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Modern approach for determination of lactulose, mannitol and sucrose in human urine using HPLC-MS/MS for studies of intestinal and upper digestive tract permeability

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Abstract

A new analytical procedure was described for the simultaneous determination of lactulose mannitol and sucrose in urine, in which HILIC chromatography and tandem mass spectrometry detection are used. Sugars are orally administered for the estimation of intestinal permeability in children digestive tract. Samples were purified by dispersive solid phase extraction (d-SPE) using Amberlite MB150 resin. Raffinose was selected as an internal standard. The chosen chromatographic separation was carried out on ZIC®-HILIC column in 10 minutes at a flow rate of 0.3 mL/min, using mixture of acetonitrile (ACN) and ammonium acetate (NH_4Ac) in water (H_2O) as the mobile phase. Within-run precision (CV) measured at three concentrations was 1.08, 0.32 and 0.49% for lactulose; 1.88, 0.47 and 0.75% for

mannitol, 2.95, 1.31 and 0.6 for sucrose. Between-run CVs were 0.75, 1.1 and 1.2% for lactulose; 1.1, 1.02 and 1.01% for mannitol; 1.17, 1.4 and 1.05% for sucrose. Analytical recovery of all three sugar probes was 95.06.–99.92%. The detection limits were: 15.94 ng/mL for lactulose, 17.10 ng/mL for sucrose and 11.48 ng/mL for mannitol. The proposed method is rapid, simple, sensitive and suitable for determination of intestinal permeability of the sugar derivatives in children.

Keywords: HILIC; intestinal permeability; tandem mass spectrometry; carbohydrate analysis; urine

1. Introduction

An efficient digestive system ensures the absorption of nutrients and water into the bloodstream. Simultaneously, it prevents the absorption into the body harmful or potentially hazardous substances. The integrity of the intestinal epithelium and its permeability is appropriate and selective in case of healthy body [1, 2]. Membranes of epithelium cells have double layer of phospholipids which is responsible for diffusion of substances soluble in lipids through small intestine cell membranes [2, 3]. Substances soluble in water are transported through spaces between cells of small intestine. Such spaces are controlled by complex protein structures known as tight junctions [2, 4]. Both types of transport are estimated by the intestinal permeability.

It is crucial to monitor and estimate the permeability of small intestine due to its relation with diseases like: coeliac disease [4, 5], Leśniowski-Crohn disease [6], allergy for proteins in cow's milk [7], cirrhosis [8], HIV infection [9, 10], chronic and acute diarrhea [11], paediatric intestinal diseases [12], cystic fibrosis [13] and damages caused by non-steroidal anti-inflammatory drugs [14].

Lactulose and mannitol represent compounds which are transported passively and are not metabolized. The lactulose/mannitol test assumes that these two compounds are treated

identically in all individual and physiological aspects [15, 16]. Sucrose permeability test is commonly accepted as a marker of upper digestive tract (stomach and duodenum) mucosal barrier damage [17, 18].

There are variety of techniques used to determine lactulose, mannitol and sucrose in urine samples. The most often used are enzymatic methods. However, estimation of concentration lactulose and mannitol is not always accurate [17-21]. Gas chromatography requires evaporation and derivatization of the analytes present in studied samples prior injection. Sample preparation is time consuming and derivatizing step requires additionally chemical reagents. Unlike enzymatic assays, it is possible to determine lactulose and mannitol simultaneously [22-25]. Determination of sugars by capillary electrophoresis is relatively rapid but requires solid phase extraction at sample preparation step. Sensitivity is relatively low with usage of UV detection thus the values of LOD are at mg/ml level which is relatively high with comparison to other methods. During analysis high pH should be maintained due to pK_a values of analytes vary from 12 to 13 [26].

Comparing with all above, HPLC is the most simple to perform, precise, rapid and easy to automate. An interesting choice for carbohydrate analysis is hydrophilic interactions liquid chromatography (HILIC) with electrospray ionisation tandem mass spectrometry (ESI-MS/MS). With this method, it is possible to detect common saccharides in extracts from plants [27]. Several HPLC procedures are available for the determination of sugars in urine [10, 12, 16, 28-30]. Some methods are based on refractive index detection [28], which has the advantage of being universal. However, this type of detector suffers from external variations such as temperature and pressure. In addition, it is not easily adaptable for routine use, and it has poor sensitivity. Therefore pre- [31] or postcolumn [32] derivatization methods have been developed in the attempt to overcome these problems. Anion-exchange chromatography in conjunction with pulsed amperometric detection has been used for the rapid and simultaneous determination of sugars in urine or plasma [9]. The high sensitivity of pulsed amperometric

detection allows injection of much diluted solutions, which leads to lower chromatographic system contamination and longer column life [33]. The detection using evaporative light-scattering detectors (ELSDs) seems to be more sensitive and easier to use than the refractive index detector, and it is compatible with gradient elution in carbohydrate analysis [16]. Electrochemical detection was applied for sugars analysis as well [34]. Specific and sensitive mass spectrometry detection in multiple reaction monitoring mode (MRM) combined with HPLC analysis was recently presented for the measurement of these compounds in urine of children affected by abdominal recurrent pain [29].

High-performance liquid chromatography coupled with tandem mass spectrometry method is presented in this paper. The advantage of this method is to identify and determine simultaneously the mannitol/lactulose ratio and sucrose concentration level in excreted urine. Proposed method is rapid, sensitive, simple to perform and reliable. HILIC was a method of choice for separation of polar compounds. Mobile phase used is mainly organic (≥70%) with amount of polar/aqueous solvent. High amount of organic solvent, due to its volatility, enhances the response of ESI-MS/MS [35, 36].

A developed method may be used as a non-invasive test for obtaining information about intestinal permeability and digestive tract permeability.

2. Materials and methods

2.1 Chemicals

Standards (lactulose, mannitol, glucose, sucrose and raffinose), ACN LC-MS grade, NH_4Ac, Amberlite MB150 resin and Whatman Puradisc™ 13 mm PTFE (2 μm pore size) syringe filters were purchased from Sigma Aldrich (St. Luis, USA). Formic acid (FA) was purchased form POCH (Gliwice, Poland). Ultrapure H_2O was prepared using HLP5 system from Hydrolab (Wiślina, Poland).

2.2 Sample collection

After 12 hours of fasting the patient empties the bladder and then drinks solution of mannitol (2 g), lactulose (10 g) and sucrose (20 g) in 250 mL of deionised water. It is recommended that patients should follow 24 h lactulose, mannitol, sucrose and raffinose free diet. To correct the endogenous presence of carbohydrates content a blank urine is collected before drinking the prepared solution. Urine is collected for the next 5 h and after first 2 h patient may drink 250 mL of mineral non-carbonated water. Chlorhexidine (0.1 mL of 1% aqueous solution) is added as antimicrobial agent to each vessel containing urine. Collected samples for longer storage than several hours are immediately frozen at -20°C.

2.3 Preparation of standards and calibration solutions

Stock solutions of lactulose, mannitol, sucrose, glucose and raffinose (raffinose was used as internal standard for tandem mass spectrometry detection) were prepared by dissolving standards in ACN/H_2O (75:25) mixture. The final concentration of four individual solutions of lactulose, mannitol, glucose and sucrose was at 20 μg/mL and raffinose at 10 μg/mL. Calibration solutions were prepared by dilution of stock solutions with ACN to obtain concentrations: 50, 100, 500, 1000, 1500 and 2000 ng/mL of each analytes. In all calibration solutions the internal standard concentration was at 500 ng/mL. Stock solutions and calibration solutions were stored at 4°C. Every two weeks new solutions were prepared.

2.4 Sample preparation

Volume of 500 μL of urine was diluted with 500 μL of deionised water in eppendorf vial. To the obtained solution 100 mg of Amberlite MB150 ion-exchange resin was added. Amberlite MB150 resin is added to eliminate from sodium ions. Sodium ions are thought to cause signal suppression from tandem mass spectrometry detector. Sample was stirred for 3 minutes and then centrifuged for 3 minutes at 5000 rpm. After centrifugation 10 μL of diluted and

centrifuged sample was transferred to a flask containing 100 μL of stock solution of internal standard. Final concentration of internal standard was 500 ng/mL. The flask was filled up with ACN to the total volume of 2 mL. For the general scheme of the protocol of sample preparation see Figure S1, Supplementary Data. Some urine samples were from patients with fairly stage of disease (including i.e. kidney damage). In such cases after last step of sample preparation a further filtration through 0.2 μm PTFE syringe filter is needed to remove denatured proteins.

2.5 Preparation of fortified samples

Urine was collected from healthy volunteers after 12 h of sucrose-, mannitol- and lactulose - free diet. Urine was collected at fasting. Specific amounts of lactulose, mannitol and sucrose were dissolved in three urine samples free from compounds of interest, to obtain 40, 200 and 500 μg/mL of each substance respectively. Fortified samples were prepared as mentioned above. Final concentrations in samples were 100, 500 and 1250 ng/mL of each substance respectively. Prepared fortified samples were analyzed by HPLC-MS/MS.

2.6 HPLC-MS/MS conditions

The HPLC-MS/MS contained Agilent (Santa Clara, USA) 1200 HPLC series pump, degasser, autosampler, column oven and Q-Trap 4000 triple quadrupole mass spectrometer from Applied Biosystems (Foster City, USA) with electrospray ionization in negative ion mode. The chromatographic separation was tested with analytical columns: 250 x 2.1mm, 5μm with pore size 200Å ZIC®-HILIC from Merck KgaA (St. Luis, Germany); 150 x 2.1mm, 5μm with pore size 100Å Ascentis Si from Supelco (St. Luis, USA); 150 x 3mm, 3μm with pore size 120Å Supelcosil LC-NH2 from Supelco (St. Luis, USA). The chromatographic separation conditions for each chosen column, parameters for the monitored ion transitions and MS/MS operation parameters are presented in the Table 1.

Tandem mass spectrometry parameters for the monitored ion transitions were obtained using 1μg/ml solutions of each substance with flow rate at 10 μl/min.

All data were collected and processed using Analyst 1.5.2 Software.

<insert Table1>

3. Results and discussion

3.1 Tandem mass spectrometry detection

Ions of compounds of interest could be detected in negative ESI mode in the presence of acetate ion. Declustering potential was the most important parameter which impacts the response form detector. The most intense signals come from the precursor ions in case of mannitol, sucrose and raffinose. In case of lactulose the most intense signal comes from the one of the fragment ion 160.9 m/z.

For MS/MS operation parameters flow injection analysis (FIA) was done using 1 μg/mL solution of each substance. Mass spectra of the three compounds under the study and spectrum of internal standard (raffinose) obtained from FIA mode are presented in Figure 1. In case of carbohydrate fragmentation, there are two types of cleavages: ring breakdown across two bonds (one of the bonds is from oxygen atom) or glycosidic bond breakdown which is linking two ring structures. Coelution, even with the usage of MRM, should be avoided because lactulose and sucrose have similar fragment ions: 179 m/z and 160 m/z which are shown in the Figure 1. In case of mannitol (sugar alcohol) cleavage is present on the molecular chain. MRM mode of MS/MS was chosen for analysis and specific ion transitions (Q for quantitative, q for qualitative) are presented in Table 1.

<insert Fig1>

3.2 Chromatographic separation

For the ZIC®-HILIC and Ascentis Si column the mobile phase consisting in the aqueous part small amount of ammonium acetate (5 mM) as additive was sufficient to obtain good peak shape and high intensities of detector signal (Figure 2A and 2B). For the Supelcosil LC-NH2, mobile phase consisting ammonium acetate in the aqueous part proved to be insufficient. The peak tailing phenomenon occurred for mannitol and lactulose (Figure 2C). To minimize peak tailing 0.05% of formic acid (FA) was added to the both components of mobile phase. This results in better peak shape, however the decrease of sensitivity was observed (Figure 2D). As mentioned before, the coelution in case of lactulose and sucrose should be avoided, even with the usage of MRM, due to the fact that both compounds form the same fragment ions.

<insert Fig2>

3.2.1 Glucose as potential interference in the studies of intestinal permeability

The separation of glucose as potential interference in the studies of intestinal permeability was done on ZIC®-HILIC column. Conditions for separation were taken from Table 1. The glucose transition is 179.1 m/z to 89.0 m/z and this type of transition is characteristic for glucose. However, secondary fragmentation of sucrose and lactulose may result in the same fragmentation pattern. Secondary fragmentation means that lactulose and sucrose fragment to 179 m/z (mass spectra are shown in the Figure 1) and such fragments may undergo subsequent fragmentation to 89 m/z. This is the reason why 89 m/z ion is produced and two coeluted peaks are visible in the same retention times of sucrose and lactulose. In the Figure 3 the chromatogram of standards (including glucose) is presented. The transition for glucose is not monitored during the analysis of samples of urine, therefore the potential interference of glucose can be eliminated due to the separation of glucose peak from the others.

<insert Fig3>

3.3 Inter-laboratory method validation

3.3.1 Linearity, LOD and LOQ

Calibration curve were made by drafting ratio of analyte peak area to internal standard peak area to analyte concentration. Calibration solutions were done from standard solutions of three analytes, as described previously. Raffinose as internal standard was introduced into each calibration solution at concentration 500 ng/mL.. Each calibration solution was analysed three times.

Test for homoscedasticity (F-test) was done to choose the best weighting for the calibration curves. Test was done at 95% confidence level with 5% rejection. The limiting F-value was taken from the table with appropriate degrees of freedom (df_1, df_2 = n-1) and is equal to 19. In this case df_1=2 and df_2=2. The standard deviations (SD) and relative standard deviations (RSD) of upper limit of quantitation (UL, C=2000 ng/mL) and lower limit of quantitation (LL, C=50 ng/mL) for every analyte chosen for this test. For the calculated ratios UL and LL see Table S1, Supplementary Data.

Test for homoscedasticity proved that, there is no need to use weighting of the curves. Ratios of SD and ratios of RSD for each set of calibration data are smaller than limiting factor. Calibration curves were constructed without weighting.

Calculation of limits of detection (LOD) and limits of quantitation (LOQ) were based on the value of standard deviation of constant term of calibration equation and slope of calibration curve. Equations of calibration curves, LOD, LOQ, correlation coefficients (R), standard deviations of slope (S_a) and standard deviations of constant terms (S_b) are presented in Table 2. Plots of calibration curves are available as Figure S2, Supplementary Material.

<insert Table2>

Despite the longer time of analysis, ZIC®-HILIC column was chosen for further experiments due to the lowest LOD and the highest correlation coefficients for each calibration curves.

3.3.2 Trueness, repeatability, intermediate precision, MDL and MQL

Trueness, repeatability and intermediate precision of the underworked method were tested with prepared fortified samples at three levels of concentration for chosen analytical column. The unfortified samples of urine were analysed to exclude the presence of the analytes of study. Three repeats were made for given three levels of fortified samples. Fortified samples were prepared accordingly to the presented protocol of sample preparation in 2.4. The method detection limit (MDL) values for analytes were calculated by multiplying the mean of sample standard deviations by Student's t-value. Degrees of freedom are 5 and the t-value is equal to 2.62. Method quantitation limit (MQL) values were obtained by multiplying MDL by 3. Data collected for trueness test are presented in Table 3.

<insert Table3>

Obtained results are satisfactory and it was proved that proposed method is suitable for analysis of lactulose, mannitol and sucrose in urine.

Repeatability study was done by analysis of one fortified sample during one day with initial concentration at 500 ng/mL of lactulose, mannitol and sucrose. Analysis by HPLC-MS/MS was repeated six times. Intermediate precision was done in next three days by analysis of the same fortified sample. Six repeats were done during each day. Recovery, standard deviations and coefficients of variation are presented in the Table 4.

<insert Table4>

In all cases recoveries were satisfactory and after sample preparation HPLC-MS/MS analysis may be performed in the next three days. Due to the high content of ACN, solution seems to be stable and decomposition of carbohydrates is reduced. Slight decrease in recovery was observed for mannitol and for sucrose. Lactulose concentration level seems to be stable along three days.

4. Analysis of real samples

Nine real samples of urine from children with diseases of digestive tract (mostly chronic intestinal inflamation, stomach and duodenum ulcer) and eight real samples of urine from healthy children were collected. All samples were prepared according to the described protocol in 2.3 and were analyzed by HPLC-MS/MS Blank urines were prepared according to the protocol as well. The content of carbohydrates in urine was insignificant, see Figure S3, Supplementary Data. Results are presented in Table 5. Concentrations below LOD in prepared samples were omitted.

<insert Table5>

The L/M ratio of recovered lactulose and mannitol was calculated. The recovered values of lactulose and mannitol were obtained by multiplying the μg/mL per volume of collected urine and divided by 10 in case of lactulose and by 2 in case of mannitol. The same calculations were made for sucrose recovery. The L/M reference range in healthy subjects is typically less than 0.03 [37]. The ratio observed in the urine samples of nine children with diseases of digestive tract was in general more elevated than the ratio in the eight healthy children. These

preliminary results must be confirmed on a higher number of well classified subjects to define ranges of values correlated to the diseases and to validate this LC-MS/MS as a diagnostic test.

5. Conclusions

Modern medicine is directed to non-invasive and patient friendly diagnosis. The developed method is simple, rapid, selective and sensitive for determination of concentration of three different sugars in human urine. Such methods are found to be useful in designation of intestinal and upper digestive tract permeability. Sample preparation step of such complex matrix like urine does not include solid phase extraction. The addition of specific amount of ion exchange resin, mixing and vortexing the sample are sufficient to obtain high recovery values. Rapid sample preparation, low solvent usage per single run and total time of analysis equal to 10 minutes are suitable when dealing with large amount of samples. ZIC®-HILIC column was chosen due to its long life and easiness of handling.

Limits of detection and limits of quantitation were based on the value of standard deviation of constant term of calibration equation and slope of calibration curve for each of the analyzed compounds. The ability of MS/MS to monitor multiple reaction ion transitions per single run gives high specificity of the method (Table 1) and allows distinguishing molecules with the same molecular mass like lactulose and sucrose. ZIC®-HILIC column provides satisfactory separation and repeatable retention times.

Presented method with rapid sample preparation step is an interesting alternative to the enzymatic assay of intestinal and upper digestive tract permeability. It proves that HPLC with ZIC®-HILIC chromatography is able to separate highly polar compounds even with the same molecular mass and ESI-MS/MS is able to identify them. Method for determination of lactulose, mannitol and sucrose in human urine may be widely applied not only to children but to adult patients as well.

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Figure captions

Fig1. Mass spectra obtained in negative mode $[M-H]^-$ of compounds under the study and their structures, including internal standard.

Fig2. Chromatogram of mixture of analytes and internal standard, detected by negative ESI-MS/MS on A) ZIC®-HILIC column (250 x 2.1mm, 5µm) with flow 300µL/min and injection volume 5µL, B) Ascentis Si column (150 x 2.1mm, 5µm) with flow 400µL/min and injection volume 5µL, C) Supelcosil LC-NH2 column (150 x 3mm, 3.5µm) with flow 500µL/min, injection volume 5µL and mobile phase consisting NH_4Ac (5mM), D) Supelcosil LC-NH2 column (150 x 3mm, 3.5µm) with flow 500µL/min, injection volume 5µL and mobile phase consisting 0.05% FA.

Fig3. Chromatogram of mixture of analytes and internal standard, detected by negative ESI-MS/MS on ZIC®-HILIC column (250 x 2.1mm, 5µm) with flow 300µL/min and injection volume 5µL, gradient programme: from 75% ACN / 25% 5mM of NH_4Ac in H_2O to 40% ACN / 60% 5mM of NH_4Ac in H_2O (pH=6.84) in 10 minutes

Table captions

Table 1. Chromatographic separation conditions for three columns (ZIC®-HILIC, Ascentis Si and Supelcosil LC-NH2), optimal parameters for the monitored ion transitions and MS/MS operation parameters.

Table 2. Data collected from calibration curves obtained from chosen columns.

Table 3. Recovery [%], standard deviation (SD), coefficient of variation (CV) [%], MDL and MQL obtained by HPLC-MS/MS analysis of fortified samples at three spiking levels of concentration in fortified samples.

Table 4. Repeatability study and intermediate precision, standard deviations and coefficients of variations are included.

Table 5. Concentrations of detected analytes in real samples.

Supplementary Data

Figure S1. General Scheme of the protocol of sample preparation.

Figure S2. Plots of obtained calibration curves.

Figure S3. A) real sample chromatogram with overlaid blank urine analysis and B) zoom of real sample chromatogram with overlaid blank urine analysis.

Table S1. Test for homoscedasticity based on limiting F-value=19 at 95% confidence level.

Table 1. Chromatographic separation conditions for three columns (ZIC®-HILIC, Ascentis Si and Supelcosil LC-NH2), optimal parameters for the monitored ion transitions and MS/MS operation parameters.

Chromatographic Separation Conditions

	ZIC®-HILIC 250 x 2.1mm, 5µm	Ascentis Si 150 x 2.1 mm ,5µm	Supelcosil LC-NH2 150 x 3mm, 3.5µm
Mobile Phase Gradient	From 75% ACN / 25% 5mM of NH_4Ac in H_2O to 40% ACN / 60% 5mM of NH_4Ac in H_2O (pH=6.84) in 10 minutes	From 80% ACN / 20% 5mM of NH_4Ac in H_2O to 65% ACN / 35% 5mM of NH_4Ac in H_2O (pH=6.84) in 6 minutes	From 75% ACN 0.05% FA / 25% H_2O 0.05% FA to 40% ACN 0.05% FA / 60% H_2O 0.05% FA in 6 minutes (pH=2.85)
Flow	300µL/min	400µL/min	500µL/min
Injection Volume	5µL		
Column Oven Temperature	25°C		
Run Time of Analysis	10 min	6 min	

Parameters for the monitored ion transitions

Name	Quantitative [Q] qualitative [q] parent ion→fragment ion	Declustering Potential [V]	Entrance Potential [V]	Collision Cell Exit Potential [V]	Collision Energy [V]
Lactulose	Q 341.0→160.9 q 341.0→100.8	-80	-10	-12 -22	-7 -15
Mannitol	Q 180.9→88.8 q 180.9→100.9	-90	-10	-20	-13 -15
Sucrose	Q 340.9→179.0 q 340.9→118.9	-115	-10	-20 -26	-13 -7
Raffinose	Q 503.1→178.8 q 503.1→220.8	-145	-10	-30 -44	-13 -15

MS/MS operation parameters

		Curtain Gas [psi]	Temperature [°C]	Nebulizer Gas [psi]	Turbo Gas [psi]
Lactulose	Q 341.0→160.9 q 341.0→100.8	20	500	20	10
Mannitol	Q 180.9→88.8 q 180.9→100.9	10	550	30	20
Sucrose	Q 340.9→179.0 q 340.9→118.9	20	500	20	10
Raffinose	Q 503.1→178.8 q 503.1→220.8	20	500	20	20
Chosen parameters		**20**	**500**	**20**	**10**

Table 2. Data collected from calibration curves obtained from chosen columns.

Analyte Name	Calibration Curve Equation (ZIC®-HILIC column)	LOD [ng/mL]	LOQ [ng/mL]	S_a	S_b	R
Lactulose	y = 0.003775x + 0.064	15.94	47.83	0.000016	0.018	0.9999
Mannitol	y = 0.0031148x + 0.037	11.48	34.43	0.0000097	0.011	0.9999
Sucrose	y = 0.0018623x + 0.0616	17.10	51.30	0.0000086	0.0077	0.9998
Analyte Name	Calibration Curve Equation (Ascentis Si)	LOD [ng/mL]	LOQ [ng/mL]	S_a	S_b	R
Lactulose	y = 0.002753x – 0.039	24.76	74.29	0.000022	0.021	0.9995
Mannitol	y = 0.001748x – 0.069	35.25	96.74	0.000018	0.017	0.9992
Sucrose	y = 0.001475x + 0.125	32.30	96.89	0.000016	0.014	0.9993
Analyte Name	Calibration Curve Equation (SUPELCOSIL LC-NH2)	LOD [ng/mL]	LOQ [ng/mL]	S_a	S_b	R
Lactulose	y = 0.002867x + 0.332	44.15	132.46	0.000041	0.038	0.9986
Mannitol	y = 0.002320x + 0.109	51.51	154.52	0.000039	0.036	0.9981
Sucrose	y = 0.001636x + 0.498	47.40	142.20	0.000025	0.024	0.9985

Table 3. Recovery [%], standard deviation (SD), coefficient of variation (CV) [%], MDL and MQL obtained by HPLC-MS/MS analysis of fortified samples at three spiking levels of concentration in fortified samples.

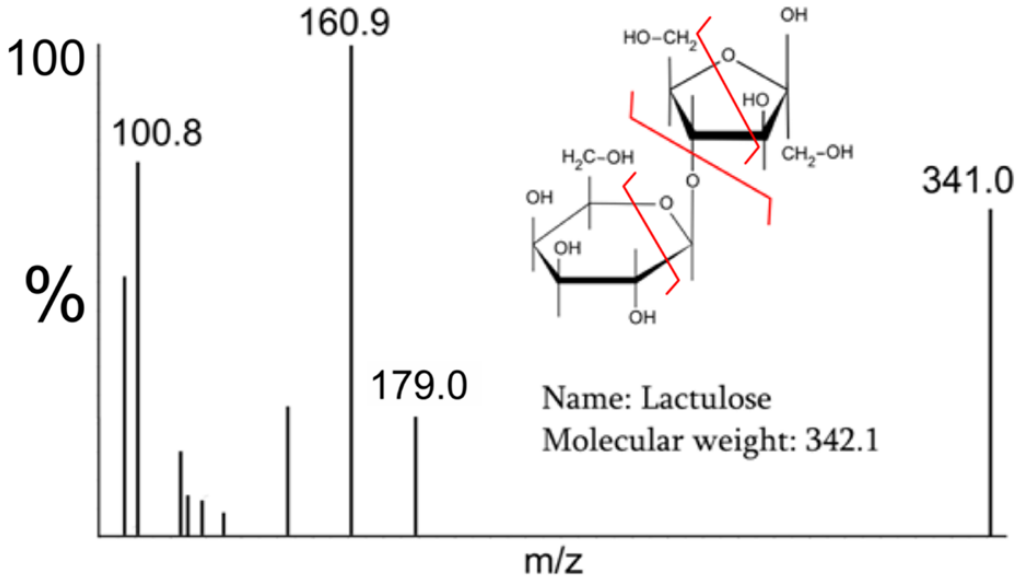
Analyte	Spiking Level [ng/mL]	Mean Recovery [ng/mL] (%) (n=3)	SD	CV [%]	MDL [ng/mL]	MQL [ng/mL]
Lactulose	100	97.56 (97.56)	1.06	1.08	7.62	22.87
	500	499.59 (99.92)	1.62	0.32		
	1250	1234.09 (98.73)	6.05	0.49		
Mannitol	100	95.06 (95.06)	1.79	1.88	11.61	34.82
	500	499.7 (99.94)	2.35	0.47		
	1250	1223.35 (97.87)	9.15	0.75		
Sucrose	100	96.56 (97.12)	2.85	2.95	14.73	44.20
	500	493.6 (98.72)	6.46	1.31		
	1250	1234.32 (98.75)	7.56	0.61		

Table 4. Repeatability study and intermediate precision, standard deviations and coefficients of variations are included.

Analyte	Day	Mean Recovery [ng/mL] (%) (n=6)	SD	CV [%]
Lactulose	1	493.91 (98.78)	3.71	0.75
	2	492.54 (98.51)	5.41	1.11
	3	494.55 (98.91)	5.92	1.23
Mannitol	1	506.42 (101.28)	5.58	1.16
	2	499.25 (99.85)	5.09	1.02
	3	494.38 (98.88)	4.97	1.01
Sucrose	1	489.79 (97.96)	5.74	1.17
	2	490.24 (98.05)	6.84	1.47
	3	484.48 (96.9)	5.11	1.05

Table 5. Concentrations of detected analytes in real samples.

Sample	Volume of sample [mL]	Concentration in urine [μg/mL] ± SD (n=3)				
		Lactulose	Mannitol	Sucrose	L/M ratio	Sucrose recovery
1	350	51.70 ± 0.61	254.47 ± 6.98	12.53 ± 1.09	0.041	0.00022
2	450	71.75 ± 0.59	144.94 ± 3.75	-	0.099	-
3	700	88.99 ± 2.65	568.91 ± 4.80	35.26 ± 1.81	0.031	0.00030
4	225	74.58 ± 0.23	222.48 ± 7.42	-	0.067	-
5	250	127.61 ± 0.78	512.84 ± 3.67	-	0.050	-
6	250	82.35 ± 0.98	199.61 ± 2.58	7.97 ± 0.21	0.083	0.00010
7	150	114.82 ± 3.53	495.53 ± 4.87	74.15 ± 2.47	0.046	0.00056
8	200	95.76 ± 0.29	454.59 ± 6.50	-	0.042	-
9	300	45.07 ± 0.16	229.45 ± 3.98	-	0.039	-
Sample (healthy volunteers)						
1	350	30.52 ± 1.21	382.78 ± 2.77	-	0.016	-
2	450	51.52 ± 0.64	515.72 ± 2.64	-	0.02	-
3	295	20.56 ± 0.18	503.05 ± 1.97	-	0.008	-
4	220	29.09 ± 0.77	524.78 ± 2.12	-	0.011	-
5	235	55.60 ± 0.61	511.32 ± 2.7	-	0.022	-
6	270	49.03 ± 0.59	500.75 ± 1.70	-	0.020	-
7	320	27.93 ± 0.46	366.22 ± 1.84	-	0.015	-
8	250	33.64 ± 0.56	395.23 ± 1.97	-	0.017	-


100
%
160.9
100.8
179.0
341.0
m/z
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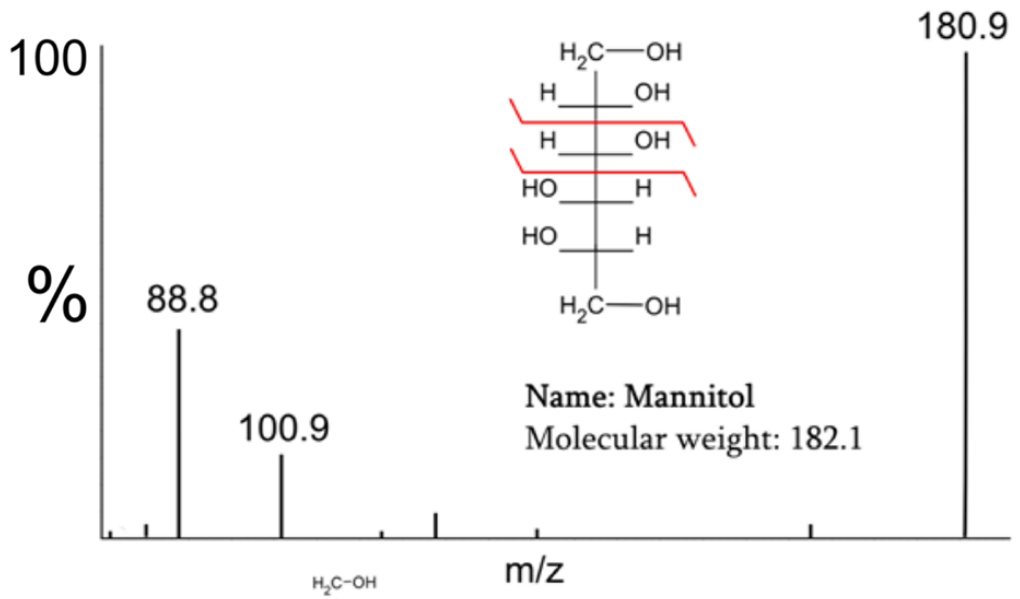
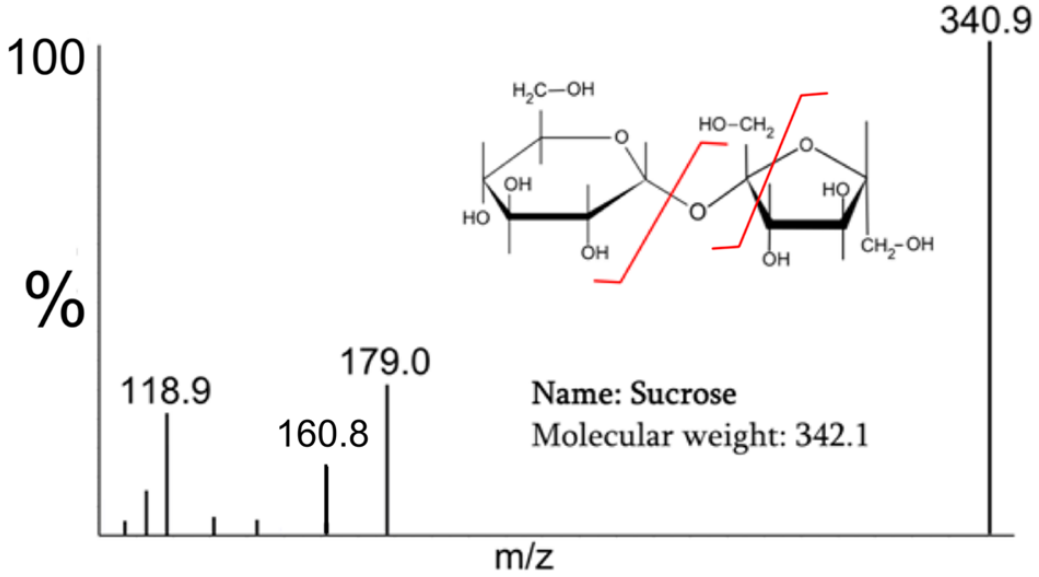
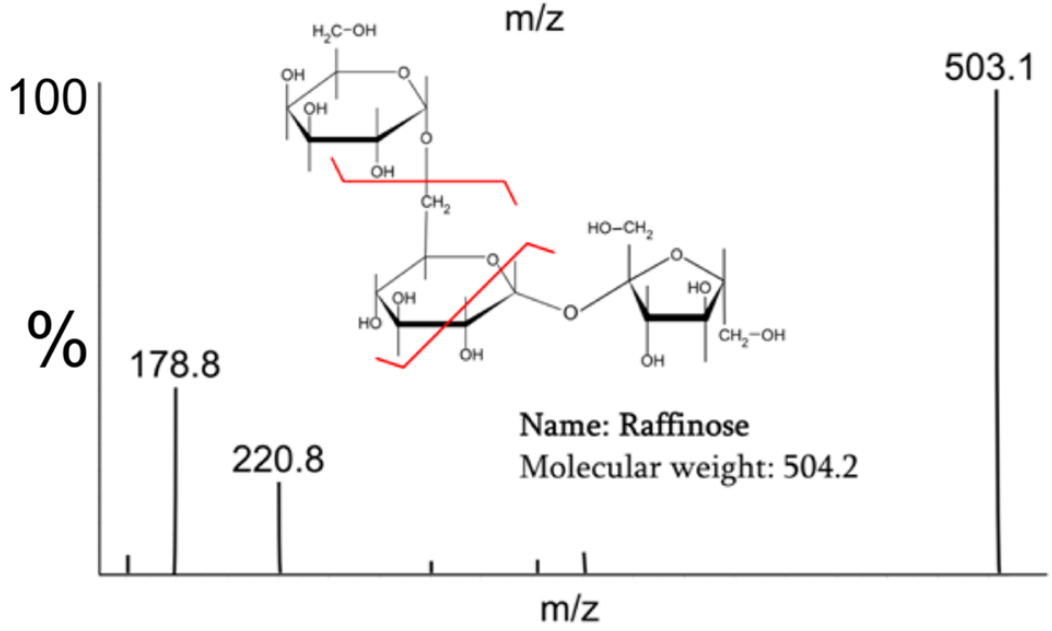
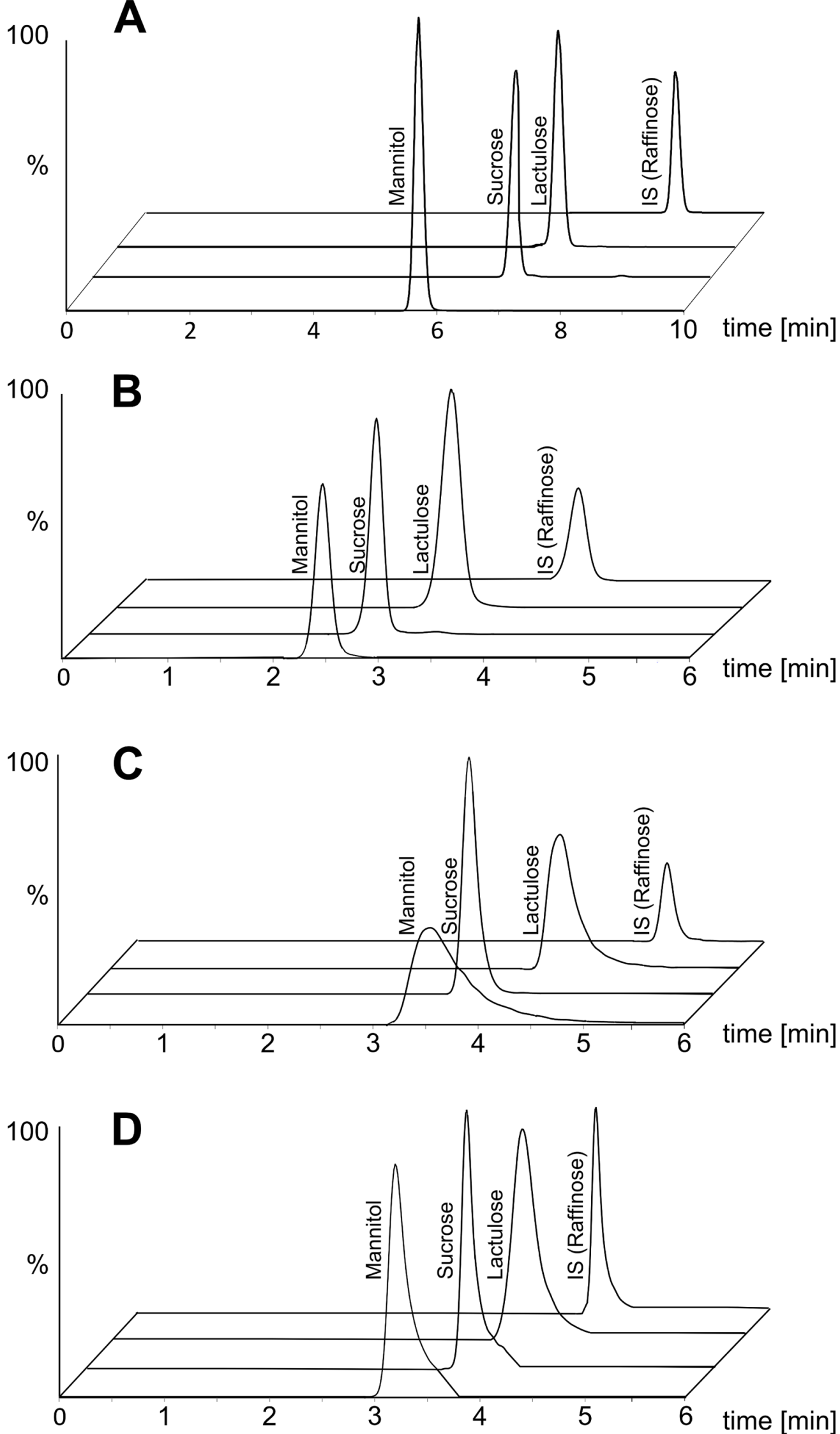
Molecular weight: 342.1


100
%
180.9
88.8
100.9
m/z
Name: Mannitol
Molecular weight: 182.1


100
%
340.9
118.9
179.0
160.8
m/z
Name: Sucrose
Molecular weight: 342.1


100
%
503.1
178.8
220.8
m/z
Name: Raffinose
Molecular weight: 504.2


A
100
%
Mannitol
Sucrose
Lactulose
IS (Raffinose)
0
2
4
6
8
10
time [min]
B
100
%
Mannitol
Sucrose
Lactulose
IS (Raffinose)
0
1
2
3
4
5
6
time [min]
C
100
%
Mannitol
Sucrose
Lactulose
IS (Raffinose)
0
1
2
3
4
5
6
time [min]
D
100
%
Mannitol
Sucrose
Lactulose
IS (Raffinose)
0
1
2
3
4
5
6
time [min]

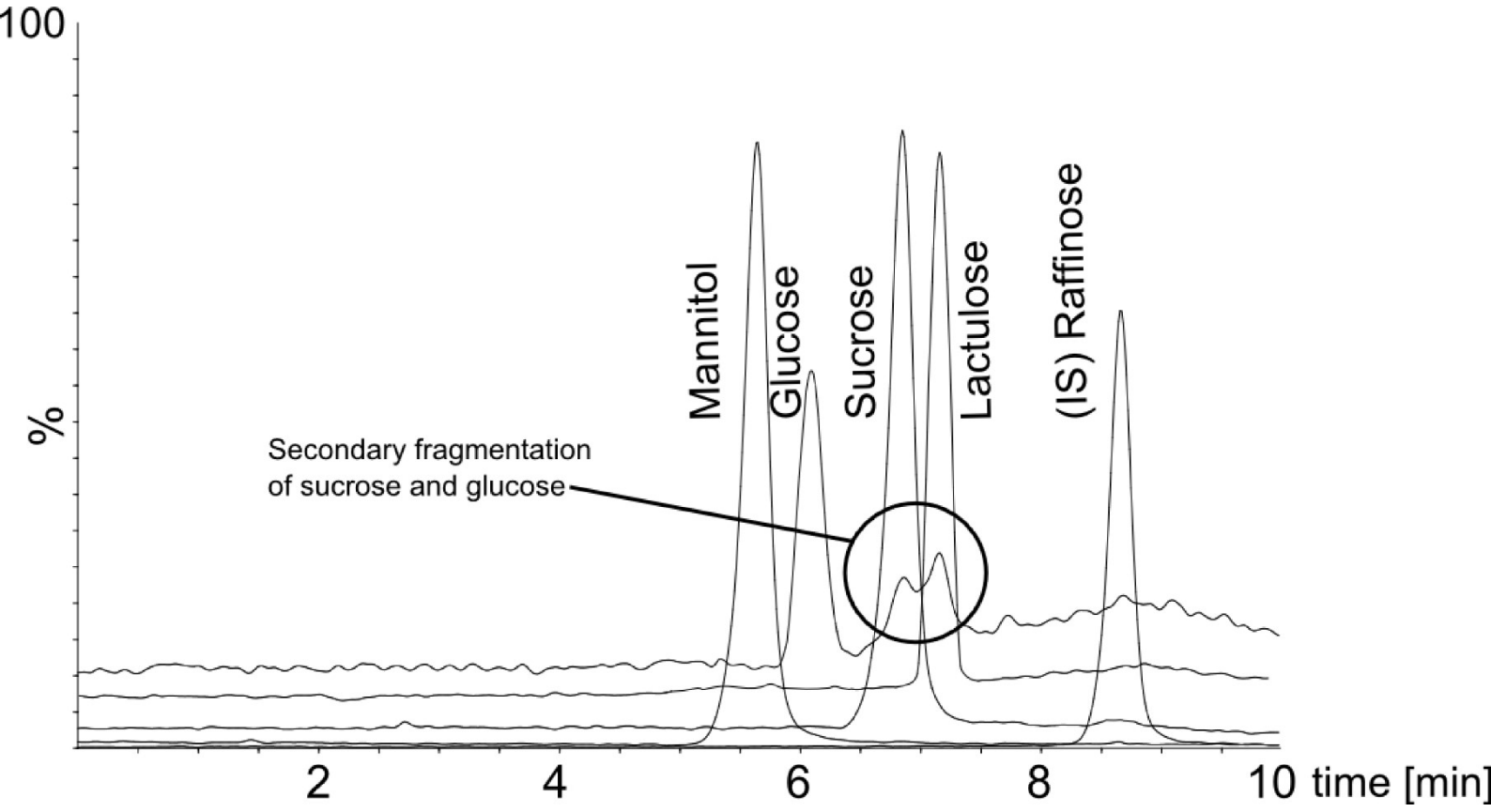

100
%
Mannitol
Glucose
Sucrose
Lactulose
(IS) Raffinose
Secondary fragmentation
of sucrose and glucose
2
4
6
8
10
time [min]

Table S1. Test for homoscedasticity based on limiting F-value=19 at 95% confidence level.

	Lactulose		**Mannitol**		**Sucrose**	
ZIC®-HILIC	SD UL 0.0091	RSD UL 0.0012	SD UL 0.0091	RSD UL 0.0015	SD UL 0.0096	RSD UL 0.0026
	SD LL 0.0083	RSD LL 0.033	SD LL 0.0053	RSD LL 0.027	SD LL 0.0032	RSD LL 0.023
Ratio	**1.20**	**0.0013**	**2.98**	**0.003**	**8.83**	**0.012**
Ascentis Si	SD UL 0.12	RSD UL 0.023	SD UL 0.084	RSD UL 0.025	SD UL 0.043	RSD UL 0.014
	SD LL 0.016	RSD LL 0.13	SD LL 0.0048	RSD LL 0.13	SD LL 0.015	RSD LL 0.11
Ratio	**7.60**	**0.018**	**17.20**	**0.20**	**2.86**	**0.13**
Supelcosil LC-NH2	SD UL 0.16	RSD UL 0.026	SD UL 0.063	RSD UL 0.013	SD UL 0.022	RSD UL 0.0057
	SD LL 0.079	RSD LL 0.21	SD LL 0.019	RSD LL 0.29	SD LL 0.094	RSD LL 0.14
Ratio	**2.03**	**0.13**	**3.25**	**0.047**	**0.23**	**0.041**