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Ultra high performance liquid chromatography–tandem mass spectrometry method for the determination of soluble milk glycans in rat serum

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ABSTRACT

The main objective of the present work was to develop and validate a multicompound method to measure soluble milk glycans (SMGs) in biological fluids such as serum. An ultra high performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) method for the identification and quantification of the following SMGs and their precursors 2′-fucosyllactose, 3′-sialyllactose, 6′-sialyllactose, lacto-N-neotetraose, N-acetylneuraminic acid, fucose, lactose and glucose in rat serum samples was set up. These analytes were separated in an Acquity UPLC BEH Amide column using acetonitrile–water gradient with ammonia as additive, in a 10 min run, and were detected and quantified using a triple quadrupole (QqQ) mass spectrometer. The mass spectrometric conditions in negative electrospray ionization mode (ESI−) were individually optimized for each analyte to obtain maximum sensitivity in the Selected Reaction Monitoring (SRM) mode. Selection of two specific fragmentation reactions for each compound allowed simultaneous quantification and identification in one run, ensuring a high specificity of the method. The limits of detection (LODs) ranged from 5 to 70 ng mL^{-1} and the limits of quantification (LOQs) from 20 to 200 ng mL^{-1}. The inter- and intra-day variability was lower than 15% and the recoveries ranged from 85% to 115%. A biological application of the method was also described, specifically the time-course of SMGs in rat serum after an oral challenge.

1. Introduction

Human milk is a complex biological fluid composed mainly of lipids, proteins, and lactose. Oligosaccharides are the third most abundant component in human milk, after lactose and lipids. They are present in large amounts, ranging from 5 to 23 g L^{-1}. The monosaccharides used for the biosynthesis of human milk glycans are glucose, galactose, N-acetylglucosamine, fucose and N-acetylneuraminic acid (SA, acronym of sialic acid). Despite these basic structures, the possible combinations of monosaccharides and plausible linkages contribute to the overall diversity and complexity of SMG structures to the point that more than 150 structurally distinct SMGs have been identified so far [1].

SMGs exert important biological effects. Namely, they have a prebiotic effect, selectively serving as a source of energy and nutrients for bacteria to colonize the infant intestine [2]. Beyond the prebiotic effects, a large body of evidence suggests that SMGs may protect against infections by mimicking the attachment sites for certain pathogens [2], influence various stages of gut maturation in vitro [3], and prevent necrotizing enterocolitis in neonatal rats [4]. SMGs were previously considered indigestible. However, it is currently known that their structure changes before reaching the colon and that they can be absorbed as they appeared in plasma of neonatal rats [5] and in urine of infants [6]. SMGs can also exert systemic effects. For instance, it has been shown that: 1) fucosylated and sialylated SMGs reduce selectin-mediated leukocyte rolling, adhesion, and activation [7,8]; 2) oligosaccharides are often found as a component of glycoproteins or glycolipids and there is evidence of a role of glycoproteins in adaptive functions of neuronal membrane components determining the efficiency of interneuronal connections [8]; and 3) sialoconjugates have been also shown to participate in the establishment of synaptic pathways, calcium transportation, binding of neurotransmitters, cell-to-cell interactions and axon regeneration [9,10].

An important tool for initiating any study on SMGs is a quantitative method to measure different SMGs in biological matrices. The separation of SMGs has been traditionally a challenge; oligosaccharides are

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polar compounds that are not well resolved by traditional reversed-phase chromatography. In addition, oligosaccharides do not generally contain strong intrinsic chromophores, resulting in low specificity and sensitivity in optical absorbance detection. Nonetheless, some studies have been published in the scientific literature for the determination of these compounds such as gel permeation chromatography [11], reverse-phase high performance liquid chromatography [12], high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC–PAD) [13,14], capillary electrophoresis [15], precolumn derivation HPLC with different detectors (ultraviolet, photodiode array ultraviolet–visible, differential refractive index detector) [16,17], and also Nuclear Magnetic Resonance Spectroscopy [18].

All of the previously published methods based on LC are limited by cumbersome protocols requiring derivatization precolumns and long-run times, which do not fully resolve the major oligosaccharides to baseline, and have low sensitivities. HPAEC–ECD improves detection limits, but requires a long analysis time. Currently, mass spectrometry offers several advantages such as greater selectivity, specificity and sensitivity. Some of these techniques involve matrix-assisted laser desorption (MALDI) or time-of-flight mass spectrometry (TOF/MS) [19–26], microfluidic chips and mass spectrometry technology (HPLC-Chip/TOF–MS) [27], and negative ion mode electrospray mass spectrometry (ESI–MS) [28,29].

Herein, we report an UHPLC–MS/MS method with negative electrospray ionization using ammonium hydroxide as additive for the separation and determination of specific carbohydrates and metabolites in serum of rats to support pharmacokinetic studies of SMGs carried out to confirm the appearance of these metabolites in serum of rats when they were administered orally. The method focused on four main SMGs: 2′-fucosyllactose (2′-FL), 3′-sialyllactose (3′-SL), 6′-sialyllactose (6′-SL), lacto-N-neo-tetraose (LNnT) and their parental mono- and disaccharides: sialic acid (SA), fucose (Fuc), lactose (Lact) and glucose (Glu). The chemical structures are shown in Fig. 1. To our knowledge, no multicomponent methods for the quantitative determination in serum samples of all the proposed compounds in a single analysis have been previously described in the literature.

2. Materials and methods

2.1. Chemicals and reagents

All reagents were analytical grade unless specified otherwise. Water (18.2 MΩ cm) was purified and filtered by a specific LC–MS filter using a Milli-Q system from Millipore (Bedford, MA, USA). SA, Fuc, Lact and Glu were supplied by Sigma-Aldrich (Madrid, Spain); 2′-FL, 3′-SL and 6′-SL were all derived from bacterial synthesis; and LNnT was synthesized from a yeast fermentation system and purified by crystallization [30]. The purity and content of each SMG was measured by high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC/PAD). Acetonitrile (MeCN) LC–MS grade, ethanol (EtOH) HPLC grade and ammonia solution 25% (v/v) eluent additive for LC–MS were from Scharlab (Barcelona, Spain).

A stock solution was prepared by weighing 0.002 g of each compound into a 10 mL flask, except for Lact and Glu, for which 0.005 g were weighed. Then, water was added up to the final volume. The solution remained stable for at least one month at 4 °C. After that, six work standard solutions were prepared for calibration purposes. The first one (WS6) was obtained by dilution to 1 mL of 100 μL of the stock solution. Then, 500 μL, 100 μL, 25 μL and 5 μL of WS6 solution were diluted to a final volume of 1 mL to obtain standards WS5, WS4, WS3 and WS2, respectively. Finally 150 μL of WS2 were diluted to 1 mL to prepare WS1. In all cases, an aqueous solution of ammonia 0.1% (v/v) was used for standard preparation. The calibration standards were injected at the beginning and end of each sample series. A quality control standard (WS4) was injected after every twenty injections. Calibration standards were freshly prepared from the original stock solution in each experiment. The stock solution was stable for 1 month at 4 °C. The working standard solutions were prepared from the stock solution for each experiment.

2.2. Apparatus and software

Detection and quantification of the studied compounds were performed using an UPLC Acquity® system from Waters (Milford, MA, USA) equipped with a binary pump, a vacuum membrane degasser, a thermostated column compartment, an autosampler, and an automatic injector. The chromatograph was connected online to a triple quadrupole mass spectrometer detector (TQD) with an electrospray ionization (ESI) interface. Three different polarity chromatographic columns were tested in order to achieve a good resolution: Acquity UPLC BEH C18 (2.1 mm × 100 mm i.d., 1.7 μm particle size), Acquity UPLC BEH HILIC (2.1 mm × 150 mm i.d., 1.7 μm particle size), and Acquity UPLC BEH Amide (2.1 mm × 100 mm i.d., 1.7 μm particle size) from Waters. MassLynx software version 4.1 was used for instrument control and for data acquisition and analysis.

Analytical balance with a precision of 0.1 mg, vortex-mixer, maximum recovery LC vials and screw caps from Waters, eVOL automated analytical syringe from SGE Analytical Science (SGE Europe, United Kingdom), and speed vac evaporator from Heraeus Instrument Thermo Scientific (Madrid, Spain) were also used.

2.3. Animal manipulation

Thirty Sprague Dawley female rats (~300 g body weight) (Charles River Laboratories, France) were used. The animals were kept in pairs in standard cages at constant room temperature (22 ± 2 °C) and 45–55% humidity under a regular 12 h light/dark schedule. Food and water were freely available. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national laws and EC policies for the Care and Use of Laboratory Animals (RD 2101-2005, 86/609/CEE).

Animals were assigned to five experimental groups ($n=6$). Group 1 received a single dose of 2′-FL (1.75×10^{-3} mol kg^{-1}); group 2, 6′-SL (1.75×10^{-3} mol kg^{-1}); group 3, 3′-SL (1.75×10^{-3} mol kg^{-1}); group 4, LNnT (1.75×10^{-3} mol kg^{-1}) and group 5, a mix of 2′-FL, 6′-SL, 3′-SL, and LNnT (4.37×10^{-4} mol of each SMGs kg^{-1}, representing 1.75×10^{-3} mol kg^{-1} in total).

All the solutions were prepared in water and were administered by intragastric gavage. The animals were fasted for 12 h and a blood sample was taken from the caudal veins (time 0). After the gavage, serial blood samples were collected at 30, 60, 90, 120, 150, 210 and 240 min. Blood samples were allowed to clot and then, centrifuged at 1800 × g for 10 min. Serum was stored at −20 °C.

2.4. Sample preparation

The initial extraction protocol was based on the procedures previously proposed in the scientific literature [18,27]. The protocol was then modified and optimized in order to improve the extraction of the analytes from serum samples. An aliquot (80 μL) of serum sample was placed into a 10 mL glass tube and extracted with a solution containing 2.0 mL of a 2:1 (v/v) mixture of chloroform and methanol, and 0.4 mL of deionized water. After shaking for 2 min in a vortex-mixer, samples were centrifuged at 2400 × g at 4 °C for 30 min. The lower chloroform layer was

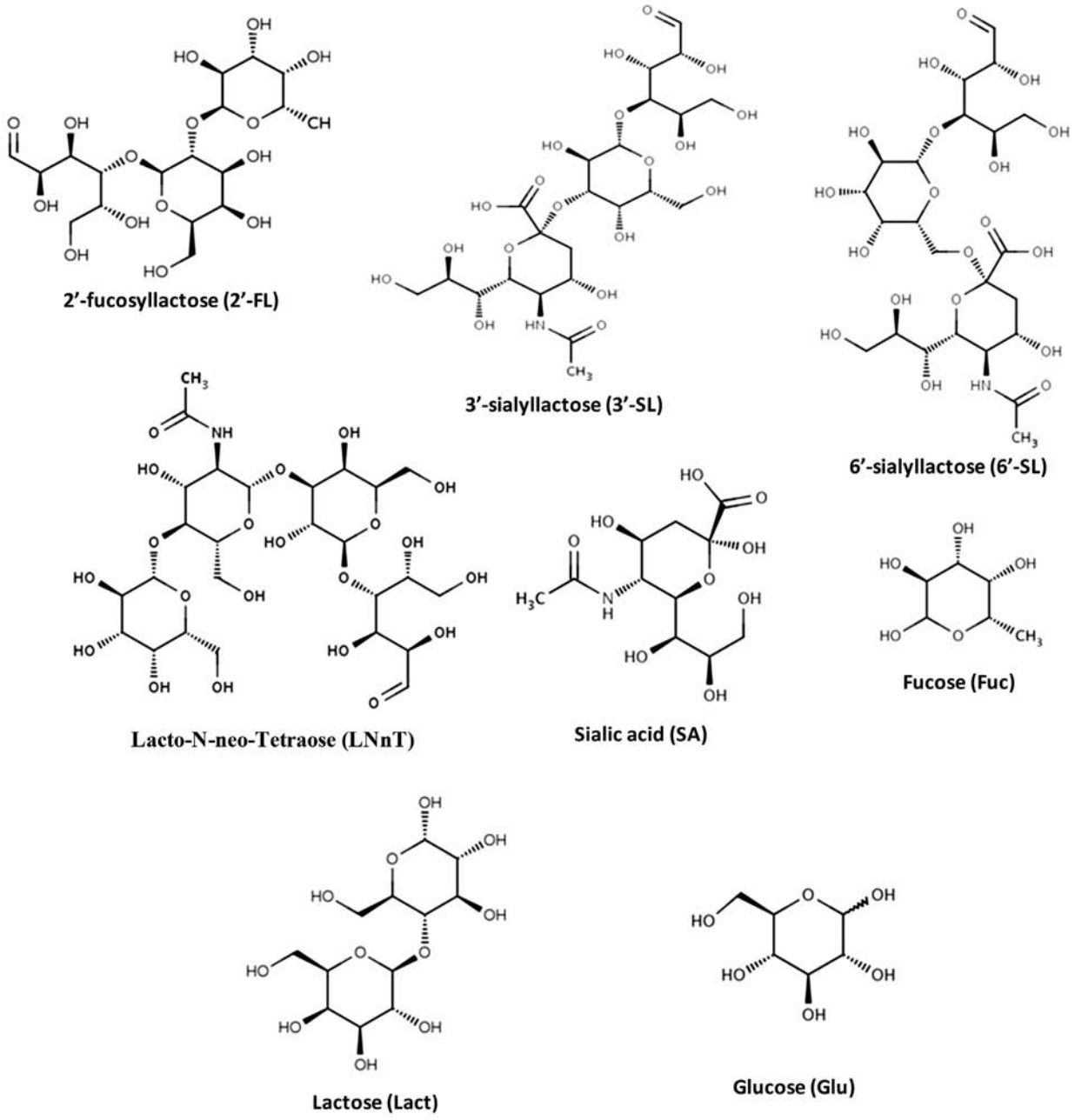

Fig. 1. Chemical structures of the human milk oligosaccharides and related compounds of interest.

discarded and the upper layer was collected. For protein precipitation, 1 mL of cold ethanol was added and the mixture was left at 4 °C overnight. Then, the solution was centrifuged at 2400 × *g* at 4 °C for 20 min and the supernatant was separated and dried in a rotary vacuum pump evaporator at 30 °C. The dried samples were re–dissolved in a total volume of 80 μL of a 0.1% (v/v) aqueous solution of ammonia and the final extract was centrifuged in a microcentrifuge at 14,600 × *g* for 10 min. The supernatant was placed in a chromatographic vial for injection into the UPLC–MS/MS system (1 μL injection volume).

2.5. Liquid chromatographic conditions

Chromatographic analysis was performed using a UPLC BEH Amide column (2.1 × 100 mm, 1.7 μm particle size) from Waters. The flow rate was 300 μL min^{-1}, the column was maintained at 25 °C, the sample at 20 °C and the injection volume was 1 μL in partial loop mode. A gradient mobile phase consisting of 0.1% (v/v) ammoniacal aqueous solution (solvent A) and 0.1% (v/v) ammonia in acetonitrile (solvent B) was used. Gradient conditions were: 0.0–3.0 min, 10–25% A; 3.0–8.0 min, 25–40% A; 8.0–8.1 min, 40–10% A; and 8.1–10.0 min 10% A to stabilize the initial conditions. The total run time was 8.1 min, and the post-delay time for reconditioning the column with 10% A was 1.9 min. Weak solvent was a mixture of 25 mL of water and 75 mL of acetonitrile, and strong solvent was a mixture of 80 mL of water and 20 mL of acetonitrile. These solutions were stable for at least one week at room temperature.

2.6. Mass spectrometric conditions

ESI was performed in negative ion mode. The tandem mass spectrometer was operated in multiple reaction monitoring (MRM)

Table 1
MS/MS parameters for ionization with ESI− in MRM mode.

Compound	Parent ion (Da)	Daughter (Da)	Dwell time (ms)	Cone (V)	Collision (eV)	Delay (ms)
Fuc	163.0	58.9	20	15	15	5
		88.9	20	15	5	5
Glu	179.0	58.8	20	13	15	5
		89.0	20	13	8	5
SA	308.1	87.0	20	25	17	5
		170.1	20	25	10	5
Lact	341.1	100.9	20	15	15	5
		161.1	20	15	8	5
2′-FL	487.2	161.0	20	20	12	5
		205.0	20	20	17	5
6′-SL	632.2	87.1	20	60	35	5
		290.1	20	60	28	5
3′-SL	632.2	87.1	20	62	30	5
		264.0	20	62	28	5
LNnT	706.2	179.0	20	28	25	5
		263.0	20	28	20	5

mode and Q1 and Q3 quadrupoles were set at unit mass resolution. The mass spectrometric conditions were optimized for each compound by continuously infusing standard solutions (0.1–1.0 mg L^{-1}). Electrospray ionization spray voltage was 3500 V. Nitrogen was used as desolvation gas at 800 L min^{-1} and as auxiliary gas in the cone at 50 L min^{-1}. The temperature of the source was 120 °C and the desolvation temperature was 400 °C. Argon (99.999% purity) was used as collision gas at an approximate rate of 0.13 mL min^{-1}. Optimized parameters for each compound are listed together with the mass transitions in Table 1. Common parameters in the single function are Inter Channel Delay, 5 ms and InterScan Time, 5 ms.

3. Results and discussion

3.1. Liquid chromatographic analysis

Based on the recommendations obtained from the scientific literature [19–22], some preliminary studies were carried out to optimize chromatographic separation and signal intensity using a standard mixture of compounds. First, an Acquity UPLC BEH C18, an Acquity UPLC BEH HILIC, and Acquity UPLC BEH Amide columns were tested in order to achieve a good separation of peaks. The amide column provided the best resolution for the studied analytes in the shortest time. Consequently, the Acquity UPLC BEH Amide column was the one we selected for further experiments.

Because our aim was to obtain higher sensitivity and selectivity in a short time, the effect of mobile phase was also studied. In order to optimize the separation and peak shapes, different solvents and additives were checked. Two organic solvents (methanol and acetonitrile) commonly used in liquid chromatography were evaluated. Acetonitrile gave better results than methanol, so we selected acetonitrile as optimum for mobile phase. Moreover, several additives (ammonia, ammonium acetate, triethanolamine, ammonium formate, acetic acid and formic acid) were also assayed. The best separation, peak shapes and ionization of the compounds were obtained with a mobile phase composed of 0.1% (v/v) of ammonia in acetonitrile as solvent A and 0.1% (v/v) of ammonia in water as solvent B. The linear gradient described previously was used.

On the other hand, in order to improve the sensitivity of the method, a study to evaluate the possibility of increasing the injection volume and to evaluate different modes of injection was also performed. Injection volumes ranging from 1 to 10 μL were studied and an extra broadening of the peaks was observed at injection volumes higher than 2 μL; therefore, a volume of 1 μL was selected. Related to injection mode, full loop required a greater amount of sample compared to partial loop, and due to the sample amount limitations, partial loop was selected.

Finally, the stability of standards and samples was also studied. The stability was studied in different days for four weeks. It was demonstrated that refrigerated standards after a week were acceptable, but refrigerated serum extracts were not. Therefore, it is advisable to prepare work solutions, standards and processed samples freshly for each experiment.

3.2. Mass spectrometric analysis

The MS/MS detection method was set up by direct infusion of each individual compound to optimize the response of the precursor ion. ESI and ESCI interfaces in positive and negative modes were evaluated. ESI interface in negative mode was selected because it showed higher sensitivity for all compounds of interest. For each compound two product ions (two reactions) were monitored: one for quantification and the other for confirmation. The most abundant transition ion was selected to obtain maximum sensitivity for quantification. The parameters optimized for the precursor ions were capillary and cone voltages, source and desolvation temperature, and desolvation gas flow. For product ions, the optimized parameters were collision energy (CE) and dwell times. The parameters selected to obtain optimum responses are presented in Table 1. Additionally, Fig. 2 shows chromatograms of a standard mixture of compounds.

3.3. Analytical performance

Analytical performance was evaluated according to the recommendations of Analytical Methods Committee [31],[30]. First, calibration curves were established using serum, plasma and solvent to check the absence of matrix effect. A Student's *t*-test was applied in order to compare the calibration curves. First, we compared the variances estimated as $S_{y/x}^2$ by means of a Snedecor's *F*-test. The Student's *t*-test showed no statistical differences among slope values for the calibration curves in all cases and, consequently, the calibration was carried out in solvent. On the other hand, although the results were very similar, serum had minor variability in replicates, for this reason serum instead of plasma was chosen as sample.

The calibration curve for each compound, was obtained in MRM mode by injecting 1 μL of different standard solutions (prepared in 0.1%, v/v, ammonia aqueous solution) in the concentration range from 0.02 to 20 μg mL^{-1} for SA, 3′-SL, 6′-SL, 2′-FL, Fuc and LNnT and 0.04–50 μg mL^{-1} for Glu and Lact. Each calibration level was made in triplicate, and analyzed twice. Table 2 shows the analytical parameters obtained.

Quality controls were injected after every 20 injections to assure the validity of the calibration curve. The predicted value expected was not to exceed ± 15% of the theoretical value.

3.4. Method validation

Validation of linearity, accuracy (precision and trueness), sensitivity, and selectivity, was performed according to the US Food and Drug Administration (FDA) guideline for bioanalytical assay validation [32].

3.4.1. Linearity

Linearity of the calibration curves was quantified by both linear coefficient of determination (% R^2) and *P*-values of the *lack-of-fit* test (% P_{lof}). The linearity for all compounds within this wide concentration range was achieved with R^2 ranging from 99.0% for

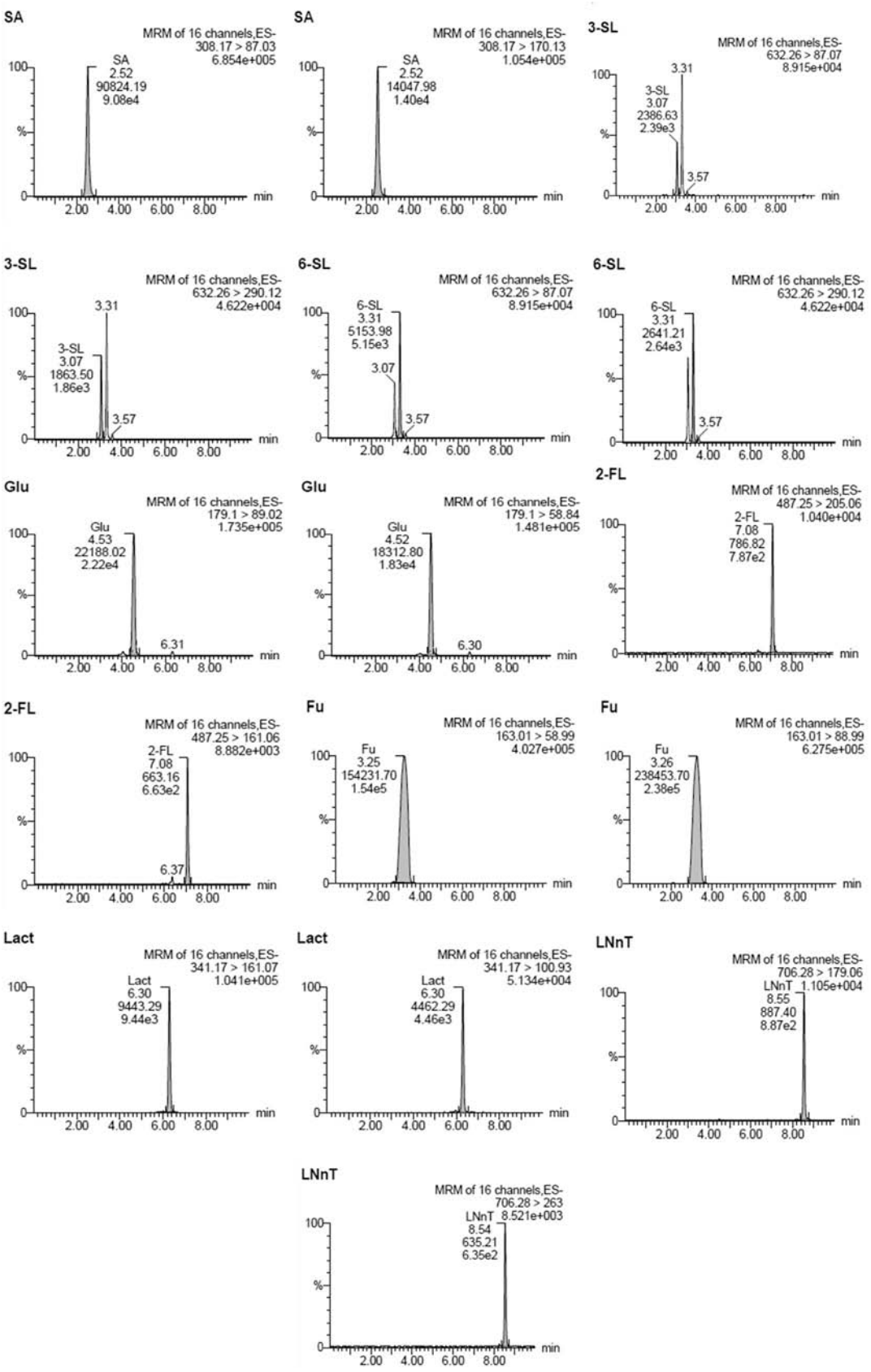

Fig. 2. Chromatogram of each analyte standard in an aqueous solution of ammonia 0.1% (v/v) (two transitions). The first transition was used for quantification and the second for confirmation at concentrations of 20 μg mL^{-1} for SA, 3′-SL, 6′-SL, 2′-FL, Fuc, LNnT and 50 μg mL^{-1} for Glu and Lact.

Table 2
Analytical and statistical parameters.

	n	*a*	*b* (mL ng^{-1})	R^2 (%)	LOD (μg mL^{-1})	LOQ (μg mL^{-1})	LDR (μg mL^{-1})	P_{lof} (%)
SA	6	15	7680	99.9	0.005	0.020	0.02–1000	95.2
Fuc	6	674	185	99.8	0.030	0.100	0.10–1000	70.7
3′-SL	6	−14	243	99.0	0.020	0.060	0.06–60	94.2
6′-SL	6	−32	237	99.4	0.020	0.060	0.06–60	17.7
Glu	6	154	150	99.5	0.030	0.100	0.10–500	91.9
Lact	6	−33	80	99.4	0.070	0.200	0.20–100	65.1
2′-FL	6	12	243	99.9	0.005	0.020	0.02–100	81.3
LNnT	6	−36	50	99.3	0.020	0.060	0.06–100	66.8

n, calibration levels; *a*, intercept; *b*, slope; R^2, determination coefficient; LOD, limit of detection; LOQ, limit of quantification; LDR, linear dynamic range; P_{lof} (%), *P*-value for *lack-of-fit* test.

3′-SL to 99.9% for SA and 2′-FL, and P_{lof} were higher than 5% in all cases. This indicated a good linearity within the stated ranges.

3.4.2. Selectivity

The specificity of the method was determined by comparing the chromatograms of blanks with those corresponding to the samples. No interferences from endogenous substances were observed at the retention times of each respective analyte (Fig. 2), which eluted at 2.97 min, 3.25 min, 3.51 min, 3.73 min, 4.43 min, 6.11 min, 6.87 min and 8.31 min. for SA, Fuc, 3′-SL, 6′-SL, Glu, Lact, 2′-FL and LNnT respectively. This finding suggested that the LC–MS/MS conditions provided sufficient selectivity.

3.4.3. Accuracy: precision and trueness

Due to the lack of certified reference materials, a recovery assay with spiked samples was performed to validate the method in terms of trueness, which was evaluated by determining the recovery of known amounts of the compounds of interest spiked into blank samples. The samples were analyzed using the proposed method and the concentration of each compound was determined by interpolation from the standard calibration curve. Recoveries were calculated by comparing the obtained amounts with the theoretical spiked amounts. As shown in Table 3, the recoveries fell between 85% and 115% in all cases.

To assure precise quantifications of the global method the intra- and inter-day precisions (as relative standard deviation, RSD) were assessed at three concentration levels for each compound. Three replicates at each level were analyzed on the same day in order to evaluate the intra-day variability. The procedure was repeated on five consecutive days to determine inter-day variability. The results of repeatability and within–laboratory reproducibility are summarized in Table 3. RSD values fell between 1.7% for 6′-SL at a concentration of 18.1 μg mL^{-1}, and 14.9% for 2′-FL at the lower concentration assayed. This last value was probably due to the fact that the concentration was close to the limit of quantification (LOQ). Therefore, all compounds were within the acceptable limits for bioanalytical method validation, which are considered $\leq$15% of the actual value, except at the LOQ, which it should not deviate by more than 20%. Precision and trueness data indicated that the methodology to determine the target compounds in serum from rat is highly accurate, precise, and robust, and that the presence of co-extracted matrix components, which typically suppress the analyte signal in mass spectrometry, did not affect the performance of the method.

3.4.4. Sensitivity

The limit of detection (LOD) and the limit of quantification (LOQ) are two fundamental parameters that need to be examined in the validation of any analytical method to determine if an analyte is present in the sample. They are based on the theory of hypothesis testing and the probabilities of false positives and false negatives. In this work, these parameters were calculated from the signal-to-noise ratio. The calculated LODs (signal-to-noise ratio=3) are in the range from 0.005 μg mL^{-1} for SA and 2′-FL to 0.07 μg mL^{-1} for Lact, and the corresponding LOQs (signal-to-noise ratio=10) ranged from 0.02 to 0.20 μg mL^{-1}, respectively. The values obtained are summarized in Table 2.

3.5. Application of the method

Samples of serum from rats were collected following the protocol previously described in Section 2.3. The objective of this experiment was to determine the time-course of each analyte when different ingredients were administered to the animals orally. Fig. 3 shows chromatograms obtained for a serum sample.

Fig. 4 shows the time-course of the four SMGs tested in this study: 2′-FL, 3′-SL, 6′-SL and LNnT in rat serum. For the purpose of simplicity this section was focused on the compounds that were administered although the mono- and disaccharides: SA, Fuc, Lact and Glu were also detected.

Both sialylated SMGs, 3′-SL and 6′-SL, in addition to 2′-FL, exhibited a similar absorptive profile: their levels peaked in the bloodstream between 30 to 60 min after the gavage. However, LNnT exhibited a significantly slower absorption rate, reaching the maximum level in plasma at 180 min after feeding. On the other hand, not all the SMGs were cleared from the blood stream with the same efficacy. Fig. 4 shows how both sialylated SMGs are almost completely removed from plasma 240 min after the challenge, whereas the plasma levels of LNnT and, specially, 2′-FL remain significantly higher at the end of the study (240 min). This fact clearly points out that each SMGs may have different mechanisms of absorption in the intestine and/or may be taken up differently by peripheral tissues or excreted by the urinary system. In that sense, it has been previously reported that there might be different mechanisms underlying the absorption of structurally different SMGs. Thus, Gnoth and collaborators reported in 2001 that while non-sialylated SMGs cross monolayers of cultured intestinal epithelial cells by receptor-mediated transcytosis and paracytosis, sialylated SMGs only use paracytosis [33]. However, it remains unknown which receptors facilitate absorption, as well as their absorption rate, appearance in the circulation and clearance from the system [34].

While it is becoming evident that SMG structure determines their specific function, understanding the metabolic fate of ingested SMGs is key in assessing their biological roles. There is little knowledge about how, when and where they are metabolized. SMGs have long been regarded as metabolically “inert” to the host, because significant amounts are excreted in feces. However, it is well known that a small percentage of SMGs is

Table 3
Recovery, precision and accuracy of target compounds in serum samples.

		Intra-day				Inter-day			
	Spiked ($\mu g\ mL^{-1}$)	Found[a] ($\mu g\ mL^{-1}$)	*R* (%)	RSD (%)	*n*	Found[a] ($\mu g\ mL^{-1}$)	*R* (%)	RSD (%)	*n*
SA	0.91	0.84	92.3	12.1	3	0.85	93.4	13.0	15
	9.05	7.73	85.4	2.4	3	7.98	88.2	5.2	15
	27.2	25.7	94.8	5.3	3	26.3	96.7	5.1	15
Fuc	11.2	9.53	85.2	3.1	3	9.57	85.6	3.8	15
	112	95.6	85.5	3.5	3	96.5	86.4	3.7	15
	335	311	92.8	4.3	3	309	92.1	4.8	15
3′-SL	0.19	0.16	88.1	7.3	3	0.18	96.2	9.1	15
	1.86	1.72	92.3	5.5	3	1.75	94.0	6.3	15
	5.59	4.76	85.0	4.9	3	5.06	90.5	8.3	15
6′-SL	0.60	0.62	96.8	7.9	3	0.59	98.1	8.8	15
	6.03	5.25	87.2	4.6	3	5.37	89.1	6.6	15
	18.1	15.5	85.8	2.1	3	15.8	87.5	1.7	15
Glu	1.10	1.17	106.3	8.0	3	1.16	105.4	9.6	15
	11.0	11.2	101.5	9.9	3	11.3	102.7	9.9	15
	33.0	34.7	103.7	4.9	3	34.3	103.7	5.1	15
Lact	1.02	0.77	85.7	9.9	3	0.90	90.0	13.6	15
	10.2	9.50	94.3	6.4	3	9.58	94.3	6.2	15
	30.5	25.9	85.2	2.6	3	24.9	81.5	2.6	15
2′-FL	0.20	0.19	95.0	14.0	3	0.19	98.8	14.9	15
	1.95	2.00	102.6	10.7	3	2.11	108.2	11.8	15
	5.86	6.61	115.0	11.1	3	6.73	114.8	12.0	15
LNnT	0.15	0.13	87.1	12.1	3	0.16	107.9	13.2	15
	1.46	1.36	93.1	4.9	3	1.49	101.6	8.6	15
	4.39	3.93	90.3	2.0	3	3.96	90.3	3.6	15

[a] Mean value; *R*, % recovery; RSD, relative standard deviation; *n*, number of determinations.

believed to be absorbed intact in the small intestine and later excreted in urine, which opens speculation on possible systemic effects, e.g. in the immune system or in the context of neuronal development [35]. These potential beneficial systemic effects of SMGs on neonatal health come from indirect evidence, namely in vitro data and the presence of SMGs excreted in urine of breast-fed, but not formula-fed infant [35].

The development of a method such as the one shown in this article, and its application to the reported experiment in rats allowed us to show that SMGs are absorbed and detected in the blood stream. Up to now, only a recent article described the appearance of 3′SL in plasma of neonatal rats [5]. No other SMGs were detected, so that the authors concluded that selective absorption occurs. There were many differences between this study and ours, with regard to the analytical method, the age of the animals, and the dosing. Despite this, our research does not support the hypothesis of a selective absorption, as the four SMGs: 2′-FL, 3′-SL, 6′-SL and LNnT, given alone or in combination, were detected in serum showing that all of these compounds can reach the systemic circulation as intact SMGs.

4. Conclusions

The determination and quantification of SMGs using UHPLC–MS/MS in serum samples was successfully performed on an Acquity UPLC BEH Amide column, using 0.1% (v/v) ammonia in acetonitrile and 0.1% (v/v) ammonia in water as mobile phase and triple quadrupole mass spectrometry detection in negative electrospray ionization mode. The analytical performance of the

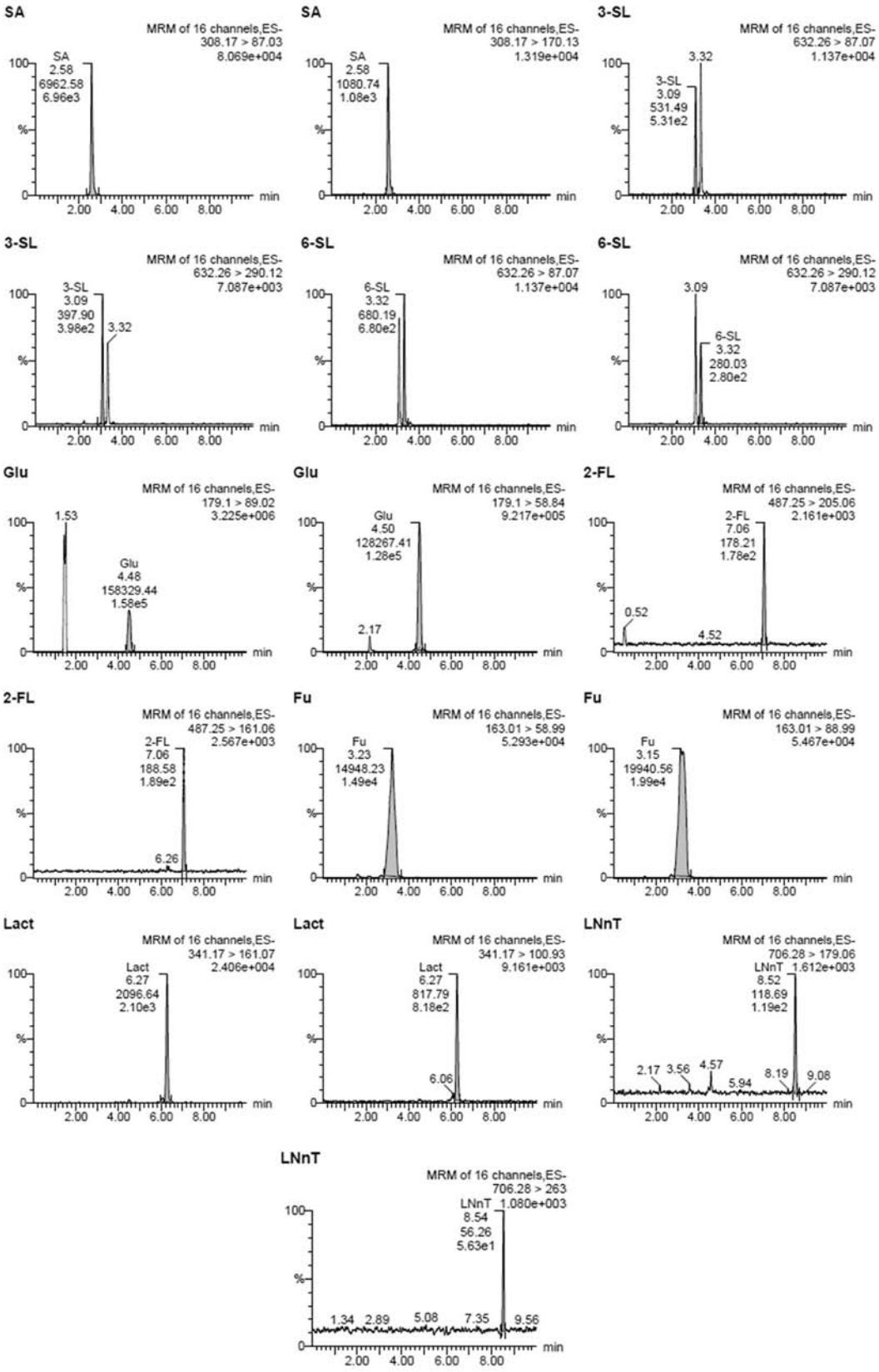

Fig. 3. Chromatograms obtained for a serum sample (two transitions). The first transition of each analyte was used for quantification and the second for confirmation.

method was validated providing a powerful tool for the simultaneous determination of eight compounds that include the most abundant SMGs and related metabolites. The simplicity and sensitivity of the new method made it possible to conduct an in vivo study in rats to demonstrate the absorption of these compounds from the intestine to the systemic circulation, which

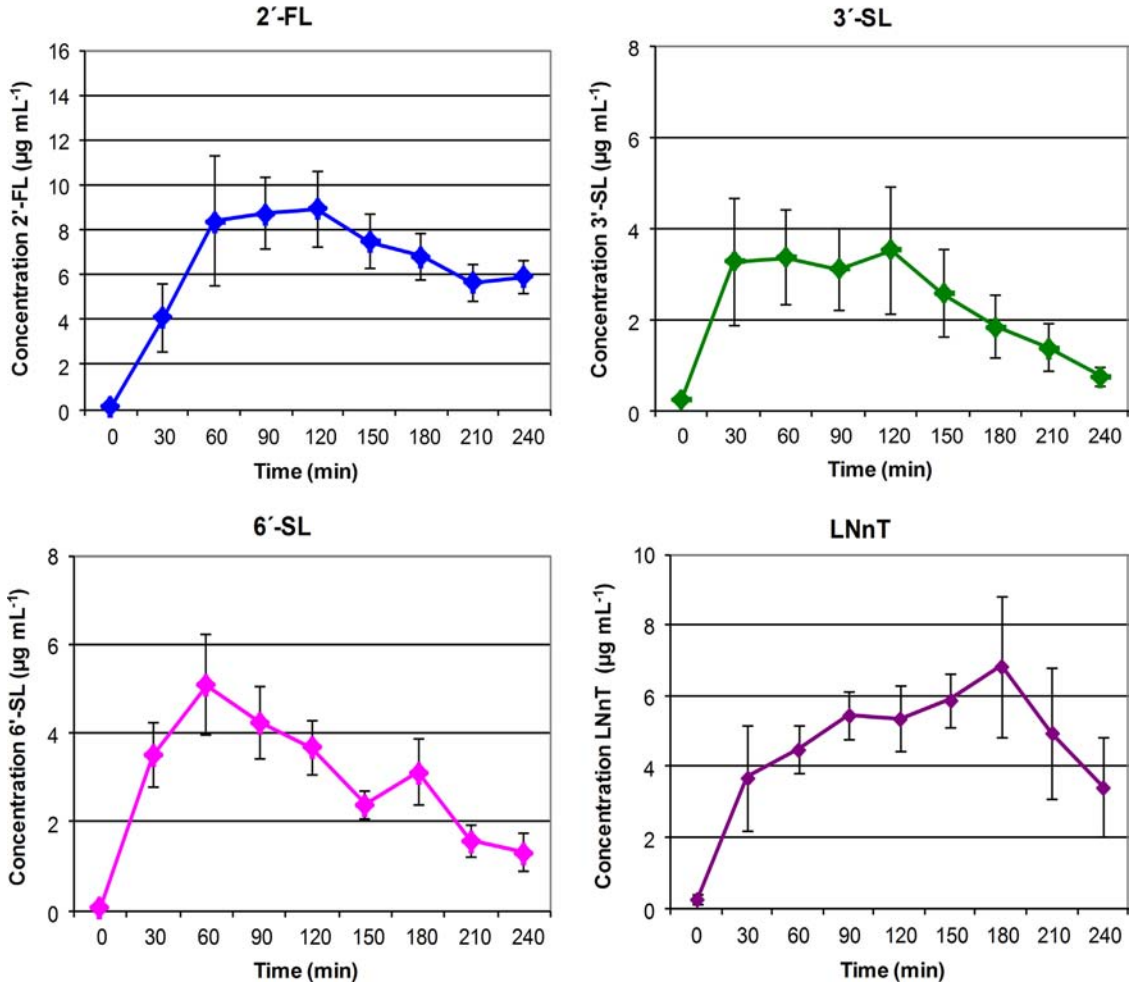

Fig. 4. Time-course for the four SMGs tested in the study: 2′-FL, 3′-SL, 6′-SL and LNnT. On the "*y*" axis is the concentration of each analyte in μg mL^{-1}, on "*x*" axis is the time expressed in minutes.

constitutes one of the first studies showing this aspect in vivo. The proposed method could also provide opportunities to study all of these components in a wide variety of situations.

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