Sequential injection analysis-based flow system for the enzymatic determination of aspartame

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Abstract

The versatility of sequential injection analysis systems as a sampling handling approach combined with the flexibility of the individual solenoid valves was conjugated for the automation of the proposed new enzymatic method for the determination of aspartame in commercial sweetener tablets. The method involves the enzymatic conversion of aspartame in hydrogen peroxide by the α-chymotrypsin–alcohol oxidase system, followed by the use of 2,2′-azinobis(3-ethylbenzthiazolin-sulfonic acid-6) (ABTS) as electron donor for peroxidase. α-Chymotrypsin and alcohol oxidase enzymes were immobilised on activated porous silica beads. No activity loss in the reactors was detected throughout 60 days. The calibration plots were linear up to 350 mg l$^{-1}$, with a detection limit of 2.16 mg l$^{-1}$ of aspartame. Relative standard deviations (R.S.D.) of 3.0 and 2.4% ($n=10$) for samples containing 25.8 and 51.9 mg l$^{-1}$ of aspartame, respectively, were obtained. The method was applied to the determination of aspartame in commercial sweetener tablets; the results obtained were compared to those provided by a HPLC method and revealed no statistical differences for a 95% confidence level.

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Keywords: Aspartame determination; SIA; Enzymatic method; Commercial sweetener tablets; α-Chymotrypsin; Alcohol oxidase; Peroxidase

1. Introduction

Aspartame (N-l-α-aspartyl-l-phenylalanine 1-methyl ester) is an artificial sweetener of low caloric value, which despite being organoleptically similar to sucrose is approximately 180 times sweeter [1]. Aspartame is currently permitted for food and beverage and/or tabletop sweetener use in more than 90 countries, which implies the development of analytical methodologies easily reproducible under normal laboratory conditions, without the need for sophisticated equipment and specially trained personnel [2].

Several spectrophotometric procedures are described in the literature to determine aspartame, but these are either time consuming or do not have the selectivity required for its determination in some commercial samples [3–5]. Chromatographic procedures [6–11] in spite of being perhaps the most commonly used for the determination of aspartame, apart from the time and cost involved per analysis, they require extensive pre-treatment of the food sample prior to the chromatographic operation. Continuous flow procedures present themselves as interesting alternatives for the automation of the different stages of an analytical process, as an increase in analytical efficiency is obtained. However, only some continuous flow procedures based on spectrophotometric detection or on the use of biosensors have been reported for the determination of aspartame. Nevertheless, the spectrophotometric methods use reagents that require either special precautions in handling and storage or need a water bath at 60°C [12–14]. The enzyme based biosensor procedures had the drawback of electrode stability, which decreased after around 250 assays [15–17]. The use of flow methodologies for the determination of aspartame with spectrophotometric detection that take advantage of the selectivity and specificity of the use of enzymes, has never been carried out. Some enzymatic batch methods [18,19] for its determination are described in the literature, but are slow procedures that involve an extensive incubation period.

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The automatisation based on flow techniques permits measurements to be carried out in short time periods in addition to leading to a reduction in the consumption of reagents and generation of effluents. Sequential injection analysis (SIA) [20] is a recent flow technique for sample and reagent manipulation that is computer controlled by user-friendly software. Robustness, versatility, simplicity, and low sample and reagent consumption are characteristic advantages of SIA.

The versatility of these systems as a sampling handling approach and the facility in the insertion of the individual solenoid valves in any place of the manifold was conigrated for the automatisation of the proposed new enzymatic method for the determination of aspartame in commercial sweetener tablets. In this method, \( \alpha \)-chymotrypsin (CHY) and alcohol oxidase (AOD) enzymes were immobilised on activated aminopropyl glass beads and packed in perspex reactors connected in series. The hydrogen peroxide produced by the action of these two enzymes reacted with the redox indicator \( 2,2' \)-azinobis(3-ethylbenzthiazolin-sulfonic acid-6) (ABTS), catalysed by the horseradish peroxidase (POD), giving a green coloured compound measured at 420 nm.

\[
\text{Aspartame} + \text{H}_2\text{O}_2 \xrightarrow{\text{immobilised CHY}} \text{l-Asp-I-Phe} + \text{methanol}
\]

\[
\text{Me-thanol} + \text{O}_2 \xrightarrow{\text{immobilised AOD}} \text{formaldehyde} + \text{H}_2\text{O}_2
\]

\[
\text{H}_2\text{O}_2 + \text{ABTS} \xrightarrow{\text{POD VI-A}} 2\text{H}_2\text{O} + \text{ABTS (oxidised form)}
\]

2. Experimental

2.1. Reagents and solutions

Aspartame, CHY (EC 3.4.21.1; Type II, bovine pancreas), AOD (EC 1.1.3.13; Hansenula species), peroxidase (EC 1.11.1.7; from Horseradish type VI-A), \( 2,2' \)-azinobis(3-ethylbenzthiazolin-sulfonic acid-6) (ABTS), 25% glutaraldehyde solution and aminopropyl glass beads (200–400 mesh, 500 Å mean pore diameter) were purchased from Sigma Chemical Co., St. Louis, MO, USA. All solutions were prepared with double deionised water (specific conductivity <0.1 \( \mu \)S cm\(^{-1} \)). As carrier stream, a buffer solution consisting of 0.1 M potassium phosphate with \( \text{pH} \) adjusted to 7.4 (with 0.4 M NaOH) was used. The reagents solution was prepared dissolving ABTS (470 mg l\(^{-1} \)) and POD (3.0 mg l\(^{-1} \)) in the phosphate buffer solution (0.1 M, \( \text{pH} \) 7.4).

The stock solution of aspartame (500 mg l\(^{-1} \)) was prepared daily in the phosphate buffer solution (0.1 M, \( \text{pH} \) 7.4). Working standard solutions were obtained by dilution of the stock solution with the same buffer.

2.2. Apparatus

The SIA based flow system used for aspartame determination (Fig. 1) consisted of a Gilson Minipuls 3 peristaltic pump equipped with PVC pump tubing (1.3 mm i.d.), a 10-port multiposition selection valve (Valco VICI C25-3180/EMHL), and a Jenway 6300 spectrophotometer with a Hellma 178.712QS 18 \( \mu \)l flow-through cell. All tubing connecting the different components of the flow system was made of Omnitft PTFE with 0.8 mm (i.d.). The analytical signals were recorded on a Linsens I260E flat-bed recorder or acquired in the computer.

Two Nresearch 161 T031 (Stow, MA, USA) valves were used. One of the valves (Fig. 1, Vs1) and a contact de-vice placed on the peristaltic pump head were introduced in the system, to control the pump’s starting point [21] in order to guarantee reproducibility in the solution volumes aspirated or propelled. The other (Fig. 1, Vs2) was used to perform the addition of the chromogenic reagent at the confluence point. All electrical devices of the manifold were computer-controlled by means of a home-made program written in Microsoft Qbasic 4.5. The computer was equipped with an Advantech PCL711B control interface card.

2.3. Enzyme immobilisation

The immobilisation procedure employed for CHY and AOD enzymes was similar to that reported by Masoom and Townshend [22]. 0.125 g of aminosolubilized glass beads were incubated in 2.5 ml of a 2.5% glutaraldehyde solution in 0.1 mol l\(^{-1} \) phosphate buffer at \( \text{pH} \) 7.4 for 1 h at room temperature, with brief nitrogen deoxygenation every 10 min for the first half hour. The activated glass beads (AGB) were washed with distilled water and 0.1 mol l\(^{-1} \) phosphate buffer at \( \text{pH} \) 7.4. Immobilisation was then performed by adding 4.5 mg of CHY or 6.4 mg of AOD to 0.125 g of the AGB. After incubation at 4 °C for 4 h in an oxygen free atmosphere, the glass beads were then filtered off and washed with distilled water and 0.1 mol l\(^{-1} \) phosphate buffer at \( \text{pH} \) 7.4. Immobilisation was then performed by adding 4.5 mg of CHY or 6.4 mg of AOD to 0.125 g of the AGB. After incubation at 4 °C for 4 h in an oxygen free atmosphere, the glass beads were then filtered off and washed with distilled water and 0.1 mol l\(^{-1} \) phosphate buffer at \( \text{pH} \) 7.4 to eliminate any non-immobilised enzyme. Thereafter, 47 mg of immobilised chymotrypsin beads (AGB–CHY) or 102 mg of immobilised AOD beads (AGB–AOD) were packed in two perspex columns of 42 mm length and 2.5 mm internal diameter, and 35 mm length and 2.5 mm internal diameter, respectively. The two immobilised enzyme reactor beads were held in position with glass wool. When not in use, the reactors were stored in 0.1 mol l\(^{-1} \) phosphate buffer at \( \text{pH} \) 7.4 and 4 °C.

2.4. Sample preparation

Our target samples were the commercial tabletip sweeteners available in the local market. Sample solutions were prepared by dissolving the required amounts of powdered tablets containing aspartame with a fixed volume of ultra-pure water, filtered through a filter paper and then...
Table 1  
Sequence of events occurring in each analytical cycle

<table>
<thead>
<tr>
<th>Step</th>
<th>Event</th>
<th>Port</th>
<th>Time (s)</th>
<th>Flow direction</th>
<th>Flow rate (ml min⁻¹)</th>
<th>Vs2 valve position</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sample aspiration</td>
<td>1</td>
<td>3</td>
<td>Aspiration</td>
<td>0.50</td>
<td>Off</td>
</tr>
<tr>
<td>2</td>
<td>Sample propelling towards the enzyme reactors with buffer</td>
<td>2</td>
<td>12</td>
<td>Propulsion</td>
<td>0.50</td>
<td>Off</td>
</tr>
<tr>
<td>3</td>
<td>Reagent mixture with the formed H₂O₂</td>
<td>2</td>
<td>33</td>
<td>Propulsion</td>
<td>0.50 ± 0.50</td>
<td>On</td>
</tr>
<tr>
<td>4</td>
<td>Propelling towards detector</td>
<td>2</td>
<td>190</td>
<td>Propulsion</td>
<td>0.50</td>
<td>Off</td>
</tr>
</tbody>
</table>

diluted with phosphate buffer solution (0.1 M, pH 7.4). These solutions were then analysed both by the proposed automatic method and the comparison method.

2.5 Analytical cycle

Initially (Table 1), a 44.6 μl sample aliquot was aspirated (step 1) to the holding coil. This was then directed towards the enzyme reactors by the carrier buffer, where the transformation of aspartame in hydrogen peroxide by the CHY–AOD system took place (step 2). Meanwhile, the Vs2 was switched-off. Following this, the Vs2 solenoid valve was activated, enabling the confluence between the formed hydrogen peroxide in the enzyme columns and 275 μl of the reagent solution containing ABTS and POD (step 3). The ABTS₄₃₈₅ green compound formed was sent to the detector at step 4, enabling its determination at 420 nm. Analysis of one sample took about 240 s corresponding to a sample frequency of about 15 samples/hour.

3. Results and discussion

3.1 Manifold configuration

In an initial phase, the configuration of the system to be optimised for the determination of aspartame was established. Given that this method involved three successive enzymatic reactions, it was necessary to guarantee that the dispersion between each reaction was minimal, to avoid any loss in sensitivity until the attainment of the final coloured product to be detected. Therefore, it was decided to, after the sampling stage, direct the sampling zone to one of the selecting valve ports, where the two columns with the immobilised CHY and AOD enzymes were sequentially placed. In case the columns were placed separately in distinct selecting valve ports, it would be necessary to carry out the propulsion of the sample to the first CHY enzymatic column, thereafter the aspiration of the product therein formed to the holding coil and only finally the direction to the AOD column and the detector. This would increase the time used in each analysis and the dispersion suffered at the level of the holding coil.

On the other hand, to guarantee the complete mixture between the sample zone that crossed the two enzymatic columns and the chromogenic reagent, a confluence was placed in the reaction coil (RC), through which the addition of a zone of ABTS and peroxidase was made. The manifold configuration presented, therefore, besides the conventional one-line SIA manifolds, another flow line for the addition of an aliquot of reagent solution at the confluence in the RC. Usually when the SIA based systems present a flow line that is not connected to the multiposition selecting valve, they incorporate another propulsion system to carry out the movement of the liquids in that additional channel. This
increases the complexity of the systems but it is therefore justified by the impossibility of co-ordinating the alternate movements of stop, backward and forward of the principal peristaltic pump connected to the holding coil with the flow in the additional flow line.

In the system presented here, and for the first time in a SIA manifold, the option was to use only one peristaltic pump with two pump tubes; one linked to the holding coil and the other linked to the additional channel. To ensure that the flow in the additional channel was not affected by the movements in the peristaltic pump at the level of the holding coil, we placed a three-way solenoid valve (Fig. 1, Vs2) in the additional channel before the confluence point. Therefore, when the solenoid valve was in one position (Fig. 1, off) the reagent solution re-circulated in closed circuit, in this way being unaffected by the movements of the peristaltic pump. When in another position (Fig. 1, on) an aliquot of reagent solution was inserted into the RC by controlling the time interval in which the solenoid valve was in this position. At the same time, the carrier and the sample zone were being propelled along the holding coil, the enzyme columns and the RC towards the confluence.

3.2. Optimisation of the operational conditions

After the basic manifold design was established, experiments were conducted to define the optimum of a number of experimental parameters related to the chemical conditions, and the operating parameters of the system.

3.2.1. α-Chymotrypsin and alcohol oxidase reactors

Several reactors of different length and diameter were tested and different quantities of packed enzyme were evaluated. The geometries of the columns were selected in order to obtain less pressure in the system and a lower degree of dispersion. The effects of the amounts of enzyme used (enzyme units) on the concentration range of aspartame analysed were then optimised, each one individually, maintaining the amount of glass beads packed as constant. Although the magnitude of the analytical signals obtained in the range of units studied (60–150 U) for CHY were not very different, only for values around 86 U or superior a linear regression line till 350 mg l\(^{-1}\) was obtained. An AOD activity of 60 U, between 40 and 80 U studied, yielded the best results under the experimental conditions employed. Lower activities were insufficient to transform concentrations of aspartame greater than 200 mg l\(^{-1}\).

The operational stability of the enzymatic reactors was quite good, enabling its use as recently prepared columns for at least 60 days. Working continuously on a daily basis, which always began with a calibration procedure, 600 determinations could be carried out with a decrease in sensitivity of less than 5%.

3.2.2. Peroxidase/ABTS reagent

Peroxidase enzyme was first tested immobilised but this option was abandoned because of the pressure obtained with the three packed reactors. As it was the less expensive enzyme and the one involved in the formation of the coloured product, it was dissolved along with the colour reagent ABTS. A concentration of 3000 U l\(^{-1}\) for peroxidase was sufficient to enable the conversion of all the hydrogen peroxide formed in the previous reactions, as higher concentrations gave rise to identical response profiles. In order to study the optimum concentration of ABTS in the reagent solution for hydrogen peroxide in the full range of concentrations, different concentrations of ABTS in the range 148–741 mg l\(^{-1}\) were tested. A 445 mg l\(^{-1}\) ABTS concentration resulted in the attainment of maximum absorbance and was used in further studies.

3.2.3. Flow rates

The flow rate of the carrier solution had a pronounced effect on the sensitivity obtained as it determined the contact time between sample and enzymes in the packed reactors thereby affecting the enzymatic conversion. A flow rate of 0.50 ml min\(^{-1}\) was selected, despite increasing the time of each analytical cycle because higher values for the carrier stream flow rate gave rise to a pronounced decrease in sensitivity (Fig. 2). The reagent flow rate selected was also 0.50 ml min\(^{-1}\) as this gave rise to a better confluence mixture between the sample plug and reagent with a minor influence on the formation of coloured product.

Regarding the influence of the RC length on sensitivity of the method, this was studied in the 50–300 cm range. It was observed that the magnitude of the analytical signal increased with the residence time for RCs lower than 150 cm. Surpassed this length the dispersion was the phenomenon that prevailed, decreasing the absorbance values obtained. Thus a 150 cm coil length was chosen for further experiments.

![Fig. 2. Effect of the flow rate of a standard solution containing 50 mg l\(^{-1}\) aspartame on the amplitude of the analytical signal (A) and on the sampling rate (B).](image-url)
3.2.4. Reagent and sample volumes

As the flow rate of the reagent solution was already established, the volume of reagent used was determined by the time the solenoid valve (Vs2) was opened. Different volumes were studied, together with the cycle time at which this volume should be inserted in the confluence point, to guarantee the complete merger with the sample zone. A volume of 275 μl was selected that was inserted in the RC 12 s after the propulsion of the sample zone through the enzyme reactors (Table 1: step 3). At that time there was efficient overlap between the two zones in the confluence, confirmed by the shape and height of the signals obtained.

The sample volume was studied between 18 and 134 μl and it was found that the signal increased markedly until 44.6 μl. Higher volumes of sample gave rise to a constant signal for the higher aspartame standards and would require more enzymes and reagent to transform the hydrogen peroxide previously produced.

3.3. Analysis of commercial products

The calibration graph was linear up to 350 mg l⁻¹ with the typical analytical curve (Fig. 3) being represented by \( A = 0.0039 + 0.0025C \), \( r = 0.9997 \), where \( A \) is the absorbance and \( C \) the concentration of aspartame in mg l⁻¹.

The precision of the proposed method was tested by 10 repeated runs of two sample solutions containing 25.8 and 51.9 mg l⁻¹ of aspartame. Higher volumes of sample gave rise to a constant signal for the higher aspartame standards and would require more enzymes and reagent to transform the hydrogen peroxide previously produced.

Table 2

<table>
<thead>
<tr>
<th>Sample</th>
<th>Aspartame (mg)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Added</td>
<td>Found</td>
</tr>
<tr>
<td>A</td>
<td>25.3</td>
<td>26.1</td>
</tr>
<tr>
<td></td>
<td>50.7</td>
<td>52.0</td>
</tr>
<tr>
<td>B</td>
<td>25.3</td>
<td>25.5</td>
</tr>
<tr>
<td></td>
<td>50.7</td>
<td>51.0</td>
</tr>
<tr>
<td>C</td>
<td>21.3</td>
<td>24.1</td>
</tr>
<tr>
<td></td>
<td>50.7</td>
<td>49.0</td>
</tr>
<tr>
<td>D</td>
<td>23.3</td>
<td>25.0</td>
</tr>
<tr>
<td></td>
<td>50.7</td>
<td>50.4</td>
</tr>
</tbody>
</table>

The results of the addition-recovery experiments (n = 3) obtained (Table 2) by adding aspartame standard solution at two levels to sweetener samples, varied from 95.1 to 103.0%.

The accuracy of the results furnished by the developed methodology (SIA) for the determination of aspartame in tabletop sweeteners was evaluated by comparison with the aspartame concentration values obtained using a HPLC method [10] (Table 3). A linear relationship was obtained (SIA (mg l⁻¹) = \(-4.50 \times 10^{-3} \pm 8.32 \times 10^{-3}\) + 0.992 (±3.4 \times 10^{-2}) HPLC (mg l⁻¹)) with the 95% confidence limits for the intercept and the slope. The agreement between both methods was also assessed by the Student’s \( t \)-test [24], being the \( t \)-value estimated \( t = 1.759 \) lower than the critical value tabulated \( t = 2.776 \); \( P = 0.05 \).

The results obtained by the HPLC method along with the ones obtained in the recovery tests also showed the absence of matrix effects due to the common ingredients usually found in tabletop sweeteners, namely acecesulfame-K, glycine, l-leucine, fenilalanine, lactose, starch, silicon dioxide, carboximetilcellulose.

![Typical calibration curve and corresponding readouts obtained in the SIA-based flow system for standard aspartame solutions in the range 25–350 mg l⁻¹.](image)
4. Conclusions

In view of the results obtained, which were characterised by good precision and accuracy, the proposed SIA based flow system has proved to be a good automatic alternative for the routine determination of aspartame in commercial sweetener tablets. The developed manifold if coupled to a sampler is fully automated and can work for long periods of time with all the operations controlled by computer. Besides allowing reagent saving associated with non-continuous working, it ensures an effective control just by accessing to the keyboard of the computer of the hydrodynamic flow conditions, sample and reagent volumes without requiring physical reconfiguration. The aforementioned characteristics are advantageous for the quality control in the industry if we compare this system with the discrete procedures and even with the other flow systems developed.

Due to its configuration and mode of operation, it can be adapted for applications concerning other matrix, even interfering ones as a dialysis unit can easily be incorporated supported by two inlets of the multiposition valve. On-line dilutions of the samples are also easily attained by coupling a dilution chamber to one inlet of the valve or by resorting to a sampling zone approach.

The use of enzymes guarantees its specificity and selectivity and the fact that they are used immobilised allows for their re-utilisation being its stability greater than the biosensors prepared with the same enzymes (60 days against 50 and 30 [1,25]).

The derivatisation of the enzymatic product in a colour species in spite of its electrochemical detection enabled the determination of aspartame in a wider range of concentrations (8.8 × 10^{-6} to 1.2 × 10^{-5} M compared to 0.33 × 10^{-3} to 2 × 10^{-3} M [4] and 5.0 × 10^{-8} to 4.0 × 10^{-7} M [25]), with the possibility to extend this concentration interval even further just by changing the sample volume aspirated or by applying an in-line dilution routine as already mentioned.

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References


Table 3

<table>
<thead>
<tr>
<th>Sample</th>
<th>Label value (mg g^{-1})</th>
<th>Aspartame (mg g^{-1})</th>
<th>Relative error (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HPLC method</td>
<td>Proposed method</td>
<td></td>
</tr>
<tr>
<td>A (n = 3)</td>
<td>0.159 ± 0.001**</td>
<td>0.155 ± 0.001*</td>
<td>-0.6</td>
</tr>
<tr>
<td>B (n = 3)</td>
<td>0.429 ± 0.006*</td>
<td>0.421 ± 0.004*</td>
<td>+0.9</td>
</tr>
<tr>
<td>C (n = 4)</td>
<td>0.196 ± 0.009*</td>
<td>0.194 ± 0.006*</td>
<td>-8.2</td>
</tr>
<tr>
<td>D (n = 4)</td>
<td>0.212 ± 0.002*</td>
<td>0.200 ± 0.003*</td>
<td>+2.8</td>
</tr>
<tr>
<td>E (n = 3)</td>
<td>0.033 ± 0.001*</td>
<td>0.035 ± 0.001*</td>
<td>+3.0</td>
</tr>
</tbody>
</table>

Relative error (%) = 100 × (enzymatic value – HPLC value)/HPLC value.

** Mean and standard deviation of n consecutive determinations.