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Analytical Methods Section

Enzymatic method for the spectrophotometric determination of aspartame

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A sensitive spectrophotometric method for the determination of aspartame is described. In this method, α-chymotrypsin and alcohol oxidase were immobilized on highly porous Amberlite IRA 938 resin by ionic binding, to allow the enzymes to be used more than once. The method involves the enzymatic conversion of aspartame into formaldehyde and hydrogen peroxide by the immobilized α-chymotrypsin and alcohol-oxidase system. Horseradish peroxidase was used for the determination of hydrogen peroxide by the formation of a chromophore with 3-methyl-2-benzo-thiazolinone hydrazone (MBTH) and *N,N*-dimethylaniline (DMA). The absorbances of the sample solutions were measured at 595 nm against a blank. The calibration graph was linear in the range 0·1–1·0 mg ml^{-1} of aspartame.

INTRODUCTION

Aspartame (*N*-L-α-aspartyl-L-phenylalanine methyl ester) is an artificial sweetener with a pronounced sucrose-like taste that organoleptically is about 200 times as sweet as sugar (Fig. 1).

Aspartame is metabolized to phenylalanine, aspartic acid, and methanol (Ranney *et al.*, 1976). Olney and Ho (1990) have shown that, under the appropriate conditions, aspartate, and excitatory amino-acid neurotransmitter, can cause neural death. The neurotoxicity of methanol in primates has been well documented (Potts *et al.*, 1955). Phenylalanine is metabolized further to tyrosine, and both amino acids are transported into the nervous system, where they can influence the disposition of monoamine transmitters such as norepinephrine and dopamine (Wurtman, 1983). Some investigators have reported that high doses of aspartame may potentiate chemical-induced seizures (Guiso *et al.*, 1988; Pinto & Maher, 1988).

Aspartame is used in table-top sweeteners, chewing gums, breakfast cereals, soft drinks, powdered drinks, and dietary foods (Yost, 1989). Its increased application in food products has given a new impetus to the development of fast and accurate methods for its determination.

However, many techniques are time-consuming and require considerable capital investment. There are several sensitive and accurate spectrophotometric and spectrofluorimetric methods for the analysis of amino acids that are applicable to the determination of aspartame (Lav *et al.*, 1988). However, they are not applicable in many instances to the analysis of foodstuffs because of the large and variable blank values obtained. High-performance liquid chromatographic methods are most commonly used for the determination of aspartame in foods (Motellier & Wainer, 1990).

However, these methods require extensive pre-treatment of the food sample prior to the chromatographic operation. An enzyme-based biosensor has been used with immobilized alcohol oxidase together with an oxygen electrode. Another enzyme-based electrode (Nikolelis & Krull, 1990) has also been used for the determination of aspartame in dietary foodstuffs by means of a carbon dioxide gas sensor.

In this work, a new enzymatic assay has been developed for the determination of aspartame. Aspartame is first cleaved by immobilized α-chymotrypsin to L-Asp-L-Phe and methanol:

$$\text{aspartame} \xrightarrow{\text{immobilized α-chymotrypsin}} \text{L-Asp-L-Phe} + \text{methanol}$$

Methanol is then oxidized by an immobilized alcohol oxidase to formaldehyde and hydrogen peroxide.

$$\text{methanol} + O_2 \xrightarrow{\text{immobilized alcohol oxidase}} \text{formaldehyde} + H_2O_2$$

As a result, horseradish peroxidase was used for the determination of hydrogen peroxide by the formation of a chromophore with 3-methyl-2-benzo-thiazolinone hydrazone (MBTH) and *N,N*-dimethylaniline (DMA).

$$H_2O_2 + \text{MBTH} + \text{DMA} \xrightarrow{\text{peroxidase}} \text{indamine dye} + 2H_2O + OH^-$$

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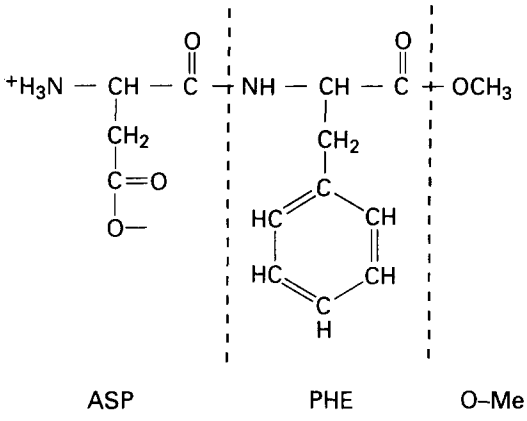

Fig. 1. The chemical structure of aspartame.

MATERIALS AND METHODS

Chemicals

Aspartame, α-chymotrypsin (EC 3.4.21.1), alcohol oxidase (EC 1.1.3.13) peroxidase (EC 1.11.1.7), Folin Ciocalteu Phenol Reagent(2N), and 3-methyl-2-benzothiazolinone hydrazone (MBTH) were purchased from Sigma Chemical Co., St. Louis, MO, USA. Amberlite IRA 938 resin was obtained from Rohm & Haas, France. *N,N*-dimethylaniline (DMA) and all other chemicals were purchased from E. Merck, Darmstadt, FRG. All chemicals were of analytical-reagent grade.

Reagents

Aspartame stock standard solution
A 1·0-mg ml^{-1} solution was freshly prepared by dissolving aspartame (25·0 mg) in phosphate buffer (0·1 M, pH 7·0) (25 ml). From this stock standard solution, working standard solutions (0·1–1·0 μg ml^{-1}) were freshly prepared by appropriate dilution with the same buffer.

Color reagent
Stock solutions of 3-methyl-2-benzo-thiazolinone hydrazone (MBTH; 1 g l^{-1}) and *N,N*-dimethylaniline (DMA; 2·5 g l^{-1}) were prepared in 0·1 M hydrochloric acid. These were stored in amber-colored glass bottles at 4°C. A working reagent was prepared fresh daily by diluting stock MBTH solution (1·25 ml), stock DMA solution (2·5 ml) and 750 U peroxidase to 50 ml with phosphate buffer (0·1 M, pH 6·0). This combined reagent was shielded from light and kept at 4°C until used.

Apparatus

A Spectronic 88 model spectrophotometer (Baush & Lomb, USA), Ultra-thermostat (Colora, FRG), and Nüve model ST 40 shaker bath (Ankara, Turkey) were used.

Enzyme immobilization

Amberlite IRA 938 resin dispersed in phosphate buffer (0·1 M, pH 7·5) was first incubated at 25°C for 24 h.

Table 1. Determination of aspartame in real samples

Sample	Aspartame content (μg ml^{-1})	Aspartame found (μg ml^{-1})	Recovery (%)
Diet cola	240·0	250·0	104·2
Sweetener tablets	1 000·0	964·0	96·4

α-Chymotrypsin immobilization
α-Chymotrypsin (960 U ml^{-1}) (10 ml) solution was added to Amberlite IRA 938 resin (1 g). The mixture was incubated by stirring at 25°C for 1 h and filtered. The excess of α-chymotrypsin was removed by washing with distilled water.

Alcohol oxidase immobilization
Alcohol oxidase (8 U ml^{-1}) (10 ml) solution was added to Amberlite IRA 938 resin (1 g). The mixture was incubated by stirring at 25°C for 1 h and filtered. The excess of enzyme was removed by washing with distilled water.

In order to make comparison of the amounts of immobilized and free enzyme. It is necessary to know the content of the enzyme bound to the Amberlite IRA 938 resin. Initial-protein concentrations of the enzyme solutions were determined by the Lowry method. The washing solutions were then combined, and amounts of protein in these solutions were estimated. The amount of enzyme on the resin was calculated by using the reduction of the protein in the washing solution.

Measurement

Sample or standard (2 ml) was transferred to a tube and mixed with resin (0·1 g) containing immobilized α-chymotrypsin. The tube was incubated by shaking at 30°C for 10 min. After incubation, the tube was centrifuged, and supernatant was removed. This was mixed with resin (0·1 g) that had immobilized alcohol oxidase, and incubated in the same way. The tube was then centrifuged, and supernatant (0·5 ml) was

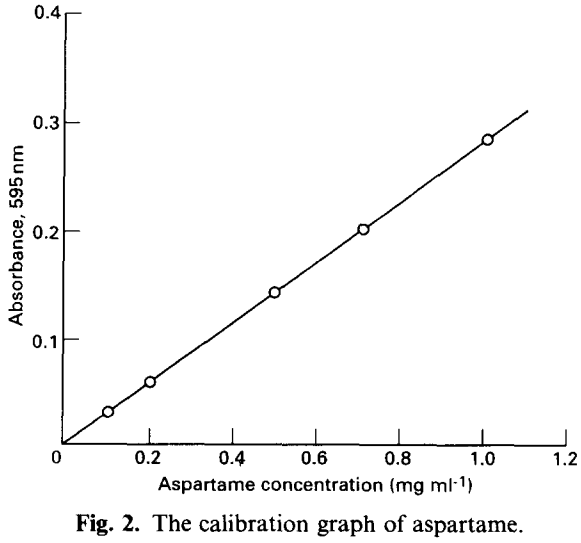

Fig. 2. The calibration graph of aspartame.

removed. This was mixed with the combined color reagent (4·0 ml). The samples were incubated at 30°C for 5 min, after which the absorbance was measured at 595 nm. Standard and blanks were included in each batch, all samples being treated in the same manner.

RESULTS AND DISCUSSION

Calibration graph and quantification limit

A series of standard aspartame solutions was subjected to the assay. Beer's law and obeyed for aspartame in the range 0·1–1 mg ml^{-1} (Fig. 2). The quantification limit was 0·1 mg ml^{-1} of aspartame.

Precision of method

The precision of the method was checked by calculating the relative standard deviation of ten replicate analyses of a tablet solution containing 0·5 mg ml^{-1} of aspartame. The relative standard deviation was 2·1%.

Applications

The contents of aspartame in real soft-drink samples (Diet Cola) and commercial sweetener tablets were determined. Each determination was carried out in triplicate. Results are given in Table 1.

CONCLUSION

The proposed enzymatic method can be used to determine aspartame with an acceptable degree of precision and accuracy. The results are comparable with those obtained by other methods. The proposed method can be performed with inexpensive instrumentation in situations in which equipment required for other methods is not available.

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