

Megazyme

GALACTOMANNAN ASSAY PROCEDURE

K-GALM 01/07

(100 Assays per Kit)



INTRODUCTION:

Galactomannans occur in Nature as the reserve polysaccharides in the endosperms of a wide range of legume seeds. These polysaccharides, in partially purified form, find widespread application as thickening and gelling (in the presence of other polysaccharides) agents in the food industry. Partially degraded guar galactomannan is used as a novel dietary fibre component.

Galactomannan is composed of a 1,4- β -linked D-mannan backbone to which single D-galactosyl units are attached to C-6 of some of the D-mannosyl residues. The major difference between galactomannans from different seed species is the ratio of D-galactose to D-mannose. However, some variation in the molecular weight of the galactomannans has also been reported. The D-galactose : D-mannose ratio of galactomannan from different varieties of a given seed species appears to be remarkably constant. In a detailed study of galactomannans purified from approx. 50 samples of carob flour from diverse sources and from a range of carob varieties, it was shown that the D-galactose : D-mannose ratio is constant; i.e.: 22 ± 1 % (w/w) D-galactose and 78 ± 1 % (w/w) D-mannose (McCleary *et al*, 1985). In a parallel study on the galactomannan from guar seed varieties, a D-galactose content of 38 ± 1 % (w/w) and D-mannose content of $62\% \pm 1$ % (w/w) was found. This observation would also appear to hold true for galactomannan from other seeds e.g. fenugreek seed, but in these cases, the studies have not been as comprehensive.

Galactomannans are quantitatively hydrolysed to D-galactose and manno-oligosaccharides by a mixture of α -galactosidase plus β -mannanase (McCleary and Matheson, 1983).

Thus, a procedure has been developed for the measurement of carob galactomannan and guar galactomannan (or in fact, possibly any other galactomannan) based on the measurement of the D-galactose content.

PRINCIPLE:

The method described in this booklet is based on the quantitative hydrolysis of galactomannan to D-galactose and manno-oligosaccharides using a combination of β -mannanase and α -galactosidase (McCleary, 1981). The released D-galactose is then quantitatively determined using a mixture of β -galactose dehydrogenase. Calculation of galactomannan content is based on a prior knowledge of the D-galactose : D-mannose ratio of the galactomannan being analysed.

In broad terms, a sample of test material (approx. 100 mg) is extracted with aqueous ethanol (80 % v/v) to remove galactosyl sucrose oligosaccharides (which, like galactomannan, contain α -linked D-galactose) and is then suspended in acetate buffer and incubated in a boiling water bath to obtain complete hydration of the galactomannan.

The viscous slurry is then cooled to 40°C and treated with β -mannanase to effect depolymerisation and complete solubilisation of the galactomannan along with a dramatic viscosity decrease. Following centrifugation, aliquots of the supernatant solution are treated with a mixture of pure α -galactosidase and pure β -mannanase to effect complete hydrolysis of the galactomannan to D-galactose and manno-oligosaccharides.

This method can be applied the measurement of galactomannan in whole milled seed, milling fractions, pure galactomannan samples or food products containing galactomannan.

The D-galactose : D-mannose contents of some commercially available, or otherwise commonly studied galactomannans are as follows:

Common name	Botanical name	Gal : Man
Cassia	<i>Cassia tora</i>	19 : 81
Carob (locust bean)	<i>Ceratonia siliqua</i>	22 : 78
Guar	<i>Cyamopsis tetragonoloba</i>	38 : 62
Lucerne (alfalfa)	<i>Medicago sativa</i>	48 : 52
Fenugreek	<i>Trigonella foenum-graecum</i>	48 : 52

KIT CONTENTS:

Kits suitable for performing 100 assays are available from Megazyme. The kits contain the full assay method plus:

- Bottle 1:** Tris/HCl buffer (25 mL, 2 M, pH 8.6) plus EDTA (40 mM) and sodium azide (0.02 % w/v) as a preservative (Buffer A). Stable for > 2 years at 4°C.
- Bottle 2:** NAD⁺ (155 mg). Stable for > 5 years at -20°C.
- Bottle 3:** β -Mannanase suspension (*A. niger*; 1.1 mL, 450 U/mL). Stable for > 4 years at 4°C.
- Bottle 4:** α -Galactosidase (guar seed, 150 U/mL) plus β -mannanase (*A. niger*; 50 U/mL) suspension, 2.2 mL. Stable for > 4 years at 4°C.
- Bottle 5:** β -Galactose dehydrogenase (200 U/mL) 2.4 mL. Stable for > 2 years at 4°C.
- Bottle 6:** D-Galactose standard solution (5 mL, 0.4 mg/mL in 0.02 % sodium azide). Stable for > 4 years at 4°C.
- Bottle 7:** Control carob galactomannan flour sample (with galactomannan content as 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 1 l mL of distilled water.
Divide into appropriately sized aliquots and store in polypropylene tubes at -20°C between use and cool during use. Stable for > 2 years at -20°C.
- 3, 4 & 5. Use the contents of bottles 3, 4 and 5 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 > 4 years at 4°C.
6. Use the contents of bottle 6 as supplied.
Stable for > 4 years at 4°C.
7. Use the contents of bottle 7 as supplied.
Stable for > 5 years at room temperature.

SOLUTIONS NOT SUPPLIED:

1. **Sodium acetate buffer (Buffer B)** (100 mM, pH 4.5).
Add 5.8 mL of glacial acetic acid (1.05 g/mL) to 900 mL of distilled water. Adjust the pH to 4.5 by careful addition of 2 M (8 g/100 mL) sodium hydroxide solution. Add 0.2 g of sodium azide. Adjust the volume to 1 litre with distilled water.
Stable for > 2 years at 4°C.
2. **Aqueous ethanol (~ 80 % v/v)**
Add 20 mL of distilled water to 80 mL of ethanol and mix.
Stable for > 5 years at room temperature in a well sealed bottle.

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 Tris/HCl buffer 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. Thermostatted hot-block heater or water bath (set at 40°C).
9. Stop clock.
10. Whatman No.1 (9 cm) filter papers.

METHOD:

1. Mill seed sample or seed fraction (e.g. carob seed) to pass a 0.5 mm screen using a Tecator Cyclotec mill (or equivalent). Commercial galactomannan flours do not require milling.
2. Accurately weigh approx. 100 mg of milled sample into a glass, round-bottomed test tube (16 x 120 mm). Tap the tube to ensure all of the sample falls to the bottom of the tube.
3. Add 5 mL of 80 % (v/v) aqueous ethanol to each tube, stir on a vortex mixer and incubate at 85-90°C for approx. 5 min. Add a further 5 mL of aqueous ethanol, again stir on a vortex mixer and centrifuge at 1,500 g for 10 min. Carefully decant the supernatant solution (discard this solution).

NOTE:

This solution contains galactosyl-sucrose oligosaccharides.

4. Resuspend the pellet in 5 mL of aqueous ethanol as in Step 3, and repeat the operations in Step 3. Again, discard the supernatant after centrifugation, and allow excess liquid to drain from the tubes by inverting on absorbent paper.
5. Resuspend the pellet in 8 mL of 100 mM sodium acetate buffer (pH 4.5) (Buffer B), and stir vigorously on a test-tube stirrer to effect complete dispersion. Immediately place the tube in a boiling water bath. After 30 seconds, remove the tube and stir it vigorously on a vortex mixer. Return the tube to the boiling water bath for a further 30 seconds, and again remove it and stir on a vortex mixer. Return the tube to the boiling water bath for 4 min. Remove the tube, stir it vigorously on a vortex mixer and place it in a water bath at 40°C.

NOTE:

These operations are performed to ensure thorough suspension and even hydration of the polysaccharide. Formation of "lumps" of partially hydrated and swollen galactomannan will hinder the ability of β -mannanase to penetrate and depolymerise the polymer

6. After 5 min at 40°C, add 10 µL of β-mannanase suspension (450 U/mL) (suspension 3) and stir the tube vigorously on a vortex mixer for 30 seconds. Incubate the tubes at 40°C for 60 min with intermittent vigorous stirring (about 2-3 times on a vortex mixer).
7. Quantitatively transfer the solution to a 25 mL volumetric flask using a water wash bottle, and adjust to volume with distilled water. Mix the flask contents thoroughly.
8. Filter an aliquot of the solution (Whatman No. 1 filter paper) or centrifuge at 1,500 g for 10 min.
9. Use this solution in the assay described below.

NOTE:

At this stage, the solution is either analysed directly, or diluted based on the predicted galactomannan content as well as the D-galactose/D-mannose ratio of the galactomannan e.g. for a sample of carob flour containing approx 30 % (w/w) galactomannan with a D-galactose/D-mannose ratio of 22 : 78, the solution is analysed directly; however for a guar flour sample with a similar galactomannan content, but with a D-galactose/D-mannose ratio of 38 : 62, the solution is diluted 2-fold with water before analysis.

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.
Final volume: 2.94 mL
Sample solution: 4-80 µg of D-galactose
Read against air (without a cuvette in the light path) or against water.

Pipette into cuvettes	Blank*	Sample
sample	0.20 mL	0.20 mL
buffer B (acetate buffer)	0.10 mL	0.10 mL
solution 4 (α-galactos./β-mannanase)	-	0.02 mL
Mix**, cap the cuvette and incubate at 40°C for 60 min. Add:		
distilled water	2.32 mL	2.30 mL
solution 1 (Tris/HCl 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 5 (β-GalDH)	0.02 mL	0.02 mL
Mix**, read the absorbances of the solutions (A ₂) at the end of the reaction. (approx. 30 min). [Reaction will take 60 min at 25°C.]		

* perform a single blank assay with each set of determinations.

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

CALCULATIONS:

Determine the absorbance difference (A₂-A₁) for both blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining

$\Delta A_{D\text{-galactose}}$

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

$$c = \frac{V \times MW}{\epsilon \times d \times v} \times \Delta A_{D\text{-galactose}} \quad [\text{g/L}]$$

where:

- V = final volume [mL]
 MW = molecular weight of the substance to be 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 D-galactose:

$$c = \frac{2.94 \times 180.16}{6300 \times 1 \times 0.2} \times \Delta A_{D\text{-galactose}} \quad [\text{g/L}]$$

$$= 0.4204 \times \Delta A_{D\text{-galactose}}$$

Thus, for galactomannan:

$$c = 0.4204 \times \Delta A_{D\text{-galactose}} \times 100/G \times 162/180 \quad [\text{g/L}]$$

$$= 37.836 \times \Delta A_{D\text{-galactose}} \times 1/G$$

where:

- 100/G = conversion from D-galactose as determined to galactomannan concentration.
 G = galactose content of the galactomannan as a percentage e.g. 22 for carob galactomannan
 38 for guar galactomannan
 48 for fenugreek and alfalfa galactomannans.
 162/180 = Adjustment from free D-galactose to anhydro-D-galactose (as occurs in galactomannan).

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 galactomannan

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

Thus, for the example detailed on pages 4 and 5 where 100 mg of sample was extracted and adjusted to a final volume of 25 mL, the weight of sample as g/L is 0.1 g/25 mL or 4 g/L.

Thus, the content of galactomannan

$$= 37.836 \times \Delta A_{D\text{-galactose}} \times 1/G \times 1/4 \times 100 \quad [\text{g}/100 \text{ g}]$$

For carob galactomannan [with 22 % (w/w) D-galactose]

$$= 37.836 \times \Delta A_{D\text{-galactose}} \times 1/22 \times 1/4 \times 100 \quad [\text{g}/100 \text{ g}]$$

$$= 43.00 \times \Delta A_{D\text{-galactose}} \quad [\text{g}/100 \text{ g}]$$

NOTE: The D-galactose 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 D-galactose is determined directly from the extinction coefficient of NADH (page 7).

REFERENCES:

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