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Development and Validation of HPLC-RID method for Determination of Sugars and Polyols

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Abstract:

The aim of the current study was to develop and validate a rapid, sensitive and accurate method for simultaneous determination of sugars (glucose, fructose, sucrose and lactose) and polyols (erythritol, mannitol, sorbitol, isomalt and xylitol) in low-calorie and no-sugar added desserts by high pressure liquid chromatography coupled with refractive index detector (RID). The best chromatographic separation was performed on Pb^{2+} Shodex Sugars SP0810 operating at 80 °C and mobile phase distilled water with flow rate 0.5 mL/min. Validation procedure includes for linearity, precision and accuracy of the method. The developed method offers excellent linearity in wide concentration range (0.1–5 mg/mL) for all tested carbohydrates with R^2>0.997. Limit of detection (LOD) and limit of quantification (LOQ) for nine analytes were in the range of 0.01–0.17 mg/mL and 0.03–0.56 mg/mL, respectively. HPLC-RID method showed very good repeatability (RSD <5 %) and reproducibility. The developed method was successfully applied for quantification of sugars and sugar alcohols in dessert foods. The results obtained from the analyzed real samples showed the possibility of the proposed method to be used for routine analysis of sugars and sugar alcohols in low-calorie, no-sugar added dessert foods and pharmaceutical formula only with one injection of the sample.

Key word: HPLC-RID method, low-calorie dessert foods, polyols, sugars

INTRODUCTION

The great variety of sweeteners and their properties allow the production of sweet foods with certain nutritional claims, such as "foods without added sugars" (Regulation 1924/2006) [1]. Applications in this area find polyols (sugar alcohols). Sugar alcohols are derived from carbohydrates in which, aldehyde or ketone group is reduced and converted into alcohol [2]. According to the European Food Legislation, polyols authorized for use in the food and beverage industry are as follows: sorbitol, mannitol, isomalt, maltitol, lactitol, xylitol and erythritol. There are no restrictions on their quantity in the dessert foods formula (Regulation EC No 1333/2008) [3]. Polyols have sweetness similar to sucrose, relatively low energy, non-insulin-dependent metabolism and non-cariogenic properties. As food additives and sweeteners, polyols are used as fillers and moisture-retaining agents [4]. They have the property and extend the shelf life of sweet-tasting products, slowing down aging and microbiological deterioration [5].

With respect to consumer health it is extremely important to control the amount of sweeteners in food and pharmaceutical products. Different analytical methods for determination have been reported for the analysis of sugars and/or sugar alcohols were used gas chromatography [6], the high-performance liquid chromatography (HPLC) [7-10] and capillary electrophoresis (CE) [11]. The major drawback of gas chromatography is time-consuming and laborious sample derivatization to trimethylsilanes or alditol acetates [12]. HPLC methods are better choice due to their simplicity, accuracy, and easy sample preparation. High performance liquid chromatography (HPLC) has become popular because it provides a rapid quantitative separation of the main sugars [13]. High-performance liquid chromatography coupled with an ultraviolet-visible (UV-VIS) detector [14], diode-array detector (DAD) [10], refractive-index detector (RID) [8, 15, 16], an evaporative light scattering detector (ELSD) [14], charged aerosol detector (CAD) [12] or pulse-amperometric detector (PAD) [8] allow a sensitive and quantitative assay of the some sugars or polyols in various formulations in the presence or absence of the active drug substance or food samples. The lack of any chromophore in sugars and sugar alcohols makes the specific UV detection unreliable or even impossible [10, 12]. HPLC method for detection of these compounds, i.e. refractive index (RI) and evaporative light scattering (ELS) detectors are applied. However, RI detection has significant limitations in sensitivity and reproducibility as well as it cannot be used in gradient elution mode [12]. But it was found sugars and polyols were rarely analyzed simultaneously. Therefore, development of instrumental method for simultaneous quantification of the most utilized polyols and sugars in foods still remained challenges. The need of fast and simple analytical HPLC methods, which can be easily applied in food analysis for sugars and sugar alcohols determination requires the choice of universal detector as RID.

Therefore, the purpose of the current study was to develop a fast and accurate HPLC-RID method for simultaneous analysis of four sugars (glucose, fructose, sucrose and lactose) and five polyols (erythritol, mannitol, sorbitol, isomalt and xylitol) in dessert foods.

MATERIAL AND METHODS

Chemicals, reagents and standards

All chemicals and reagents used are pure for analysis. Analytical grade sugars (D-glucose, D-fructose, sucrose, D-lactose) and polyols (erythritol, sorbitol, isomalt, mannitol

and xylitol) all contents > 99%) were purchased from Sigma Aldrich. All aqueous solutions were prepared using ultra-pure water.

Equipment

Chromatographic experiments were performed on high-performance liquid chromatograph HPLC Elite Chrome Hitachi, equipped with pump LC-20 AD, column thermostat, refractive index detector Chromaster 5450 (WVR, Hitachi) and software. The HPLC separation was carried out on Shodex® Sugar SP0810 (300 mm × 8.0 mm i.d.) column with Pb^{2+} and a guard column Shodex SP - G (5 μ m , 6 × 50 mm). The mobile phase was filtered under vacuum through a 0.2 μm membrane filter (Sartorius AG, Goettingen, Germany). All samples before injection were filtered through ISO Lab (Germany) filters with a diameter of 4 mm and a pore size of 0.45 μm. The volume of the injected sample was 20 μL.

Standard solutions preparation

Standards solutions were prepared by dissolving 1.0000 g of each individual analyte in ultra-pure water in separate 100.00 mL volumetric flasks. The standard working solutions were daily prepared by appropriate dilution from the individual stocks. The stock solutions and diluted standard solutions were stored in glass volumetric flasks in the dark at 4 °C.

Linearity

Linearity was established by triplicate injections of four different concentrations levels (0.5, 1.0, 2.5, 5.0 mg /mL) of the standards obtained by dilution in water of the standards mixture. The calibration curve for each sugars or polyols was obtained by plotting the concentration of compound versus the area of the respective peaks.

Optimization of the chromatographic separation

Mobile phase flow rate

The optimum flow rate of the mobile phase should provide good separation, high sensitivity, and short analysis time. The optimization of the flow rate was carried out by injecting the same concentration of mixed standard solution at various flow rates from 0.5 to 1 mL/min.

Column temperature

The optimization of the column temperature was carried out by injecting the same concentration of mixed standard solutions at various temperatures from 80 to 85 °C.

Method validation

The sensitivity, repeatability and reproducibility of the HPLC-RID method are also determined according to the International Conference on Harmonization guidelines [17]. The sensitivity of the method was determined in terms of the detection limit (LOD) and quantitation limit (LOQ). Detection limit and quantitation limit were estimated for each of examined compounds. The value was calculated from the standard deviation (SD) of response and the slope of the curve (S) by means of the equations: LOD = 3.3(SD/S) and LOQ = 10(SD/S), where SD: standard deviation of the detector response; S: slope of the calibration curve [9].

Precision

The precision was evaluated by repeatability and reproducibility of the method. The intra-day repeatability was assessed by determining the relative standard deviation (RSD %) of the areas obtained from the injection of six replicates of the standard solutions (D-glucose, D-fructose, sucrose, D-lactose, erythritol, sorbitol, isomalt, mannitol and xylitol each of them in concentration 2.5 mg/mL) on the same day. In the evaluation of reproducibility (interday repeatability), the same standard with concentration 2.5 mg/mL was injected one time per day during a period of six non-consecutive days.

Accuracy

Accuracy was calculated by the recovery obtained for each compound at two concentration levels (0.1 and 0.2 g/100 g). These concentrations were added in a known mass at two levels in previously analyzed creams.

Sample Preparation

Based on a basic dessert cream formula, the formulations of individual samples obtained with an equivalent amount of polyols have been developed. The composition of the creams is shown in Table. 1

For convenience, samples are S-sample with sugar, E-sample with erythritol, Is-isomalt sample, K-sample with xylitol, sample So- with sorbitol and sample M- with maltitol. Creams are made in laboratory conditions by boiling to 80 °C and then cooled under refrigeration conditions for 60 minutes.

Sample preparation for HPLC analysis

The preparation of sample for analysis was done by following the method for determination of sugars according to Lurie et al. [18]. In brief 10 g sample (dessert cream) was dissolved in 50 cm^3 distilled water at temperature 50 °C and then was placed in water bath at 60 °C for 15 min. The sample was precipitaed by addition of NaOH and $ZnSO_4$. The solution was made up to 200 mL and then was filtrated. The samples solutions were kept at room temperature until their analysis. The sample solutions were filtered through 0.45 μm membrane filters before injection. The obtained peaks were identified by comparing the retention times. For the quantification of the analyzed carbohydrates the peak areas of the resulting chromatogram were integrated.

Statistics

All statistical analyses were performed using ANOVA Microsoft Excel.

RESULTS AND DISCUSSION

The current study presents the development and optimization HPLC-RID method for analysis of glucose, fructose, sucrose and lactose and polyols (erythritol, mannitol, sorbitol, isomalt and xylitol) in dessert foods in a single run with minimal sample preparation using only distilled water as mobile phase. Therefore, this inexpensive and non-toxic solvent has the advantage in a comparison of reported previously HPLC methods for sugars and polyols using acetonitrile in high concentration 80% [10, 13]. In addition, the column temperature was influenced strongly on separation. In our previous research many temperatures were tested for separation of sugars [9]. It was found that Shodex® Sugar SP0810 column operated better when the

temperature is higher than 75 °C. Therefore, in this study the column temperature was tested at 80 and 85 °C. Results showed that with increasing temperature the resolution of peaks decreased. Based on symmetry peak and the peak area, which were the basic criteria of choice, the very effective and good separation of sugars and polyols was obtained at optimal temperature 80 °C and it was used in all our further experiments. The variation of flow rate showed that with increasing to 1 mL/min the resolution lower.

The best HPLC-RID separation conditions were as follows: column temperature 80 °C, temperature of RID 35 °C and mobile phase distilled water with flow rate 0.5 mL/min. The duration of each run for analysis of these nine compounds was 50 min. With flow rate 0.5 mL/min on Shodex® Sugar SP0810 column the following retention times for analyzed compounds was detected: 1. sucrose (15.90 min); 2. lactose (17.12 min), 3. glucose (18.62 min), 4. fructose (23.85 min), 5. maltitol (26.57 min), 6. erhytritol (27.55 min), 7. isomalt (30.52 min), 8. xylitol (43.22 min) and 9. sorbitol (47.14 min) (Fig. 1).

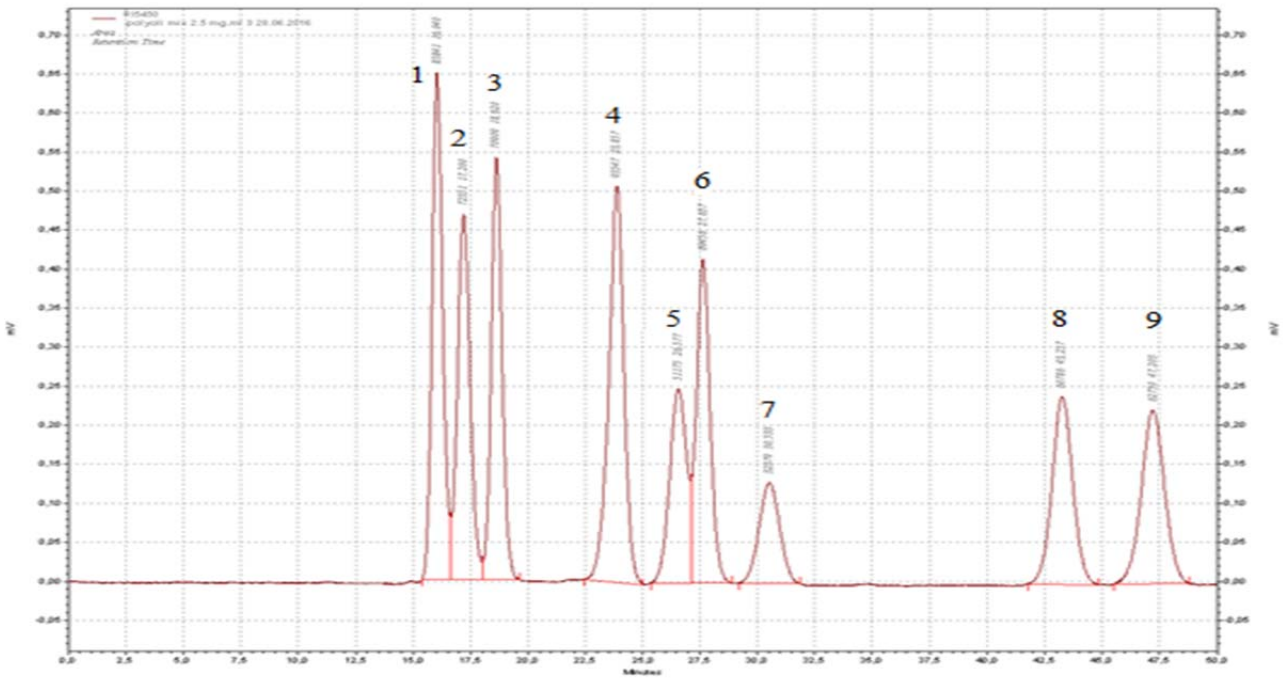

Fig. 1. HPLC chromatograms of sugars and polyols, where 1-sucrose; 2-lactose; 3-glucose; 4-fructose; 5-maltitol; 6-erhytritol; 7-isomalt; 8-xylitol; 9-sorbitol with d. H_2O at flow rate 0.5 mL/min, column temperature 80 °C and RID operating temperature 35 °C

Table 1. Receipt for dessert cream preparation, %

Ingredients	Sample					
	S	E	Is	K	So	M
Sucrose	14.2	-	-	-	-	-
Erythritol	-	14.2	-	-	-	-
Isomalt	-	-	14.2	-	-	-
Xylitol	-	-	-	14.2	-	-
Sorbitol	-	-	-	-	14.2	-
Maltitol	-	-	-	-	-	14.2
Milk	52.7	52.7	52.7	52.7	52.7	52.7
Cream	31.5	31.5	31.5	31.5	31.5	31.5
Starch	1.4	1.4	1.4	1.4	1.4	1.4
Other additives	0.2	0.2	0.2	0.2	0.2	0.2
Total	100	100	100	100	100	100

S-sample with sugar, E-sample with erythritol, Is-isomalt sample, K-sample with xylitol, sample So- with sorbitol and sample M- with maltitol

Table 2. Linearity of sugars and polyols (n = 3) analyzed by RI detection

Compound	Retention time, min	Standard curve[a] (Linearity form 0.5 to 5 mg/mL)	R^{2b}	RSD[c],%
Sucrose	15.90	Y=27050x+207.93	0.9996	1.22
Lactose	17.12	Y=26367x+3002.9	0.9967	0.46
Glucose	18.62	Y=26733x+1731.2	0.9981	0.50
Fructose	23.85	Y=36588x+1338.3	0.9994	0.60
Maltitol	26.57	Y=21072x-546.2	0.9994	0.52
Erythritol	27.55	Y=27365x+549.27	0.9986	1.09
Isomalt	30.52	Y=12937x+440.22	0.9981	2.81
Xylitol	43.22	Y=24578x-70.092	0.9996	0.71
Sorbitol	47.14	Y=26083x-899.52	0.9995	0.55

[a]*x*: concentration (mg/mL); *Y*: peak area. [b]Determination coefficient, [c]RSD-relative standard deviation

Table 3. Limit of detection (LOD) and quantification limits (LOQ) in sugars and polyols by RI detection

Compound	LOD, mg/mL	LOQ, mg/mL
Sucrose	0.06	0.22
Lactose	0.14	0.48
Glucose	0.09	0.31
Fructose	0.13	0.46
Maltitol	0.17	0.56
Erythritol	0.01	0.03
Isomalt	0.08	0.26
Xylitol	0.07	0.22
Sorbitol	0.17	0.56

Table 4. Precision of the instrument

Compound	Repeatability (n=6)		Reproducibility (n=6)	
	Mean ± SD	RSD, %	Mean ± SD	RSD, %
Sucrose	2.46±0.12	3.0	3.01±0.20	6.5
Lactose	2.60±0.12	4.8	2.52±0.19	7.5
Glucose	2.56±0.11	4.4	2.50±0.17	6.7
Fructose	2.50±0.09	3.4	2,43±0.17	7.0
Maltitol	2.43±0.10	4.0	2.37±0.16	6.8
Erythritol	2.50±0.14	5.6	2.43±0.19	8.0
Isomalt	2.48±0.09	3.5	2.41±0.15	6.2
Xylitol	2.45±0.10	4.1	2.38±0.16	6.8
Sorbitol	2.41±0.10	4.2	2.36±0.15	6.5

SD – standard deviation; RSD –relative standard deviation

Table 5. Quantities of sugars and polyols In the analyzed finished dessert creams, %

Compounds	Sample					
	S	E	Is	So	K	M
Sucrose	15.1	3.2	3.9	3.8	3.9	3.9
Lactose	2.8	2.9	2.8	2.5	2.7	2.5
Erythritol	-	14.8	-	-	-	-
Isomalt	-	-	14.8	-	-	-
Sorbitol	-	-	-	15.5	-	-
Xylitol	-	-	-	-	15.0	-
Maltitol	-	-	-	-	-	15.5

Table 6. Sugar and polyols recovery rates added to dessert creams

Compound	Recovery, %	
	Level 1	Level 2
Sucrose	98	106
Lactose	91	101
Glucose	93	102
Fructose	96	103
Maltitol	95	109
Erythritol	98	104
Isomalt	97	104
Xylitol	97	105
Sorbitol	98	109

Validation Of The Proposed Method

The analytical method was fully validated by evaluating linearity, limit of detection (LOD), limit of quantification (LOQ), precision and accuracy. The data concerning method validation are summarized in Tables 2, 3 and 4.

Linearity

Under the chromatographic conditions described above, a linear relationship between the peak areas and the concentrations of sugars and polyols (0.5; 1.0; 2.5; 5.0 mg/mL) was found.

The regression equations of the standard curves were given in Table 2, where Y is the area; x is the concentration of the corresponding standard, mg/mL. Each point of the line was generated by three repeated injections of standard solutions at four concentration levels. The developed method is characterized by a good linearity in the range of 0.5 to 5 mg/mL, with determination coefficients over 0.997 typically specified in method validation protocols [13, 19] and RSD in the range of 1-3% (Table 2).

Limit of detection and limit of quantitation

The estimation of the LOD and LOQ were done based on the calibration curve, using 3.3 SD/b and 10 SD/b, respectively, where SD is standard deviation of the response (b - intercept) and a is a slope of calibration curve according to ICH guidelines [17]. The LOD and LOQ values determined for all investigated compounds (Table 3). LOD and LOQ for nine analytes were in the range of 0.03–0.56 mg/mL, respectively. The lowest LOQ and LOD values were found for erythritol better than values obtained with CAD [12]. The limits of detection and quantification values indicate high sensitivity of the system, as some of the data were near to the reports for sugars, sorbitol and xylitol [12, 13].

Precision

The precision was expressed as relative standard deviation (RSD%=SD/mean×100). The relative standard deviation (RSD) of the calibration standards (n = 6) for intra-day precision (repeatability) and inter-day (intermediate) precision ranged from 3.0 to 5.5% and between 6.2 and 8.0 %, respectively (Table 4). In articles for simultaneous analysis of sugars and polyols some authors found repeatability values below the maximum acceptable limits for the validation of chromatographic methods [12, 13, 20]. In our case the presented HPLC-RID method establishes that RSD % values less than 5 that are acceptable and below 10 % (for reproducibility). Our data were in accordance with Petkova et al., Grembecka et al., Zielinski et al. [8, 12, 13] for method validation requirements.

The results from the chromatographic analysis of prepared dessert creams were summarized in Table 5. It was found that lactose level was in range from 2.5 to 2.9 %, sucrose content was less than 4 % in all creams with added polyols.

Accuracy

The lowest levels of recovery of the sugars and polyols were observed in the creams containing the lowest concentration of the standards added. The lowest level of recovery of lactose was found for the lowest concentration of the added standard. The lowest level of recovery with the addition of the highest concentration of standard was observed only for D-glucose (Table 6) and this coincides with report of Zielinski et al., [13] for fruit juices. All the results found in the present study are within the acceptable limits for the validation of chromatographic methods [8, 13].

CONCLUSION

The developed HPLC-RID method was precise, reproducible and enough sensitive for simultaneous quantitative evaluation of glucose, fructose, sucrose, lactose, erythritol, mannitol, maltitol, sorbitol and xylitol in dessert foods. The method enabling the simultaneous determination of these sugars and polyols in food products by HPLC-RID is demonstrated for the first time. The chromatographic separation and detection procedures were successfully applied for their evaluation in dessert foods.

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CONFLICTS OF INTERESTS

All authors declare no conflicts of interests.

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