Raffinose

UV-method

for the determination of raffinose in foodstuffs and other materials

Cat. No. 10 428 167 035

Test-Combination for 32 determinations
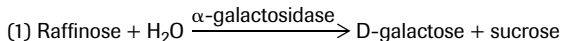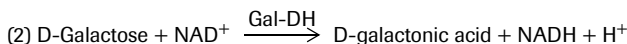
Principle (Ref. 1)

Raffinose is hydrolyzed at pH 4.5 to D-galactose and sucrose in the presence of the enzyme α-galactosidase (1).

(1) Raffinose + H_2O $\xrightarrow{\text{α-galactosidase}}$ D-galactose + sucrose

D-Galactose is oxidized by nicotinamide-adenine dinucleotide (NAD) to D-galactonic acid in the presence of the enzyme galactose dehydrogenase (Gal-DH) (2).

(2) D-Galactose + NAD^+ $\xrightarrow{\text{Gal-DH}}$ D-galactonic acid + NADH + H^+

The amount of NADH formed in the above reaction is stoichiometric to the amount of raffinose. The increase in NADH is determined by means of its light absorption at 334, 340 or 365 nm.

This principle allows the determination of raffinose besides sucrose and D-glucose.

The Test-Combination contains

1. Bottle 1 with approx. 320 mg lyophilizate, consisting of:
   citrate buffer, pH approx. 4.5; NAD, approx. 28 mg
2. Bottle 2 with approx. 1.6 ml suspension α-galactosidase, approx. 36 U
3. Bottle 3 with approx. 34 ml solution, consisting of:
   potassium diphosphate buffer, pH approx. 8.6
4. Bottle 4 with approx. 1.6 ml suspension β-galactose dehydrogenase, approx. 30 U

Preparation of solutions

1. Dissolve contents of bottle 1 with 8.0 ml redist. water.
2. Use suspension of bottle 2 undiluted.
3. Use solution of bottle 3 undiluted.
4. Use suspension of bottle 4 undiluted.

Stability of reagents

The contents of bottle 1 are stable at 2-8°C (see pack label).
   Solution 1 is stable for 3 months at 2-8°C, for 5 months at −15 to −25°C. Bring solution 1 to 20-25°C before use.
The contents of bottles 2, 3 and 4 are stable at 2-8°C (see pack label).
   Bring solution 3 to 20-25°C before use.

Procedure

Wavelength[1]:   340 nm, Hg 365 nm or Hg 334 nm
Glass cuvette[2]: 1.00 cm light path
Temperature: 20-25°C
Final volume: 3.400 ml
Read against air (without a cuvette in the light path) or against water
Sample solution: 3-250 µg raffinose/assay[3] (in 0.100-0.500 ml sample volume)

If the sample solution contains free D-galactose, this D-galactose is to be determined in a separate assay **without suspension 2** (D-galactose sample).

Pipette into cuvettes	Blank raffinose sample	Raffinose sample	Blank D-galactose-sample	D-Galac-tose-sample
solution 1*	0.200 ml	0.200 ml	0.200 ml	0.200 ml
sample solution**	-	0.100 ml	-	0.100 ml
suspension 2	0.050 ml	0.050 ml	-	-
redist. water	0.100 ml	-	0.150 ml	0.050 ml
Mix*, and incubate for 15 min at 20-25°C. Add:				
solution 3	1.000 ml	1.000 ml	1.000 ml	1.000 ml
redist. water	2.000 ml	2.000 ml	2.000 ml	2.000 ml
Mix***, read absorbances of the solutions after approx. 2 min (A_1). Start reaction by addition of:				
suspension 4	0.050 ml	0.050 ml	0.050 ml	0.050 ml
Mix***, wait until the reaction has stopped (approx. 20 min) and read absorbances of the solutions (A_2).				

* Pipette solution 1, suspension 2, redist. water and the sample solution, each, onto the bottom of the cuvette and mix by gentle swirling. When using a plastic spatula, remove it from the cuvette only directly before measuring absorbance A_1.

** Rinse the enzyme pipette or the pipette tip of the piston pipette with sample solution before dispensing the sample solution.

*** For example, with a plastic spatula or by gentle swirling after closing the cuvette with Parafilm (trademark of the American Can Company, Greenwich, Ct., USA)

Determine the absorbance differences (A_2-A_1) for blanks and samples. Subtract the absorbance differences of the blanks from the absorbance differences of the corresponding samples.

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

It follows $\Delta A_{D-galactose}$ (from "D-galactose sample") and

$\Delta A_{raffinose + D-galactose}$ (from "raffinose sample").

The difference of these values stands for $\Delta A_{raffinose}$.

The measured absorbance differences should, as a rule, be at least 0.100 absorbance units to achieve sufficiently precise results (see "Instructions of performance of assay" and "Sensitivity and detection limit", pt. 3).

Calculation

According to the general equation for calculating the concentration:

$$c = \frac{V \times MW}{\varepsilon \times d \times v \times 1000} \times \Delta A \text{ [g/l]}$$

V   = final volume [ml]

v   = sample volume [ml]

MW = molecular weight of the substance to be assayed [g/mol]

d   = light path [cm]

ε   = extinction coefficient of NADH at:
       340 nm = 6.3   [l × $mmol^{-1}$ × cm^{-1}]
       Hg 365 nm = 3.4   [l × $mmol^{-1}$ × cm^{-1}]
       Hg 334 nm = 6.18   [l × $mmol^{-1}$ × cm^{-1}]

It follows for raffinose:

$$c = \frac{3.400 \times 504.5}{\varepsilon \times 1.00 \times 0.100 \times 1000} \times \Delta A_{raffinose} = \frac{17.15}{\varepsilon} \times \Delta A_{raffinose} \text{ [g raffinose/l sample solution]}$$

for D-galactose:

$$c = \frac{3.400 \times 180.16}{\varepsilon \times 1.00 \times 0.100 \times 1000} \times \Delta A_{D-galactose} = \frac{6.125}{\varepsilon} \times \Delta A_{D-galactose} \text{ [g D-galactose/l sample solution]}$$

If the sample has been diluted during preparation, the result must be multiplied by the dilution factor F.

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1 The absorption maximum of NADH is at 340 nm. On spectrophotometers, measurements are taken at the absorption maximum; if spectralline photometers equipped with a mercury vapor lamp are used, measurements are taken at a wavelength of 365 nm **or** 334 nm.
2 If desired, disposable cuvettes may be used instead of glass cuvettes.
3 See instructions for performance of assay

When analyzing solid and semi-solid samples which are weighed out for sample preparation, the result is calculated from the amount weighed:

$$\text{Content}_{\text{raffinose}} = \frac{c_{\text{raffinose}} \ [\text{g/l sample solution}]}{\text{weight}_{\text{sample}} \ \text{in g/l sample solution}} \times 100 \ [\text{g/100 g}]$$

$$\text{Content}_{\text{D-galactose}} = \frac{c_{\text{D-galactose}} \ [\text{g/l sample solution}]}{\text{weight}_{\text{sample}} \ \text{in g/l sample solution}} \times 100 \ [\text{g/100 g}]$$

1. Instructions for performance of assay

The amout of raffinose present in the assay has to be between 5 μg and 250 μg (measurement at 365 nm) or 3 μg and 130 μg (measurement at 340, 334 nm), respectively. In order to get a sufficient absorbance difference, the sample solution is diluted to yield a raffinose concentration between 0.5 and 2.5 g/l or 0.25 and 1.3 g/l, respectively.

Dilution table

Estimated amount of raffinose per liter		Dilution with water	Dilution factor F
measurement at			
340 or 334 nm	365 nm		
< 1.3 g	< 2.5 g	-	1
1.3-13 g	2.5-25.0 g	1 + 9	10
13-130 g	25.0-250 g	1 + 99	100

If the measured absorbance difference (ΔA) is too low (e.g. < 0.100), the sample solution should be prepared again (weigh out more sample or dilute less strongly) or the sample volume to be pipetted into the cuvette can be increased up to 0.500 ml. Adjust sample solution to pH 4.2-4.8, if necessary. The volume of water added after incubation must then be reduced so as to obtain the same final volume in the assays for sample and blank. The new sample volume v must be taken into account in the calculation.

2. Specificity

Besides raffinose, α-galactosidase also hydrolyzes other α-galactosides, e.g. galactinol, melibiose, and stachyose. Substances with β-galactoside linkages, such as lactose are not hydrolyzed and do not interfere with the assay, even when present in high concentrations.

Apart from D-galactose, Gal-DH also oxidizes L-arabinose. Small amounts of L-arabinose only occur in foodstuffs, if it has been released from its natural glycosidic linkage due to chemical and enzymatic influences.

In the analysis of commercial raffinose, results of approx. 99 % have to be expected.

3. Sensitivity and detection limit (Ref. 1.2)

The smallest differentiating absorbance for the procedure is 0.005 absorbance units. This corresponds to a maximum sample volume v = 0.500 ml and measurement at 340 nm of a raffinose concentration of approx. 3 mg/l, resp. 1 mg D-galactose/l sample solution (if v = 0.100 ml, this corresponds to 14 mg raffinose/l, resp. 5 mg D-galactose/l sample solution).

The detection limit of 5 mg raffinose/l, resp. 2 mg D-galactose/l is derived from the absorbance difference of 0.010 (as measured at 340 nm) and a maximum sample volume v = 0.500 ml.

4. Linearity

Linearity of the determination exists from approx. 3 μg raffinose + D-galactose/assay (5 mg raffinose + D-galactose/l sample solution; sample volume v = 0.500 ml) to 250 μg raffinose + D-galactose/assay (2.5 g raffinose + D-galactose/l sample solution; sample volume v = 0.100 ml).

5. Precision

In a double determination of raffinose using one sample solution, a difference of 0.010 to 0.015 absorbance units may occur in the presence of D-galactose in the sample. With a sample volume of v = 0.100 ml and measurement at 340 nm, this corresponds to a raffinose concentration of approx. 25-40 mg/l. (If the sample is diluted during sample preparation, the result has to be multiplied by the dilution factor F. If the sample is weighed in for sample preparation, e.g. using 1 g sample/100 ml = 10 g/l, a difference of 0.25-0.4 g/100 g can be expected.)

In a double determination of D-galactose using one sample solution, a difference of 0.005 to 0.010 absorbance units may occur. With a sample volume of v = 0.100 ml and measurement at 340 nm, this corresponds to a D-galactose concentration of approx. 5-10 mg/l. (If the sample is diluted during sample preparation, the result has to be multiplied by the dilution

factor F. If the sample is weighed in for sample preparation, e.g. using 1 g sample/100 ml = 10 g/l, a difference of 0.05-0.1 g/100 g can be expected.)

The following data for the determination of raffinose and D-galactose have been published in the literature:

x = 39.8 mg/l			CV = 2.6 %	(Ref. 1.1)
x = 130 μg/assay	raffinose solution		CV = 0.26 %	
x = 220 μg/assay	raffinose solution		CV = 0.43 %	(Ref. 1.2)
x = 25 μg/assay	D-galactose solution		CV = 0.95 %	
x = 50 μg/assay	D-galactose solution		CV = 0.64 %	(Ref. 1.4)

Milk and milk products:
D-galactose: r = 0.10 × (content$_{\text{D-galactose}}$ in g/100 g) g/100 g
R = 0.12 × (content$_{\text{D-galactose}}$ in g/100 g) g/100 g (Ref. 2.1)

6. Recognizing interference during the assay procedure

6.1 If the conversion of D-galactose has been completed according to the time given under "Procedure", it can be concluded in general that no interference has occurred.

6.2 On completion of the reaction, the determination can be restarted by adding D-galactose (qualitative or quantitative): if the absorbance is altered subsequent to the addition of the standard material, this is also an indication that no interference has occurred.

The reaction cannot be restarted with raffinose as, subsequent to altering the reaction conditions from pH 4.5 to pH 8.6 ("change of the buffer"), raffinose is no longer cleaved.

6.3 Operator error or interference of the determination through the presence of substances contained in the sample can be recognized by carrying out a double determination using two different sample volumes (e.g. 0.100 ml and 0.200 ml): the measured differences in absorbance should be proportional to the sample volumes used.

When analyzing solid samples, it is recommended that different quantities (e.g. 1 g and 2 g) be weighed into 100 ml volumetric flasks. The absorbance differences measured and the weights of sample used should be proportional for identical sample volumes.

The use of "single" and "double" sample volumes in double determinations is the simplest method of carrying out an assay control in the determination of raffinose.

6.4 Possible interference caused by substances contained in the sample can be recognized by using an internal standard as a control: in addition to the sample, blank and standard determinations, a further determination should be carried out with sample **and** assay control solution in the same assay. The recovery can then be calculated from the absorbance differences measured.

6.5 Possible losses during the determination can be recognized by carrying out recovery tests: the sample should be prepared and analyzed with and without added standard material. The additive should be recovered quantitatively within the error range of the method.

7. Reagent hazard

The reagents used in the determination of raffinose are not hazardous materials in the sense of the Hazardous Substances Regulations, the Chemicals Law or EC Regulation 67/548/EEC and subsequent alteration, supplementation and adaptation guidelines. However, the general safety measures that apply to all chemical substances should be adhered to.

After use, the reagents can be disposed of with laboratory waste, but local regulations must always be observed. Packaging material can be disposed of in waste destined for recycling.

8. General information on sample preparation

In carrying out the assay:
Use **clear, colorless and practically neutral liquid samples** directly, or after dilution according to the dilution table, and of a volume up to 0.500 ml;
Filter **turbid solutions** (use membrane filter if necessary);
Degas **samples containing carbon dioxide** (e.g. by filtration);
Crush or homogenize **solid or semi-solid samples,** extract with water or dissolve in water and filter if necessary; resp. remove turbidities or dyestuffs by Carrez clarification;
Deproteinize **samples containing protein** with Carrez reagents;
Extract **samples containing fat** with hot water (extraction temperature should be above the melting point of the fat involved). Cool to allow the fat to separate, make up to the mark, place the volumetric flask in an ice bath for 15 min and filter; alternatively clarify with Carrez-solutions after the extraction with hot water;
Break **emulsions** with Carrez-solutions.

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Carrez clarification:

Pipette the liquid sample into a 100 ml volumetric flask whick contains approx. 60 ml redist. water, or weigh sufficient quantity of the sample into a 100 ml volumetric flask and add approx. 60 ml redist. water. Subsequently, carefully add 5 ml Carrez-I-solution (potassium hexacyanoferrate(II) (ferro-cyanide), 85 mM = 3.60 g $K_4[Fe(CN)_6] \times 3\ H_2O$/100 ml) and 5 ml Carrez-II-solution (zinc sulfate, 250 mM = 7.20 g $ZnSO_4 \times 7\ H_2O$/100 ml). Adjust to pH 7.5-8.5 with sodium hydroxide (0.1 M; e.g. 10 ml). Mix after each addition. Fill the volumetric flask to the mark, mix and filter.

9. Application examples

Determination of raffinose in molasses, syrup and other products of sugar manufacture

Before starting the assay, precipitate quantitatively any galactinol which may be present in the sample by addition of basic lead acetate. For performance of the sample preparation see Ref. 2.3. In the analysis of e.g. molasses, a part of raffinose is adsorbed to the precipitate (see Ref. 2.4).

Determination of raffinose in soybean flour and flour of cereals

Accurately weigh approx. 2-5 g of the ground and homogenized sample into a 100 ml volumetric flask and heat with approx. 50 ml water for 30 min in a water-bath at 60°C. Stir occasionally (alternatively a magnetic stirrer can be used). For clarification add 5 ml Carrez-I-solution (3.60 g potassium hexa-cyanoferrate(II), $K_4[Fe(CN)_6] \times 3\ H_2O$/100 ml), 5 ml Carrez-II-solution (7.20 g zinc sulfate, $ZnSO_4 \times 7\ H_2O$/100 ml) and 10 ml sodium hydroxide (0.1 M). After each addition mix or stir, respectively, cool to 20-25°C, fill up to the mark with water, and filter. Use the clear, possibly slightly turbid solution diluted, if necessary, for the assay.

Soybean flour contains up to 10 % raffinose; flours of cereals contain up to 0.3 % raffinose.

10. Further applications

The method may also be used when analyzing biological samples.

Determination of raffinose in fermentation samples and cell culture media

Place the sample (after centrifugation, if necessary) in a water-bath at 80°C for 15 min to stop enzymatic reactions. Centrifuge and use the supernatant (diluted according to the dilution table, if necessary) for the assay. Alternatively, deproteinization can be carried out with Carrez-solutions. See the above-mentioned examples.

Homogenize gelatinous agar media with water and treat further as described.

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Raffinose assay control solution

The assay control solution serves as a control for the enzymatic determina-tion of raffinose in foodstuffs and other materials.

Reagents

Raffinose penta-hydrate (M = 594.52 g/mol; contains 84.9% raffinose), AR grade

Preparation of the assay control solution

Accurately weigh approx. 150 mg raffinose penta-hydrate to the nearest 0.1 mg into a 100 ml volumetric flask, fill up to the mark with redist. water, and mix thoroughly (corresponds to approx. 1.3 g raffinose/l).

Prepare assay control solution freshly before use. The assay control solution may be frozen in portions.

Application:

1. Addition of raffinose assay control solution to the assay mixture:
Instead of sample solution the assay control solution is used for the assay. (For the calculation of results use the molecular weight of the assay control material weighed in for analysis.)
The measurement of the assay control solution is not necessary for calcula-ting the results.

2. Restart of the reaction, quantitatively:
A restart of the reaction with assay control solution after completion of the reaction cannot be performed as under the assay conditions (potassium diphosphate buffer, pH 8.6) raffinose is not cleaved. If necessary, restart with 0.050 ml of a D-galactose solution (0.5 g/l).
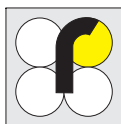
3. Internal standard:
The assay control solution can be used as an internal standard in order to check the determination for correct performance (gross errors) and to see whether the sample solution is free from interfering substances:

Pipette into cuvettes	Blank raffinose sample	Raffinose sample	Raffinose standard	Raffinose sample + raffinose standard
solution 1	0.200 ml	0.200 ml	0.200 ml	0.200 ml
suspension 2	0.050 ml	0.050 ml	0.050 ml	0.050 ml
sample solution	-	0.100 ml	-	0.050 ml
assay control sln.	-	-	0.100 ml	0.050 ml
redist. water	0.100 ml	-	-	-
Mix. Continue as described in the pipetting scheme under "Procedure". Follow the instructions given under "Instructions for performance of assay" and the footnotes.				

The recovery of the standard is calculated according to the following formula:

$$\text{recovery} = \frac{2 \times \Delta A_{\text{sample + standard}} - \Delta A_{\text{sample}}}{\Delta A_{\text{standard}}} \times 100\ [\%]$$