

# Megazyme

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## RAFFINOSE-SERIES OLIGOSACCHARIDES ASSAY PROCEDURE

RSO 8/98

FOR THE MEASUREMENT OF  
RAFFINOSE-SERIES  
OLIGOSACCHARIDES,  
SUCROSE AND GLUCOSE  
IN SEED MATERIALS



## INTRODUCTION:

Grain legumes are an important component of both human and livestock diets. Oligosaccharides of the raffinose-series (namely raffinose, verbascose and stachyose) are major components in many food legumes<sup>1</sup>, and the antinutritional activity of grain legumes is frequently associated with the presence of these oligosaccharides<sup>2</sup>. Raffinose-series oligosaccharides are not hydrolysed in the upper gut due to the absence of  $\alpha$ -galactosidase. In the lower intestine they are metabolised by bacterial action, producing methane, hydrogen and carbon dioxide, which lead to flatulence and diarrhoea. Raffinose-series oligosaccharides are thus a factor limiting the use of grain legumes in monogastric diets<sup>3</sup>.

Several solvents have been employed for the extraction of raffinose-series oligosaccharides from legume-seed flours. These are generally water/alcohol mixtures. Before (or concurrent with) extraction, it is vital that endogenous  $\alpha$ -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 raffinose-series oligosaccharides have been achieved using an array of chromatographic procedures, however many of these methods are, at best, semiquantitative. 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  $\alpha$ -galactosidase to galactose and sucrose. Biochemical kits for the measurement of raffinose are commercially available. The  $\alpha$ -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 galactose and sucrose.

## PRINCIPLE:

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

## KIT CONTENTS:

Kits contain sufficient reagent for 120 determinations of glucose, sucrose and raffinose-series oligosaccharides i.e. they contain the full assay method plus:

1.  $\alpha$ -Galactosidase (*A. niger*; 2 mL, 500 U/mL) in ammonium sulphate. Store at 4°C.
2. Invertase (yeast; 6 mL; 100 U/mL) in 50% glycerol plus bovine serum albumin (BSA; 1 mg/mL) and sodium benzoate (0.2%). Store at 4°C.
3. Glucose Determination Reagent (GOPOD; high purity). Store dry at 4°C. Long term storage at -20°C.
4. Glucose Reagent Buffer (concentrate). Store at 4°C.
5. Glucose Standard Solution. Store at room temperature.
6. Control Soy-Flour Sample (containing glucose, sucrose and raffinose-series oligosaccharides).

## ENCLOSED ENZYMES:

- A. Invertase** [6 ml, 100 U/ml on sucrose (10 mM, pH 4.5, 40°C)].  
Stabilised solution.

Dilute an aliquot (1.0 mL) to 12 mL with Buffer I [50 mM sodium acetate buffer, (pH 4.5)]. Store frozen between use.

- B.  $\alpha$ -Galactosidase** [2 ml, 500 U/ml on *p*-nitrophenyl  $\alpha$ -D-galactopyranoside (10 mM, pH 4.5, 40°C)].  
Stabilised solution.

Add the entire contents of this vial (2 ml, 1,000 Units) to 21 mL of Buffer I. Then add 2 ml of invertase (100 U/ml) and mix the contents thoroughly by careful inversion. Divide the solution into aliquots of suitable volume and store these at -20°C between use.

## ENCLOSED REAGENTS:

- A. Glucose Determination Reagent (GOPOD)** (for 1 litre).

Reagent concentrations after dissolution in buffer:

Glucose oxidase	> 12,000 U/litre.
Peroxidase	> 650 U/litre.
4-Aminoantipyrine	0.4 mM.

## **B. Glucose Reagent Buffer** (concentrate) (50 ml).

Dilute the entire contents to 1 litre with distilled water and use to dissolve the Glucose Determination Reagent (GOPOD).

Divide this reagent (GOPOD Reagent) into aliquots of desired volume for storage.

Stability:	2-3 months at 4°C
	12 months at -20°C

### **ENCLOSED STANDARD:**

Glucose Standard Solution (100 mg/0.1 ml in 0.2% benzoic acid; i.e. 0.556 mmoles in 0.1 ml).

### **ENCLOSED CONTROL FLOUR:**

Soybean flour containing glucose, sucrose and raffinose-series oligosaccharides (RSO) The concentrations of these in millimoles per 100 grams are shown on the vial label (e.g. Glc = 0.67).

### **BUFFER:**

**Sodium acetate buffer** (50 mM, pH 4.5).

Add glacial acetic acid (2.9 ml) to 900 mL of distilled water. Adjust to pH 4.5 using 1 M sodium hydroxide. Adjust the volume to 1 litre. Store at 4°C. Sodium azide (0.2g) can be added as a preservative.

#### **NOTE:**

**Sodium azide is a toxic chemical and should be treated accordingly. It is added to buffers (after pH adjustment) solely as a preservative. It can be deleted from buffer recipes, but buffers should then be stored at 4°C.**

### **EQUIPMENT (RECOMMENDED):**

1. Glass test tubes (round bottomed; 16 x 120mm and 18 x 150mm).
2. Micro-pipettors, e.g. Gilson Pipetman 200 microlitre and 100 microlitre.
3. Positive displacement pipettor e.g. Eppendorf Multipipette®
  - with 12.5 Combitip® [to dispense 1.0 mL aliquots of invertase (in 50% glycerol)].
  - with 5.0 ml Combitip® (to dispense 0.2 ml aliquots of diluted invertase and invertase/a-galactosidase mixtures).

4. Analytical balance.
5. Spectrophotometer set at 510 nm.
6. Vortex mixer (we recommend the Thermolyne Maxi-Mix II).
7. Thermostated water bath (set at 50.0°C).
8. Boiling water bath (set at 84-88°C).
9. Bench centrifuge (capable of speeds of 1,000g).
10. Stop clock.

## **CONTROLS AND PRECAUTIONS:**

1. The time of incubation with GOPOD reagent is not critical, but should be at least 20 min. The colour formed should be measured within 60 min.
2. With each set of determinations, reagent blanks and glucose controls [0.556 mmoles (i.e. 100 mg) quadruplicate] should be included.
  - a. The reagent blank consists of 0.4 mL of 50 mM sodium acetate buffer (Buffer I) + 3.0 ml GOPOD Reagent.
  - b. The glucose control consists of 0.1 ml of glucose standard solution (100 mg/0.1 ml) + 0.3 mL of Buffer I + 3.0 ml GOPOD Reagent.
3. With each set of determinations a control flour is included.
4. With each new batch of GOPOD Reagent, the time for maximum colour formation with 100 mg (0.556 mmoles) of glucose standard should be checked. This is usually about 15 min.

## **ASSAY PROCEDURE:**

### **Enzyme Inactivation and Sugar Extraction:**

1. Flour sample ( $0.50 \pm 0.01$  g) is accurately weighed into a glass test-tube (18 x 150 mm) and treated with 5 ml ethanol (95% v/v).
2. The tube is incubated at 84-88°C in a deep-fry cooker (containing water) and allowed to reflux for 5 min. (This treatment inactivates endogenous enzymes.)
3. The tube contents are transferred quantitatively to a 50 ml volumetric flask and the volume is adjusted to the mark with sodium acetate buffer (50 mM, pH 4.5) (Buffer I). The sample is allowed to extract over 15 min. and the slurry is thoroughly mixed.

4. An aliquot (approximately 5 ml) of this solution/slurry is transferred to a glass test-tube (16 x 120 mm, suitable for centrifugation at 1,000 g).
5. This solution is treated with 2 ml of chloroform, mixed vigorously on a vortex mixer for 15 sec and is then centrifuged (1,000 g) for 10 min.

**This treatment removes most of the lipids from the aqueous phase. These lipids are extracted into the chloroform, which is the lower phase on centrifugation. Insoluble plant material tends to concentrate between the phases.**

6. The upper aqueous solution (Solution A) is used directly in analyses.

**Assay for Glucose, Sucrose and Raffinose-Series Oligosaccharides:**

1. Aliquots (0.2 mL) of Solution A are treated as follows:
  - 0.2 ml of Soln A + 0.2 ml Buffer I [Glucose] . . . . . A
  - 0.2 ml of Soln A + 0.2 ml invertase [Sucrose + Glucose] . . . . . B
  - 0.2 ml of Soln A + 0.2 ml  $\alpha$ -galactosidase + invertase [Glucose + Sucrose + Raffinose-series oligosaccharides] . . . . . C
2. All solutions are incubated at 50°C for 20 min.
3. 3.0 mL of GOPOD Reagent is added to solutions A, B, and C, and these, as well as the Reagent Blank and the Glucose Controls are incubated with at 50°C for 20 min.
4. The absorbance at 510 nm for each sample is measured against the Reagent Blank:

Absorbances:      DA = GOPOD absorbance for A  
                               DB = GOPOD absorbance for B  
                               DC = GOPOD absorbance for C

**The reagent blank consists of 0.4 mL of 50mM sodium acetate buffer (Buffer A) plus 3.0 mL of GOPOD Reagent. The glucose control consists of 0.1 mL of glucose standard solution (0.556 mmoles/0.1mL (100 mg/0.1 mL) plus 0.3 mL of Buffer I and 3.0mL of GOPOD Reagent.**

## CALCULATIONS:

### Glucose, millimoles/100 grams:

$$\begin{aligned} &= \Delta A \times F \times 250 \times 200 \times \frac{1}{1000} \\ &= \Delta A \times F \times 50 \end{aligned}$$

### Sucrose, millimoles/100 grams:

$$\begin{aligned} &= (\Delta B - \Delta A) \times F \times 250 \times 200 \times \frac{1}{1000} \\ &= (\Delta B - \Delta A) \times F \times 50 \end{aligned}$$

### Raffinose-series oligosaccharides, millimoles/100 grams:

$$\begin{aligned} &= (\Delta C - \Delta B) \times F \times 250 \times 200 \times \frac{1}{1000} \\ &= (\Delta C - \Delta B) \times F \times 50 \end{aligned}$$

### where:

$\frac{\Delta A}{0.2}$  = GOPOD absorbance for 0.2 ml of samples + acetate buffer.

$\frac{\Delta B}{0.2}$  = GOPOD absorbance for 0.2 ml of samples + invertase.

$\frac{\Delta C}{0.2}$  = GOPOD absorbance for 0.2 ml of samples +  $\alpha$ -galactosidase and invertase.

F = a factor to convert from absorbance to mmoles of glucose  
=  $\frac{0.556 \text{ (micromoles of glucose)}}{\text{GOPOD absorbance for 0.556 mmoles of glucose}}$

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

200 = conversion from 0.5 to 100 grams of sample.

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

The concentrations of 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 raffinose-series 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.

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

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

Raffinose-series oligosaccharides (RSO) (g/100 g flour)  
= (RSO)/100 g x MW/1000.

**where:**

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

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

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

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