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Development and validation of a UPLC-MS/MS method for the quantification of sugars and non-nutritive sweeteners in human urine

Marlies Diepeveen-de Bruin [1], Walid Maho [1], Marion E.C. Buso, Novita D. Naomi, Elske M. Brouwer-Brolsma, Edith J.M. Feskens, Michiel G.J. Balvers [*]

Division of Human Nutrition and Health, Wageningen University & Research, P.O. Box 17, 6700 AA Wageningen, the Netherlands

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ABSTRACT

High-intensity sweeteners ('sweeteners'), such as sucralose, saccharine, acesulfame, cyclamate and steviol, are replacing sugars in many food products, but biomarker-based data on their population-wide exposure, as well as analytical methods that can quantify urinary concentrations of sugars and sweeteners simultaneously, are lacking. Here, we developed and validated an ultra-pressure liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) method to quantify glucose, sucrose, fructose, sucralose, saccharine, acesulfame, cyclamate and steviol glucuronide in human urine. Urine samples were prepared by a simple dilution step containing the internal standards in water and methanol. Separation was achieved on a Shodex Asahipak NH2P-40 hydrophilic interaction liquid chromatography (HILIC) column using gradient elution. The analytes were detected using electrospray ionization in negative ion mode, and selective reaction monitoring was optimized using the [M−H]- ions. Calibration curves ranged between 34 and 19,230 ng/mL for glucose and fructose, and 1.8 to 1,026 ng/mL for sucrose and the sweeteners. The method has acceptable accuracy and precision, which depends on the application of appropriate internal standards. Storage of urine samples in lithium monophosphate gives the best overall analytical performance, and storage at room temperature without any preservatives should be avoided since this leads to reduced glucose and fructose concentrations. With the exception of fructose, all analytes were stable throughout 3 freeze–thaw cycles. The validated method was applied to human urine samples, demonstrating quantifiable concentrations of the analytes which were in the expected range. It is concluded that the method has acceptable performance to quantitatively determine dietary sugars and sweeteners in human urine.

1. Introduction

Sugars, including the monosaccharides glucose and fructose and the disaccharide sucrose, are naturally present in fruits and vegetables and commonly added to processed foods and drinks. Although sugars are important energy substrates for the human body, overconsumption is harmful and an important risk factor for the development of obesity and type 2 diabetes [1–3]. Recent efforts by the industry to reduce the intake of sugars while maintaining the taste of food products have led to the replacement of sugars by so-called 'sweet taste enhancers' or 'sweeteners' [4,5]. Various sweeteners are commonly found in food products, such as sucralose, saccharine, cyclamate, acesulfame K and steviol. Although these sweet taste enhancers are generally considered safe for human consumption from the toxicological viewpoint, epidemiological data on their long-term health effects is still limited [6,7]. To gain more insight in the potential impact of this shift in consuming sweet taste

Abbreviations: ACN, acetonitrile; CID, collision-induced dissociation; ESI, electrospray ionization; GC, gas chromatography; HILIC, hydrophilic interaction liquid chromatography; LC-MS, liquid chromatography coupled to mass spectrometry; LLOQ, lower limit of quantification; LOD, limit of detection; *m/z*, mass-to-charge ratio; MeOH, methanol; MQ, milli-Q water; S/N, signal-to-noise; SRM, selective reaction monitoring; UPLC-MS/MS, ultra-pressure liquid chromatography coupled to tandem mass spectrometry.

* Corresponding author at: Division of Human Nutrition and Health, Wageningen University & Research, P.O. Box 17, 6700 AA, Wageningen, the Netherlands.
E-mail addresses: marlies.diepeveen-debruin@wur.nl (M. Diepeveen-de Bruin), walid.maho@wur.nl (W. Maho), marion.buso@wur.nl (M.E.C. Buso), novita.naomi@wur.nl (N.D. Naomi), elske.brouwer-brolsma@wur.nl (E.M. Brouwer-Brolsma), edith.feskens@wur.nl (E.J.M. Feskens), michiel.balvers@wur.nl (M.G.J. Balvers).
[1] both authors contributed equally.

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enhancers instead of sugars, further studies monitoring long-term health effects of dietary sugars and sweeteners are of utmost importance. This is particularly the case for the above-mentioned sweeteners, which are known to be commonly consumed [5,8]. However, monitoring exposure is challenging. Commonly used research tools to characterize dietary intake, such as food frequency questionnaires and food diaries, have certain limitations [9]. These self-reported methods depend on the memory recall of the participant, and the participant may (un)intentionally give inaccurate answers. In addition, self-reported methods depend on the quality of food composition databases, which do not always contain a full characterization of sweeteners for all commonly consumed food products. Therefore, alternative approaches to assess the exposure to dietary sugars and sweeteners are urgently needed. Biomarkers of nutritional status may offer a more objective measure of exposure to sugars and sweeteners [9]. Previously, urinary sucrose and fructose excretion were investigated for their potential to act as predictive biomarkers for sugar intake [10], and this has also been investigated for sweeteners [11]. The rationale behind this is that a (fixed) proportion of the ingested amount of sugars and/or sweeteners is excreted in the urine, and therefore urinary excretion serves as a proxy for dietary intake. This may apply to sucralose, saccharine, cyclamate, acesulfame K and steviol, which can be excreted via the urine [11]. However, not all sweeteners are excreted via the urine. For instance, aspartame is fully metabolized into methanol and the amino acids aspartic acid and phenylalanine, and will therefore not be excreted in the urine at normal consumption levels, so a urinary biomarker approach may not be applicable to all sweeteners [5].

Several methods are available to determine urinary sugars, such as enzyme assays [12] or methods that use gas chromatography (GC) [13]. These methods require derivatization (with GC) or chemical reactions (enzyme assays), which can be laborious and could limit specificity. With liquid chromatography coupled to mass spectrometry (LC-MS), it is possible to detect sugars and sweeteners without derivatization or chemical reactions, which could increase the sensitivity and specificity of the test. Already several LC-MS based methods have been reported for sugars [14–18], but to date only one method for sweeteners in urine is reported in the literature [19]. In addition, currently no method is published that allows for the simultaneous quantification of a wider panel of sugars and sweeteners using a single analytical method. Such a more comprehensive method would be preferable over methods that detect single (groups of) analytes, since it would reduce the amount of sample that is required while increasing the amount of data from it. Since sugars such as fructose and sucrose are increasingly replaced by sweeteners, it is particularly interesting to analyze both classes of compounds from a single sample, as well as to allow comparison to previously published studies that validated these sugars as predictive biomarkers of intake [9,10]. Analyzing sugars and sweeteners in urine is challenging. On column separation of glucose and fructose is required since they have the same precursor ion mass and fragmentation pattern. Separation of saccharides is typically performed using hydrophilic interaction liquid chromatography (HILIC), which is known to be sensitive for disturbances in sample pH or water content [20]. The sweeteners contain elements that are normally not present in natural saccharides, such as nitrogen and sulphur (e.g. acesulfame K, saccharin, cyclamate) or chlorine (e.g. sucralose), or have a molecular structure that is not typical for mono- and disaccharides (e.g. steviol glucuronide). In addition, depending on the level of dietary intake, the concentrations of the sugars and sweeteners in urine may be in the ng/mL range, which demands for sensitive analytical equipment. Moreover, as urine is one of the primary excretion routes of the body, it also contains many polar metabolites that could potentially interfere with the analysis.

The current paper presents the method development and validation of an ultra-pressure liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) based method for the simultaneous quantification of glucose, fructose, sucrose, acesulfame K, cyclamate, saccharine, steviol glucuronide and sucralose in human urine.

2. Materials and methods

2.1. Chemicals, materials, and reagents

Standards for glucose, fructose, sucrose and saccharin, and the internal standard 13C6-glucose, were obtained from Sigma-Aldrich (Munich, Germany). Sodium cyclamate was from Acros Organics (Geel, Belgium), and acesulfame potassium was from Supelco (Merck, Amsterdam, the Netherlands). Steviol acyl glucuronide and the internal standards saccharin-d4, sucralose-d6 and 13C6-sucrose were obtained from Toronto Research Chemicals (Toronto, Canada). Sodium cyclamate-d11 was from CDN Isotopes (Quebec, Canada).

Acetonitrile (ACN), methanol (MeOH), and water (all ULC/MS grade) were obtained from Biosolve (Valkenswaard, the Netherlands). Ammonium acetate was obtained from Sigma Aldrich and 25% ammonia solution was obtained from Merck. Ascorbic acid, boric acid and lithium monophosphate were from Sigma-Aldrich. Urine that was needed for the purpose of method development and validation was obtained from coworkers who volunteered to give urine.

2.2. Preparation of stock solutions and calibration standards

All stocks solution were first prepared by dissolving the standards and internal standards in water at a concentration of ~ 1 mg/mL for acesulfame and steviol acyl glucuronide, or 10 mg/mL for the other analytes. Further dilutions were prepared in 80% ACN – 20% water. The concentration range for the calibration standards was based on previously published work that presented data on daily excretion and/or concentrations of sugars and sweeteners in urine [11,21]. Based on this and the 20x sample dilution during sample preparation (see section 2.3), the calibration curve ranged between 34 and 19,230 ng/mL for glucose and fructose, and 1.8 to 1,026 ng/mL for all other compounds.

2.3. Sample preparation

To 50 μL of human urine, 40 μL of internal standard working solution, and 110 μL ULC/MS grade water containing 10% v/v ammonia was added. Subsequently, 800 μL ULC/MS grade MeOH is added (total final volume 1000 μL; 20x dilution), and the sample was vortexed. The sample was then centrifuged for 1 min at 10,000 rpm. After centrifugation, the supernatant was transferred to a LC vial for analysis.

2.4. UPLC-MS/MS analysis of urine extracts

The extracts were analyzed on an Acquity H-class Plus UPLC coupled to a Xevo TQ-S micro tandem mass spectrometer (Waters Chromatography Europe BV, Etten-Leur, the Netherlands). Chromatography was performed on a Shodex Asahipak NH2P-40 2D column (Showa Denko Europe GmbH, Munich, Germany; 150 × 2.0 mm, 4 μm). The column temperature was set at 55 °C, the samples were kept at 5 °C, and 2 μL extract was injected on column. LC separation was performed using ULC/MS grade water containing 1.0 mM ammonium acetate and 0.1% ammonia (eluent A) and 100% ACN (eluent B). The following gradient was applied with a flow of 0.25 mL/min: 0 – 8 min, 80% B; 8 – 8.1 min, 50% B; 8.1 – 12 min, 50% B; 12 – 12.1 min, 80% B; 12.1 – 20 min, 80% B. The needle wash solution was 80% ACN – 20% MQ water. Electrospray ionization in negative ion mode (ESI-neg) was used for all analytes and internal standards. The MS parameters, such as capillary voltage and collision-induced dissociation settings (CID), were tuned by individually infusing all analytes to optimize product ion signal intensity in selective reaction mode (SRM). The MS was operated in with the following settings: capillary voltage 2 kV, cone voltage 10 V, desolvation temperature 600 °C, source temperature 150 °C, desolvation gas flow 700 L/hr, cone gas flow 120 L/hr. The SRM settings are presented in Table 1. Data acquisition and processing was performed using MassLynx version 4.2 SCN 1001 (Waters).

Table 1
m/z values for precursor and product ions, collision energies and retention times for the analytes and their internal standards. * cyclamate uses acesulfame-d4 as internal standard; ** fructose uses glucose-d6 as internal standard; *** steviol acyl glucuronide uses saccharine-d4 as internal standard.

Analyte	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (V)	Retention time (min)
Acesulfame	162	82	20	5.6
Acesulfame-d4	166	86	20	5.6
Cyclamate *	178	80	25	6.3
Glucose	179	89	10	3.8
Fructose **	179	89	10	3.0
Glucose-d6	185	92	10	3.8
Sucrose	341	89	18	5.4
Sucrose-13C6	347	92	20	5.4
Sucralose	395	35	20	2.1
Sucralose-d6	401	35	20	2.1
Saccharine	182	42	25	7.2
Saccharine-d4	186	42	25	7.2
Steviol acyl glucuronide***	493	317	25	9.4

2.5. Method validation

2.5.1. Calibration, linearity, sensitivity and carry-over

Quantification was performed against a 7-point calibration curve (see section 2.2) which was generated using regression analysis with $1/x^2$ weighing. The lower limit of quantification (LLOQ) and limit of detection (LOD) were determined from spiked human urine samples. The LLOQ was calculated as the extract concentration that would give a peak with a signal-to-noise (S/N) ratio of 10 in human urine extracts, and for the LOD this was the extract concentration that would give a peak with S/N = 3. This approach was preferred over basing LLOQ and LOD based on injections from standard solutions, which contain less noise and would overestimate sensitivity in urine samples. Carry-over was determined by injecting a blank sample after the highest calibrator. Carry-over was found acceptable if the peak area in the blank was $\leq$ 0.5% of the peak of the highest calibrator.

2.5.2. Matrix effect, accuracy and precision

Matrix effect was determined by comparing the internal standard areas from (pre-spiked) extracted human urine samples to calibration standards. This approach was deemed equivalent to the typical approach to work with post-spiked samples, since the sample extraction procedure only consisted of a dilution and centrifugation step and did not include further steps (such as solid phase extraction or sample drying) where it can be expected that analytes are lost. The between-day accuracy and precision were determined by spiking known amounts to pooled human urine. Urine was spiked at the three levels (n = 5 per level per day, for 3 days) that covered the range of the calibration curve. Glucose and fructose were spiked at extract concentrations of 75, 615 and 6,250 ng/mL, and the other analytes were spiked at 4, 33 and 333 ng/mL. These values were chosen based on previous literature reports that presented data on urinary excretion of the target analytes [19,21]. In addition, unspiked urine samples were also analyzed (n = 5 per day, for 3 days) to determine the endogenous presence of the target analytes. Each analytical batch contained the validation samples for that day and a duplicate calibration curve. The endogenous concentrations of the target analytes in the unspiked samples were used to determine the theoretical target values of the spiked sample to be able to calculate accuracy and precision. Accuracy was calculated as [(measured concentration in spiked urine sample)/(measured endogenous concentration in unspiked sample + spiked concentration) *100%]. Precision was calculated as [RSD = SD/mean *100%]. Accuracy and precision were calculated for each day separately (within-day) and from this the average was calculated as between-day accuracy and precision. Accuracies between 85 and 115% and precision < 15% were generally considered acceptable.

2.5.3. Effect of preservatives & stability

Human urine is typically collected, processed and stored in the presence of anti-microbial agents. It was therefore investigated whether commonly used preservatives, such as lithium monophosphate (8.3 g/L), boric acid (1.5 g/L) and ascorbic acid (0.65 g/L), or their combination, would affect the performance of the method. Urine containing the above-mentioned preservatives was spiked (615 ng/mL for glucose and fructose, 33 ng/mL for the other analytes; n = 3) and the accuracy and precision were evaluated. In addition, the effect of storage temperature and time was evaluated. The analytes were spiked to pooled urine or water (n = 3), and were stored for 24 h or 7 days at either RT, 4–8 °C or −80 °C. Comparison was made against fresh samples that were immediately extracted. Finally, the effect of (repeated) freeze–thaw cycles was investigated by subjecting spiked urine samples to 1, 2 or 3 freeze–thaw cycles at −80 °C. Accuracies between 85 and 115% were generally considered acceptable.

2.6. Analysis of urinary sugars and sweeteners in human urine

To demonstrate the suitability of the method and to explore the concentrations of the analytes in urine, 24-hour urine samples were collected from 20 volunteers from an existing human study, the Nutrition Questionnaires Plus (NQPlus) Study [22]. In this study, participants completed food frequency questionnaires (FFQ) and 24-hr urine samples were collected. The FFQ data was used to select the 20 participants from which urine samples were analyzed; 15 participants with the highest self-reported consumption of low-calorie beverages, and 5 participants with no self-reported consumption. Urine was stored in the presence of 8.3 g/L lithium monophosphate and stored at −80 °C until analysis. This study was previously approved by the medical ethical committee of Wageningen University (registration number NL34775.081.10) and conducted according to the principles of the Declaration of Helsinki. All participants provided their written informed consent.

3. Results and discussion

3.1. Method development and optimization

Various HILIC columns were evaluated for their ability to achieve separation with acceptable peak shapes for the analytes. The best results were obtained with the Shodex Asahipak column, which was the only column that showed separation and promising peak shapes using isocratic elution with 20% MQ – 80% ACN with 0.1% ammonia, which was in accordance with information provided by the column manufacturer. It is common for HILIC methods to depend on additional buffers, such as ammonium acetate or ammonium formate, to achieve separation [20]. Based on manufacturer recommendations, we initially continued with the 20% MQ – 80% ACN − 0.1% ammonia solution, since this would reduce preparation steps when making LC solutions. However, the isocratic elution yielded unacceptable results with urine extracts, giving poor peak shapes and inconsistent retention times. Gradient elution was also explored but this required long (e.g. $\geq$ 30 min) pre-run equilibration times to achieve stable retention times. Thus, the addition of 1–10 mM of ammonium acetate to the eluent was explored. The addition of 1.0 mM ammonium acetate was critical to achieve acceptable separation and peak shapes of the analytes in urine extracts. The retention times of the analytes in urine extracts however showed variability with isocratic elution, which improved when using gradient elution (see section 2.4 for final protocol), and which further improved when ammonia was added to the sample extraction solvent. The column temperature was set at 55 °C to keep the pressure below the specified maximum column pressure of 120 bar with the optimized gradient elution method. See Fig. 1A for SRM chromatograms from standards and Fig. 1B. for SRM chromatograms from human urine..

Next, different sample dilution factors and solvents were compared for their ability to dilute any interferences while maintaining extract

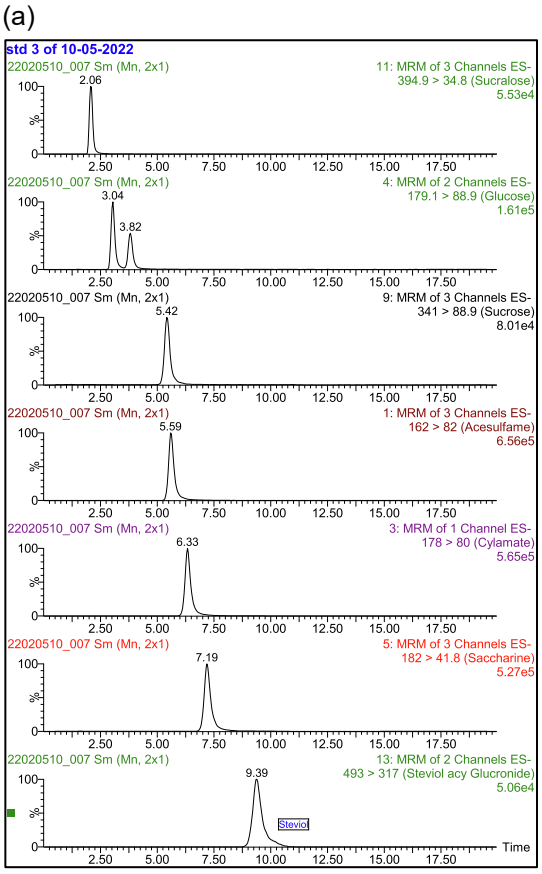

Fig. 1A. SRM chromatograms of standard solution mixture containing glucose and fructose at 1,923 ng/ml, and sucralose, sucrose, acesulfame, cyclamate, saccharin and steviol glucuronide at 103 ng/ml.

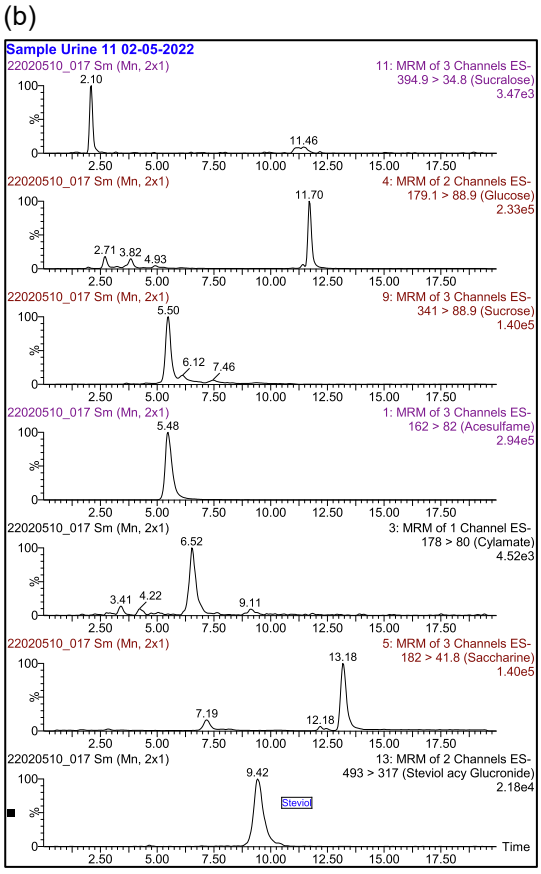

Fig. 1B. SRM chromatograms of the target analytes in a pooled, non-spiked urine sample.

concentrations that fall in the detection range of the instrument as well as maintaining acceptable peak shape and chromatographic separation. Optimal reproducibility for retention time and analyte quantification were obtained when the samples were diluted 20-fold with MQ water (containing the internal standard) and MeOH with ammonia to a final concentration of 80% MeOH (data not shown). ACN also gave reproducible retention times, but showed a higher degree of variation when quantifying concentrations of analytes in replicate samples. Therefore, MeOH was preferred for the final sample extraction method.

Optimization of the MS parameters was performed to maximize signal intensities for precursor and product ions. The optimization yielded precursor ions with *m/z* ratios that corresponded with the [M−H]- ions, which was in agreement with the ESI-neg ionization mode (see Table 1 for precursor and product ion *m/z* values, collision energies and retention times). With the optimized MS settings and sample preparation, the method was sufficiently robust and sensitive to detect endogenous sugars and sweeteners as [M−H]- ions in human urine in the ng/mL range. This has also been reported by others for the sweeteners or for sucrose [19,21]. Metal adduct formation has been observed in positive ion mode mass spectrometric analyses of carbohydrates, which has an impact on ionization and MS detection [23]. For instance, sodium adducts have been reported even when no sodium has been added to LC solutions [24]. Sodium adducts may be undesirable if the signal may be split between e.g. protonated and sodiated ions, and therefore other metal salts, such as cesium [14,25] or lithium [26] salts, may be added to improve robustness and sensitivity in positive ion mode ESI. The present method was capable of sensitive and robust analysis without adduct detection. It is preferable to detect the native (de-) protonated ions because it simplifies the interpretation of MS data and reduces the amount of critical elements (e.g. addition of salts in the eluent) of the method.

We aimed to implement deuterated fructose as an internal standard, which would likely have improved the accuracy and precision for fructose. However, we found that the internal standard had significant amounts of impurities that caused peaks in the SRM chromatograms of other analytes. A newly ordered fructose stock revealed the same interferences. Therefore, it was decided to use glucose-d6 as the internal standard for fructose. In the absence of a commercially available deuterated internal standard for steviol acyl glucuronide, it was decided to use saccharine-d4 as its internal standard since these compounds had nearly similar retention times. For cyclamate, acesulfame-d4 was used as the internal standard for the same reason.

3.2. Method validation and performance

3.2.1. Standard curves, sensitivity and carry-over

Calibration curves were made fresh daily for 3 different days from duplicate analysis of calibrators using regression analysis with $1/x^2$ weighing. The calibration range for glucose and fructose ranged from 34 to 19,230 ng/mL, and ranged between 1.8 and 1,026 ng/mL for all other analytes. The r^2 values from these calibration curves were between 0.967 and 0.999, with deviations between actual and calculated concentrations typically $< 20\%$. Values for the LOD and LLOQ in the urine matrix are presented in Table 2. The linearity and sensitivity of our method are comparable or an improvement over previously published methods. For instance, Abreu et al. reported a quantification range for sucrose from 34 ng/mL using LCMS, where our method can quantify sucrose in urine extracts at 3.6 ng/mL [15]. Similarly, our method is far more sensitive for quantifying sucrose and fructose compared to enzymatic assays that were previously used, reporting LOD values of 1000 ng/mL [12]. In addition, the linear quantification range for the

Table 2
LOD, LLOQ and matrix effect for the analytes.

Analyte	LOD (ng/mL)	LLOQ (ng/mL)	Matrix effect (%)
Acesulfame	0.07	0.2	73.6
Cyclamate	0.22	0.7	–
Glucose	12.0	40.4	98.5
Fructose	23.0	76.0	–
Sucrose	1.1	3.6	84.2
Sucralose	0.6	1.8	44.4
Saccharine	0.1	0.3	91.2
Steviol acyl glucuronide	0.7	2.3	–

sweeteners in our method starts at 1.8 ng/mL and LLOQs < 2.3 ng/mL, where Logue et al. report a linear quantification range starting from 10 ng/mL [19].

Carry-over was investigated by injecting a blank sample after the highest calibrator. Carry-over for sucrose was 0.13%, and for all other analytes no peaks were detected in the blank sample. Thus, it is concluded that carry-over is negligible.

3.2.2. Matrix effect

Matrix effect was evaluated by comparing internal standard areas from spiked urine samples to clean internal standard solutions. Data is presented in Table 2, with values < 100% indicating that there is a loss of signal in the urine extract compared to the standards. The matrix effect was most pronounced for sucralose (44.4% signal in urine compared to standard solution) and was negligible for glucose (98.5%). The matrix effect is deemed acceptable considering the simple sample preparation, but also indicates that the use of isotope-labeled internal standards is required in order to compensate for differences in ionization. Matrix effect was not evaluated for fructose, steviol glucuronide and cyclamate since their internal standards were not available, and hence they are corrected using glucose-d6, saccharine-d4, and acesulfame-d4 (see section 3.1). Based on the results on accuracy (see section 3.2.3), it is concluded that these internal standards result in accurate results for these analytes and that matrix effect therefore is comparable.

3.2.3. Within- and between-day accuracy and precision

Within- and between-day accuracy and precision were determined by spiking pooled human urine at 3 concentrations on 3 different days. Within-day results are presented in Supplementary Table S1. The between-day results are presented in Table 3.

The between-day accuracies ranged between 85.5% and 112.4%, which is within the widely accepted 85–115% criteria. Most between-day precision values were ≤ 15%, with some values between 15 and 20% and in one instance 24.0% was observed. Precision values of > 15% were only observed for analytes that do not have their own internal standard in the assay (e.g. fructose, steviol glucuronide) or for analytes that were spiked at the lowest concentration level (cyclamate). Unfortunately, there is no deuterated standard available for steviol glucuronide, and also no suitable internal standard for fructose was found (see section 3.1). It is likely that the precision would improve if suitable internal standards are available for steviol glucuronide and fructose, and this should be addressed in future studies. With these limitations in mind, we accept the borderline precision for fructose and steviol glucuronide, and we conclude that the method has acceptable accuracy and reproducibility for glucose, sucrose and the other sweeteners in urine.

3.2.4. Effect of preservatives & stability

To test the effect of commonly used anti-microbial treatments, the effect of storing human urine in the presence of lithium monophosphate, boric acid and ascorbic acid was investigated. The results are displayed in Fig. 2. Glucose, cyclamate, sucrose, acesulfame, saccharine and steviol glucuronide were not affected by any of the preservatives as

Table 3
between-day accuracy and precision. Human urine was spiked at 3 concentration levels in 5-fold prior to extraction and analysis, which was performed on 3 different days.

Analyte	Spiked concentration (ng/mL)	Accuracy (% of target)	Precision (RSD, %)
Acesulfame	4	99.9	2.6
	33	98.4	2.2
	333	96.9	2.6
Cyclamate	4	99.9	18.1
	33	105.0	8.3
	333	112.4	7.0
Glucose	75	96.0	2.1
	615	90.9	1.9
	6250	92.4	3.2
Fructose	75	–	–
	615	86.1	24.0
	6250	85.5	16.0
Sucrose	4	96.5	3.4
	33	96.4	2.8
	333	95.5	5.0
Sucralose	4	103.5	14.8
	33	100.4	7.0
	333	104.1	6.4
Saccharine	4	96.4	3.0
	33	96.7	4.0
	333	96.4	3.1
Steviol acyl glucuronide	4	107.3	5.6
	33	104.7	15.8
	333	103.4	16.1

indicated by accuracies that were between 85% and 104% and precision that was < 10%. For fructose, ascorbic acid led to a accuracy of 125% whereas the other preservatives had deviations < 20% of the expected value. It was observed that the precision for sucralose went up to 18.3% when stored with boric acid, but remained < 15% with lithium monophosphate or ascorbic acid while also having acceptable accuracies of 106.8 and 108.4%, respectively. Similarly, in the presence of the mix of preservatives, the accuracy of sucralose also went up to 118%. It is therefore concluded that storage of human urine with lithium monophosphate (up to 8.3 g/L) does not negatively impact the methods' performance and gives the best overall performance.

To investigate storage stability, the analytes were spiked to urine and water, and the effect of time and storage temperature was evaluated. Storage in water and urine at 4–8 °C or at −80 °C for 1 or 7 days did not lead to marked changes in the concentrations of the analytes, since all values were between 85% and 116.5% of the expected concentrations. This is in agreement with other data on sweeteners in urine stored with boric acid [19]. However, when stored in urine at room temperature, reductions to < 10% of the expected values were observed for glucose and fructose. Issues with stability of sugars in urine kept at room temperature have been reported before, with one report indicating acceptable stability when urine was kept at room temperature while using boric acid, and with stable values for fructose and sucrose when samples are stored for up to 1.5 years at −20 °C in the presence of boric acid [21]. From these results, it can be concluded that the analytes are stable in urine when stored at 4–8 °C or −80 °C, whereas glucose and fructose are unstable when stored at room temperature without the presence of antimicrobial treatment. Therefore, storage of urine at room temperature without antimicrobial treatment should be avoided. Based on our results and the work of others, it is recommended to collect urine samples in the presence of lithium monophosphate, which ensure the stability of the analytes even when urine is kept at room temperature. This is relevant in research settings where participants are instructed to collect and store 24-hour urine, which is potentially performed under sub-optimal preanalytical conditions. Future studies should characterize the long-term stability of sweeteners in urine under various conditions.

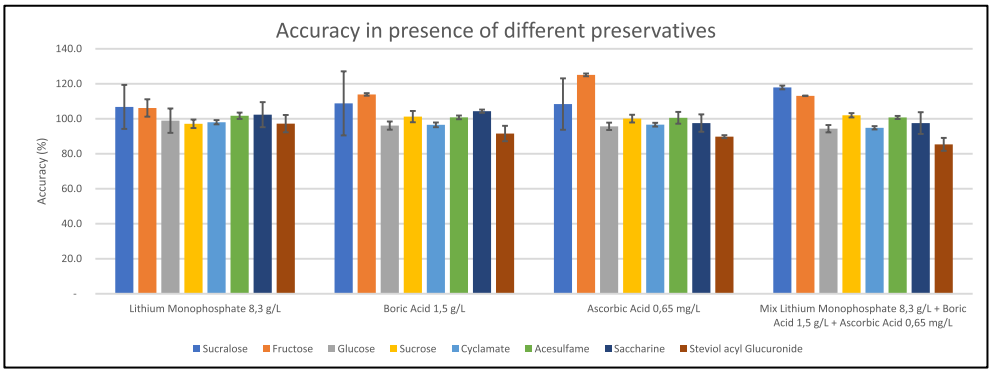

Fig. 2. the effect of anti-microbial treatments on the method performance. Urine was stored with lithium monophosphate, boric acid, ascorbic acid or their mix, and was subsequently spiked with the analytes prior to sample analysis.

The effect of repeated freeze–thaw cycles was investigated by using spiked urine samples. Either 1, 2 or 3 freeze–thaw cycles led to a minor increase of fructose in the sample as indicated by an accuracy between 121.5 and 121.9 % compared to the expected value. A report from others concluded that fructose in urine was stable after freeze-thawing when spiked at 50 μmol/L, which is equivalent to 9,000 ng/mL [14]. Here, we spiked at 615 ng/mL, which may explain the difference. For all other analytes, no effect of freeze–thaw cycles was observed as indicated by accuracies that ranged between 85 and 115%. It is therefore concluded that with the exception for fructose, the analytes are stable for up to 3 freeze–thaw cycles.

3.3. Application of the method to human urine samples

Urine samples from 20 volunteers were analyzed to demonstrate the suitability of the method as well as to explore the concentrations of the sugars and sweeteners in human urine samples from participants with no to very high self-reported intakes of low-calorie beverages. Data is presented in Table 4. The results are corrected for sample preparation dilution, thus representing true urine concentrations. In addition, the analyte concentrations were multiplied with the 24-hour urine volume, thus giving the total daily excretion of the analytes. To the best of our knowledge, this is the first report to present both urinary concentrations as well as total daily excretion values for sugars and sweeteners. It is important to know the urinary concentrations, since the volume of 24-hour urine samples can show considerable variation, from as low as < 1 L to values in excess of 3 L. The total volume will have effect on the ultimate urinary concentration, which will subsequently have an impact on the required analytical procedures and sensitivity to quantify the analytes. Sucrose was detected in all samples, and glucose and fructose could not be detected in 2 out of the 20 samples. All concentrations of sugars were in the calibrated range. The sweeteners were less frequently found in the samples. Cyclamate and sucralose were detected in 12 out of 20 samples, acesulfame and saccharine in 15 out of 20 samples, and steviol glucuronide was only detected in 6 out of 20 samples. The concentrations of sucralose and steviol acyl glucuronide were lower compared to the other analytes. This is in line with expectations, since sucralose has limited absorption from the gastro-intestinal tract, leading to lower bioavailability and urinary excretion [27]. The reported analyte concentrations show considerable variation, which was particularly observed for the sweeteners, where values ranged from undetectable to the μg/mL range. This wide range of concentrations and excretion rates was also expected given the selection of the samples, which was from participants with highest or no self-reported consumption of sweeteners. In line with this, the total amount of daily excreted amounts in the 24-hour urine samples also showed variation, with values of < 5 mg/day for sucralose and steviol glucuronide, and values in excess of 300 mg/day for fructose. This is in agreement with previously published literature showing e.g. sucrose excretion up to 100 mg/day [21]. The excreted daily amounts of sucralose and steviol glucuronide were lower compared to the other sweeteners, which has also been reported by others [11]. Occasionally, urinary extract concentrations exceeded the upper limit of the calibration curve, which were extrapolated here. This was for instance observed for one of the 12 cyclamate values, and two of the 20 sucrose values. The majority of samples were however within the calibrated range or below detection limits of the assay. Higher sample dilution factors or a higher calibrator range may be required if high urinary concentrations are expected.

Table 4
concentrations and excreted amounts of sugars and sweeteners in 24-hour human urine samples. Twenty samples were analyzed using the analytical method. The column 'Occurence' indicates in how many out of the 20 samples the analytes were detected, 'Concentration range' presents the concentrations as found in human urine, and 'excreted amount range' presents the total excreted amount of analyte in the 24-hour urine sample. N.D. = not detected.

Analyte	Occurrence	Concentration range (ng/mL)	Excreted amount range (mg/day)
Acesulfame	15/20	N.D. – 40,340	N.D. – 89.6
Cyclamate	12/20	N.D. – 43,220	N.D. – 133.4
Glucose	18/20	N.D. – 42,900	N.D. – 113.6
Fructose	18/20	N.D. – 95,440	N.D. – 369.4
Sucrose	20/20	120 – 95,520	0.4 – 245.3
Sucralose	12/20	N.D. – 3,230	N.D. – 4.9
Saccharine	15/20	N.D. – 10,360	N.D. – 28.0
Steviol acyl glucuronide	6/20	N.D. – 2,120	N.D. – 3.2

4. Conclusion

A new UPLC-MS/MS based method for the quantification of sugars (glucose, fructose and sucrose) and sweeteners (sucralose, saccharine, acesulfame, cyclamate and steviol glucuronide) has been developed and successfully validated. The method has sufficient sensitivity and is accurate and precise. In addition, the method is robust, showing compatibility with various preserving agents that are recommended to stabilize the analytes during collection and storage. The method was capable of quantifying the target analytes in a set of real-life 24-hour urine samples, where concentrations as low as in the ng/mL range could be adequately quantified. With this method, we were able to demonstrate that the majority of samples contain detectable concentrations of sweeteners. We conclude that the validated method is well suited to quantify the concentrations of sugars and sweeteners in human urine.

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CRediT authorship contribution statement

Marlies Diepeveen-de Bruin: Investigation, Methodology, Validation, Writing – review & editing. **Walid Maho:** Investigation, Methodology, Validation, Writing – review & editing. **Marion E.C. Buso:** Conceptualization, Investigation, Writing – review & editing. **Novita D. Naomi:** Conceptualization, Investigation, Writing – review & editing. **Elske M. Brouwer-Brolsma:** Conceptualization, Project administration, Writing – review & editing. **Edith J.M. Feskens:** Conceptualization, Funding acquisition, Project administration, Writing – review & editing. **Michiel G.J. Balvers:** Conceptualization, Methodology, Validation, Writing – original draft.

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 (Marlies Diepeveen-de Bruin, Walid Maho, Marion E.C. Buso, Novita D. Naomi, Elske M. Brouwer-Brolsma, Michiel G.J. Balvers).

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Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2023.123741.

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