Raffinose

H. C. Siegfried de Whalley

Methods for the determination of raffinose are mainly used with sugar beet, beet juice, crude beet sugar and less pure syrups including sugar beet molasses. Their application to extracts of other plant material occasionally requires slight modifications, but on the whole the principle remains the same.

Principle

Raffinose (the trisaccharide of glucose, galactose and fructose) is hydrolysed by invertase to fructose and the disaccharide, melibiose. Melibiase hydrolyses melibiose to glucose and galactose. The optical rotation and the ability to reduce Fehling's solution changes during the hydrolysis:

(1)	Raffinose·5 H_2O	$\xrightarrow{\text{invertase}}$	fructose + melibiose	
$[\alpha]_D$:	+105.0		−92.0	+143.0
Reduction of Fehling's solution (invert sugar = 1.0):	—		0.97	0.60

(2)	Melibiose	$\xrightarrow{\text{melibiase}}$	glucose + galactose	
$[\alpha]_D$:	+143.0		+52.5	+81.0
Reduction of Fehling's solution (invert sugar = 1.0):	0.60		1.03	0.97

Consequently, raffinose can be determined by two different methods:

A. by means of the change in optical rotation or

B. by means of the change in the reducing value, for example, with Fehling's solution.

Sugar beet contain large amounts of sucrose together with smaller amounts of glucose and fructose. Plant extracts may contain other sugars which can be attacked by hydrolases. It is therefore expedient to identify the compounds contained in the samples and the reaction products by paper chromatography[1] before evaluating the results obtained with methods A or B. After preliminary separation by paper chromatography the raffinose spots can be extracted with water and hydrolysed enzymatically.

The enzymatic determination of the glucose (see p. 117) formed after the action of melibiase on melibiose is hardly used at present.

A. Determination of the Optical Rotation

This method is recognized in the USA by A.O.A.C.[2] and internationally by the I.C.U.M.S.A.[3]. The rotation before the hydrolysis (I), after the action of invertase (II) and after the action of melibiase (III) is measured.

---

[1] Ref.[3]; *H. C. S. de Whalley, N. Albon* and *D. Gross*, Analyst *76*, 287 [1951].

[2] Official Methods of Analysis of the Association of Official Agricultural Analysts. 8th ed. Washington, D. C., USA, 1955, p. 542.

[3] I.C.U.M.S.A., International Commission for Uniform Methods of Sugar Analysis: Proc. 12tt, Session, Washington (1958). I.C.U.M.S.A., Tate and Lyle Research Laboratories, Keston, Kenh England, p. 93.

Reagents and Solutions

1. Basic lead acetate*) (sp. gr. 1.25)
   Dissolve 9.5 to 10.5 g. lead acetate in distilled water and make up to 100 ml. The amount of salt to be dissolved is governed by the lead content.
2. Ammonium dihydrogen phosphate, $NH_4H_2PO_4$
3. Invertase**)
4. Invertase-melibiase enzyme mixture***)
   preparation, see Appendix, p. 98.

Purity of the enzyme preparations

The enzyme preparations must be checked to see whether they have any optical rotation. If so, this must be allowed for in the calculations. The invertase preparation must be completely free from melibiase. To test for this, incubate raffinose with the enzyme and examine the products by paper chromatography[1]. There should be no galactose spot.

Stability of the solutions

The lead acetate solution should be protected from atmospheric CO_2. The invertase solution is stable for a year at 5° C.

Procedure

Preliminary treatment of the sample

Dissolve the sample, for example, *ca.* 30 g. sugar beet molasses, in a little distilled water. Add 10 ml. basic lead acetate solution (reagent 1), shake thoroughly, dilute with distilled water to 250 ml. and filter or centrifuge. Add 0.6 g. $NH_4H_2PO_4$ to the filtrate or supernatant, stir very thoroughly and filter or centrifuge. The filtrate or supernatant is designated as solution (a) in the following.

Enzymatic reaction and measurements

Pipette into three 100 ml. volumetric flasks:

I	II	III
50 ml. solution (a)	50 ml. solution (a) 0.5 ml. invertase solution (reagent 3)	50 ml. solution (a) 0.2 g. enzyme mixture (reagent 4)

Allow to stand overnight at room temperature. When the hydrolysis is complete [no change in the rotation of (II) and (III) or on paper chromatography[1], no raffinose in (II) or melibiose

*) For comparative studies on sugar beet products, preparations which fulfil the specifications of the I.C.U.M.S.A.[4] should be used. Therefore the dry, basic lead acetate should contain not less than 75% PbO and not more than 1.5% water.

**) Purified, soluble product from Distillers Co. Ltd., London, England, obtainable from Sugar Manufacturers Supply Co., c/o International Sugar Journal, Central Chambers, The Broadway, London W. 5. It can also be prepared according to[5].

***) The invertase-melibiase enzyme mixture is obtainable already prepared from Bios Laboratories, Inc., 17 West 60th Street, New York 13, N. Y., USA.

[4] I.C.U.M.S.A.: Proc. 10th Session (1949), p. 31; 11th Session (1954), p. 52; 12th Session (1958), p. 45.

[5] Polarimetry, Saccharimetry and the Sugars. Circular C 440. National Bureau of Standards, Washington, D.C., U.S.A., p. 146; *A. Bertho* and *W. Grassmann:* Laboratory Methods of Biochemistry. Macmillan, London 1938.

in (III)] dilute all three solutions to 100 ml. with distilled water and determine the rotations. Wavelength: sodium D line (5892.5 Å); temperature 20°C; 1 dm. tube.

Calculations

On hydrolysis 1 g. melibiose gives 0.527 g. glucose and galactose. The corresponding specific rotations $[\alpha]_D^{20}$ are:

for melibiose: +143°
for glucose: +52.5°
for galactose: +81°

During the hydrolysis of melibiose the specific rotation decreases from +143° to $0.527 \times 52.5 + 0.527 \times 81 = +70.4°$, *i.e.* by 72.6°. Accordingly, with 1 g. melibiose/100 ml. and a 1 dm. polarimeter tube, the rotation decreases by $72.6 \times \frac{1}{100} = 0.726°$.

That means that a difference of 0.726 in the optical rotation of solutions II and III corresponds to 1 g. melibiose in solution II and 1.474 g. raffinose or 1.737 g. raffinose pentahydrate in solution I. Therefore

$$\frac{\alpha_{II} - \alpha_{III}}{0.76} \times 1.474 = \text{g. raffinose/solution I}$$

or

$$\frac{\alpha_{II} - \alpha_{III}}{0.726} \times 1.737 = \text{g. raffinose pentahydrate/solution I}$$

In the sugar industry the rotation is measured according to the International Sugar Scale[6] in °S. 1° rotation of the Na-D-line = 2.88850°S. This relation is obtained as follows: 26 g. sucrose/100 ml. water in a 2 dm. tube rotate the Na-D-line by 34.62°. This value is arbitrarily set to equal 100°S. If the rotations of solutions II and III are measured in °S (with a 2 dm. tube), then the following calculation applies: with 1 g. melibiose/100 ml. and a 2 dm. tube the rotation decreases on hydrolysis by

$$72.6 \times \frac{1}{100} \times 2 = 1.452° = 4.18°S$$

and therefore:

$$\frac{°S_{II} - °S_{III}}{4.18} \times 1.474 = \text{g. raffinose/solution I}$$

or

$$\frac{°S_{II} - °S_{III}}{4.18} \times 1.737 = \text{g. raffinose pentahydrate/solution I}$$

Example

Sugar beet molasses (32.5 g.) was analysed. The measurements were made at 20°C with a 1 dm. tube:

for solution II $\alpha = -0.687°$
for solution III $\alpha = -0.744°$

Therefore:

$$\alpha_{II} - \alpha_{III} = (-0.687) - (-0.744) = 0.744 - 0.687 = 0.057$$

$$\frac{\alpha_{II} - \alpha_{III}}{0.726} \times 1.474 = \frac{0.057}{0.726} \times 1.474 = 0.116 \text{ g. raffinose/solution I}$$

or

$$\frac{\alpha_{II} - \alpha_{III}}{0.726} \times 1.737 = \frac{0.057}{0.726} \times 1.737 = 0.136 \text{ g. raffinose pentahydrate/solution I}$$

[6] Ref. [2], p. 536.

If the optical rotation is measured in °S with a 2 dm. tube, then:

for solution II $\alpha = -3.97$°S

for solution III $\alpha = -4.30$°S

Therefore

$$°S_{II} - °S_{III} = (-3.97) - (-4.30) = 4.30 - 3.97 = 0.33$$

$$\frac{°S_{II} - °S_{III}}{4.18} \times 1.474 = \frac{0.33}{4.18} \times 1.474 = 0.116 \text{ g. raffinose/solution I}$$

or

$$\frac{°S_{II} - °S_{III}}{4.18} \times 1.737 = \frac{0.33}{4.18} \times 1.737 = 0.136 \text{ g. raffinose pentahydrate/solution I}$$

B. Determination of the Reducing Value

Analogous to method A the reducing value is measured before and after the action of invertase and invertase + melibiase. In this case it is not necessary to carry out the preliminary lead precipitation (for the removal of coloured compounds which interfere with the measurement of optical rotation). The reducing value is determined titrimetrically with Fehling's solution.

Reagents and Solutions

1. Fehling's solution[7]
   a) Dissolve 69.28 g. $CuSO_4 \cdot 5\ H_2O$ in distilled water and make up to 1000 ml.
   b) Dissolve 346 g. potassium-sodium tartrate (Rochelle salt, $KNaC_4H_4O_6 \cdot 4\ H_2O$) and 100 g. NaOH in distilled water and make up to 1000 ml. Just before use mix equal volumes of solutions a) and b).
2. Invertase (see p. 94)
3. Invertase-melibiase enzyme mixture (see p. 94).
4.—8. Standard preparations of invert sugar (glucose + fructose, 1:1), glucose, fructose, melibiose and galactose. All as aqueous solutions (0.25% w/v).

Stability of the solutions

All the solutions are stable practically indefinitely.

Procedure

Preliminary treatment of the sample

Dissolve the sample, for example, *ca.* 25 g. sugar beet molasses, in a little distilled water, make up to volume in a 250 ml. volumetric flask, mix thoroughly and filter. The filtrate = solution (a).

Standardization of the Fehling's solution

Before the assay of the samples it is necessary to determine the relative reducing values of the sugars which occur in the assay mixture with Fehling's solution. The values depend on the assay conditions.

Titrate 10 ml. portions of the Fehling's solution in 400 ml. flasks with aqueous solutions of glucose, fructose, melibiose and galactose (0.25% w/v). According to *Lane* and *Eynon*[7] 10 ml. Fehling's solution requires 20.4 ml. invert sugar solution (0.25% w/v).

---

[7] *J. H. Lane* and *L. Eynon*, J. Soc. chem. Ind. *42*, 32 T, 143 T, 463 T [1923]; *44*, 150 T [1925]; *46*, 434 T [1927]; *50*, 85 T [1931].

Therefore: $\frac{20.4}{\text{ml. sugar solution required}}$ = relative reducing value of the sugar.

Generally, the values are as follows (compared to invert sugar = 1.0): glucose 1.03, fructose 0.97, melibiose 0.60 and galactose 0.97.

Assay

I. Titrate 10 ml. Fehling's solution (or more according to the invert sugar content of the sample) in a 400 ml. flask with solution (a). Titre: x ml.

II. To 50 ml. solution (a) in a 1000 ml. volumetric flask, add 4 drops acetic acid and 0.5 ml. invertase. Incubate for 40 min. at 50°C and dilute to 1000 ml. with distilled water. Titrate 25 ml. Fehling's solution in a 400 ml. flask with this solution. Titre: y ml.

III. Treat 50 ml. solution (a) like II, but add 200 mg. invertase-melibiase enzyme mixture (reagent 3) instead of the invertase solution. Titrate 25 ml. Fehling's solution with this solution. Titre: z ml.

Calculations

The raffinose content of solution (a) can be calculated from the ml. of mixture II and III required to titrate the Fehling's solution. For this purpose the Tables of *Lane* and *Eynon*[7] are used. If these are not available, the result can be obtained approximately from the following formula:

$$489.9\left(\frac{1}{z}-\frac{1}{y}\right) = \text{g. raffinose pentahydrate/50 ml. solution (a).}$$

This formula is obtained as follows: the hydrolysis of 342 g. melibiose yields 180 g. glucose + 180 g. galactose. The reducing power of 342 g. melibiose is equivalent to $342 \times 0.6 = 205.2$ g. invert sugar. The reducing power of 180 g. glucose + 180 g. galactose is equivalent to $180 \times 1.03 + 180 \times 0.97 = 360$ g. invert sugar. The reducing value of mixture II and mixture III after enzymatic hydrolysis must differ by $360 - 205.2 = 154.8$ g. invert sugar, if mixture II contains 342 g. melibiose/1000 ml. With 1 g. melibiose/1000 ml. mixture II the reducing value differs by $\frac{154.8}{342} = 0.452$ g. invert sugar.

According to *Lane* and *Eynon*[7] 20.4 ml. of a 0.25% invert sugar solution are required to titrate 10 ml. Fehling's solution. Therefore 10 ml. Fehling's solution is equivalent to 0.051 g. invert sugar, and 25 ml. Fehling's solution is equivalent to 0.1275 g. invert sugar.

If y ml. of mixture II are required per 25 ml. Fehling's solution, this corresponds to a reducing value of

$$\frac{0.1275}{y} \times 1000 \text{ g. invert sugar/1000 ml. mixture II.}$$

If z ml. of mixture III are required per 25 ml. Fehling's solution this corresponds to a reducing value of

$$\frac{0.1275}{z} \times 1000 \text{ g. invert sugar/1000 ml. mixture III.}$$

From this it follows that:

$$\frac{0.1275 \times 1000}{0.452}\left(\frac{1}{z}-\frac{1}{y}\right) = \text{g. melibiose/1000 ml. mixture II}$$

or

$$\frac{0.1275 \times 1000 \times 594}{0.452 \times 342}\left(\frac{1}{z}-\frac{1}{y}\right) = 489.9\left(\frac{1}{z}-\frac{1}{y}\right) = \text{g. raffinose pentahydrate/50 ml. solution (a)}$$

(594 = molecular weight of raffinose pentahydrate, 342 = molecular weight of melibiose).

Appendix

Preparation of the invertase-melibiase enzyme mixture

Suck 11.5 litres brewer's yeast dry on a Buchner funnel. About 5.5 kg. compressed yeast containing 25% dry weight is obtained. Add this to 3000 ml. toluene and allow to stand for 7 days with occasional stirring (autolysis). Filtration (2 days) through large fluted filter papers yields about 3000 ml. filtrate. Mix the residue with 1200 ml. water, allow to stand overnight, filter and combine the filtrate with the first. Ultrafilter the mixture with stirring until there is only 600 ml. above the filter. Add 6 ml. acetic acid to this residue, allow to stand overnight and filter through fluted filter papers. Ultrafilter the filtrate with stirring until there is only 600 ml. above the filter.

The ultrafilter is prepared from nitrocellulose (6 g.) dissolved in a mixture of absolute alcohol (50 ml.) and absolute ether (50 ml.).