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Simultaneous analysis of aspartame and its hydrolysis products of *Coca-Cola Zero* by on-line postcolumn derivation fluorescence detection and ultraviolet detection coupled two-dimensional high-performance liquid chromatography

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ABSTRACT

An innovative two-dimensional high-performance liquid chromatography system was developed for the simultaneous analysis of aspartame and its hydrolysis products of *Coca-Cola Zero*. A C8 reversed-phase chromatographic column with ultraviolet detection was used as the first dimension for the determination of aspartame, and a ligand-exchange chromatographic column with on-line postcolumn derivation fluorescence detection was employed as the second dimension for the analysis of amino acid enantiomers. The fluorimetric derivative reagent of amino acid enantiomers was *o*-phthaldialdehyde. The hydrolysis of aspartame in *Coca-Cola Zero* was induced by electric-heating or microwave heating. Aspartame was quantified by the matrix matched external standard calibration curve with a linear concentration range of 0–50 μg mL^{-1} (r^2 = 0.9984). The limit of detection (LOD) and the limit of quantification (LOQ) were 1.3 μg mL^{-1} and 4.3 μg mL^{-1}, respectively. The amino acid enantiomers was analyzed by the matrix matched internal standard calibration method (D-leucine as the internal standard) with a linear concentration range of 0–10 μg mL^{-1} (r^2 = 0.9988–0.9997). The LODs and LOQs for L- and D-aspartic acid and L- and D-phenylalanine were 0.16–0.17 μg mL^{-1} and 0.52–0.55 μg mL^{-1}, respectively, that was 12–13 times more sensitive than ultraviolet detection. The overall analysis accuracy for aspartame and amino acid enantiomers was 90.2–99.2% and 90.4–96.2%, respectively. The overall analysis precision for aspartame and amino acid enantiomers was 0.1–1.7% and 0.5–6.7%, respectively. Generally, the extent of aspartame hydrolysis increases with the increase of electro-thermal temperature, microwave power, and the duration of hydrolysis time. D-aspartic acid and D-phenylalanine can be observed with the electro-thermal racemization at the hydrolysis temperature 120 °C for 1 day and only D-aspartic acid can be observed at the hydrolysis temperature 90 °C for 2 and 3 days. For the microwave induced hydrolysis, only L-aspartic acid was detected at the power 560 W for 1 min and 320 W for 3 min.

1. Introduction

The artificial sweetener, aspartame (L-aspartyl-L-phenylalanine methyl ester) has long been commonly used as a substitute for either table sugar or sugar in low-calorie meals, soft drinks, chewing-gums, and frozen deserts. Recently, there is a trend to mix aspartame with other kinds of additive such as preservatives and antioxidants to yield synergistic effects in food and cosmetics [1]. Therefore, aspartame is important in food and cosmetics industry. Medicinally, the design of aspartame is partially due to coping with diseases associated with the consumption of carbohydrates. In anatomy, aspartame has been used as a model compound to identify and recognize the active site of the sweet taste receptor [2].

However, aspartame is hydrolyzed naturally under the influence of temperature and pH to generate L-aspartic acid, L-phenylalanine, L-phenylalanine methyl ester, L-aspartyl-phenylalanine, etc. [3,4] The hydrolysis products, L-aspartic acid and L-phenylalanine, may further undergo racemization to form D-aspartic acid and D-phenylalanine by high temperature [5,6], pH [6], and enzyme [7]. L-form amino acids are nutrients for organisms and occur naturally while D-form amino acids are usually few and useless in nature. However, in recent years some D-amino acids have been found in mammals including humans [8,9] that play important biological functions [10–13]. Nevertheless, there are inborn hereditary diseases related with amino acids such as phenylketonuria. In phenylketonuria, phenylalanine is abnormally metabolized to toxic phenylpyruvic acid due to the absence of phenylalanine hydrox-

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ylase by gene defect. The accumulation of phenylpyruvic acid in child causes mental retardation. It seems that the only effect of aspartame hydrolysis would be on the patient of phenylketonuria.

Analytical methods [14–16] including high-performance liquid chromatography (HPLC) [17,18] have been reported for quantitative analysis of amino acid. However, HPLC coupled with different detection methods [19–29] has been widely used for the analysis of amino acids. Particularly, chiral HPLC [30–34] can separate amino acid enantiomers most efficiently. Within the category of chiral chromatography, ligand-exchange chromatography (LEC) [33–35] with stationary phase containing single amino acid enantiomeric functional group to complex the metal ions has been shown to be the most powerful method for the simultaneous separation of a pool of amino acid enantiomers. While the simultaneous analysis of amino acid enantiomers and dipeptide aspartame with LEC was found difficult because of the long retention time of aspartame.

Amino acids and peptides can be separated very easily by the reversed-phase chromatography [34] and can be detected by highly sensitive fluorescence detector with various fluorescent derivative reagents [23,24,27,29,36–40]. However, the separation of amino acid enantiomers is difficult with the reversed-phase chromatography. In order to resolve completely and simultaneously a wide range of analytes containing amino acid enantiomers in complicated matrix, two-dimensional HPLC (2-D HPLC) [41–44] that uses on-line column-switching technique [45] to combine a reversed-phase column and a ligand-exchange column is found usefully to solve this problem. In the literature, the less sensitive UV detector was traditionally used for LEC to analyze amino acid enantiomers. Since the less sensitive UV detector cannot be applied for trace amino acid enantiomer analysis, it is urgent to develop a highly sensitive fluorescence detection coupled LEC for amino acid enantiomers analysis.

The purpose of this work aims the development of a 2-D HPLC system for simultaneous determination of aspartame and its thermal hydrolysis and racemization products, amino acid enantiomers, in *Coca-Cola Zero* and the use of the most common fluorescent derivative reagent *o*-phthaldialdehyde (OPA) for developing an on-line postcolumn derivation fluorescence detection coupled LEC to improve the detection sensitivity for amino acid enantiomers.

2. Experimental

2.1. Chemicals and materials

The enantiomers of amino acid, D-aspartic acid (D-Asp, 99%), L-phenylalanine (L-Phe, 99%), D-phenylalanine (D-Phe, 98%), D-leucine (D-Leu, 99%), sodium tetraborate decahydrate (99.5%), 2-mercaptoethanol (99%) were purchased from Sigma (St. Louis, USA). *o*-Phthaldialdehyde (OPA, 98%) was purchased from Alfa Aesar (Karlsruhe, Germany). Aspartame (L-aspartyl-L-phenylalanine methyl ester) was obtained from ICN Biochemicals Inc. (OH, USA). L-Aspartic acid (L-Asp, 99%), methanol (MeOH) of HPLC grade, copper (II) sulfate ($CuSO_4$, 98%) were all bought from Merck (Darmstadt, Germany). Plastic bottled (600 mL) and aluminum canned (330 mL) *Coca-Cola Zero* were obtained from local supermarket. Ultrapure water (18.2 MΩ) was prepared first from tap water by reverse osmosis and distillation once then treated with Barnstead ultrapure system (Dubuque, IA, USA).

2.2. Standards and sample preparation

One thousand microgram per milliliter each of standard solutions of amino acids were prepared by dissolving $CuSO_4$ in pure water. Then, they were stored in the refrigerator or further diluted to the desired concentration for use. The sample of *Coca-Cola Zero* was degassed in ultrasonic bath for 10 min and the degassed sample (pH 2.12) was filtered through a 0.2 μm pore-size membrane filter and stored in the refrigerator for later use.

2.3. Amino acid derivation reagent

The amino acid derivation reagent was prepared freshly each day by dissolving 2.2 mg of OPA in 1.0 L of 0.01 M sodium tetraborate decahydrate buffer (pH = 9.5) to make an OPA concentration of 16 mM. Then, 1.0 mL of 2-mercaptoethanol was added. The pH of the borate buffer was adjusted by 5 mM NaOH solution. To avoid the possible decay of the derivation reagent by irradiation of light, the reagent was kept in a dark bottle.

2.4. Electro-thermal hydrolysis of Coca-Cola Zero

The thermal hydrolysis of pretreated *Coca-Cola Zero* sample was performed with a specially designed electric-heating micro-reactor that can control the hydrolysis temperature precisely to one tenth of a Celsius degree. The heating-block type micro-reactor was made with stainless steel that contains 20 screw capped wells of 1.5 mL in volume. One milliliter *Coca-Cola Zero* sample was placed in one of the wells of the micro-reactor and heated to a specific temperature (37, 60, 90, and 120 °C) for a period of 1–5 days. After hydrolysis was completed the reactor was cooled to room temperature. The sample was pipetted, the volume was measured, and 2 mM $CuSO_4$ solution was added to make the sample volume to the original 1 mL. The sample was further diluted 5 folds with 2 mM $CuSO_4$ (pH 2.86) and analyzed by the 2-D HPLC.

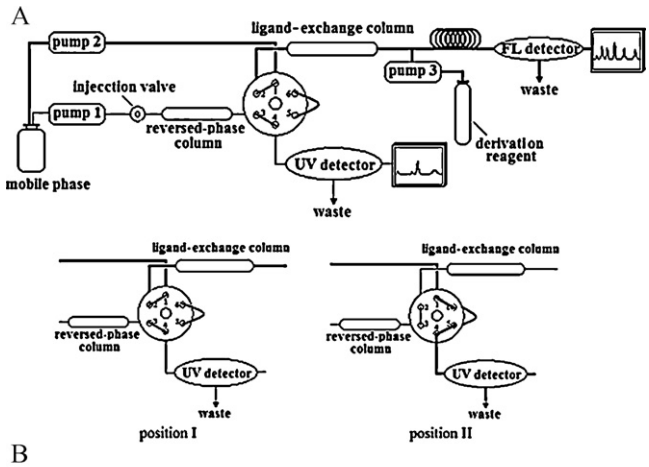
2.5. Microwave hydrolysis of Coca-Cola Zero

A volume of 5 mL pretreated *Coca-Cola Zero* sample was placed into a 25 mL volumetric flask. The volumetric flask was sealed with non-adsorptive cotton and put in a home-used microwave oven. The cola sample was hydrolyzed at different microwave powers (320, 560, and 800 W) for a period of 1 or 3 min. After hydrolysis was completed, the volumetric flask was allowed to cool to room temperature. Then 2 mM $CuSO_4$ solution was diluted to the mark (a 5-fold dilution) and analyzed by the 2-D HPLC.

2.6. Analysis of aspartame in Coca-Cola Zero by standard addition calibration method

Six 5 mL of pretreated cola samples were put in respective 25 mL volumetric flasks. Six 1000 μg mL^{-1} aspartame standard solutions of different volumes, 0.0, 0.25, 0.50, 0.75, 1.0, and 1.25 mL, were added respectively to the six cola samples. Each of aspartame standard added cola sample solutions was diluted to the mark using 2 mM $CuSO_4$ solution. The dilution makes the final standard aspartame concentration to 0, 10, 20, 30, 40, and 50 μg mL^{-1} respectively in each of the six cola samples. The six 5-fold diluted aspartame standard added cola samples were analyzed with the 2-D HPLC system by a reversed-phase chromatographic column Zorbax Eclipse XDB-C8 (150 × 4.6 mm i.d., 5 μm, Agilent, Waldbronn, Germany). The guard column was Zorbax Eclipse XDB-C8 (12.5 × 4.6 mm i.d., 5 μm). The column temperature was maintained at 50 °C. The mobile phase was 2 mM $CuSO_4$/methanol (80/20, v/v) at 1.00 mL/min. The UV detection wavelength was 254 nm. The aspartame content in the cola sample was found from the *x*-intercept of the standard addition calibration curve [46].

A



B

Stage	Time (min)	Valve position
1	0.0 - 1.2	I
2	1.2 - 5.8	II
3	5.8 - 65.0	I

Fig. 1. (A) The schematic diagram of the two-dimensional high-performance liquid chromatography system. Position I: the reversed-phase column and the ligand-exchange column in parallel, position II: the reversed-phase column and the ligand-exchange column in series. (B) The time and duration for operating the column-switching at different stage and corresponding valve position.

2.7. Simultaneous analysis of aspartame and amino acid enantiomers by 2-D HPLC system

The 2-D HPLC system consisted of a Shimadzu LC-9A pump (Tokyo, Japan), a Shimadzu SPD-6A UV detector operated at 254 nm, a Shimadzu CTO-6A column oven, an Aglient 1100 series quaternary pump (Waldbronn, Germany), an Aglient 1100 series fluorescence detector operated at λ_{ex} of 340 nm and λ_{em} of 450 nm, a Micro-tech ultra-plus II pump (CA, USA) as the on-line delivery of the derivation reagent, a 1 m long post-column derivation loop, and a Rheodyne 7725i injection valve with a 20 μL sampling loop (Cotati, USA). The analytical column for the reversed-phase separation of aspartame was Zorbax Eclipse XDB-C8 (150 × 4.6 mm i.d., 5 μm, Agilent, Waldbronn, Germany). The guard column was Zorbax Eclipse XDB-C8 (12.5 mm × 4.6 mm i.d.). The LEC column, Chirex 3126 D-penicillamine column (250 mm × 4.6 mm, i.d., 5 μm) with a guard column (30 mm × 4.6 mm, i.d.) from Phenomenex (Torrance, USA), was used for the amino acid enantiomer separation. The reversed-phase column and the ligand-exchange column were coupled parallel by a six-port switching valve (Rheodyne 7000, Cotati, USA) to form the 2-D HPLC system. The chromatographic data processing software for analyzing the reversed-phase separation of UV detection was obtained from Scientific Information Service Corporation (SISC, Taipei, Taiwan) and stored in a P-III 500 computer (Acer, Taipei, Taiwan). The chromatographic data processing software (ChemStation) for ligand-exchange separation of amino acid enantiomers and fluorescent detection was obtained from Agilent and stored in an AMD-400 computer (Gigabyte Technology, Taipei, Taiwan). The detailed configuration of the 2-D HPLC system for simultaneous detection and analysis of aspartame and amino acid enantiomers with UV and fluorescence detector is shown in Fig. 1A.

The operation procedures for analysis of thermally hydrolyzed cola sample were as follows: for stage 1, as the column switching valve was in position I, the cola sample was loaded and injected; soon after the injection, the switching valve was turned to position II to cut and deliver amino acids produced by the hydrolysis of aspartame to the LEC column for separation and detection (stage 2); as all of the amino acid enantiomers were delivered to the LEC column, the switching valve was switched back to position I; at stage 3, aspartame in the cola sample was separated by the reversed-phase column and detected by UV detector, the chromatographic run was then stopped for the next sample injection. The optimal time and duration for operating the column switching of the 2-D HPLC system was found by using the statistical ANOVA procedure [47] according to the variation of peak areas of amino acid enantiomers and was summarized in Fig. 1B. During the chromatographic run the two analytical columns were maintained at 50 °C. The mobile phase for both columns was the same (2 mM $CuSO_4$/methanol, 80:20, v/v) and at 1.0 mL min^{-1}.

2.8. Analysis of aspartame with 2-D HPLC by matrix matched external calibration method

External calibration method was used for the quantitative analysis of aspartame in the sample of thermally hydrolyzed *Coca-Cola Zero* with the 2-D HPLC system. Owing to the complexity of matrix of the cola sample, the external calibration curve for aspartame must be prepared by matching the matrix to assure the analysis accuracy. Therefore, standard aspartame concentrations of 0, 1.0, 2.5, 5.0, 10.0, 20.0, 30.0, 40.0, and 50.0 μg mL^{-1} were prepared by using 5-fold diluted *Coca-Cola Zero* with 2 mM $CuSO_4$ diluent. Each of the standard aspartame solutions was then analyzed 4 times with the 2-D HPLC system of UV detection. The average peak area of each of the standard was corrected with the average background peak area (signal of 0 μg mL^{-1} standard). The corrected signal of each of the standard was then used to obtain the calibration curve with the linear least-square regression method of Excel software.

2.9. Analysis of amino acid enantiomer with 2-D HPLC by matrix matched internal standard calibration method

The quantitative analysis of amino acid enantiomers obtained from the thermal hydrolysis of aspartame in *Coca-Cola Zero* was via the matrix matched internal standard calibration method. The internal standard used for the analysis was D-leucine which has an elution retention time roughly central for the four possible amino acid enatiomer products, L- and D-aspartic acid and L- and D-phenylalanine. The cola sample was diluted 5-fold with 2 mM $CuSO_4$ solution and was used as the matrix in the preparation of the internal standard calibration curves. The standard concentrations for the four amino acid enantiomers were 0, 2.5, 5.0, 10.0, and 20.0 μg mL^{-1} for ultraviolet detection and 0, 0.25, 0.5, 0.75, 1.25, 2.5, 5.0, and 10.0 μg mL^{-1} for fluorescence detection. The concentration of internal standard in each of the amino acid enantiomer standard solutions was fixed at 4 μg mL^{-1}. Each of the amino acid enantiomer standard solutions was analyzed 4 times with the 2-D HPLC system of either UV or fluorescent detection. The average peak area ratio of each of the four amino acid enantiomers to the internal standard was used to obtain the internal standard calibration curve by the linear least-square regression method of Excel software.

3. Results and discussion

3.1. Selection of mobile-phase and concentration of postcolumn derivation reagent for 2-D HPLC system

The separation of amino acid enantiomers was through a ligand-exchange column that contains D-penicillamine stationary phase and uses a mobile phase of aqueous Cu^{2+} ion solution. The Cu^{2+} ion can form complexes with amino acid enantiomers both on the stationary phase and in the mobile phase. The ligand (amino

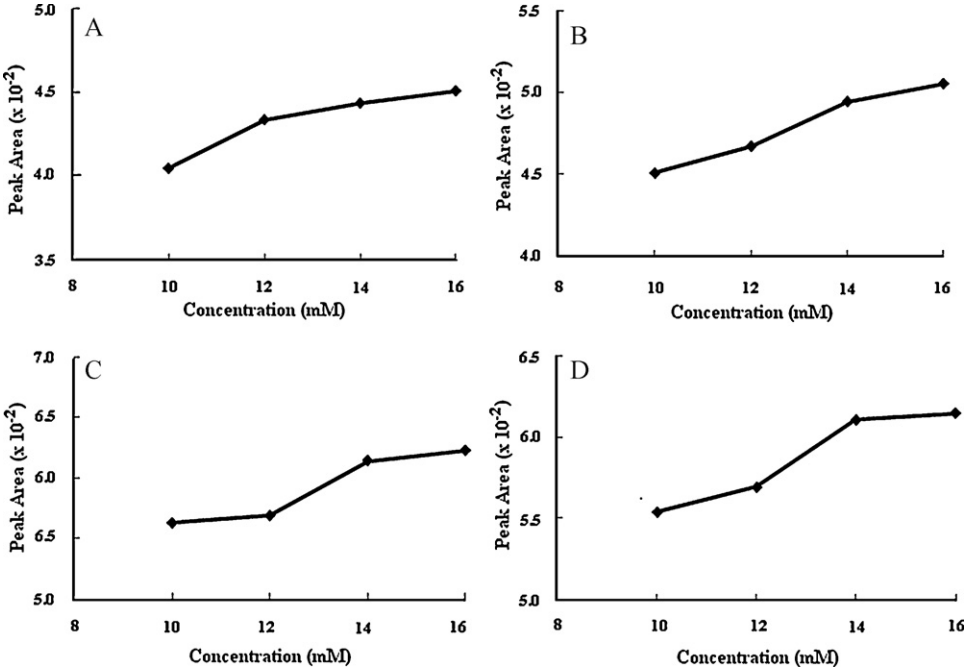


Fig. 2. The variation of the fluorescence detection peak area for amino acid enantiomer at different concentration of fluorescence derivative reagent OPA: (A) L-aspartic acid, (B) D-aspartic acid, (C) L-phenylalanine, and (D) D-phenylalanine.

acid enantiomers) can exchange between the stationary phase and the mobile phase. However, due to different stability of the complexes formed with different amino acids and their L- and D-enantiomers, the ligand exchange rate was different for different amino acid enantiomers, thus, a separation of different amino acid enantiomers can be achieved for analysis. Although a best separation for the four amino acid enantiomers (L- and D-aspartic acid and L- and D-phenylalanine) can be obtained with the use of 2 mM Cu^{2+} ion as the mobile phase, the retention time was too long, i.e. over 200 min for phenylalanine. The use of organic modifier, methanol, can decrease the retention time, however, the composition of methanol is limited to 30% by volume so as not to deteriorate the stationary phase. In order to protect the column and obtain a reasonable retention time for analysis, a mobile phase composition of 2 mM Cu^{2+} ion–methanol (80:20, v/v) was employed. With this kind of mobile phase composition, the four amino acid enantiomer products can be well separated within 70 min. In addition, the choice of this kind of mobile phase is also suitable for the reversed-phase separation of aspartame and its thermal hydrolysis products (amino acids) in cola sample. Therefore, there is only one kind of mobile phase applied for the two different kinds of separation column of the 2-D HPLC system.

In order to make successful fluorescence detection with the postcolumn fluorescent derivation of the amino acid enantiomer-Cu^{2+} ion complexes after the LEC separation, we found that the concentration of the OPA must be high enough to compete with the complexing agent Cu^{2+} ion for the amino acid enantiomers in the complexes. Experimental results showed that as the concentration of OPA is 4 times larger than the concentration of the Cu^{2+} ion in the mobile phase, an obvious fluorescence signal can be observed. The fluorescence signal increases as the concentration of OPA increases for the four amino acid enantiomers which is shown in Fig. 2. However, the increase of the fluorescence signal was small as the concentration of the fluorescent derivation reagent OPA was increased to a concentration of 16 mM. Therefore, 16 mM OPA was used for the postcolumn fluorescent derivation of the amino acid enantiomers to produce a highly sensitive fluorescent detector.

3.2. Optimal column-switching times for 2-D HPLC system

Fig. 3 illustrates the reversed-phase separation for 5-fold diluted cola sample spiked with 20 μg mL^{-1} aspartame and each of the four amino acid enantiomers (L-Asp, D-Asp, L-Phe, D-Phe) and 4 μg mL^{-1} D-leucine (the internal standard). The chromatogram was detected with UV detector at the switching-valve position I. Since polar L- and D-aspartic acid (k' = 0.486 and N = 421 plates) are eluted quickly out from the reversed-phase column, the best initial column-switching time used to transfer the aspartic acid to the LEC column with the switching-valve position II was tested at three time points (1.1, 1.2, and 1.3 min) and the second column-switching time was fixed at 5.8 min. The second column-switching

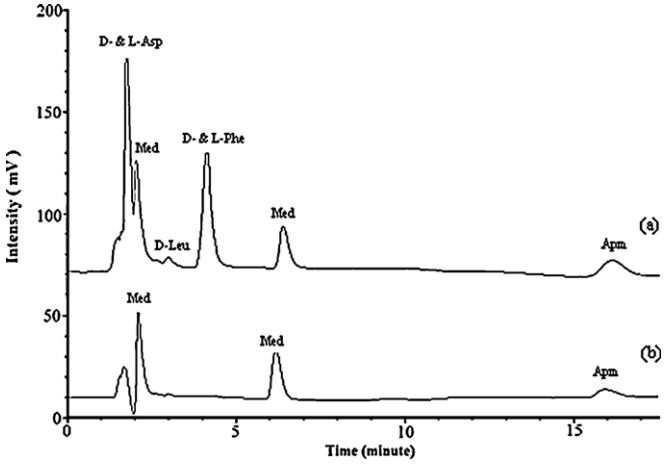


Fig. 3. The reversed-phase chromatography of the 5-fold diluted cola sample with the 2-D HPLC at switching valve position I: (a) with standard addition of D- and L-aspartic acid, D- and L-phenylanaline, D-leucine, and aspartame; (b) plain sample without any standard addition. Column: Zorbax Eclipse XDB-C8; column temperature: 50 °C; mobile phase: 2 mM copper(II) sulfate–methanol (80:20, v/v); flow rate: 1 mL min^{-1}; ultraviolet detector at λ = 254 nm. Peak: Apm = aspartame, L-Asp = L-aspartic acid, D-Asp = D-aspartic acid. D-Leu = D-leucine, Med = cola medium, L-Phe = L-phenylalanine, D-Phe = D-phenylalanine.

time makes the switching-valve back to position I and allows L- and D-phenylalanine (k' = 2.52 and N = 768 plates) be transferred to the LEC column but the rest of the components of cola sample including aspartame (k' = 12.72 and N = 1982 plates) remained in the reversed-phase column for separation. Comparison of average peak areas and their standard deviations for the four amino acid enantiomers with statistical ANOVA procedure [47] at the three tested column-switching times indicates that the initial optimal time for changing the valve position I to position II was 1.2 min.

Similarly, after fixing the initial column-switching time at 1.2 min, the optimal second column-switching time that switches the valve position II back to position I and allows aspartame remained in the reversed-phase column for separation was tested at three different times (5.7, 5.8, and 6.0 min). The results tested with ANOVA procedure showed that the second optimal column-switching time was 5.8 min. The column-switching time and its corresponding valve position are summarized in Fig. 1B.

3.3. LOD and LOQ for aspartame and amino acid enantiomer

The matrix matched external standard calibration method was used for the quantitative analysis of aspartame in thermally hydrolyzed cola sample which is separated by the reversed-phase chromatography with ultraviolet detection. The linear equation for the matrix matched external standard calibration curve and its corresponding linear correlation coefficient (r^2) are listed in Table 1. The standard calibration curve had a linear concentration range of 0–50 μg mL^{-1}. The limit of detection (LOD) and the limit of quantification (LOQ) found for aspartame from the standard calibration curve of five lowest concentrations of standard (0, 1.0, 2.5, 5.0, and 10.0 μg mL^{-1}) [47] were 1.3 μg mL^{-1} and 4.3 μg mL^{-1}, respectively.

The quantitative analysis of amino acid enantiomers produced from the thermal hydrolysis of aspartame in cola sample with LEC was made by the matrix matched internal standard calibration method. The detection method was either post-column OPA derivation fluorescence detection or UV detection. The LODs and LOQs of amino acid enantiomers were estimated from the matrix matched internal standard calibration curve by five lowest concentrations of standard, 0. 0.25, 0.5, 0.75, and 1.25 μg mL^{-1}, for fluorescence detection and 0, 2.5, 5.0, 10.0, and 20.0 μg mL^{-1}, for UV detection. The concentration of the internal standard, L-leucine, was kept at 4 μg mL^{-1}. The linear equation of the four internal standard calibration curves, their corresponding linear correlation coefficients, and the LODs and LOQs were listed in Table 1. It is obvious that the sensitivity of LEC with fluorescent detection was 12–13 times better than that of the UV detection. The improvement of sensitivity was small as compared to the sensitivity of amino acid analysis with fluorescent detection by the reversed-phase chromatography that was in general about 10^3 folds better than the UV detection. Probably, the quenching effect of Cu^{2+} ion in the mobile phase of LEC should be mostly counted for the aforementioned observation. Although the enhancement of sensitivity for fluorescence detection coupled LEC was not as large as we expect for the common sensitivity of fluorescent detection for amino acid analysis with reversed-phase chromatography, this is the first time technically to prove that the more sensitive fluorescent detection can be coupled to LEC.

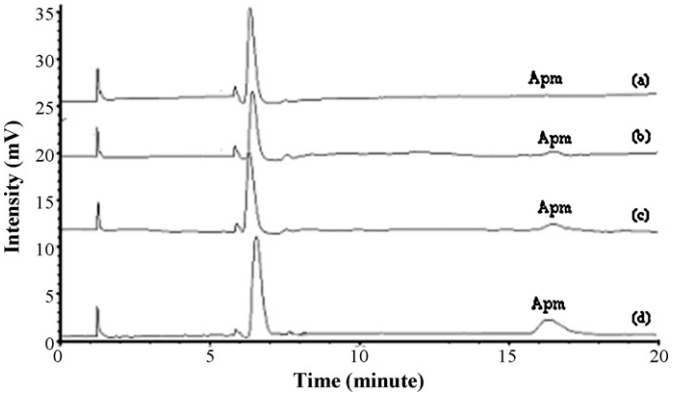


Fig. 4. The reversed-phase chromatogram of the residue aspartame in thermal hydrolyzed *Coca-Cola Zero* with the 2-D HPLC at switching valve position I of the third stage: (a) 120 °C for one-day reaction, (b) 90 °C for 3-day reaction, (c) 60 °C for 5-day, and (d) 30 °C for 5-day reaction. The chromatographic conditions are the same as describe in Fig. 3.

3.4. Analysis of aspartame thermal hydrolysis with electrically heated micro-reactor by 2-D HPLC system

Before the thermal hydrolysis of cola sample, the original content of aspartame in cola sample was determined by the standard addition calibration method using reversed-phase column and UV detection. The analytical results for four different batches (different manufacturing date) of plastic bottled *Coca-Cola Zero* sample were 79.6 ± 3.7, 117.6 ± 1.6, 81.5 ± 3.5, and 89.9 ± 2.2 μg mL^{-1} and for one aluminum canned *Coca-Cola Zero* sample was 86.6 ± 1.9 μg mL^{-1}. The results show that the content of aspartame in cola sample maybe adjusted for regions or times by *Coca-Cola* Company. For the following study, we used the plastic bottled *Coca-Cola Zero* sample containing 117.6 ± 1.6 μg mL^{-1} aspartame.

Fig. 4 illustrates the result of the electro-thermal hydrolysis of aspartame in cola sample with the 2-D HPLC system. These chromatograms show that the extent of hydrolysis increases with an increase of temperature while the time needed to get a certain degree of hydrolysis is shorter. Table 2 shows the detailed analysis results for thermal hydrolysis of aspartame and its amino acid enantiomer products at different reaction temperatures and different periods. Aspartame was almost entirely hydrolyzed in just one day with an electro-thermal temperature 120 °C, however, this situation did not happen at lower temperatures such as 90, 60, and 37 °C.

Fig. 5 illustrates the production of amino acid enantiomers from the hydrolysis of aspartame for four different temperatures with

Table 1
The linear equation and linear correlation coefficients of the matrix matched external standard calibration curve of aspartame and the matrix matched internal standard calibration curves of the four amino acid enantiomers and their corresponding limit of detection and limit of quantification.

Analytes	UV detector				Fluorescence detector			
	Linear equation	r^2	LOD (μg mL^{-1})	LOQ (μg mL^{-1})	Linear equation	r^2	LOD (μg mL^{-1})	LOQ (μg mL^{-1})
Aspartame	$y = 7981.6x - 3401.5$	0.9984	1.3	4.3				
L-Aspartic acid	$y = 0.2151x - 0.1566$	0.9915	2.5	8.4	$y = 0.4826x - 0.0230$	0.9997	0.2	0.6
D-Aspartic acid	$y = 0.2021x - 0.1214$	0.9915	2.5	8.5	$y = 0.5021x - 0.0466$	0.9994	0.2	0.6
L-Phenylalanine	$y = 0.2052x - 0.1525$	0.9911	2.6	8.6	$y = 0.4357x - 0.0051$	0.9990	0.2	0.5
D-Phenylalanine	$y = 0.2007x - 0.1528$	0.9912	2.6	8.6	$y = 0.4301x - 0.0153$	0.9988	0.2	0.5

Table 2
The quantitative analysis results for the electric heating thermal hydrolysis of aspartame and the thermal racemization of its amino acid enantiomeric products in *Coca-Cola Zero*.

Reaction temperature (°C)	Reaction time (day)	L-Asp[a]		D-Asp[a]		L-Phe[a]		D-Phe[a]		Aspartame[a]		Asp	Phe
		Conc. ($\mu g\,mL^{-1}$)	RSD (%)	Conc. ($\mu g\,mL^{-1}$)	RSD (%)	Conc. ($\mu g\,mL^{-1}$)	RSD (%)	Conc. ($\mu g\,mL^{-1}$)	RSD (%)	Conc. ($\mu g\,mL^{-1}$)	RSD (%)	Yield (%)	Yield (%)
120[b]	1	–	–	2.7 ± 0.1	3.7	9.3 ± 0.2	2.2	2.2 ± 0.1	4.5	–	–	2.6	14.0
120	1	14.1 ± 0.1	0.7	8.6 ± 0.1	1.2	22.8 ± 0.2	0.9	2.2 ± 0.1	4.5	–	–	19.3	21.3
90	1	17.7 ± 0.1	0.6	–	–	7.3 ± 0.1	1.4	–	–	20.6 ± 0.2	1.0	15.1	6.2
90	2	21.9 ± 0.1	0.5	3.2 ± 0.1	3.1	11.1 ± 0.2	1.8	–	–	14.0 ± 0.1	0.7	21.3	9.4
90	3	17.6 ± 0.1	0.6	4.0 ± 0.1	2.5	8.4 ± 0.1	1.2	–	–	6.0 ± 0.1	1.7	18.4	7.1
60	1	3.1 ± 0.1	3.2	–	–	–	–	–	–	81.5 ± 0.5	0.6	2.6	–
60	2	4.3 ± 0.2	4.7	–	–	–	–	–	–	69.2 ± 0.6	0.9	2.8	–
60	3	5.6 ± 0.1	1.8	–	–	2.2 ± 0.1	4.5	–	–	58.9 ± 0.2	0.3	2.5	1.8
60	4	7.7 ± 0.1	1.3	–	–	2.9 ± 0.1	3.4	–	–	44.5 ± 0.1	0.2	6.5	2.5
60	5	10.9 ± 0.2	1.8	–	–	2.9 ± 0.1	3.4	–	–	37.0 ± 0.2	0.5	9.3	2.5
37	3	1.5 ± 0.1	6.7	–	–	–	–	–	–	99.3 ± 0.1	0.1	1.3	–
37	4	2.0 ± 0.1	5.0	–	–	–	–	–	–	95.0 ± 0.1	0.1	1.7	–
37	5	2.5 ± 0.1	4.0	–	–	–	–	–	–	90.3 ± 0.1	0.1	2.1	–

[a] Number of measurement = 4 (n = 4).
[b] Reaction of 100 $\mu g\,mL^{-1}$ standard aspartame aqueous solution.

different reaction periods. The enantio-separation of LEC for L- and D-aspartic acid and L- and D-phenylalanine is efficient. The enantioselectivity (α) and the resolution (R_S) between L- and D-aspartic and between L- and D-phenylalanine calculated from chromatogram a were 1.33 and 1.51 and 1.37 and 4.16, respectively. Results in Table 2 show that there were obviously more D-aspartic acid (k' = 1.78 and N = 1156) and D-phenylalanine (k' = 6.40 and N = 2869) produced at the highest temperature of 120 °C than those low temperatures. This phenomenon indicates the energy needed for the racemization of L- and D-amino acid enantiomer is larger than the hydrolysis of aspartame. In addition, the thermal degradation of amino acids or reactions of amino acid with other substances such as caramel by Maillard reaction [48] may occur in the cola sample. These side-reactions are also accounted for the lower yields of the racemization. Correspondingly, thermal hydrolysis of 100 $\mu g\,mL^{-1}$ standard aspartame in aqueous solution was performed that indicates L-phenylalanine (k' = 4.67 and N = 6623) is more stable than L-aspartic acid (k' = 1.33 and N = 1244) for thermal degradation and the thermal racemization of L-aspartic acid is more easily performed than L-phenylalanine. It seems that the complex medium of cola sample may protect the hydrolysis product, amino acid, from thermal degradation.

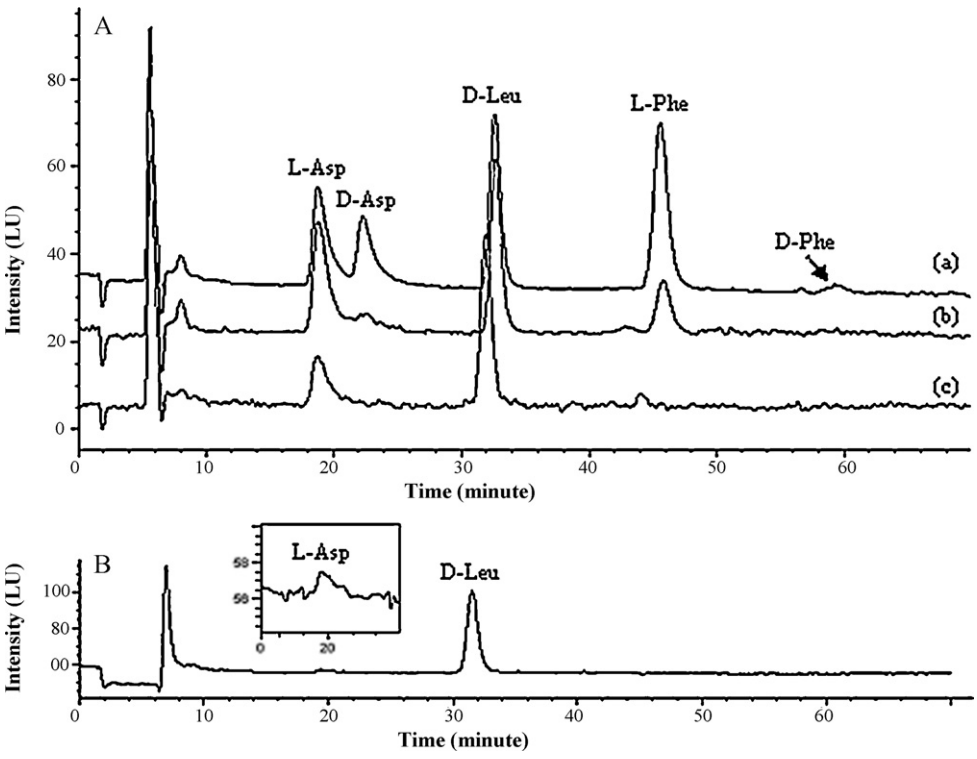


Fig. 5. (A) The LEC chromatogram of the 2-D HPLC for the amino acid enantiomer products after thermal hydrolysis of aspartame in *Coca-Cola Zero*: (a) hydrolysis at 120 °C for one day reaction, (b) hydrolysis at 90 °C for 3-day reaction, and (c) hydrolysis at 60 °C for 5-day reaction. (B) The LEC chromatogram of 2-D HPLC for the amino acid enantiomer products after thermal hydrolysis of aspartame in *Coca-Cola Zero* performed at 37 °C and five-day reaction period. Column: Chirex 3126 D-penicillamine column, column temperature: 50 °C, mobile phae: 2 mM copper(II) sulfate–methanol (80:20, v/v), flow rate: 1 mL min^{-1}, fluorescence detector (λ_{ex} = 340 nm and λ_{em} = 450 nm). Peak: L-Asp = L-aspartic acid, D-Asp = D-asparticacid, D-Leu = D-leucine (internal standard), L-Phe = L-phenylalanine, D-Phe = D-phenylalanine.

Table 3
The quantitative analysis results of the microwave heating thermal hydrolysis of aspartame and its hydrolysis amino acid enantiomeric products in *Coca-Cola Zero*.

Power (W)	Reaction time (min)	L-Asp[a]		D-Asp[a]		L-Phe[a]		D-Phe[a]		Aspartame[a]		Asp	Phe
		Conc. ($\mu g\,mL^{-1}$)	RSD (%)	Conc. ($\mu g\,mL^{-1}$)	RSD (%)	Conc. ($\mu g\,mL^{-1}$)	RSD (%)	Conc. ($\mu g\,mL^{-1}$)	RSD (%)	Conc. ($\mu g\,mL^{-1}$)	RSD (%)	Yield (%)	Yield (%)
800	3	–	–	–	–	–	–	–	–	12.8 ± 0.1	0.8	–	–
800	1	–	–	–	–	–	–	–	–	34.4 ± 0.1	0.3	–	–
800[b]	1	–	–	–	–	–	–	–	–	37.6 ± 0.1	0.3	–	–
560	3	–	–	–	–	–	–	–	–	16.0 ± 0.1	0.6	–	–
560[b]	3	–	–	–	–	–	–	–	–	18.0 ± 0.1	0.6	–	–
560	1	1.7 ± 0.1	5.9	–	–	–	–	–	–	37.4 ± 0.1	0.3	2.1	–
320	3	1.9 ± 0.1	5.2	–	–	–	–	–	–	46.2 ± 0.2	0.4	2.3	–

[a] Number of measurement = 4 ($n = 4$).
[b] Reaction of 80 $\mu g\,mL^{-1}$ standard aspartame aqueous solution.

Results (Table 2) for thermal hydrolysis of aspartame performed at 90 °C and reacted for 1–3 days implies that L-phenylalanine seems to react more easily with other substances in the medium of cola sample. Therefore, the thermal hydrolysis of aspartame in cola sample favors the production of both L- and D-aspartic acid at temperature 90 °C. Also, at 90 °C, which is still high enough, the formation of D-aspartic acid can be aided with long hydrolysis time.

For thermal hydrolysis of aspartame in cola sample performed at 60 °C, where the energy is not high enough, only L-aspartic acid and L-phenylalanine can be accumulated after a reaction period of 3–5 days. The thermal energy at this temperature cannot overcome the activation energy of thermal racemization between L- and D-amino acid for both aspartic acid and phenylalanine.

The thermal hydrolysis of aspartame in cola sample was also done at a lower temperature of 37 °C (a simulated temperature of human body). At this temperature a very small amount of L-aspartic acid can be detected for a long reaction period of 3–5 days. Results in Table 2 show that most of aspartame in cola sample was still preserved in good form. This result also means that people may not worry about the occurrence of the hydrolysis of aspartame in the cola drink at the usual body temperature.

The RSD values for those replicate quantitative measurements shown in Table 2 were all lower than 1.7% for aspartame analysis by UV and were from 0.5% to 6.7% for fluorescence detection of amino acid enantiomers that demonstrates a very good analysis precision for the determination of aspartame and amino acid enantiomers with the 2-D HPLC system. The accuracy for the analysis of aspartame and amino acid enantiomers with the 2-D HPLC system was found through the percentage recovery of the spiked experiments [45]. The spike recovery for aspartame with UV detection was 90.2–100.8% and the spike recovery for L- and D-aspartic acid and L- and D-phenylalanine was 90.4–96.2% that also shows very good overall analysis accuracy (90.2–99.2%) for the 2-D HPLC system.

Our results shown here are consistent with the literature [5,6] that is the extent of aspartame hydrolysis increases with an increase of temperature and the length of time elapsed. In the literature, the rate of hydrolysis can be speeded by acid which plays the role of catalyst. There was also more D-aspartic acid produced than D-phenylalanine produced by the racemization in an acidified medium (3 and 6 M HCl) and at a hydrolysis temperature 110 °C for 1 day [5]. Using bottled *CoCa* light as a sample, Chen et al. [6] also showed that hydrolysis and racemization of aspartame can be occurred at room temperature (25 °C) and pH 2.0.

3.5. Analysis of microwave induced aspartame thermal hydrolysis with 2-D HPLC system

Nowadays there are a lot of microwave heating ready-to-eat foods and drinks sold in the supermarkets which may contain aspartame. Therefore, in order to understand the effect of microwave heating on the hydrolysis of aspartame, plastic bottled *Coca-Cola Zero* containing 81.5 ± 3.5 $\mu g\,mL^{-1}$ of aspartame was used as a model sample to study the thermal hydrolysis of aspartame with pulsed microwave heating at three different output microwave powers, 320, 560, and 800 W for either 1 or 3 min. Table 3 summarizes the quantitative results determined by the 2-D HPLC system. It is obvious that the extent of aspartame hydrolysis in cola sample increases as the microwave output power increases. However, in most of the cases, the hydrolysis products, amino acid enantiomers were not found. Only a very small amount of L-aspartic acid can be detected at the microwave output power of either 320 W for 3 min operation or 560 W for 1 min operation. This phenomenon could be due to the instantaneous extremely high temperature produced by the pulsed microwave power to trigger a rapid thermal hydrolysis of aspartame and the subsequent thermal degradation of amino acids. In contrast to the thermal hydrolysis of aspartame with electric heating, aspartic acid seems more stable than phenylalanine in *Coca-Cola Zero* matrix under the microwave heating.

Stenberg et al. [5] also performed the microwave hydrolysis of aspartame in acidified aqueous medium (3 and 6 M HCl) under 700 W or 300 W for 1, 2, 3, 6, or 10 min. The percentage of aspartame hydrolysis was in the range 82–100% and the racemization for L-aspartic acid and L-phenylalanine was in the range 0.19–4.83% and 0.12–3%, respectively. The results are quite different from ours mainly because the significant difference of medium used for the experiment. In our study, comparison of the results for microwave induced thermal hydrolysis of aspartame in cola sample and in aqueous solution performed at either 800 W for 1 min or 560 W for 3 min shows that the complex matrix of cola sample is able to protect aspartame from being hydrolyzed.

The analysis precision of microwave induced hydrolysis for the residue aspartame and the only hydrolysis product of L-aspartic acid was lower than 0.8% and 5.9%, respectively. These results demonstrated a good analysis precision for the determination aspartame and the trace amino acid enantiomers with the 2-D HPLC system. The spike recovery for aspartame and L-aspartic acid was between 91.3–93.5% and 90.1–93.5%, respectively, that also shows very good analysis accuracy for the 2-D HPLC system.

4. Conclusions

A 2-D HPLC system was developed by on-line coupling a ligand-exchange chromatographic column and a reversed-phase chromatographic column which can be used for studying the thermal hydrolysis of dipeptide ester aspartame in *Coca-Cola Zero*. This is the first report on the determination of L- and D-aspartic acid and L- and D-phenylalanine using a ligand-exchange column with on-line postcolumn OPA derivation for fluorescent detection. The analysis sensitivity of amino acid enantiomers in cola sample with

the fluorescence detection coupled ligand-exchange chromatography was 12–13 times better than the traditional UV detection. The analytical results of the electro-thermal and microwave induced hydrolysis of aspartame in cola sample imply that the hydrolysis of aspartame in *Coca-Cola Zero* increases with the increase of temperature and the increase of hydrolysis time and there are small extent of thermal racemization of L-amino acid to its corresponding D-amino acid at high temperature. At body temperature, the extent of aspartame hydrolysis is negligible. Overall, the simultaneous analysis of aspartame and amino acid enantiomers in cola sample by the 2-D HPLC system exhibits high sensitivity, accuracy, and precision.

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