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Measuring Lactulose and Mannitol Levels Using Liquid Chromatography Coupled with Tandem Mass Spectrum: Application to Clinical Study of Intestinal Epithelium Barrier Function

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Supplementary File

The mass analyzer used in this work was a triple quadrupole, being the most used in quantitative analysis [1]. The ionization method used determines how the sample material is transferred to the mass spectrometer [2]. The ionization source was Electrospray Ionization (ESI), being the most used source for coupling with the mass spectrum [3]. Another advantage of the HLPC-MS/MS system is the speed in the individual analysis of the samples when compared to the liquid chromatography system with Pulsed Amperometric Detection (HPLC-PAD), where the run lasts 35 minutes and in the present system, only 10 minutes. Thus, reducing the race time to less than a third. With this, a greater number of runs can be done in a shorter time interval, thus increasing the dosage robustness in the LC-MS/MS platform, giving another advantage to this system.

Due to the characteristics of the described process, ESI applies very well to compounds of medium and high polarity, hence its extensive applicability in the pharmaceutical, food, natural products, among others [4-6]. The automatic acquisition mode used was the Multiple Reaction Monitoring (MRM), indicated for quantitative analysis [7]. The first step in developing the MRM method is to determine the best conditions for analyzing the analytes of interest, as described by Iglesias [8]. The ability of MS/MS to monitor multiple reaction ion transitions per single run gives the method

high specificity [9]. We observed the MS/MS method to be highly specific for the detection of single product ions for lactulose, mannitol and sorbitol, as demonstrated in this work and reported by other authors [10]. The five selected product ions were obtained from high collision energies. According to Kind, et al. low collision energies preserve precursor ions and only a few product ions are observed. Increasing the collision energy promotes the abundance of product ions towards lower m/z ranges and at the same time decreases the abundance of the precursor ion.

The HILIC-ZIC® column was chosen because it offers greater retention of compounds such as lactulose, mannitol and sorbitol as described by several authors [11]. The HILIC-ZIC® column also offered satisfactory separation and reproducible retention times for lactulose, mannitol and sorbitol in the standards and analyzed samples as reported by Kubica, et al. Liquid chromatography with the HILIC-ZIC® column can separate polar compounds with the same molar mass and mass spectrometry is able to identify them. We were able to observe the separation of mannitol and sorbitol compounds, even with the same molar masses, due to the ability of mass spectrometry to separate through the mass/charge ratio (m/z) and the precursor ion/product ion ratio in the triple quadrupole, confirming the advantage of the LC-MS/MS system against the isolated HPLC-PAD in the specification of the analyte of interest. The column provided reproducible separation and retention time, as reported by Kubica, et al. The use of retention time is highly recommended for high confidence compound identification [12].

We observed better ionization of ions through the negative mode using formic acid as an additive compound. Formic acid is known as a positive mode additive, but it can also be used as a negative ionization mode additive [14]. 0.1% formic acid is considered a mobile phase additive aiding the ionization of analytes. It is volatile and must be used in low concentration, to avoid interference in the analyte ionization process, a process known as ionization suppression, as described by other authors such as Iglesias and Trufelli, et al. [15].

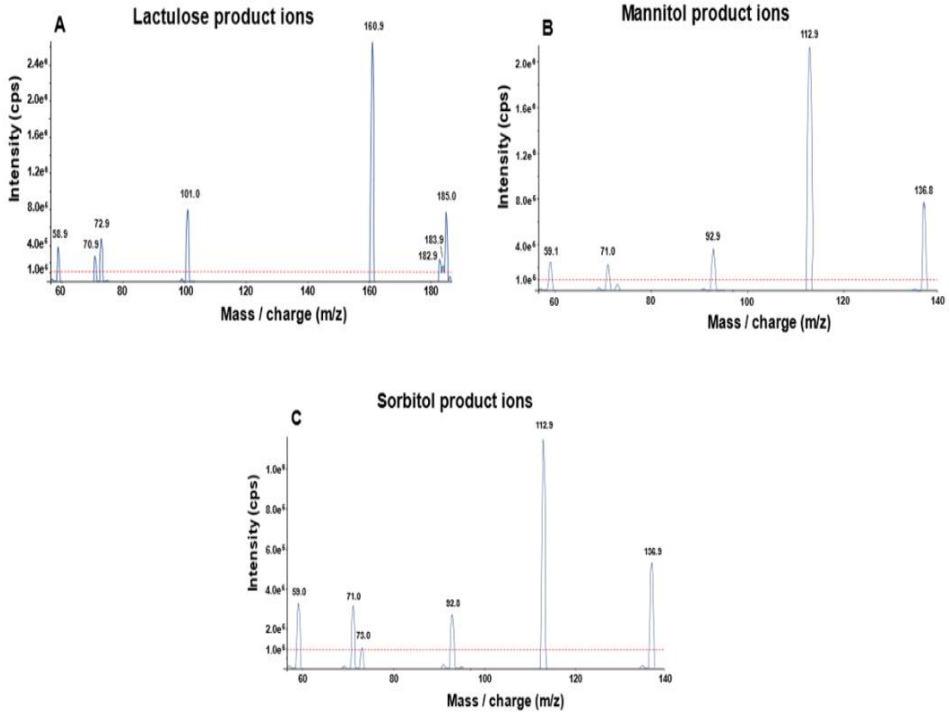
The presence of Formic Acid (FA) in the mobile phase under different concentrations changed its pH, varying according to the added concentration. The higher the concentration of AF added, the lower the pH of the mobile phase. Mobile phase with pH below 6 did not show good ionization. The mobile phase with the addition of 0.05% of FA showed better ionization and pH close to neutral (pH=6.84). Gan, et al. also observed that changes in pH or organic percentage can significantly increase ESI ionization [16]. According to Van Wijck et al. the application of a weaker acid, such as formic acid, increases the disaccharide response, but strongly reduces the monosaccharide response [17]. Differently from the above, we found in the presence of AF 0.05% an improvement in the response of both lactulose and mannitol.

During the mobile phase definition process, we identified several changes in ionization according to the mobile phase gradient. There were several attempts until we arrived at the gradient that would provide the best ionization. As electrospray efficiency depends primarily on mobile phase composition, the optimal eluent composition for proper chromatographic separation is sometimes inadequate to achieve maximum electrospray response [18]. As described by Lostia, et al. the present method, based on fragment ion identification, overcomes many of the problems encountered in the analysis of carbohydrates in biological fluids and can be considered a useful automated tool to study intestinal functions and the modification of the functional gastrointestinal

barrier, both in pediatrics and also in adult diseases such as celiac disease, Crohn's disease and inflammatory bowel disease, allowing accurate patient discrimination also for dietary restrictions.

The matrix effect should always be evaluated when validating quantitative LC-MS/MS methods, especially in complex matrices such as urine [19]. One of the biggest problems of analysis through mass spectrometry is to minimize the matrix effect [20]. One of the strategies adopted that can minimize this effect is sample purification or a more efficient chromatographic separation [21]. We performed the matrix effect interference analysis by the reference chemical substance (SQR) addition method, in which known amounts of SQR were added to the sample. We observed that the retention time of the analytes remained without significant variations, within the range of 2% of the times in the standard as described by Nunez, Moyano and Galceran. The recovery ranged between 84.23 and 135.27.

We observed the suppression of ionization at almost all concentrations, being greater at the concentration of 6000 ng/mL in the three analytes. In our method the reported ion suppression remained between 8%-30%. However, suppression did not affect the accuracy and precision of the assay as the signals for lactulose and mannitol were above the signals for LD and LQ (Supplementary Figure 1).



Supplementary Figure 1: Spectrograms obtained in negative ionization mode for lactulose, mannitol and sorbitol product ions. Product ion intensity data were obtained by direct injection into the QTRAP 5500 mass spectrometer ABSciex (Framingham, MA, USA) at a concentration of 10 ng/mL The spectrograms show the five main product ions of each of the lactulose (A) mannitol (B) and sorbitol (C), respectively, of higher intensity selected in the third pole of the quadrupole system of the mass spectrometer. The red dashed line indicates the minimum intensity limit (cps) acceptable for the product ions.

Time (minutes)	Eluent A (%)	Eluent B (%)
0	25	75
5	90	10
7	90	10
7.5	25	75
10	25	75
Note: The run started with 75% acetonitrile in 0.05% formic acid (eluent B) at 25% H_2O in 5mM ammonium acetate (eluent A; pH=6.84) in 5 min, equilibrated for 2 min, returning to 75% eluent B in 0.5 min and re-equilibrating for 2.5 min before the next injection.		

Supplementary Table 1: Mobile phase gradient for liquid chromatography system.

Analytes	Precursor ions[a] (m/z)	Product ions[b] (m/z)	TEM[c] (°C)[i]	GS_1[d] (psi)[j]	CUR[e] (psi)	GS_2[f] (psi)	IS[g] (V)[k]	CAD[h]
Lactulose	341.016	160.952	500	50	20	40	-3500	Median
		184.939	700		25	50		High
		58.947	450		20	45		High
		100.89	550		20	45		High
		73.008	450		20	45		High
Mannitol	180.932	112.798	450	50	20	50	-4000	Median
		136.813	700	45	30	45	-4500	Median
		58.994	500	50	22	50	-4500	Median
		71.009	550	50	22	50	-4000	High
		89.033	450	50	25	50	-3500	High
Sorbitol	180.935	112.912	650	50	30	45	-4500	Median
		58.924	450	40	20	40	-3500	High
		136.927	650	50	20	50	-4500	High
		70.973	450	40	20	40	-3500	High
		92.879	700	40	20	45	-4500	Median
Note: [a]=The Q precursor ion of analytes; [b]=Fragment ions; [c]=Temperature at the source; [d]=Nebulizer gas (helps in droplet formation); [e]=Gas flow between orifice and gas curtain; [f]=Heating gas (GS2 and TEM help with desolvation); [g]=Ions spray (voltage), which directs the formed ions to the analyzer; [h]=Gas collision (helps in the fragmentation of precursor ions to form product ions); [I]=Celsius temperature unit; [J] =Pound force per square inch; [k]=Voltage.								

Supplementary Table 2: Automatic parameters for precursor and product ions from lactulose, mannitol and sorbitol, through Flow Injection Analysis (FIA).

Analytes	Precursor ions (m/z)	Product ions (m/z)	DP[a] (V)[f]	EP[b] (V)	CE[c] (V)	CXP[d] (V)	Intensitye (cps)
Lactulose	341.016	160.952	-75	-10	-12	-11	499000
		184.939			-48	-3	420000
		58.947			-48	-9	324000
		100.89			-22	-9	301000
		73.008			-36	-9	281000
Mannitol	180.932	112.798	-60	-10	-10	-9	230000
		136.813			-16	-9	193000
		58.994			-26	-9	124000
		71.009			-26	-5	122000
		89.033			-18	-7	109000
Sorbitol	180.935	112.912	-75	-10	-26	-9	225000
		58.924			-16	-7	150000
		136.927			-26	-9	143000
		70.973			-26	-9	131000
		92.879			-24	-11	107000
Note: [a]=Decomposition potential (voltage applied to the orifice to avoid clustering of ions); [b]=Entry potential and [c]=Collision energy; [d]=Collision cell output potential; [e]=Intensity of fragment ions by MRM; [f]=Volt (voltage measurement).							

Supplementary Table 3: Mass spectrum monitoring and operational parameters of precursor ions and product ions of lactulose, mannitol and sorbitol, through Multiple Reaction Monitoring (MRM).

Analytes	Precursor ions[a] (m/z)	Product ions[b] (m/z)	TEM[c] (°C)[i]	GS1[d] (psi)[j]	CUR[e] (psi)	GS2[f] (psi)	IS[g] (V)[k]	CAD[h]
Lactulose	341.016	160.952	500	50	20	40	-3500	Median
		184.939	700		25	50		High
		58.947	450		20	45		High
		100.89	550		20	45		High
		73.008	450		20	45		High
Mannitol	180.932	112.798	450	50	20	50	-4000	Median
		136.813	700	45	30	45	-4500	Median
		58.994	500	50	22	50	-4500	Median
		71.009	550	50	22	50	-4000	High
		89.033	450	50	25	50	-3500	High
Sorbitol	180.935	112.912	650	50	30	45	-4500	Median
		58.924	450	40	20	40	-3500	High
		136.927	650	50	20	50	-4500	High
		70.973	450	40	20	40	-3500	High
		92.879	700	40	20	45	-4500	Median
Note: [a]=The Q precursor ion of analytes; [b]=Fragment ions; [c]=Temperature at the source; [d]=Nebulizer gas (helps in droplet formation); [e]=Gas flow between orifice and								

gas curtain; [f]=Heating gas (GS2 and TEM help with desolvation); [g]=Ions spray (voltage), which directs the formed ions to the analyzer; [h]=Gas collision (helps in the fragmentation of precursor ions to form product ions); [i]=Celsius temperature unit; [j] =Pound force per square inch; [k]=Voltage.

Supplementary Table 4: Automatic parameters for precursor and product ions from lactulose, mannitol and sorbitol, through Flow Injection Analysis (FIA).

Analytes (precursor ion / product ions; m/z unit)	**Calibration curve equation**	**LDa (ng/mL)**	**LQb (ng/mL)**	**Rc (ng/mL)**
Lactulose (341.016/58.947)	$y=1.15e^{4}x +1.33e^{6}$	0.0055	0.0168	0.991
Mannitol (180.932/71.009)	$y=4.46e^{4}x +6.42e^{6}$	0.0003	0.0010	0.993
Sorbitol (180.935/58.924)	$y=5.03e^{4}x +7.10e^{6}$	0.0031	0.0001	0.995
Note: [a]=The detection limit; [b]=Limit of quantification; [c]=Correlation coefficient.				

Supplementary Table 5: Linearity, Limit of Detection (LD), Limit of Quantification (LQ) of the method in the LC-MS/MS system for analysis of the excretion of lactulose, mannitol and sorbitol sugars.

	Initial concentration[a] (ng/mL)	**Concentration obtained[b] (ng/mL) (n=3)**	**Recovery[c] (%)**	**SD[d]**	**CV[e] (%)**
Lactulose (341.016/58.947)	100	95.7	95.7	1.71	1.8
	500	499.2	99.8	6.15	1.2
	1000	994.8	99.4	20.83	2.1
Mannitol (180.932/71.009)	100	112.9	112.9	11.06	9.8
	500	661.2	132.2	8.63	1.3
	1000	1116.4	111.6	18.71	1.7
Sorbitol (180.935/58.924)	100	90.4	90.4	2.25	2.5
	500	568.6	113.7	33.08	5.8
	1000	988.2	98.8	45.82	4.6
Note: [a]=Concentration in fortified samples; [b]=Concentration obtained through the average of the values obtained by the equation of the calibration curve of the spiked samples adding the standards in the samples of urine of volunteers; [c]=% recovery; [d]=Standard deviation and [e]=Coefficient of variation.					

Supplementary Table 6: Repeatability of the method in the LC-MS/MS system to analyze the excretion of lactulose, mannitol and sorbitol sugars.

Analytes (mass/charge; m/z)	**Day**	**Initial concentration[a]**	**Concentration obtained[a] (ng/mL) (n=6)**	**Recovery[b] (%)**	**SD[c]**	**CV[d] (%)**
Lactulose (341.016/58.947)	1	500	621.6	124.3	41.1	6.6
	2		504.7	100.4	11.8	2.3
	3		607.7	121.5	52.3	8.6

Mannitol (180.932/71.009)	1	500	531.4	106.3	46.5	8.7
	2		650.7	130.1	31.3	4.8
	3		698.8	139.8	30.2	4.2
Sorbitol (180.935/58.924)	1	500	575.6	115.1	50.2	8.7
	2		564.2	112.8	34.8	6.1
	3		657.8	131.6	104.0	5.8
Note: [a]=Concentration in fortified samples; [b]=Percentage of recovery of concentrations obtained through the equation of the calibration curve in fortified samples adding the standards in the samples of urine of volunteers; [c]=% recovery of mean concentrations obtained on different days; [d]=Standard deviation and coefficient of variation.						

Supplementary Table 7: Intermediate precision of the method in the LC-MS/MS system for analysis of lactulose, mannitol and sorbitol in urine samples.

Analytes	**Concentration[a] (ng/mL)**	**Samples**	**Intensity of analytes[b] (cps; N=3)**	**SD[c]**	**CV[d] (%)**
Lactulose	750	FU	54176.67	1552.23	3.0
		Standard	51866.67	1521.26	2.9
	1500	UF	76046.67	5877.77	6.6
		Standard	95003.33	8227.71	8.7
	3000	FU	130410.00	10424.17	6.7
		Standard	174466.67	21185.92	12.1
	6000	UF	245300.00	7031.59	2.5
		Standard	383666.67	31187.55	8.1
Mannitol	750	FU	12523333	519839.7	4.2
		Standard	23706667	973721.4	6.6
	1500	FU	13000000.00	253245.6	1.1
		Standard	24000000.00	1769755	6.3
	3000	FU	41350000.00	727461.3	1.8
		Standard	49000000.00	1545930	3.2
	6000	FU	71253333.33	1912599	2.7
		Standard	84000000.00	1553094	1.8
Note: [a]=Concentration of analytes in standard solutions and in fortified urine; [b]=Mean intensities of lactulose and mannitol analytes; [c]=Standard deviation; [d]=Coefficient of variation.					

Supplementary Table 8: Intensity of lactulose and mannitol analytes in standard solutions and fortified urine samples. Fortified Urine samples (FU) obtained from known concentrations of analytes in urine samples.

Analyte's concentrations (ng/mL)	**Analytes**					
	Mannitol			**Lactulose**		
	Retention time[a] (min)	**Retention time FU[b] (min)**	**Recovery (%)[c]**	**Retention time (min)**	**Retention time FU (min)**	**Recovery (%)**
750	6.87	6.83	99.4	7.18	7.13	99.3

1500	6.86	6.83	99.6	7.17	7.14	99.6
3000	6.84	6.82	99.7	7.16	7.13	99.6
6000	6.84	6.82	99.7	7.16	7.12	99.4
750	6.81	6.81	100.0	7.13	7.12	99.9
1500	6.82	6.81	99.9	7.14	7.12	99.7
3000	6.81	6.81	100.0	7.13	7.12	99.9
6000	6.81	6.80	99.9	7.14	7.11	99.6
750	6.8	6.84	100.6	7.12	7.14	100.3
1500	6.81	6.81	100.0	7.12	7.12	100.0
3000	6.82	6.81	99.9	7.14	7.12	99.7
6000	6.81	6.81	100.0	7.13	7.11	99.7
Note: [a]=Retention time obtained with the standard solution; [b]=Retention time obtained with the fortified urine; [c]=% recovery.						

Supplementary Table 9: Retention time of lactulose and mannitol in standard solutions and fortified urine samples. Retention time reproduced on standard and Fortified Urine (FU) samples.

Analytes	**Concentration[a] (ng/mL)**	**Recovery[b] (%)**	**EM[c](%)**
Lactulose	750	99.8	4.4
	1500	93.9	-8.9
	3000	88.8	-22.0
	6000	135.3	-30.8
Mannitol	750	84.7	-11.7
	1500	84.2	-10.8
	3000	85.2	-11.8
	6000	84.4	-12.4
Note: [a]=Known concentration added to standard solutions and urine samples; [b]=Percentage obtained through the average of analyte concentrations; [c]=Percentage expressed in suppression of analytes concentrations.			

Supplementary Table 10: Recovery and matrix effect of lactulose and mannitol in fortified urine. Fortified urine obtained by adding known concentrations of the analyte to the urine of volunteers in triplicate.

Source	**Type III sum of squares**	**df**	**Mean square**	**F**	**Sig.**	**Partial eta squared**
Corrected model	7.569[a]	3	2.523	11.812	0.000	0.330
Intercept	83.187	1	83.187	389.479	0.000	0.844
Age (months)	0.000	1	0.000	0.002	0.966	0.000
Groups	3.134	2	1.567	7.336	0.001	0.169
Error	15.378	72	0.214			
Total	365.935	76				
Corrected total	22.947	75				

Note: [a]R squared=0.330 (adjusted R squared=0.302).

Supplementary Table 11: Unidirectional Analysis of Covariance (ANCOVA) between the experimental groups, independent variable and in the dependent variable, lactulose: Mannitol excretion rate (Napierian logarithm of lactulose: Mannitol rate) to correct the covariate age of children.

Source	Type III sum of squares	df	Mean square	F	Sig.	Partial eta squared
Corrected model	5,824[a]	2	2,912	18,365	,000	,542
Intercept	54,566	1	54,566	3,44,130	,000	,917
Agem	,002	1	,002	,015	,904	,000
CEFCC	3,166	1	3,166	19,967	,000	,392
Error	4,915	31	,159			
Total	2,03,211	34				
Corrected Total	10,739	33				
Note: [a]= R Squared=, 542 (adjusted R squared=, 513).						

Supplementary Table 12: Unidirectional Analysis of Covariance (ANCOVA) between the experimental groups, independent variable and in the dependent variable, lactulose: Mannitol excretion rate (Napierian logarithm of lactulose: Mannitol rate) to correct the covariate age of children.

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