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VALIDATION OF AN ANALYTICAL METHOD FOR THE QUANTIFICATION OF 1-KESTOSE, GLUCOSE AND SUCROSE IN THE REACTION PRODUCTS OF A FRUCTOSYLTRANSFERASE

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ABSTRACT

The enzymatic synthesis of fructooligosaccharides (FOS) from sucrose by the action of fructosyltransferases produces a mixture of different carbohydrates. The concentration of each carbohydrate involved must be quantified to follow its variation as the kinetics of the reaction progresses. This requires a method such as high-performance liquid chromatography (HPLC) with refractive index detector (IR). The aim of this work was to validate the HPLC-IR analytical method for the quantification of total and individual carbohydrates in mixtures of 1-kestose, sucrose and glucose. For this study, the validation parameters recommended by the FDA (Food and Drug Administration), the ICH (International Council for Harmonisation) and the EMEA (European Medicines Agency) were followed. The method was able to quantify carbohydrates with adequate levels of precision, accuracy and linearity in ranges of 1.6-8.7, 4.3-22.0 and 7.0-41.0 mg/mL for glucose, 1-kestose and sucrose, respectively. The lowest limit of detection was for glucose (0.2 mg/mL) and the highest for sucrose (0.8 mg/mL), while the lowest and highest limit of quantification were also for glucose (0.6 mg/mL) and sucrose (1.8 mg/mL). The detection (0.7 mg/mL) and quantification (1.4 mg/mL) limits for 1-kestose were intermediate. The HPLC-IR method was used to calculate the concentration of the carbohydrates involved in the reaction kinetics of a recombinant fructosyltransferase (Sa1-SSTrec). This analytical technique can be implemented for the analysis of samples from the FOS production process at both laboratory and industrial scale, but also the technique is useful to detect 1-kestose in functional foods based in fruit juices.

Keywords: carbohydrates quantification, HPLC-RI, system suitability, 1-kestose

INTRODUCTION

Fructooligosaccharides (FOS) are short-chain soluble fructans considered functional fibers (**Paineau *et al.*, 2014; Franco-Robles and Lopez, 2015**). FOS are typical prebiotics with proven health-promoting effects in humans and animals associated with selective enhancement of beneficial bacteria in the intestinal microbiota, such as *Bifidobacterium* and *Lactobacillus spp* (**Roberfroid, 2007; Ose *et al.*, 2018**). Other benefits associated with FOS consumption are reduction of serum cholesterol, increased absorption of calcium and magnesium, prevention of colon cancer, and production of B vitamins. In addition, FOS are non-cariogenic sugars, low in calories, sweet tasting and exhibit antioxidant activity through free radical scavenging (**Perna *et al.*, 2018; Cunha *et al.*, 2019; Kaplan and Hutkins, 2000; Pereira and Gibson, 2002; Maiorano *et al.*, 2020; Faria *et al.*, 2021**). Among the FOS, 1-kestose has shown strong bifidogenic activity which has been associated with multiple beneficial effects on the host, such as an increase in cecal butyrate level and a decrease in serum insulin level (**Tochio *et al.*, 2016**). The superiority of 1-kestose over mixed FOS in selective stimulatory activity on beneficial microbiota (**Suzuki *et al.*, 2006; Tochio *et al.*, 2018**) suggests the potential of 1-kestose as a prebiotic to improve host health. FOS can be incorporated into other products such as soft drinks, juices or jams that become functional foods or can be consumed directly as nutraceuticals.

Sa1-SSTrec is a recombinant fructosyltransferase from tall fescue (*Schedonorus arundinaceus*) constitutively expressed in *Pichia pastoris*. Either free Sa1-SSTrec or the immobilized recombinant yeast react with sucrose to produce 1-kestose and nystose, the less polymerized FOS. As the enzymatic reaction progresses the fructosyl group of sucrose is transferred to 1-kestose and the concentration of nystose increases, furthermore 1-kestose acts as a fructosyl donor which regenerates sucrose and a small amount of fructose appears reflecting the onset of 1-kestose hydrolysis (**Hernández *et al.*, 2018; Pérez *et al.*, 2021**). To follow the time course of transfructosylations and hydrolysis caused by Sa1-SSTrec a method is required that enables quantification of the carbohydrates involved in the reaction. One of the most widely used analytical assays is molecular exclusion chromatography using high performance liquid chromatography (HPLC) coupled to a refractive index (IR) detector. Molecular exclusion can show the molecular weight distribution in the effluent, which can be associated with a known molecular weight of appropriate molecular weight standards (**Yan, 2014**). However, the application of this analytical assay requires it evaluation using the validation of parameters recommended by the FDA (Food and Drug Administration), ICH (International Council for Harmonisation) and EMEA (European Medicines Agency): precision, accuracy, linearity, range, limit of detection, limit of quantification and specificity.

The objective of this work was to validate the chromatographic method (HPLC-IR) as a simple and fast method to be used in the quantification of carbohydrates during the production of FOS.

MATERIALS Y METHODS

Reagents

The carbohydrates 1-kestose, sucrose, glucose and nystose used as standards in the HPLC were supplied by Sigma-Aldrich (Sigma, St. Louis, MO, USA). The rest of the reagents used were supplied by Applichem (Germany).

The recombinant sucrose-sucrose 1-fructosyltransferase (Sa1-SSTrec) enzyme was obtained from the Research and Development laboratory of the Center for Genetic Engineering and Biotechnology of Sancti Spíritus (**Hernández *et al.*, 2018**).

High Performance Liquid Chromatography coupled to an Index Refraction Detector (HPLC-IR)

An HPLC system (Lachrom Merck Hictachi®, Germany) coupled to a Knauer Differential-Refractometer refractive index detector (model 2300, Germany) and a manual injector with a 20 µL loop was used. Chromatographic separation was performed using an Aminex HPX 42-C 300 mm x 7.8 mm column (BioRad, Richmond, USA) placed inside a column oven (model L-7350), with Carbo C precolumn (BioRad, Richmond, USA) and 50 µL Hamilton® syringe. The runs were performed at a working flow rate of 0.5 mL/min, a pressure of 52 ± 2 bar and a working temperature of 85 ± 2°C. The solvent used as mobile phase was deionized and degassed miliQ water for HPLC. The analog output of the detector is connected to an NI USB-6008 (National instrument data acquisition interface) device that provides eight analog input channels, with a high-speed USB interface connected to a computer that allows signal recording using AdqUSB4 software. The recorded data were exported to the ezData software (www.chemilab.net), for integration and calculation of retention time, height, peak width at half height and area under the curve. The parameters recommended by the FDA, ICH and EP (European Pharmacopoeia) were used to validate the analytical assays: for system

suitability (retention times repeatability, number of theoretical plates, resolution between peaks, peak/valley ratio and peak symmetry) and for validation (linearity, precision, accuracy, range, specificity, limit of detection and quantification).

Samples preparation

Individual standard solutions at 100 mg/mL of sucrose, 1-kestose, glucose and nystose were used for standard preparation. These solutions were combined according to Table 1 and simulate the possible concentrations of the main carbohydrates involved in the Sa1-SSTrec reaction. The standards were assayed by 3 analysts, 2 days each and 3 replicas per analyst.

Table 1 Carbohydrates concentrations (mg/mL)

Standard	Nystose (GF_3)	1-kestose (GF_2)	Sacarose (GF)	Glucose (G)
1	0	0	40	0
2	0	4.3	34	1.6
3	0	8.8	28	3.1
4	0	13.2	22	4.7
5	0	17.6	15	6.3
6	0	22.0	10	7.9
7	4	19. 5	8	8.4
8	6	18.2	7	8.7

System suitability testing

Retention time repeatability

Retention time data were provided by ezData and MATLAB software (version R2015a). The mean, standard deviation and relative standard deviation (% RSD) were calculated. Repeatability was evaluated based on the relative standard deviation values obtained from the retention times (t_R) for each sugar, equation (1). Acceptance criterion ≤ 1%.

$$RSD_{t_R}(\%) = \frac{100}{\bar{y}} * \sqrt{\frac{\sum (y_i - \bar{y})^2}{n-1}} \qquad \text{(Ec.1)}$$

Where y_i is individual values expressed as peak area, peak height or ratio or areas by the internal standardization method; $\bar{y}$ is mean of individual values; and n is number of individual values.

Number of theoretical plates (N)

Column performance (efficiency) was determined from data obtained under isothermal and isocratic conditions. The number of theoretical plates was calculated using equation (2), values of t_R and W_h are expressed in the same units. The acceptance criterion was ≥ 2000 according to manufacturer (BioRad, Richmond, USA).

$$N = 5.54 * (\frac{t_R}{W_h})^2 \qquad \text{(Ec.2)}$$

Where N is number of theoretical plates; t_R is peak retention time (min) and W_h is peak width at mid-height (min).

Resolution between two peaks (R_s)

The resolution between two peaks corresponding to reference standard was calculated using the following equation (3). Acceptance criteria ≥ 1.86 according to the manufacturer (BioRad, Richmond, USA).

$$R_s = 1.18 * \frac{(t_{R2} - t_{R1})}{W_{h1} + W_{h2}} \qquad \text{(Ec.3)}$$

Where R_s is chromatographic column resolution; t_{R1}, t_{R2} are peak retention time (min) and w_{h1}, w_{h2} are peak width at mid-height (min)

Peak/valley ratio (p/v)

Peak/valley ratio is a criterion for related substances when the baseline does not achieve separation between 2 peaks. It was calculated using the following equation

$$p/v = \frac{H_p}{H_v} \qquad \text{(Ec.4)}$$

Where Hp is height above the baseline extrapolated to the lower summit peak; Hv is height above the baseline extrapolated to the lowest curve point separating the lowest and highest peaks.

Peak symmetry (As)

The peak symmetry factor was calculated using the following equation (5).

$$As = \frac{W_{0.05H_p}}{2d} \qquad \text{(Ec.5)}$$

Where $W_{0.05Hp}$ is peak width to one-twentieth of peak height; d is distance between the perpendicular dropped from the maximum peak height and the inflection point at one-twentieth of the peak height.

Acceptance criterion: between 0.8 to 1.5 (**EUROPEAN PHARMACOPOEIA 7.0**)

Carbohydrates quantification validation

Precision

The method precision was evaluated as both, intra-assay (repeatability) and inter-assay precision (includes variability due to experimental error, analysts and days). The factors, analysts and test days, were statistically analyzed using a hierarchical design. Three replicas were used for each standard curve point (concentration vs. area under the curve). The estimation of variance components for the concentration variable, related to each factor analyzed, was performed using the Maximum Likelihood Restricted (MVR) method.

Acceptance criteria: the intra-assay and inter-assay coefficients of variation (CV) for each concentration of glucose, sucrose and 1-kestose should be ≤ 20% at the minimum point from the curve and ≤ 15% at the rest of the points.

Accuracy

For each concentration value of the standard curves, all data were taken from the area under the curve (3 analysts, 2 days each and 3 replicas per analyst). The concentration, the relative error (comparison of the calculated value with the nominal value) and the 95% confidence intervals for the mean of calculated concentrations were determined. Acceptance criteria: the relative errors for each glucose, sucrose and 1-kestose concentration should be ≤ 20% at the minimum point of the curve and ≤ 15% at the rest of the points.

Linearity

For each nominal concentration value of standard curves, all calculated concentration data were taken (3 analysts, 2 days each and 3 replicas per analyst) and the concordance between the nominal concentration value (independent variable) for each point with the real concentration obtained by calculating the area under the curve was evaluated by means of a linear regression analysis. Acceptance criteria: The coefficient of determination (R2) should be ≥ 0.98. The slope of the regression curve must be statistically different from zero (t-test, α=0.05). The value of the intercept should not be statistically different from zero (t-test, α=0.05). Both variables must fit the linear model (significant ANOVA, α=0.05).

Range

Considering the accuracy, precision and linearity results obtained for the calculated concentrations of each type of carbohydrate, the values that fulfilled the acceptance criteria were taken as the limit values of the range. Acceptance criteria: The minimum value of the range will be taken as the point on the curve where both the relative error and the CV are ≤ 20% and the maximum value will be taken as the point where both the relative error and the CV are ≤ 15%. Both values must be in the linear range.

Detection and quantification limit

The determination was performed from the data of the calibration curves of each carbohydrate. The detection limit was calculated from the value of the standard error of the intercept multiplied by 3.29, as recommended by the ICH (limit value of area under the curve). The quantification limit was calculated from the standard error value of the intercept multiplied by 10, as recommended by the ICH (minimum quantifiable value of area under the curve).

Specificity

For the experimental evaluation of this parameter, standards for each carbohydrate type were prepared in the same way as above, but 10 µL of the respective buffers (100 mM sodium phosphate pH 6, 100 mM citrate pH 5, 100 mM sodium phosphate + heat-inactivated Sa1-SSTrec at a final concentration of 9 U/mL) were added instead of 120 µL of water and 110 µL were added and compared with the carbohydrates dissolved in water. One enzyme unit (U) represents the amount of Sa1-SSTrec releasing 1 µmol of glucose per minute at initial reaction rates in a 1.75 mol/L sucrose solution in 100 mM sodium acetate buffer, pH 5.5, at 30°C. Each analyzed concentration was performed in triplicate. From the calibration curve, the concentrations of each type of carbohydrate were recalculated. The recoveries were measured for each point tested with respect to a control (reaction mixture in water without the potentially interfering compound). Acceptance criteria: It was considered that there was no interference when the recovery for each point tested was in the range of 80 to 120 %.

Robustness

The assay performance was evaluated against small changes introduced to the optimized method. Checking of this assessment will be the result obtained according to the established procedure for the test. Acceptance criteria: It will be considered that there was no variation when the recovery for each point tested is in the range between 80 and 120 %.

RESULTS AND DISCUSSION

HPLC-IR performance evaluation

In this work, validation of the HPLC-IR method for quantification of the main carbohydrates involved in an optimized reaction of the Sa1-SSTrec enzyme was performed. Figure 1 shows the chromatographic profile corresponding to standard 7 (Table 1) where the peaks corresponding to the FOS nystose and 1-kestose appear, followed by the disaccharide sucrose and the monosaccharide glucose, in that order. The chromatogram shows a good separation of the analytes, which allows, in a single chromatographic run of 25 min, the quantification of the different concentrations of each carbohydrate in samples from FOS synthesis.

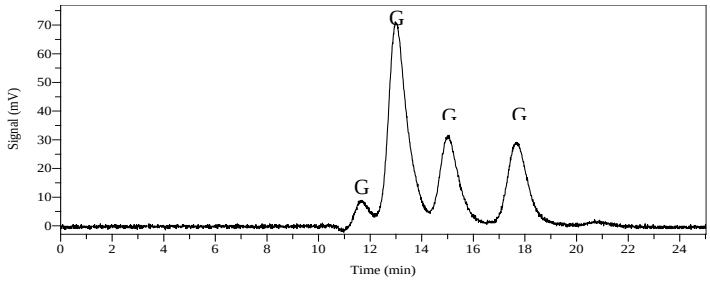

Figure 1 Chromatographic profile obtained from standard 7. Nystose (GF_3), 1-kestose (GF_2), Sucrose (GF), Glucose (G).

In order to guarantee an adequate performance of the HPLC-IR, system suitability tests were performed, since they represent an integral part of the analytical method. The EUROPEAN PHARMACOPOEIA 7.0 states that compliance with system suitability criteria is required throughout the chromatographic procedure.
Table 2 shows the results obtained in the evaluation of instrumental precision by analyzing the repeatability of retention times and peak areas of the molecules used. Since the relative standard deviation values were less than 1%, it was shown that it is possible to minimize the potential errors associated with manual sample injection (low variability in chromatographic injection). These results are similar to those reported by **Correia *et al.*, 2014 and Quiñones-García *et al.* in 2015**.

Table 2 Retention times and peak areas repeatability

	Retention times (min)		peak areas (mV/mseg)	
	$t_R \pm$ SD	%RSD	m ± SD	%RSD
1-Kestose	12.92 ± 0.02	0.18	51.58 ± 2.34	4.55
Sucrose	14.95 ± 0.03	0.22	39.28 ± 2.58	4.55
Glucosa	17.55 ± 0.05	0.34	23.71 ± 2.99	12.53

Legend: %RSD relative standard deviation; m mean, SD standard deviation *H/2* half of peak height

Other parameters commonly used in system performance evaluation were analyzed. The number of theoretical column plates is 2144.46 ± 69.41, which is within the range specified by the BioRad manufacturer. The resolution factor between 1-kestose (GF_2)/Sucrose (GF) peaks was 1.51 ± 0.05. The peak symmetry factor was in the range between 0.8 and 1.5; therefore, the precision becomes more reliable. These data show efficiency in the separation of the analytes that make up the sample, therefore, the chromatographic system is adequate for its use in FOS quantification.

HPLC carbohydrate quantification method validation

To validate the carbohydrate mixture quantification resulting from the transfructosylation reaction, known concentrations of the major components sucrose, glucose and 1-kestose were evaluated. Although nystose appears at the end of the reaction, it was not taken into account for validation since it is a minority component. The following statistical parameters were calculated with data from three experiments obtained on two non-consecutive days by three different analysts: precision, accuracy, linearity, range, limit of detection, limit of quantification and specificity.

Precision

Table 3 shows the variance components and the intra-assay and inter-assay variation coefficients for the carbohydrates analyzed. The coefficients of variation, for each concentration, for both inter and intra-assay precision was less than 20% at the minimum point of the curve and less than 15% at the remaining points. Considering these results, it was demonstrated that the HPLC-IR carbohydrate quantification method is accurate for the three carbohydrates quantified under the conditions studied. Similar results were reported by **Correia *et al.* (2014)** for fructooligosaccharides quantification by the HPLC-IR method.

Table 3 Intra- and inter-assay precision

		Variance components			Variation coefficient (%)	
Nominal concentration (mg/mL)	Average concentration (mg/mL)	Analyst	Day	Error	Intra-assay precision	Inter-assay precision
Glucose						
1.6	1.7	0.000	0.016	0.026	9.2	11.7
3.1	3.0	0.000	0.010	0.064	8.4	9.1
4.7	4.5	0.029	0.000	0.064	5.6	6.8
6.3	6.3	0.029	0.000	0.049	3.5	4.5
7.9	8.0	0.000	0.000	0.094	3.8	3.8
8.4	8.2	0.000	0.000	0.127	4.3	4.3
8.7	8.9	0.029	0.018	0.056	2.7	3.6
1-kestose						
4.3	4.3	0.000	0.087	0.051	5.3	8.7
8.8	8.4	0.095	0.161	0.033	2.2	6.4
13.2	13.1	0.000	0.143	0.078	2.1	3.6
17.6	17.5	0.000	0.302	0.038	1.1	3.3
22	22.4	0.046	0.162	0.395	2.8	3.5
19.5	19.7	0.000	0.000	2.029	7.2	7.2
18.2	17.1	0.333	0.184	0.028	1.0	4.3
Sucrose						
40	39.9	0.000	0.000	0.338	1.5	1.5
34	35.6	0.000	0.000	0.726	2.4	2.4
28	27.3	0.004	0.000	0.082	1.0	1.1
22	20.9	0.000	0.000	0.089	1.4	1.4
16	14.9	0.000	0.000	0.052	1.5	1.5
10	9.8	0.002	0.000	0.065	2.6	2.6
8	9.0	0.000	0.000	0.428	7.3	7.3
7	7.6	0.001	0.013	0.037	2.5	3.0

Accuracy

For all tested concentrations of the three carbohydrates of interest: glucose, 1-kestose and sucrose (Table 4) the relative error is below 20% so that the method of carbohydrate quantification by HPLC-IR is accurate for the three quantified

carbohydrates. Correia and co-workers in 2014 achieved a satisfactory accuracy with a 6% relative error. The results concerning the recovery evaluation show general recoveries between 80 and 120%. These results are in agreement with the values reported by **Borromei et al., (2010)**.

Table 4 Accuracy analysis for the three carbohydrates tested at different concentrations

Nominal concentration (mg/mL)	Average concentration (mg/mL)	Relative error (%)	CI 95%
Glucose			
1.6	1.7	9.3	1.5 - 1.8
3.1	3.1	-0.8	2.8 - 3.2
4.7	4.5	-4.5	4.4 - 4.7
6.3	6.3	-0.7	6.1 - 8.2
7.9	8.0	1.4	7.8 - 8.2
8.4	8.2	-2.1	8.0 - 8.5
8.7	8.9	2.3	8.7 - 8.8
1-Kestose			
4.3	4.3	-0.4	4.1 - 4.4
8.8	8.4	-4.3	8.2 - 8.6
13.2	13.1	-0.6	12.9 - 13.3
17.6	17.5	-0.4	17.3 - 17.8
22	22.4	1.8	22.0 - 22.8
19.5	19.7	1.1	19.0 - 20.4
18.2	17.1	-6.0	16.9 - 17.3
Sucrose			
40	39.9	-0.3	39.6 - 40.2
34	35.6	4.7	35.2 - 36.0
28	27.3	-2.5	27.1 - 27.4
22	20.9	-5.0	20.8 - 21.1
16	14.9	-6.9	14.8 - 15.0
10	9.8	-2.0	9.7 - 10.0
8	9.0	12.5	8.6 - 9.3
7	7.6	8.6	7.5 - 7.7

Legend: CI: confidence interval for the mean concentration.

Linearity

HPLC-IR method linearity, for glucose, 1-kestose and sucrose concentration calculation, was evaluated by means of the curve calibration obtained by linear regression, considering the peak area for each carbohydrate concentration. Figure 2 shows the regression analysis corresponding to glucose, 1-kestose and sucrose. The determination coefficient (R^2) was in all three cases ≥ 0.98. The concentration variability of glucose, 1-kestose and sucrose (98, 98 and 99 %, respectively), is explained by the linear regression model. Regression curve slopes were found to be significantly different from zero ($p \leq 0.05$). The intercepts were statistically equal to zero ($p \geq 0.05$) (Table 5). From these results it is concluded that calculated concentration values are practically equal to the expected concentration. All statistical evaluations show that the method of carbohydrate quantification by HPLC-IR is linear in the concentration range studied for the three carbohydrates quantified.

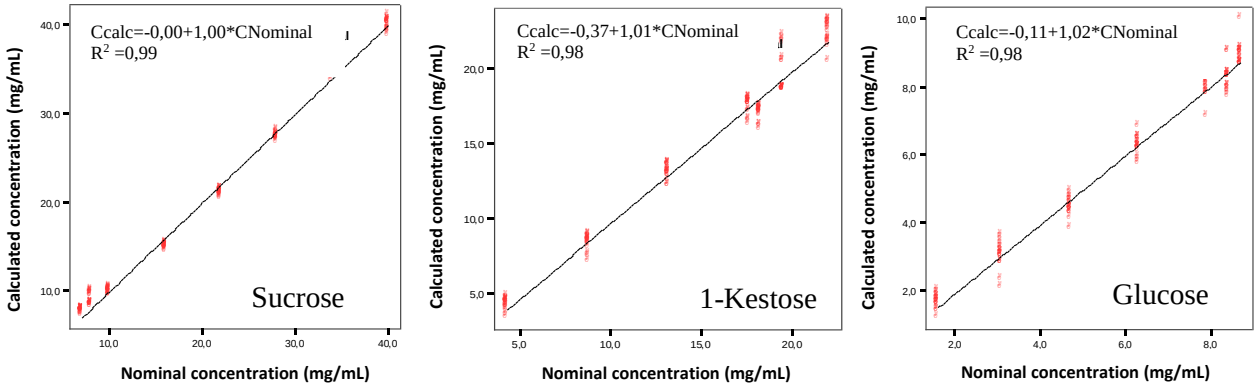

Figure 3 Calculated concentration vs. actual concentration behavior. Ccal: calculated concentration; CNominal: nominal concentration.

Table 5 Linearity statistical analysis and regression analysis parameters

	Glucose		1-kestose		Sucrose	
Parameter	**Estimate**	**p-value**	**Estimate**	**p-value**	**Estimate**	**p-value**
Intercept	-0.114	0.12	-0.370	0.074	-0.003	0.989
Slope	1.017	≥0.000	1.013	≥0.000	1.000	≥0.000
R^2	0.984	-	0.98	-	0.992	-
ANOVA	-	≥0.000	-	≥0.000	-	≥0.000

Range, detection and quantitation limit

The range allows selecting the concentration interval for which the method is able to quantify carbohydrates with adequate levels of precision, accuracy and linearity. The acceptable accuracy, precision and linearity points were taken into account. For accuracy a relative error ≤ ±15% and up to 20% at the minimum point was considered and for precision a variation coefficient ≤15% and up to 20% at the minimum point was considered. In addition, the linearity analysis demonstrated linear behavior for all points tested. The detection and quantification limits were determined from the intercept error of the regression equation using SPSS. For determination of detection limit, the standard error multiplied by 3.29 was converted to concentration through the regression equation, while for the quantification limit; the standard error was multiplied by 10. The results are summarized in Table 6.

The highest range of quantification was obtained for sucrose and the lowest for glucose. The lowest detection and quantification limits correspond with sucrose and the highest with glucose. 1-kestose can be quantified up to a limit of 1.4 mg/mL by this HPLC-IR system.

Table 6 Range, detection and quantification limits

	Range (mg/mL)	Standard error	Detection limit (mg/mL)	Quantification limit (mg/mL)
Glucose	1.6 - 8.7	0.3827	0.8	1.8
1-Kestose	4.3 - 22.0	0.2056	0.7	1.4
Sucrose	7.0 - 41.0	0.1770	0.2	0.6

Specificity

A specificity evaluation was performed, as a measure of the ability of the assay to detect the carbohydrates analyzed in the presence of different buffers and Sa1-SSTrec. As shows Table 7, the recovery for each of the points of the standard curve tested is in the range of 80 to 120 %, so it can be said that there was no interference, except for glucose at the minimum point.

Table 7 Specificity analysis at maximum, intermediate and minimum concentrations of each quantified carbohydrate

GF	Mean Concentration (mg/mL)				CI 95%				Recovery (%)		
	Control	pH 5	pH 6	pH 6+E	Control	pH 5	pH 6	pH 6+E	pH 5	pH 6	pH 6+E
34	33.6	34.7	34.9	34.3	33.2-34.0	34.4-35.0	34.4-35.3	33.7-35.0	103.7	103.7	102.2
16	16.0	16.3	16.5	16.2	15.4-16.6	15.4-17.3	14.8-18.1	15.3-17.2	103.3	103.3	101.4
7	6.6	7.1	7.1	7.0	6.2-7.01	6.4-7.8	6.6-7.6	6.7-7.3	107.5	107.5	106.3
G	**Mean Concentration (mg/mL)**				**CI 95%**				**Recovery (%)**		
	Control	**pH 5**	**pH 6**	**pH 6+E**	**Control**	**pH 5**	**pH 6**	**pH 6+E**	**pH 5**	**pH 6**	**pH 6+E**
1.6	2.6	1.4	1.3	1.4	2.4-2.7	0.7-2.2	1.1-1.4	1.0-1.8	54.5	49.3	56.0
6.3	6.9	6.3	6.3	6.1	6.4-7.3	5.1-7.5	5.7-5.9	5.6-6.6	92.0	92.0	88.3
8.7	8.4	8.8	9.0	7.8	7.3-9.0	8.3-9.3	8.1-9.9	7.6-7.9	100.0	107.2	92.6
GF_2	**Mean Concentration (mg/mL)**				**CI 95%**				**Recovery (%)**		
	Control	**pH 5**	**pH 6**	**pH 6+E**	**Control**	**pH 5**	**pH 6**	**pH 6+E**	**pH 5**	**pH 6**	**pH 6+E**
4.3	4.8	4.2	4.3	4.3	3.8-5.8	3.4-5.0	4.1-4.4	3.9-4.9	87.5	89.0	90.0
17.6	18.4	16.9	17.5	17.5	18.1-18.6	16.4-17.3	15.6-19.4	15.7-19.2	91.7	95.0	94.9
18.2	18.7	17.0	17.4	17.1	17.8-19.5	16.2-17.8	17.3-17.6	16.0-18.2	91.1	93.4	91.6

GF: sucrose; G: glucose; GF_2:1-kestose; pH 6+E: pH 6 buffer with 1-SST enzyme; CI: confidence interval for the mean concentration.

The retention times of the carbohydrates included in the reaction mixture are in agreement with the retention times of reference standard specific molecules. In all cases it was observed that there is no additional chromatographic peak not sufficiently resolved from the peaks corresponding to the reference standard, so it is concluded that the method under the conditions studied is specific.

Robustness

The 2005 ICH Q2 (R1) standard states that robustness evaluation should be considered during the development phase and depends on the procedure type under study. For liquid chromatography the standard states as typical variations, the influence of pH and the mobile phase composition. For the separation of carbohydrates the mobile phase used was water; the stability of pH and conductivity was controlled by a distillation-deionization-ultrafiltration equipment (Siemens Ultra Clear RO). Another variation that can influence robustness is the use of different batches and/or column suppliers; in this work only the Aminex HPX 42-C column (BioRad, Richmond) was used. Temperature and flow rate were as recommended by the column supplier and were not varied during determinations. The oven and pump (Merck-Hitachi) were calibrated by the CIGB Havana Metrology Department and qualified accordingly. The 1-kestose, sucrose and glucose standards were always from the same supplier (Sigma-Aldrich). Since no variations were introduced in the established method, no robustness analysis was performed.

Glucids detection and quantification in the Sa1-SSTrec reaction

The validated HPLC-IR method was applied to follow the variation of carbohydrate concentrations during the Sa1-SSTrec enzyme reaction. Figure 4 shows the chromatographic profiles obtained at the different reaction times.

From the regression curves obtained with different standards, the main carbohydrate concentrations of the reaction were calculated. Table 8 shows the areas under curve for each glucid and the calculated concentration. Equimolar proportions of glucose and 1-kestose were produced until 65-70% of the initial substrate was consumed. From this point, the remaining fructosyl of sucrose was also transferred to 1-kestose producing nystose, which was not further elongated throughout the incubation period. The yield of 1-kestose continued to increase until sucrose depletion reached ~80% after 90 minutes of reaction. At 90 minutes of reaction the highest concentration of 1-kestose is reached which decreases at 120 minutes as it is used as a substrate for nystose synthesis. This analytical methodology will allow establishing the optimal conditions to obtain the maximum yield of 1-kestose and to identify the moment to stop the reaction to maximize the specific production of 1-kestose.

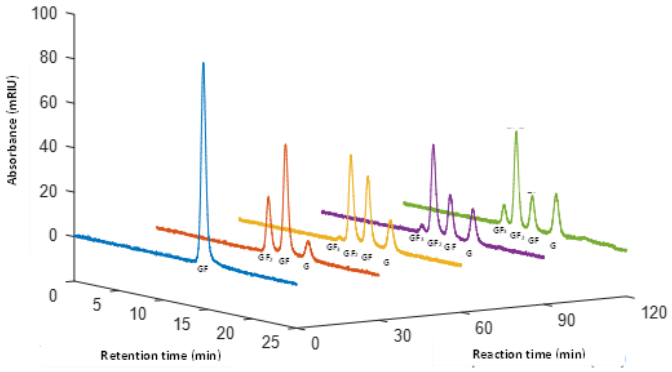

Figure 4 Chromatographic profiles obtained at different times of the Sa1-SSTrec reaction. 9 U/mL of enzyme in a solution of 1.75 M sucrose in 0.1 M sodium acetate buffer, pH 5.5 at 45°C was used. GF_3: nystose, GF_2: 1-kestose, GF: sucrose, G: glucose

1-kestose has shown superior bifidogenic activity than other FOS, which has been associated with several beneficial effects on the host. This demonstrates the potential of 1-kestose as a prebiotic, hence the importance of increasing the yields of this particular FOS during its production. The validation developed in this study demonstrated that the simple HPLC-IR method could be used accurately for the simultaneous quantification of 1-kestose, glucose and sucrose present in samples collected during FOS synthesis using Sa1-SSTrec. Accurate and precise quantification of the carbohydrate composition would allow better nutritional formulation of functional foods to provide prebiotic effects. But, to guarantee the prebiotic effect, 1-kestose should be stable in the food and is needed its quantification. To approach the application of the validated method in the detection and quantification of 1-kestose in beverages, we added a known concentration of 1-kestose to three different fruits juices.

Table 8 Variation of carbohydrate concentrations in the Sa1-SSTrec reaction

	Area (mV/mseg)			Concentration (g/L)		
Time (min)	1-kestose	Sucrose	Glucose	1-kestose	Sucrose	Glucose
0	1.7	57.5	1.4	0.0	599.7	0.0
30	21.5	29.7	8.9	225.7	301.6	70.4
60	28.3	17.4	11.8	307.9	169.6	102.4
90	29.7	13.1	13.2	325.3	123.7	117.4
120	28.4	10.8	14.4	308.8	98.8	130.0

Table 9 Sugar composition of juices

		Guava	Mango	Tomato
Natural carbohydrates (g/L)	GF_2	0	0	0
	GF	6.8	23.1	0
	G	5.0	4.6	8.9
	F	12.4	14.7	14.3
Added GF_2 (g/L)	GF_2	8.0	13.0	8.0
Mean concentration Detected	GF_2 (g/L)	8.1	13.3	7.9
CI 95%		7.4-8.7	12.4-14	7.2-8.6
Recovery (%)		101.2	102.3	98.7

GF: sucrose; G: glucose; GF_2:1-kestose; CI: confidence interval for the mean concentration.

The validated HPLC-IR method was applied for determination of the sugars in fresh juices of guava, mango and tomato and in those mixed with FOS. Mainly three kinds of sugar were detected (sucrose, glucose, and fructose) in the composition of the juices, except in tomato where sucrose was not detected (Table 9). The absence of interfering peaks in the retention time window of 1-kestose was observed by analyzing the independent samples of the evaluated fruits juices. Once added a known concentration of FOS to the juices we determine the content of 1-kestose in juices samples. In all the cases there was an agreement between the expected concentration of 1-kestose and the concentration detected in the sample (Table 9). The separation and quantitative analysis of 1-kestose, glucose and sucrose conducted by the HPLC-IR provides a fast and reliable method for qualitative and quantitative analysis of 1-kestose in fruits and vegetables juices, it can help to follow the stability of FOS in a variety of products to guarantee the prebiotic effect.

CONCLUSIONS

The validation developed in this study showed that the simple HPLC-RI method was accurate, precise, linear and specific according to ICH, EMEA and FDA standards. The validated analytical method allowed following the variation of the concentration of 1-kestose, glucose and sucrose present in the FOS synthesis reaction using the Sa1-SSTrec enzyme. But also could be used to detect the carbohydrates in other matrices.

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