

RAFFINOSE/SUCROSE/ GLUCOSE

ASSAY PROTOCOL

K-RAFGL

08/23

(120 Assays per Kit)



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 anti-nutritional activity of grain legumes is frequently associated with the presence of these oligosaccharides.² Galactosyl-sucrose oligosaccharides are not hydrolysed in the upper gut due to the absence of α -galactosidase. In the lower intestine they are metabolised by bacterial action, producing methane, hydrogen and carbon dioxide, which lead to flatulence and diarrhoea. Galactosyl-sucrose oligosaccharides are thus a factor limiting the use of grain legumes in monogastric diets.³

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

Identification and quantification of the extracted galactosyl-sucrose oligosaccharides have been achieved using an array of chromatographic procedures, however many of these methods are, at best, semi-quantitative. Chromatographic procedures employing high performance liquid chromatography and low-pressure liquid chromatography (using Bio-Gel P2) are quantitative, but can be time consuming, particularly in the area of sample preparation.

It is well known that raffinose, stachyose and verbascose are hydrolysed by α -galactosidase to D-galactose and sucrose. Biochemical kits for the measurement of raffinose are commercially available. The α -galactosidase used in these kits (from green coffee beans) rapidly hydrolyses raffinose, but acts quite slowly on stachyose and verbascose, and thus does not give complete hydrolysis of these oligosaccharides under the incubation conditions recommended. In contrast, the enzyme used in the current procedure (from *Aspergillus niger*) readily and rapidly catalyses complete hydrolysis of raffinose, stachyose and verbascose to D-galactose and sucrose.

PRINCIPLE:

Galactosyl-sucrose oligosaccharides are hydrolysed to D-galactose, D-glucose and D-fructose using α -galactosidase and invertase. The D-glucose is then determined using glucose oxidase/peroxidase reagent. The method does not distinguish between raffinose, stachyose and verbascose, but rather measures these as a group. Since one mole of each of the raffinose-series oligosaccharides contains one mole of D-glucose, the concentrations are presented on a molar basis. Free sucrose and D-glucose in sample extracts are determined concurrently.

KITS:

Kits suitable for performing 120 assays of D-glucose, sucrose and raffinose-series oligosaccharides are available from Neogen. The kits contain the full assay method plus:

- Bottle 1:** α -Galactosidase suspension (*A. niger*; 2 mL) in ammonium sulphate.
Store at 4°C. See individual label for expiry date.
- Bottle 2:** Invertase solution (yeast; 6 mL) containing sodium azide (0.02%) as a preservative.
Store at 4°C. See individual label for expiry date.
- Bottle 3:** **GOPOD Reagent Buffer.** Buffer (50 mL, pH 7.4), *p*-hydroxybenzoic acid and sodium azide (0.09% w/v).
Store at 4°C. See individual label for expiry date.
- Bottle 4:** **GOPOD Reagent Enzymes.** Glucose oxidase plus peroxidase and 4-aminoantipyrine. Freeze-dried powder.
Store below -10°C. See individual label for expiry date.
- Bottle 5:** D-Glucose standard solution (5 mL, 1.0 mg/mL) in 0.2% (w/v) benzoic acid.
Store sealed at room temperature. See individual label for expiry date.
- Bottle 6:** Soy-Flour Reference Sample (containing glucose, sucrose and galactosyl-sucrose oligosaccharides).
Store sealed at room temperature. See individual label for expiry date.

PREPARATION OF REAGENT SOLUTIONS/SUSPENSIONS:

1. Add the entire contents of **bottle 1** (α -Galactosidase) to 21 mL of **buffer 1** (see page 3). Then add 2 mL of the contents of **bottle 2** (invertase) and mix the contents thoroughly by careful inversion. Divide the solution into aliquots of suitable volume (approx. 5 mL) and store in polypropylene tubes below -10°C. This is **solution 1 (diluted α -Galactosidase and invertase)**.
Stable for ≥ 2 years below -10°C.
2. Dilute 1.0 mL of the contents of **bottle 2** to 12 mL with **buffer 1**. This is **solution 2 (diluted invertase)**.
Stable for ≥ 2 years below -10°C.
3. Dilute the contents of the **GOPOD Reagent Buffer** bottle to 1 L with distilled water (this is **solution 3**). Use immediately.

NOTE:

1. On storage, salt crystals may form in the concentrated GOPOD Reagent Buffer. These must be completely dissolved when this buffer is diluted to 1 L with distilled water.
2. This buffer contains 0.09% (w/v) sodium azide. This is a poisonous chemical and should be treated accordingly.

4. Dissolve the contents of the **GOPOD Reaction Enzymes** bottle in 20 mL of **solution 3** and quantitatively transfer this to the bottle containing the remainder of **solution 3**. Cover this bottle with aluminium foil to protect the enclosed reagent from light. This is **Glucose Determination Reagent (GOPOD Reagent)**. Stable for ≥ 1 month when stored at 4°C or ≥ 12 months below -10°C .

If this reagent is to be stored in the frozen state, preferably it should be divided into aliquots. Do not freeze/thaw more than once. When the **GOPOD reagent** is freshly prepared it may be light yellow or light pink in colour. It may develop a stronger pink colour upon storage at 4°C . The absorbance of this solution should be less than 0.05 when read against distilled water.

5 & 6. Use the contents of **bottles 5** and **6** as supplied.

REAGENTS REQUIRED (NOT SUPPLIED):

1. **Chloroform**

Analytical reagent grade (CAS number: 67-66-3).

2. **95% Ethanol (v/v).**

95% Ethanol (v/v) reagent grade, or similar.

BUFFER 1 (NOT SUPPLIED):

Sodium acetate buffer (50 mM, pH 4.5).

Add 2.9 mL of glacial acetic acid to 900 mL of distilled water. Adjust to pH 4.5 using 1 M sodium hydroxide. Adjust the volume to 1 L. Store at 4°C . This is **buffer 1**.

EQUIPMENT (RECOMMENDED):

1. Glass test tubes (round bottomed; 16 x 120 mm [suitable for centrifugation at 1,000 g] and 18 x 150 mm).
2. Micro-pipettors, e.g. Gilson Pipetman 100/200 μL .
3. Positive displacement pipettor, e.g. Eppendorf Multipette®
 - with 5.0 mL Combitip® (to dispense 0.2 mL aliquots of diluted invertase and invertase/ α -galactosidase mixtures).
 - with 12.5 mL Combitip® (to dispense 1.0 mL aliquots of invertase).
4. Analytical balance.
5. Spectrophotometer (set at 510 nm).
6. Vortex mixer (e.g. IKA® Yellowline Test Tube Shaker TTS2).
7. Thermostated water bath (set at 50°C).
8. Boiling water bath (set at $84-88^{\circ}\text{C}$).
9. Bench centrifuge (capable of 1,000 g).
10. Stop clock.

CONTROLS AND PRECAUTIONS:

1. Time of incubation with **GOPOD reagent** is not critical but should be at least 20 min. Colour formed should be measured within 60 min.
2. With each set of determinations, reagent blanks and D-glucose controls [0.556 μ moles (i.e. 100 μ g) quadruplicate] should be included.
 - a. The reagent blank consists of 0.4 mL of **buffer 1** + 3.0 mL GOPOD Reagent.
 - b. The glucose control consists of 0.1 mL of **bottle 5** (D-glucose standard solution, 100 μ g/0.1 mL) + 0.3 mL of **buffer 1** + 3.0 mL **GOPOD Reagent**.
3. With each new set of determinations, the reference sample should be included.

ASSAY PROCEDURE:

Enzyme Inactivation and Sugar Extraction:

1. Accurately weigh 0.50 ± 0.01 g of flour sample into a glass test-tube (18 x 150 mm) and add 5 mL of ethanol (95% v/v).
2. Incubate the tube in a water bath at 84-88°C for 5 min (this treatment inactivates endogenous enzymes).
3. Quantitatively transfer the tube contents to a 50 mL volumetric flask and adjust the volume to the mark with **buffer 1**. Allow the sample to extract over 15 min and then mix thoroughly.
4. Transfer 5 mL of this solution/slurry to a glass test-tube (16 x 120 mm).
5. Add 2 mL of chloroform to this solution, mix vigorously on a vortex mixer for 15 sec and centrifuge (1,000 g) for 10 min.

NOTE: This treatment removes most of the lipids from the aqueous upper phase. Insoluble plant material tends to concentrate between the phases.

6. Use the upper aqueous solution (**solution A**) directly for analysis.

Assay for Glucose, Sucrose and Raffinose-Series Oligosaccharides:

1. Treat 0.20 mL aliquots of **solution A** as follows:
 - 0.2 mL of **solution A** + 0.2 mL **buffer 1** [D-Glucose]..... = **A**
 - 0.2 mL of **solution A** + 0.2 mL **solution 2 (diluted invertase)**
[D-Glucose + Sucrose] = **B**
 - 0.2 mL of **Solution A** + 0.2 mL **solution 1 (diluted α -galactosidase + invertase)**
[D-Glucose + Sucrose + Galactosyl-sucrose oligosaccharides] = **C**

2. Incubate all solutions at 50°C for 20 min.
3. Add 3.0 mL of **GOPOD Reagent** to solutions **A, B** and **C**, as well as to the Reagent Blank, Reference sample and the D-glucose controls, and incubate all at 50°C for 20 min.
4. Read the absorbance of all solutions against the Reagent Blank at 510 nm:

Absorbances: ΔA = GOPOD absorbance for **A**
 ΔB = GOPOD absorbance for **B**
 ΔC = GOPOD absorbance for **C**

CALCULATIONS:

D-Glucose, millimoles/100 grams:

$$= \Delta A \times F \times 250 \times 200 \times \frac{1}{1000}$$

$$= \Delta A \times F \times 50$$

Sucrose, millimoles/100 grams:

$$= (\Delta B - \Delta A) \times F \times 250 \times 200 \times \frac{1}{1000}$$

$$= (\Delta B - \Delta A) \times F \times 50$$

Raffinose-series oligosaccharides (RSO), millimoles/100 grams:

$$= (\Delta C - \Delta B) \times F \times 250 \times 200 \times \frac{1}{1000}$$

$$= (\Delta C - \Delta B) \times F \times 50$$

where:

ΔA = GOPOD absorbance for 0.2 mL of samples + **buffer 1**.

ΔB = GOPOD absorbance for 0.2 mL of samples + **solution 2 (diluted invertase)**.

ΔC = GOPOD absorbance for 0.2 mL of samples + **solution 1 (diluted α -galactosidase and invertase)**.

F = a factor to convert from absorbance to μ moles of glucose

$$= \frac{0.556 \text{ (}\mu\text{moles of glucose)}}{\text{GOPOD absorbance for } 0.556 \text{ }\mu\text{moles of glucose}}$$

250 = conversion to 50 mL of extract (i.e. to 0.5 g of sample).

200 = conversion from 0.5 to 100 g of sample.

$\frac{1}{1000}$ = conversion from μ moles to millimoles.

1000

The concentrations of D-glucose and sucrose can be represented as millimoles/100 g, or can simply be calculated as g/100 g of flour, as shown below. However, it is not possible to calculate galactosyl-sucrose oligosaccharides as g/100 g of flour because these oligosaccharides are a mixture of raffinose, stachyose and verbascose. If the major component of this mixture for a given seed material is known, then it is possible to use the molecular weight of this compound and calculate an approximate value in grams/100 grams of flour.

D-Glucose (g/100 g flour) = D-Glucose (millimoles)/100 g x 0.1799.

Sucrose (g/100 g flour) = Sucrose (millimoles)/100 g x 0.3425.

Galactosyl-sucrose oligosaccharides (GSO) (g/100 g flour)
= (GSO)/100 g x MW/1000.

where:

0.1799 = the MW of D-glucose (180)/1000 mg of D-glucose.

0.3425 = the MW of sucrose (342)/1000 mg of sucrose.

MW/1000 = the average MW for GSO/1000 mg of RSO.

REFERENCES:

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4. McCleary, B. V., Charnock, S. J., Rossiter, P. C., O'Shea, M. F., Power, A. M. & Lloyd, R. M. (2006). Measurement of carbohydrates in grain, feed and food. *J. Sci. Food. Agric.*, **86**, 1648-1661.



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