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Determination of complex polysaccharides by HPAE-PAD in foods: Validation using accuracy profile☆

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ABSTRACT

The increasing supplementation of foods with carbohydrates substitutes and the growing regulatory requirements for controlling these products, turn into the necessary development and validation of accurate analytical control techniques. This paper presents the simultaneous validation of two close analytical procedures for the determination of sucralose and fructooligosaccharides (FOS) in fruit juices using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD). This study consisted in applying the accuracy profile procedure with a three-level validation experimental design. Decision criteria, namely acceptability limits (±10%) and proportion of result contained in the calculated tolerance intervals (80%), were decided on a consensus basis with end-users, whereas no official references were available. In conclusion, the proposed analytical procedures were validated over the selected validation domains for fruit juices and came out on very capable techniques. Validation strategy was purposely oriented towards the ease of use in routine and the liability of the methods rather than extreme performances. This objective is consistent with this of contract laboratories which need to reach a known level of guarantee for the results which they produce. In that respect, accuracy profile represents a very convenient tool to ascertain such a goal.

1. Introduction

In search of sugar substitutes and reduction calories contents in diet, several synthetic di- and polysaccharides were developed for their interesting nutritional properties and their possible use as effective food additives. Considering their growing use by food industry and the possible nutritional claims related to their incorporation into foods, it was necessary to develop fully validated analytical methods in order to control the actual levels of these ingredients in human foods. This paper presents the validation studies to two types of these products:

- sucralose (or trichlorogalactosaccharose) which is a low-calorie sweetener used worldwide in many foods, beverages, and nutritional products;
- fructooligosaccharides (FOS) which are probitotics presenting several positive nutritional effects.

Sucralose, also known as 4,1′,6′-trichlorogalactosucrose, was discovered in 1976 and jointly developed by Tate & Lyle PLC and McNeil Nutritionals, LLC. It is industrially produced by selectively substituting three hydroxyl groups of sucrose by chlorine atoms, according to a patented multi-step process. This change produces a sweetener that has no calories, yet is 600 times sweeter than sucrose. Final molecule illustrated in Fig. 1 is 1,6-dichloro-1,6-dideoxy-β-D-fructofuranosyl-4-chloro-4-deoxy-α-D-galactopyranoside and has a molecular weight of 397.64. Many toxicological studies concluded to the absence of toxicity of this molecule and many regulation bodies agreed for its use as food additive. A special issue of the journal *Food and Toxicological Chemistry* presents an extensive review of major nutritional and toxicological studies devoted to sucralose [1].

The Codex General Standard for Food Additives (GFSA) on-line database proposes several maximum provision limits for the addition of sucralose into many food categories [2]. They are ranging from 120 up to 5000 mg/kg for chewing-gums. When dealing with fruit juices or fruit-based preparations, the limits are around 200 up to 300 mg/kg.

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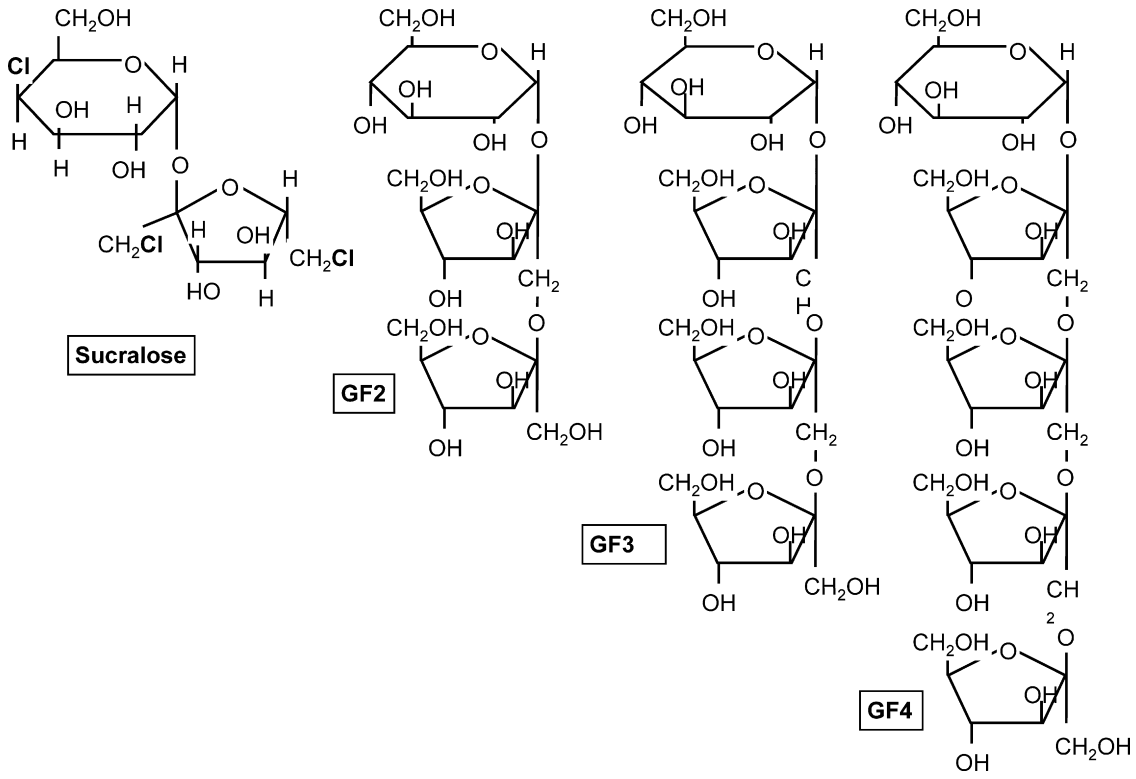

Fig. 1. Sucralose and FOS molecules.

Considering analytical aspect several methods were developed for nutritional studies, for instance urinary lactulose and mannitol have been quantified by several methods including paper and thin layer chromatography, enzymatic assays, gas chromatography and HPLC [3]. Exploratory analysis of commercial sweeteners by infrared spectroscopy (FTIR) and principal component analysis (PCA) was also described [4]. In a recent paper, Morlock and Prabha [5] present a review of available methods for the determination of sucralose in milk. They conclude in favour of planar chromatography for its cost-effectiveness but underline the advantage of anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) to get rid of interfering background, in comparison with other HPLC methods.

Fructooligosaccharides (FOS) refer to a class of naturally occurring non-digestible polysaccharides present in a wide variety of plants and, consequently, present in human diet for centuries. Likewise fibres, they are non-digestible and pass through the human digestive tracts virtually unchanged. On the one side, whereas they provide almost no calories and approximately have one-half the sweetness of sugar, they are used as sweeteners. On the other side, their probiotics potency was demonstrated as promoting the development of bifidobacteria in human colon [6]. While several nutritional claims are associated to FOS, they are being added to a variety of food products. The recommended daily dosage in human diet ranges from 500 to 3000 mg. From a chemical viewpoint, FOS have been defined as a mixture of:

- 1-kestose (1-kestotriose or GF2);
- nystose (1,1-kestotetraose or GF3);
- 1^F-β-fructofuranosylnystose (1,1,1-kestopentaose or GF4) [7].

The molecular structures of each type of polymer unit are presented in Fig. 1.

Traditionally a variety of techniques have been used to analyse for FOS in foods. An extensive review is available in [8], including gel permeation chromatography, high-performance liquid chromatography (HPLC) and gas chromatography (GC). By far the most used procedure for analysis of FOS is HPAE-PAD [9]. However, these numerous methods were developed for toxicological or nutritional experiments and applied to biological fluids or high concentration preparations. But, there is a lack for routine analytical methods applicable to the control of foods such as consumed.

When considering published HPLC methods for the determination of sugars and sugar alcohols used as sweeteners in food products, an abundant literature is available. Most accepted methods include various types of columns and detectors, such evaporative light scattering, refractive index, diode array or mass spectrometers. They all present various drawbacks and advantages. Because our prospect was to develop a routine method based on a single operating device equipped with a unique column, acceptable for all considered analytes, it was decided to select HPAE-PAD.

In that context, we selected two published methods using the same chromatographic system, i.e. HPAE-PAD and anion-exchange resin column:

- for sucralose, published by Clarke et al. [10];
- for FOS, published by Hogarth et al. [7].

The goal of this paper is to describe the validation of these two methods, initially developed for research purposes, using a new validation procedure called accuracy profile, in the prospect of assessing a routine food control technique.

2. Experimental and chromatographic conditions

2.1. General

Measurements for all analytes were performed on a Dionex ICS 3000 (Dionex, Sunnyvale, CA) chromatograph equipped with a Pulsed Amperometric Detector (PAD) with a working gold electrode operating in the integrated amperometric mode. The unique column used was a PA1 CarboPac anion-exchange resin column (25 cm × 4 cm)/guard column (5 cm × 4 mm) in a temperature-controlled jacket at 25 °C. Chromatographic equipment was controlled by Chromeleon® software Version 6.80 and validation data processed using an in-house Microsoft Excel add-in.

General equipment for sample and standard solution preparation included:

- laboratory blender with stainless steel blending cups;
- a source of 18 MΩ deionised water;
- class A volumetric flasks and pipettes, general glasswar;
- balances with a precision of ±1 mg for food samples and ±0.1 mg for pure reagents;
- Whatman filter paper 190 mm;
- a filtering syringe with cellulose acetate, porosity 0.45 μm and diameter 25 mm (Chromafil RC-45/25, Macherey-Nagel).

Two stability studies were performed for each analyte in order to assess the shelf-life of stock solutions. They consisted in the same experimental design with triplicate measurements at 0, 7, 15 and 30 days. Eventual significant degradation was estimated by ANOVA.

2.2. Sucralose

2.2.1. Reagents and reference chemicals

Analytical grade sucralose was provided from LGC Standards (Molsheim, France) and kept at +4 °C. Because of possible degradation after 7 days, standard stock solution of sucralose (10 g/L) was daily prepared by weighing 100 ± 3 mg of sucralose into a 10-mL volumetric flask and diluted up to final volume with deionised water. All calibration standard solutions were also daily prepared from an intermediate stock solution, prepared by diluting initial stock solution at 1:20 (v/v) in a volumetric flask. The solvents used for elution medium were:

- Solvent A, 100 mM sodium hydroxide/1 M sodium acetate;
- Solvent B, 250 mM sodium hydroxide;
- Solvent C, 18 MΩ deionised water.

2.2.2. Sample preparation

Sample preparation procedure was only applied to liquid samples, such as soft drinks. It consisted in diluting an accurately weighed 0.5 g of liquid sample in 100 mL of deionised water. This solution was filtered on the 0.45 μm cellulose acetate membrane, and 25 μL of this filtrate was directly injected in HPAEC-PAD. When necessary, filtrate can be diluted in order to present a concentration within the calibration range.

2.2.3. Chromatographic conditions

The chromatographic separation conditions were adapted and optimised from a previously published operating procedure [10]. Elution was achieved by a mixture of solvents A, B and C in the following proportions 7.5/13.0/79.5 (v/v/v) and delivered at 1.0 mL/min. The PAD detector was operated using a gold electrode with a silver–silver chloride electrode at 2.0 μA in pulsed mode with potential settings following the so-called "carbohydrate waveform", starting at +0.1 V with a maximum of +0.6 V over a period of time of 0.50 s.

2.2.4. Calibration and quantification

Standard solutions were daily prepared from intermediate stock solution at 1.5, 2.5 and 7.5 mg/L by convenient dilution. For quantification purposes, signals were recorded as peak surfaces and measurements were directly integrated using Chromeleon® Chromatography Management System. Linear calibration models were calculated for each day using either Ordinary Least-Squares (OLS) or Weighted Least-Squares (WLS), in order to verify the influence of heteroscedadicity of responses on the accuracy of the results. Concentration C in the filtrate was calculated by inverse prediction using the daily calibration model. Final result was expressed as

$$X = \frac{C \times V}{m} \quad (1)$$

where X was sucralose concentration in sample in g/100 g, C was sucralose concentration in filtrate in g/100 g, V was final dilution volume (mL), and m sample aliquot in g. For quality control, every 12 samples a standard solution was injected in order to control any possible variation of the sensitivity.

2.3. Fructooligosaccharides

2.3.1. Reagents and reference chemicals

Reagent-grade FOS standards of GF2, GF3, and GF4 were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Three separate stock solutions of 1-kestose, nystose and 1^F-β-fructofuranosylnystose at 10 mg/mL were directly prepared by weighing and diluted in a 7:100 (v/v) 2-propanol/water mixture. According to the results of the stability study, these stock solutions were kept during 1 week at +4 °C. Later on, calibration solutions were daily prepared from the intermediate composite stock solution that contained the three analytes at 0.5 mg/L each in deionised water. The solvents used for elution medium were:

- Solvent D, 100 mM sodium hydroxide/600 mM sodium acetate;
- Solvent E, 200 mM sodium hydroxide;
- Solvent F, 18 MΩ deionised water.

2.3.2. Sample preparation

Liquid samples, mainly fruit juices and soft drinks, were diluted in such way that FOS component concentrations would fall within the range of the calibration curve. Typically, for fruit juices 1 mL was diluted to 100 mL with deionised water. Finally, before injection samples are filtered through 0.45 μm cellulose acetate filters. In all cases, 25 μL of filtrate was directly injected in the chromatograph.

2.3.3. Chromatographic conditions

The chromatographic separation conditions were adapted and optimised from a previous publication [7]. Elution was achieved by a mixture of solvents D, E and F mixed according to the following gradient proportions (v/v/v) and delivered at 1.0 mL/min:

Time (min)	D (%)	E (%)	F (%)
0.0	0	50	50
2.0	2	49	49
20.0	16	42	42
25.0–35.0	0	50	50

The PAD detector was also operated using a gold electrode with a silver–silver chloride electrode at 2.0 μA in pulsed mode with potential settings under the same conditions than for sucralose. And the same quality control procedure was applied that consisted in injecting a standard calibration solution every 12 samples.

2.3.4. Calibration and quantification

Standard solutions were daily prepared from intermediate stock solution at 2.5, 5.0, and 15 mg/L of GF2, GF3 and GF4 by convenient dilution. Quantification was achieved by Chromeleon software using the same equation than (1).

3. Statistical processing: accuracy profile

Accuracy of measurement is defined in the International Vocabulary of Metrology (VIM) as "the closeness of agreement between a measured quantity value and a true quantity value of the measurand". In other terms, it characterises a result rather than a method. In that respect it is valuable to validate a method on the basis of the accuracy of the measurements it is able to produce rather than the method by itself.

It is possible to assess accuracy in a global way according to the use of accuracy profile coming from the concept of acceptability limit. Accuracy profile is a decision-making graphical tool aiming to help the analyst in deciding whether an analytical procedure gives accurate results. It consists in simultaneously combining in one single graphic tolerance intervals (TI) and acceptability limits (noted $\pm\lambda$). TI is a concept introduced by statisticians in the early 1940s and represents an interval that covers a certain percentage of a distribution with a given probability. It was widely used for developing control chats in industry. For instance, a tolerance interval can be claimed to contain an expected proportion of 80% of future measurements. TI must not be confounded with confidence interval which is only devoted to a statistical parameter, such as a mean or a reproducibility standard deviation. In that respect TI is well adapted to the goal of validation; obviously, end-users need a guarantee on individual results and not on the standard deviation of the theoretical distribution of data.

Accuracy profile has been introduced by a standardisation body related to pharmaceutical industry – Societé Française des Sciences et Techniques Pharmaceutiques (SFSTP) – in order to easily evaluate the capability of the method to quantify samples with a known accuracy and a risk fixed according to the objective of an analytical method. This goal must be put in the perspective of the fitness-for-purpose of a method. Too many validation procedures are only limited to the calculation of statistical criteria but do not propose effective techniques to decide whether a method is able to accurately quantify a measurand. The ambition of the accuracy profile approach is to bring this understanding. General references on accuracy profile are available in a series of papers which present the theory and applications of this new validation procedure [11–13].

The application of accuracy profile can be summarised as a 10-step procedure:

1. Define the validation domain of the method of analysis in terms of concentration levels and its objective in terms of an acceptability criterion noted $\pm\lambda$. It is usual to select one single value of λ for whole validation domain, but it is possible to have different values depending on the level of concentration.
2. Select K validation samples with known concentration levels, called hereafter reference values and noted T. Several techniques exist to select these samples: reference materials, spiked samples, surrogate samples, etc. The concentration levels of the various validation samples must cover the whole validation domain.
3. Define the experimental design for validation. The validation design is fully characterised by the number of series I, the number of replicates by series and level J, and the number of concentration levels of the validation samples K. The number of validation assays is equal to $I \times J \times K$. These assays are performed under the condition of intermediate precision and are used to calculate trueness and precision criteria and compute the tolerance intervals.
4. Define the experimental design for calibration. The calibration design is fully characterised by the number of series I which is similar to the number of series of the validation design, the number of replicates by series and level J', and the number of concentration levels of the calibration solutions K'. The number of calibration assays is equal to $I \times J' \times K'$.
5. Collect validation and calibration data. Any deviation from the planned experiments must be reported.
6. Collect the recovered concentrations in the validation samples. Fit calibration model for each series (day) and calculated inverse predicted concentrations. These concentrations are noted x_{ijk}, where i is the series number, j the replicate number and k the level number.
7. For each level k, compute the validation criteria, namely the intermediate precision standard deviation s_R and the bias.
   - The basic principle behind the calculation of s_R is that all x_{ijk} replicates of one level are modelled according to a random effect ANOVA. This classical statistical procedure is fully described in ISO 5725-2 standard [14]. Total variance is split into the within-laboratory variance s_r^2 (or repeatability variance) and the between-laboratory variance s_L^2. Finally, the intermediate precision standard deviation for the k level is

$$s_{(k)R} = \sqrt{s_{(k)L}^2 + s_{(k)r}^2} \tag{2}$$

   - Compute $\bar{\bar{x}}_{(k)}$ the global average of measurements and the trueness criterion as a recovery yield for k level:

$$R_{(k)}\% = \frac{\bar{\bar{x}}_{(k)}}{T_{(k)}} \times 100 \tag{3}$$

   - For each level, compute the limits of the β-content tolerance interval (TI) (for details see [12]). TI is defined as the interval where the expected proportion (with an error risk α) of future results will fall is β. TI can be expressed as

$$\left[\bar{\bar{x}}_{(k)} \pm k_{(k)M} \times s_{(k)R}\right] \tag{4}$$

8. For each level k, compute the relative limits of TI to reference value T, i.e.

$$\left[\frac{(\bar{\bar{x}}_{(k)} \pm k_{(k)M} \times s_{(k)R})}{T_{(k)}} \times 100\right] \tag{5}$$

9. Make a graphical representation of computed results as follows:
   - on the horizontal axis report reference values $T_{(k)}$;
   - on the vertical axis expressed as %, simultaneously report for each level, the recovery yields (Eq. (3)), the acceptability limits $\pm\lambda$ and the relative limits of TIs (Eq. (5)).
10. Interpret the graphics and give final conclusion on the validity of the method or propose complementary study.

This sequence will be used to present the results collected in this study.

4. Results

4.1. Validation protocols

Both categories of analytes – sucralose and FOS – are simultaneously presented, although analytical methods slightly differ and all analytes cannot be determined at the same time. But, many experimental features are the similar in both validation studies. Typical chromatograms are illustrated in Figs. 2 and 3. They demonstrate

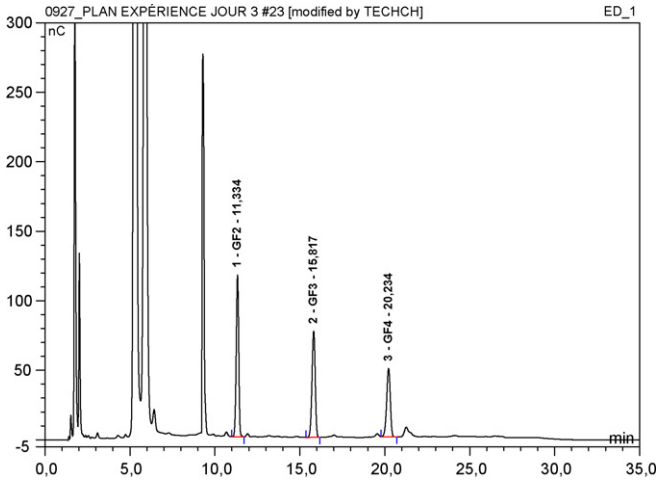

Fig. 2. HPAEC-PAD chromatogram of FOS components in validation sample (fruit juice) spiked at 7.5 mg/L level. Peak surface expressed in arbitrary units.

that chromatographic conditions have been optimised in order to obtain a very satisfactory peak resolution. The selectivity of the method was tested by visually comparing chromatograms of different blank samples, supplemented matrices and standard solutions. No interferences were observed at the retention times of sucralose and FOS.

4.1.1. Validation domains and acceptability limits

The study was focused on fruit juices and soft drinks, and regulatory references can be used to assess the validation domains. Considering sucralose, on the one hand, European directive 2003/115/EC provides maximum dosage level of 300 mg/L for "milk and milk-derivative-based or fruit juice based, energy-reduced or with no added sugar", on the other hand, Joint Expert Committee of Codex Alimentarius (JECFA) defines maximum levels of incorporation for fruit nectars at 400 mg/L [15]. When dealing with FOS, it is generally recognised as safe by FDA and no maximum incorporation level was defined for soft drinks or fruit juices [16]. It is admitted that tolerable dosages range from 500 to 750 mg daily in the total diet.

Finally, the validation domains were established after a discussion with the result end-users and range from 1.5 up to 7.5 mg/L for sucralose, and from 2.5 up to 15.0 mg/L for FOS. For other food categories than fruit juices, adequate dilution can be applied in order to fall within these limits. Selected calibration domains were similar to validation domains for each analyte.

Considering the acceptability limits, in absence of any official reference, decision was also taken after consulting end-users. They were set at ±10% for all analytes. On the other hand, the expected proportion of data contained into the TI was set at 80%. This means that the methods can be claimed as validated if the analyst can guarantee that, at least, 80% of measurements will fall between ±10% of the actual value of the sample.

4.1.2. Selection of validation samples and preparation of spiked samples

The same commercial fruit juice was used as a validation sample for both validation studies. Preliminary measurements demonstrated it was free from sucralose and FOS. Requested concentration levels were obtained by spiking this unique sample with convenient volumes of the intermediate stock solutions. The operation was repeated each day in order to take into account this source of uncertainty in the global error of measurement.

4.1.3. Experimental designs

Validation and calibration designs both consisted in 3 days, 3 replicates and 3 levels of concentration. Altogether the number of trials was 27. These values were selected as a good compromise between the total number of analyses that can be achieved over one day and the cost of the validation study. Validation and calibration measurements were collected on the same days. All measurements were made by Institut Scientifique d'Hygiène Alimentaire (ISHA) which is an accredited contract laboratory specialised in food chemistry. Standard operating procedure was also developed by ISHA in the framework of its quality management system.

4.1.4. Quantification

Chromeleon® integration system was able to simultaneously express the instrumental response as peak surface and peak height. Both types of recordings were used and compared. Quantification was directly achieved, either from peak surface, or peak height as described in *Calibration and quantification* section.

4.2. Accuracy profiles

4.2.1. Sucralose

Accuracy profiles for sucralose, as illustrated in Fig. 4. When considering this figure, it can be concluded that the method is valid for sucralose quantification because all TIs are comprised within the acceptability limits of ±10%. Let us recall that the decision rule is to

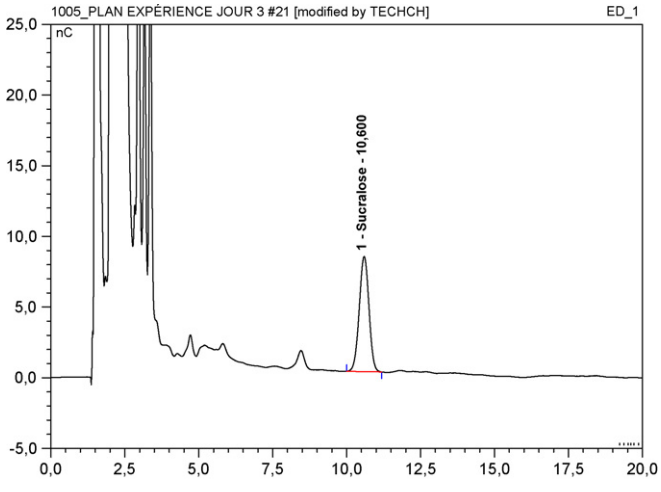

Fig. 3. HPAEC-PAD chromatogram of sucralose in spiked fruit juice sample at 7.5 mg/L Peak surface expressed in arbitrary units.

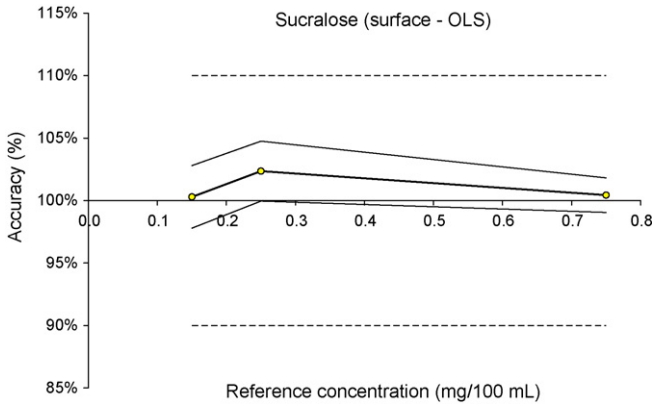

Fig. 4. Accuracy profile of sucralose.

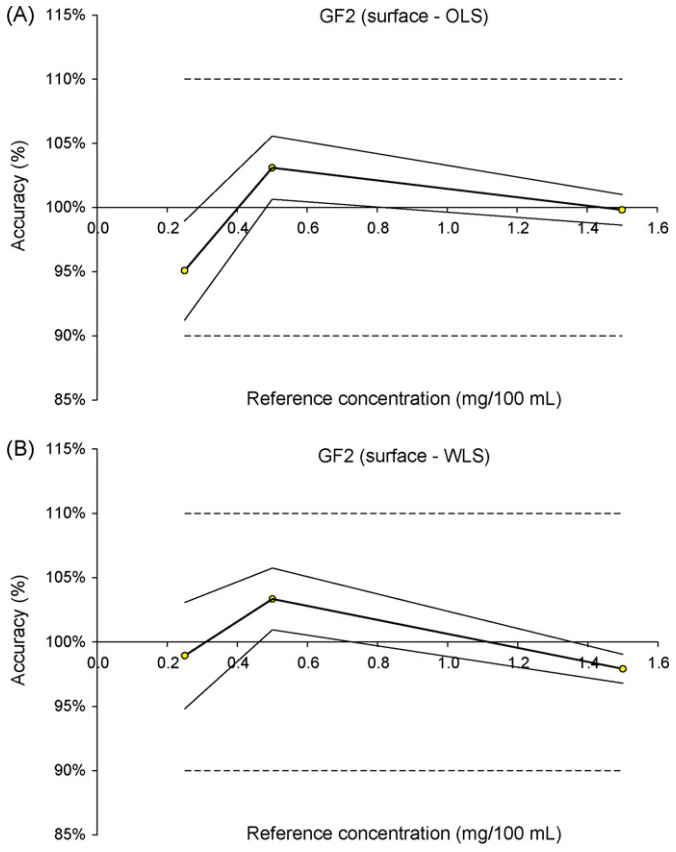

Fig. 5. Comparison of different accuracy profiles for GF2 using linear calibration models estimated by Ordinary Least-Squares (OLS) or Weighted Least-Squares (WLS) regression: (A) peak surface and OLS; (B) peak surface and WLS.

consider a method as valid as far as it is capable produce at least β% of future results between $(1 \pm \lambda)$% around the reference true value. Whereas the TI is very narrow it can be concluded that sucralose can be easily quantified by HPAEC-PAD, and the method is very capable over the studied validation range of 0.15–0.75 mg/L. In this case the Limit Of Quantification (LOQ) is equal to the lowest limit of the validation domain, i.e. 0.15 mg/L, but if required better performance could be achieved by widening this domain towards lower concentration.

4.2.2. Fructooligosaccharides

Fig. 5 presents two accuracy profiles for GF2 (1-kestose or 1-kestotriose) computed from the same experimental assays but under different conditions: for Fig. 5A, daily linear calibration models were estimated by OLS; for Fig. 5B, calibration models were fitted by WLS.

When considering Fig. 5, it can be concluded that the method is valid when both GF2 quantification techniques are used. The application WLS for estimating model coefficients is illustrated in Fig. 5B and slightly improves the capability of the analytical method, because TIs are narrower and better centred around the 100% recovery line. But the use of WLS for routine measurements makes the method more complicated for operators. Whereas, Chromeleon management software is only able to apply OLS it would require an intermediate calculation step to routinely use WLS. Therefore, it was decided to simply use OLS for routine analysis. As an illustration, Table 1 puts together the raw recovered concentrations calculated under these conditions and the TI and acceptability limits that were used to compute accuracy profile of Fig. 5A. A relationship between the intermediate precision and the concentration

Table 1
Results of the validation design for GF2 expressed in mg/100 mL and quantified from peak surface and OLS calibration models. Values are recovered concentrations from blank fruit juice spiked samples and spiking levels are used as reference values. Accuracy profile data are calculated using $\beta = 80\%$ for tolerance interval probability content.

Days	Levels	Reference value	Replicate 1	Replicate 2	Replicate 3
Day 1	A	0.25	0.233	0.233	0.232
	B	0.50	0.518	0.515	0.516
	C	1.50	1.507	1.506	1.494
Day 2	A	0.25	0.236	0.238	0.243
	B	0.50	0.496	0.514	0.518
	C	1.50	1.471	1.490	1.504
Day 3	A	0.25	0.244	0.242	0.239
	B	0.50	0.523	0.521	0.517
	C	1.50	1.497	1.500	1.506

Accuracy profile data	Levels		
	A	B	C
Average recovered concentration	0.238	0.516	1.497
Intermediate precision standard deviation	0.0051	0.0080	0.0118
Relative intermediate precision standard deviation (%)	2.16	1.54	0.79
Recovery yield (%)	95.09	103.11	99.82
TI lower limit (%)	91.22	100.65	98.62
TI upper limit (%)	98.96	105.57	101.02
Acceptability lower (%)	90.00	90.00	90.00
Acceptability upper (%)	110.00	110.00	110.00

can be observed as precision relative standard deviation is changing from 2.2% to 0.8%. Likewise sucralose, LOQ is equal to the lowest level of the validation domain, i.e. 0.25 mg/L. But, it seems obvious that lower LOQ could be achieved by extending the validation to lower concentration.

The goal of this comparison between both regression techniques was to demonstrate that accuracy profile can also be used to set some final conditions of the standard operating procedure that cannot always be assessed during the development of the method.

Fig. 6 illustrates the accuracy profile of GF3 (nystose or 1,1-kestotetraose). Using the same decision rule, the proposed analytical method can be claimed as valid for this analyte and LOQ fixed at 0.25 mg/L. This analyte does not bring any further comment and confirm the fitness of the analytical technique to the goal at stake.

When considering the accuracy profile of GF4 (1^F-β-fructofuranosylnystose or 1,1,1-kestopentaose) in Fig. 7, the situation is rather different and the method cannot be alleged

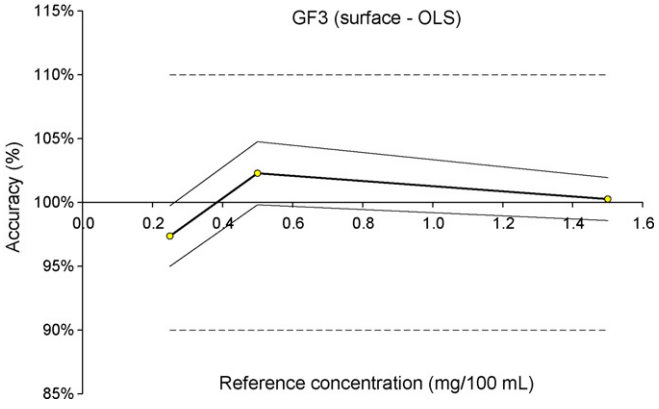

Fig. 6. Accuracy profile for GF3 using peak surface and OLS linear calibration model.

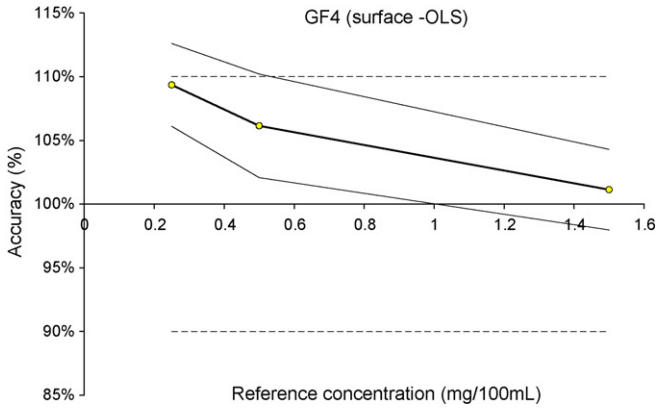

Fig. 7. Accuracy profile for GF4.

as valid because of an important systematic bias. This bias can be slightly reduced by using WLS but this solution is not fully satisfactory as far as the chosen validation domain is not yet wholly valid.

4.2.3. Assessing correction factor

This situation illustrates an interesting feature of accuracy profile. This statistical tool can be used for decision-making but also for revealing some ways to improve an operating procedure, which are not available during the development step. The existence of a relationship between precision and concentration is well established and was underlined for GF2 and the criteria presented in Table 1. But Fig. 7 shows that there is also a relationship between the trueness and the concentration in the case of GF4.

If we assume that the linearity is correct, a global model can be proposed to describe the recovered concentrations X as a function of the reference values T:

$$X = a_0 + a_1 T + E \quad (6)$$

In this equation a_0 represents a systematic bias, also called *additive bias* because it produces a constant shift of the recovered values, a_1 a *multiplicative bias* that modifies the proportionality between the recovered concentration and the reference value, and E a residual random error [17]. When unbiased method, $a_0 = 0$ and $a_1 = 1$.

Fig. 8 illustrates this relationship for GF4. Because, X can be considered as a dependent variable and T as explicative variable, the coefficients of Eq. (6) can be estimated by OLS regression. It comes $a_0 = 0.03$ and $a_1 = 0.99$.

If we want to combine these results with the observed accuracy profile of Fig. 7, it is necessary to divide both members of Eq. (6) by

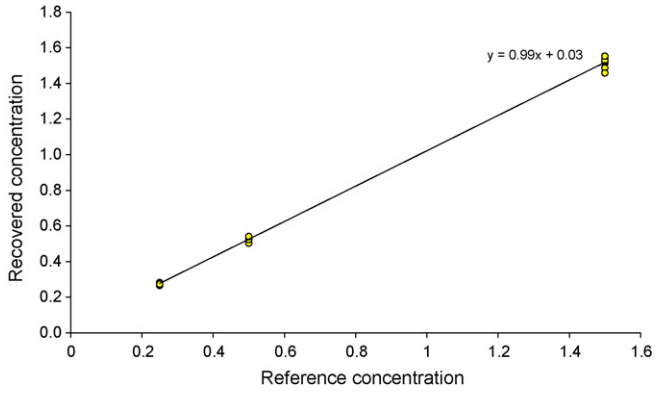

Fig. 8. Linearity of GF4 based on the recovered concentrations.

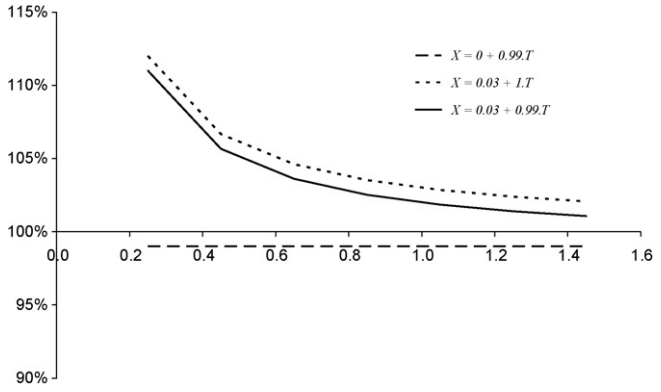

Fig. 9. Simulation of the role of the coefficients of the multiplicative and additive bias on the shape of the accuracy profile.

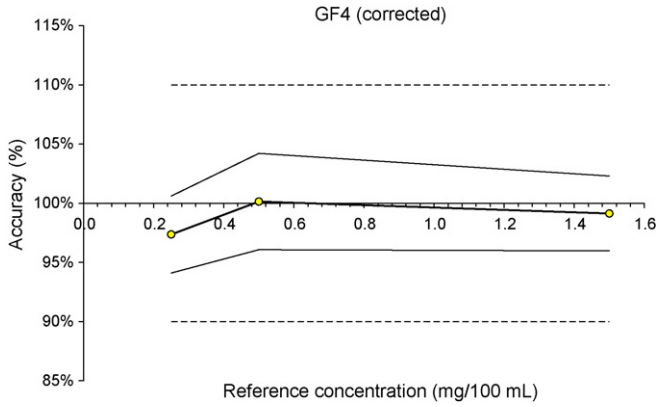

Fig. 10. Accuracy profile for GF4 after subtracting the additive bias.

T; resulting equation is no more linear and it will necessary to be carefully when interpolating:

$$\frac{X}{T} = \frac{a_0}{T} + a_1 + \frac{E}{T} \quad (7)$$

In order to understand the role of each coefficient, the best way consists in simulating their respective influence as illustrated in Fig. 9. Three simulation models are drawn using respectively the value of a_0, the value of a_1 and the combination of both. When comparing these curves to the observed accuracy profile, it seems obvious that a_0 plays the major role in the curvature of the recovery.

Therefore, it can be decided to simply correct all recovered concentrations by a constant of −0.03 mg/L and recalculate the accuracy profile. A new accuracy profile can then be drawn after applying this correction factor; it is illustrated in Fig. 10. It indicates that the method can also be claimed as valid for GF4 by introducing this correction in the expression of the final result and modifying the operating procedure in consequence.

The origin of this additive bias can be variable but, with chromatographic methods using the peak surface as measured signal, it generally caused by the integrator's algorithm when applied to poorly resolved or too large peaks. GF4 is well resolved but is eluted after 20 min (Fig. 2). Elution conditions could be modified in order to reduce this bias, but it was decided to simply correct measurements and keep the operating procedure as it is.

5. Conclusion

Sucralose and FOS are now widely used in food industry and it is necessary to have accurate methods to control the actual levels of

incorporation into foods. The development of accurate methods for this type of new molecules represents a challenge for food chemists. Consumer social demand for better controlled foods and regulatory requirements are more and more demanding, although small amounts of these molecules are incorporated and do not present any hazards. But, better estimation of the global consumer exposure to synthetic molecules through their diet is a compulsory step for quantitative risk assessment.

On the one hand, accuracy profile represents and easy way to deciding whether an analytical technique is well fitted to the purpose of end-user. On the other hand it is very convenient tool for analyst to make final decisions on the operating procedure that will be applied for routine measurements. For instance, we have seen with GF4 that accuracy may help to determine a correction factor that improves the accuracy of the method. Such a decision could not easily be made during the development of the method because an additive bias has not the same influence when the concentration varies. The calculation technique which considers each level of concentration separately is a very convenient way to exhibiting such a systematic bias. Therefore, accuracy profile is a decision-making tool but also a diagnosis tool that helps in understanding the possible weaknesses of an analytical method and proposing solutions to bypass them. It must also be underlined that accuracy profile is well adapted to in-house single laboratory validation.

In general, the described operating procedures that are based on HPAEC-PAD are very capable and well adapted to the goals of users. Lower LOQs may have been determined by increasing the domain of validation but, due to the actual level of incorporation of these additives, it was unnecessary explore lower concentration levels. In conclusion, the validation study was focused on the validation of the methods rather than extreme performance capacity. For instance, several decisions, such as using OLS instead of WLS, were made in order to design operating procedures that will be easy to apply in a routine laboratory. This strategy was purposely decided and accuracy profile provided a good approach to apply it.

This study presents the specific validation study for fruit juices. However, sucralose and FOS are used as ingredients for many other food matrices. When extending the domain of application of the operating procedures to other food matrices, a simple revalidation study can be applied that consists in making complementary measurements for one single level of concentration under intermediate precision condition (several days). Thereafter, an unique TI can be calculated and added into the accuracy profiles which have already been obtained. If inconsistent results would be obtained full validation procedure should be applied for these new matrices.

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