View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by JRC Publications Repository

EUROPEAN COMMISSION
DIRECTORATE-GENERAL
Joint Research Centre

Institute for Reference Materials and Measurements

Method-specific certification of free sugars and starch/glucose in two artificial food materials
BCR-644
BCR-645

C. Briffaut, J.-M. Lansiaux, M. Marcault, G.N. Kramer, T. Linsinger, B.M. Gawlik, A. Bernreuther, A. Lamberty, A. Boenke

Report EUR 20987

The mission of IRMM is to promote a common European measurement system in support of EU policies, especially health and consumer protection, environment, agriculture, internal market and industrial standards.

European Commission
Directorate-General Joint Research Centre
Institute for Reference Materials and Measurements

Contact information
European Commission
Directorate-General Joint Research Centre
Institute for Reference Materials and Measurements
Retieseweg 111
B-2440 Geel • Belgium

Tel.: +32 (0)14 571211
Fax: +32 (0)14 590406

http://www.irmm.jrc.be
http://www.jrc.cec.eu.int

Legal Notice
Neither the European Commission nor any person acting on behalf of the Commission is responsible for the use which might be made of the following information.

A great deal of additional information on the European Union is available on the Internet.
It can be accessed through the Europa server
http://europa.eu.int

EUR Report 20987
Luxembourg: Office for Official Publications of the European Communities

ISBN 92-894-6871-8

© European Communities, 2004

Reproduction is authorised provided the source is acknowledged

Printed in Belgium

European Commission

BCR information
REFERENCE MATERIALS

Method-specific certification of free sugars and starch/glucose in two artificial food materials BCR-644 BCR-645

C. Briffaut, J.-M. Lansiaux, M. Marcault

Laboratoire des Douanes de Paris
F-75003 PARIS (France)

G.N. Kramer, T. Linsinger, B.M. Gawlik, A. Bernreuther
A. Lamberty

European Commission, DG Joint Research Centre
Institute for Reference Materials and Measurements
B-2440 GEEL (Belgium)

A. Boenke

European Commission, DG RTD
Standards, Measurements and Testing
B-1049 BRUSSELS (Belgium

Report EUR 20987 EN

Institute for Reference Materials and Measurements

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

ABSTRACT

This report describes the work performed to prepare two reference materials containing sugars, starch and starch degradation products, and certify them by two methods of analysis. The first method consists in the determination of free sugars (sucrose, fructose and lactose) in food by liquid chromatography. The second method consists in an enzymatic hydrolysis of starch and starch degradation products to glucose, and then in the determination of glucose by liquid chromatography. The materials are powders consisting in mixtures of milk powder, starch, sugars and dextrins. The reference materials can only be used to check the application of the methods A and B described in the Annex C of the report.

Certified values were accompanied by an expanded uncertainty according to the requirements laid down in the Guide for the Expression of Uncertainty in Measurement (GUM) [1].

BCR-644 (1). Results are expressed g/100 g dry mass

Certified property	*Certified value(2)*	*Uncertainty(3)*	*No. of data sets*
Free fructose (F)	16.2	1.1	7
Free sucrose (S)	10.81	0.25	8
Free lactose (L)	15.85	0.29	7
Starch/glucose (Z)(4)	35.1	1.2	8

BCR-645 (1). Results are expressed g/100 g dry mass

Certified property	*Certified value(2)*	*Uncertainty(3)*	*No. of data sets*
Free sucrose (S)	26.2	0.8	9
Free lactose (L)	27.8	0.6	8
Starch/glucose (Z)(4)	25.2	0.9	9

(1) The results are specific to the method described in this report

(2) These values are unweighted means of accepted means obtained independently by 7 different laboratories

(3) Expanded uncertainty with a coverage factor of k = 2 according to the GUM [1]

(4) Expressed as glucose

LIST OF ABBREVIATIONS AND SYMBOLS

CV	variation coefficient of the standard deviation
CV_r	variation coefficient of the repeatability
CV_R	variation coefficient of the reproducibility
df	degree of freedom
EC	Enzyme Commission
F	fructose
HPLC	high performance liquid chromatography
IRMM	Institute for Reference Materials and Measurements
k	coverage factor
L	lactose
LGC	Laboratory of the Government Chemist
OJ	Official Journal of the European Community
RM	reference material
R(EEC)	Regulation of the European Economic Community
S	sucrose
s_{bb}	estimate of ubb obtained from the variation coefficients of the homogeneity study
SD	standard deviation
S/G	starch/glucose: starch and starch degradation products, expressed as starch
u_{bb}	uncertainty contribution resulting from the homogeneity study
u_{char}	uncertainty contribution resulting from the batch characterisation
U_{CRM}	expanded uncertainty of the certified value
u_{lts}	uncertainty contribution resulting from the long-term stability study
Z	glucose obtained after complete hydrolysis of starch and starch degradation products

TABLE OF CONTENTS

1 INTRODUCTION

1.1 Need for a reference material

The determination of what is called "s*tarch/glucose*" is required for the application of EU Regulations related to import and export [2,3].

These regulations introduce the notion of "s*tarch/glucose*" which is the result of the hydrolysis of starch or of its hydrolysis products (dextrins, maltodextrins, corn syrups, maltose) to glucose, in order to have an evaluation of a set of products derived from maize. Maize and its derivatives are subjected to particular import taxes or export refunds (restitutions) when incorporated in food products classified under different codes of the *Combined Nomenclature*. The concerned codes belong to the headings 1704, 1806, 1901, 1905, 2004, 2005, 2008 and 2106 of the *Harmonised System*. They are described with full details in the regulation (EC) 1460/96 [4]. The taxation is based on an additional code. Setting up this code requires the determination of starch/glucose, sucrose, milk fat and milk proteins.

1.2 Choice of reference materials

As "*starch/glucose*" must be determined in any kind of foodstuff, the choice of the matrix is important. It seemed necessary to use matrixes containing important quantities of powder milk, as this product appears frequently in analysed products and the experience shows that it was a sensitive factor in the evaluation of the tested methods. A too strong hydrolysis may lead to the partial transformation of lactose to glucose and galactose, and consequently to a too high result. It appeared too that it was advisable to have materials including the different elements contributing to "*starch/glucose*".

For these reasons, the first material (BCR-644) is made of milk powder (26 % milk fat), fructose, sucrose and maltose; the second (BCR-645) is made of milk powder (skimmed), sucrose and dextrins. Fructose is included for the reason that, when present, sucrose may produce by hydrolysis equivalent quantities of glucose and fructose. It is therefore necessary to subtract from the determined glucose an equivalent quantity of fructose. Sucrose and lactose are not really necessary for the determination of "*starch/glucose*", but their determination is required to establish the additional code. The lactose mass content is an indication of the accuracy of the declared amounts of other milk products, generally required on the same materials, and may then be determined with the same method. A common reference is therefore useful.

1.3 Organisation of the project

A preliminary interlaboratory study of two materials was performed to evaluate 4 analytical methods. Two materials suitable to serve as Reference Materials were prepared, studied for homogeneity and stability and analysed for certification. Table 1.1 provides an overview of the approach followed.

Table 1.1 - Overview of the approach followed to prepare and finally certify two RMs

Optimisation of methods	*Interlaboratory study*	*Evaluation of results*	*Preparation of 2 RMs*
Feb 1995	Jun 1996	Sep 1997	Sep 1996 to Jan 1997
Homogeneity –Stability	*Certification*	*Evaluation*	

Jun 1997 to Jun 1998	Sep to Oct 1998	Jan 1999

2 PARTICIPANTS

2.1 Preparation of reference materials

- European Commission, DG JRC, Institute for Reference Materials and Measurements (IRMM), Geel BE

2.2 Tests for homogeneity and stability

- Laboratoire Interrégional des Douanes, Paris FR

2.3 Preliminary interlaboratory studies

- Dipartimento Dogane- Direzione Centrale per l'Analisi Merceologica, Rome IT
- Finnish Customs Laboratory, Espoo FI
- Force Instituttet, Bronby DK
- General Chemical State Laboratory- Direction D of Chemical Services, Athens GR
- Laboratoire des Douanes et Accises, Louvain BE
- Laboratoire Interrégional des Douanes, Bordeaux FR
- Laboratoire Interrégional des Douanes, Lyon FR
- Laboratoire Interrégional des Douanes, Paris FR
- Laboratoire Interrégional des Douanes, Villeneuve d'Asq FR
- Laboratorio da Direccao, Geral das Alfandegas, Lisboa PT
- Laboratorium Belastingdienst, Amsterdam NL
- Laboratory of the Government Chemist (LGC), Teddington UK
- State Laboratory, Abbotstown IE
- Zolltechnische Prüfungsstelle und Lehranstalt, Frankfurt DE
- Zolltechnische Prüfungsstelle und Lehranstalt, Hamburg DE
- Zolltechnische Prüfungsstelle und Lehranstalt, Köln DE
- Zolltechnische Prüfungsstelle und Lehranstalt, München DE

2.4 Certification of the materials

- Dipartimento Dogane- Direzione Centrale per l'Analisi Merceologica, Rome IT
- Finnish Customs Laboratory, Espoo FI
- Laboratoire Interrégional des Douanes, Lyon FR
- Laboratoire Interrégional des Douanes, Paris FR
- Laboratoire Interrégional des Douanes, Villeneuve d'Asq FR
- Laboratorium Belastingdienst, Amsterdam NL
- State Laboratory, Abbotstown IE
- Zolltechnische Prüfungsstelle und Lehranstalt, Berlin DE
- Zolltechnische Prüfungsstelle und Lehranstalt, Frankfurt DE
- Zolltechnische Prüfungsstelle und Lehranstalt, Hamburg DE
- Zolltechnische Prüfungsstelle und Lehranstalt, Köln DE
- Zolltechnische Prüfungsstelle und Lehranstalt, München DE

2.5 Evaluation of the results

- European Commission, DG RTD, Brussels BE
- Laboratoire Interrégional des Douanes, Paris FR

- European Commission, DG JRC, Institute for Reference Materials and Measurements (IRMM), Geel BE

3 PREPARATION OF THE MATERIALS

The two candidate RMs were prepared using starch, milk powder and sugars: these products were homogenised in de-mineralised water and freeze dried. The freeze dried materials were then crushed, ground to fine powders (particle size <180 µm) and sampled. The composition, moisture content and top particle size of the materials used for the preparation of the RMs are reported in Tables 3.1 and 3.2.

Table 3.1 - Composition, moisture content and top particle size of the base materials for BCR-644

Material	*Mass (%)*	*Top particle size (µm)*	*Moisture content (%)*
Starch	30	180	12.15
Milk powder (high fat mass fraction)	40	435	3.78
Fructose	15	875	0.04
Sucrose	10	875	0.03
Maltose	5	515	5.51

Table 3.2 - Composition, moisture content and top particle size of the base materials for BCR-645

Material	*Mass (%)*	*Top particle size (µm)*	*Moisture content (%)*
Milk powder (low fat mass fraction)	50	435	5.36
Sucrose	25	875	0.03
Maltodextrin	25	735	3.47

Flow charts describing the preparation of the two candidate RMs are shown in Fig. 3.1 and 3.2.

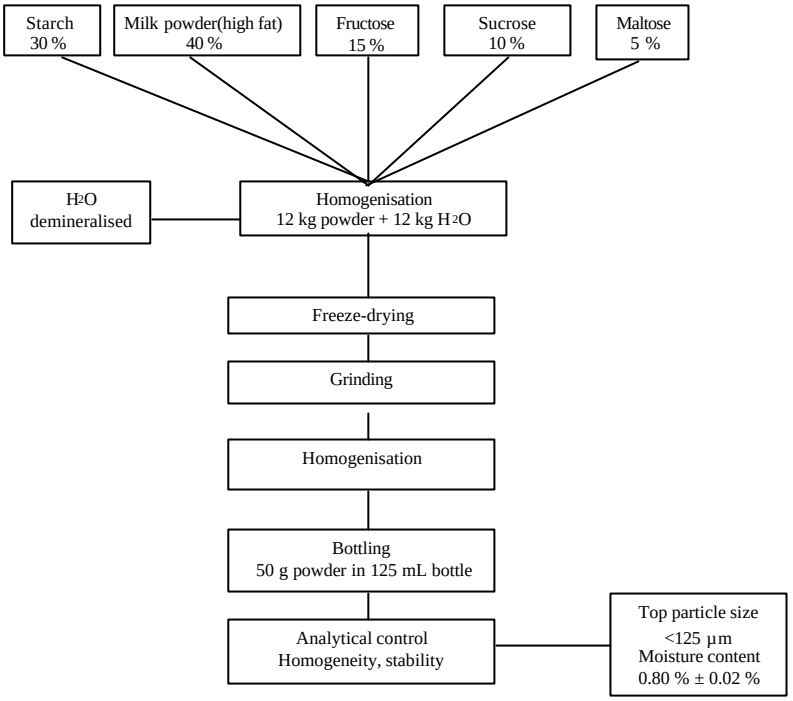

Figure 3.1 – Preparation of BCR-644

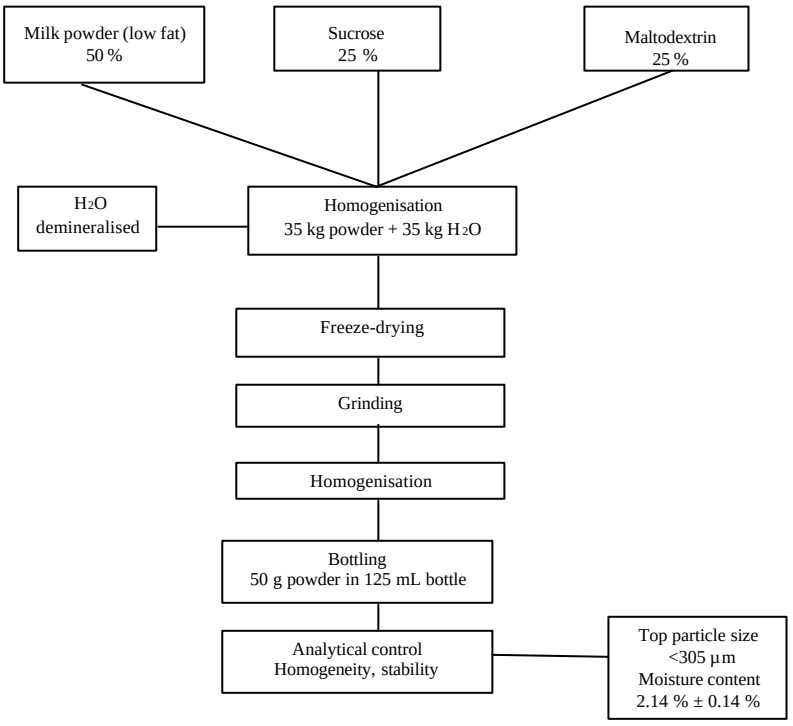

Figure 3.2 – Preparation of BCR-645

The final materials consist of about 2400 bottles of approximately 50 g each of BCR-644 and BCR-645.

4 TESTING OF THE MATERIALS

4.1 Repeatability of the method

The repeatability of the two methods A and B (Annex C) was determined. Ten test portion sizes each of about 5 g of one material BCR-644 were used for the determination of free sugars (method A); 10 test portion sizes of about 2.4 g of one material BCR-645 were used for the determination of starch/glucose. Information on the repeatability of the method used is reported in the Table 4.1.

Table 4.1 - Repeatability of the methods

	Z	*S*	*F*	*L*
$\overline{X}$ (g/100 g)	24.18	9.58	16.12	14.07
± 1 SD (g/100 g)	0.24	0.12	0.16	0.29
CV (%)	1.0	1.3	1.0	2.1
n	10	10	10	10

4.2 Homogeneity determination

4.2.1 Sampling the materials

Eighty bottles of each material were received at the Laboratoire des Douanes de Paris on the 19th March 1997. They were stored at –20 °C until required for testing.

4.2.2 Statistical design of the homogeneity study

Each of the two RMs was analysed in two series: method A (free sugars) in order to determine fructose (F), sucrose (S) and lactose (L) in BCR-644, and sucrose and lactose in BCR-645; method B in order to determine glucose after hydrolysis (Z). For each RM, 19 different units were analysed, each one in duplicate, the order of analysis being different from the order of filling of the bottles (number of the bottle).

Test portions of 5 g (BCR-644) and 4 g (BCR-645) were used for the determination of free sugars (method A). Test portions of 2.2 g (BCR-644) and 2.4 g (BCR-645) were used for the determination of hydrolysed glucose (method B). Unsatisfying chromatographic peaks were obtained with lower test portions.

4.2.3 Results of the homogeneity testing

The results of these determinations, including overall, between and within bottles standard deviations, are shown in Tables 4.2 and 4.2 and graphically presented in Fig. 4.1 to 4.7. In each of these graphs, three horizontal lines correspond to the mean value and to the mean value ± 2 SD, respectively.

The results for fructose (BCR-644) were corrected taking into account the degradation of fructose occurring in the experimental conditions of the chromatography, depending on the nature of the substrate.

This correction is performed by considering the results of fructose in the order of analysis, determining by linear regression the coefficients of: $F = an + b$, n being the order of analysis, and

finally by applying to each result a correction: F' = F – an.

Table 4.2 - Homogeneity study of BCR-644

	Sucrose (S)	*Lactose (L)*	*Fructose (F)*	*Starch/glucose (Z)*
± 1 SD (g/100 g)	0.091	0.431	0.09	0.343
CV (%)	0.9	2.0	0.6	1.1
between unit ± 1 SD (g/100 g)	0.086	0.476	0.07	0.377
within unit ± 1 SD (g/100 g)	0.055	0.039	0.029	0.036
between unit CV (%)	0.8	3.1	0.5	1.2
within unit CV (%)	0.5	0.3	0.2	0.1

Table 4.3 - Homogeneity study of BCR-645

	Sucrose (S)	*Lactose (L)*	*Starch/glucose (Z)*
± 1 SD	0.49	0.418	0.264
CV (%)	2.0	1.6	1.1
between unit ± 1 SD (g/100 g)	0.362	0.444	0.359
within unit ± 1 SD (g/100 g)	0.221	0.458	0.96
between unit ± CV (%)	1.5	3.3	1.0
within unit ± CV (%)	0.9	1.9	0.4

An $F_{0.95}$-test on corresponding variances shows that there is no significant difference between them and the repeatability of the applied methods. Table 4.4 shows the results of the $t_{0.95}$-test calculated, based on regressions performed on the data against both bottle number and order of analysis.

The $t_{0.95}$-test values cited are the ratios of the estimated slopes from the regressions divided by their standard errors. The expression "df" indicates the number of degrees of freedom associated with the regression, $t_{0.95}$ and $t_{0.99}$ are the reference values of the test taken from the table of Student.

Table 4.4 - Regression of data against bottle number and testing order

Material	*Analyte*	*df*	$t_{0.95}$	$t_{0.99}$	*t bottle number*	*t testing number*
BCR-644	Fructose	19	1.73	2.55	0.818	0.028
	Sucrose	19	1.73	2.55	1.109	1.404
	Lactose	19	1.73	2.55	0.533	0.568
	Z	19	1.73	2.55	1.055	0.754
BCR-645	Sucrose	19	1.73	2.55	0.723	0.924
	Lactose	19	1.73	2.55	0.321	2.079
	Z	19	1.73	2.55	0.794	0.838

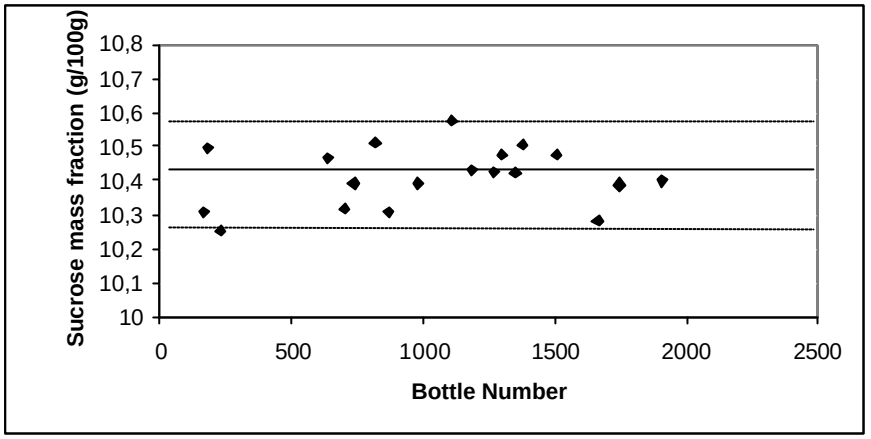

Figure 4.1 - BCR-644 – Results of the homogeneity study for free sucrose (S)

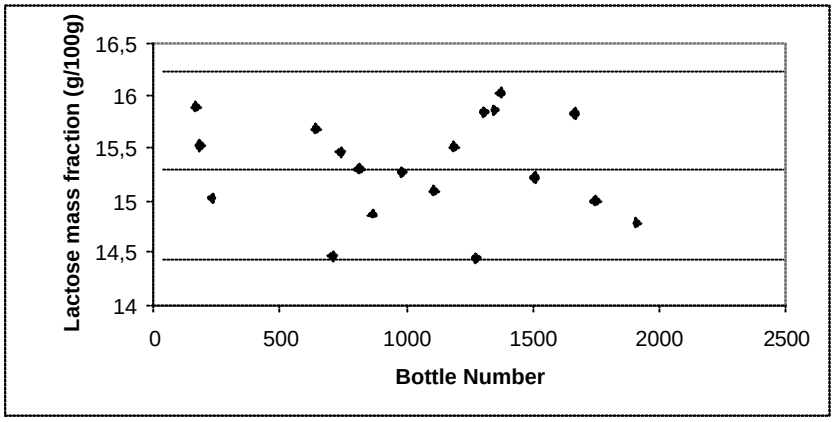

Figure 4.2 - BCR-644 – Results of the homogeneity study for free lactose (L)

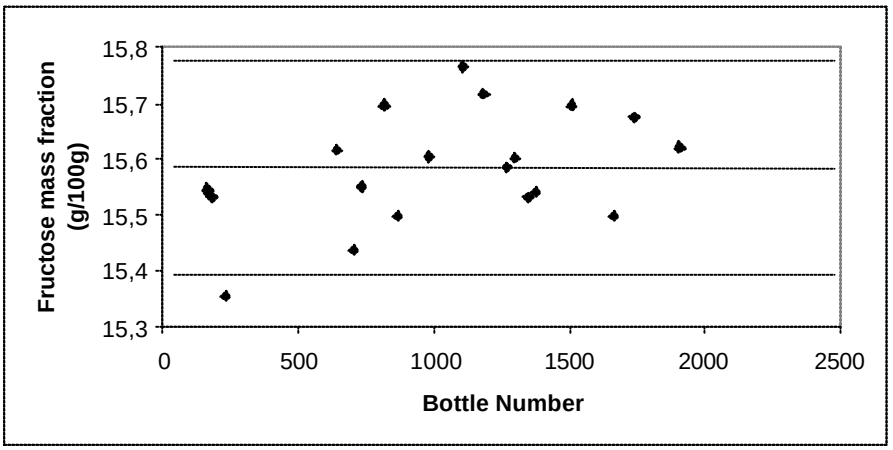

Figure 4.3 - BCR-644 – Results of the homogeneity study for free fructose (F)

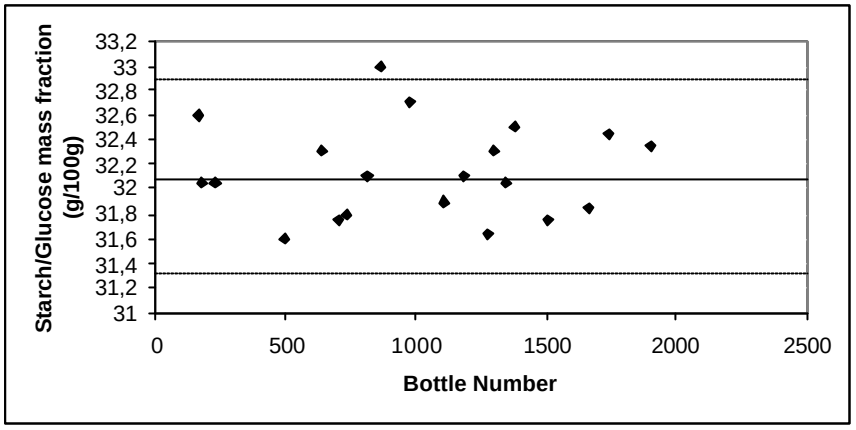

Figure 4.4 - BCR-644 – Results of the homogeneity study for starch/glucose (Z)

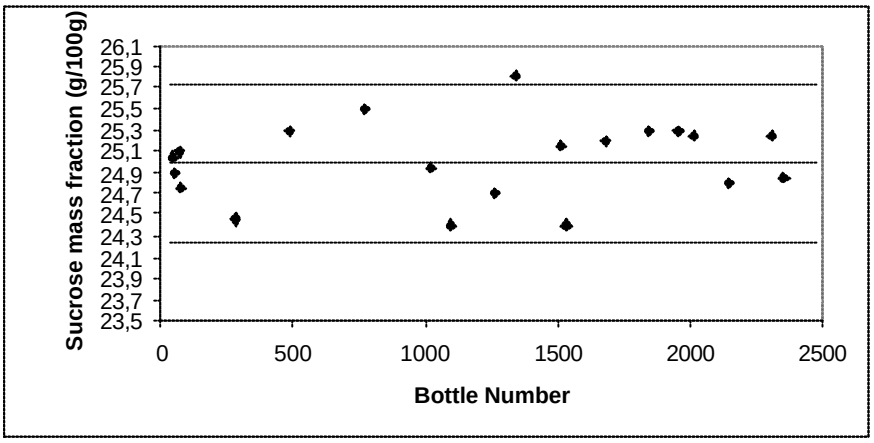

Figure 4.5 - BCR-645 – Results of the homogeneity study for free sucrose (S)

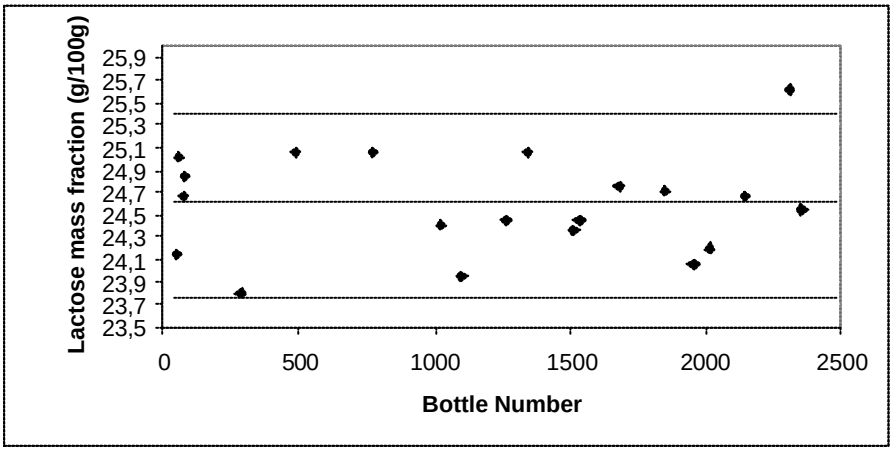

Figure 4.6 - BCR-645 – Results of the homogeneity study for free lactose (L)

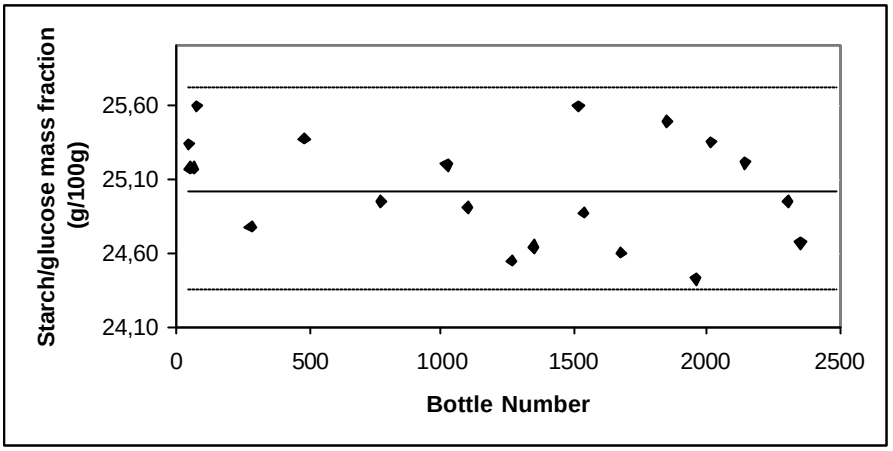

Figure 4.7 - BCR-645 – Results of the homogeneity study for starch/glucose (Z)

The trend analysis shows a significant trend due to the analysis order for lactose in BCR-645 at the 95 % level, and none for any of the RMs at the 99 % level neither due to the filling sequence. The homogeneity of BCR-645 was confirmed by examining the results of the certification exercise. For this reason, the laboratories participating in this exercise performed the measurements in the order of the individual unit identification number (i.e. "nested design"). An $F_{0.95}$-test was made to compare the standard deviations of the within- and between-units homogeneity. This test confirmed the homogeneity for all free sugars measured and starch/glucose. An uncertainty contribution for homogeneity was included in the expanded uncertainty accompanying the certified value.

4.3 Stability study

The stability study was planned by setting at the time zero two bottles of each RM at -18, 4 and 25 °C. Other bottles were placed in a special oven at 40 °C. The bottles were placed back to -18 °C until analysis after 3, 6 and 9 months. Analyses were made after 3, 6, 9 and 12 months on two bottles corresponding to each temperature. Bottles kept at 40 °C were analysed after 6, 9 and 12 months. The results considered for the time zero are the results obtained during the homogeneity study. The individual results are reported in Tables B1 and B2 (Annex B). Aside the raw data, a normalisation was made based on the values obtained at –18 °C. The graphs represented in Fig. 4.8 to 4.28 show the variation of the normalised values in function of time. The three lines represent the mean value and the mean value ± 1 SD (SD obtained during the homogeneity study). An uncertainty contribution for stability was included in the expanded uncertainty accompanying the certified value.

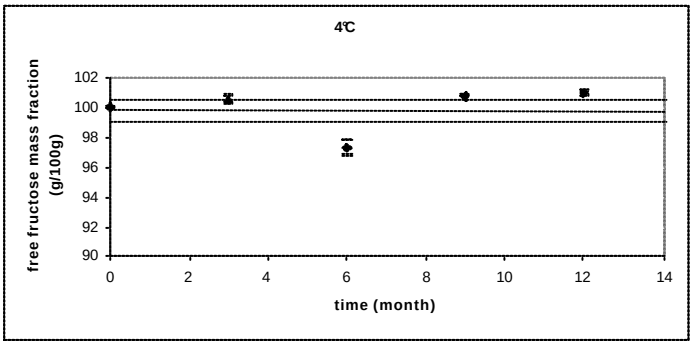

Figure 4.8 - BCR-644 – Results of stability measurements for fructose (F) stored at +4 °C

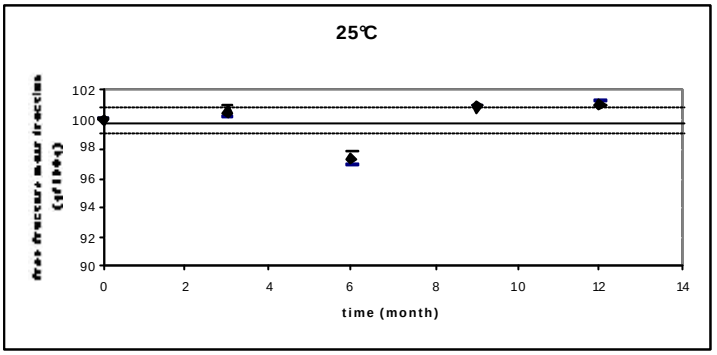

Figure 4.9 - BCR-644 – Results of stability measurements for fructose (F) stored at 25 °C

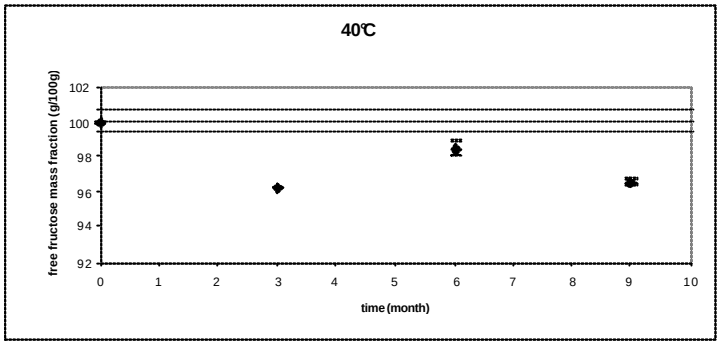

Figure 4.10 – BCR-644 – Results of stability measurements for fructose (F) stored at 40 °C

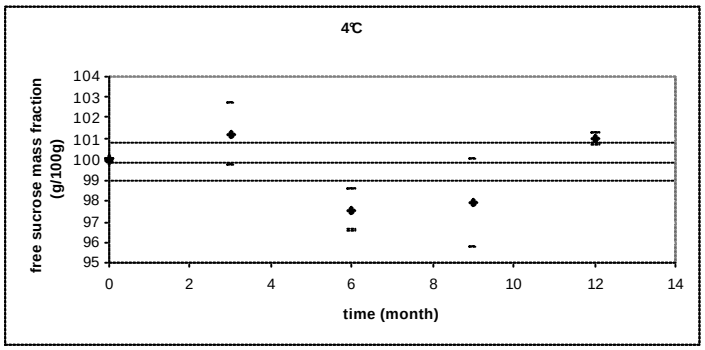

Figure 4.11 - BCR-644 – Results of stability measurements for sucrose (S) stored at +4 °C

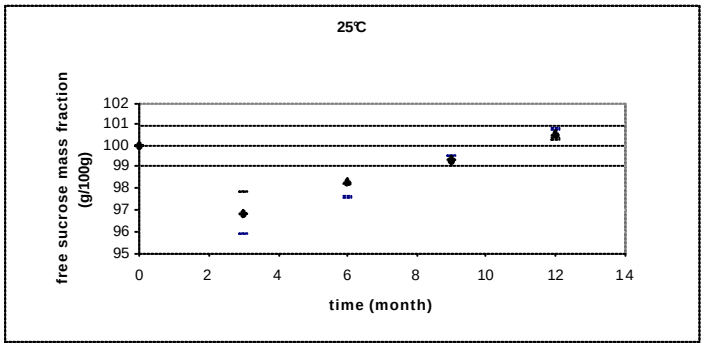

Figure 4.12 - BCR-644 – Results of stability measurements for sucrose (S) stored at 25 °C

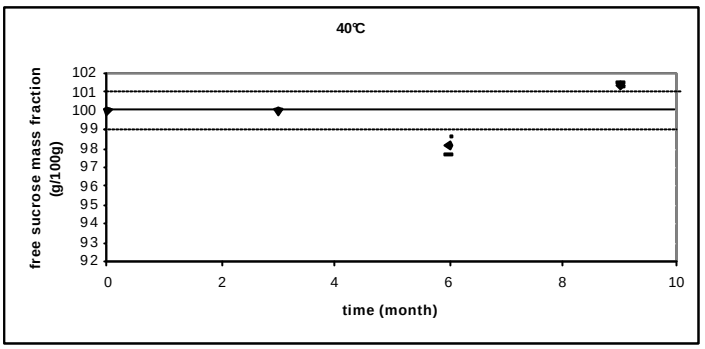

Figure 4.13 - BCR-644 – Results of stability measurements for sucrose (S) stored at 40 °C

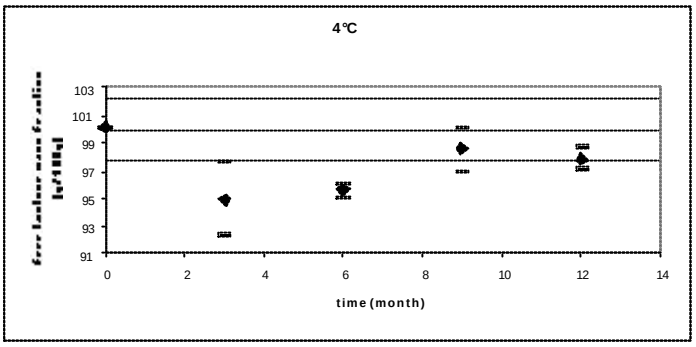

Figure 4.14 - BCR-644 – Results of stability measurements for lactose (L) stored at +4 °C

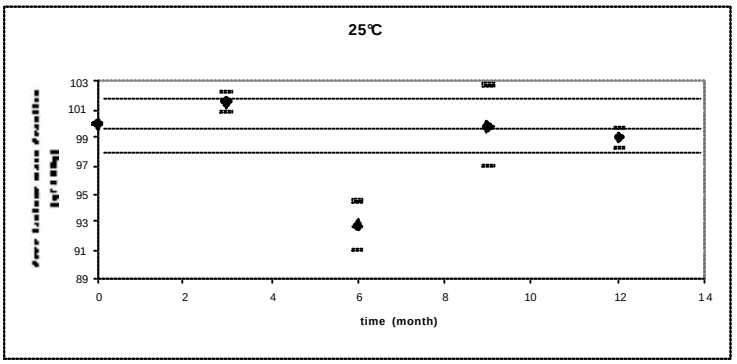

Figure 4.15 - BCR-644 – Results of stability measurements for lactose (L) stored at 25 °C

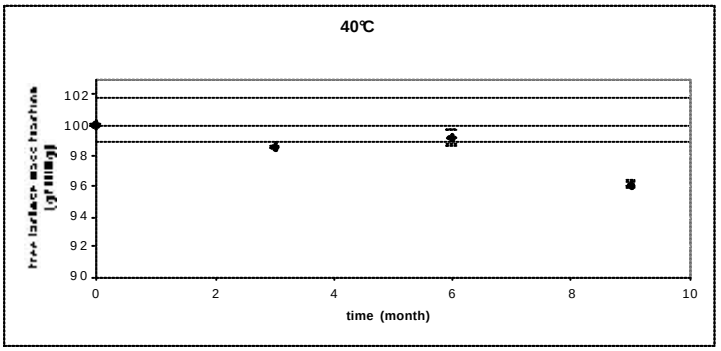

Figure 4.16 - BCR-644 – Results of stability measurements for lactose (L) stored at 40 °C

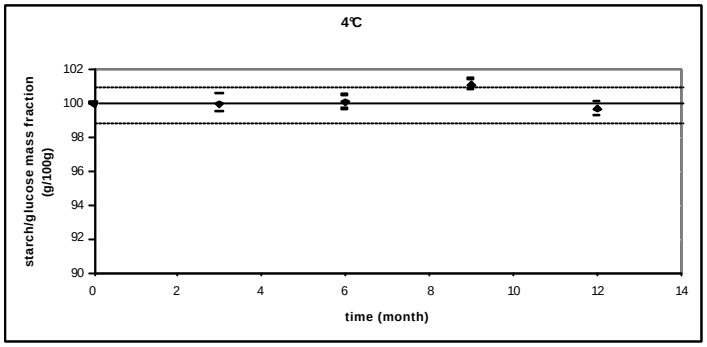

Figure 4.17 - BCR-644 – Results of stability measurements for starch/glucose (Z) stored at +4 °C

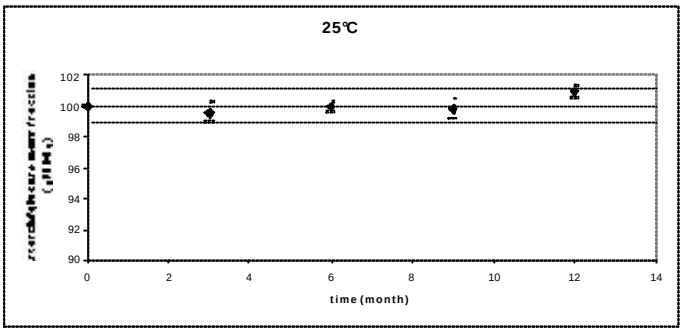

Figure 4.18 - BCR-644 – Results of stability measurements for starch/glucose (Z) stored at 25 °C

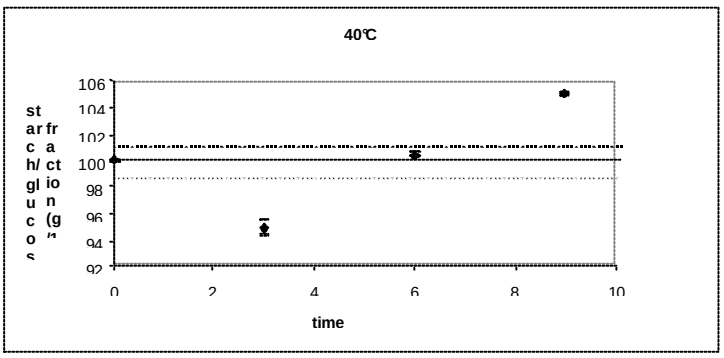

Figure 4.19 - BCR-644 – Results of stability measurements for starch/glucose (Z) stored at 40 °C

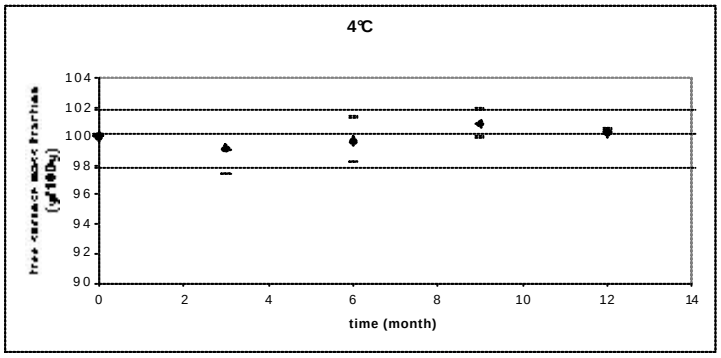

Figure 4.20 - BCR-645 – Results of stability measurements for sucrose (S) stored at +4 °C

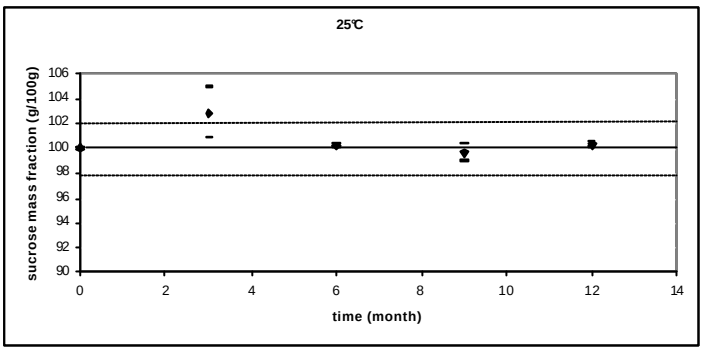

Figure 4.21 - BCR-645 – Results of stability measurements for sucrose (S) stored at 25 °C

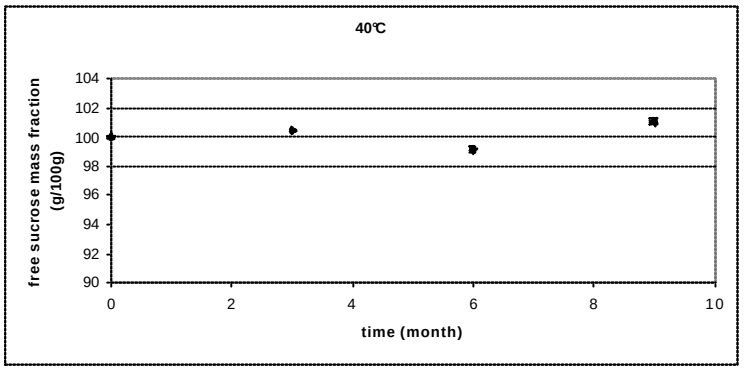

Figure 4.22 - BCR-645 – Results of stability measurements for sucrose (S) stored at 40 °C

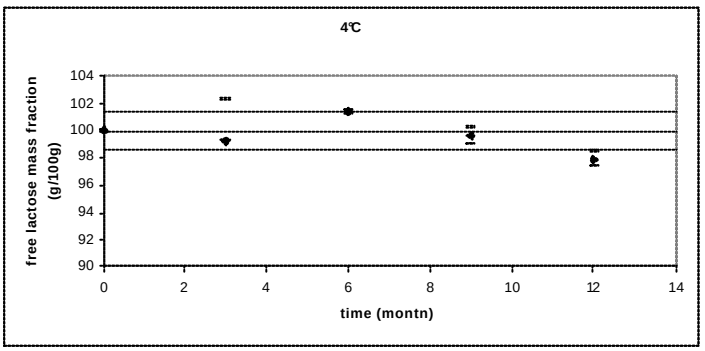

Figure 4.23 - BCR-645 – Results of stability measurements for lactose (L) stored at +4 °C

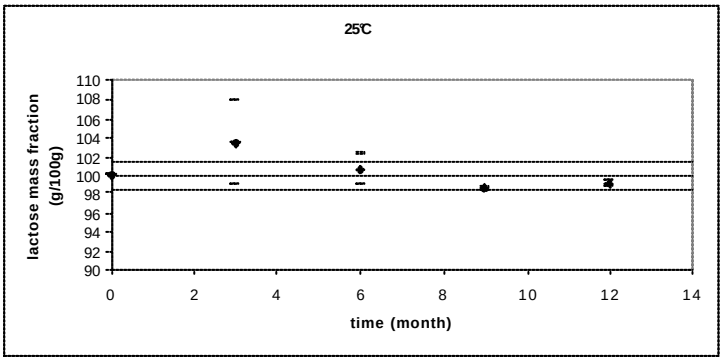

Figure 4.24 - BCR-645 – Results of stability measurements for lactose (L) stored at 25 °C

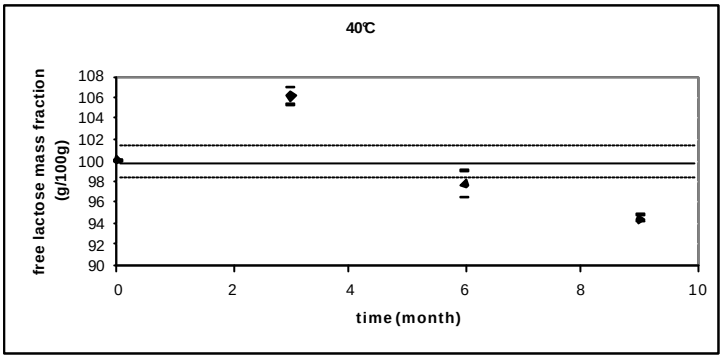

Figure 4.25 - BCR-645 – Results of stability measurements for lactose (L) stored at 40 °C

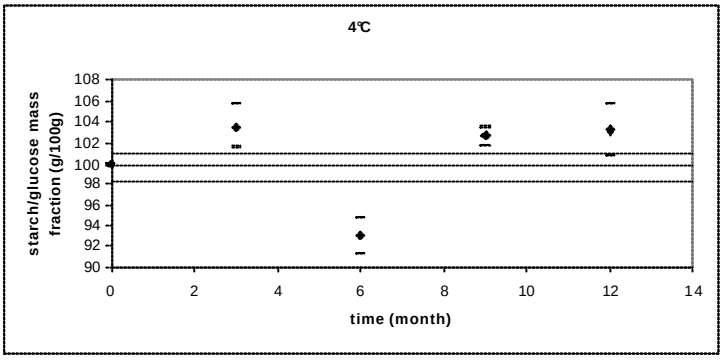

Figure 4.26 - BCR-645 – Results of stability measurements for starch/glucose (Z) stored at +4 °C

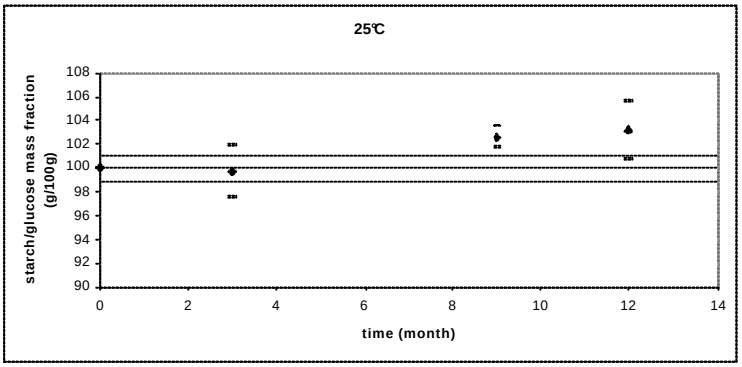

Figure 4.27 - BCR-645 – Results of stability measurements for starch/glucose (Z) stored at 25 °C

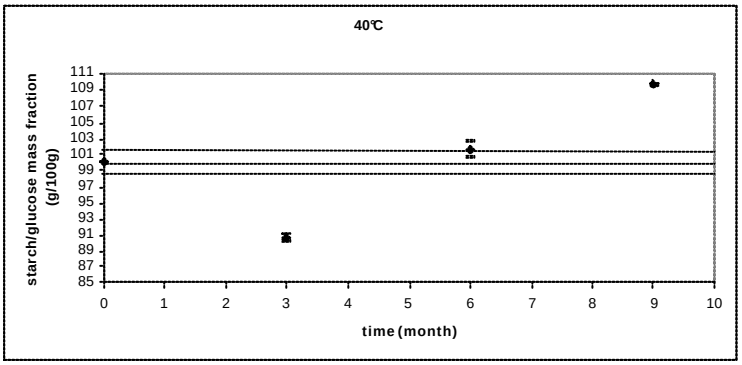

Figure 4.28 - BCR-645 – Results of stability measurements for starch/glucose (Z) stored at 40 °C

These normalised results show no instability except for starch/glucose (Z) in BCR-644 and -645 at 40 °C after 3 months storage. In addition, an instability of lactose was detected at 40 °C after 9 months in BCR-645 and fructose at 40 °C in BCR-644. However, all materials allow normal

postal shipment.

5 PRELIMINARY INTERLABORATORY STUDIES

5.1 First studies

Many studies have been made on this subject since the publication of an official method for the determination of starch/glucose [2]. In 1988, a ring test was organised by LGC (UK). The conclusion was that, in this official method, the dissolution of the materials in the presence of soda lead to the partial destruction of higher condensation products of glucose (e.g. maltose, maltotriose, etc.).

In 1990, a ring test was again organised by the LGC (UK) using a method based on the hydrolysis of samples with HCl. The results showed problems in the presence of lactose: In one material containing about 28 g/100 g of lactose, the found mass fraction of starch/glucose was 20.6 g/100 g with a reproducibility standard deviation of 6.4 g/100 g and a relative standard deviation of 31 %. The reason was that HCl hydrolysed lactose into glucose and galactose. In addition, this transformation was not complete.

In 1991, The Customs Laboratory of Paris (FR) organised another ring test in the network of French customs laboratories. It was based on a double enzymatic hydrolysis of starch: dissolution of the material and a liquefaction with an enzyme at 100 °C, followed by a final hydrolysis to glucose using amyloglucosidase. It was observed that for a material similar to the preceding one (with 16 g/100 g of lactose and 33 g/100 g of starch/glucose), the reproducibility standard deviation for starch/glucose was ± 4 g/100 g and the reproducibility coefficient of variation was 11 %.

5.2 Fourth interlaboratory study

A series of ring tests was realised in 1993, on 8 commercial products, using a method proposed by the Customs Laboratory of Paris (FR). 18 European laboratories participated. The participants were asked to determine in each material:

- Free sugars (sucrose, fructose, glucose, lactose) using an HPLC method.
- Starch/glucose with an enzymatic hydrolyse of starch and HPLC determination of glucose. Two protocols were used with an internal and an external standard.

The methods used and the main results have been described in a memorandum prepared by EC, DG III [5]. The mean values for hydrolysed glucose, repeatability and reproducibility are presented in the Table 5.1.

Table 5.1 - Results of the 4th interlaboratory study for hydrolysed glucose

	Petit Beurre	*Bonbons*	*Chocolate*	*Beans*	*Pudding powder*	*Biscuit*	*Baby food*
	A	*B*	*C*	*E*	*F*	*G*	*H*
Mean (g/100 g)	58.94	50.24	1.92	70.66	102.16	53.84	38.65
CV_r (%)	2.3	1.7	13.6	1.3	1.2	1.5	2.0
CV_R (%)	5	15	40	4	8	4	6

The results were not found satisfactory for chocolate and "*bonbons*" because of the very low level of

starch. In the material “bonbons” which is mainly made of sucrose and corn syrup, it was easy to see that, for some laboratories, fructose was produced during the enzymatic hydrolysis, because of a partial inversion of sucrose. If one assumes that an equivalent quantity of glucose is produced, the real quantity of glucose derived from starch can be calculated by Z' = Z – Fr' (where Z is glucose after hydrolysis and "' " is used for sugars after hydrolysis). Corrected in that manner, the values for this product are reported in Table 5.2.

After examination of these results it was decided to further improve the method and to produce certified reference materials for its correct application.

Table 5.2 - Z' results for "Bonbon B"

	Bonbon B
mean (starch) (g/100 g)	37.77
CV_r (%)	2
CV_R (%)	4

5.3 Fifth Interlaboratory study

Two artificial materials called 351J and 351K were prepared by the IRMM for this study. They consisted on two blends of starch, milk powder, sugars and maltodextrins. The materials 351J and 351K were analysed by 16 European Customs laboratories in June 1996. Each laboratory received two units with a different reference number. They also received 4 methods descriptions referred to as A, B, C and D. These methods were:

- Method A:

  Determination of free sugars by HPLC.

- Method B:

  Enzymatic hydrolyse of starch to glucose and determination of glucose by HPLC.

- Method C:

  Enzymatic hydrolyse of starch to glucose and then determination of glucose by an enzymatic method.

- Method D:

  Enzymatic hydrolyse of starch to glucose and inversion of sucrose to glucose and fructose by HPLC on ion exchange column. In this method, initial sucrose and invert sucrose may be calculated directly.

The laboratories had to use the method A and at least one of the methods B, C or D. They had to make two determinations on each received unit. The results are summarised in Table 5.3.

Table 5.3 – Results of the interlaboratory study on the materials 351J and 351K

	Method	*Analyte*	*Number of data sets (p)*	*Mean of means (g/100 g)*	*Repeatability CV (%)*	*Reproducibility CV (%)*	*Theoretical value(g/100 g)*
J	A	Free fructose (F)	14	16.06	2	4	16.2
J	A	Free sucrose (S)	14	10.66	2	5	10.8
K	A	Free sucrose (S)	14	10.16	2	6	10.4
J	A	Free lactose (L)	14	15.63	4	7	16.2
K	A	Free lactose (L)	16	39.22	3	8	
J	B	Glucose (Z)	14	36.09	4	7	35.2
K	B	Glucose (Z)	13	16.62	4	11	
J	B	Glucose (Z-F'+F)	14	35.40	3	5	35.2
K	B	Glucose (Z-F'+F)	13	15.39	4	7	
J	C	Glucose (Z)	10	35.84	1	4	
K	C	Glucose (Z)	6	15.99	2	3	
J	D	Glucose (Z)	8	40.70	1	3	
K	D	Glucose (Z)	7	21.13	1	6	
J	D	Starch	7	31.92	1	4	
K	D	Starch	8	13.85	2	8	
J	D	Fructose	9	21.01	2	5	
K	D	Fructose	8	5.60	3	14	

6 CERTIFICATION EXERCISE

6.1 Analytical methods used

Participating laboratories applied three methods for the determination of starch/glucose. In these methods, a preliminary step was the determination of free sugars (glucose and fructose). The three methods started with the hydrolysis of starch in two steps: first, liquefaction of starch with a thermostable enzyme at 100 °C; second, hydrolysis into glucose with amyloglucosidase at 60 °C. For the end-determination step of formed glucose, three approaches were used:

- HPLC separation of sugars on an "aminopropyl" column with refractive index detection;
- Enzymatic determination of glucose;
- HPLC separation of sugars on an ion-exchange column with refractive index detection.

The first approach was chosen for the reason that few laboratories use ion-exchange columns and that the fully enzymatic method does not provide correct results in every case.

The determination of starch/glucose (S/G) was realised in two steps:

- Determination of free glucose and fructose (G, F);
- Determination of glucose after hydrolyse (Z).

As the determination of free glucose and fructose was needed for the calculation of starch/glucose, the amount of these analytes was required. An HPLC method using an aminopropyl column was applied. The laboratories were asked to measure lactose, as the amount of this sugar can also be obtained during the same analysis.

The methods referred as method A (free sugars) and method B (hydrolysed glucose, Z) are described in details in the Annex C. The calibrants used are all of analytical grade, with purity higher than 99 %.

6.2 Preliminary controls to be performed by the participants

As it is important to verify that the used enzymes have full activity, each participant had to control this activity, using pure starch as indicated in the method (paragraph n°6, method B in the Annex C). It was asked to verify that the difference of the results obtained by the Ewers method (D72/199 EEC) [5] was not greater than 2 %.

Each participant had to make 6 complete measurements on one material (BCR-644 or -645) to verify that the repeatability was not higher than 2.5 % for free sugars and 3 % for hydrolysed glucose.

6.3 Performance of certification measurements

The materials were distributed to the participants in the middle of September 1998, the results were received by the end of October 1998. Each laboratory received 4 units (bottles) of BCR-644 and 4 units of BCR-645. Each laboratory was asked to make 6 true replicates on each material, taken from 2 or 3 different bottles in regular individual unit identification number in order to follow the nested design approach.

6.4 Expression of results

The results had to be expressed on dry matter, with at least 3 significant figures.

6.5 Technical evaluation

All participants met to discuss the outcome of the certification exercise. All the data were carefully evaluated. No data were rejected on statistical grounds, but only on identified technical grounds.

All laboratories except L4, L8 and L11 confirmed that the materials were analysed in order of increasing bottle number; the results were entered in the same order. Laboratory L4 did not perform the certification measurements according to the numerical order of the units; the laboratory L8 analysed only BCR-645 in numerical order. Laboratory L11 did not provide any information on this. As a consequence, it was decided to discard the results of the laboratories L4 and L11 for both materials and the results of laboratory L8 for BCR-644, only. However, the set of results for L3, L8 and L11 were accepted as supporting data. Laboratory L13 used a different method. It was agreed that its results would be used as supporting data. The method used by this laboratory is the "C" method described in Annex C. The results accepted for certification are presented as diagrams in Annex D.

6.5.1 Results for BCR-644 - free fructose (F)

Laboratories L2 and L3 had results considered as stragglers according to the Cochran test. Laboratory L3 confirmed that it had difficulties with determination, having a high level of noise, and agreed to discard its results for this property. As no technical reason could be identified for laboratory L2, its results were kept.

6.5.2 Results for BCR-644 - free sucrose (S)

There were no outlier and no straggler detected. All sets of results were accepted on technical grounds.

6.5.3 Results for BCR-644 - free lactose (L)

One result of laboratory L5 was reported as an outlier. It was discarded because there was a high level of noise on the chromatogram.

6.5.4 Results for BCR-644 - starch/glucose (Z)

One result of the laboratory L6 was considered as an outlier. As the laboratory had indicated that an integration problem occurred, all the data of this laboratory for this analyte were rejected.

6.5.5 Results for BCR-645 - free lactose (L)

Laboratory L3's results were considered as outliers by the Cochran test. It was agreed to omit these results, as there was a high level of noise and a poor baseline on the chromatograms.

6.5.6 Results for BCR-645 - free sucrose (S)

Outliers were noted in the data sets from laboratory L9. As no technical explanation could be identified, the data were retained.

6.5.7 Results for BCR-645 - hydrolysed glucose (Z')

The results of laboratory L10 were rather low which lead to the fact that the mean values for hydrolysed glucose (Z') showed an about normal (acceptable at the 99 % confidence level) distribution. No technical reason could explain this.

6.6 Statistical Evaluation

Statistical analysis was carried out on the results accepted on technical grounds using software provided by the Standards, Measurements and Testing Programme. No data were rejected on statistical grounds but only on identified grounds so that there was no risk of discarding data which was more correct than data which was retained.

The following statistical tests were applied to the submitted and accepted data:

- Kolmogorov-Smirnov-Lilliefors test to assess the conformity of the distributions of individual results and of laboratory means to normal distributions,
- Nalimov test to detect "outlying values in the population of individual results and in the population of laboratory means,
- Cochran test to detect "outlying" values in the laboratory variances (s_i^2),
- Bartlett test to assess the overall consistency of the variance values obtained in the participants laboratories,
- one way analysis of variance ($F_{0.05}$-test) to compare and estimate the between- and within-laboratory components of the overall variance of all individual results.
- Snedecor F-test to check whether the between- laboratories standard deviation is significant,
- Scheffe's multiple t-test to check the compatibility of the data sets two by two.

Pertinent information derived from the normality test and the Nalimov test applied to laboratory mean populations are reported in Tables 6.7.1 and 6.7.2 as well as the outcome of Cochran and Bartlett tests on the laboratory variances.

For Cochran and Nalimov tests a value is called an "outlier" when the hypothesis that it belongs to the populations of the results considered can be rejected with a 0.01 risk of error. For a "straggler" the risk lies between 0.01 and 0.05. Summaries of the statistical data for BCR-644 and -645 are given in Tables 6.7.1 and 6.7.2.

Table 6.1 - Summary of statistical data for BCR-644. Certified property expressed in g/100 g on dry matter. Tests performed at the 0.05 and 0.01 significance levels.

Property	*Free fructose*	*Free sucrose*	*Free lactose*	*Starch/glucose*
Number of data sets	7	8	7	7
Number of individual data	42	48	42	42
All data sets compatible two by two (Scheffe's multiple t-test) (1)	No	No	No	No
Outlying data sets (Dixon test, Nalimov t-test) (1)	No	No	No	No
Outlying variances (Cochran test) (1)	Yes	No	No	No
Mean of data set means	16.1791	10.8140	15.8522	35.1010
Within-data sets SD	0.1579	0.1373	0.3438	0.4413

Property	*Free fructose*	*Free sucrose*	*Free lactose*	*Starch/glucose*
Between-data sets SD	0.3143	0.2908	0.2892	1.1128
Variances homogeneous (Bartlett test) (1)	No	About	Yes	Yes
?SD of mean of means	0.3209	0.2962	0.3215	1.1273
Data set means normally distributed? (Kolmogorov-Smirnov-Lilliefors test) (1)	Yes	Yes	Yes	Yes
Half width of the 95 % confidence interval of the mean of means	0.2967	0.2476	0.2973	1.0426
Half width of the 95 % tolerance interval of the mean of means	0.73	0.71	0.78	2.77

Table 6.2 - Summary of statistical data for BCR-645. Certified property expressed in g/100 g on dry matter. Tests performed at the 0.05 and 0.01 significance levels.

Property	*Free sucrose*	*Free lactose*	*Starch/glucose*
Number of data set	9	8	9
Number of individual data	54	48	54
All data sets compatible two by two (Scheffe's multiple t-test) (1)	No	No	No
Outlying data sets (Dixon test, Nalimov t-test) (1)	No	No	Yes
Outlying variances (Cochran test) (1)	Yes	No	No
Mean of data set means	26.1674	27.8264	25.1879
Within-data sets SD	0.3298	0.4305	0.4170
Between-data sets SD	0.5276	1.2926	1.0924
Variances homogeneous (Bartlett test) (1)	No	Yes	No
?SD of mean of means	0.5447	1.3045	1.1055
Data set means normally distributed? Kolmogorov-Smirnov-Lilliefors test) (1)	Yes	Yes	About
Half width of the 95 % confidence interval of the mean of means	0.4186	1.0905	0.8498
Half width of the 95 % tolerance interval of the mean of means	1.25	3.08	2.56

7 CERTIFIED VALUES AND THEIR UNCERTAINTIES

7.1 Calculation of the certified values and their uncertainties

The certified value is calculated as the mean of means. The expanded uncertainty is calculated on the basis of the quadratic addition of the uncertainty contribution resulting from the homogeneity study (u_{bb}), the stability uncertainty (u_{lts}) from the stability study based on the standard deviation of the slope of the regression line at +4 °C for each property (Table 7.1), and the standard error of the mean of means of certification trial (u_{char}) (Tables 6.1 and 6.2). The contribution from the homogeneity study was estimated as:

$$u_{bb} \approx s_{bb} = \sqrt{CV^2_{between} - CV^2_{within}}$$

using the data of Tables 4.2 and 4.3.

Table 7.1 - Mean of means and standard deviation (SD) of the slope of the stability regression line and its corresponding CV (%) for different properties in BCR-644 and -645 at +4 °C

	BCR-644; +4 °C				*BCR-645, +4 °C*		
	Sucrose	*Fructose*	*Lactose*	*Z*	*Sucrose*	*Lactose*	*Z*
SD of the slope g/100 g	0.014	0.017	0.022	0.054	0.044	0.043	0.042
Mean of means	10.3	15.6	15.0	33.3	25.2	25.9	24.7
CV (%)	0.1359	0.1100	0.1467	0.1622	0.1746	0.1660	0.1700

The combined and expanded uncertainties (Table 7.2) according to the GUM [1] for BCR-644 and -645 were then obtained as:

$$U_{CRM} = k \cdot \sqrt{u^2_{char} + u^2_{bb} + u^{\;2}_{lts}}\;;\; k=2$$

Table 7.2 - U_{CRM} (%) and uncertainty budget for each property in BCR-644 and -645 except free fructose in BCR-645, which was not certified. (Z) as glucose, including starch degradation products expressed as glucose.

	Uncertainty (rel. %)	*Free fructose (F)*	*Free sucrose (S)*	*Free lactose (L)*	*Starch/glucose(Z)*
BCR-644	U_{CRM}	6.36	2.32	1.82	3.42
	u_{char}	0.75	0.97	0.77	1.21
	u_{bb}	3.09	0.62	0.46	1.20
	u_{lts}	0.11	0.14	0.15	0.16
BCR-645	U_{CRM}	Not certified	2.79	6.35	3.47
	u_{char}		0.69	1.66	1.46
	u_{bb}		1.20	2.70	0.92
	u_{lts}		0.17	0.17	0.17

7.2 Certified values and their uncertainties

Based on the observed mean values and the expanded uncertainties the following certified values are obtained:

Table 7.2 - The certified values and their uncertainties(1) for BCR--644 and -645. Results are expressed in g/100 g as dry matter. (Z) as glucose, including starch degradation products expressed as glucose. Rounding was done according to ISO 31-0 [7]

	Free fructose (F)	*Free sucrose (S)*	*Free lactose (L)*	*Starch/glucose (Z)*
BCR-644	16.2 ± 1.1	10.81 ± 0.25	15.85 ± 0.29	35.1 ± 1.2
BCR-645	not certified	26.2 ± 0.8	27.8 ± 0.6	25.2 ± 0.9

(1) The uncertainty is expressed as combined uncertainties of characterisation, homogeneity study and stability testing according to the GUM [1]. A coverage factor of k = 2 has been used.

7.3 Supporting data

The laboratories L4, L11 and L13, L8 for BCR-644 only, sent results that were not used for the certification. These results are reported as supporting data. The laboratory L13 used a different method, described in Annex C (method C). The results are reported too as supporting data in Tables 7.3.1 and 7.3.2.

Table 7.3 – BCR-644 – Supporting data. Results are expressed in g/100 g as dry matter. (Z) as glucose, including starch degradation products expressed as glucose.

	Free fructose (F)	*Free sucrose (S)*	*Free lactose (L)*	*Starch/glucose (Z)*	*Methods*
L4	15.73 ± 0.18	10.13 ± 0.13	15.29 ± 0.23	33.83 ± 0.70	
L8	16.96 ± 0.14	11.05 ± 0.19	17.45 ± 0.33	37.18 ± 0.30	Annex C, A-B
L11	15.61 ± 0.20	10.96 ± 0.23	15.69 ± 0.30	34.02 ± 0.37	
L13	15.48 ± 0.05	9.60 ± 0.06	15.53 ± 0.17	35.00 ± 0.10	Annex C, C

Table 7.4 – BCR-645 – Supporting data. Results are expressed in g/100 g as dry matter. (Z) as glucose, including starch degradation products expressed as glucose.

	Free sucrose (S)	*Free lactose (L)*	*Starch/glucose (Z)*	*Methods*
L4	10.13 ± 0.13	15.29 ± 0.23	33.83 ± 0.70	
L8	11.05 ± 0.19	17.45 ± 0.33	37.18 ± 0.30	Annex C, A-B
L11	10.96 ± 0.23	15.69 ± 0.30	34.02 ± 0.37	
L13	9.60 ± 0.06	15.53 ± 0.17	35.00 ± 0.10	Annex C, C

8 INSTRUCTIONS FOR USE

BCR-644 and -645 can only be used to check the determination of the mass fraction of "*starch/glucose*", required for the application of the regulations 4056/87 and 4154/87 [2,3] on the basis of the method reported in Annex C.

BCR-644 can only be used to control the application of the method described in the regulations 4154/87 and 4056/87 in case of samples having a high (e.g. 40g/100 g) content of milk with high (e.g. 26 g/100 g) fat content. It will enable the verification of the efficiency of the hydrolysis conditions in case of difficult materials.

For BCR-644, the recommended minimum test portion size for the different analytical methods should be 5 g for the determination of free sugars and 2.2 g for the determination of starch/glucose.

BCR-645 may be used to control the application of the same method, in case of samples containing ingredients such as maltodextrins or milk with low fat content.

For BCR-645, the recommended minimum test portion size for the different analytical methods should be 4 g for the determination of free sugars and 2.4 g for the determination of starch/glucose.

8.1 Use of the certified values

These materials may be used to check the precision and the trueness of the laboratory measurement process according to ISO Guide 33 [8].

8.1.1 Assessment of precision

The precision of the measurement process is assessed by comparing the within-laboratory standard deviation determined during the certification step. All necessary equations are listed in detail in ISO Guide 33 [8].

8.1.2 Assessment of trueness

The trueness of the measurement process is checked by comparing the average $\bar{x}$ of n measurement results with the certified value, μ. The criterion for acceptance of the results is as follows:

$$-a_2 - 2\,\sigma_D \leq \bar{x} - \mu \leq a_1 + 2\,\sigma_D$$

- a_1 and a_2 are adjustment values chosen by the experimenter according to economical or technical limitations or stipulations.
- $2\,\sigma_D$ is the long term within-laboratory standard deviation of the user's method.

However, if the reference material is used for confirming a calibration, the value to be used for each parameter is the certified mean value with the uncertainty at the 95 % confidence level.

8.2 Correction for moisture content

The certified values are expressed on a dry matter basis. The dry mass measurement is to be

made in duplicate on a separate sample of the contents of the bottle immediately before the analytical test portion is taken. It is important that the samples for the dry matter and the analytical test portions are weighted at the same time to eliminate inaccuracies due to moisture pick up in very dry samples. The dry mass measurement is made by desiccation at 102 ± 1 °C until a constant mass is obtained on a test portion size weighted to the mg.

9 REFERENCES

[1] ISO-IEC-BIPM-IFCC-IUPAC-IUPAP-OIML (1993). *Guide to the Expression of Uncertainties in Measurement (GUM)*, Geneva, Switzerland (ISBN 92-67-10188-9).

[2] Commission of the European Communities, Commission Regulation (EEC) 4154/87 of 22 December 1987 laying down the methods of analysis and other technical provisions necessary for the implementation of Regulation (EEC) 3033/80 laying down the trade arrangements applicable to certain goods resulting from the processing of agricultural products, *Official Journal of the European Communities*, **L392**/87, 19-28.

[3] Commission of the European Communities, Commission Regulation (EEC) 4056/87 of 22 December 1987 laying down the methods of analysis and other technical provisions necessary for the implementation of Regulation (EEC) 3035/80 laying down general rules for granting export refunds on certain agricultural products exported in the form of goods not covered by Annex II to the Treaty, and the criteria for fixing the amount of such refunds, *Official Journal of the European Communities*, **L379**/87, 29-30.

[4] Commission of the European Communities, Commission Regulation (EC) 1460/96 of 25 July 1996 establishing the detailed rules for implementing the preferential trade arrangements applicable to certain goods resulting from the processing of agricultural products, as provided for in Article 7 of Council Regulation (EC) 3448/93, *Official Journal of the European Communities*, **L187**/96, 18-32.

[5] Commission of the European Communities. Directorate General Industry III/E/2. *Memorandum by G. Van Daele on improvements and validation of Analytical Methodologies for EC Customs Analysis.*

[6] Commission of the European Communities – Third Commission Directive 72/199/EEC of 27 April 1972 establishing Community methods of analysis for the official control of feedingstuffs. *Official Journal of the European Communities*, **L123**/72, 6-9.

[7] ISO-Standard 31 (1992). *Quantities – Part 0: General principles.* International Organization for Standardization, Geneva, Switzerland.

[8] ISO Guide 33 (1989). *Uses of certified reference materials*. International Organization for Standardization, Geneva, Switzerland.

10 ACKNOWLEDGEMENTS

Support from Prof. Dr. T. Gough (King's College London, London, GB) for the editing of the report and the certificates is gratefully acknowledged.

11 ANNEX A – HOMOGENEITY DATA

Table A.1 – BCR-644: Raw data for free sugars and starch/glucose obtained during the homogeneity study. Results are expressed in g/100 g.

Unit	*Free sucrose(S)*			*Free lactose(L)*			*Free fructose(F)*			*Unit*	*Starch/glucose(Z)*		
			mean			mean			mean				mean
1302	10.48	10.47	10.475	15.68	16	15.84	15.61	15.59	15.6	1906	32.5	32.1	32.3
165	10.35	10.27	10.31	16.08	15.7	15.89	15.55	15.54	15.545	18	32.7	32.5	32.6
1378	10.47	10.54	10.505	15.98	16.04	16.01	15.53	15.55	15.54	1663	32.6	32.4	32.5
227	10.25	10.26	10.255	14.72	15.33	15.025	15.40	15.31	15.355	818	32.1	32	32.05
1906	10.45	10.35	10.4	14.95	14.61	14.78	15.63	15.61	15.62	1347	32.8	31.9	32.35
707	10.34	10.3	10.32	14.38	14.53	14.455	15.43	15.44	15.435	1743	31.9	31.6	31.75
818	10.5	10.52	10.51	15.28	15.33	15.305	15.69	15.70	15.695	1587	32	32.2	32.1
1273	10.43	10.42	10.425	14.26	14.64	14.45	15.59	15.58	15.585	627	31.6	31.7	31.65
1743	10.38	10.4	10.39	15.12	14.88	15	15.68	15.67	15.675	1378	32.4	32.5	32.45
1505	10.48	10.47	10.475	15.36	15.09	15.225	15.67	15.72	15.695	178	31.7	31.8	31.75
982	10.38	10.41	10.395	15.31	15.21	15.26	15.59	15.62	15.605	2262	32.7	32.7	32.7
1347	10.43	10.41	10.42	15.69	16	15.845	15.54	15.52	15.53	1222	32.2	31.9	32.05
1106	10.54	10.61	10.575	15.1	15.08	15.09	15.75	15.78	15.765	1024	31.9	31.9	31.9
1665	10.3	10.27	10.285	15.89	15.76	15.825	15.47	15.52	15.495	1273	31.9	31.8	31.85
640	10.43	10.5	10.465	15.74	15.62	15.68	15.64	15.59	15.615	2383	32.3	32.3	32.3
178	10.36	10.63	10.495	15.54	15.5	15.52	15.56	15.50	15.53	982	32	32.1	32.05
738	10.36	10.43	10.395	15.48	15.43	15.455	15.51	15.59	15.55	707	31.7	31.9	31.8
1183	10.46	10.4	10.43	15.75	15.26	15.505	15.69	15.74	15.715	1503	32.1	32.1	32.1
867	10.29	10.33	10.31	15.07	14.68	14.875	15.47	15.52	15.495	387	33	33	33
498										98	31.6		31.6

Table A.2 – BCR-645: Raw data for free sugars and starch/glucose obtained during the homogeneity study. Results are expressed in g/100 g.

Unit	*Free sucrose(S)*			*Free lactose(L)*			*Starch/glucose(Z)*		
			mean			*mean*			*mean*
772	25.6	25.4	25.5	25.7	24.4	25.05	24.85	25.03	24.94
280	24.2	24.7	24.45	23.9	23.7	23.8	24.71	24.84	24.78
1100	24.3	24.5	24.4	23.8	24.1	23.95	24.93	24.87	24.90
1954	25.4	25.2	25.3	24.3	23.8	24.05	24.52	24.32	24.42
1534	24.7	24.1	24.4	24.4	24.5	24.45	24.77	24.96	24.87
1265	24.8	24.6	24.7	24.1	24.8	24.45	24.64	24.44	24.54
1345	25.9	25.7	25.8	24.9	25.2	25.05	24.56	24.72	24.64
2143	24.6	25	24.8	24.3	25	24.65	25.13	25.28	25.21
1676	25.3	25.1	25.2	24.8	24.7	24.75	24.60	24.61	24.61
1018	25	24.9	24.95	24.6	24.2	24.4	25.09	25.30	25.20
70	25.4	24.8	25.1	24.6	24.7	24.65	25.21	25.13	25.17
1512	25.3	25	25.15	24.4	24.3	24.35	25.55	25.64	25.60
48	25.2	24.9	25.05	24.4	23.9	24.15	25.37	25.30	25.34
485	25.2	25.4	25.3	25.8	24.3	25.05	25.30	25.46	25.38
55	25	24.8	24.9	24.4	25.6	25	25.16	25.19	25.18
1847	25.4	25.2	25.3	24.9	24.5	24.7	25.52	25.44	25.48
2352	25.1	24.6	24.85	24.8	24.3	24.55	25.22	24.10	24.66
78	24.8	24.7	24.75	24.8	24.9	24.85	25.66	25.52	25.59
2015	25.2	25.3	25.25	24.3	24.1	24.2	25.35	25.34	25.35
2307	25.2	25.3	25.25	26	25.2	25.6	24.97	24.92	24.95

12 ANNEX B – STABILITY DATA

Table B1 – Stability of free sugars and starch/glucose in BCR-644 using methods A and B.

TIME OF STORAGE (Months)	Free Fructose mass fraction (g/100g)						Free Sucrose mass fraction (g/100g)					
	STORAGE TEMPERATURE -18°C											
	1	% of -18°C value	2	% of -18°C value	mean value	% of -18°C value	1	% of -18°C value	2	% of -18°C value	mean value	% of -18°C value
0					16,20	100,00					9,70	100,00
3	15,55	100,00	15,9	100,00	15,73	100,00	10,40	100,00	10,15	100,00	10,28	100,00
6	15,75	100,00	15,85	100,00	15,80	100,00	10,25	100,00	10,35	100,00	10,30	100,00
9	16	100,00	16,05	100,00	16,03	100,00	10,55	100,00	10,65	100,00	10,60	100,00
12	15,27	100,00	15,29	100,00	15,28	100,00	10,20	100,00	10,15	100,00	10,18	100,00
	STORAGE TEMPERATURE 4°C											
	1	% of -18°C value	2	% of -18°C value	mean value	% of -18°C value	1	% of -18°C value	2	% of -18°C value	mean value	% of -18°C value
0					16,20	100,00					9,70	100,00
3	15,85	100,79	15,7	99,84	15,78	100,32	10,25	99,76	10,55	102,68	10,40	101,22
6	15,25	96,52	15,45	97,78	15,35	97,15	9,95	96,60	10,15	98,54	10,05	97,57
9	15,95	99,53	16,05	100,16	16,00	99,84	10,15	95,75	10,60	100,00	10,38	97,88
12	15,45	101,11	15,4	100,79	15,43	100,95	10,30	101,23	10,25	100,74	10,28	100,98
	STORAGE TEMPERATURE 4°C											
	1	% of -18°C value	2	% of -18°C value	mean value	% of -18°C value	1	% of -18°C value	2	% of -18°C value	mean value	% of -18°C value
0					16,20	100,00					9,70	100,00
3	15,75	100,16	15,85	100,79	15,80	100,48	9,85	95,86	10,05	97,81	9,95	96,84
6	15,3	96,84	15,45	97,78	15,38	97,31	10,05	97,57	10,20	99,03	10,13	98,30
9	16,15	100,78	16,15	100,78	16,15	100,78	10,55	99,53	10,50	99,06	10,53	99,29
12	15,45	101,11	15,4	100,79	15,43	100,95	10,25	100,74	10,20	100,25	10,23	100,49
	STORAGE TEMPERATURE 4°C											
	1	% of -18°C value	2	% of -18°C value	mean value	% of -18°C value	1	% of -18°C value	2	% of -18°C value	mean value	% of -18°C value
0					16,20	100,00					9,70	100,00
3	15,2	18,99	15,2	120,17	15,20	96,20	10,30	100,00	10,30	100,00	10,30	100,00
6	15,85	37,44	15,7	233,64	15,78	98,44	10,35	97,64	10,45	98,58	10,40	98,11
9	14,77	58,90	14,72	385,47	14,75	96,50	10,33	101,52	10,30	101,23	10,32	101,38

TIME OF STORAGE (Months)	Free Lactose mass fraction (g/100g)						Hydrolysed Glucose (Z) mass fraction (g/100g)					
	STORAGE TEMPERATURE -18°C											
	1	% of -18°C value	2	% of -18°C value	mean value	% of -18°C value	1	% of -18°C value	2	% of -18°C value	mean value	% of -18°C value
0					14,30	100,00					32,20	100,00
3	16,65	100,00	15,85	100,00	16,25	100,00	32,40	100,00	32,75	100,00	32,58	100,00
6	15,85	100,00	15,6	100,00	15,73	100,00	34,75	100,00	34,75	100,00	34,75	100,00
9	15,05	100,00	14,75	100,00	14,90	100,00	33,05	100,00	33,05	100,00	33,05	100,00
12	15,45	100,00	15,25	100,00	15,35	100,00	32,05	100,00	33,20	100,00	32,63	100,00
	STORAGE TEMPERATURE 4°C											
	1	% of -18°C value	2	% of -18°C value	mean value	% of -18°C value	1	% of -18°C value	2	% of -18°C value	mean value	% of -18°C value
0					14,30	100,00					32,20	100,00
3	15,00	92,31	15,85	97,54	15,43	94,92	32,40	99,46	32,75	100,54	32,58	100,00
6	14,95	95,07	15,10	96,03	15,03	95,55	34,90	100,43	34,65	99,71	34,78	100,07
9	14,90	100,00	14,45	96,98	14,68	98,49	33,30	100,76	33,50	101,36	33,40	101,06
12	14,90	97,07	15,15	98,70	15,03	97,88	32,40	99,31	32,65	100,08	32,53	99,69
	STORAGE TEMPERATURE 25°C											
	1	% of -18°C value	2	% of -18°C value	mean value	% of -18°C value	1	% of -18°C value	2	% of -18°C value	mean value	% of -18°C value
0					14,30	100,00					32,20	100,00
3	16,4	100,92	16,6	102,15	16,50	101,54	32,20	98,85	32,65	100,23	32,43	99,54
6	14,85	94,44	14,3	90,94	14,58	92,69	34,60	99,57	34,85	100,29	34,73	99,93
9	14,45	96,98	15,3	102,68	14,88	99,83	32,75	99,09	33,20	100,45	32,98	99,77
12	15,3	99,67	15,1	98,37	15,20	99,02	32,80	100,54	33,05	101,30	32,93	100,92
	STORAGE TEMPERATURE 40°C											
	1	% of -18°C value	2	% of -18°C value	mean value	% of -18°C value	1	% of -18°C value	2	% of -18°C value	mean value	% of -18°C value
0					14,30	100,00					32,20	100,00
3	15,5	98,57	15,5	98,57	15,50	98,57	32,80	94,39	33,20	95,54	33,00	94,96
6	14,7	98,66	14,85	99,66	14,78	99,16	33,15	100,30	33,25	100,61	33,20	100,45
9	14,76	96,16	14,72	95,90	14,74	96,03	34,25	104,98	34,30	105,13	34,28	105,06

Table B2 – Stability of free sugars and starch/glucose in BCR-645 using methods A and B.

TIME OF STORAGE (Months)	**Free sucrose mass fraction (g/100g)**						**Free lactose mass fraction (g/100g)**					
STORAGE TEMPERATURE -18°C												
	1	% of -18°C value	2	% of -18°C value	mean value	% of -18°C value	1	% of -18°C value	2	% of -18°C value	mean value	% of -18°C value
0					24,99	100,00					24,56	100,00
3	23,70	100,00	24,75	100,00	24,23	100,00	24,95	100,00	25,10	100,00	25,03	100,00
6	25,45	100,00	25,50	100,00	25,48	100,00	25,30	100,00	25,25	100,00	25,28	100,00
9	26,00	100,00	26,45	100,00	26,23	100,00	26,05	100,00	27,30	100,00	26,68	100,00
12	24,90	100,00	24,88	100,00	24,89	100,00	27,35	100,00	27,08	100,00	27,22	100,00
STORAGE TEMPERATURE 4°C												
	1	% of -18°C value	2	% of -18°C value	mean value	% of -18°C value	1	% of -18°C value	2	% of -18°C value	mean value	% of -18°C value
0					24,99	100,00					24,56	100,00
3	23,6	3,00	24,45	100,93	24,03	99,17	24,10	96,30	25,60	102,30	24,85	99,30
6	25	6,00	25,8	101,28	25,40	99,71	25,65	101,48	25,60	101,29	25,63	101,38
9	26,7	9,00	26,2	99,90	26,45	100,86	26,75	100,28	26,40	98,97	26,58	99,63
12	24,91	12,00	25	100,44	24,96	100,26	26,50	97,37	26,80	98,48	26,65	97,92
STORAGE TEMPERATURE 25°C												
	1	% of -18°C value	2	% of -18°C value	mean value	% of -18°C value	1	% of -18°C value	2	% of -18°C value	mean value	% of -18°C value
0					24,99	100,00					24,56	100,00
3	25,4	104,85	24,4	100,72	24,90	102,79	27,00	107,89	24,75	98,90	25,88	103,40
6	25,5	100,10	25,55	100,29	25,53	100,20	25,85	102,27	25,00	98,91	25,43	100,59
9	25,95	98,95	26,3	100,29	26,13	99,62	26,25	98,41	26,30	98,59	26,28	98,50
12	25	100,44	24,9	100,04	24,95	100,24	26,90	98,84	27,05	99,39	26,98	99,12
STORAGE TEMPERATURE 40°C												
	1	% of -18°C value	2	% of -18°C value	mean value	% of -18°C value	1	% of -18°C value	2	% of -18°C value	mean value	% of -18°C value
0			% of -18°C value	% of -18°C value	24,99	100,00					24,56	100,00
3	25,6	100,49	25,6	100,49	25,60	100,49	27,00	106,82	26,60	105,24	26,80	106,03
6	26,05	99,33	25,95	98,95	26,00	99,14	26,40	98,97	25,70	96,34	26,05	97,66
9	25,1	100,84	25,2	101,25	25,15	101,04	25,8	94,80	25,60	94,07	25,70	94,43

TIME OF STORAGE (Months)	**Hydrolysed Glucose (Z) mass fraction (g/100g)**					
STORAGE TEMPERATURE -18°C						
	1	% of -18°C value	2	% of -18°C value	mean value	% of -18°C value
0					24,63	100,00
3	24,80	100,00	24,00	100,00	24,40	100,00
6	27,50	100,00	27,20	100,00	27,35	100,00
9	23,50	100,00	22,70	100,00	23,10	100,00
12	23,06	100,00	23,32	100,00	23,19	100,00
STORAGE TEMPERATURE 4°C						
	1	% of -18°C value	2	% of -18°C value	mean value	% of -18°C value
0					24,63	100,00
3	24,75	101,43	25,75	105,53	25,25	103,48
6	25,00	91,41	25,90	94,70	25,45	93,05
9	23,90	103,46	23,50	101,73	23,70	102,60
12	24,50	105,65	23,35	100,69	23,93	103,17
STORAGE TEMPERATURE 25°C						
	1	% of -18°C value	2	% of -18°C value	mean value	% of -18°C value
0					24,63	100,00
3	24,85	101,84	23,80	97,54	24,33	99,69
6	22,80	83,36	23,30	85,19	23,05	84,28
9	23,90	103,46	23,50	101,73	23,70	102,60
12	24,50	105,65	23,35	100,69	23,93	103,17
STORAGE TEMPERATURE 40°C						
TIME OF STORAGE (Weeks)	1	% of -18°C value	2	% of -18°C value	mean value	% of -18°C value
0					24,63	100,00
3	24,7	90,31	24,9	91,04	24,80	90,68
6	23,7	102,60	23,25	100,65	23,48	101,62
9	25,45	109,75	25,45	109,75	25,45	109,75

13 ANNEX C – METHODS FOR SUGARS AND STARCH/GLUCOSE

METHOD A

DETERMINATION OF FREE SUGARS (GLUCOSE, FRUCTOSE, SUCROSE, MALTOSE, LACTOSE) CONTENT OF PROCESSED FOOD PRODUCTS

1 Scope and field of application

The method allows determination of the content of free sugars in processed food products

2 Principle

The sugars in the sample are extracted by using water and measured by HPLC with external calibration.

3 Reagents

Water used should be double-distilled or equivalent grade. Chemicals should be of analytical grade.

3.1 D-Fructose (anhydrous)

3.2 D-Glucose (anhydrous)

3.3 Sucrose (anhydrous)

3.4 Maltose (monohydrate)

3.5 Lactose (monohydrate)

3.6 Acetonitrile

3.7 Carrez I solution (dissolve 23.8 g zinc acetate dihydrate and 3 g glacial acetic acid in water and make up to 100 mL).

3.8 Carrez II solution (dissolve 10.8 g potassium II hexacyanoferrate trihydrate in water and make up to 100 mL).

4 Apparatus

4.1 Usual glass laboratory equipment

4.2 Filter, disposable, 0.2 µm

4.3 Magnetic stirrer

4.4 High-performance liquid chromatography equipment including

- 0.2 µm filter for mobile phase solvents
- high-pressure pump
- injector, manual or automatic
- detector, refractive index type
- integrator recorder
- column heater
- a precolumn and analytical column capable of separating the 5 sugars (3.1 to 3.5) with a chromatographic resolution of the peaks of the least-well separated compounds of R= 1.R is traditionally defined by the equation

$$R = \frac{2\Delta t}{w_1 + w_2}$$

Where Δt is the interval between the apexes of the two peaks in question and w_1 and w_2 are the base widths of the peaks, measured in the same units.

This separation can be achieved using an aminopropyl column, for example, with the proportion of water to acetonitrile being adjusted around the ratio of 15:85, depending on the state of the column.

NB1: All the solutions injected into the chromatograph must be filtered just before injection using a 0.2 µm porosity filter (disposable or non-disposable).

5 Procedure

5.1 Preparation of calibration solution; calibration

Weigh out to the nearest milligram a 0.5 g sample of each of the sugars 3.1 to 3.5 in the 100 mL calibrated flask. Add 50 mL of double-distilled water. Shake until the sugars are completely dissolved and make up to the mark on the flask. Kept refrigerated, the solution will remain stable for 5 days.

5.2 Resolution test

Following stabilisation of the chromatographic equipment, inject 20 µL of the standard sugar solutions (5.1). After elution of all the components, check the resolution in the manner described in 4.4. In order for the calculation to be valid, it is advisable to increase considerably the speed at which the paper travels through the recorder. This test should be carried out if the performance of the column begins to suffer because of wear.

NB2: The test also makes it possible to assess the performance of a new column by calculating the number of theoretical plateaus for a given component, using the equation

$$N_{eff} = 16\left(\frac{t_r}{w}\right)^2$$

Where t_r is the retention time of the component and w the base width of its peak, with both values expressed in the same unit. A record of the result should be kept in order to assess subsequent wear on the column.

5.3 Preparation of the laboratory samples

Before testing, samples should be homogenised using the method (grinder, mixer, processor) most suited to their consistency.

5.4 Preparation of solutions

Weigh out to the nearest milligram a sample of between 1 g and 5 g (depending on the declared or presumed sugar content) in a 200 mL calibrated flask. Add approximately 150 mL water. Leave at ambient temperature for at least one hour, swirling from time to time. Add, if necessary (particularly in the presence of proteins) in succession, 1 or 2 mL of the Carrez I and II solutions (3.7 and 3.8). Adjust the level of the solution, swirl and filter all or part of the solution (4.2). The filtrate is the test solution.

5.5 Chromatography of calibration and test solutions

As the refractive index detector provides a peak area response which is proportional to the concentration of each of the sugars, the calibration should be carried out as follows: inject 20 µL (or 0.5 µL accordingly to the equipment) standard solution (5.1), and repeat at the beginning of each series of tests. Check the uniformity of this calibration as time progresses by injecting some of the standard solution (5.1) once for every 5^{th} or 6^{th} injection of test solution.

Analyse the test solutions in the same conditions as for the calibration, with two injections of 20 µL of each solution.

NB3: If the sugar concentration in the samples differs widely from that of each of the calibration solutions, prepare a new, more suitable, standard by adjusting the mass weighed out.

5.6 Calculation and expression of results

Calculate the G-F-S-M-L content (in g) of each of the sugars in g/100 g of sample, using the formula

$$G\ (\text{or } F, S, M, L) = \frac{A_i \cdot V \cdot C_i}{a_i \cdot m}$$

where A_i is the peak area for sugar i in the test solution;
a_i is the peak area for sugar i in the calibration solution;
C_i is the concentration of sugar i (in g) in the calibration solution (in g/100 mL);
V is the volume (in mL) in which sample m (in g) was diluted.

The sugar content is expressed in term of anhydrous glucose, fructose and sucrose, and of lactose and

maltose monohydrates.

METHOD B

DETERMINATION OF THE AMOUNT OF STARCH AND ITS DEGRADATION PRODUCTS, INCLUDING GLUCOSE, IN PROCESSED FOOD PRODUCTS

1 Scope and field of application

(a) The method allows the determination of the content of starch and its degradation products, including glucose, i.e. "*starch/glucose*".

(b) The "*starch/glucose*" content referred to above is equal to the value A as calculated according to 5.4 of this method.

2 Principle

The starch in the sample is degraded into water-soluble dextrins and oligosaccharides by the action of a first, thermally stable, enzyme (a-amylase). This takes place in a water bath. The degradation products are then completely hydrolysed to glucose by a second enzyme (amyloglucosidase) at 60 °C. The freed glucose is measured by high-performance liquid chromatography using external calibration.

3 Reagents

Water used should be double-distilled or equivalent grade. Chemicals should be of analytical grade.

3.1 Thermally stable a-amylase solution

Enzyme solution having a-amylase activity of at least 50 U/mg, retaining this activity at 100 °C. Commercial product such as A3306 (Sigma) or thermamyl 60 L (Novo-Nordisk) generally fits.

3.2 Dilute acetic acid (ca. 5 % glacial acetic acid in water).

3.3 Amyloglucosidase (EC 3.2.1.3) from *Aspergillus niger*. Its activity must be checked every month.

3.4 Amyloglucosidase (3.3) solution, 1500 U/mL, prepared immediately before use.

3.5 Carrez I solution (dissolve 23.8 g zinc acetate dihydrate and 3 g glacial acetic acid in water and make up to 100 mL).

3.6 Carrez II solution (dissolve 10.8 g potassium II hexacyanoferrate trihydrate in water and make up to 100 mL).

3.7 HPLC-grade acetonitrile

3.8 D-Glucose (anhydrous)

4 Apparatus

4.1 Usual glass laboratory equipment

4.2 Water bath at 100 °C

4.3 Magnetic stirrer with bath, maintained at 60 °C by thermostat

4.4 High-performance liquid chromatography equipment, including

- high-pressure pump
- injector, manual or automatic
- detector, refractive index type
- integrator recorder
- column heater
- a column suitable for analysing glucose (the column used for determining free sugar content, for example)

4.5 Inert disposable filter, 0.2 µm porosity

4.6 pH meter

5 Procedure

5.1 Preparation of the laboratory samples

Before testing, samples should be homogenised using the method (grinder, mixer, processor) most suited to their consistency. Step 5.2.1. should be omitted for liquid or entirely water-soluble samples.

In a 250 mL Erlenmeyer flask, weigh out to the nearest mg a mass of the sample likely on hydrolysis to yield approximately 0.2 g glucose. Add 100 mL water.

5.2 Preparation of test solutions

5.2.1 Liquefaction of starch

Add 0.2 mL of the thermally stable a-amylase solution (3.1). Cover with aluminium foil, swirl, place in the water bath (4.2) and leave for one hour, swirling once per minute for the first 5 minutes. Remove from the water bath and leave to cool at ambient temperature.

5.2.2 Hydrolysis

Adjust the pH to between 4.6 and 4.8 with the dilute acetic acid (3.2). Place a magnetised bar in the flask and place the flask in the water bath (at 60 °C) with the stirrer on. Add 1 mL of the enzyme solution (3.4) and leave to take effect for 30 min with the stirrer at moderate speed.

Transfer the content of the flask, when cool, into a 200 mL calibrated flask. Add, if necessary (particularly in the presence of proteins) in succession, 1 or 2 mL of the Carrez I and II solutions (3.5 and 3.6). Adjust the level of the solution; swirl and filter all or part of the solution (see 4.4). The filtrate is the test solution.

5.3 Chromatography of test solutions

Determine the glucose content of the test solutions using the same method applied for the determination of free sugar content. As fructose, sucrose and lactose should be left intact by this enzymatic hydrolysis, care should be taken:

- to wait for any lactose peak to emerge;

- to carry out chromatography under the best possible conditions of resolution between the glucose and fructose peaks (R = 1).

5.4 Calculation and expression of results

Calculate the total glucose content (Z) (after hydrolysis) in g/100 g of unaltered sample (the result is in the form of anhydrous glucose content), as shown in 5.6 of the method for free sugars (method A).

The starch/glucose content A of the sample, expressed in g/100 g, is given by one or other of the formulae below:

$$A = (Z - F) \cdot 0.9$$

If the free glucose content G is greater than or equal to the free fructose content F,

$$A = (Z - G) \cdot 0.9$$

If the free glucose content is less than the free fructose content F. The values for G and F are determined by HPLC accordingly to the method for free sugars (method A).

6 Observation

The control of the efficacy of the enzymes is very important. The best method to verify this efficacy is:

Before a new series of analysis or after the acquisition of new reagents or every 6 months, proceed to the analyse of pure starch with this method and with the Ewers method (D.72/199/EC, ECOJ n°L 123, 29/5/72. The difference between the two results should not exceed 2 %. If the result of the enzymatic method is too weak, the activity of one (or two) enzyme is probably insufficient.

METHOD C

DETERMINATION OF THE AMOUNT OF STARCH AND ITS DEGRADATION PRODUCTS , INCLUDING GLUCOSE , IN PROCESSED FOOD PRODUCTS

1 Purpose and fields of application

The method permits the determination of the starch content, its degradation products including glucose, hereafter referred as "starch" and sucrose, invert sugar and iso-glucose hereafter referred to as sucrose.

2 Principle

Starch is hydrolysed to glucose using thermostable a–amylase and amyloglucosidase. Sucrose is hydrolysed to fructose and glucose using ß-fructosidase (invertase). The glucose and fructose are determined using HPLC and a cation exchange column (Ca form).

3. Reagents

Use double-distilled water.

3.1 Thermally stable a-amylase solution

Enzyme solution having a-amylase activity of at least 50 U/mg, retaining this activity at 100 °C. Commercial products such as A3306 (Sigma) or thermamyl 60 L (Novo-Nordisk) generally fit.

3.2 Solution of amyloglucosidase/ß-fructosidase (immediately before use, dissolve approximately 10 mg of amyloglucosidase (EC 3.2.1.3) (6 U/mg) and 1 mg ß-fructosidase/mL in water).

3.3 Carrez I solution (dissolve 23.8 g zinc acetate dihydrate and 3 g glacial acetic acid in water and make up to 100 mL).

3.4 Carrez II solution (dissolve 10.6 g potassium II hexacyanoferrate trihydrate in water and make up to 100 mL).

3.5 D-Fructose (anhydrous)

3.6 D-Glucose (anhydrous)

3.7 Dilute acetic acid (ca. 5 % glacial acetic acid in water)

3.8 HPLC standard solution

Weigh to the nearest mg 0.1 g each of fructose and glucose into a 100 mL volumetric flask. Add 50 mL of water, shake to dissolve sugars and make up to the mark. Solution is stable for 5 days in the fridge.

4 Apparatus

4.1 Magnetic stirrer with water bath at 60 °C.

4.2 Water bath at 100 °C.

4.3 Magnetic rods.

4.4 High-performance liquid chromatography equipment including

- Refractive index detector
- Column heater capable of maintaining column at 90 °C or at column manufacturers recommended temperature
- Cation exchange column calcium form

5 Method

5.1 Starch is hydrolysed using thermostable a-amylase into dextrins and then hydrolysed into glucose units with amyloglucosidase. Sucrose is hydrolysed into fructose and glucose.

Before testing, samples should be homogenised using the method (grinder, mixer, processor) most suited to their consistency. Steps 5.1.3 and 5.1.4 should be omitted for liquid or entirely water-soluble samples.

5.1.1 Accurately weigh between 0.2 and 1 g of sample, depending on expected starch/sugar content, into a

250 mL Erlenmeyer flask.

5.1.2 Add ca. 100 mL of water and swirl to disperse the sample.

5.1.3 Add 0.1 mL thermostable a-amylase (3.1).

5.1.4 Cover with aluminium foil and place in a boiling water bath for 60 min.

5.1.5 After cooling, adjust pH between 4.6 and 4.8 using dilute acetic acid. Depending on the column, the sodium salt of the acid may interfere with a peak of interest, so it is necessary to check with a blank that there is no interference. Salts of inorganic acids elute much earlier than organic acids, so a suitable acid can be selected for a given column.

5.1.6 Place in the water bath (4.1) with a magnetic stirrer (4.2) at 60 °C, add 1 mL of enzyme solution (3.2.) and allow to react for 30 min whilst stirring continuously.

5.1.7 After cooling, transfer quantitatively to a 200 mL graduated flask, add 3 mL each of Carrez I and Carrez II solution (Note: DO NOT exceed this quantity of Carrez solutions as it will interfere with the chromatography; on some columns the peak due to Carrez may co-elute with some of the peaks of interest in which Carrez solutions should not be used) and make up to the mark with water, shake and filter. The filtrate is the test solution.

5.2 Quantitative determination of glucose and fructose HPLC

5.2.1 HPLC conditions

The following conditions have been found to be successful:

Mobile phase:	HPLC grade water
Column temperature:	90 °C
Flow rate:	0.4 mL/min
Injection volume:	20 µL

5.2.2 Calibration

Use solution (3.8) to calibrate the HPLC equipment. The glucose and fructose peaks should be completely resolved.

5.2.3 Chromatography of test solutions

Filter solution through a 0.2 µm filter and inject 20 µL into the chromatograph under the same conditions as the standard solution.

5.2.4 Calculation of fructose and glucose contents

Calculate the glucose and fructose contents of the sample using the following formula:

$$\textit{glucose or fructose in g/100 g} = \frac{a_i \cdot V \cdot C_i}{A_i \cdot M}$$

where A_i is the peak area for sugar i in the standard solution;
a_i is the peak area for sugar i in the test solution;
V is the volume of the test solution (in mL);
M is the weight (in g) of the test sample;
C_i is the concentration of sugar i in test solution (in g/100 mL).

6. Calculation of starch and sucrose contents

The starch/glucose content is given by the following formula:

starch = (glucose – fructose) x 0.9

The sucrose/invert/isoglucose content is given by the following formula:

sucrose = fructose x 1.9

In the rare cases where free fructose content is declared to exceed free glucose, it will be necessary to

determine the free fructose and glucose contents of the sample and calculate the correction factor D and determine the starch and sucrose contents using the following formulae:

$$D = (\text{free fructose}) - (\text{free glucose})$$

$$\text{starch} = (\text{glucose} - \text{fructose} + D) \times 0.9$$

$$\text{sucrose} = (\text{fructose} - D) \times 1.9$$

14 ANNEX D –ACCEPTED RESULTS

Table D.1 – BCR-644: Bar-charts (mean of means and 95 % confidence interval) of results for free fructose accepted for certification.

DATA SET	NUM	REPLICATES.....			MEAN	ST.DEV
L1	6	15.9520	15.8110	15.8060		
		15.6340	15.8310	15.7850	15.8032	0.1019
L2	6	16.1050	15.9100	15.6000		
		16.3600	16.3400	15.8800	16.0325	0.2941
L3	6	16.1680	16.2260	15.6780		
		15.7470	16.6600	16.2110	16.1150	0.3599
L4	6	15.8150	15.4440	15.8280		
		15.5510	15.8230	15.8920	15.7255	0.1819
L5	6	16.0490	15.9790	15.9960		
		15.9780	15.9400	15.8800	15.9703	0.0567
L6	6	16.4370	16.5890	16.6260		
		16.5040	16.5050	16.8180	16.5798	0.1347
L8	6	17.1170	17.0780	17.0400		
		16.7940	16.9550	16.7980	16.9637	0.1405
L9	6	16.0570	16.1220	15.7740		
		16.0800	15.9530	15.9830	15.9948	0.1249
L10	6	16.4500	16.7500	16.4500		
		16.6000	16.9500	16.6500	16.6417	0.1908
L11	6	15.3880	15.7750	15.8980		
		15.4530	15.6240	15.5140	15.6087	0.1967
L12	6	16.1570	16.2510	16.2340		
		16.1520	16.3030	16.2920	16.2315	0.0649
L13	6	15.5281	15.4985	15.5169		
		15.4761	15.4643	15.3832	15.4879	0.0476

Table D.2 – BCR-644: Bar-charts (mean of means and 95 % confidence interval) of results for free lactose accepted for certification.

DATA SET	NUM	REPLICATES.....			MEAN	ST.DEV
L1	6	15.4110	15.3000	15.8210		
		16.0990	16.0530	16.1040	15.7980	0.3599
L2	6	15.8650	15.5850	15.2300		
		15.9100	15.5950	15.4800	15.6108	0.2519
L3	6	15.9070	16.0330	16.3980		
		15.5280	15.8450	16.4590	16.0283	0.3524
L4	6	15.4340	15.1320	15.2190		
		14.9360	15.5100	15.5000	15.2885	0.2318
L5	5	16.3410	16.4650	16.3820		
		16.4130	16.2540		16.3710	0.0795
L6	6	15.1660	15.7020	15.8050		
		15.7730	15.6370	15.4850	15.5947	0.2388
L8	6	17.0620	17.3210	17.6460		
		17.1610	17.5800	17.9490	17.4532	0.3332
L9	6	15.3290	15.6520	15.1680		
		15.8130	15.4890	15.5980	15.5082	0.2323
L10	6	16.1500	16.8500	16.4000		
		16.1500	17.1500	15.8000	16.4167	0.4997
L11	6	15.7350	16.0230	15.1770		
		15.7950	15.5270	15.8990	15.6927	0.3024
L13	5	15.6580	15.6114	15.6815		
		15.4432	15.2360		15.5260	0.1672

Table D.3 – BCR-644: Bar-charts (mean of means and 95 % confidence interval) of results for free sucrose accepted for certification.

DATA SET	NUM	REPLICATES.....			MEAN	ST.DEV
L1	6	10.5290	10.5750	10.4330		
		10.3010	10.4020	10.5940	10.4723	0.1135
L2	6	10.9550	10.7200	10.6900		
		10.8600	10.8500	10.6300	10.7842	0.1233
L3	6	10.3640	10.7270	11.0030		
		10.9750	10.7730	10.8440	10.7810	0.2314
L4	6	10.2040	9.9490	10.0410		
		10.0680	10.2940	10.2190	10.1292	0.1302
L5	6	10.5930	10.7800	10.7700		
		10.7550	10.7630	10.6460	10.7178	0.0784
L6	6	10.9640	11.4930	11.4040		
		11.3890	11.3150	11.2190	11.2973	0.1874
L8	6	11.2370	11.0010	10.9920		
		11.2980	11.0330	10.7550	11.0527	0.1947
L9	6	10.4800	10.4030	10.4700		
		10.5820	10.2350	10.6180	10.4647	0.1372
L10	6	11.3000	11.2000	11.2000		
		11.1500	11.0500	11.1500	11.1750	0.0822
L11	6	10.7120	10.7140	11.0370		
		10.9720	10.9870	11.3320	10.9590	0.2312
L12	6	10.7550	10.7990	10.8390		
		10.8470	10.7910	10.8890	10.8200	0.0477
L13	5	9.5690	9.6448	9.6723		
		9.5013	9.6227		9.6020	0.0607

Table D.4 – BCR-644: Bar-charts (mean of means and 95 % confidence interval) of results for starch/glucose accepted for certification.

DATA SET	NUM	REPLICATES.....			MEAN	ST.DEV
L1	6	34.2600	34.5580	34.7450		
		33.8000	34.6040	34.2150	34.3637	0.3440
L2	6	34.5600	35.2800	35.3750		
		34.1800	34.0700	34.2150	34.6133	0.5778
L3	6	35.7920	35.7950	35.8640		
		35.1920	36.4980	35.4710	35.7687	0.4390
L4	6	34.4700	33.2290	33.2640		
		33.0860	34.5120	34.4100	33.8285	0.6995
L5	6	35.4410	35.2450	35.1000		
		35.2250	35.0780	34.9770	35.1777	0.1627
L6	6	33.2360	32.4150	33.6330		
		33.5210	33.2820	33.3880	33.2458	0.4330
L8	6	36.9650	37.6100	36.8450		
		37.2470	37.4550	36.9870	37.1848	0.3035
L9	6	36.3510	35.9310	35.4400		
		35.4910	35.2720	37.0050	35.9150	0.6629
L10	6	33.4500	33.2000	33.5000		
		32.6500	33.7000	33.0000	33.2500	0.3821
L11	6	33.8290	34.1330	34.3390		
		33.4790	34.4890	33.8800	34.0248	0.3696
L12	6	36.2190	36.3080	36.6860		
		36.5510	36.8860	37.0610	36.6185	0.3263
L13	5	35.0529	34.9404	34.9293		
		34.9060	35.1735		35.0004	0.1003

Table D.5 – BCR-645: Bar-charts (mean of means and 95 % confidence interval) of results for free sucrose accepted for certification.

DATA SET	NUM	REPLICATES.....			MEAN	ST.DEV
L02	6	25.3010	25.1040	25.5430		
		25.4060	25.5030	24.9410	25.2997	0.2361
L03	6	25.6240	26.0420	26.0950		
		26.4730	26.1050	25.7750	26.0190	0.2951
L04	6	25.7120	25.6460	25.6710		
		25.7430	25.4980	25.6150	25.6475	0.0863
L05	6	26.6220	26.5910	26.3100		
		26.2660	26.0970	26.5620	26.4080	0.2142
L06	6	26.0000	26.3600	26.4000		
		25.8100	26.4900	26.5350	26.2658	0.2926
L07	6	26.7740	27.3170	27.0590		
		26.6630	26.7370	26.8630	26.9022	0.2446
L08	6	25.5310	25.7060	25.2280		
		25.5510	26.9690	25.4280	25.7355	0.6246
L09	6	27.2000	27.2500	27.0500		
		26.0400	27.0500	26.6500	26.8733	0.4594
L10	6	26.4320	26.1050	26.5080		
		25.4360	26.2040	26.2810	26.1610	0.3844
L11	6	27.3070	26.9190	26.4810		
		26.3530	27.3650	27.0060	26.9052	0.4167
L12	6	26.3920	26.2090	26.4770		
		26.3370	26.0920	26.6290	26.3560	0.1909
L13	6	25.9664	25.9660	25.8677		
		25.5951	26.4322	26.4958	26.0539	0.3159

Table D.6 – BCR-645: Bar-charts (mean of means and 95 % confidence interval) of results for free lactose accepted for certification.

DATA SET	NUM	REPLICATES.....			MEAN	ST.DEV
L02	6	29.3890	29.2370	28.7450		
		28.9490	28.8360	29.4120	29.0947	0.2891
L03	6	28.3340	27.4180	27.6370		
		27.5870	27.5020	26.9820	27.5767	0.4386
L04	6	28.0820	28.0260	28.3270		
		27.9960	28.4540	28.5660	28.2418	0.2408
L05	6	28.5720	26.6360	28.7860		
		30.3000	27.7680	26.7740	28.1393	1.3810
L06	6	27.5500	26.8850	27.7400		
		26.1500	27.7100	26.8950	27.1550	0.6256
L07	6	30.3460	30.3970	29.9770		
		30.2610	30.7930	29.8950	30.2782	0.3230
L08	6	26.3290	26.0590	26.2750		
		26.1670	27.4650	26.6540	26.4915	0.5176
L09	6	26.9000	26.9500	26.4500		
		27.3500	26.6000	26.0500	26.7167	0.4513
L10	6	27.1500	27.0410	27.4310		
		26.9920	27.4860	27.4870	27.2645	0.2296
L11	6	25.4750	25.2200	24.7580		
		24.9960	25.6080	25.9030	25.3267	0.4189
L12	6	26.8130	26.6650	27.3830		
		27.2130	26.6080	27.6590	27.0568	0.4261
L13	6	26.5906	26.6174	26.5531		
		25.9766	26.7388	26.8208	26.5495	0.2720

Table – BCR-645: Bar-charts (mean of means and 95 % confidence interval) of results for starch/glucose accepted for certification.

DATA SET	NUM	REPLICATES.....			MEAN	ST.DEV
L02	6	24.8740	25.0810	25.1680		
		24.6570	23.1230	24.2900	24.5322	0.7593
L03	6	25.4340	25.2600	25.3800		
		25.3150	25.2150	25.3990	25.3338	0.0853
L04	6	25.3100	25.3050	24.9240		
		25.6410	25.6460	25.6970	25.4205	0.2993
L05	6	24.7290	25.2700	25.4480		
		25.1010	25.1300	26.3620	25.3400	0.5542
L06	6	25.7850	24.8650	25.9350		
		24.9600	25.5750	24.7450	25.3108	0.5150
L07	6	25.2410	25.2620	25.2100		
		25.3990	24.9600	25.3680	25.2400	0.1559
L08	6	27.0630	26.5190	26.2820		
		26.5620	27.2430	27.0520	26.7868	0.3826
L09	6	23.2000	23.2000	22.5000		
		22.5500	22.5500	22.4000	22.7333	0.3656
L10	6	20.0410	21.4950	20.4750		
		20.1090	19.0230	21.7080	20.4752	0.9991
L11	6	24.0440	23.8430	23.9020		
		22.8630	23.7450	23.9090	23.7177	0.4299
L12	6	25.9330	26.0350	25.8550		
		25.8790	26.1140	26.1480	25.9940	0.1233
L13	6	25.4475	25.5564	25.4491		
		25.2291	25.9311	25.9166	25.5883	0.2564

European Commission

EUR 20987 – DG Joint Research Centre, Institute for Reference Materials and Measurements –
Method-specific certification of free sugars and starch/glucose in two artificial food materials
Authors: C. Briffaut, J.-M. Lansiaux, M. Marcault, G.N. Kramer, T. Linsinger, B.M. Gawlik, A. Bernreuther, A. Lamberty, A. Boenke
Luxembourg: Office for Official Publications of the European Communities
2004 – 48 pp. –21.0 x 29.7 cm
Scientific and Technical Research series
ISBN 92-894-6871-8

Abstract

This report describes the work performed to prepare two reference materials containing sugars, starch and starch degradation products, and certify them by two methods of analysis. The first method consists in the determination of free sugars (sucrose, fructose and lactose) in food by liquid chromatography. The second method consists in an enzymatic hydrolysis of starch and starch degradation products to glucose, and then in the determination of glucose by liquid chromatography. The materials are powders consisting in mixtures of milk powder, starch, sugars and dextrins. The reference materials can only be used to check the application of the methods A and B described in the Annex C of the report.
Certified values were accompanied by an expanded uncertainty according to the requirements laid down in the Guide for the Expression of Uncertainty in Measurement (GUM) [1].

The mission of the Joint Research Centre is to provide customer-driven scientific and technical support for the conception, development, implementation and monitoring of European Union policies. As a service of the European Commission, the JRC functions as a reference centre of science and technology for the Community. Close to the policy-making process, it serves the common interest of the Member States, while being independent of commercial and national interests.

EUROPEAN COMMISSION
DG JOINT RESEARCH CENTRE
Joint Research Centre

15 LA-NA-20987-EN-C

Publications Office
Publications.eu.int