Megazyme

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L-ARABINOSE & D-GALACTOSE (*Rapid*)

ASSAY PROCEDURE

K-ARGA 04/20

(*115 Manual Assays per Kit) or
(1150 Auto-Analyser Assays per Kit) or
(1150 Microplate Assays per Kit)

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

Patented: US 7,785,771 B2 and EP1 828 407 (GB, FR, IE, DE)

INTRODUCTION:

Enzymic kits for the determination of L-arabinose and D-galactose are very slow. This is due to the low rate of natural chemical "mutarotation" between the α- and β-anomeric forms of these monosaccharides. Only the β-form is recognised by β-galactose dehydrogenase. In incubations containing NAD^+, L-arabinose or D-galactose and β-galactose dehydrogenase, there is a very rapid initial increase in absorbance due to the consumption of β-L-arabinose or β-D-galactose, and this is followed by a very slow approach to the end-point. This very slow approach results from the very low rate of chemical "mutarotation" of the α-anomeric conformation of these monosaccharides to the respective β-anomer. Using technology developed by Megazyme (patented), a galactose mutarotase has now been incorporated into the assay format to rapidly catalyse this rate-limiting mutarotation step. The result is very rapid analysis times of approximately 6 min for D-galactose and approximately 12 min for L-arabinose (Figure 1, page 6).

PRINCIPLE:

In the currently described procedure interconversion of the α- and β-anomeric forms of L-arabinose and D-galactose is catalysed by galactose mutarotase (GalM) (1, 2).

$$\text{(1)}\quad \alpha\text{-L-Arabinose} \xleftrightarrow{\text{(GalM)}} \beta\text{-L-arabinose}$$

$$\text{(2)}\quad \alpha\text{-D-Galactose} \xleftrightarrow{\text{(GalM)}} \beta\text{-D-galactose}$$

The β-L-arabinose and β-D-galactose are oxidised by NAD^+ to L-arabinonic acid and D-galactonic acid in the presence of β-galactose dehydrogenase (β-GalDH) at pH 8.6 (3, 4).

$$\text{(3)}\quad \beta\text{-L-Arabinose} + NAD^+ \xrightarrow{\text{(β-GalDH)}} \text{L-arabinonic acid} + NADH + H^+$$

$$\text{(4)}\quad \beta\text{-D-Galactose} + NAD^+ \xrightarrow{\text{(β-GalDH)}} \text{D-galactonic acid} + NADH + H^+$$

The amount of NADH formed in this reaction is stoichiometric with the amount of L-arabinose or D-galactose. It is the NADH which is measured by the increase in absorbance at 340 nm.

SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

The assays are specific for L-arabinose and D-galactose. The smallest differentiating absorbance for the assay is 0.010 absorbance units. This corresponds to 0.288 mg/L of L-arabinose or 0.346 mg/L of D-galactose of sample solution at the maximum sample volume of 2.00 mL. The detection limit is 0.577 mg/L of L-arabinose or

0.692 mg/L of D-galactose, which is derived from an absorbance difference of 0.020 with the maximum sample volume of 2.00 mL.

The assay is linear over the range of 4 to 80 µg of L-arabinose or D-galactose 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 0.144 to 0.288 mg/L of L-arabinose or 0.173 to 0.346 mg/L of D-galactose 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 L-arabinose or D-galactose has been completed within 12 min at room temperature, it can be generally concluded that no interference has occurred. However, this can be further checked by adding L-arabinose or D-galactose (approx. 40 µg in 0.1 mL) to the cuvette on completion of the reaction. A significant increase 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 L-arabinose or D-galactose to the sample in the initial extraction steps.

Since divalent metal ions inhibit the β-galactose dehydrogenase employed in this assay, EDTA is included in buffer 1.

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 115 assays in manual format (or 1150 assays in auto-analyser or microplate formats) are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: Buffer (25 mL, pH 8.6) plus sodium azide (0.02% w/v) as a preservative.
Stable for > 2 years at 4°C.

Bottle 2: NAD^+. Lyophilised powder.
Stable for > 5 years below -10°C.

Bottle 3: β-Galactose dehydrogenase plus galactose mutarotase suspension, 2.4 mL.
Stable for > 2 years at 4°C.

Bottle 4: L-Arabinose standard solution (5 mL, 0.4 mg/mL in 0.02% w/v sodium azide).
Stable for > 2 years; store sealed at 4°C.

Bottle 5: D-Galactose standard solution (5 mL, 0.4 mg/mL in 0.02% w/v sodium azide).
Stable for > 2 years; store sealed at 4°C.

PREPARATION OF REAGENT SOLUTIONS (SUPPLIED):

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

2. Dissolve the contents of bottle 2 in 12 mL of distilled water. **Stable for ~ 4 weeks 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).

3. Use the contents of bottle 3 as supplied. Before opening for the first time, shake the bottle to remove any enzyme that may have settled on the rubber stopper. Subsequently store the bottle in an upright position. **Swirl the bottle to mix contents before use.**
Stable for > 2 years at 4°C.

4 & 5. Use the contents of bottles 4 & 5 as supplied.
Stable for > 2 years; store sealed at 4°C.

NOTE: The L-arabinose or D-galactose standard solutions (bottle 4 & 5) are 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. To test the assay using the standard solution, add 0.1 mL (40 µg L-arabinose or D-galactose) of standard solution to the assay cuvette in place of the sample. The concentration of L-arabinose or D-galactose is determined directly from the extinction coefficient of NADH (page 5).

EQUIPMENT (RECOMMENDED):

1. Volumetric flasks (50 mL, 100 mL and 500 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 5.0 mL Combitip® (to dispense 0.2 mL aliquots of buffer 1 and 0.1 mL aliquots of NAD^+ solution).
   - with 25 mL Combitip® (to dispense 2.0 mL aliquots of distilled water).
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 No. 1 (9 cm) filter papers.

A. MANUAL ASSAY PROCEDURE:

Wavelength: 340 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: ~ 25°C to 37°C
Final volume: 2.42 mL
Sample solution: 4-80 µg of L-arabinose or D-galactose per cuvette (in 0.10-2.00 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)	2.10 mL	2.00 mL
sample	-	0.10 mL
solution 1 (buffer)	0.20 mL	0.20 mL
solution 2 (NAD^+)	0.10 mL	0.10 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 3 min and start the reactions by addition of:		
suspension 3 (β-GalDH/GalM)	0.02 mL	0.02 mL
Mix*, read the absorbance of the solutions (A_2) at the end of the reaction (~ 12 min for L-arabinose or ~ 6 min for D-galactose). If the reaction has not stopped after 13 min for L-arabinose or 7 min for D-galactose, continue to read the absorbances at 1 min intervals until the absorbances remain the same over 1 min**.		

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

** if the absorbance A_2 increases constantly, extrapolate the absorbance to the time of addition of suspension 3.

CALCULATION (Manual Assay Procedure):

Determine the absorbance difference (A_2-A_1) for both blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining ΔA.

The value of ΔA should be at least 0.100 absorbance units to achieve sufficiently accurate results.

The concentration of L-arabinose or D-galactose can be calculated as follows:

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

where:

V = final volume [mL]
MW = molecular weight of L-arabinose or D-galactose [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]

It follows for L-arabinose:

$$c = \frac{2.42 \times 150.13}{6300 \times 1.0 \times 0.1} \times \Delta A_{L\text{-arabinose}} \quad [g/L]$$

$$= 0.5767 \times \Delta A_{L\text{-arabinose}} \quad [g/L]$$

It follows for D-galactose:

$$c = \frac{2.42 \times 180.16}{6300 \times 1.0 \times 0.1} \times \Delta A_{D\text{-galactose}} \quad [g/L]$$

$$= 0.6920 \times \Delta A_{D\text{-galactose}} \quad [g/L]$$

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) L-arabinose or D-galactose is calculated from the amount weighed as follows:

Content of L-arabinose or D-galactose:

$$= \frac{c \text{ [g/L sample solution]}}{\text{weight}_{sample} \text{ [g/L sample solution]}} \times 100 \quad [g/100\ g]$$

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

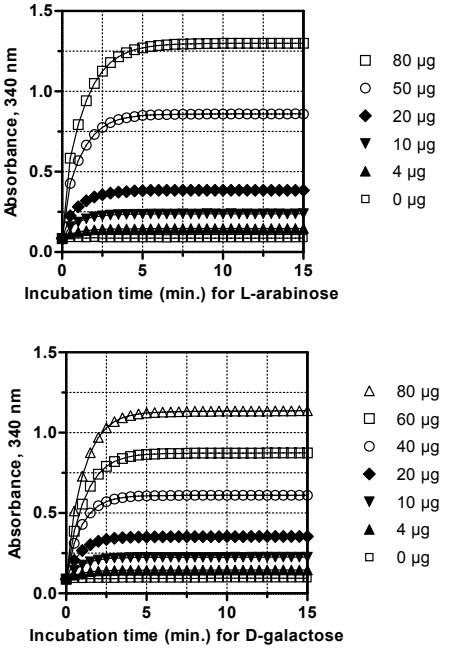

Figure 1. Increase in absorbance at 340 nm on incubation of 0-80 µg of L-arabinose or D-galactose with NAD^+ in the presence of β-galactose dehydrogenase (β-GalDH) at pH 8.6 at 25°C using 1 cm path-length cuvettes (Manual Format; page 4).

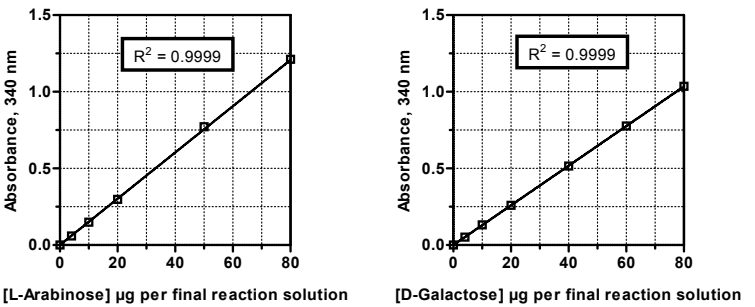

Figure 2. Calibration curves showing the linearity of **K-ARGA** from 0-80 µg of L-arabinose or D-galactose. The reactions used to generate this calibration curve were performed at 25°C using 1 cm path-length cuvettes (Manual Format; page 4).

B. AUTO-ANALYSER ASSAY PROCEDURE:

NOTES:

1. The Auto-Analyser Assay Procedure for L-arabinose or D-galactose 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 L-arabinose or D-galactose **either a single point standard or a calibration curve must be performed concurrently using the same batch of reagents.**

Preparation of R1:

Reagent preparation is performed as follows:

Component	Volume
distilled water solution 1 (buffer) solution 2 (NAD^+)	42.0 mL 4.6 mL 2.3 mL (after adding 12 mL of H_2O to bottle 2)
Total volume	48.9 mL

Preparation of R2:

Component	Volume
distilled water suspension 3 (β-GalDH/GalM)	5.40 mL 0.46 mL
Total volume	5.86 mL

EXAMPLE METHOD:

R1:	0.200 mL
Sample:	~ 0.01 mL
R2:	0.025 mL
Reaction time:	~ 12 min for L-arabinose or ~ 6 min for D-galactose at ~ 25°C to 37°C
Wavelength:	340 nm
Prepared reagent stability:	> 7 days when refrigerated
Calculation:	endpoint
Reaction direction:	increase
Linearity:	up to ~ 0.77 g/L of L-arabinose or D-galactose using 0.01 mL sample volume

C. MICROPLATE ASSAY PROCEDURE:

NOTES:

1. The Microplate Assay Procedure 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 L-arabinsoe or D-galactose **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 to 37°C
Final volume: 0.242 mL
Linearity: 0.4-8 µg of L-arabinose or D-galactose per well (in 0.01-0.20 mL sample volume)

Pipette into wells	Blank	Sample	Standard
distilled water	0.210 mL	0.200 mL	0.200 mL
sample solution	-	0.010 mL	-
standard solution	-	-	0.010 mL
solution 1 (buffer)	0.020 mL	0.020 mL	0.020 mL
solution 2 (NAD^+)	0.010 mL	0.010 mL	0.010 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 3 min and start the reactions by addition of:			
suspension 3 (β-GalDH/GalM)	0.002 mL	0.002 mL	0.002 mL
Mix*, read the absorbance of the solutions (A_2) at the end of the reaction (~ 12 min for L-arabinose or ~ 6 min for D-galactose). If the reaction has not stopped after 13 min for L-arabinose or 7 min for D-galactose, continue to read the absorbances at 1 min intervals until the absorbances remain the same over 1 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 L-arabinose or D-galactose present in the cuvette (i.e. in the 0.1 mL of sample being analysed) should range between 4 and 80 µg. The sample solution must therefore be diluted sufficiently to yield a lactose concentration between 0.04 and 0.8 g/L.

Dilution Table

Estimated concentration of L-arabinose or D-galactose (g/L)	Dilution with water	Dilution factor (F)
< 0.8	No dilution required	1
0.8-8.0	1 + 9	10
8.0-80	1 + 99	100
> 80	1 + 999	1000

If the value of ΔA 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 to 2.0 mL, making sure that the sum of the sample and distilled water components in the reaction is 2.10 mL and using the new sample volume in the equation.

2. Sample clarification.

a. Solutions:

Carrez I solution. Dissolve 3.60 g of potassium hexacyanoferrate (II) $\{K_4[Fe(CN)_6].3H_2O\}$ (Sigma cat. no. P9387) in 100 mL of distilled water. Store at room temperature.

Carrez II solution. Dissolve 7.20 g of zinc sulphate ($ZnSO_4.7H_2O$) (Sigma cat. no. Z4750) in 100 mL of distilled water. Store at room temperature.

Sodium hydroxide (NaOH, 100 mM). Dissolve 4 g of NaOH in 1 L of distilled water. Store at room temperature.

b. Procedure:

Pipette the liquid sample into a 100 mL volumetric flask which contains approx. 60 mL of distilled water, or weigh sufficient quantity of the sample into a 100 mL volumetric flask and add 60 mL of distilled water. Carefully add 5 mL of Carrez I solution, 5 mL of Carrez II solution and 10 mL of NaOH solution (100 mM). Mix after each addition. Fill the volumetric flask to the mark, mix and filter.

3. General considerations.

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

(b) Acidic samples: if > 0.1 mL of an acidic sample is to be used undiluted (such as wine or fruit juice), the pH of the solution should

be increased to approx. 8.6 using 2 M NaOH, and the solution incubated at room temperature for 30 min.

(c) Carbon dioxide: samples containing a significant amount of carbon dioxide, such as beer, should be degassed by increasing the pH to approx. 8.6 with 2 M NaOH and gentle stirring, or by stirring with a glass rod.

(d) Coloured samples: an additional sample blank, i.e. sample with no β-GalDH/GalM, may be necessary in the case of coloured samples.

(e) Strongly coloured samples: if used undiluted, strongly coloured samples should be treated by the addition of 0.2 g of PVPP/10 mL of sample. Shake the tube vigorously for 5 min and then filter through Whatman No. 1 filter paper.

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

(g) 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 at 60°C. Adjust to room temperature and fill the volumetric flask to the mark with water. Store on ice or in a refrigerator for 15-30 min to allow separation of the fat 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 Carrez reagents.

(h) Samples containing protein: deproteinise samples containing protein by adding an equal volume of ice-cold 1 M perchloric acid with mixing. Centrifuge at 1,500 *g* for 10 min and neutralise the supernatant with 1 M KOH. Alternatively, clarify with Carrez reagents.

SAMPLE PREPARATION EXAMPLES:

(a) Determination of D-galactose in milk, cream or yogurt.
Accurately weigh approx. 1 g of milk, cream, yogurt or condensed milk into a 100 mL volumetric flask, add approx. 60 mL of distilled water, mix and store at 50°C for 15 min with occasional swirling. Add 2 mL of Carrez I solution and mix. Add 2 mL of Carrez II solution and mix. Add 4 mL of 100 mM NaOH solution and mix vigorously. Dilute to volume with distilled water and mix thoroughly. Filter an aliquot of the solution through Whatman No. 1 filter paper. Discard the first few mL of filtrate. Use the clear filtrate (sample solution) in the assay. *Typically, for milk, cream and yogurt no dilution is required and a sample volume of 0.2 mL is satisfactory; for condensed milk a dilution of 1:3 and sample volume of 0.2 mL are satisfactory.*

(b) Determination of D-galactose in cheese and chocolate.

Add 10 g of grated cheese or 0.5 g of grated chocolate to a 200 mL beaker. Add approx. 60 mL of distilled water and a stirrer bar and mix on a magnetic stirrer at 50°C for approx. 15 min. Add 2 mL of Carrez I solution and mix. Add 2 mL of Carrez II solution and mix. Add 4 mL of 100 mM NaOH solution and mix vigorously. Quantitatively transfer the solution to a 100 mL volumetric flask and dilute to volume with distilled water. Mix thoroughly and filter an aliquot of the solution through Whatman No.1 filter paper. Discard the first few mL of filtrate. Use the clear filtrate (sample solution) in the assay. *Typically, for chocolate and most cheeses no dilution is required and a sample volume of 0.2 mL is satisfactory; however for red cheddar cheese a dilution of 1:10 and sample volume of 0.2 mL are satisfactory.*

(c) Determination of L-arabinose or D-galactose in polysaccharides and fibrous plant material.

Mill plant material or polysaccharide to pass a 0.5 mm screen using a Retsch centrifugal mill, or similar. Accurately weigh approx. 100 mg of material into a Corning® screw-cap culture tube (16 x 125 mm). Add 5 mL of 1.3 M HCl to each tube and cap the tubes. Incubate the tubes at 100°C for 1 h. Stir the tubes intermittently during the incubation. Cool the tubes to room temperature, carefully loosen the caps and add 5 mL of 1.3 M NaOH. Quantitatively transfer the contents of the tube to a 100 mL volumetric flask using distilled water and adjust the volume to 100 mL with distilled water. Mix thoroughly by inversion and filter an aliquot of the solution through Whatman No. 1 filter paper or centrifuge at 1,500 *g* for 10 min. *Typically, no further dilution is required and a sample volume of 0.1 mL is satisfactory.*

REFERENCE:

1. Fukimura, Y. (1988). D-Galactose. *"Methods of Enzymatic Analysis"* (Bergmeyer, H. U., ed.), 3rd ed., **Vol. VI**, pp. 288-296, VCH Publishers (UK) Ltd, Cambridge, UK.
2. Sturgeon, R. J. (1988). L-Arabinose. *"Methods of Enzymatic Analysis"* (Bergmeyer, H. U., ed.), 3rd ed., **Vol. VI**, pp. 427-431, VCH Publishers (UK) Ltd, Cambridge, UK.

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