Enzymatic spectrophotometric reaction rate determination of aspartame

Kata T. Trifković[1], Lucyna Łękawska-Andrinopoulou[2], Branko M. Bugarski[1], Constantinos A. Georgiou[2]

[1]*Faculty of Technology and Metallurgy, University of Belgrade, Belgrade, Serbia*
[2]*Chemistry Laboratory, Agricultural University of Athens, Athens, Greece*

Abstract

Aspartame is an artificial sweetener of low caloric value (approximately 200 times sweeter than sucrose). Aspartame is currently permitted for use in food and beverage production in more than 90 countries. The application of aspartame in food products requires development of rapid, inexpensive and accurate method for its determination. The new assay for determination of aspartame was based on set of reactions that are catalyzed by three different enzymes: α-chymotrypsin, alcohol oxidase and horseradish peroxidase. Optimization of the proposed method was carried out for: *i*) α-chymotrypsin activity; *ii*) time allowed for α-chymotrypsin action, *iii*) temperature. Evaluation of the developed method was done by determining aspartame content in "diet" drinks, as well as in artificial sweetener pills.

Keywords: aspartame determination, enzymatic method, α-chymotrypsin.

Available online at the Journal website: http://www.ache.org.rs/HI/

SCIENTIFIC PAPER

UDC 664.162:543.42.577

Hem. Ind. **69** (4) 355–359 (2015)

doi: 10.2298/HEMIND140526049T

Aspartame (*N*-L-α-aspartyl-L-phenylalanine, 1--methyl ester) is a low-calorie artificial sweetener that is about 200 times sweeter than table sugar. It is composed of two amino acids (aspartic acid and phenylalanine) and methanol [1,2]. Aspartame is used in variety of beverages and foods, as well as in tabletop sweeteners, pharmaceuticals and supplements [3]. There is a concern regarding safety of use of aspartame in human diet. However, numerous studies have shown that aspartame is non-toxic and safe [4]. Also, there are lists of artificial high-intensity sweeteners approved for utilization in European Union, as well as in USA, and both lists contain aspartame [5,6].

The increased utilization of aspartame resulted in need for development of fast and efficient methods for its determination [2,7–11]. There are a number of methods described in literature, but most of them are time-consuming and expensive. This particularly refers to HPLC methods where prior to the determination of aspartame, samples need to be subjected to an extensive pre-treatment [7]. Furthermore, numerous spectroscopic methods have also been examined, such as those based on ninhydrin [12] and *N*-bromsuccinil-imide-metol-sulfanilamide [13]. However, these methods are often not suitable for aspartame determination due to the large and variable blank values [7]. The modern analytical methodologies employ enzymes in the determination techniques as a result of their selectivity and the tendency to replace the hazardous chemical substances, leading to the cleaner processes and sustainable chemistry [14].

After all being said, there is a real exigency for development of rapid, inexpensive and accurate method for aspartame determination, which will overcome the listed deficiency of existing methods. This paper describes a new assay for determination of aspartame, based on following reaction cascade:

$$\text{Aspartame} + H_2O \xrightarrow{\alpha\text{–Chymotrypsin}} \rightarrow \text{L-Asp-L-Phe} + H^+ + CH_3OH \quad (1)$$

$$CH_3OH + O_2 \xrightarrow{\text{Alcohol oxidase}} HCHO + H_2O_2 \quad (2)$$

$$2H_2O_2 + \text{Phenol} + \text{4-aminoantipyrine} \xrightarrow{\text{Horseradish peroxidase}} \rightarrow \text{4-}N\text{-}(p\text{-benzoquinoneimine)-antipyrine} + 4H_2O \quad (3)$$

In the reaction (1) methanol is released by α-chymotrypsin (α-CHY). Methanol is subsequently oxidized by alcohol oxidase (AO) creating hydrogen peroxide, Eq. (2), that reacts further in a reaction catalyzed by horseradish peroxidase (HRP), Eq. (3). During that reaction 4-*N*-(*p*-benzoquinoneimine)-antipyrine, coloured product is formed, and the formation is followed spectrophotometrically at 505 nm.

MATERIALS AND METHODS

Aspartame stock solution, 33.3 mM. An amount of 9.79 g of aspartame was placed in 1000 mL volumetric flask, and volume was brought up using phosphate buffer (pH 7.4).

Correspondence: K.T. Trifković, Faculty of Technology and Metallurgy, University of Belgrade, Karnegijeva 4, 11000 Belgrade, Serbia; E-mail: ktrifkovic@tmf.bg.ac.rs

L. Łękawska-Andrinopoulou, Chemistry Laboratory, Agricultural University of Athens, 75 Iera Odos, 11855 Athens, Greece; E-mail: lekawska@aua.gr

Paper received: 26 May, 2014

Paper accepted: 17 June, 2014

Enzyme working solution. Phenol, 7.5 mM; 4-amino -antipyrine, 2.50 mM; KH_2PO_4, 0.1 M; AO 1 U/mL, HRP 40 U/mL. The amounts of 0.706 g of phenol, 0.508 g of 4-aminoantipyrine and 13.608 g of KH_2PO_4 were placed in 800 mL of deionized water and pH was adjusted to 7.5, using 1 M NaOH. After adjustment of pH, 1000 U of AO and 40,000 U of HRP were added and prepared solution was transferred to a 1000 mL volumetic flask and brought up to the volume [15].

α-Chymotrypsin solution. 80 U/mL, an amount of 8000 U of *α*-CHY was placed in a 100 mL volumetric flask and brought up to the volume using deionized water.

Calibration standard aspartame solutions. 5.00, 10.00, 20.00, 30.00 and aspartame solutions used as samples: 1.00, 2.00, 7.00, 15.00, 25.00, 35.00 mM aspartame. All samples were prepared placing the appropriate volume of stock aspartame solution in 100 mL of phosphate buffer.

Phosphate buffer. 0.1 M, an amount of 13.61 g of KH_2PO_4 was dissolved in 900 mL of deionized water, and pH was adjusted to 7.4 using 1 M NaOH; the solution was placed in 1000 mL volumetric flask and brought up to the volume using deionized water.

Spectrophotometer: Jasco V-550.

Experimental procedure

An aliquot of 0.5 mL of standard or sample was placed in a 3 mL quartz cuvette in the spectrophotometer. Solution containing 12 U of *α*-chymotrypsin was pipetted into cuvette, with repeated flushing of the pipette tip to evenly disperse the enzyme added, and pre-incubated for 2 min for reaction (1) to proceed. Then, an aliquot of 2.5 mL of enzyme working solution was added in the cuvette and the software for kinetic measurement was started at 505 nm wavelength. Blank samples were prepared as described above, using the same amount of phosphate buffer instead of aspartame solution. Reaction rates were calculated in units of mA s^{-1}. Further on, the calibration curve was constructed.

To check the precision, the reaction rate from the 15 mM aspartame solution was measured ten times using *α*-CHY solution of 12 U.

RESULTS AND DISCUSSION

Optimization of the assay

Optimization of α-CHY activity

In order to check the influence of the activity of *α*-CHY used, the optimization test was conducted. The *α*-CHY activity was varied in range from 8 to 32 U. As can be seen from Table 1, analytical signal increased along *α*-CHY activity. The increase from 12 to 32 U was 7.2%. This small analytical signal increase did not justify the increase of the enzyme cost, therefore 12 U was selected for further experiments.

Optimization of pre-incubation time

In reaction (1), aspartame is cleaved by *α*-chymotrypsin to L-Asp-L-Phe and methanol. The goal was to determine the optimum time for this reaction. Results in Table 2 showed that analytical signal increased along pre-incubation time. While increasing pre-incubation time by 12 min, analytical signal increase was just 7.4%. In this respect, 2 min pre-incubation time was selected.

Interesting result shown in both Tables 1 and 2 is that blank signal was almost zero in most of the cases.

Table 1. Effect of α-CHY activity; 33.3 mM aspartame, 2 min pre-incubation time, 20.2 °C

α-Chymotrypsin activity, U	Aspartame slope value ± *SE* mA s^{-1}	Correlation coefficient R^2	Blank (water) slope value ± *SE* mA s^{-1}
8	4.07±0.02	0.995	–0.014±0.002
12	4.40±0.02	0.996	–0.003±0.002
16	4.58±0.02	0.996	0.002±0.002
20	4.65±0.02	0.997	–0.004±0.002
24	4.67±0.02	0.997	0.009±0.002
28	4.74±0.02	0.997	0.001±0.002
32	4.75±0.02	0.997	0.000±0.002

Table 2. Effect of pre-incubation time; 33.3 mM aspartame, 12 U α-CHY, 20.2 °C

Pre-incubation time, min	Aspartame slope value ± *SE* mA s^{-1}	Correlation coefficient R^2	Blank (water) slope value ± *SE* mA s^{-1}
2	4.72±0.02	0.997	0.015±0.002
6	4.85±0.02	0.997	0.006±0.002
10	4.97±0.02	0.997	0.008±0.002
14	5.07±0.02	0.997	0.011±0.002

Temperature effect

Temperature effect was investigated by adjusting temperature of reaction (1) in a water bath. Results shown in Figure 1 were as expected – reaction rate increased along temperature.

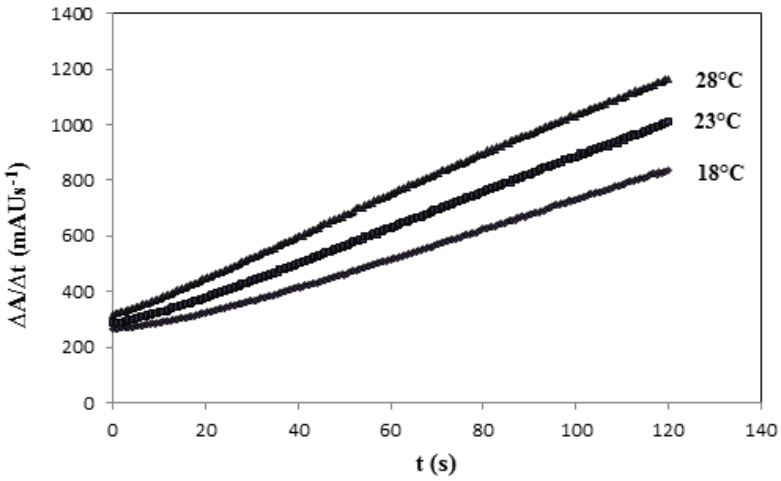

Figure 1. Temperature influence on reaction rate.

Calibration curve

Based on experimental data obtained for dependence of reaction rate on aspartame concentration, calibration curve was constructed (Figure 2).

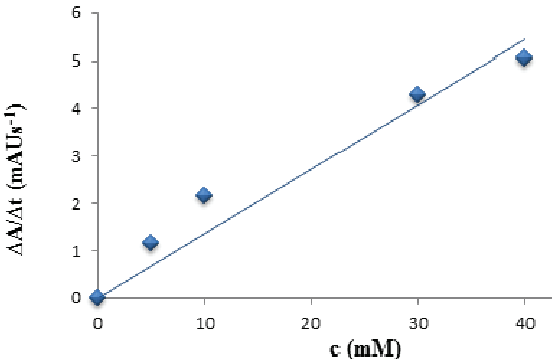

Figure 2. Calibration curve.

Equation of the calibration curve:

$$\frac{\Delta A}{\Delta t}[\text{mA s}^{-1}] = (0.14 \pm 0.013)c \text{ , } R^2 = 0.9690 \quad (4)$$

Standard solution analysis

In order to check the accuracy of developed assay, the concentration of aspartame in solutions of known concentrations was determined. The results are shown in Table 3.

The limit of detection (LOD) for the developed assay was calculated as follows:

$$LOD = \frac{3.3S_a}{b} \quad (5)$$

where S_a stands for standard deviation of the response, while b stands for the slope of the calibration curve.

The value for *LOD* was found to be 9.9 mM. Results clearly indicate that this assay needs further improvement towards lowering the detection limit using a more sensitive technique, such as fluorimetry or chemiluminescence.

Table 3. Analysis of standard solutions

Taken, mM	Found, mM
1.00	1.52
2.00	3.75
7.00	11.74
15.00	22.44
25.00	31.09
35.00	36.25

Reproducibility of the results

Table 4 presents results for 10 consecutive measurements of the 15 mM aspartame solution.

The mean reaction rate, calculated from experimental results, was 2.98±0.087 mA s^{-1} (n= 10).

Determination of aspartame in real food samples

After basic development and optimization of the assay, an attempt was made to determine the aspartame concentration in commercial products Coca-Cola Zero and Canderel sweetener pills. Main problem that occurred was low concentration of aspartame in the sample. It was hard to obtain accurate signal as the aspartame concentration was close to the detection limit (9.9 mM). Namely, proposed method requires relatively high aspartame concentration in sample, in

Table 4. Reproducibility of the results for the 15 mM aspartame solution

No of measurement	Reaction rate ± *SE*, mA s^{-1}	Correlation coefficient, R^2
1	2.928±0.009	0.998
2	2.925±0.008	0.998
3	2.845±0.008	0.998
4	2.906±0.009	0.997
5	3.087±0.007	0.999
6	3.039±0.009	0.998
7	2.936±0.008	0.998
8	3.053±0.008	0.998
9	3.105±0.009	0.998
10	2.952±0.008	0.998

order to get the measurable response, as can be seen from analysis of standard solutions. Thus, preparation of 33.3 mM aspartame Canderel solution requires dissolution of 24 Canderel pills in 20 mL of deionized water. Additionally, in order to get rid of the interference of caramel color of Coca-Cola it was necessary to dilute samples at 1:5. In this way, aspartame concentration was even lower, so it could not be measured spectrophotometrically.

CONCLUSION

The determination of aspartame, particularly in food products, is mandatory from health and legal aspects. Therefore, the development of new assay for aspartame determination is highly recommended. The proposed method showed good results in samples containing high aspartame concentrations. However, for the samples containing lower aspartame content, the method requires further optimization. That could be achieved through enhancing the sensitivity of the detection. For that purpose, chemiluminescence as method for detection could provide good results, as it can go down to 10 nM [16].

Acknowledgements

Work is performed within LeanGreenFood Marie Curie Initial Training Network funded by FP7/2007--2013 under grant agreement No. 238084; FP7-REGPOT 2009-1 Project Nanotech FTM, under grant agreement No.245916, as well as Ministry of Education, Science and Technological Development, Republic of Serbia, Project No. III46010, 2011-2014.

REFERENCES

[1] R.G. Ranney, J.A. Operman, E. Muldoon, F.G. McMahon. Comperative metabolism of aspartame in experimental animals and humans. J. Toxicol. Environ. Health **2** (1976) 441–451.

[2] D. Odaci, S. Timur, A. Telefoncu, Carboxyl esterase-alcohol oxidase based biosensor for the aspartame determination, Food Chem. **84** (2004) 493–496.

[3] L.F. Capitan-Vallvey, M.C. Valencia, E.A. Nicolas, Flow-through spectrophotometric sensor for the determination of aspartame in low-calorie and dietary products, Anal. Sci. **20** (2004) 1437–1442.

[4] FAO/WHOEC, Aspartame: Specifications for Identity and Purity of Carrier Solvents, Emulsifier and Stabilizers, Enzyme Preparations, Flavoring Agents, Food Colors, Sweetening Agents and Other Food Additives, Rome, 1982.

[5] European Commission, Directive 2006/52/EC of European Parliament and of the Council of 5 July 2006 amending Directive 95/2/EC on food additives other than colors and sweeteners and Directive 94/35/EC on sweeteners for use in foodstuffs, Off. J. Eur. Un. **L024** (2004) 10.

[6] A. Zygler, A. Wasik, J. Namiesnik, Analytical methodologies for determination of artificial sweeteners in foodstuffs, Trend Anal. Chem. **28** (2009) 1082–1102.

[7] E. Dinckaya, M. Cagin, A. Telefoncu, Enzymatic method for the spectrophotometric determination of aspartame, Food Chem. **50** (1994) 95–97.

[8] V. Pereira, O. Fatibello-Filho, Flow-injection spectrophotometric determination of L-ascorbic acid in pharmaceutical formulations with on-line solid-phase reactor containing copper (II) phosphate, Anal. Chim. Acta **366** (1998) 55–62.

[9] K. Wrabel, K. Wrabel, Determination of aspartame and phenylalanine in diet soft drinks by high-performance liquid chromatography, J. Chromatogr., A **773** (1997) 163–167.

[10] O.W. Lau, S.F. Luk, W.M. Chan, Spectrophotometric determination of aspartame in soft-drinks with ninhydrin as reagent, Analyst **113** (1988) 765–768.

[11] S. Motellier, I.W. Wainer, Direct stereochemical resolution of aspartame stereoisomers and their degradation products by high-performance liquid chromatography on a chiral crown ether-based stationary phase, J. Chromatogr. **516** (1990) 365–373.

[12] I.C. Vieira, O. Fatibello-Filho, Determinação espectrofotométrica de aspartame em produtos dietéticos

usando ninidrina como reagent, Quim. Nova **18** (1995) 250–254.

[13] U.V. Prasad, T.E. Divakar, C.S.P. Sastry, M.V.Rao, O.P. Kapur, New methods for determination of aspartame, Food Chem. **28** (1988) 269–278.

[14] C.I.C. Silvestre, P.C.A.G. Pinto, M.A. Segundo, M.L. M.F.S. Saraiva, J.L.F.C. Lima, Enzyme based assays in a sequential injection format: A review, Anal. Chim. Acta **689** (2011) 160–177.

[15] A.M.G. Vasilarou, C.A. Georgiou, Enzymatic spectrophotometric reaction rate determination of glucose in fruit drinks and carbonated beverages. An analytical chemistry laboratory experiment for food science-oriented students, J. Chem. Edu. **77** (2000) 1327–1329.

[16] E.G. Vasiliou, Y.M. Makarovska, I.A. Pneumatikos, N.V. Lolis, E.A. Kalogeratos, E.K. Papadakis, C.A. Georgiou, Hydrogen peroxide assessment in exhaled breath condensate: condensing equipment – rapid flow injection chemiluminescence method, J. Braz. Chem. Soc. **18** (2007) 1040–1047.

IZVOD

ENZIMATSKO–SPEKTROFOTOMETRIJSKI TEST ZA ODREĐIVANJE ASPARTAMA

Kata T. Trifković[1], Lucyna Łękawska-Andrinopoulou[2], Branko M. Bugarski[1], Constantinos A. Georgiou[2]

[1]*Tehnološko–metalurški fakultet, Univerzitet u Beogradu, Beograd, Srbija*
[2]*Chemistry Laboratory, Agricultural University of Athens, Athens, Greece*

(Naučni rad)

Aspartam je veštački zaslađivač niske kalorijske vrednosti, približno 200 puta slađi od saharoze. Aspartam se trenutno koristi u industriji hrane i pića kao zamena za šećer u preko 90 zemalja u svetu, pa je stoga nužno imati razvijenu metodologiju i dobru analitiku za njegovo određivanje. Metode koje se najčešće koriste za određivanje aspartama su obično dugotrajne, skupe i zahtevaju značajna ulaganja u opremu, kao i komplikovan pred-tretman uzoraka. Stoga je neophodno razviti brz, ekonomičan, osetljv i precizan test za njegovu detekciju. U ovom radu je predložen test za određivanje aspartama koji se zasniva na nizu reakcija katalizovanih upotrebom tri enzima: *α*-himotripsin, alkoholna oksidaza i peroksidaza iz rena. Tokom reakcione kaskade dolazi do transformacije aspartama u tri reakciona koraka, od kojih je svaki enzimski katalizovan, α-himotripsinom, alkoholnom oksidazom i peroksidazom iz rena, redom. Preko nekoliko reakcionih međuproizvoda (methanol i vodonik-peroksid) aspartam se transformiše do obojenog produkta, čije se formiranje prati spektrofotometrijski, a zatim se na osnovu apsorbance obojenja dalje preračunava koncentracija aspartama prisutna u ispitivanom uzorku. Eksperimentalni postupak je jednostavan i efikasan, a njegova preciznost dobra. Optimizacija predloženog testa izvršena je u cilju određivanja optimalne: *i*) aktivnosti *α*-himotripsina; *ii*) vremena pre-inkubacije; *iii*) temperature pri kojoj se odvija reakcija. Predloženi test ocenjen je određivanjem sadržaja aspartama u dijetalnim pićima (Coca-Cola Diet) i veštačkim zaslađivačima (Candarel tablete), kao i procenom njegovog praga osetljivosti. Dobijeni rezultati ukazuju na neophodnost optimizacije metode detekcije, u cilju omogućavanja određivanja nižih koncetracija aspartama prisutnih u uzorku.

Ključne reči: Određivanje aspartama • Enzimatska metoda • *α*-Himotripsin