradation; Analysis; Capillary electrophoresis

1. Introduction

Aspartame (*N*-L-α-aspartyl-L-phenylalanine methyl ester, α-L-Asp-L-PheOMe) is a low calorie sweetner used in numerous foods, beverages and pharmaceuticals. Although relatively stable in the dry state at low temperatures the compound undergoes pH- and temperature-dependent degradation in solution (Fig. 1). The reactions include cyclization as well as ester and peptide bond hydrolysis [1,2]. Intramolecular aminolysis yields

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[2] Dedicated to Prof. Dr Gottfried Blaschke on the occasion of his 60th birthday.

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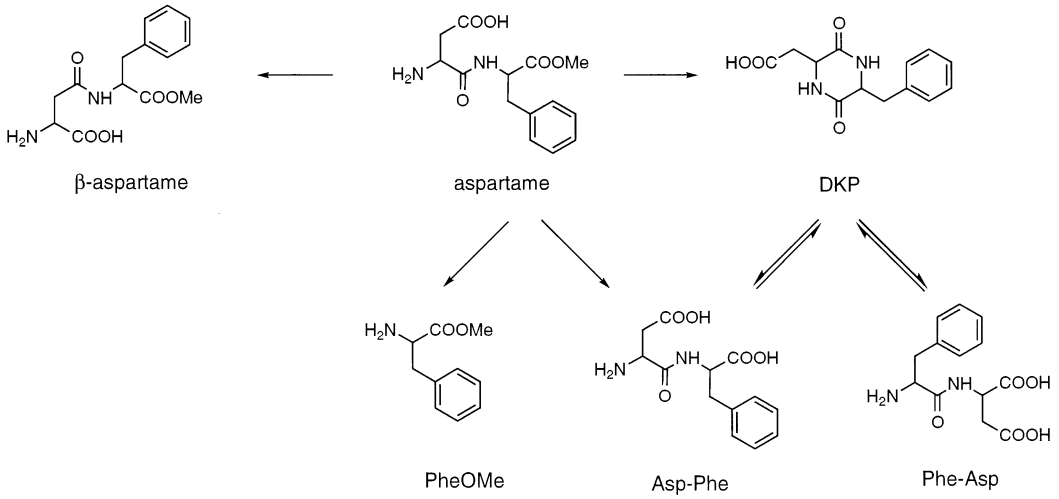

Fig. 1. Major degradation pathways of aspartame.

5-benzyl-3,6-dioxo-2-piperazineacetic acid (DKP) as the major degradation product. Ring opening results in the isomeric dipeptides L-phenylalaninyl-L-aspartic acid (Phe-Asp) and L-asparaginyl-L-phenylalanine (Asp-Phe). The latter can also result from direct hydrolysis of the methyl ester of aspartame. Cleavage of the amide bond yields L-phenylalanine methyl ester (PheOMe). The ultimate degradation products are L-aspartic acid (Asp) and L-phenylalanine (Phe). Additionally, formation of the isomeric β-aspartame (β-L-Asp-L-PheOMe) has been reported [3].

Moreover, racemization of Asp and Phe residues has been described for aspartame and its degradation products in aqueous solutions [4,5] leading to epimeric dipeptides. The racemization was primarily attributed to epimerization of DKP. The half-lives of the racemization of Asp and Phe were found to be dependent on the pH of the solution.

Capillary electrophoresis (CE) has become an important analytical technique complimentary to HPLC and GC that is especially suitable for the analysis of polar compounds. Although the determination of aspartame by CE has been described [6,7] to date no report on the analysis of the degradation products has been published in the literature using this technique.

2. Experimental

2.1. Chemicals

Aspartame was a gift from NutraSweet AG (Zug, Switzerland). β-Aspartame, L-Asp-L-Phe, L-Phe-L-Asp, L-Phe-Gly, L-PheOMe, L-Phe, and Z-D/L-Asp were obtained from Bachem AG (Heidelberg, FRG), salicylic acid from Caelo (Hilden, FRG). DKP was synthesized according to the literature [8]. The diasteromeric pairs α-L-Asp-L-PheOMe/α-D-Asp-L-PheOMe and β-L-Asp-L-PheOMe/β-D-Asp-L-PheOMe were prepared from Z-D/L-Asp and L-PheOMe according to [9]. The composition of the product was determined by HPLC on a LiChrospher 60 RP select B column (125×4.6 mm, 5 mm) (Knaur, Berlin, FRG) with 5 mM sodium phosphate (pH 3.5) containing 5 mM heptanesulfonic acid-acetonitrile (80:20, v/v) as mobile phase and a flow rate of 1.0 ml min^{-1}. All other chemicals were purchased from commercial sources at the highest purity

available. Buffers and solutions were prepared in double destilled, deionized water, filtered (0.47 μm) and degassed by sonication.

2.2. Capillary electrophoresis

All experiments were performed on a Beckmann P/ACE 2100 equipped with a diode arrray detector set to 215 nm (Waldbron, FRG). Fused-silica capillaries 50 μm inner diameter (Beckmann, Waldbron, FRG) were used. Detection was carried out 7 cm from the cathodic end. Sample solutions were introduced into the capillary at the anodic end by hydrodynamic injections at a pressure of 0.5 p.s.i. for 3 s. The separations were performed at 20°C.

The two finalized separation conditions were employed. Assay 1 was performed in a 47/40 cm capillary with 50 mM borate buffer, pH 9.35. The applied voltage was 20 kV. In between runs the capillary was rinsed with 0.1 M NaOH for 2 min and with the run buffer for another 2 min. Salicylic acid was used as internal standard. Assay 2 was performed in a 37/30 cm capillary. The run buffer was 50 mM phosphate, pH 2.70, the applied voltage was 18.5 kV. Between analyses the capillary was flushed with 0.1 M phosphoric acid for 2 min and with the run buffer for another 2 min. L-Phe-Gly served as internal standard. The peak area ratio method was applied for the calibration curves.

2.3. Incubations

Aspartame (25 mg ml^{-1}) was incubated in 50 mM phosphate buffer pH 3.0 and 7.0, respectively, at 70 ± 2°C. At selected time intervals 100 μl aliquots were added to 200 μl water containing 22 μg ml^{-1} of the respective internal standard and analyzed by CE. Concentrations were calculated by the peak area ratio method.

3. Results and discussion

3.1. Capillary electrophoresis

An electropherogram of a standard mixture using a run buffer with pH 9.35 is shown in Fig. 2. The analysis is complete within 10 min. Due to the lack of a chromophore Asp cannot be detected. At pH 9.35 the carboxyl groups of the compounds are fully ionized. Thus, apart from the size of the molecules the migration order can be explained based on the pK_a values of the amino groups. Values of 9.1, 7.1, and 7.9 have been reported for Phe [10], PheOMe [11] and aspartame [11], respectively, while the pK_a's of dipeptides generally range between 8.0 and 8.3 [10,12]. PheOMe migrates as an uncharged species with the endoosmotic flow (EOF) followed by Phe, β-aspartame and aspartame which bear a partial negative charge at pH 9.35. DKP does not have an amino group and the carboxyl function is fully ionized. Phe-Asp and Asp-Phe with two carboxylate groups migrate last.

An alternative method for the analysis of the compounds was developed using 50 mM phosphate buffer, pH 2.70. A typical electropherogram is displayed in Fig. 3. As the amino groups are protonated at pH 2.70, the migration order is determined by the pK_a values of the carboxyl functions. For example, PheOMe has a positive charge and migrates first while Phe due to the partially deprotonated carboxyl group ($pK_a = 2.0$ [10]) has a lower electrophoretic mobility. β-aspartame is apparently more acidic than aspartame ($pK_a = 3.2$ [11]) and, thus, migrates slower. DKP as a neutral species does not migrate to the detector at the cathodic end of the capillary within a

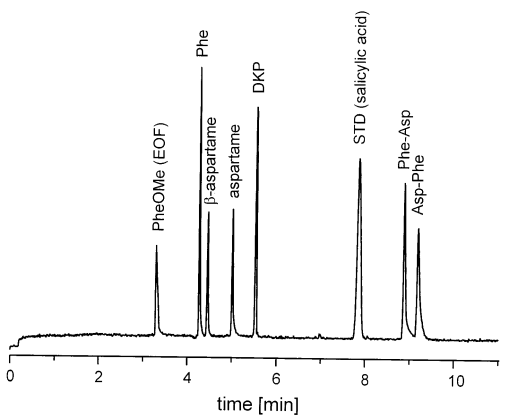

Fig. 2. Electropherogram of standards analyzed using 50 mM borate buffer, pH 9.35, as described in Experimental.

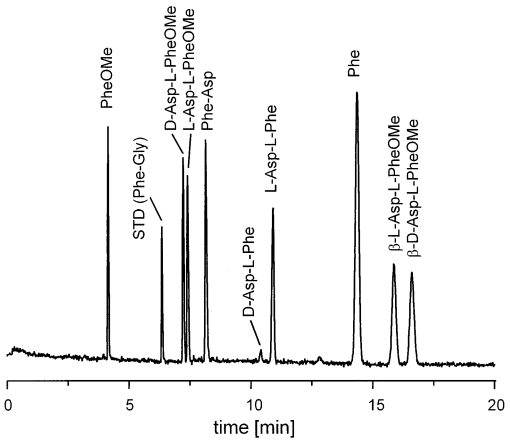

Fig. 3. Electropherogram of standards analyzed using 50 mM phosphate buffer, pH 2.70, as described in Experimental. The synthesized diastereomeric mixture of α-L-Asp-L-PheOMe/α-D-Asp-L-PheOMe and β-L-Asp-L-PheOMe/β-D-Asp-L-PheOMe was injected instead of commercial aspartame and β-aspartame.

reasonable time frame due to the negligible EOF at this pH.

Under these conditions resolution of the diastereomers of aspartame and β-aspartame was observed due to small differences in the pK_a values of the diastereomers. The assignment of the migration order of aspartame (α - L - Asp - L-PheOMe) and the diasteromeric α - D - Asp - L-PheOMe as well as the β-isomers, β - L - Asp-L-PheOMe and β-D-Asp-L-PheOMe, was performed using commercial aspartame, β-aspartame and the synthesized D/L-diastereomers as reference compounds. Interestingly, within the aspartame diastereomers the D/L-epimer migrates in front of the L/L-epimer, while within the β-aspartame diastereomers an opposite migration order is observed. The L/L-epimer migrates faster than the D/L-epimer. At pH 2.7 an epimeric impurity (D-Asp-L-Phe) could be also detected in commercial L-Asp-L-Phe (Fig. 3). Identification was performed using synthetic D/L-Asp-L-Phe.

The dependence of the resolution of the diastereomers on the pH of the run buffer is summarized in Table 1. While the resolution of the epimeric β-isomers exhibited a plateau between pH 2.3 and 2.7, resolution of the aspartame diastereomers peaked at pH 3.0. With increasing pH of the run buffer the carboxyl groups display a higher degree of ionization resulting in the

Table 1
Dependence of migration time and resolution of the diastereomers of aspartame and β-aspartame

pH	aspartame			β-aspartame		
	t_1 (min)[a]	t_2 (min)[b]	R_S^c	t_1 (min)[a]	t_2 (min)[b]	R_S^c
2.0	5.46	5.53	<0.5	7.80	8.04	1.77
2.2	5.93	6.03	<0.5	10.22	10.69	2.41
2.3	6.29	6.43	0.85	12.20	12.80	2.72
2.5	6.51	6.67	1.03	13.46	14.12	2.55
2.6	7.16	7.36	1.24	16.33	17.20	2.67
2.7	7.53	7.74	1.51	17.63	18.52	2.76
2.9	8.93	9.26	1.56	24.20	25.45	2.40
3.0	10.38	10.77	2.70	29.10	30.51	2.02
3.2	12.22	12.72	1.73	32.03	33.26	1.31
3.5	11.17	11.53	0.92	18.42[d]		—

[a] Migration time of the faster migrating diastereomer.
[b] Migration time of the slower migrating diastereomer.
[c] The resolution was calculated using the equation

$$Rs = \frac{1.18(t_2 - t_1)}{b^{0.5}(1) + b^{0.5}(2)}$$

where t_1 and t_2 are the migration times of the first and last migrating diastereomer, and $b^{0.5}(1)$ and $b^{0.5}(2)$ are the half height widths of the respective peaks.
[d] No separation of the diastereomers observed.

Table 2
Linearity and slope of the regression curves obtained for CE analysis at pH 9.35 and 2.70, respectively, for the concentration range between 5 μg ml^{-1} and 1 mg ml^{-1}

Compound	pH 9.35		pH 2.70	
	r^2	Slope · 10^3	r^2	Slope · 10^2
aspartame[a] (L-Asp-L-PheOMe)	0.996	4.348	0.999	1.662
D-Asp-L-PheOMe	—	—	1.000	1.471
DKP[a]	1.000	4.838	—	—
Asp-Phe	1.000	4.246	0.999	1.228
Phe-Asp	1.000	5.218	1.000	1.511
PheOMe	0.990	6.113	0.999	1.586
Phe	0.999	6.975	0.999	2.065
β-aspartame (β-L-Asp-L-PheOMe)	0.998	4.838	0.999	1.422
β-D-Asp-L-PheOMe	—	—	1.000	1.405

[a] Determined for a concentration range of 5 μg ml^{-1} to 5 mg ml^{-1}.

concomitant increase of the migration times (Table 1) due to the negligible EOF at these pH values. The increased mobility of the compounds at pH 3.5 can be attributed to an increase of the EOF at this pH. However, this led to a loss of the resolution of the diastereomers. Overall, pH 2.7 was selected as a compromise between resolution and analysis time in the acidic pH range.

3.2. Reproducibility

Using salicylic acid as internal standard at pH 9.35 and Phe-Gly as internal standard at pH 2.70 good linearity of the detector response was observed for all compounds in a concentration range of 5 μg ml^{-1} up to 1 mg ml^{-1} (Table 2). Aspartame and DKP were evaluated up to a concentration of 5 mg ml^{-1}. The detection limit of α-D-Asp-L-PheOMe in aspartame (α-L-Asp-L-PheOMe) was 0.5% of the L/L-epimer.

The precision and accuracy of the CE assays was determined using concentrations of about 13, 150 and 450 μg ml^{-1} of the compounds. Each sample was analyzed 4 times on 3 different days. For intraday analysis, the relative standard deviation of the ratios of the area of the compounds to the area of the internal standards varied between 1.3% and 3.6% for the analysis at pH 9.35, except for PheOMe, which showed deviations of up to 10.3%. R.S.D.s between 1 and 2.5% were found at pH 2.70. For the determinations on three different days (interday precision) the R.S.D.s ranged from 2.2 to 9.1% for CE at pH 9.35 and from 1.5 to 5.2% at pH 2.70.

3.3. Degradation of aspartame in solution

In order to evaluate the applicability of the CE assays to study the degradation of aspartame incubations of the compound in 50 mM posphate buffer at pH 3 and 7 were performed. Typical electropherograms of the solutions following heating to 70°C for 48 h are shown in Fig. 4 and Fig. 5. The peaks were identified by spiking with the reference compounds. Additional degradation products were observed but could not be identified due to the lack of reference substance. The time-dependency of the concentration of the products in the incubations is displayed in Fig. 6 and Fig. 7. As described by several previous studies, aspartame was less stable at pH 7 than at pH 3 [1,2,5]. At both pH values, the degradation was primarily due to the formation of DKP. Compared to incubations at pH 7, higher concentrations of β-aspartame, PheOMe and Phe were found at pH 3.

In degraded samples of aspartame a large discrepancy was observed between the concentration of PheOMe determined at pH 9.35 and pH 2.70. Generally, higher values were found at pH 9.35. This can be attributed to additional neutral compounds other than PheOMe migrating with the

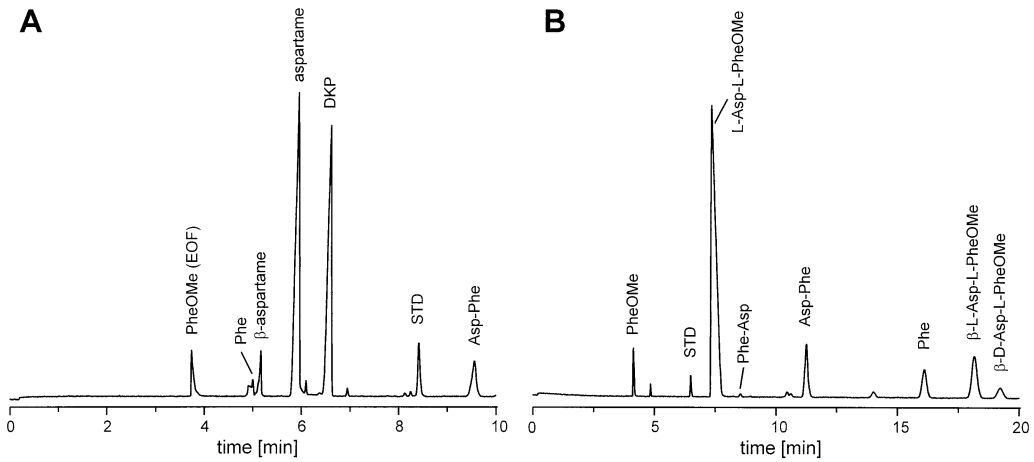

Fig. 4. Typical electropherogram of a solution of aspartame following incubation in 50 mM phosphate buffer, pH 3.0, at 70°C for 48 h. (A) Incubations analyzed with borate buffer, pH 9.35; (B) incubations analyzed with phosphate buffer, pH 2.70.

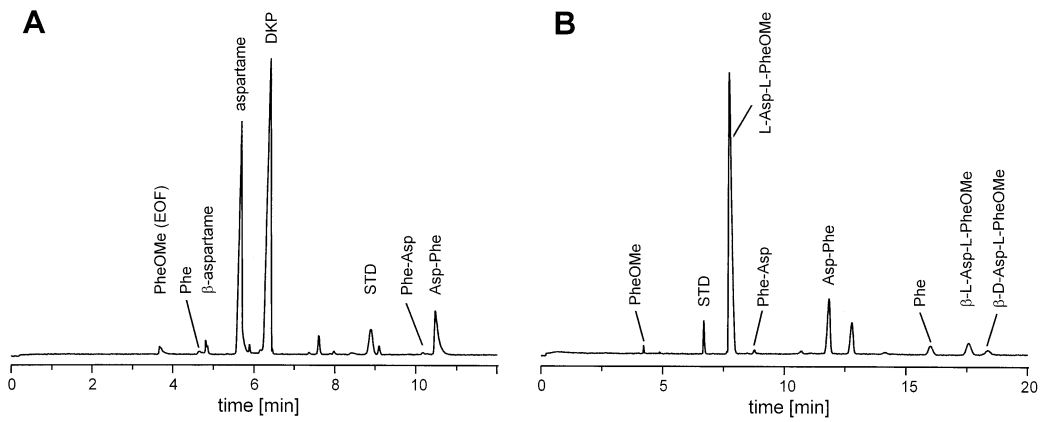

Fig. 5. Typical electropherogram of a solution of aspartame following incubation in 50 mM phosphate buffer, pH 7.0, at 70°C for 48 h. (A) Incubations analyzed with borate buffer, pH 9.35; (B) incubations analyzed with phosphate buffer, pH 2.70.

EOF. Combined with the relatively poor linearity of the calibration curve (Table 2) and the large relative standard deviations the determination of PheOMe at pH 2.70 resulted in more reliable data.

While no epimerization of aspartame was observed at either pH, analysis of the diastereomeric composition of β-aspartame revealed an approximate 20% epimerization at any time point studied (data not shown). No significant difference was observed between epimerization at pH 3 and at pH 7. Isomerization of aspartame to β-aspartame proceeds presumably via a succinimide intermediate [3] as described for peptides containing Asp [13]. The succinimide is especially prone to racemization [13,14]. The same process should be assumed for the epimerization of β-aspartame observed in our studies. The degree of epimerization of 20% determined for β-aspartame is in the same range as the approximate 30% epimerization of Asp described for a hexapeptide [14]. Interestingly, no significant epimerization could be observed for aspartame.

Only the diastereomers but not the enantiomers of aspartame and β-aspartame could be resolved by capillary electrophoresis using the run buffer

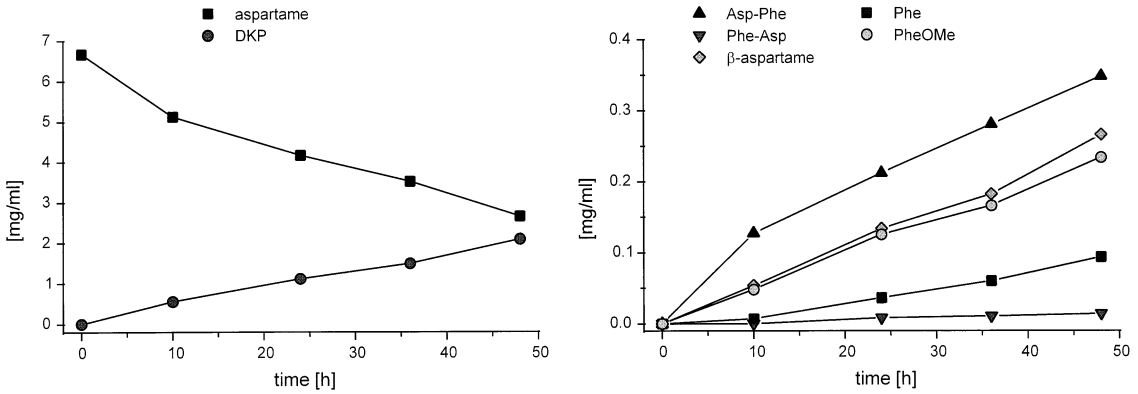

Fig. 6. Time course of the concentration of aspartame and its degradation products following incubation at pH 3.0 and 70°C.

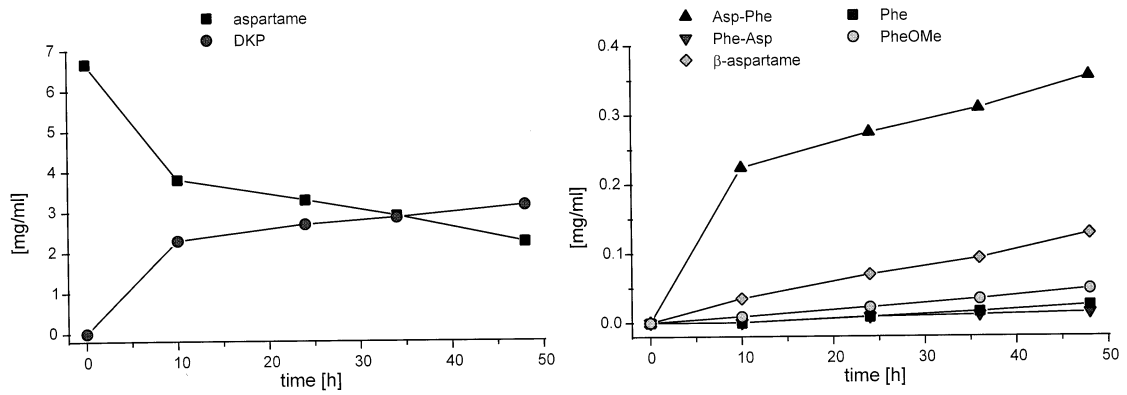

Fig. 7. Time course of the concentration of aspartame and its degradation products following incubation at pH 7.0 and 70°C.

pH 2.70. Thus, it cannot be excluded that the peaks of the L/L-enantiomers also contain the D/D-isomers and that the respective diasteromeric D/L-isomer contains its L/D-enantiomer. However, as Asp is especially prone to racemization [13] the epimerization observed in this study should be primarily due to the racemization of Asp instead of racemization of Phe.

4. Conclusion

Two sensitive and reproducible capillary electrophoretic assays were developed for the determination of aspartame and its degradation products. The concomitant resolution of the Asp-containing dipeptide derivatives in the acidic pH range enables the quantitation of the epimers during the degradation processes.

Acknowledgements

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