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A facile HPLC method for optical purity and quantitative measurements of phenylalanine from the hydrolyzed aspartame under different pH and temperature after its derivatization with a fluorescent reagent

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Summary. In this paper, the artificial sweetener aspartame is deliberately hydrolyzed under different pH and temperature in the matrix, and time period for the hydrolysis. The HPLC analysis is then performed to quantitatively measure the amount and the optical purity of phenylalanine produced as a result of hydrolysis in the matrix after its functionalization with a fluorescent reagent. The results show that the amount of phenylalanine in the matrix is affected by the pH variation during the hydrolysis and found increased in low pH conditions. High temperature or long time periods for the decomposition also increases the amount, which indicates that beverages and foods containing aspartame as a sweetener may not be safe for phenylketonuria patients to consume if they are stored under these conditions. Conversely, the optical purity of phenylalanine, expressed as the percentage of D-enantiomer, is not affected by pH variations. However, it decreases as the length of time elapsed is increased or surrounding temperature is elevated during the decomposition.

Keywords: Aspartame – Optical purity – Phenylalanine – Teicoplanin – Fluorescent reagent

Introduction

Aspartame, a chemically synthesized dipeptide, was developed in 1965 and introduced to USA in 1983 as an artificial sweetener in food industry under the brand name ''NutraSweet''. It tastes sweet as sucrose. However, its sweetness is 180 times more and thus routinely consumed in low-calorie meals, soft drinks (i.e., carbonated and fruit-juice beverages), candy, chewing gums, and frozen desserts for dietary reduction of sugars and calories (Feng et al., 1999). When added to food or soft drinks, the aspartame stability is subject to the influence of pH, temperature and light intensity within the matrix, which in turn causes the decomposition and thus raises the safety concerns (Kim et al., 1997). The study reported previously has indicated that aspartame is most stable at pH 4.0 (Sabah et al., 1998). Lowering the pH value to 3.1 at room temperature decreases the sweetness to two-thirds of the original level. However, the degradation is observed at pH >6.0 or <1.0 as described previously (Stenberg et al., 2001). It is suspected that the degraded components such as L-aspartic acid, L-phenylalanine and diketopiperazine are optically active molecules and will racemize. Note that the patients with phenylketonuria disease do not metabolize the phenylalanine and are supposed to avoid consuming it in the daily diet.

To monitor the concentration of aspartame and other artificial sweeteners such as dihydrochalcone (NHDC), many analytical methods have been developed including spectrophotometry, enzymatic analysis, capillary electrophoresis and chromatography (Bernard et al., 1996; Kazimierz and Katarzyna, 1997; Sabah et al., 1998; Stenberg et al., 2001; Zhu et al., 2005). Among them, the most frequently used method on a routine basis is high-performance liquid chromatographic separation with UV and spectrofluorimetric detection for its great availability and expansibility. However, only few techniques are reported for monitoring the quantity of phenylalanine present in the matrix due to the hydrolysis of aspartame under different storage conditions (e.g., pH, temperature and light intensity, etc.) or in the urine as the metabolite after digestion (Sabah et al., 1998; Stenberg et al., 2001; Zhu et al., 2005). This is in part because the separation of aspartame and phenylalanine is still challenging for some of the techniques such as ion chromatography under isocratic elution (Stenberg et al., 2001). The importance of analyzing phenylalanine during the routine neonatal

screening for patients with phenylketonuria disease has been mentioned. Notice that aspartame after digestion and hydrolysis is not the only source for phenylalanine (Chen, 1996). Many others have been reported, which could be referenced to phenylketonuria patients as the diet guide for staying healthy.

In this report, a facile HPLC technique is developed to analyze the amount and optical purity of the degraded component of aspartame in diluted acidic solution such as phenylalanine after its derivatization with a fluorescent reagent. The effects of pH, temperature variations in the matrix and length of time elapsed during the hydrolysis on the quantity and the optical purity of phenylalanine are also explored. This method is applied to analyze the amount of phenylalanine present in the commercial daily products such as diet Coke and chewing gum as well.

Materials and methods

Apparatus

The HPLC system used to carry out all analyses is a Hitachi model L-7100 linked to an UV (L-7420) or a fluorescent detector (L-2480). The UV detection wavelength is set at 266 nm in the HPLC resolution study. In the enantiomeric purity determination according to the previous results (Hsien and Chen, 2006), the excitation and emission wavelengths for the fluorescence detection are set at 266 and 520 nm, respectively. All calculations based on the peak area are processed on a Hitachi model D-2500 data integrating station. A 20 µl injection valve (Rheodyne) is used. The optical purity analysis of phenylalanine after its derivatization with the FCC reagent is carried out at ambient temperature (∼28 °C) and at a flow rate of 1.0 mL/min on a teicoplanin bonded chiral stationary phase (250 × 4.6 mm i.d., 5 µm particle diameter, supplied by Advanced Separation Technologies, Whippany, NJ, USA) using nonaqueous methanol-based solvent mixture as the mobile phase (Hsien and Chen, 2006). The achiral separation and quantitative determination of components in aspartame hydrolysis study under a variety of pH and temperature as 9-fluorenylmethyl chloroformate (FMOC) derivatives is performed on a C_{18} column (150 × 4.6 mm i.d., 5 µm particle diameter, supplied by Vercopak, Taiwan, ROC) using a mixture of acetonitrile and double distilled water as the mobile phase. The water needs to be acidified with glacial acetic acid (HOAC) at a ratio of 495/2 by volume (v/v) before use.

Chemicals

All chemicals used in this study are purchased from Sigma (St. Louis, MO, USA) and Aldrich (Milwaukee, WI, USA), respectively. Aspartame is from TCI (Tokyo, Japan). All HPLC grade solvents (acetonitrile, methanol, triethylamine, glacial acetic acid, etc.) are obtained from Fisher Scientific (Pittsburgh, PA, USA) and Merck Taiwan Ltd. (Taipei, Taiwan, ROC). The double filtered and distilled water is used in all cases.

Preparation and derivatization of samples

To simulate the digestion in stomach under the pH of gastric juice, the weighed aspartame is first dissolved in diluted hydrochloric acid solution (0.01 M) for hydrolysis. During the degradation, factors such as the temperature and time elapsed are carefully controlled to study how the total amount and the optical purity of the degraded product such as phenylalanine are affected. Once the degradation is completed, the acidic solution is first neutralized before being dried out under low temperature and reduced pressure. The residue is collected, dissolved in alkaline medium and then

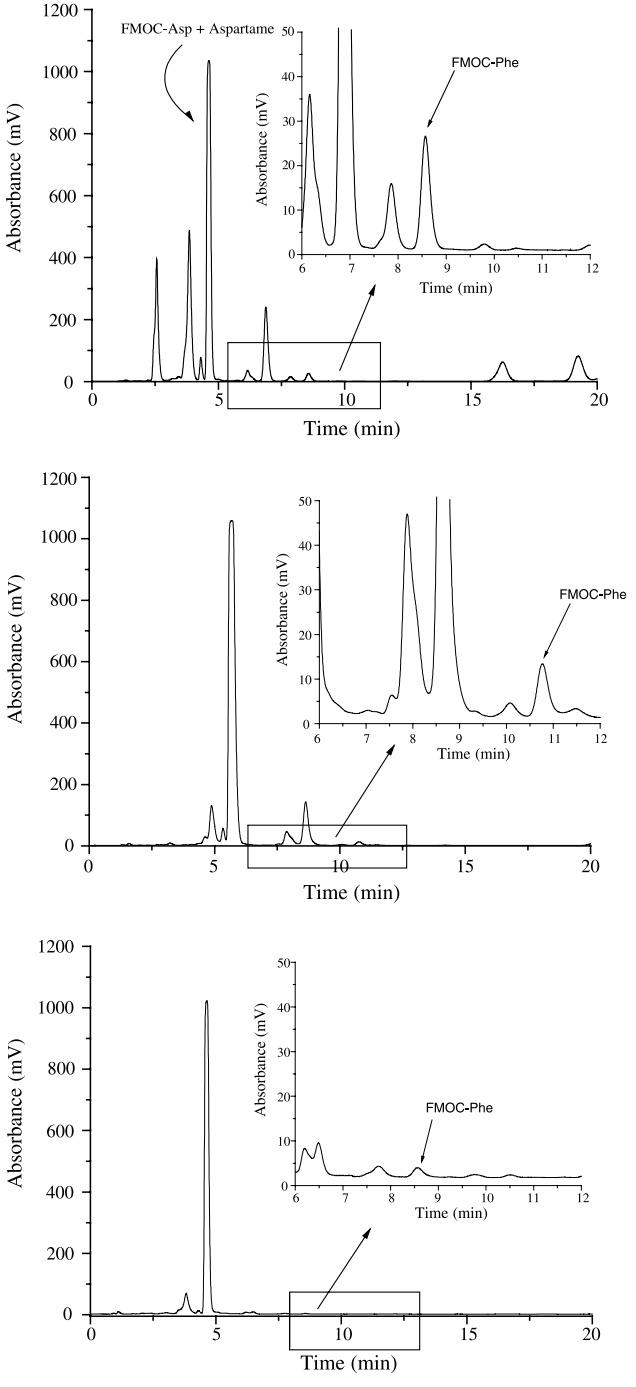

Fig. 1. Chromatograms showing the achiral resolution of degraded components from aspartame after its hydrolysis at a temperature of 60 °C in acidic (top), neutral (middle) and basic (bottom) medium in FMOC derivatization form on a 15 cm C_{18} column using a solvent mixture of acetonitrile and water (top: 60/40, middle: 55/45, bottom: 60/40) as the mobile phase. The water is first acidified with acetic acid before use. For clarity, a small portion of chromatogram is magnified in each case to show the peak for FMOC-Phe derivative

derivatized with 9-fluorenone-4-carbonyl chloride (FCC) reagent immediately for optical purity analysis according to the procedure reported previously to minimize the racemization process due to bacterial contamination upon exposure to air (Hsien and Chen, 2006). In this case, the degradation temperature is set at 100 °C.

In the case of quantitative determination of phenylalanine produced during the hydrolysis, the same preparation procedure is followed except that the derivatizing reagent FMOC is used.

Chromatographic conditions and analyses

The achiral separation of FMOC derivatized aspartame after hydrolysis is performed on a 15 cm C_{18} column with a solvent mixture of acetonitrile, HPLC grade water and glacial acetic acid. As shown in Fig. 1, FMCO-Phe is well separated from FMOC-Asp and other degraded components of aspartame on a 15 cm C_{18} column in the reversed-phase mode. The amount of FMOC-Phe is quantitatively determined based on the peak area calculation and by extrapolating a calibration curve created with FMOC-Phe standard as shown in Fig. 2. The correlation and linearity is considered to be good enough for the quantitative measurement in this study.

In studying the effects of hydrolysis temperature and time period for the hydrolysis on the amount of phenylalanine, the weighed aspartame in diluted hydrochloric acid solution (0.01 M) is placed in the water bath with temperature carefully controlled by a thermostat. Once the hydrolysis is completed, the solution is first neutralized, dried out and then re-dissolved in an alkaline medium before the derivatization with FMOC reagent.

In the enantiomeric ratio determination, the matrix is treated with ethyl ether after hydrolysis and derivatization to recover the FCC-Phe derivative. The ethyl ether layer is collected, evaporated under low temperature and reduced pressure. The residue is then re-dissolved in proper amount of nonaqueous methanol for enantiomeric analysis on a teicoplanin bonded chiral stationary phase using the methanol-based mobile phase as described previously (Hsien and Chen, 2006). The enantiomeric composition of FCC-Phe derivative is determined by integrating the peak area and is expressed as the percentage of D-enantiomer. All separations are carried out at a flow rate of 1.0 ml/min and at ambient temperature (~28 °C) using the methanol-based mobile phase. The mobile phase used in the resolution is 95 MeOH/5 EE/0.2 HOAC/0.4 TEA by volume (v/v). The ACN, MeOH, HOAC, TEA and EE are abbreviations for acetonitrile, methanol, acetic acid, triethylamine and ethyl ether, respectively. The fluorescence detection is employed in this case.

Results and discussion

The achiral resolution for degraded components of aspartame on a 15 cm C_{18} column with a solvent mixture of acetonitrile, HPLC grade water and glacial acetic acid after the derivatization with FMOC reagent is shown in Fig. 1. As can be seen, FMCO-Phe derivative is well separated from FMOC-Asp and other degraded components of aspartame on a 15 cm C_{18} column in the reversed-phase mode. For clarity, a small portion of chromatogram is magnified to show the peak for FMOC-Phe derivative. Note that FMOC-Phe derivative is present in all three chromatograms for the resolution of degraded components as a result of hydrolyzing aspartame in acid (top), neutral (middle) and basic (bottom) mediums. The differ-

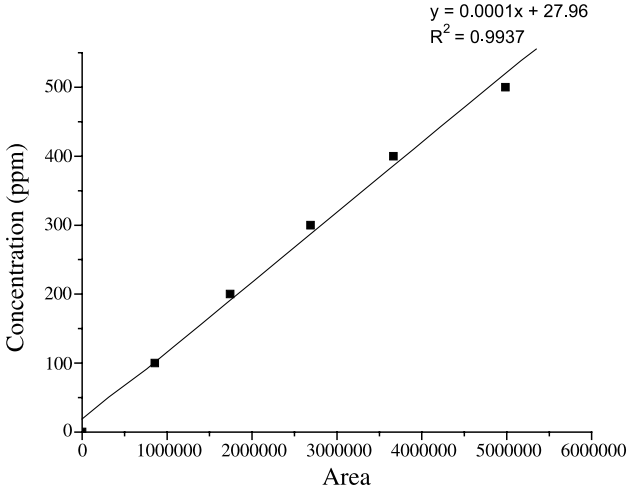

Fig. 2. The curve calibrated with the standard of FMOC-Phe. As can be seen, the calibration curve is obtained with great linearity and good correlation. The ratio of acetonitrile and water in the mobile phase is 70–30

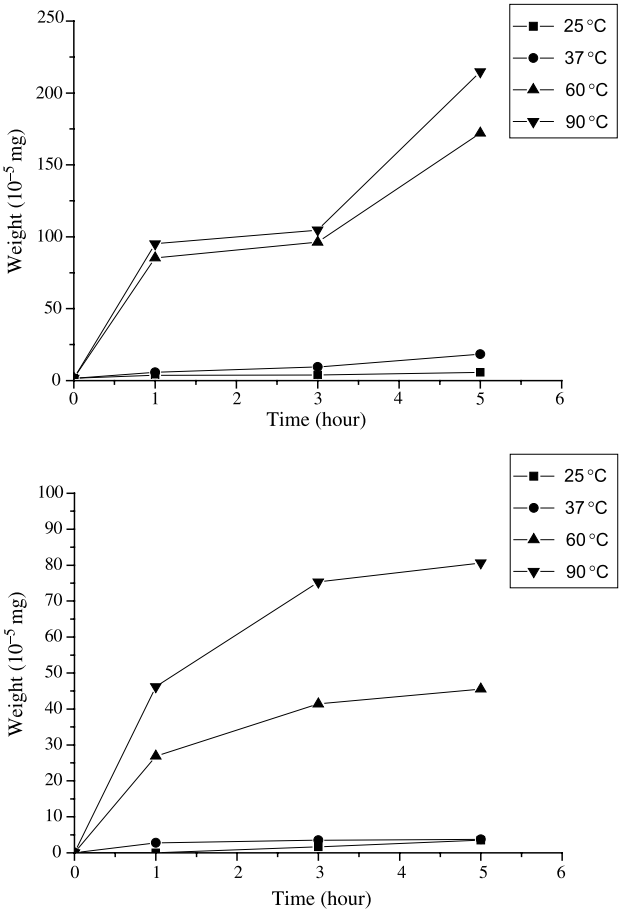

Fig. 3. The effect of temperature and length of time elapsed during the hydrolysis in acidic (top) and distilled water (bottom) mediums on the amount of phenylalanine present in the matrix. A solvent mixture of acetonitrile and water (60/40) is used as the mobile phase in this study

ence among them is in the amount of FMOC-Phe derivative produced in the matrix after the degradation. Upon comparison, it clearly indicates that the largest quantity is obtained if the hydrolysis is carried out in acidic medium. As the pH of matrix rises to be in the basic region, the hydrolysis of aspartame seems to be suppressed judging from the peak area for FMOC-Phe derivative. This conclusion is consistent with the results reported previously (Hire et al., 1954; Liardon and Jost, 1981; Liardon and Ledermann, 1986; Manning, 1970; Stenberg et al., 2001). Figure 3 shows that, when the length of time elapsed during the hydrolysis increases, the corresponding increase in the amount of FMOC-Phe is observed in acidic (top) and neutral (bottom) mediums. This increasing trend is also observed in all mediums when the temperature for hydrolysis is elevated (the results for basic medium are not shown). Under high surrounding temperature and acidic environment, aspartame is expected to decompose more significantly based on the results shown above. Consequently, food and beverages with aspartame added as the artificial sweetener should avoid being stored under these conditions.

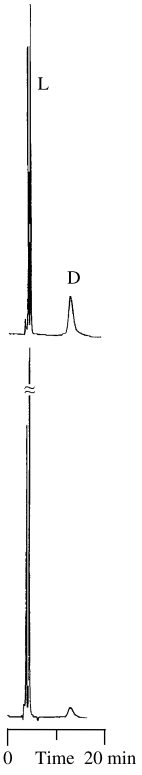

Fig. 4. The chromatograms for the chiral resolution of commercial Phe (top) and Phe (bottom) from the decomposed aspartame in FCC derivatization form at a temperature of 40 °C on a teicoplanin phase using the methanol-based mobile phase described in the experimental section. The % D-enantiomer of phenylalanine is calculated to be 0.51 based on the peak area in this particular case. The y-axis represents the fluorescence intensity

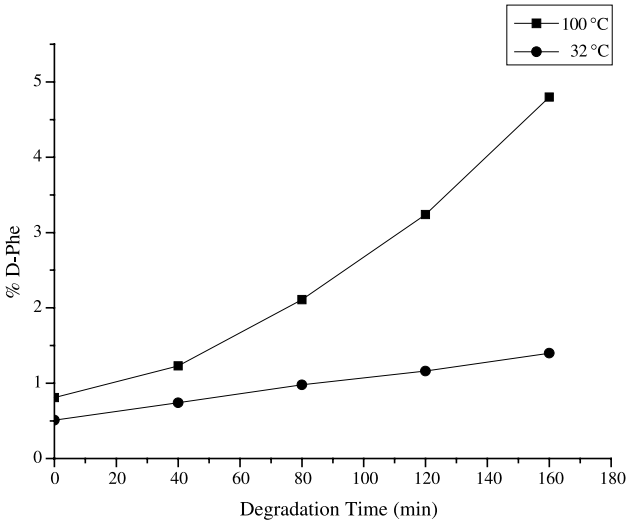

Fig. 5. The effect of temperature and length of time elapsed during the hydrolysis in acidic medium on the percentage of D-enantiomer of phenylalanine present in the matrix in FCC derivatization form. The mobile phase used is the same as that in Fig. 4

To determine the optical purity of phenylalanine as the FCC derivative, the degraded components of aspartame are prepared for the HPLC resolution according to the procedure described before (Hsien and Chen, 2006). The trends in Fig. 4 show that high hydrolysis temperature and long time period for the hydrolysis both lower the optical purity (i.e., high percentage of D-phenylalanine) in acidic medium. In either neutral or basic conditions, no detectable racemization is found even under the elevated temperature environment. A typical chromatogram showing the small percentage of D-phenylalanine in the presence of prevailing L-phenylalanine is demonstrated in Fig. 5. In this particular case, the percentage of D-phenylalanine is calculated to be 0.51 based on the peak area at a hydrolysis temperature of 40 °C. In summary, these results indicate the optical purity of phenylalanine from the aspartame degraded in acidic medium is changed to the storage conditions. Lowering the surrounding temperature and shortening the shelf life of the aspartame-sweetened food and beverages appear to be the effective approach of decreasing the percentage of D-enantiomer produced in the matrix. According to the conclusion drawn above, more D-phenylalanine is expected to produce in stomach due to the acidic hydrolysis by gastric juice. This increase will become even more significant when the surrounding temperature is elevated and the time period for the process is extended. The racemization of amino acids (e.g., phenylalanine and aspartic acid) due to the action of micro-

wave in two diluted HCl solutions (i.e., 3 and 6 M) has been reported (Stenberg et al., 2001). The results showed that more racemization was observed in 3 M HCl solution. However, the racemization appeared not as significant in the conventional hydrolysis using hot plate as the heat source. Oppositely, more racemization is observed in our study when the hydrolysis is carried out in the further diluted HCl solution (0.01 M). It seems that factors other than those mentioned in previous study may be involved in the process of amino acid inversion (Zhu et al., 2005).

This method is also applied to analyze the amount of phenylalanine present in some daily products with aspartame added as the artificial sweetener such as soft drinks, and chewing gum etc. and proved to be facile. The results including the quantity and the optical purity of phenylalanine found in these samples at room temperature are summarized in Table 1. Figure 6 shows the chromatogram

Table 1. Amount and percentage of D-enantiomer of phenylalanine present in some daily products containing aspartame as the artificial sweetener

Sample	Phe amount ($\times 10^5$ mg)[a]	% D-Phe[a]
CoCa light (bottled)	11.56/10 ml	0.51
Extra chewing gum	3.77/piece[b]	Not detectable
Suntory[c]	8.04/15 ml	0.78
NutraSweet	Not found	Not detectable

[a] Average of four determinations
[b] Average weight for each chewing gum is 1.52 g
[c] An amino acid-based soft drink

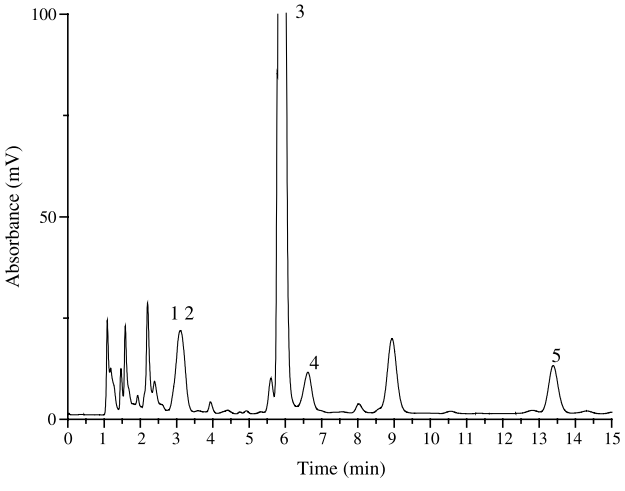

Fig. 6. The chromatogram showing the achiral resolution of ingredients present in the amino acid-based beverage. As can be seen, the peaks for phenylalanine and aspartame are well resolved among other amino acids on a 15 cm C_{18} column after the derivatization with FMOC reagent. The ratio of acetonitrile and water in the mobile phase is 70/30. Peaks 1–5 are identified as arginine, lysine, aspartame, proline and phenylalanine by the comparison with the standards, respectively

for HPLC resolution of an amino acid-based beverage (Suntory) after its FMOC derivatization on a 15 cm C_{18} phase in the reversed-phase mode. As can be seen, phenylalanine is separated from aspartame and several other amino acids and is only found in liquid or soft samples at storage temperature (i.e., not in NeutraSweet). The presence of phenylalanine is very likely due to the photodecomposition (Kim et al., 1997). Interestingly, the racemization is also observed only in those liquid samples. It appears that the energy needed to cause the amino acid inversion is accumulated more easily and effectively in the liquid phase, rather than in the solid phase (Stenberg et al., 2001).

Conclusion

The HPLC method developed to analyze the artificial sweetener aspartame that is deliberately hydrolyzed under different pH and temperature in the matrix, and time period for the hydrolysis after its functionalization with a fluorescent reagent is proved to be facile. This method is also versatile since it employs the ordinary LC system with the UV or fluorescent detection and can do the chiral/achiral separations and determinations simultaneously if coupling two LC systems in series.

The results show that the amount of phenylalanine is affected by pH, temperature and time period for hydrolyzing aspartame in the matrix. High temperature or long time period for the decomposition process also increases the amount, which indicates that beverages and foods containing aspartame as a sweetener may not be safe for phenylketonuria patients to consume if they are stored under these conditions. Conversely, the optical purity of phenylalanine, expressed as the percentage of D-enantiomer, is not affected by pH variations. However, it decreases as the length of time elapsed is increased or temperature is elevated during decomposition. Note that the D-enantiomer of phenylalanine is not metabolized by the human body.

Acknowledgement

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