Journal of Chromatography B 1242 (2024) 124216

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/jchromb

Development and validation of an LC-MS/MS method for the quantification of oral-sugar probes in plasma to test small intestinal permeability and absorptive capacity in the domestic cat (*Felis catus*)

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ARTICLE INFO

Keywords:
Intestinal permeability
Absorptive capacity
LC-MS/MS
Differential sugar absorption test
Tandem mass spectrometry
Plasma

ABSTRACT

A novel method for quantifying the concentration of lactulose, rhamnose, xylose, and 3-O-methylglucose (3-OMG) in cat plasma using liquid chromatography-mass spectrometry (LC-MS) was developed. Domestic male cats (n = 13) were orally dosed with a solution containing the four sugars to test the permeability and absorptive capacity of their intestinal barrier. Plasma samples were taken 3 h later and were prepared with acetonitrile (ACN), dried under N_2, and reconstituted in 90 % ACN with 1 mM ammonium formate. Stable isotope labelled ^{13}C standards for each analyte were used as internal standards. Chromatographic separation was conducted using a Phenomenex Luna NH2 column with a gradient elution system of deionized water and 90 % ACN with 1 mM ammonium formate at 300 μL/min for 13 min total analysis time. Recovery trials were conducted in triplicate over three days with RSD values (%) for each day ranging from 1.2 to 1.4 for lactulose, 5.4 – 6.0 for rhamnose, 3.3 – 5.5 for xylose, and 2.6 – 5.6 for 3-OMG. Inter-day variations for each analyte were not different ($p > 0.05$). Limit of detection and quantification were 0.2 and 0.7 μg/mL for lactulose, 0.8 and 2.4 μg/mL for rhamnose, 0.6 and 1.8 μg/mL for xylose, and 0.3 and 1.1 μg/mL for 3-OMG, respectively. Plasma sugar concentrations recovered from cats were above the limit of quantification and below the highest calibration standard, validating the use of this method to test intestinal permeability and absorptive capacity in cats.

1. Introduction

The intestinal barrier plays a critical role in maintenance of normal physiologic processes of the digestive system, maintaining homeostasis in the gastrointestinal tract. It enables the selective permeability of molecules, allowing for absorption of nutrients and water, but prohibiting the passage of pathogens and other potential toxins. These molecules can cross the barrier through different paracellular, transcellular, carrier-mediated active transport, and ATP-dependent mediated active transport pathways, depending on their size and charge. The paracellular route is mediated by tight junctions between epithelial cells which facilitate the transport of ions, water, and larger hydrophilic compounds, in contrast to the transcellular route which permits small hydrophilic and lipophilic compounds to pass through the plasma membrane of the epithelial cell. Active transport pathways are reserved for the larger nutrients and molecules that require energy to move across the epithelial barrier, a characteristic also known as the absorptive capacity [1].

Abbreviations: 3-OMG, 3-O-methylglucose; SAT, sugar absorption test; AAFCO, Association of American Feed Control Officials; L:R, lactulose: rhamnose; X:G, xylose: 3-O-methylglucose.

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https://doi.org/10.1016/j.jchromb.2024.124216
Received 15 May 2024; Received in revised form 19 June 2024; Accepted 21 June 2024
Available online 22 June 2024

Ageing domestic cats experience a reduced ability to digest nutrients, specifically fat and protein, with no clear cause [2]. The relationship between age and intestinal permeability has not been examined in cats, offering a potential new perspective for investigation of this phenomenon. There is conflicting evidence whether intestinal permeability increases with age in other species, where it seems to be supported in fruit flies, mice, rats, and baboons, but not in dogs and humans [3–8]. Exploring the potential relationship between age and intestinal permeability will enhance our fundamental understanding of the domestic cat's gastrointestinal system, enabling researchers to investigate interventions that could ultimately enhance the quality of life for senior cats.

A common method to quantify the permeability and absorptive capacity of the small intestine is the differential sugar absorption test (SAT). A differential SAT involves administering a monosaccharide and a disaccharide, such as rhamnose and lactulose respectively, to measure transcellular and paracellular transport, respectively [9–12]. Non-metabolisable monosaccharides, xylose and 3-O-methylglucose (3-OMG), can be orally administered as well to determine the mucosal absorptive capacity [13,14]. Several studies have evaluated intestinal permeability in the cat, using varying SAT methodologies as the basis of investigation. These studies have all used different doses of disaccharides and monosaccharides and chose to analyse urine, which is harder to collect aseptically than plasma. Additionally, the sugar concentration in the urine was quantified using varying methods including high-performance liquid chromatography (HPLC), gas–liquid chromatography (GLC), and enzyme assay [11,15–18]. Emms *et al.* (1982) did however analyse the concentration of D-xylose in plasma to determine absorptive capacity in the cat using a method described by Trinder (1975) involving phloroglucinol [19,20]. Unfortunately, while Emms *et al.* (1982) and Randell *et al.* (2001) both successfully orally syringed the sugar solution to cats without sedation, these two studies do not clearly explain the protocol, making it hard to duplicate, especially given the great differences in the dose of probes used between them [11,19].

The differential SAT can also be performed using serum and plasma samples, as shown in multiple studies in humans and dogs [10,13,21–24]. Obtaining sterile urine and free catch urine samples are difficult in cats since cystocentesis is invasive as well as challenging to do on a partially filled bladder and they do not urinate on command, thus blood-based intestinal permeability tests in cats may be easier to conduct. A pilot study using sugars lactulose, rhamnose, xylose, and 3-OMG in the domestic cat was undertaken to determine the feasibility of using this multi-sugar procedure in a larger-scale study. Due to the lack of replicable and reliable oral sugar probe and intestinal permeability studies completed in cats, this study was also required to develop and evaluate the methodology and the sensitivity of the proposed laboratory analysis of feline blood samples. The aim of this study was to develop a quantitative LC-MS/MS method measuring the oral sugar probes in plasma using stable isotope standards. Benefits of such a method include less invasive and more reliable sampling, and thus ethically less expensive means of evaluating intestinal permeability and absorptive capacity in cats.

2. Materials and methods

2.1. Ethics

The preliminary validation pilot study and subsequent research trial were both approved by the Massey University Animal Ethics Committee (MUAEC 22/69 and 23/14) which meets the requirements of the Animal Welfare Act [25]. Cats selected for each experiment continued to be provided with their normal commercially retorted wet cat food (Heinz Wattie's Ltd., Hastings, New Zealand) which is complete and balanced according to Association of American Feed Control Officials (AAFCO) requirements for adult cats. They were fed *ad libitum* daily in their enclosures leading up to the trial and fasted in metabolic cages, as described by Hendriks *et al.* (1999) [26], for 12 h before sugar probe ingestion with access to fresh water at all times.

2.2. Animal studies

This study was split into two parts. The first trial aimed to determine the feasibility and palatability of giving cats a single oral dose of sugar solution and was then followed by a second pilot trial whereby the detection of the sugar probes in plasma was verified.

In order to assess the feasibility of dosing adult cats a sugar solution containing 0.07 g/mL lactulose, 0.02 g/mL rhamnose, 0.07 g/mL xylose, and 0.02 g/mL 3-OMG (1160 mOsm) was mixed in purified water. Six, neutered, male adult (n = 6) domestic shorthair cats aged 5.0 to 8.9 years (mean ± SD, 5.8 ± 1.8 years) and weighing from 3.7 to 5.2 kg (4.3 ± 0.6 kg) received a single oral dose of 2 mL/kg.

All cats tolerated the dosing protocol well and the full dose was administered. From this, the pilot study was undertaken. Following an overnight fast, 2 mL of blood was taken from 13 healthy male adult cats (mean age ± SD (range), 2.5 ± 0.8 (1.3–3.4) years) via jugular venepuncture (T_0). The cats were then dosed with 2 ml/kg of the sugar probe solution (0.07 g/mL lactulose, 0.02 g/mL rhamnose, 0.07 g/mL xylose, and 0.02 g/mL 3-OMG; osmolarity 1160 mOsm). After three hours, 2 mL blood samples were collected again from the opposite jugular vein, and the cats were permitted to return to their regular enclosures where they had free access to both food and water.

The dose of the sugar solution made of lactulose, rhamnose, xylose, and 3-OMG was extrapolated from previous studies in cats and dogs [8,11,13,19,21,27,28]. The sugar solution had an osmolality of 1160 mOsm, which is under the threshold of 1500 mOsm when hypertonic solutions begin to increase permeability [29]. The final blood samples were collected from the cats 3 h after oral dosing based on findings from multiple studies that blood collected at this time is the most consistent between samples [13,21,30].

2.3. Chemicals and standards

$^{13}C_{12}$ lactulose was purchased from Santacruz Biotechnology Inc (Santa Cruz, USA), $^{12}C_6$ 3-OMG from Toronto Research Chemicals (Toronto, Canada), $^{13}C_5$ xylose, $^{13}C_6$ 3-OMG, and $^{13}C_6$ rhamnose from Omicron Biochemicals Inc (South Bend, USA), ^{12}C lactulose, ^{12}C rhamnose, ^{12}C xylose, acetonitrile (HPLC grade), and ammonium formate (HPLC grade) from Sigma-Aldrich (Auckland, New Zealand). Ultrapure water was prepared with a Milli-Q® ultrapure water system (Merck, Auckland, New Zealand) with a resistivity of 18.2 MΩ.

A mixed internal standard solution containing the four ^{13}C labelled sugars was made from stock solutions of individual ^{13}C sugars in 90 % ACN to yield a final concentration of 50 μg/mL. Similarly, a mixed standard of unlabelled sugars was made from stock solutions of individual target sugars in 90 % ACN to yield a final concentration of 1 mg/mL. This mixed standard, along with the mixed internal standard solution, were used to make the calibration standards (in 90 % ACN) for calculating concentration and testing linearity (Table 1) and preparing fortified samples for recovery and repeatability experiments. A seven-point calibration series was prepared containing 0, 1, 5, 10, 20, 50 or 100 μg/mL of the target unlabelled sugars, and 5 μg/mL of the ^{13}C labelled sugars.

2.4. Sample preparation

Whole blood was transferred to a K2 EDTA 5.4 mg vacutainer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and spun at 3000x*g* at 4 °C for 15 min. Plasma was then aliquoted into micro-centrifuge tubes and frozen at −80 °C until analysis.

Plasma samples (200 μL) were thawed, then mixed with ice cold acetonitrile (ACN; 590 μL) and spiked with an internal standard mixture (10 μL) containing 50 μg/mL of each of the four ^{13}C labelled sugars.

Table 1
Parallel reaction monitoring inclusion list.

Name	Mass (*m/z*)	Isolation width (*m/z*)	Adduct	Start	End	(N) CE
Xylose	149	2	$[M-H]^-$	1.5	6	10
$^{13}C_5$ xylose	154	2	$[M-H]^-$	1.5	6	10
Rhamnose	163	2	$[M-H]^-$	1.5	6	10
$^{13}C_6$ rhamnose	169	2	$[M-H]^-$	1.5	6	10
3-OMG	239	2	$[M + HCO_2]^-$	1.5	6	10
$^{13}C_6$ 3-OMG	245	2	$[M + HCO_2]^-$	1.5	6	10
Lactulose	387	2	$[M + HCO_2]^-$	6	11	10
$^{13}C_{12}$ lactulose	399	2	$[M + HCO_2]^-$	6	11	10

Abbreviations: 3-OMG = 3-O-methylglucose; NCE = normalised collision energy.

Samples were then vortexed thoroughly, incubated (−20 °C, 60 min), vortexed again, and centrifuged (14,000xg, 10 min, 4 °C). Then, 100 μL of purified water was added to each sample, vortexed, and centrifuged (14,000xg, 10 min, 4 °C) for a second time. A fixed volume of the supernatant (800 μL) was removed and then dried under nitrogen at 35 °C, and the dry extract was reconstituted in 100 μL 90 % ACN with 1 mM ammonium formate.

2.5. Preparation of fortified samples

Plasma samples collected before oral administration of sugar solution (T_0) were pooled into a composite sample and aliquots were spiked with a known amount of the target sugars, then subjected to the extraction protocol (as described in section 2.4). The experiment was conducted using no spike, low spike, and high spike plasma samples in triplicate. No spike samples were unaltered blank plasma matrix, low spike samples were 5 μL of mixed sugar standard (^{12}C) with 995 μL of blank plasma (concentration: 5 μg/mL), and high spike samples were 25 μL of mixed sugar standard (^{12}C) with 975 μL of blank plasma (concentration: 25 μg/mL). Blank plasma was unaltered T_0, fasted plasma samples that served as a control.

2.6. LC-MS/MS conditions

LC-MS/MS was carried out using a Thermo Scientific Accela HPLC system coupled to a Q Exactive Orbitrap mass spectrometer with electrospray ionisation (ESI) (Thermo Scientific, New Zealand). Samples were introduced to the HPLC system via a PAL autosampler (injection volume = 5 μL), and chromatography was performed using a Phenomenex Luna NH2 column (2 x 150 mm, 3 μm, Phenomenex, New Zealand) with a gradient elution of deionized water with 1 mM ammonium formate (eluent A) and 90 % ACN with 1 mM ammonium formate (eluent B) at a flow rate of 300 μL/min. The gradient elution program was as follows: 100 % B (0 – 0.5 min), 100 – 85 % B (0.5 – 3.5 min), 85 % B (3.5 – 11 min), and a re-equilibration period at 100 % B for 3 min (11 – 13 min). The first 1.5 min of the chromatographic run was diverted to waste and data was acquired from 1.5 – 11 min. The mass spectrometer was operated in negative ionisation mode and data was acquired in full scan MS1 mode (50 – 500 *m/z*) and parallel reaction monitoring (PRM) mode monitoring the MS2 using dynamic first mass for all samples. The ESI source was operated with a spray voltage of 4.5 kV, capillary temperature of 350 °C, sheath gas flow of 20 L/min, aux gas flow of 10 L/min and sweep gas flow of 5 L/min. PRM mode was operated with the inclusion list and relevant parameters in Table 1.

Data acquisition and processing was carried out using Thermo Xcalibur software (version 3.1.66.10; Thermo Fisher Scientific). Regression statistics and standard deviation of the equation were calculated using Microsoft Excel (Office 360, Microsoft Corporation, Redmond, Washington, United States).

2.7. Calculations

Concentration of sugars in the sample (Ci) was calculated by using the concentration measured by the instrument (Cf) and adjusting for factors introduced during sub-sampling (Sf) and during extraction such as the pre-concentration factor (Pf). The equation is shown in equation (1) below:

$$Ci = \frac{Cf^{*}\frac{1}{Sf}}{Pf} = \frac{Cf^{*}\frac{9}{8}}{2} = \frac{Cf^{*}9}{8^{*}2} = \frac{9Cf}{16} \quad (1)$$

3. Results

Quantification for xylose, 3-OMG, and lactulose was performed using MS1 accurate mass peak areas, including molecular ions and any reliable adduct ions to increase sensitivity. MS1 masses 161.0434 and 167.0634 were observed in authentic standards and target samples for ^{12}C 3-OMG and ^{13}C 3-OMG, respectively, and have been previously used to quantify lactulose [31]. ^{12}C rhamnose and ^{13}C rhamnose were solely characterised and quantified in MS2. Exact ions used for quantitation are shown in Table 2 with a mass tolerance of 10.0 ppm. Identifications confirmed by monitoring the MS2 spectra for characteristic fragment ions (Fig. 1) and retention time compared to authentic standards.

3.1. Tandem mass spectrometry detection

MS2 spectra of the four analytes along with their internal standards are presented in Fig. 1. MS2 fragmentation patterns for ^{12}C xylose, rhamnose, 3-OMG, and lactulose are presented in Fig. 2, including predicted fragment ions from the MetFrag tool [32]. It is important to note that ^{13}C standards follow the same fragmentation pattern, but exhibit a mass shift relative to the number of ^{13}C atoms present.

3.2. Chromatographic separation

Chromatographic separation of the sugars was successfully achieved between ≈3.5 and 9 min. While the peaks for both analyte and internal standard of xylose and rhamnose suffered some minor tailing, this did not affect linearity, recovery, or repeatability for these sugars (Table 3). Chromatograms of all 4 analytes and the 4 internal standards in the level

Table 2
Ions measured for each analyte.

Analyte	Ion	MS1 (*m/z*)	MS2 (*m/z*)
^{12}C xylose	$[M-H]^-$	149.0432	
	$[M + HCOO]^-$	195.0488	
	$[M + Cl]^-$	185.0202	
^{13}C xylose	$[M-H]^-$	154.0600	
	$[M + HCOO]^-$	200.0657	
	$[M + Cl]^-$	190.0370	
^{12}C 3-OMG		161.0434	
	$[M + HCOO]^-$	239.0751	
	$[M + Cl]^-$	229.0462	
^{13}C 3-OMG		167.0634	
	$[M + HCOO]^-$	245.0951	
	$[M + Cl]^-$	235.0666	
^{12}C lactulose	$[M-H]^-$	341.1061	
	$[M + HCOO]^-$	387.1112	
	$[M + Cl]^-$	377.0825	
^{13}C lactulose	$[M-H]^-$	353.1464	
	$[M + HCOO]^-$	399.1519	
	$[M + Cl]^-$	389.1230	
^{12}C rhamnose			59.0123
^{13}C rhamnose			61.0190

Abbreviations: 3-OMG = 3-O-methylglucose.

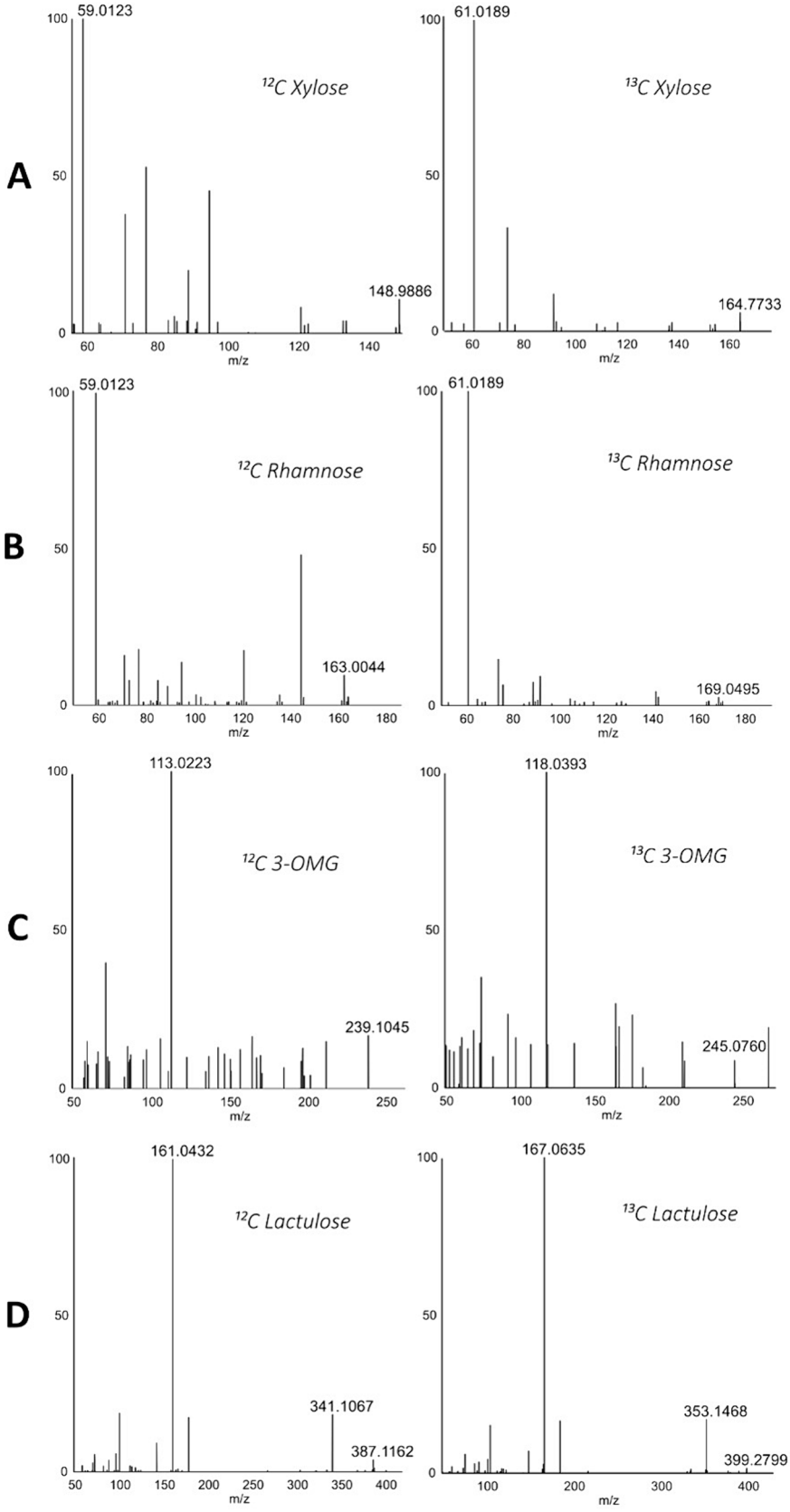

Fig. 1. MS2 spectra for (A) xylose, $^{13}C_5$ xylose, (B) rhamnose, $^{13}C_6$ rhamnose, (C) 3-O-methylglucose, $^{13}C_6$ 3-O-methylglucose, and (D) lactulose, $^{13}C_{12}$ lactulose.

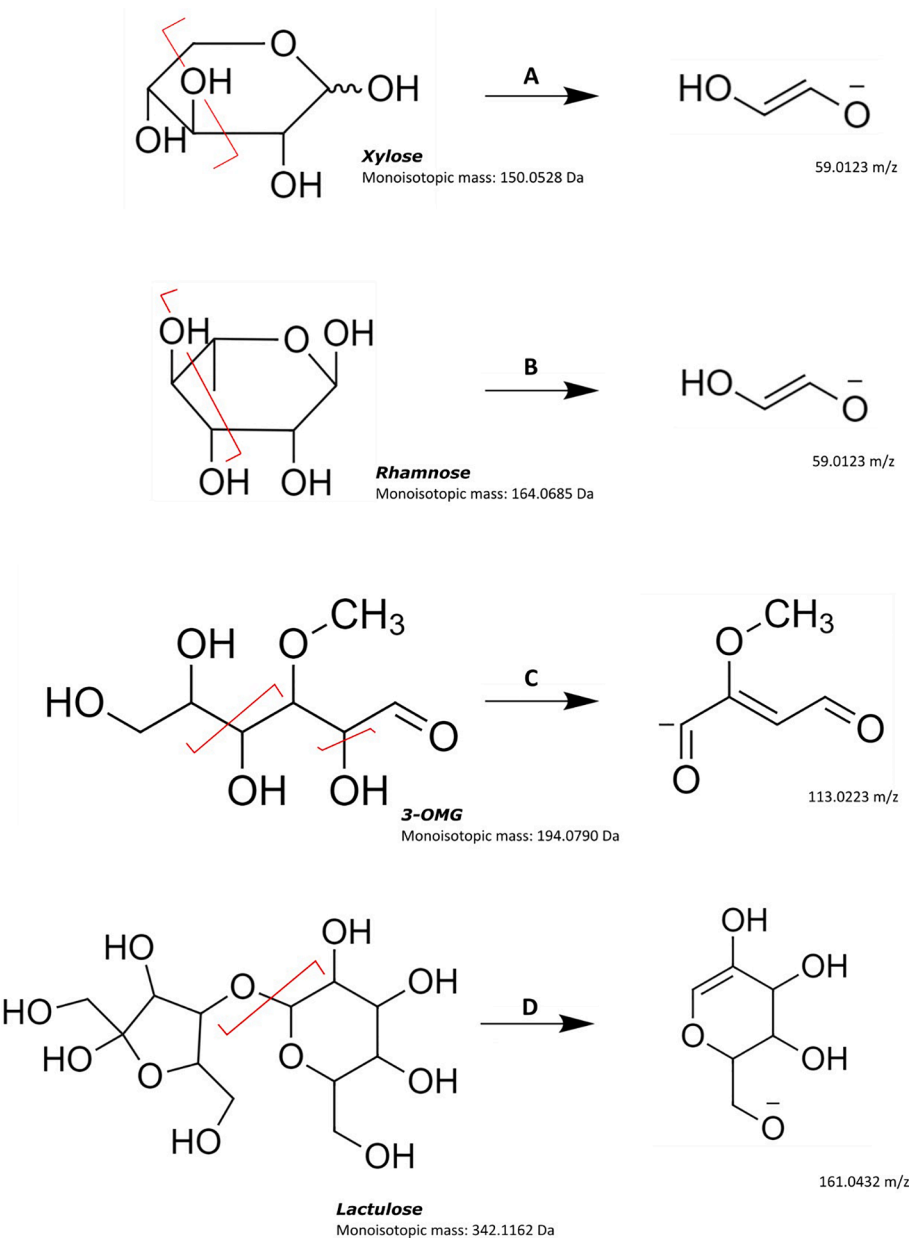

Fig. 2. (A) Xylose, (B) rhamnose, (C) 3-O-methylglucose (3-OMG), and (D) lactulose fragments used for identification and quantification. Fragments predicted using the in silico fragmenter tool, MetFrag [32].

Table 3
Linearity, limit of detection and limit of quantitation.

Analyte	Equation	R^2	LOD (μg/mL)	LOQ (μg/mL)
Lactulose	$y = 0.171145x - 0.0475757$	0.9997	0.2	0.7
Rhamnose	$y = 0.136588x + 0.0198997$	0.9990	0.8	2.4
Xylose	$y = 0.134708x + 0.0888231$	0.9991	0.6	1.8
3-OMG	$y = 0.155246x - 0.0730001$	0.9995	0.3	1.1

Abbreviations: LOD = limit of detection; LOQ = limit of quantitation; 3-OMG = 3-O-methylglucose.

4 calibration standard mixture (concentration of ^{13}C internal standards at 5 μg/mL and target analytes at 20 μg/mL) are shown in Fig. 3.

3.3. Linearity, LOD, and LOQ

Limit of Detection (LOD) and Limit of Quantitation (LOQ) for each sugar was calculated by using the standard deviation of the response of the blank (σ) and the slope (S) of the calibration curve. LOD and LOQ were calculated from these values using equations (2) and (3) respectively. The linearity and sensitivity values for the method are summarised in Table 3.

$$LOD = 3.3 \times \frac{\sigma}{S} \quad (2)$$

$$LOQ = 10 \times \frac{\sigma}{S} \quad (3)$$

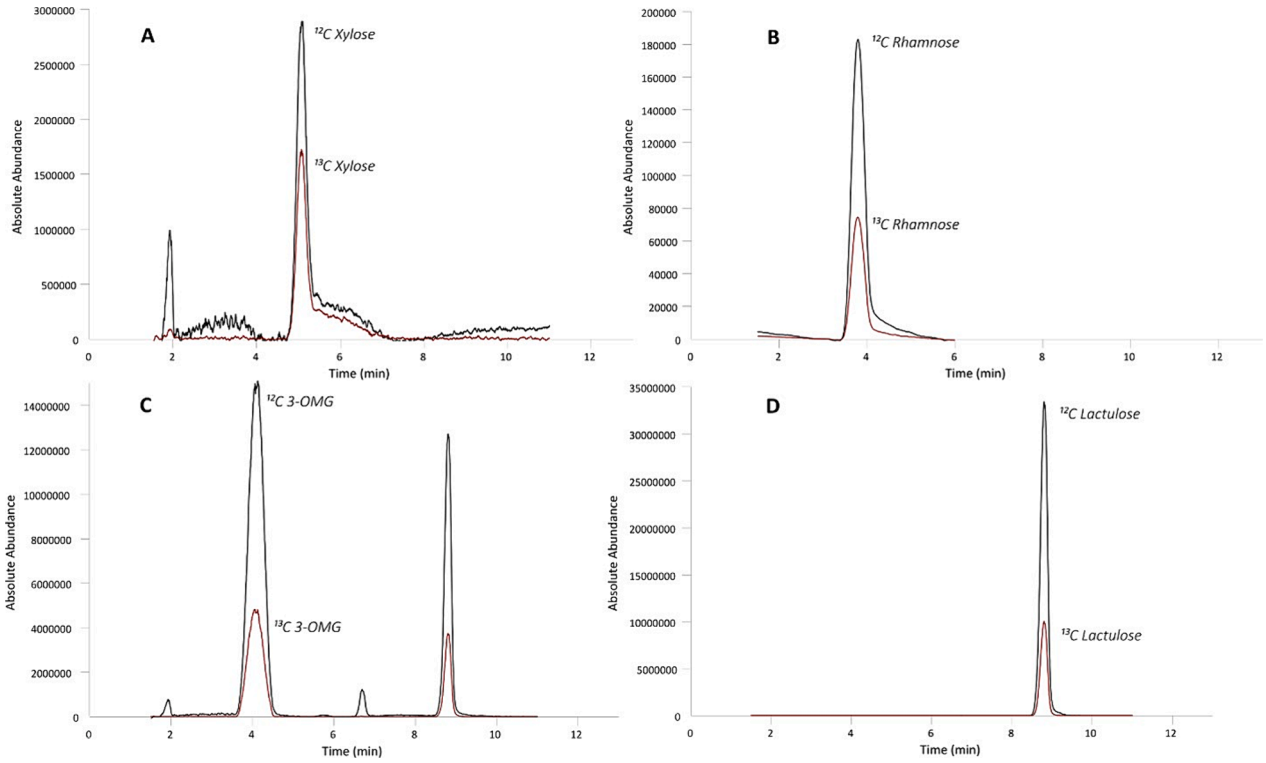

Fig. 3. Chromatograms of analytes and internal standards, (A) xylose, (B) rhamnose, (C) 3-O-methylglucose (3-OMG), and (D) lactulose, in a calibration standard mixture containing 20 μg/mL detected by negative ionisation electrospray and MS/MS. Concentration of ^{13}C internal standards are 5 μg/mL.

3.4. Accuracy, intermediate precision, and repeatability

Trueness, repeatability, and intermediate precision of the method were tested using the spiked samples at two concentration levels (low and high, 5 and 25 μg/mL respectively) and unfortified T_0 samples. The two spike levels and matrix blank were each analysed in triplicate within the same day and across three days. The difference in mean recovery was significantly different based on concentration ($p < 0.05$). Trueness and repeatability of this method met the acceptance criteria (RSD < 10 % and inter-day variation with $p > 0.05$) for the analysis of samples from the main study (Table 4). The mean and intermediate precision of the intestinal permeability (L:R) and absorptive capacity (X:G) was calculated for each spike level and the combined total (Table 5).

Table 4
Repeatability study, mean recovery, standard deviation, relative standard deviation, and significance of inter-day variation.

Analyte	Day	Mean ER* (%)	SD	RSD	Inter-day Variation (ANOVA)
Lactulose	Day1	112.2	1.6	1.4	
	Day2	111.7	1.4	1.2	
	Day3	112.5	1.6	1.4	
	All Days	112.1	1.5	1.3	p = 0.66
Rhamnose	Day1	106.2	5.7	5.4	
	Day2	105.7	6.3	6.0	
	Day3	107.7	5.9	5.5	
	All Days	106.5	5.7	5.3	p = 0.84
Xylose	Day1	109.6	6.0	5.5	
	Day2	109.0	5.6	5.1	
	Day3	109.5	3.6	3.3	
	All Days	109.4	4.9	4.4	p = 0.97
3-OMG	Day1	97.7	5.5	5.6	
	Day2	97.5	5.3	5.4	
	Day3	99.7	2.6	2.6	
	All Days	98.3	4.5	4.6	p = 0.67

*Number of samples used to calculate the mean per day was 6 (3 samples from each spike level) Abbreviations: ER = extraction recovery; 3-OMG = 3-O-methylglucose.

Table 5
Mean and intermediate precision of intestinal permeability (L:R) and absorptive capacity (X:G).

Measure	Spike Level	Mean	RSD
L:R	Low	1.1	4.4
	High	1.0	0.7
	Total	1.1	5.2
X:G	Low	1.1	2.8
	High	1.1	3.2
	Total	1.1	2.9

Abbreviations: L:R = lactulose: rhamnose; X:G = xylose: 3-O-methylglucose.

Recovery was calculated based on the concentration measured in the spiked sample (C_s), blank sample (C_b), and the theoretical known concentration (C_a). C_s and C_b were calculated as described in section 2.7, and the extraction recovery percentage (ER%) was then estimated by equation (4):

$$ER\% = \left(\frac{C_s - C_b}{C_a}\right) * 100 \quad (4)$$

4. Analysis of experimental samples

The concentrations of the oral-dosed sugars measured in plasma, normalised by dose administered to each cat, are displayed in Table 6. Equations used to calculate L:R and X:G ratios are shown in equations (4) and (5), respectively, using the concentration of the sugar quantified in the plasma and the amount of the sugar ingested by each cat in the sugar solution. Concentrations of analytes reported in the samples were adjusted based on the recoveries presented in Table 4. The concentration

Table 6
Analyte concentration of real samples.

Cat ID	Dose (mL)	Lactulose (μg/mL)	Rhamnose (μg/mL)	Intestinal Permeability (L:R)	Xylose (μg/mL)	3-OMG (μg/mL)	Absorptive Capacity (X:G)
1	10.2	8.42	3.92	0.61	53.87	26.09	0.59
2	9.8	31.36	14.97	0.60	83.09	34.99	0.68
3	7.8	7.08	3.64	0.56	48.87	27.40	0.51
4	8.7	56.76	23.05	0.70	86.61	46.06	0.54
5	7.7	35.69	14.42	0.71	75.30	35.87	0.60
6	9.2	8.65	5.06	0.49	48.61	27.03	0.51
7	8.9	17.64	6.43	0.78	47.21	25.31	0.53
8	9.1	22.97	16.18	0.41	92.35	49.29	0.54
9	6.7	5.54	3.79	0.42	42.50	17.53	0.69
10	8.6	17.26	11.05	0.45	51.96	30.70	0.48
11	8.5	10.32	4.41	0.67	51.67	27.22	0.54
12	8.3	26.28	11.20	0.67	59.97	32.66	0.52
13	6.8	15.06	11.44	0.38	82.89	42.43	0.56

Abbreviations: L:R = lactulose: rhamnose; 3-OMG = 3-O-methylglucose; X:G = xylose: 3-O-methylglucose.

of all analytes in samples exceeded their respective LOQ and were within the calibration range (0 – 100 μg/mL), deeming this method fit for purpose. Urinary ratios have previously been observed as being directly comparable to plasma and serum ratios [10,21], but have never been compared in the domestic cat. The intestinal permeability values, the lactulose to rhamnose ratio, obtained in this study showed higher permeability than previously documented urinary ratios. Earlier reported values varied between 0.03 and 0.53, with a median of approximately 0.44, which is lower than the present range of 0.38 – 0.78, with a median of 0.60 [11,15–18]. The absorptive capacity values, the xylose to 3-OMG ratio, range from 0.48 to 0.69 in the present study, with a median of 0.50, which is lower than the only other reported xylose to 3-OMG ratio of 0.70 in a urine-based study by Johnston *et al.* (2001). The values obtained in this study could be different from the literature due to a variety of factors, including but not limited to, age, environment, neuter status, genetics, diet, and osmolarity of solution.

$$L:R = \frac{(LactuloseRecovered)}{(RhamnoseRecovered)} = \frac{\left(\frac{LactuloseConcentration}{LactuloseIngested}\right)}{\left(\frac{RhamnoseConcentration}{RhamnoseIngested}\right)} \quad (4)$$

$$X:G = \frac{(XyloseRecovered)}{(3-OMGRecovered)} = \frac{\left(\frac{XyloseConcentration}{XyloseIngested}\right)}{\left(\frac{3-OMGConcentration}{3-OMGIngested}\right)} \quad (5)$$

5. Discussion

The aim of this study was to develop a LC-MS/MS method for quantifying the intestinal permeability and absorptive capacity of cats using the simultaneous analysis of oral-dosed sugars in plasma 3 h after dosing. Lactulose, rhamnose, xylose, and 3-OMG were all detected in plasma 3 h following a single oral dose, indicating this method is highly suitable for future use examining impacts on intestinal permeability and absorptive capacity from factors such as diet and ageing in the domestic cat.

The extraction process was quick, and simple to complete. The use of stable isotope standards for each target sugar ensured high quantitative accuracy and precision [33]. The selectivity of the high-resolution mass spectrometry and PRM and MS/MS fragmentation patterns provided the high degree of sensitivity and selectivity required to work in plasma as opposed to urine as is commonly used. Previous studies evaluating sugar probe concentrations in canine plasma used gas chromatography-mass spectrometry (GC–MS) due to its increased sensitivity [13,21]. However, sugars are non-volatile compounds and require chemical derivatisation to be measured using GC–MS. This process not only adds complexity to the sample preparation, but may also cause unstable or incomplete derivatisation in the case of some compounds, generating multiple sugar peaks due to the presence of their isomers [34] and therefore incorrect concentration calculations. Derivatisation steps are often characterised for variability, which can be influenced by many factors, for example, by moisture [34]. To overcome the challenges linked to GC–MS, the initial testing of six cats was completed to verify that the samples could be quantified within the detection limits of LC-MS.

Cats were fasted for 12 h before the baseline venepuncture and administration of the oral sugar solution. While the fasting method clearly demonstrated no other sugars present at detectable levels in the plasma that could act as interfering peaks, if this was an issue then utilising MS/MS would help to differentiate between isobaric compounds based on their fragmentation patterns. The baseline samples produced no peaks within the retention times that were tested, which was not surprising since the cats were fasted. This also confirmed that these sugars are not endogenously produced at detectable levels within the cat.

The dose of the sugar solution made of lactulose, rhamnose, xylose, and 3-OMG was extrapolated from previous studies in cats and dogs [8,11,13,19,21,27,28]. The sugar solution had an osmolality of 1160 mOsm, which is under the threshold of 1500 mOsm when hypertonic solutions begin to increase permeability [29]. The final blood samples were collected from the cats 3 h after oral dosing based on findings from multiple studies that blood collected at this time is the most consistent between samples [13,21,30].

Diarrhoea is a potential issue when consuming high levels of sugar, however this was not observed in any of the cats after ingestion of the hyperosmolar sugar solution. The sugar solution made in the current study was made to be hyperosmolar for multiple reasons. Firstly, the solution needed to be given in a dose that could be easily administered to the cats and therefore, needed to be concentrated to lower the volume of solution being orally syringed. Secondly, a previous study in dogs used a 1560 mOsm solution of lactulose and mannitol to successfully determine intestinal permeability [24], comparable to the dose used in the current study. Lastly, hyperosmolar solutions have been said to provide a better discrimination between a healthy and a damaged small intestinal mucosal barrier [35–38]. The reasoning behind this increase in lactulose permeation is unknown, however authors have speculated that a hyperosmolar solution may influence paracellular transport via tight junctions or cause solvent drag [35,36]. Although the dose used in this study did not reach the level of hyperosmolarity proposed to increase permeation of lactulose, the use of a hyperosmolar solution as opposed to an *iso*-osmolar one used in previous literature with cats may explain the higher lactulose recovery rates in comparison to previous urinary studies [11,15–18]. The dose may therefore pose a challenge for directly comparing results to previous trials measuring feline intestinal permeability, but it paves the way for exciting opportunities and progress in future research within this area. This new LC-MS/MS method should be applied to a larger sample size which will help in determining a

reference range for lactulose to rhamnose and xylose to 3-OMG ratio when measured in plasma using this dose.

6. Conclusions

The LC-MS/MS method developed here for measuring sugars used in intestinal permeability assays was shown to be sensitive, reliable, and reproducible for a small volume of plasma. It was successful in its ability to be minimally invasive, fast, selective, and sensitive for the accurate and precise quantification of four different test sugars in plasma using stable isotope internal standards. The development and application of this method is the first reported quantitative analysis of these sugars used for permeability assays in feline plasma. It proves to be a potential tool to analyse intestinal permeability and absorptive capacity in cats.

Funding

This work was supported by the New Zealand Ministry of Business, Innovation and Employment (MBIE) through the Strategic Science Investment Fund (AgResearch contract A27685: Intestinal function in aging cats) and Massey University.

Data availability statement

The data that support the findings of this study are available from the corresponding author, KP, upon reasonable request.

CRediT authorship contribution statement

Keely Patterson: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Karl Fraser:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Daniel Bernstein:** Writing – review & editing, Validation, Methodology, Data curation. **Emma N. Bermingham:** Writing – review & editing, Supervision, Project administration, Methodology, Conceptualization. **Karin Weidgraaf:** Resources, Investigation. **Anna Kate Shoveller:** Writing – review & editing, Supervision, Project administration, Conceptualization. **David Thomas:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

We wish to thank Massey University Centre for Feline nutrition staff Dr. Margreet Hekman and Ms. Rachael Richardson for their assistance with blood collection, and senior technician, Milson Francis (AgResearch Ltd.) for his assistance sourcing and ordering all the sugars needed for this study.

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