Megazyme

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RAFFINOSE/ D-GALACTOSE

ASSAY PROCEDURE

K-RAFGA 08/18

(*120 Assays per Kit)

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

INTRODUCTION:

Grain legumes are an important component of both human and livestock diets. Galactosyl-sucrose oligosaccharides (raffinose, stachyose and verbascose) are major components in many food legumes,[1] and the antinutritional activity of grain legumes is frequently associated with the presence of these oligosaccharides.[2] However, recent data now suggest a beneficial role as prebiotics. Raffinose is usually the major galactosyl-sucrose oligosaccharide present in most legume seeds.[3] It is also a significant component in sugar-beet molasses.

Several solvents can be employed for the extraction of galactosyl-sucrose oligosaccharides from milled legume-seed. These are generally water/alcohol mixtures. Before, or concurrent with, extraction it is essential that endogenous α-galactosidase and invertase are inactivated. This can be achieved by refluxing a sample of the ground material in ethanol or in an aqueous ethanol mixture before the flour is subjected to extraction.

PRINCIPLE:

Raffinose is hydrolysed to D-galactose and sucrose by α-galactosidase (α-GAL) (1). α-GAL also hydrolyses other α-galactosides such as stachyose, verbascose and galactinol [1-O-(α-D-galactosyl)-*myo*-inositol], if present. The enzyme does not cleave β-linked galactose, as in lactose.

$$\text{(1)}\quad \text{Raffinose} + \text{Stachyose} + \text{Verbascose} + H_2O \xrightarrow{(\alpha\text{-galactosidase})} \text{D-galactose} + \text{sucrose}$$

Interconversion of the α- and β-anomeric forms of D-galactose is catalysed by galactose mutarotase (GalM) (2).

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

The β-D-galactose is oxidised by NAD^+ to D-galactonic acid in the presence of β-galactose deyhydrogenase (β-GalDH) at pH 8.6 (3).

$$\text{(3)}\quad \beta\text{-D-Galactose} + NAD^+ \xrightarrow{(\beta\text{-GalDH})} \text{D-galactonic acid} + NADH + H^+$$

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

The measurement of raffinose as D-galactose liberated is an alternative to the measurement as D-glucose liberated through the action of α-galactosidase plus β-fructosidase [see Megazyme "Raffinose/Sucrose/D-Glucose Assay Kit" (**K-RAFGL**) in the assay kits section of the Megazyme website (**www.megazyme.com**)]. Each procedure has advantages and limitations.

SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

As well as raffinose, α-galactosidase also hydrolyses other α-galactosides, such as stachyose and verbascose. This is a limitation of all enzyme based assays for raffinose. If, for example, a sample also contains stachyose, for each mol of stachyose hydrolysed, two mol of D-galactose are released. When calculated as raffinose (for which one mol of D-galactose is released), this will lead to an overestimation of the raffinose content, the level of which will depend on the stachyose content. Galactinol is also hydrolysed but, if desired, this can be removed by a semi-specific precipitation with basic lead acetate.[4,5]

β-GalDH also oxidises L-arabinose. While L-arabinose occurs frequently in plant polysaccharides, only small quantities of free L-arabinose are found in plant extracts or foodstuffs.

In the analysis of commercial raffinose, results of approximately 99% should be expected.

The smallest differentiating absorbance for the assay is 0.010 absorbance units. This corresponds to 10.5 mg of D-galactose/L of sample solution with a sample volume of 0.20 mL. The detection limit is 21.0 mg of D-galactose/L, which is derived from an absorbance difference of 0.020 and a sample volume of 0.20 mL.

The assay is linear over the range of 4 to 83 µg of D-galactose per assay (i.e. approx. 12 to 250 µg of raffinose 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 0.20 mL, this corresponds to a D-galactose concentration of 5.25 to 10.5 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 D-galactose has been completed within 40 min at 25°C or 20 min at 40°C, it can be generally concluded that no interference has occurred. However, this can be further checked by adding 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 raffinose or D-galactose to the sample in the initial extraction

steps. Complete hydrolysis of raffinose is confirmed by adding raffinose to the extract solution before α-galactosidase treatment.

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 120 assays are available from Megazyme. The kits contain the full assay method plus:

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

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

Bottle 3: D-Galactose dehydrogenase plus galactose mutarotase suspension (2.5 mL).
Stable for > 2 years at 4°C.

Bottle 4: α-Galactosidase (pH 4.6), lyophilised powder.
Stable for > 4 years below -10°C.

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

Bottle 6: Raffinose control powder (~ 4% w/w raffinose in mannitol; the exact concentration is stated on the vial label).
Stable for > 5 years at room temperature.

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 12.2 mL of 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).
3. Use the contents of bottle 3 as supplied. Before opening for the first time, shake the bottle to remove any protein that may have

settled on the rubber stopper. Subsequently, store the bottle in an upright position.
Stable for > 2 years at 4°C.

4. Dissolve the contents of bottle 4 with 12 mL of distilled water. Divide into aliquots of approx. 3 mL. Store below -10°C in polypropylene tubes between use and on ice during use.
Stable for > 2 years below -10°C.

5. Use the contents of bottle 5 as supplied.
Stable for > 2 years at 4°C.

6. Accurately weigh 0.500 g of the contents of bottle 6 into a 25 mL volumetric flask. Adjust to the mark with distilled water and mix the contents thoroughly. Divide into 10 mL aliquots and store below -10°C.
Stable for > 2 years below -10°C.

EQUIPMENT (RECOMMENDED):

1. Volumetric flasks (25, 50 mL and 100 mL).
2. Glass test tubes (16 x 120 mm and 18 x 150 mm).
3. Disposable plastic cuvettes (1 cm light path, 3.0 mL).
4. Micro-pipettors, e.g. Gilson Pipetman® (20 µL, 100 µL and 200 µL).
5. Positive displacement pipettor, e.g. Eppendorf Multipette®
   - with 5.0 mL Combitip® (to dispense 0.1 mL aliquots of α-galactosidase solution, 0.1 mL of NAD^+, 0.2 mL of Buffer 1 and 0.1, 0.2 and 0.3 mL of distilled water).
   - with 25 mL Combitip® (to dispense 2.0 mL aliquots of distilled water).
6. Analytical balance.
7. Spectrophotometer set at 340 nm.
8. Vortex mixer (e.g. IKA® Yellowline Test Tube Shaker TTS2).
9. Thermostated hot-block heater set at 40°C (optional).
10. Stop clock.
11. Whatman No. 1 (9 cm) filter papers.

PROCEDURE:

Wavelength: 340 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: optimally 40°C in a dry hot-block heater or in the spectrophotometer, but otherwise ~ 25°C
Final volume: 2.62 mL
Sample solution: 3-250 µg of raffinose per cuvette (in 0.10-0.20 mL sample volume)
Read against air (without a cuvette in the light path) or against water

If the sample contains free D-galactose (D-Gal), it must be determined in a separate assay without solution 4 (free D-Gal sample) (see below).

Pipette into cuvettes	Blank raffinose + free D-Gal	Raffinose + free D-Gal sample	Blank free D-Gal	free D-Gal sample
sample solution	-	0.20 mL	-	0.20 mL
distilled water	0.20 mL	-	0.30 mL	0.10 mL
solution 4 (α-GAL)	0.10 mL	0.10 mL	-	-
Mix* and incubate for 20 min (NOTE: before pipetting solution 4, first warm it to 25-30°C). Add:				
solution 1 (buffer)	0.20 mL	0.20 mL	0.20 mL	0.20 mL
distilled water	2.00 mL	2.00 mL	2.00 mL	2.00 mL
solution 2 (NAD^+)	0.10 mL	0.10 mL	0.10 mL	0.10 mL
Mix** and read absorbances of the solutions (A_1) after approx. 3 min and start the reactions by addition of:				
suspen. 3 (β-GalDH)/GalM	0.02 mL	0.02 mL	0.02 mL	0.02 mL
Mix** and read the absorbances of the solutions (A_2) at the end of the reaction (approx. 40 min at 25°C or 20 min at 40°C). If the reaction has not stopped after 20 min (i.e. in incubations at 40°C), continue to read the absorbances at 5 min intervals until the absorbances remain the same over 5 min.				

* pipette sample solution, distilled water and solution 4 into the bottom of the cuvette and mix by gentle swirling.

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

CALCULATIONS:

Determine the absorbance differences (A_2-A_1) for blanks and samples. Subtract the absorbance difference of the blank from the absorbance difference of the corresponding sample as follows:

$$\Delta A = (A_2\text{-}A_1)_{sample} - (A_2\text{-}A_1)_{blank}$$

$\Delta A_{raffinose + free\ D\text{-}galactose}$ is obtained from the "raffinose + free D-Gal" sample, and $\Delta A_{free\ D\text{-}galactose}$ is obtained from the "free D-Gal" sample.

Determination of raffinose

$$\Delta A_{raffinose} = \Delta A_{raffinose + free\ D\text{-}galactose} - \Delta A_{free\ D\text{-}galactose}$$

The values of $\Delta A_{raffinose + free\ D\text{-}galactose}$ and $\Delta A_{free\ D\text{-}galactose}$ should as a rule be at least 0.100 absorbance units to achieve sufficiently accurate results.

The concentration of free D-galactose and raffinose 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 the substance assayed [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 free D-galactose:

$$c = \frac{2.62 \times 180.16}{6300 \times 1 \times 0.2} \times \Delta A_{free\ D\text{-}galactose} \quad [g/L]$$

$$= 0.3746 \times \Delta A_{free\ D\text{-}galactose} \quad [g/L]$$

for raffinose:

$$c = \frac{2.62 \times 504.5}{6300 \times 1 \times 0.2} \times \Delta A_{raffinose} \quad [g/L]$$

$$= 1.049 \times \Delta A_{raffinose} \quad [g/L]$$

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 free D-galactose

$$= \frac{c_{\text{free D-galactose}}\ [\text{g/L sample solution}]}{\text{weight}_{\text{sample}}\ [\text{g/L sample solution}]} \times 100 \quad [\text{g/100 g}]$$

Content of raffinose

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

SAMPLE PREPARATION:

1. Sample dilution.

The amount of D-galactose present in the cuvette (i.e. in the 0.2 mL of sample being analysed) should range between 4 and 83 µg (i.e. raffinose in the sample taken should range between approx. 12 and 250 µg). The sample solution must therefore be diluted sufficiently to yield a raffinose concentration of between 0.2 and 1.25 g/L.

Dilution Table

Estimated concentration of raffinose (g/L)	Dilution with water	Dilution factor (F)
< 1.25	No dilution required	1
1.25-12.5	1 + 9	10
12.5-125	1 + 99	100

If the value of $\Delta A_{\text{free D-galactose}}$ or $\Delta A_{\text{raffinose}}$ 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 1.00 mL making sure that the sum of the sample, distilled water and solution 4 components in the hydrolysis reaction is 1.10 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 an acidic sample is to be used undiluted (such as red wine or coloured 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 carbon dioxide 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, should be performed in the case of coloured samples.

(e) Strongly coloured samples: if used undiluted, strongly coloured samples should be treated by the addition of 1 g/100 mL of polyvinylpolypyrrolidone (PVPP). Stir for 2 min and then filter.

(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. 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 the assay. Alternatively, clarify with Carrez reagents.

(h) Samples containing protein: deproteinise samples containing protein with Carrez reagents.

SAMPLE PREPARATION EXAMPLES:

(a) Determination of raffinose in milled seed material.

Accurately weigh approx. 0.50 g of milled seed (to pass a 0.5 mm screen) into glass test-tubes (18 x 150 mm). Add 5 mL of ethanol (95% v/v) to each tube and incubate at 84-88°C for 5 min (this treatment inactivates endogenous enzymes). Quantitatively transfer the tube contents to a 50 mL volumetric flask and adjust the volume to the mark with 50 mM sodium acetate buffer (pH 4.5). Allow the sample to extract over 15 min with occasional swirling. Transfer an aliquot (approx. 5 mL) of this slurry to a glass test tube (16 x 120 mm). Add 2 mL of chloroform, mix vigorously on a vortex mixer for 15 s and centrifuge at 1,500 *g* for 10 min [this treatment removes most of the lipids from the aqueous phase into the chloroform (lower phase); insoluble plant material tends to concentrate between the phases]. Analyse the upper (aqueous) phase. Dilute according to the dilution table, if required. *Typically, no dilution is required and a sample volume of 0.2 mL will be satisfactory.*

(b) Determination of raffinose in milled seed material (alternative extraction/clarification procedure).

Accurately weigh approx. 1.00 g of ground and homogenised sample into a 50 mL volumetric flask. Add 6 mL of ethanol (95% v/v) and incubate the flask in a boiling water bath for approx. 20 min with occasional stirring. Cool to room temperature and add 30 mL of distilled water with mixing. Carefully add 3 mL of Carrez I solution, 3 mL of Carrez II solution and 6 mL of NaOH solution (100 mM). Mix after each addition. Fill the volumetric flask to the mark, mix and filter. Dilute according to the dilution table, if required. *Typically, no dilution and a volume of 0.2 mL will be required.*

(c) Determination of raffinose in sugar-beet molasses or syrup.

Before starting the assay, quantitatively precipitate any galactinol present in the sample by the addition of basic lead acetate,[4,5] and then proceed as described in point (b) above.

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