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Simplified Enzymatic High-Performance Anion Exchange Chromatographic Determination of Total Fructans in Food and Pet Food—Limitations and Measurement Uncertainty

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A simplified method to determine total fructans in food and pet food has been developed and validated. It follows the principle of AOAC method 997.08, i.e., high-performance anion exchange chromatographic (HPAEC) determination of total fructose released from fructans (F_f) and total glucose released from fructans (G_f) after enzymatic fructan hydrolysis. Unlike AOAC method 997.08, calculation of total fructans is based on the determination of F_f alone. This is motivated by the inherent difficulty to accurately determine low amounts of G_f since many food and pet food products contain other sources of total glucose (e.g., starch and sucrose). In this case, a correction factor g can be used (1.05 by default) to take into account the theoretical contribution of G_f. At levels >5% of total fructans and in commercial fructan ingredients, both F_f and G_f can and should be accurately determined; hence, no correction factor g is required. The method is suitable to quantify total fructans in various food and pet food products at concentrations ≥0.2% providing that the product does not contain other significant sources of total fructose such as free fructose or sucrose. Recovery rates in commercial fructan ingredients and in selected food and pet food ranged from 97 to 102%. As part of a measurement uncertainty estimation study, individual contributions to the total uncertainty (u) of the total fructan content were identified and quantified by using the validation data available. As a result, a correlation between the sucrose content and the total uncertainty of the total fructan content was established allowing us to define a limit of quantitation as a function of the sucrose content. One can conclude that this method is limited to food products where the sucrose content does not exceed about three times the total fructan content. Despite this limitation, which is inherent to any total fructan method based on the same approach, this procedure represents an excellent compromise with regard to accuracy, applicability, and convenience.

KEYWORDS: Fructans; inulin; fructanase; inulinase; high-performance anion exchange chromatography; measurement uncertainty

INTRODUCTION

Fructans are oligomeric and polymeric carbohydrates composed of β-linked fructose monomers. According to the type of linkage [$\beta(2\rightarrow1)$ or $\beta(2\rightarrow6)$], one distinguishes between inulin or levan type fructans. Inulin type fructans are the storage carbohydrates of many plants of the *Compositae* family, in particular of chicory root (*Cichorium intybus*). Graminan type fructans of mixed $\beta(2\rightarrow1)$ and $\beta(2\rightarrow6)$ structure are found in low concentrations in grains of several grasses and cereals, in particular in rye and wheat.

Inulin is composed of polydisperse linear chains of $\beta(2\rightarrow1)$-linked fructose (F) moieties, generally bearing a terminal glucose (G) unit. The molecular structure of inulin type fructans is therefore usually referred to as GF_n ($n = 3$ to ∼60). In inulin from chicory, F_m fructans (without terminal glucose) coexist with their GF_n homologues. Fructooligosaccharides (FOS) or oligofructose refer to the low molecular weight fraction of inulin ($n = 2-9$).

Because of the lack of the enzyme fructanase in the human digestive system, fructans are considered as nondigestible oligosaccharides. For this reason, they are classified as soluble dietary fiber from a nutritional and legal point of view in most countries worldwide. In addition, they are claimed to have a beneficial impact on the gut flora (prebiotic effect) (*1*). Bacterial species thought to be selectively promoted through the consumption of prebiotics are in particular *Bifido* bacteria and *Lactobacillus* strains.

For both reasons, commercial inulin and FOS preparations are used as functional ingredients and added to an increasing number of food products (*2*). Typically, the amount used in a food application is <5%. In pet food, dried chicory root may be incorporated as a fructan ingredient providing generally up to about 1.0% of added total fructans.

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It is important to check the amount of fructans in food products, e.g., for labeling or supporting a prebiotic claim. Specific analytical methods for the determination of fructans are required because FOS and inulin are not recovered as dietary fiber by the classical methods for total dietary fiber determination. Because of the relatively low stability of the $\beta(2\rightarrow1)$ bonding, heat processing or prolonged storage tend to depolymerize fructans into either shorter chains or straightforward to fructose. This degradation process is strongly accelerated with increasing acidity, temperature, and water activity. Stability is therefore another important aspect of fructan monitoring.

There are two fundamentally different approaches to quantify total fructans in food products, which can be classified as "direct" and "indirect" methods. The direct approach consists of monitoring the fructan (fingerprint) profile, by separating fructans according to their degree of polymerization (DP). Various chromatographic methods for the separation and determination of fructo-oligosaccharides in food products have been described. The most important one is high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) given the high-resolution power and sensitivity of this technique (*3*, *4*). Because of the limited availability of commercial fructan standards, only the three fructo-oligosaccharides 1-kestose (GF_2), nystose (GF_3), and fructosyl-nystose (GF_4) may be quantitatively determined. Several papers deal with the quantitation of GF_2, GF_3, and GF_4 in various types of food and other matrixes (*5*, *6*). If the fructan ingredient used in a food application is known and available for analysis, the amount of fructan ingredient may be determined by using the fructan ingredient itself as an external standard. However, an important restriction to this approach is the fact that the fructan profile in the food product may be altered through processing or storage. Matrix interferences or the presence of other oligosaccharides (maltodextrins, cereal fructans) may also compromise the quantitative use of fructan marker peaks.

The indirect approach consists of an enzymatic hydrolysis and depolymerization of fructans followed by quantitation of the released monosaccharides fructose and glucose. Methods based on the enzymatic hydrolysis of fructans using the enzyme β-fructosidase (inulinase) have been reported in the literature, the most important one being AOAC method 997.08 (*7*). In this approach, the total fructan concentration (c_f) is obtained as the sum of fructose released from fructans (F_f) and glucose released from fructans (G_f) by using a correction factor (k) for the water uptake during hydrolysis:

$$c_f = k \cdot (F_f + G_f) \quad (1)$$

The enzyme used in this protocol (Novo's fructozyme) also quantitatively hydrolyzes sucrose and to some extent lactose and α-glucans (starch and maltodextrins). Therefore, not only free fructose and free glucose but also other sources of total fructose and glucose, in particular sucrose and lactose, have to be determined to correct the amounts of total F and G measured after depolymerization accordingly. Sucrose is determined in the first chromatographic run together with free fructose and glucose. Starch and maltodextrins are hydrolyzed, and the amount of glucose released from α-glucans is determined in a second chromatographic run. Finally, to determine the amount of glucose released from lactose, free galactose and total galactose are monitored as well, the latter together with total fructose and total glucose after fructan hydrolysis in the third chromatographic run. As a result, the presence of other sources of fructose (e.g., free fructose, sucrose) and glucose (free glucose, sucrose, starch, maltodextrins, lactose) may strongly interfere and thus considerably reduce the accuracy of this approach, especially at low levels of fructan incorporation. This is, for instance, the case of fructan-fortified infant formulas or cereals and pet food that may contain high amounts of starch and/or sucrose.

A simplified version of the AOAC method would therefore not only save time and cost (two HPAEC runs instead of three) but also overcome the problem of determining low amounts of G_f in many fructan food applications. Given that most food and pet food products provide less than 5 g/100 g of total fructans, G_f may actually better be estimated from the determination of F_f. Accordingly, a correction factor g can be used instead:

$$c_f = k \cdot g \cdot F_f \quad (2)$$

The g factor is given by the composition of the fructan ingredient. The F_f/G_f ratio depends on the mean DP and ratio of fructan molecules with and without end-standing glucose moieties. g can in principle be determined for each individual fructan ingredient, but a F_f/G_f ratio close to 20:1 is observed in most commercial fructan ingredients. This suggests the use of a correction factor of 1.05 by default.

MATERIALS AND METHODS

Materials. Ultrapure 18 MΩcm dematerialized water was obtained from a Milli-Q-system from Millipore (U.S.A.). Sodium hydroxide, 50% (w/w) aqueous solution (NaOH, Nr. 7067) was purchased from J. T. Baker (U.S.A.). Sodium acetate anhydrous, MicroSelect quality (NaOAc, Nr. 71179) was purchased from Fluka (Germany). Potassium hydroxide pellets (KOH, Nr. 105033), hydrochloric acid (1 mol/L HCl Nr. 109057), and acetic acid glacial 100% (AcOH, Nr. 100063) were purchased from Merck. Glucose, galactose, fructose, and sucrose were purchased from Fluka. Inulinase was purchased from MEGAZYME (Ireland) (fructanase mixture, 2000 U/mL) and was used as is.

Sample Preparation. All samples were homogenized either by using a Polytron homogenizer (wet products) or by thorough grinding in a mortar or a mill. Subsequently, a test portion of 2.5 g of dry sample or 5.0 g of wet sample (m) was weighed, to the nearest 0.1 mg, into a 100 mL beaker containing a mixing rod. About 40 mL of boiling water was added to the sample. When the resulting solution was too viscous, the sample portion was reduced to about 1.0−1.5 g. The pH was immediately measured under mild agitation. If necessary, it was adjusted with 0.05 mol/L HCl or 0.05 mol/L KOH to lie between 6.5 and 8.0.

Free Carbohydrates Assay (A_1). The sample solution or suspension was quantitatively transferred into a 100 mL (V_1) volumetric flask, and the beaker was rinsed with boiling water. The flask was placed in a water bath for 15 min at 85 ± 2 °C under continuous stirring. Once cooled to room temperature, the content was filled up to the mark with water and mixed. Part of this solution or suspension (50 mL) was used to determine free carbohydrates (assay A_1) and, if necessary, diluted with water (D_1) to be within the linear range of the detector. When the sample was not fully dissolved, the sample extract was shaken vigorously and transferred into a glass bottle, and the aliquot for dilution was taken by means of a micropipet under vigorous mixing on a magnetic stirrer.

Total Carbohydrates Assay (A_2). A 10.0 mL (V_2) amount of solution V_1 was pipetted into a glass tube with a screw cap. When the sample was not fully dissolved, the aliquot was taken as described above. Subsequently, 10.0 mL of acetate buffer (pH 4.5, prepared by dissolving 3.61 g of NaOAc and 3.2 mL of glacial AcOH in 1000 mL of water) was added. If necessary, the pH was adjusted with 0.05 mol/L HCl or 0.05 mol/L KOH solution to 4.5 ± 0.05. Subsequently, 100 μL of enzyme suspension was added (200 μL for commercial fructan ingredients). The mixture was incubated in a water bath at 60 ± 2 °C for precisely 30 min under mild stirring. Timing (30 min) was started once the reaction mixture had reached 60 °C. Once cooled to room temperature, the solution was transferred into a 100 mL (V_3) volumetric

Table 1. Elution Profile for the Determination of Relevant Carbohydrates for Total Fructan Analysis

run time (min)	eluents (%) water	300 mM NaOH	100 mM NaOH	steps
0.00	95	0	5	
0.10	95	0	5	start acquisition
42.0	95	0	5	stop acquisition
42.1	0	100	0	start cleanup
52.0	0	100	0	stop cleanup
52.1	95	0	5	start reequilibration
62.0	95	0	5	stop reequilibration

flask. If necessary, this solution or suspension was diluted with water (D_2) as described above to be within the linear range of the detector.

HPAE Chromatography. Chromatography was performed on a DX 500 Dionex system (Sunnyvale, CA) consisting of a GP 40 gradient pump, a postcolumn delivery system or DQP postcolumn pump, and an ED 40 electrochemical detector working in PAD mode, equipped with a gold working electrode. The three pulse potentials and durations were as follows: $E_1 = 0.05$ V ($t_1 = 400$ ms), $E_2 = 0.75$ V ($t_2 = 200$ ms), and $E_3 = -0.15$ V ($t_3 = 400$ ms). Eluents were degassed with helium by a Dionex degas module for at least 20 min. The sample and standard solutions were injected by an AS3500 Autosampler (Thermo Separation Products, U.S.A.) equipped with a 20 μL injection loop. Sugars were separated on a pellicular anion exchange resin (CarboPac PA1 guard column, 50 mm × 4 mm, followed by a CarboPac PA1 analytical column, 250 mm × 4 mm). The column was periodically washed with 300 mM NaOH. Chromatographic peaks were integrated using a PeakNet work station. The conditions were as follows: injection volume, 20 μL; flow rate, 1.0 mL/min; postcolumn addition, 300 mM NaOH at a flow rate of 0.6 mL/min; room temperature. Carbohydrates in the sample solution were identified and quantified by comparison with retention times and areas of corresponding peaks obtained for the standard solution. The elution profile is shown in **Table 1**.

Carbohydrate Standard Solution. A 100 mg (m_{ST}) amount of each carbohydrate to be determined (fructose, glucose, sucrose, and galactose) was weighed, to the nearest 0.1 mg, into a 100 mL (V_{ST}) volumetric flask, dissolved, and made up to the mark with freshly distilled water. This standard solution was diluted 25 times (D_{ST}) to obtain individual carbohydrate concentrations of 0.04 mg/mL.

Calculation and Expression of Results. The free and total sugar contents were calculated according to the equations used in AOAC method 997.08 (*7*). The fructose content released from fructans (F_f), expressed in percentage by weight, was calculated according to the following formula:

$$F_f = \left(F_{tot} - F_{free} - \frac{S}{1.9}\right) \quad (3)$$

where F_{tot} = amount of total fructose, in percent; F_{free} = free fructose content, in percent; and S = free sucrose content, in percent. The term S/1.9 represents the amount of fructose released by the hydrolysis of sucrose.

Finally, the total fructan content, expressed in percentage by weight, is calculated according to eq 2:

$$c_f = k \cdot g \cdot F_f \quad (2)$$

where c_f = total fructan concentration; k = correction factor for water uptake during hydrolysis [k is needed to correct for the water uptake during hydrolysis and depends on the average fructan chain length. For FOS preparations, a k factor of 0.925 is conventionally used, and for most inulin preparations, a k factor of 0.910 is used (*1*)]; and g = correction factor for the theoretical contribution of G_f.

For total fructan contents >5 g/100 g and commercial fructan ingredients, G_f was taken into account (see formulas in ref *7*) and the total fructan content was calculated according to eq 1.

RESULTS AND DISCUSSION

Method Optimization. The simplification and optimization of the method with regard to AOAC method 997.08 focused in particular on the following points: (i) influence of the presence of maltodextrins and starch on the inulinase activity and (ii) use of a correction factor for the theoretical amount of G_f.

Influence of the Presence of Maltodextrins and Starch on the Inulinase (Fructanase) Activity. Novo's Fructozyme, the enzyme used in AOAC method 997.08, is known to have some important side activities besides its exo- and endoinulinase activity (*8*). For instance, it is contaminated with α-galactosidase, pectinase, and cellulase/β-glucanase, which may lead to an increased release of glucose from other sources than fructans and hence to an overestimation of G_f. Megazyme's fructanase mixture is claimed to be devoid of most of these side activities (*9*).

We did not observe any significant difference in terms of total fructan content between both enzymes' fructanase activities in the analysis of several commercial fructan ingredients (results not shown). To evaluate whether the α-glucan hydrolysis step in AOAC method 997.08 may be skipped without influencing the activity of the inulinase (Megazyme), several products were comparatively analyzed with and without this step. The results are shown in **Table 2**.

As expected, even with Megazyme's fructanase mixture, the presence of starch during the fructan hydrolysis step leads to considerable overestimation of the fructan content if the calculation is based on both F_f and G_f. On the other hand, the presence of starch during the fructan hydrolysis step has no negative impact on the recovery of F_f. The α-glucan hydrolysis step can therefore be omitted, and the result of c_f can be based on F_f only according to eq 2.

Correction Factor g for the Theoretical Amount of G_f. If the fructan ingredient used in a finished product is available for analysis according to eq 1, g can be determined according to the following equation:

$$g = \frac{F_f + G_f}{F_f} \quad (4)$$

Table 3 shows the g factors for selected fructan ingredients. Ideally, this factor should be determined for each specific ingredient. However, the analysis of most commercial fructan ingredients refined from chicory root showed typically an F_f/G_f ratio close to 20:1, which corresponds to a g factor of 1.05. Therefore, a suitable correction factor g to be used by default appears to be 1.05. It should be borne in mind that g may actually be very different from 1.05, especially in the case of Actilight, which is exclusively composed of the three short chain fructans GF_2 to GF_4.

Limit of Quantitation. Given the contribution of sucrose to the amount of total fructose, the question of the limit of quantitation has to be addressed in a different way for sucrose-free products and for sucrose-containing products. For sucrose-free products (milk powder, pet food), spiking experiments demonstrated (based on a minimum recovery of 90%, results not shown) that total fructans can be accurately determined using the proposed method down to at least 0.2 g/100 g. For products containing sucrose, the limit of quantitation is directly dependent on the amount of sucrose. The relationship between the amount of sucrose and the limit of quantitation is discussed in the next paragraph.

Measurement Uncertainty (MU). The MU of the proposed method was estimated based on the approach recommended by

Table 2. Comparative Analysis under Conditions of AOAC Method 997.08, with and without the α-Glucan Hydrolysis Step

		with α-glucan hydrolysis step				without α-glucan hydrolysis step			
product	k	F_f[a] (g/100 g)	G_f[a] (g/100 g)	$F_f \cdot k$ (g/100 g)	$(F_f + G_f) \cdot k$ (g/100 g)	F_f[a] (g/100 g)	G_f[a] (g/100 g)	$F_f \cdot k$ (g/100 g)	$(F_f + G_f) \cdot k$ (g/100 g)
milk powder spiked with 5% Raftiline HP	0.910	4.95	0.15	**4.51**	**4.68**	4.73	0.22	**4.31**	**4.51**
whole wheat meal	0.910	1.03	0	**0.94**	**0.94**	1.01	2.87	**0.91**	**3.51**
dry dog food with 8% chicory	0.910	4.31	0.22	**3.92**	**4.12**	4.06	1.39	**3.69**	**4.96**

[a] Mean of duplicate determination.

Table 3. g Factors of Selected Fructan Ingredients

product	F_f (g/100 g)	G_f (g/10 g)	g
Raftiline HP	101.5	4.18	1.04
Raftilose P95	95.3	3.71	1.04
dried chicory root	57.5	5.29	1.09
Actilight P950	69.6	25.4	1.37

the ISO and Eurachem Guides (*10*, *11*). It is divided into five steps (**Table 4**).

The aim of a MU estimation study is to determine a standard deviation of uncertainty or standard uncertainty $u(y)$ related to the result of the measurand y. If individual contributions of random and systematic effects (a, b, c, ...) are independent, the total uncertainty is obtained according to the general formula:

$$\frac{u(y)}{y} = \sqrt{\left[\frac{u(a)}{a}\right]^2 + \left[\frac{u(b)}{b}\right]^2 + \left[\frac{u(c)}{c}\right]^2 + \dots} \qquad (5)$$

Eventually, the MU should be expressed as a confidence interval or expanded uncertainty $U(y)$ around the result y obtained. We have recently applied this approach to the estimation of the MU of the HPAE chromatographic determination of free and total carbohydrates in soluble coffee (*12*).

Step 1: Description of the Measurement Procedure—Flowchart of the Method. Scheme 1 shows the flowchart of the method.

Step 2: Specification of the Measurand—Relationship between the Measurand and the Variables. Combining eqs 2 and 3, the total fructan concentration c_f (g/100 g) (without determination of G_f) can be expressed as follows:

$$c_f = k \cdot g \cdot \left(F_{tot} - F_{free} - \frac{S}{1.9}\right) \qquad (6)$$

and the relative standard uncertainty of the amount of fructose released from fructans (F_f) is

$$\frac{u(F_f)}{F_f} = \frac{\sqrt{[u(F_{tot})^2 + u(F_{free})^2 + u(S)^2]}}{\left(F_{tot} - F_{free} - \frac{S}{1.9}\right)} \qquad (7)$$

Accordingly, the relative standard uncertainty of c_f can be determined as follows:

$$\frac{u(c_f)}{c_f} = \sqrt{\left[\frac{u(k)}{k}\right]^2 + \left[\frac{u(g)}{g}\right]^2 + \left[\frac{u(F_f)}{F_f}\right]^2} = \sqrt{\left[\frac{u(k)}{k}\right]^2 + \left[\frac{u(g)}{g}\right]^2 + \left(\frac{\sqrt{[u(F_{tot})^2 + u(F_{free})^2 + u(S)^2]}}{\left[F_{tot} - F_{free} - \frac{S}{1.9}\right]}\right)^2} \qquad (8)$$

Free carbohydrates (F_{free} and S) are determined in the assay A_1. For the purpose of MU estimation, the purity of the carbohydrate standard (P_{ST}) as well as the recovery of free carbohydrates (R_{free}) need to be taken into consideration. Their concentration $c_{freecarb}$ (g/100 g) is therefore obtained according to the following equation:

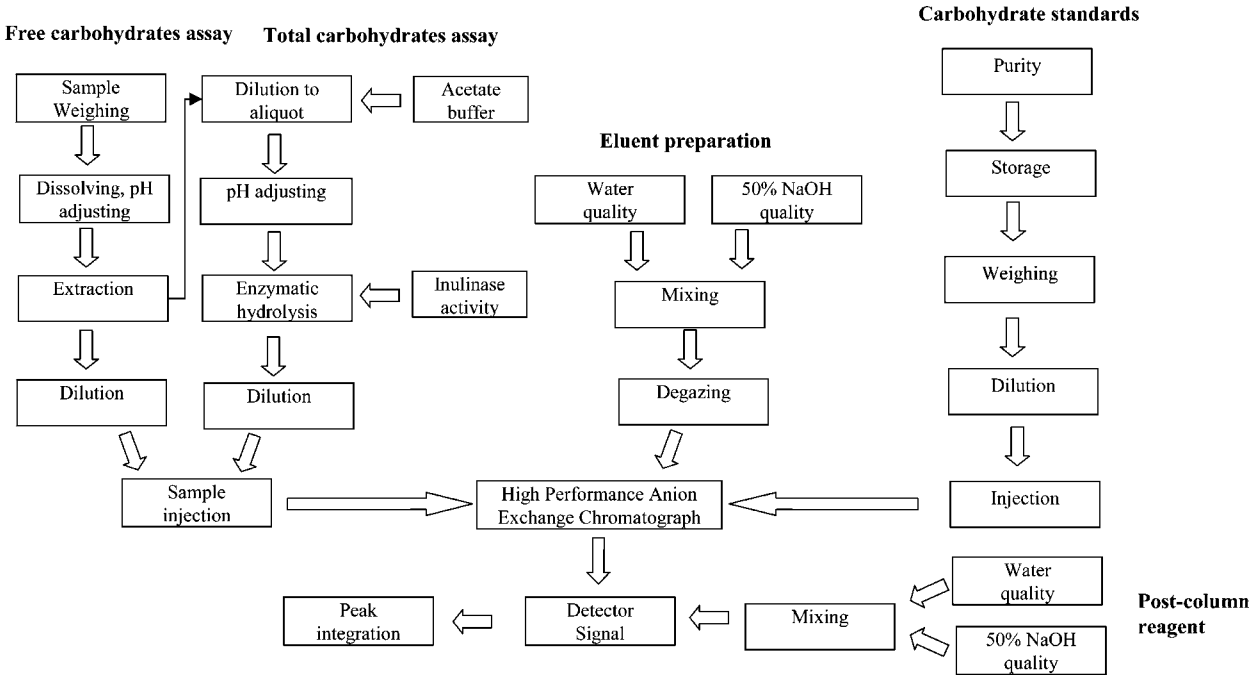
$$c_{freecarb} = \frac{A_1 \cdot m_{ST} \cdot V_1 \cdot D_1 \cdot 100}{A_{ST} \cdot m \cdot V_{ST} \cdot D_{ST}} \cdot \frac{P_{ST}}{R_{free}} \qquad (9)$$

where A_1 = peak area of the carbohydrate measured in the sample solution, assay A_1; A_{ST} = peak area of the carbohydrate measured in the standard solution; m_{ST} (g) = weight of the carbohydrate in the standard solution, in grams; m (g) = weight of the test portion, in grams; V_1 (mL) = volume of the sample solution, assay A_1; V_{ST} (mL) = volume of the standard solution; D_1 = dilution factor of the sample solution, assay A_1; and D_{ST} = dilution factor of the standard solution.

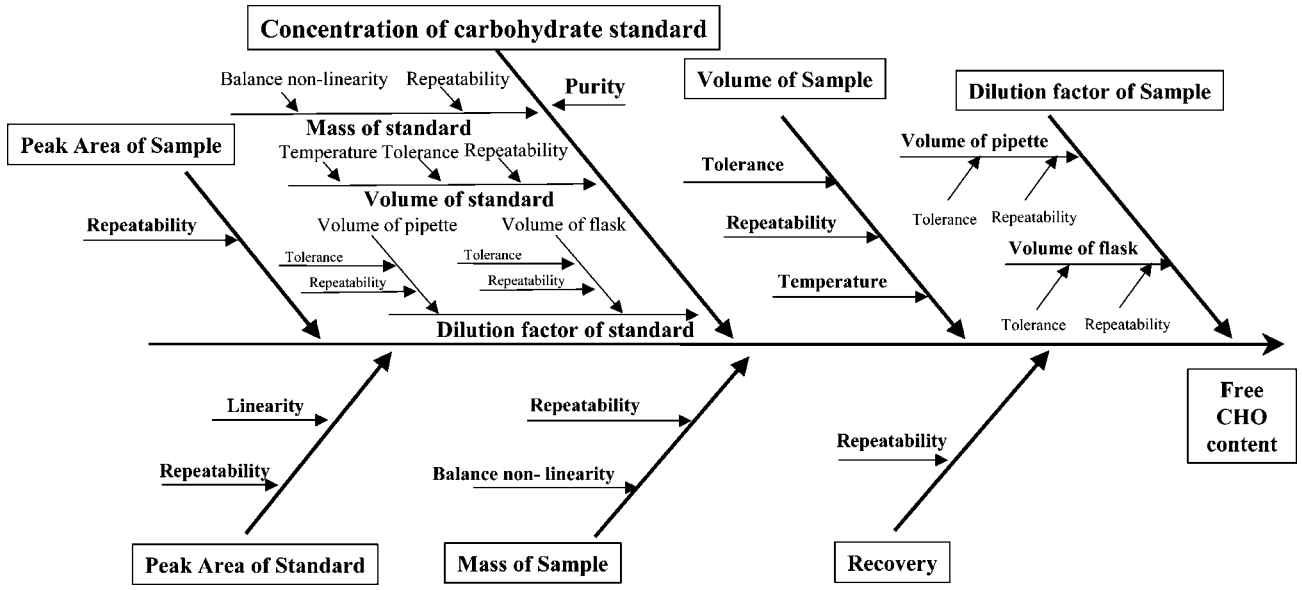
Table 4. Summary of the Procedure for the Estimation of MU

step	objective	how to do it
1	description of the measurement procedure	Produce a flowchart with a description of all steps to be included in the measurement (analytical) procedure. All conversions (manipulations) should appear separately.
2	specification of the measurand	Define the measurement model, which is the relationship between the measurand and the variables of the measuring (analytical) procedure. Write the main equation of the analytical method.
3	identification of uncertainty sources	Identify and show the uncertainty sources for each variable by means of a cause and effect diagram based on the main equation.
4	quantitation of uncertainties	Quantify the identified uncertainties. Tabulate them in an uncertainty budget table.
5	combination of uncertainties	Combine the uncertainties according to error propagation rules to yield the total uncertainty of the measurand.

Scheme 1. Flow Chart of the Enzymatic HPAEC Determination of Total Fructans



Scheme 2. Cause and Effect Diagram for the Determination of Free Carbohydrates



The concentration of each carbohydrate (c_{ST}) in the mixed standard solution used for calibration is given as:

$$c_{ST} = \frac{m_{ST} \cdot P_{ST}}{V_{ST} \cdot D_{ST}} \quad (10)$$

Therefore, eq 9 can be simplified to:

$$c_{freecarb} = \frac{A_1 \cdot c_{ST} \cdot V_1 \cdot D_1 \cdot 100}{A_{ST} \cdot m \cdot R_{free}} \quad (11)$$

Similarly, the concentrations of total carbohydrates ($c_{totcarb}$, fructose, and glucose) determined in assay A_2 are obtained according to eq 12:

$$c_{totcarb} = \frac{A_2 \cdot c_{ST} \cdot V_1 \cdot V_3 \cdot D_2 \cdot 100}{A_{ST} \cdot m \cdot V_2 \cdot R_{tot}} \quad (12)$$

where A_2 = peak area of the carbohydrate measured in the sample solution, assay A_2; V_2 (mL) = volume of the aliquot taken from sample solution, assay A_1; V_3 (mL) = volume of the sample solution, assay A_2; D_2 = dilution factor of the sample solution, assay A_2; and R_{tot} = recovery of total carbohydrates.

To determine the standard uncertainties of c_{free} and $c_{totcarb}$, individual uncertainty contributions related to each of the variables need to be identified and quantitatively estimated.

Step 3: Identification of Uncertainty Sources—Cause and Effect Diagram. According to eq 11, the cause and effect diagram can be drawn for the determination of free carbohydrate (**Scheme 2**). The repeatability of the individual carbohydrate determination is known because samples are analyzed in duplicate. The repeatability takes into account all operations regarding the sample (mass, volume, dilution, injection, peak area, and peak integration). It can therefore be extracted and dealt with separately. It is also suitable to deal with linearity

Scheme 3. Refined Cause and Effect Diagram for the Determination of Free Carbohydrates

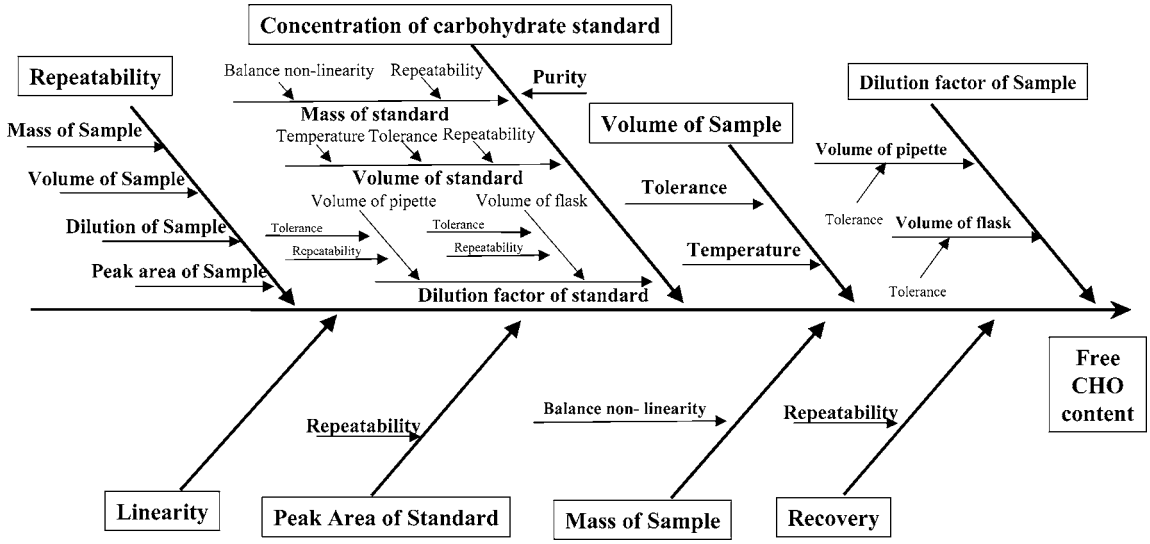


Table 5. Relative Repeatability Standard Deviations RSD(r) of the Duplicate Determination of Free and Total Carbohydrates as a Function of the Carbohydrate Concentration

	range (g/100 g)			
	<0.2	0.2–1.0	1.0–10	>10
free carbohydrates	10%	4%	2.5%	1.5%
total galactose	20%	5%	1.5%	
total glucose			1.5%	
total fructose		2%	1.5%	0.5%

and repeatability of the peak area of the carbohydrate standard separately. Accordingly, a refined cause and effect diagram can be drawn (**Scheme 3**). Similarly, a refined cause and effect diagram can be set up for the total carbohydrates assay (not shown).

Step 4: Quantitation of Individual Uncertainties. Precision (Repeatability). Using HPAEC-PAD, the repeatability varies with the type of carbohydrate analyzed (mono- or disaccharide), its retention time (larger peaks with increasing retention times), and its concentration. The sample dilution factor and the condition of the working electrode also have an impact of the repeatability. The duplicate results obtained on 114 analysis of different products (23 for total glucose) were statistically analyzed.

For free sugars, only a differentiation according to the carbohydrate concentration range has been made, thus neglecting the other influences. For total sugars, a differentiation according to the type of carbohydrate and its concentration range is made. The values of relative standard deviation of repeatability RSD(r) or $u(r)/r$ observed in our laboratory are summarized in **Table 5**.

Trueness. Four different fructan preparations were analyzed according to the proposed method. Results were calculated according to eq 1, that is taking into account the contribution of total glucose (G_f). The results (**Table 6**) were compared to their theoretical fructan content [The theoretical fructan content was calculated from the free sugar content (determined as part of this method), the moisture content (determined by Karl Fischer), and estimates of ash, proteins, and fat (according to specifications).] to determine the recovery. Recoveries ranging between 97 and 102% were found.

To check the recovery in a finished food and in a pet food, a milk powder and a dry dog food (both sucrose-free) product were spiked with Raftilose P95 and Raftiline ST, respectively. [Milk powder, respectively, 3, 6, and 9%; dog food, 0.25, 0.5, 1.0, 2.5, and 5%. Results were calculated according to eq 2 (not shown).] Again, recoveries between 97.5 and 102.5% were achieved (except for the dog food spiked with 0.25% where the recovery was 92%).

We therefore assumed the total fructan recovery R both in fructan ingredients and finished products to be $100 \pm 2.5\%$ over the a total fructan range of 0.5−100 g/100 g. To transform this interval into a relative standard deviation of recovery RSD(R), a normal distribution is assumed. Division by 1.96 leads to an RSD(R) of 1.28%. For lower total fructan values (0.25−0.5 g/100 g), the recovery R can be assumed to be $100 \pm 8\%$ (rectangular distribution, division by square root of 3, resulting RSD(R) = 4.62%) [A rectangular (or uniform) distribution around x is assumed if no information of the level of confidence of x is available. All values between $x \pm$ tolerance interval are equally likely to be true. A triangular distribution around x is assumed if values close to x are more likely than those close to the limits of the tolerance interval.]

Calibration with Mixed Carbohydrate Standard Solution. Three points have to be considered to discuss the uncertainty related to the calibration of the chromatographic system using a mixed carbohydrate standard solution: the repeatability of the carbohydrate standard peak area (integration), the uncertainty linked to the lack of linearity, and the uncertainty of the carbohydrate standard concentration.

Repeatability of the Carbohydrate Standard Peak Area (r_{areaST}). Performing HPAEC-PAD analysis of carbohydrates, the electrochemical response (peak area) is subject to variations within a series of injections. Another source of uncertainty is the repeatability of the integration of the peak area. A typical sample series is composed of an injection of the mixed carbohydrate standard solution, followed by up to six sample injections (e.g., three samples in duplicate) and another standard solution. All six sample chromatograms are integrated using the mixed standard preceding the sample series.

To quantitatively evaluate the variation of the detector's response within one series of injections, we monitored the differences in the peak areas of each carbohydrate in two mixed standard solutions in a row, that is separated by six sample injections. Eighteen pairs of mixed standard chromatograms were analyzed in this way, and the relative repeatability standard deviations of the peak areas for each individual carbohydrate

Table 6. Total Fructan Recovery in Commercial Fructan Preparations

				fructan content			
product	F_f (g/100 g)	G_f (g/100 g)	F_f/G_f	k	$(F_f + G_f) \cdot k$ (g/100 g)	theoretical (g/100 g)	recovery (%)
Raftilose P95[a]	94.8	3.7	25.6	0.925	91.1	91.6	97–100
Raftiline HP[a]	101.3	4.2	24.2	0.905	95.4	95.0	99–102
Raftiline ST[b]	89.6	8.2	11.0	0.91	89.0	89.7	99
Actilight P950[b]	69.6	25.4	2.75	0.94	89.3	91.5	98

[a] Mean of five determinations in duplicate. [b] Mean of one determination in duplicate.

Table 7. Carbohydrate Specific Relative Uncertainties Related to the Calibration

carbohydrate	RSD(r_{areaST}) (%)	RSD(lin_{ST}) (%)	RSD(c_{ST}) (%)
galactose	1.8	2.0	0.8
glucose	1.0	2.0	0.8
sucrose	1.7	3.0	0.8
fructose	2.4	3.0	0.8

standard RSD(r_{areaST}) were determined. The RSD(r_{areaST}) values found were 1.0% for glucose and 2.4% for fructose (see **Table 7**).

Uncertainty of Linearity. Calibration is done by injecting a mixed standard solution at one single concentration for each carbohydrate (external one point calibration). It is therefore imperative to check the linearity of the response for each carbohydrate in order to define a suitable concentration working range.

As a general tendency, the linear range of the response of carbohydrates on the PAD is relatively limited. At high carbohydrate concentrations, the detector tends to saturate and the response (ratio peak area over concentration) decreases rapidly. To determine the appropriate working range, 10 mixed carbohydrate standard solutions at 10 different carbohydrate concentrations were analyzed and the resulting peak areas for each carbohydrate were recorded. Subsequently, a plot of the ratio of peak area over concentration against the concentration was done. Fructose and sucrose tend to have a "less linear behavior" with PAD than other carbohydrates, that is their response actually very slightly decreases over the whole concentration range analyzed. We defined maximum acceptable relative standard deviation of the ratio peak area over concentration for each carbohydrate standard, RSD(lin_{ST}). They can be considered as a compromise between acceptable precision and working range, keeping in mind that the calibration model is not perfect. Thus, RSD(lin_{ST}) values ranging between 2.0 and 3.0% were used as criteria to define the lower and upper limit of the working range (**Table 7**). At the same time, these values express the uncertainty related to the linear calibration model.

Figure 1 shows the plot obtained for fructose. To define the working range while complying with the RSD(lin_{ST}) previously defined of 3.0%, the three highest fructose standard concentrations had to be discarded.

Carbohydrate Concentration in the Mixed Standard Solution (c_{ST}). The carbohydrate standard solution is a mixture of the individual carbohydrate standards at the same concentrations (0.04 mg/mL). The same solution is used in the "free" and in the "total" carbohydrate assay.

To calculate the uncertainty of the concentrations of the mixed carbohydrate standard solution, the following contributions were taken into account (see eq 10): (i) m_{ST}, mass of the carbohydrate standard (100 mg); (ii) P_{ST}, purity of the carbohydrate standard (≥99%); (iii) V_{ST}, volume of the undiluted mixed carbohydrate standard solution (100 mL); and (iv) D_{ST}, dilution of this solution (dilution factor 25).

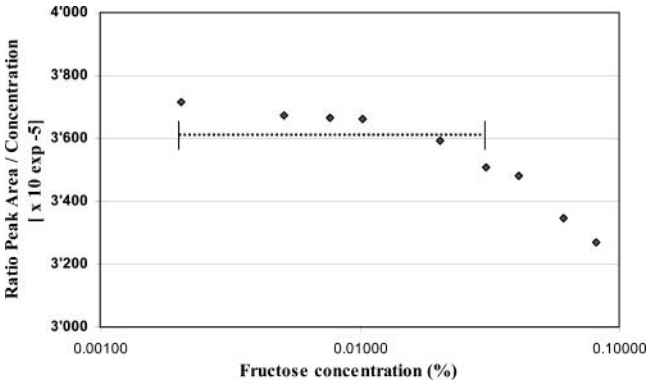


Figure 1. PAD response of fructose and linear range defined (dotted line).

We have previously demonstrated that the contributions of the determinations of m_{ST} and V_{ST} are negligible, whereas those of P_{ST} and D_{ST} are both about 0.6%. Accordingly, the standard uncertainty of c_{ST} is approximately:

$$u(c_{ST}) = \sqrt{\left[\frac{u(P_{ST})}{P_{ST}}\right]^2 + \left[\frac{u(D_{ST})}{D_{ST}}\right]^2} = 0.8 \qquad (13)$$

Uncertainty Budget of Calibration with Mixed Carbohydrate Standard Solution. The individual contributions to the relative standard uncertainty related to the calibration with the standard solution are summarized in **Table 7**.

Remaining Biases (Sample Solution and Dilution Factor). The relative uncertainties related to the sample weight m and the sample solution volume V_1 have been shown to be negligible. The relative standard uncertainty of an overall dilution factor of 10 (10 mL pipetted into a 100 mL volumetric flask) has been shown to be about 0.45%. Accordingly, the relative standard uncertainty of a dilution factor of 100 (two subsequent dilutions by 10, as generally used in assay A_2) is about 0.65%.

Combination of Standard Uncertainties—Total Uncertainty Budget. The total uncertainty budget of the HPAEC determination of free and total carbohydrates is summarized in **Table 8** (The dilution factor $V_3/V_2 \cdot D_2$ applies only to the total carbohydrate assay.).

Determination of the MU. The relative standard uncertainty of a free carbohydrate concentration is calculated as follows:

$$\frac{u(c_{freecarb})}{c_{free\ carb}} = \sqrt{\left[\frac{u(r)}{r}\right]^2 + \left[\frac{u(c_{ST})}{c_{ST}}\right]^2 + \left[\frac{u(lin_{ST})}{lin_{ST}}\right]^2 + \left[\frac{u(r_{areaST})}{r_{areaST}}\right]^2} \qquad (14)$$

The relative standard uncertainty of a total carbohydrate concentration is calculated as follows:

$$\frac{u(c_{totcarb})}{c_{totcarb}} = \sqrt{\left[\frac{u(r)}{r}\right]^2 + \left[\frac{u(c_{ST})}{c_{ST}}\right]^2 + \left[\frac{u(lin_{ST})}{lin_{ST}}\right]^2 + \left[\frac{u(r_{areaST})}{r_{areaST}}\right]^2 + \left[\frac{u(V_3/V_2 \cdot D_2)}{V_3/V_2 \cdot D_2}\right]^2} \qquad (15)$$

Examples. *Case Study 1: Sucrose-Free Milk Powder Containing About 3.0% of Fructans.* This product is used as a quality

Table 8. Total Uncertainty Budget of the HPAEC Determination of Free and Total Carbohydrates

				uncertainty	
parameter	symbol	value	unit	$u(x)$	$u(x)/x$
		repeatability			
$c < 0.2$	r	depends on the	g/100 g	depends on the	10.0%
$0.2 < c < 1.0$		concentration		concentration	4.0%
$1.0 < c < 10$					2.5%
$c > 10$					1.5%
		calibration			
concentration standard	c_{ST}	0.04	mg/mL	0.00033	0.8%
linearity	lin_{ST}	depends on the		depends on the	depends on the
peak area standard	r_{areaST}	carbohydrate		carbohydrate	carbohydrate
dilution factor	$V_3/V_2 \cdot D_2$	100		0.636	0.64%

Table 9. Uncertainty Budget of a Total Fructose Content of 3.24 g/100 g

				uncertainty	
parameter	symbol	value	unit	$u(x)$	$u(x)/x$
repeatability:					
$1.0 < c < 10$	r	3.24	g/100 g	0.046	1.5%
calibration:					
concentration standard	c_{ST}	0.04	mg/mL	0.000326	0.8%
linearity	lin_{ST}	lin_{ST}			3.0%
peak area standard	r_{areaST}	r_{areaST}			2.4%
dilution factor	$(V_3/V_2) \cdot D_2$	100		0.64	0.64%
total fructose content	***c***	**3.24**	**g/100 g**	**0.1376**	**4.25%**

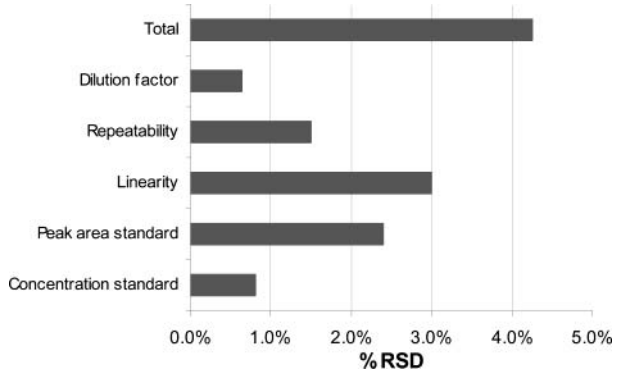


Figure 2. Uncertainty budget of a total fructose content of 3.24 g/100 g.

control (QC) sample in our laboratory. The mean value of eight determinations under intermediate reproducibility conditions was 3.10 g/100 g of total fructans. On the basis of the standard deviation of the mean of the duplicates (0.16 g/100 g), control limits of ± 0.40 g/100 g (multiplication by 2.58) had been defined for its use as QC sample in our laboratory. This experimental control limit was compared to the calculated MU of a determination in duplicate.

After analysis, the milk powder is found to contain 0.04 g/100 g of sucrose, 0.09 g/100 g of free fructose, and 3.24 g/100 g of total fructose. **Table 9** and **Figure 2** summarize the individual contributions and the total standard uncertainty of a total fructose content of 3.24 g/100 g [$u(c_f)/c_f = 4.25\%$]. The overall repeatability of the determination, the repeatability of the integration of the external carbohydrate standards, and the uncertainty related to the linear calibration model used account for the major part of the standard uncertainty of c_f.

Similarly, the total uncertainties determined for free fructose [$c = 0.04$ g/100 g, $u(c) = 0.0042$, and $u(c)/c = 10.6\%$] and sucrose [$c = 0.09$ g/100 g, $u(c) = 0.0097$, and $u(c)/c = 10.7\%$] were determined.

The amount of fructose released from fructans (F_f) is calculated according to eq 6. It is interesting to determine the individual contributions of each carbohydrate to F_f. The standard uncertainty related to the determination of total fructose is the only relevant contribution to the standard uncertainty of F_f (**Table 10**). The total fructan content of the milk powder is calculated according to eq 2: $c_f = 3.03$ g/100 g (with $k = 0.925$ and $g = 1.05$).

Table 10. Uncertainty Budget of a F_f Content of 3.12 g/100 g in Milk Powder

				uncertainty	uncertainty contribution to F_f
parameter	symbol	value	unit	$u(x)$	$u(x)/x$
sucrose	S	0.04	g/100 g	0.0042	0.14%
free fructose	F_{free}	0.09	g/100 g	0.0097	0.31%
total fructose	F_{tot}	3.24	g/100 g	0.1376	4.40%
fructose released from fructans	F_f	3.12	g/100 g	0.1380	4.42%

Table 11. Uncertainty Budget of a Total Fructan Content of 3.03 g/100 g in Milk Powder

				uncertainty	
parameter	symbol	value	unit	$u(x)$	$u(x)/x$
fructose released from fructans	F_f	3.12	g/100 g	0.1380	4.42%
correction factor	k	0.925		0.0058	0.63%
correction factor	g	1.05		0.0058	0.55%
total fructans ($k \cdot g \cdot F_f$)	c_f	3.03	g/100 g	0.1351	4.47%
recovery ($c_f > 0.5$ g/100 g)	R	100	%	1.28	1.28%
total fructans corr. R	$\boldsymbol{c_{fcorr.R}}$	**3.03**	**g/100 g**	**0.1420**	**4.67%**

Its standard uncertainty is determined according to eq 8. However, this uncertainty needs to be corrected for the standard uncertainty of the total fructan recovery, and eq 8 becomes

$$\frac{u(c_{fcorr.R})}{c_{fcorr.R}} = \sqrt{\left[\frac{u(R)}{R}\right]^2 + \left[\frac{u(c_f)}{C_f}\right]^2} = \sqrt{\left[\frac{u(R)}{R}\right]^2 + \sqrt{\left[\frac{u(k)}{k}\right]^2 + \left[\frac{u(g)}{g}\right]^2 + \left\{\frac{\sqrt{[u(F_{tot})^2 + u(F_{free})^2 + u(S^2]}}{\left(F_{tot} - F_{free} - \frac{S}{1.9}\right)}\right\}^2}} \quad (16)$$

The uncertainty intervals of both the k and the g factor were arbitrarily estimated to be 0.01. To transform these intervals into standard deviations, a rectangular distribution is assumed (division be square root of 3).

Accordingly, the following uncertainty budget for a total fructan content of 3.03 g/100 g is obtained (**Table 11** and **Figure**

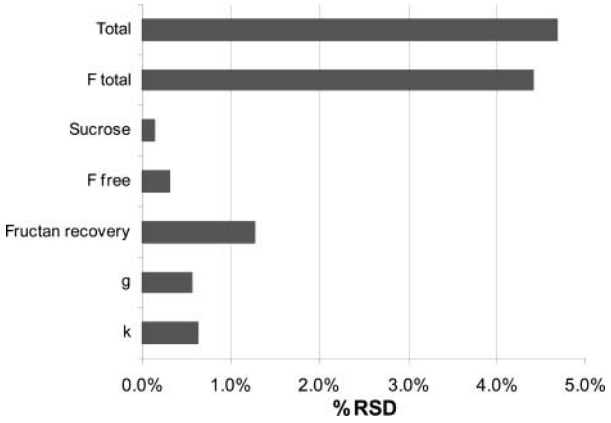


Figure 3. Uncertainty budget of a total fructan content of 3.03 g/100 g (sucrose-free milk powder).

Table 12. Uncertainty Budget of a F_f Content of 3.05 g/100 g in an Infant Formula

parameter	symbol	value	unit	uncertainty $u(x)$	uncertainty contribution to F_f $u(x)/x$
sucrose	S	10.3	g/100 g	0.3964	13.0%
free fructose	F_{free}	0.08	g/100 g	0.0086	0.3%
total fructose	F_{tot}	8.55	g/100 g	0.3636	11.9%
fructose released from fructans	F_f	3.05	g/100 g	0.5379	17.64%

3). The standard uncertainty related to the contribution of totalfructose represents the only relevant source of the total uncertainty.

The thus determined standard uncertainty has to be multiplied by 2 to obtain the "expanded uncertainty" U, which designs a confidence interval in which the probability to find the "true" value is 95% (Multiplication by 2 has been suggested by the Eurachem guide to transform relative uncertainties into 95% confidence levels.). Accordingly, the result of the total fructan determination in the milk powder should be reported as:

$$c_f = 3.03 \pm 0.28 \text{ g/100 g} \quad (17)$$

This corresponds to an expanded uncertainty of ±9% of the actual value. As a conclusion, the calculated expanded uncertainty is about of the same order as the experimental control limit based on the intermediate reproducibility study (±0.40 g/100 g). It appears that the intermediate reproducibility could be used as a fair approximation to estimate the MU of the present method applied to this sample.

Case Study 2: Infant Formula Containing 10% Sucrose and 3.0% of Fructans. After analysis, the infant formula is found to contain 10.3 g/100 g of sucrose, 0.08 g/100 g of free fructose, and 8.55 g/100 g of total fructose. The $u(c)/c$ values calculated according to eqs 14 and 15 for these three carbohydrates are 3.85, 10.7, and 4.25%, respectively.

As can be seen from **Table 12**, because of the high amount of sucrose and its contribution to the amount of total fructose, both the contribution of sucrose and the total fructose become major sources of the uncertainty of F_f.

The total fructan content of the infant formula is calculated according to eq 2, and its standard uncertainty again is calculated according to eq 16. Accordingly, the following uncertainty budget for a total fructan content of 2.96 g/100 g is obtained (**Table 13** and **Figure 4**). In this case, the determinations of

Table 13. Uncertainty Budget of a Total Fructan Content of 2.96 g/100 g in an Infant Formula

parameter	symbol	value	unit	uncertainty $u(x)$	uncertainty $u(x)/x$
fructose released from fructans correction factor	F_f	3.05	g/100 g	0.5379	17.64%
water uptake	k	0.925		0.0058	0.63%
correction factor for G_f	g	1.05		0.0058	0.55%
total fructans ($k \cdot g \cdot F_f$)	c_f	2.96	g/100 g	0.5230	17.66%
recovery ($c_f > 0.5$ g/100 g)	R	100	%	1.28	1.28%
total fructans corr. R	$\boldsymbol{c_{fcorr.R}}$	**2.96**	**g/100 g**	**0.5245**	**17.71%**

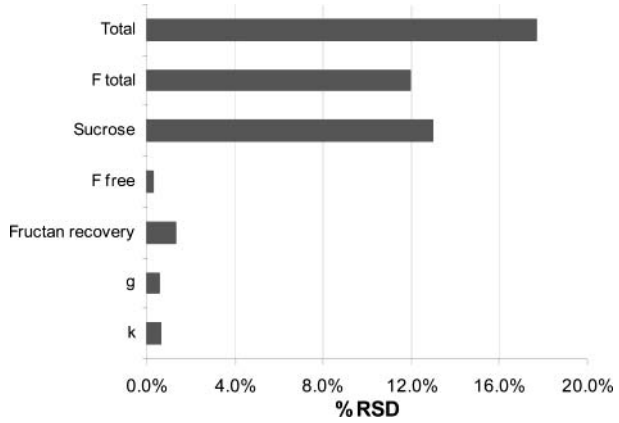


Figure 4. Uncertainty budget of a total fructan content of 2.96 g/100 g (sucrose-rich infant formula).

sucrose and total fructose account almost exclusively for the total uncertainty of c_f.

Accordingly, the result of the total fructan determination in the infant formula containing should be reported as:

$$c_f = 2.96 \pm 1.05 \text{ g/100 g} \quad (18)$$

This corresponds to an expanded uncertainty of ±35% of the actual value.

Relationship between Total Fructan and Sucrose Content. A simulation of the expanded MU that can be expected at different total fructan levels as a function of the sucrose content was carried out. For this purpose, a product was assumed to contain no free fructose, and a k factor of 0.925 and a g factor of 1.05 were used. A limit of quantitation of total fructans in the presence of sucrose can be deduced if one defines a maximum acceptable expanded MU of the actual value. For instance, assuming an expanded standard uncertainty of ±33% of the actual value as a criterion to define the limit of quantitation, a linear relationship between total fructan and sucrose content is obtained (**Figure 5**). In other words, on the basis of this criterion, the limit of quantitation of total fructans by this method (without taking into account G_f) can be approximated by the following equation:

$$\text{LoQ } (c_f) = 0.3 \cdot \text{sucrose content} \quad (19)$$

It should be kept in mind that a similar relationship exists in principle for any total fructan method based on the same approach (that is, independent determinations of sucrose and total fructose), even if it obviously also depends on the analytical method used to determine these carbohydrates.

CONCLUSION

On the basis of the principle of AOAC method 997.08, a simplified method for the determination of total fructans in food

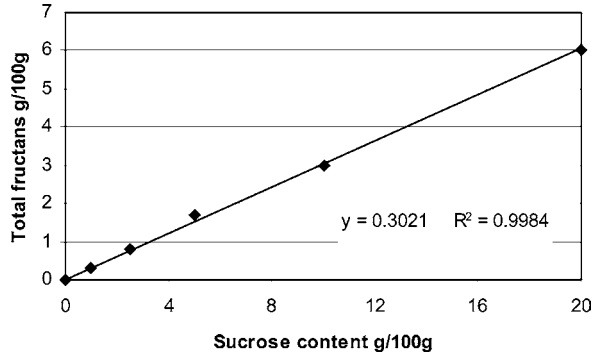


Figure 5. Relationship between total fructan and sucrose for a given expanded uncertainty of ±33% of the total fructan amount.

and pet food has been developed and validated. The method is suitable to quantify total fructans at any concentration ≥0.2% (without sucrose) in food and pet food products as well as in fructan ingredients (raw materials and preparations). It is important to keep in mind that no distinction is made between inulin and graminan type fructans; in other words, cereal fructans will contribute to the overall amount of fructans determined in a finished product.

In the proposed method, the result is based on the determination of F_f alone (fructose released from fructans upon enzymatic hydrolysis) for products containing less than about 5% of total fructans, which covers the vast majority of actual fructan applications in finished food and pet food products. This is justified by the inherent difficulty to accurately determine low amounts of G_f (glucose released from fructans) by difference in most fructan-containing food products. To take into account the theoretical contribution of G_f, the use of a correction factor g is proposed. A suitable correction factor to be used by default is 1.05, which corresponds to a F_f/G_f ratio of 20 as found in most commercially available fructan preparations. The method also allows us to determine precisely g if the fructan ingredient used in the food product analyzed is known and available for analysis.

As part of a MU estimation study, the method's performance criteria have been evaluated. The recovery, both in fructan preparations and in finished food and pet food products, ranged from 97 to 102%. Following the recommendations of the Eurachem guide, all relevant contributions to the total standard uncertainty of the total fructan result have been identified and quantified. With regard to the HPAE chromatographic determination of fructose, glucose, and sucrose, the most relevant sources of the total standard uncertainty are the repeatability of the determination in duplicate (between 1 and 3%), the repeatability of the integration of the external carbohydrate standards (1.0−2.4%), and the uncertainty related to the linear calibration model used (2−3%).

However, given that the total fructan result is obtained by difference, the case of products where fructans are the only sources of total fructose to be determined has to be distinguished from that where other carbohydrates contribute to the total fructose amount (mainly sucrose). In products without sucrose, the relative standard uncertainty $u(c_f)/c_f$ is typically about 4.5% (expanded uncertainty U about ±9% of the actual result). Interestingly, the calculated MU for the in-house quality control sample used in our laboratory is of about the same order as the control limit determined based on an intermediate reproducibility study.

In products containing sucrose, the MU is directly related to the [fructans to sucrose] ratio. As an approximation, we could demonstrate that this method (and in principle any total fructan method based on the same approach) is not appropriate to quantify fructans with reasonable accuracy in products containing an amount of sucrose that is about three times (or more) that of total fructans.

To improve the accuracy of the determination of F_f in the presence of significant amounts of sucrose (e.g., in infant formulas), sucrose can be selectively hydrolyzed and the resulting free sugars reduced to sugar alcohols by borohydride prior to the enzymatic fructan hydrolysis step (*9*). However, the undesired reduction of reducing F_m type fructans in this alternative method is another problem and requires a sound knowledge of the composition of the fructan ingredient to be analyzed (use of correction factor).

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