Megazyme

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ASPARTAME

ASSAY PROCEDURE

K-ASPTM 08/18

(*50 Assays per Kit) or
(500 Microplate Assays per Kit) or
(500 Auto-Analyser Assays per Kit)

** The number of tests per kit can be doubled if all volumes are halved*

INTRODUCTION:

Aspartame is a dipeptide sweetener comprising L-aspartate and methyl esterified L-phenylalanine (Asp-Phe-O-Me). At 200 times the sweetness of sucrose, and with a structure similar to dipeptides found in a normal diet, its use in low calorie carbonated drinks and other foodstuffs has been widely accepted since the early 1980s. However, due to its L-phenylalanine content, consumption of this compound must be strictly monitored by those suffering from phenylketonuria. There is also a significant level of concern voiced by some who believe this sweetener poses serious health problems relating to the three components, L-aspartic acid, L-phenylalanine and methanol, though there is, as yet, no evidence to support such claims.

PRINCIPLE:

The detection of aspartame requires three enzyme reactions, after an initial treatment at high pH to remove the methyl group from the L-phenylalanine residue (1).

$$\text{(1)}\quad \text{Asp-Phe-O-Me} \xrightarrow{\text{(pH 12.5)}} \text{Asp-Phe} + \text{MeOH}$$

After the methyl group has been removed, the product aspartame acid is hydrolysed in the first enzymic reaction catalysed by a specific dipeptidase (PepM), to free L-aspartate and L-phenylalanine (2).

$$\text{(2)}\quad \text{Asp-Phe} + H_2O \xrightarrow{\text{(PepM)}} \text{L-aspartate} + \text{L-phenylalanine}$$

The L-aspartate is subsequently converted to oxaloacetate and L-glutamate in the presence of 2-oxoglutarate and the enzyme glutamate-oxaloacetate transaminase (GOT) (3).

$$\text{(3)}\quad \text{L-Aspartate} + \text{2-oxoglutarate} \xrightarrow{\text{(GOT)}} \text{L-glutamate} + \text{oxaloacetate}$$

Finally, the oxaloacetate is converted to L-malate and NAD^+, in the presence of NADH and the enzyme L-malate dehydrogenase (L-MDH) (4).

$$\text{(4)}\quad \text{Oxaloacetate} + \text{NADH} + H^+ \xrightarrow{\text{(L-MDH)}} \text{L-malate} + NAD^+$$

The amount of NAD^+ formed in the above coupled reaction pathway is stoichiometric with the amount of aspartame. It is NADH consumption which is measured by the decrease in absorbance at 340 nm.

SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

The assay is specific for aspartame and its breakdown products, aspartame acid and aspartic acid. Due to the order of addition of the enzymes, free aspartic acid in the extract is measured before hydrolysis of the dipeptide by PepM. Additionally, free aspartame acid (demethylated aspartame) can be quantified by analysing the sample before demethylation.

The smallest differentiating absorbance for the assay is 0.005 absorbance units. This corresponds to 0.29 mg/L of sample solution at the maximum sample volume of 2.00 mL. The detection limit is 0.57 mg/L which is derived from an absorbance difference of 0.010 with the maximum sample volume of 2.00 mL.

The assay is linear over the range of 10 to 150 µg of aspartame per assay. In duplicate determinations using one sample solution, an absorbance difference of 0.005 to 0.010 may occur. With a sample volume of 2.00 mL, this corresponds to an aspartame concentration of approx. 0.29 to 0.57 mg/L of sample solution. If the sample is diluted during sample preparation, the result is multiplied by the dilution factor, F. If in sample preparation, the sample is weighed, e.g. 10 g/L, a difference of 0.02 to 0.05 g/100 g can be expected.

INTERFERENCE:

If the conversion of the liberated aspartame acid has been completed within the time specified in the assay (approx 5 min.), it can be generally concluded that no interference has occurred. However, this can be further checked by adding aspartame acid (approx. 80 µg in 1.0 mL) to the cuvette on completion of the reaction. A significant decrease in the absorbance should be observed.

Interfering substances in the sample being analysed can be identified by including an internal standard. Quantitative recovery of this standard would be expected. Losses in sample handling and extraction are identified by performing recovery experiments, i.e. by adding aspartame to the sample in the initial extraction steps.

SAFETY:

The general safety measures that apply to all chemical substances should be adhered to.
For more information regarding the safe usage and handling of this product please refer to the associated SDS that is available from the Megazyme website.

KITS:

Kits suitable for performing 50 assays in manual format (or 500 assays in auto-analyser format or 500 assays in microplate format) are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: Buffer (12 mL, pH 8.0) plus 2-oxoglutarate and sodium azide (0.02% w/v) as a preservative.
Stable for > 2 years at 4°C.

Bottle 2: (x2) NADH plus stabiliser.
Stable for > 5 years below -10°C.

Bottle 3: L-Malate dehydrogenase plus glutamate-oxaloacetate transaminase suspension (1.1 mL).
Stable for > 2 years at 4°C.

Bottle 4: Peptidase M suspension (1.1 mL).
Stable for > 2 years at 4°C.

Bottle 5: Aspartame control powder (~ 1 g, ~ 2.5% w/w aspartame on an "as is" basis. The exact concentration of aspartame is given on the vial label).
Stable for > 5 years when stored dry at 4°C.

PREPARATION OF REAGENT SOLUTIONS/SUSPENSIONS:

1. Use the contents of bottle 1 as supplied.
Stable for > 2 years at 4°C.

2. Dissolve the contents of bottle 2 in 5.5 mL distilled water. **Stable for > 1 year at 4°C** or stable for > 2 years below -10°C (to avoid repetitive freeze/thaw cycles, divide into appropriately sized aliquots and store in polypropylene tubes). **Do not** dissolve the contents of the other bottle until required.

3 & 4. Use the contents of bottles 3 and 4 as supplied. Before opening for the first time, shake the bottles to remove any protein that may have settled on the rubber stopper. Subsequently, store the bottles in an upright position.
Stable for > 2 years at 4°C.

5. Dissolve 0.40 g of the aspartame control powder in 80 mL of water at approx. 50°C. Cool to room temperature and adjust volume to 100 mL and mix. Take 40 mL, adjust the pH to 12.5 by dropwise addition of 1 M NaOH and stir the solution for 10 min to demethylate. Adjust the pH to approx. 8.0 with 1 M HCl and wash the pH probe with a minimum volume of water. Transfer the solution to a 50 mL volumetric flask, adjust to volume and mix. Use 1.0 mL of this solution in the assay. Divide into 10 mL aliquots and store below -10°C.
Stable below -10°C for > 2 years.

NOTE: The aspartame standard solution is only assayed where there is some doubt about the accuracy of the spectrophotometer being used or where it is suspected that inhibition is being caused by substances in the sample. The concentration of aspartame is determined directly from the extinction coefficient of NADH (see page 5 and 6).

EQUIPMENT (RECOMMENDED):

1. Volumetric flasks (50 mL and 100 mL).
2. Disposable plastic cuvettes (1 cm light path, 3.0 mL).
3. Micro-pipettors, e.g. Gilson Pipetman® (20 µL and 200 µL).
4. Positive displacement pipettor, e.g. Eppendorf Multipette®
   - with 25 mL Combitip® (to dispense 1.0-2.0 mL aliquots of distilled water).
   - with 5.0 mL Combitip® [to dispense 0.2 mL aliquots of buffer (bottle 1) and NADH solution].
5. Analytical balance.
6. Spectrophotometer set at 340 nm.
7. Vortex mixer (e.g. IKA® Yellowline Test Tube Shaker TTS2).
8. Stop clock.
9. Whatman GF/A glass fibre (9 cm) filter papers.
10. Hot-plate magnetic stirrer.

A. MANUAL ASSAY PROCEDURE:

Wavelength: 340 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: ~ 25°C
Final volume: 2.44 mL
Sample solution: 10-150 µg of aspartame (as aspartame acid) per cuvette (in 1.0 mL sample volume)

Read against air (without a cuvette in the light path) or against water

Pipette into cuvettes	Blank	Sample
distilled water (at ~ 25°C)	2.00 mL	1.00 mL
sample	-	1.00 mL
solution 1 (buffer mixture)	0.20 mL	0.20 mL
solution 2 (NADH)	0.20 mL	0.20 mL
suspension 3 (L-MDH/GOT)	0.02 mL	0.02 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 4 min and start the reactions by addition of:		
suspension 4 (PepM)	0.02 mL	0.02 mL
Mix*, read the absorbances of the solutions (A_2) at the end of the reaction (approx. 5 min).		

* for example with a plastic spatula or by gentle inversion after sealing the cuvette with a cuvette cap or Parafilm®.

CALCULATION:

Determine the absorbance difference (A_1-A_2) for both blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining $\Delta A_{aspartame}$. The value of $\Delta A_{aspartame}$ should as a rule be at least 0.100 absorbance units to achieve sufficiently accurate results.

The concentration of aspartame can be calculated as follows:

$$c = \frac{V \times MW}{\varepsilon \times d \times v} \times \frac{50}{40} \times \Delta A \quad [g/L]$$

where:

V = final volume [mL]
MW = molecular weight of aspartame [g/mol]

ε = extinction coefficient of NADH at 340 nm
= 6300 [l x mol^{-1} x cm^{-1}]
d = light path [cm]
v = sample volume [mL]
50/40 = volume correction due to dilution in the demethylation step

It follows for aspartame:

$$c = \frac{2.44 \times 294.31}{6300 \times 1.0 \times 1.0} \times \frac{50}{40} \times \Delta A_{aspartame} \quad [g/L]$$

$$= 0.1425 \times \Delta A_{aspartame} \quad [g/L]$$

If the sample has been diluted during preparation, the result must be multiplied by the dilution factor, F.

When analysing solid and semi-solid samples which are weighed out for sample preparation, the content (g/100 g) is calculated from the amount weighed as follows:

Content of aspartame

$$= \frac{c_{aspartame}\ [g/L\ sample\ solution]}{weight_{sample}\ [g/L\ sample\ solution]} \times 100 \quad [g/100\ g]$$

For example:

With the aspartame control powder, 0.4 g is dissolved in 100 mL of water (i.e. 4 g/L) and 40 mL of this is taken for demethylation.

Thus, content of aspartame:

$$= \frac{c_{aspartame}\ [g/L\ sample\ solution]}{4\ (g/L)} \times 100 \quad [g/100\ g]$$

NOTE: These calculations can be simplified by using the Megazyme ***Mega-Calc***™, downloadable from where the product appears in the Megazyme web site (www.megazyme.com).

B. AUTO-ANALYSER ASSAY PROCEDURE:

NOTES:

1. The Auto-Analyser Assay Procedure for aspartame can be performed using either a single point standard or a full calibration curve.
2. For each batch of samples that is applied to the determination of aspartame **either a single point standard or a calibration curve must be performed concurrently using the same batch of reagents.**

Reagent preparation is performed as follows:

Preparation of R1:

Component	Volume
bottle 1 (buffer) bottle 2 (NADH) bottle 3 (L-MDH/GOT) distilled water	2 mL 2 mL (after adding 11 mL of H_2O to bottle 2) 0.2 mL 16.5 mL
Total volume	20.7 mL

Preparation of R2:

Component	Volume
bottle 4 (PepM) distilled water	0.2 mL 2.4 mL
Total volume	2.6 mL

EXAMPLE METHOD:

R1:	0.200 mL
Sample:	~ 0.01 mL
R2:	0.025 mL
Reaction time:	~ 5 min at 37°C
Wavelength:	340 nm
Prepared reagent stability:	> 2 days when refrigerated
Calculation:	endpoint
Reaction direction:	decrease
Linearity:	up to 1.44 g/L of aspartame using 0.01 mL sample volume

C. MICROPLATE ASSAY PROCEDURE:

NOTES:

1. The Microplate Assay Procedure for aspartame can be performed using either a single point standard or a full calibration curve.
2. For each batch of samples that is applied to the determination of aspartame **either a single point standard or a calibration curve must be performed concurrently using the same batch of reagents.**

Wavelength: 340 nm
Microplate: 96-well (e.g. clear flat-bottomed, glass or plastic)
Temperature: ~ 25°C
Final volume: 0.244 mL
Linearity: 1-15 µg of aspartame per well (in 0.1 mL sample volume)

Pipette into wells	Blank	Sample	Standard
distilled water	0.200 mL	0.100 mL	0.100 mL
sample solution	-	0.100 mL	-
standard solution	-	-	0.100 mL
solution 1 (buffer)	0.020 mL	0.020 mL	0.020 mL
solution 2 (NADH)	0.020 mL	0.020 mL	0.020 mL
suspension 3 (L-MDH/GOT)	0.002 mL	0.002 mL	0.002 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 4 min and start the reactions by addition of:			
suspension 4 (PepM)	0.002 mL	0.002 mL	0.002 mL
Mix*, read the absorbances of the solutions (A_2) at the end of the reaction (approx. 5 min).			

* for example using microplate shaker, shake function on a microplate reader or repeated aspiration (e.g. using a pipettor set at 50-100 µL volume).

CALCULATION (Microplate Assay Procedure):

$$g/L = \frac{\Delta A_{sample}}{\Delta A_{standard}} \times g/L \text{ standard} \times F$$

If the sample is diluted during preparation, the result must be multiplied by the dilution factor, F.

SAMPLE PREPARATION:

1. Sample dilution.

The amount of aspartame present in the cuvette (i.e. in the 1.0 mL of sample being analysed) should range between 10 and 150 µg. The sample solution must therefore be diluted sufficiently to yield an aspartame concentration between 0.005 and 0.15 g/L.

Dilution Table

Estimated concentration of aspartame (g/L)	Dilution with water	Dilution factor (F)
< 0.15	No dilution required	1
0.15-1.5	1 + 9	10
1.5-15	1 + 99	100
> 15	1 + 999	1000

If the value of $\Delta A_{aspartame}$ is too low (e.g. < 0.100), weigh out more sample or dilute less strongly. Alternatively, the sample volume to be pipetted into the cuvette can be increased up to 2.0 mL, making sure that the sum of the sample and distilled water components in the reaction is 2.0 mL and using the new sample volume in the equation.

2. Sample clarification.

Carrez reagents are commonly used for sample clarification, but they cannot be used to clarify solutions for aspartame determination. These reagents not only reduce the rate of hydrolysis of aspartame acid by PepM, but also give an underestimation of aspartame by as much as 50%. It would appear that the reagents cause some precipitation of the aspartame.

For samples containing high levels of fat, e.g. chocolate, a combination of charcoal and chloroform treatment can be used to clarify aqueous solutions/suspensions (see recommended procedure for chocolate).

3. General considerations.

(a) Liquid samples: clear, slightly coloured and approximately neutral, liquid samples can be used directly in the assay.

(b) Coloured samples: an additional sample blank, i.e. sample with no PepM, should be performed in the case of coloured samples.

(c) Strongly coloured samples: if used undiluted, strongly coloured samples should be treated by the addition of 1 g/100 mL of activated charcoal. Stir for 2 min and then filter through Whatman GF/A glass fibre filter paper.

(d) Solid samples: homogenise or crush solid samples in distilled water and filter if necessary.

(e) Samples containing fat: extract such samples with hot water at a temperature above the melting point of the fat, e.g. in a 100 mL volumetric flask. Adjust to 20°C and fill the volumetric flask to the mark with water. Store on ice or in a refrigerator for 15-30 min and then filter. Discard the first few mL of filtrate, and use the clear supernatant (which may be slightly opalescent) for assay. Alternatively, clarify with chloroform (see chocolate samples).

(f) Samples containing protein: such samples generally do not cause problems.

4. Demethylation of Aspartame.

(a) Sample extraction:

Liquid samples: clear, slightly coloured and approximately neutral, liquid samples can be used directly for demethylation.

Solid samples: homogenise solid and semi-solid samples, such as powders, candies, jams and chocolate, extract the aspartame in distilled water and filter (see specific examples).

(b) Demethylation:

Add 40 mL of aspartame containing solution or extract to a 100 mL beaker. Add a magnetic stirrer bar and adjust the pH of the stirring solution to 12.5 by dropwise addition of 1 M NaOH at approx. 25°C. Monitor the pH change with a pH meter. Allow the solution to react for 10 min, and then adjust the pH to ~ 8.0 with 1 M HCl (if the pH is over-adjusted, use the 1 M NaOH to readjust). This process converts aspartame to aspartame acid. Transfer the solution to a 50 mL volumetric flask and adjust to volume with water.

If this solution is clear and only slightly coloured, it can be used directly in the assay. Otherwise, decolourise with charcoal and filter.

SAMPLE PREPARATION EXAMPLES:

(a) Determination of aspartame in soda drinks and fruit juices.

Add 40 mL of soda drink or juice to a 100 mL beaker. Add a magnetic stirrer bar and adjust the pH of the stirring solution to 12.5 by dropwise addition of 1 M NaOH at approx. 25°C while monitoring with a pH meter. Allow the solution to react for 10 min, and then adjust the pH to approx. 8.0 with 1 M HCl (if the pH is over-adjusted, use the 1 M NaOH to readjust). Wash the pH electrode with a few mL of water to remove all sample solution, and adjust the volume to 50 mL and mix thoroughly. Analyse clear and slightly

coloured solutions directly. Filter turbid solutions. Decolourise strongly coloured solutions by adding 0.1 g of activated charcoal. Stir for 2 min and filter through Whatman GF/A glass fibre filter paper. Dilute as required using the dilution table. Use 1.0 mL for assay.

(b) Determination of aspartame in artificial sweeteners, candies and mints.

Dissolve 2 g of the powder or the ground solid in 80 mL of distilled water by stirring. Adjust the volume to 100 mL. Take 40 mL of this solution, demethylate as described above and adjust the volume to 50 mL. Filter if necessary and dilute according to the dilution table, if required. Use 1.0 mL for assay.

(c) Determination of aspartame in chewing gum.

Accurately weigh approx. 4 g of representative chewing gum sample into a 100 mL Duran® bottle. Add 20 mL of toluene and 40 mL of distilled water, cap the bottle and vigorously stir the slurry on a magnetic stirrer for approx. 20 min (until the gum is fully dispersed). Centrifuge the suspension at 1,500 *g* in sealed polypropylene tubes, and carefully remove the upper phase (toluene) and discard with waste solvents. Transfer the lower phase (aqueous) to a 100 mL volumetric flask and adjust to volume with distilled water. Transfer 40 mL to a 100 mL beaker. Add a magnetic stirrer bar and adjust the pH of the stirring solution to 12.5 by dropwise addition of 1 M NaOH at room temperature while monitoring with a pH meter. Allow the solution to react for 10 min, and then adjust the pH to approx. 8.0 with 1 M HCl (if the pH is over-adjusted, use the 1 M NaOH to readjust). Wash the pH electrode with a few mL of water to remove all sample solution, adjust the volume to 50 mL and mix thoroughly. Use 1.0 mL for assay.

(d) Determination of aspartame in jam.

Add 20 g of aspartame containing jam to 70 mL of water in a 200 mL beaker. Stir vigorously over 20 min at approx. 25°C to dissolve. Quantitatively transfer to a 100 mL volumetric flask and adjust to volume. Transfer to a 200 mL beaker and add 1 g of activated charcoal. Stir for 2 min and filter through Whatman GF/A glass fibre filter paper. Transfer 40 mL of the filtrate to a 100 mL beaker, add a magnetic stirrer bar and adjust the pH of the stirring solution to 12.5 by dropwise addition of 1 M NaOH at approx. 25°C. Monitor the pH change with a pH meter. Allow the solution to react for 10 min, and then adjust the pH to approx. 8.0 with 1 M HCl. Rinse the pH electrode with a few mL of water, adjust volume to 50 mL and mix. Use 1.0 mL for assay.

(e) Determination of aspartame in chocolate.

Accurately weigh approx. 1 g of a representative chocolate sample into a 100 mL volumetric flask and add 80 mL of distilled water. Heat to 60°C in a water bath over 20 min and shake the container regularly to ensure complete dispersion of the chocolate. Fill the volumetric flask to the mark. Transfer the solution to a 200 mL beaker, add 2 g of activated charcoal, stir for 2 min and filter through Whatman GF/A glass fibre filter paper. Add 40 mL of the filtrate to a 100 mL beaker, demethylate the aspartame as described on page 10 and adjust to 50 mL. At this stage, the solution is still slightly turbid. This turbidity can be removed by one of two procedures. ***Procedure 1:*** centrifuge aliquots of the solution at 12,000 *g* for 10 min in a microfuge. ***Procedure 2:*** to 5 mL of the solution in glass centrifuge tubes (16 x 120 mm) add 2 mL of chloroform and mix vigorously on a vortex mixer for 1 min. Cap the tubes and centrifuge at 1,500 *g* for 10 min. Use 1.0 mL aliquots of the upper (aqueous) phase for assay. With this solution, completion of the determination reaction takes approx. 12 min (Note: the usual time is approx. 5 min). Allow the reaction to continue until there is no further change in absorbance, or until the rate of change of absorbance is constant (creep rate).

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