Enzymatic Determination of Aspartame in Carbonated Beverages

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The determination of aspartame in carbonated beverages was investigated by enzymatic method. Conditions for determining aspartame were as follows: time of hydrolysis by 6 N HCl, 5 h at 110°C; concentration and pH of 2-amino-2-methyl-1,3-propanediol-HCl buffer, 200 mM and 7.5; reaction temperature, 37°C; reaction time, 5 h; GOT, 0.655 IU/ml; MDH, 0.234 IU/ml; α -ketoglutarate; 0.615 mM; concentration of NADH, 0.1 mM. The method gave high recoveries (93.9–102.1%) of aspartame to amount added (0.058–0.339 mg/ml) into carbonated beverages. The minimum limit of determination was 6 μ g/ml.

Keywords — enzymatic determination; aspartame; carbonated beverage; aspartame hydrolysis

Aspartame (α -L-aspartyl-L-phenylalanine methyl ester) is a low-caloric and artificial sweetener that is approximately 200 times sweeter than sugar. The sweetener was approved for use in carbonated beverages by the Food and Drug Administration in the United States in 1983 and was approved as a food additive in Japan the same year. Although the quantity of aspartame content in foods has not been yet limited, it is necessary to establish a method to determine the amount present in carbonated beverages.

Methods of quantitative analysis by amino acid analyzer,^{1,2)} HPLC³⁻⁵⁾ and GLC⁶⁾ to determine aspartame content in foods have been reported.

This note reports investigation of an enzymatic method for determination of aspartame based on the conditions of the presence of other additives in carbonated beverages. Aspartame is a peptide composed of L-aspartic acid and L-phenylalanine. If the peptide is quantitatively hydrolyzed, aspartame will be determined by measuring the amount

of aspartic acid by enzymatic method.⁷⁾ The relevant reactions are: aspartame hydrolysis L-aspartate + L-phenylalanine, L-aspartate + α -ketoglutarate GOT oxaloacetate + L-glutamate, oxaloacetate + NADH + H⁺ MDH L-malate + NAD⁺, where GOT is glutamate oxaloacetate transaminase and MDH is malate dehydrogenase. The amount of aspartame present is proportional to the decrease in absorbance of NADH at 340 nm. The method is limited to the determination of aspartame in foods which contain no protein. Suitable conditions of hydrolysis by HCl, pH and concentration of buffer, reaction time and temperature were evaluated.

Experimental

Reagents — (1) Buffer Solution: Na₂B₄O₇-HCl, Na₂B₄O₇ + KCl-HCl, Tris-HCl and 2-amino-2-methyl-1,3-propanediol-HCl (AMP-HCl) buffers were adjusted with hydrogen chloride, and KH₂PO₄-NaOH buffer was adjusted with sodium hydroxide. The buffers were 200 mM solution of pH 7.5.

(2) NADH Solution: β -NADH was obtained from Sigma Chemical Co., Ltd. and was grade III; approx. 98% pure. A stock solution was prepared by adding 2.43 mg of β -NADH to 1 ml of distilled and deionized water.

(3) Enzyme Solution: MDH (suspension, from bovine heart, 2570 IU/mg) and GOT (suspension, type I from porcine heart, 240 IU/mg) were obtained from Sigma Chemical Co., Ltd. Stock solutions of MDH and GOT were prepared by adding 3 μ l and 10 μ l, respectively, to 1 ml of water.

(4) Sample: Cola, cider and apple soda were commercial products. Apple soda was a gift from Ajinomoto Co., Inc.

(5) Others: α -Ketoglutaric acid (2.97 mg) was mixed with 5.94 ml of glycerol and the mixture was adjusted to 10 ml with water.

Procedure — One-tenth of 1 ml of sample was degassed and hydrolyzed with 0.5 ml of 6 N HCl in a small test tube sealed hermetically at 110°C for 5 h. After centrifugation of the resulting product, the precipitation was washed with water several times. The supernatant and the washings were combined and were dried using a rotary evaporator to completely remove hydrogen chloride.

AMP-HCl buffer was accurately added to a 10-ml volumetric flask in which the dry substance was put. The solution obtained was a prepared sample solution. Into a clean dry cuvette, 1.0 ml of buffer solution, 1.0 ml of α -ketoglutaric acid-glycerol mixture, 0.1 ml of NADH, 0.1 ml of GOT and 1.0 ml of prepared sample solution were pipetted and mixed. The mixture was kept at 37°C; the absorbance (E_1) being measured at 340 nm. Then, 0.1 ml of MDH was added to the cuvette and the mixture was kept for 5 h at 37°C; the absorbance (E_2) was measured at 340 nm. Aspartame in the sample was determined using the calibration curve (relative coefficient, 0.999) over concentrations from 0 to 0.08 mM.

Results and Discussion

1. Hydrolysis of Aspartame

Since hydrolysis of aspartame could not be catalyzed by carboxypeptidase, aminopeptidase or pepsin, it was attempted in hydrogen chloride (Table I).

The results of decomposition rate and coefficient of variation indicated the most appropriate concentration to be 6 N.

TABLE I. Hydrolysis of Aspartame at Different Concentrations of Hydrogen Chloride

Concentration	Decomposition rate (%)	Coefficient of variation ($n=6$)
1 N HCl	94.7	7.7
3 N HCl	92.9	8.7
6 N HCl	97.3	4.1
9 N HCl	94.1	8.8

Hydrolysis conditions: temperature, 110°C; time, 5 h; initial concentration of aspartame, 3.4 mM.

TABLE II. Comparison of Absorbance due to Formation of NAD in Various Buffers

Buffer	ΔE^a
$\text{Na}_2\text{B}_4\text{O}_7\text{—HCl}$	Not possible
$\text{Na}_2\text{B}_4\text{O}_7 + \text{KCl—HCl}$	Not possible
$\text{KH}_2\text{PO}_4\text{—NaOH}$	0.022
(Tris) hydroxymethyl aminomethane—HCl	0.312
2-Amino-2-methyl-1, 3-propanediol—HCl	0.527

a) $\Delta E = E_1 - E_2$. E_1 is the absorbance under the following conditions: 0.1mM solution of aspartic acid dissolved in buffer, 1 ml; 200 mM buffer, 1 ml; α -ketoglutaric acid-glycerol mixture, 1ml; GOT, 0.1ml; NADH 0.1ml in a cuvette. E_2 is the absorbance after adding MDH, 0.1 ml to the cuvette.

Fig. 1. Rate of Decomposition of Aspartame at Given Elapsed Time

2. Suitable Conditions for Enzymatic Reaction

Absorbance due to the formation of NAD was measured in various buffers to select a

Fig. 2. Effects of Buffer Concentration (A), pH (B) and Temperature (C) on NADH Decrease

concentration of aspartic acid, 0.1 mM.

buffer suitable to the enzymatic reaction (Table II). The AMP-HCl buffer was selected.

Since rate of decomposition of aspartame reached 100% at approx. 4 h, as shown in Fig. 1, adequate hydrolysis of aspartame was set at 5 h. Decrease in rate of decomposition with time over approx. 10 h can be explained by the formation of methylester.⁸⁾

Absorbance slightly decreased with increased concentration or pH of AMP-HCl buffer (Fig. 2). A concentration of 200 mM and pH of 7.5 of AMP-HCl buffer were desirable because of the highly stable enzymatic reaction.

Absorbance linearly increased with increasing temperature (Fig. 2). Based on the enzyme stability, the temperature was set at 37°C.

Time to reach equilibrium of the enzymatic reaction was 40 min for aspartic acid solution and 5 h for prepared sample solution. Sodium benzoic acid, caffeine and sucrose were found in the carbonated beverages, and seemed to slow up the enzymatic reaction.

3. Recovery of Aspartame

Amounts of aspartame contained in three kinds of carbonated beverages and recoveries of added aspartame are shown in Table III; they were from 0.116 to 0.196 mg/ml. The amounts added were 0.058 to 0.339 mg/ml. The recovery of aspartame in the samples was in the range of 93.9 to 102.1%. Recoveries of 94.9–98.8%,²⁾ 99.9%,³⁾ 90–97%⁴⁾ and 97%⁶⁾ were reported for the added amounts of 0.05 mg/g,²⁾ 0.1 mg/g,³⁾ 0.10–1.00 mg/g⁴⁾ and 0.10–0.50 mg/g,⁶⁾ respectively. Their determination limits were 5 μ g/g,³⁾ 10 μ g/g⁴⁾ and 2 μ g/g.⁶⁾ Our limit was 6 μ g/ml. Aspartame in carbonated beverages containing no protein was determined by the enzymatic method which did not require expensive apparatus and obtained the same degree of recovery and determination limit as other methods.^{1–6)}

TABLE III. Recovery of Aspartame from Carbonated Beverages

Sample	Amount added ^{a)} (mg/ml)	Found (mg/ml)	Recovery (%)	Coefficient of variation ($n=6$)
Cola	0.000	0.158		9.0
	0.088	0.246	99.8	8.4
	0.201	0.363	102.1	5.7
Cider	0.000	0.116		6.4
	0.058	0.170	93.9	10.8
	0.108	0.225	100.9	5.6
Apple soda	0.000	0.196		6.9
	0.169	0.365	99.9	4.9
	0.339	0.522	96.1	2.9

a) Amounts of aspartame added were approx. 50 to 170% the amount found initially in each carbonated beverage.

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