Received: 29 June 2021 | Revised: 20 January 2022 | Accepted: 24 January 2022

DOI: 10.1002/bmc.5350

RESEARCH ARTICLE

Biomedical Chromatography WILEY

Development and validation of an LC–MS/MS method for the quantification of artificial sweeteners in human matrices

Eva Greibe[1] | Magnus Leth-Møller[2,3] | Sofie Stampe[2,3] | Per Ovesen[3] | Michael Pedersen[2] | Elke Hoffmann-Lücke[1]

[1]Department of Clinical Biochemistry, Aarhus University Hospital, and Institute for Clinical Medicine, Health, Aarhus University, Denmark

[2]Comparative Medicine Laboratory, Aarhus University, Denmark

[3]Department of Gynecology and Obstetrics, Aarhus University Hospital, Steno Diabetes Centre Aarhus, and Institute for Clinical Medicine, Health, Aarhus University, Denmark

Correspondence
Eva Greibe, Department of Clinical Biochemistry, Aarhus University Hospital, and Institute for Clinical Medicine, Health, Aarhus University, Aarhus, Denmark.
Email: evagreib@rm.dk

Funding information
A.P. Møllers Foundation; Department of Clinical Biochemistry, Aarhus University Hospital; Helga and Peter Kornings Foundation; P. Carl Petersens Foundation; Novo Nordisk Foundation

Abstract

Artificial sweeteners are widely used as substitutes for sugar. The sweeteners are generally considered safe, however their whereabouts during pregnancy and lactation and the effect on child development are poorly explored. There is a need for new tools to measure these substances during pregnancy and lactation. Here, we describe the development and validation of a sensitive liquid chromatography–tandem mass spectrometry method for the simultaneous quantification of acesulfame, cyclamate, saccharin and sucralose in human plasma, umbilical cord blood, amniotic fluid and breast milk. The samples were prepared by protein precipitation and separated on a Luna Omega Polar C_{18} column (2.1 × 50 mm, 1.6 μm). Electrospray ionization in negative mode and multiple reaction monitoring were used to monitor the ion transitions. The validated concentration ranges were from 1 to 500 ng/ml (10–500 ng/ml for sucralose). Interassay precisions were all ≤15% and the accuracies were within ±15%. Stability, linearity, dilution integrity, carryover and recovery were also examined and satisfied the validation criteria. Finally, this analytical method was successfully applied on spiked samples of plasma, umbilical cord blood, amniotic fluid and breast milk, proving its suitability for use in clinical studies on artificial sweeteners, including during pregnancy and lactation.

KEYWORDS
artificial sweetheners, lactation, LC–MS/MS, method validation, pregnancy

1 | INTRODUCTION

Artificial sweeteners are used as a sugar substitute in food and beverages, including baked goods and soft drinks, in pharmaceuticals and even in sanitary products, such as toothpaste (Lange et al., 2012; Schiffman & Rother, 2013). The artificial sweeteners provide no or negligible calories but are much sweeter than sugar (Lange et al., 2012). Their use is becoming more widespread as consumers are increasingly concerned about obesity and dental decay caused by the consumption of natural sugars. In addition, artificial sweeteners are increasingly popular among diabetics as an alternative to sugar (Mejia & Pearlman, 2019). Pregnant women are often encouraged to replace sugary foods with 'light' products containing artificial sweeteners as maternal obesity and diabetes have been linked to childhood obesity. In general, artificial sweeteners are considered safe, but a recent study suggests that the consumption of 'light' products during pregnancy may increase the risk of infant obesity in the first year of life (Azad et al., 2016), and others have found an association with

childhood obesity at the age of 7 years (Zhu et al., 2017). These studies suggest that artificial sweeteners have an impact on the developing fetus, but the underlying mechanism remains unsolved. More studies are needed to understand the transport and accumulation of artificial sweeteners during pregnancy and lactation, and for this purpose there is a need for sensitive methods for the quantification of artificial sweeteners in human sample material related to pregnancy and lactation.

Acesulfame potassium, sodium cyclamate, saccharin and sucralose are all commonly used artificial sweeteners that are approved for use in many countries. Most methods for quantification of artificial sweeteners are designed for industrial use on commercial products or foods (Chang & Yeh, 2014), rather than for direct measurement on human samples. Zhang et al. (2016) presents the development and validation of an LC–MS/MS method for the measurement of acesulfame, cyclamate and saccharin in human blood and urine, and Sylvetsky et al. (2017) reports a study on sucralose in human plasma. Rother et al. (2018) presents a method for the quantification of acesulfame potassium and sucralose in human breast milk. To the best of our knowledge, no studies have previously attempted to develop an LC–MS/MS method for the simultaneous quantification of acesulfame, cyclamate, saccharin and sucralose for use on human material. The identification and quantification of these artificial sweeteners in the mother and fetal compartment are valuable for understanding not only maternal exposure during pregnancy, but also elimination and transport to the fetus.

The aim of the present study was to develop and validate an LC–MS/MS method for the simultaneous measurement of acesulfame, cyclamate, saccharin and sucralose in a single run in pregnancy-related human matrices (blood, umbilical cord blood, and amniotic fluid) and in breast milk. In order to demonstrate the usability of our method, we have analyzed samples from pregnant and lactating women with and without spiking with artificial sweeteners.

2 | MATERIALS AND METHODS

An LC–MS/MS multiple reaction method (MRM) for the quantification of acesulfame, cyclamate, saccharin and sucralose in plasma was developed. The assay design was further modified for quantification in amniotic fluid and breast milk. The methodological details are outlined below.

2.1 | Chemicals and reagents

Acesulfame (acesulfame potassium salt; acesulfame K; CAS no. 55589-62-3; MW 201.24) was purchased from Celanese Corporation (Sulzbach, Germany). Cyclamate (sodium salt of cyclamic acid; CAS no. 139-05-09; MW 201.22) was purchased from Golden Time Chemical Co. (Jiangsu, China). Saccharin (sodium saccharin; CAS no. 6155-57-3; MW 241.19) was purchased from Kaifeng Xinghua Fine Chemical Co. (Kaifeng, China). Sucralose (CAS no. 56038-13-2; MW 397.64) was purchased from Anhui Jinhe Industrial Co. (Anhui, China). Acesulfame-d4 potassium salt, cyclamic acid-d11, saccharin-d4 and sucralose-d6 were purchased from Toronto Research Chemicals (North York, Ontario, Canada). Stock quality control (QC) concentrations of acesulfame, cyclamate and saccharin in soft drink were purchased from Fapas (York, UK). LCMS-grade acetonitrile, isopropanol and water were purchased from VWR International (Soeborg, Denmark), and methanol and formic acid were purchased from Merck (Soeborg, Denmark).

2.2 | Preparation of stock solutions, calibration standards and quality control samples

Stock solutions of acesulfame, cyclamate, saccharin and sucralose were prepared by dissolving 1 mg of accurately weighed standard in water to a total concentration of 1 mg/ml. Intermediary stocks were prepared by diluting the primary stocks (1 mg/ml) in water to a stock concentration of 1 μg/ml. The stock solutions were stored at 4°C.

The intermediary stock solutions were used to prepare standard matrix-matched calibration samples for analysis in: (a) plasma/umbilical cord blood/amniotic fluid, by dilution with human EDTA plasma tested negative for presence of artificial sweeteners; and (b) breast milk, by dilution with human breast milk tested negative for presence of artificial sweeteners. The final concentrations of acesulfame, cyclamate and saccharin standard calibration samples were 0 (blank), 1, 5, 10 and 50 ng/ml, whereas the final concentrations of sucralose standard calibration samples were 0 (blank), 10, 15, 25 and 50 ng/ml. The standard calibration samples were stored at 4°C until use.

Separate intermediary stock solutions for QC samples were prepared as described for standard calibration samples, and further diluted in EDTA plasma (plasma, umbilical cord blood and amniotic fluid) or breast milk. For acesulfame, cyclamate and saccharin, the QC levels were 10 and 34 ng/ml. For sucralose, the QC levels were 20 and 40 ng/ml. The QC samples were stored at 4°C until use.

Stock solutions of the internal standards (IS) were prepared by dissolving 1 mg of accurately weighed standard in methanol to a total concentration of 1 mg/ml. The IS stock solutions were stored at −20°C (acesulfame, cyclamate and sucralose) or 4°C (saccharin). The IS working solution (IS_{WS}) with all four sweeteners was made from the stock solutions (1 mg/ml) with water–methanol (1:1, v/v) and stored at 4°C until use.

2.3 | Sample pre-treatment

For analysis in plasma, umbilical cord blood, amniotic fluid and breast milk, a volume of 100 μl of sample material (blank, calibration standard, QC, or sample) was mixed with 400 μl of methanol in a 2-ml micro tube to precipitate proteins. The samples were then centrifuged for 10 min at 10,000*g* at room temperature. Clear supernatants (400 μl) were transferred to a 1.2 ml 96-well storage plate

(Thermo Scientific, Roskilde, Denmark). The supernatants were then evaporated under a gentle stream of nitrogen gas at 40°C. After approximately 40 min, dry extracts were obtained. The dry extracts were reconstituted with 100 µl 0.1% (v/v) formic acid in water and 20 µl IS_{WS} was added. The plate was then sealed and mixed for 5 min at 2,000 rpm. A volume of 10 µl was injected into the LC–MS/MS system.

2.4 | Instrumentation

Liquid chromatography was performed on an Agilent 1290 Infinity Series System (Agilent, Glostrup, Denmark) and mass spectrometric detection was carried out on an Agilent 6470 Triple Quad mass spectrometer (Agilent, Glostrup, Denmark), which was equipped with an electrospray ionization (ESI) source. Analytical separation was performed on a Luna Omega Polar C_{18} column (2.1 × 50 mm, 1.6 µm; Phenomenex, Vaerloese, Denmark) at a temperature of 30°C controlled by a column heater.

2.5 | Liquid chromatography and mass spectrometry

Extracted samples were analyzed using reversed-phase liquid chromatography. The mobile phases were composed of: (A) 0.1% (v/v) formic acid in water and (B) a gradient of 0.1% (v/v) formic acid in acetonitrile. The gradient ran linearly from 3% to 100% B in 4.0 min after injection before the mobile phase was returned to 3% B (4.1 min). The flush period was followed by a 1.4 min re-equilibration of the column until the end of the chromatographic run (5.5 min) followed by a 1.5 min post time. The flow rate was 0.3 ml/min, and the total run time for one sample was 7.0 min. The column temperature was set to 30°C and the autosampler temperature was set to 10°C. The needle was cleaned between samples with 75% methanol in water, and the back of the pump seal was rinsed with 10% isopropanol in water.

Mass spectrometry (MS) detection was achieved on an Agilent 6470 Triple Quad mass spectrometer (Agilent, Glostrup, Denmark). The ESI source operated in negative mode with mass spectrometry parameters as follows: nebulizer pressure (nitrogen), 55 psi; nozzle voltage, −300 V; capillary voltage, −4,200 V; drying gas flow, 11 L/min; drying gas temperature, 290°C; sheath gas flow, 12 L/min; and sheath gas temperature, 400°C. The acquisition was performed in MRM mode. Dwell time at each transition was 25 ms. An overview of the applied mass spectrometer settings, including analyte specific settings, is shown in Table 1.

2.6 | Data analysis

All data used for qualification were collected and processed by MassHunter version B.09 (Agilent, Glostrup, Denmark). A linear regression of each calibration curve was applied with concentration being the explanatory variable and the peak area ratio (y) of the analyte to IS. The calibration settings were performed with a linear curve fit without weighing factor. As criteria of acceptance, all calibrator standards had to be within ±15% from the nominal concentration in each analytical run, and the correlation coefficient (R^2) had to be >0.98.

2.7 | Method validation

For validation of the developed method, linearity, dilution integrity, carryover, lower limit of detection (LLOD), limit of quantification (LOQ), upper limit of quantification (ULOQ), precision (CV), extraction efficiency, recovery and stability were assessed.

The linearity of the developed methods was assessed over their respective calibration ranges from 1 to 50 ng/ml (acesulfame, cyclamate and saccharin) and from 10 to 50 ng/ml (sucralose). The calibration curves were performed with five concentrations (including blank). The acceptable criteria for the calibration curves are outlined in Section 2.6. To analyze samples at a concentration above the calibration range (>50 ng/ml), the dilution integrity was assessed using a 500 ng/ml stock solution with the artificial sweeteners. The stock solution was used to make a dilution series (100, 75, 50, 25, 10 and 5%) that was measured in three replicates for evaluation by linear regression. The validation criteria were that both precision and deviation from the nominal concentrations for the means of the three

TABLE 1 Optimized mass spectrometric parameters for the sweeteners and internal standards

Compounds	Precursor ion, *m/z*	Product ion, *m/z*	Fragmentor voltage, V	Collision energy, V	Cell accelerator voltage, V	Retention time, min	Polarity
Acesulfame	162.0	82.0	70	16	5	~1.36	Negative
Acesulfame-d4	166.0	86.0	70	16	5	~1.36	Negative
Cyclamate	178.1	79.9	135	30	5	~1.66	Negative
Cyclamate-d11	189.3	79.9	135	30	5	~1.66	Negative
Saccharin	182.1	42.0	115	30	5	~1.65	Negative
Saccharin-d4	186.2	42.0	115	30	5	~1.65	Negative
Sucralose	395.1	359.1	135	10	5	~1.90	Negative
Sucralose-d6	403.1	366.6	135	10	5	~1.90	Negative

replicates should be ≤15% from the nominal concentration, and that the correlation coefficient (R^2) should be >0.98.

Carryover was assessed in combination with the linearity studies by running two subsequent blank samples after the 100% stock solution (500 ng/ml). The concentration of analyte in the blank samples should be below the LLOD to rule out carryover. The LLOD was assessed as the mean value of 35 replicates of blank samples plus five times the relative SD value of the blanks. The LOQ was determined as the concentration with a signal-to-noise ratio of 10. The ULOQ was defined as the highest concentration with a known CV.

The precision of the assay was determined at four different concentration levels. For acesulfame, cyclamate and saccharin, these concentrations where 1, 10 (low QC), 34 (high QC) and 500 ng/ml (ULOQ). For sucralose, these concentrations were 10, 20 (low QC), 40 (high QC) and 500 ng/ml. For the low and high QCs, analysis was performed in three replicates in six runs ($n = 18$). For the other concentrations, analysis was performed in three replicates in five runs ($n = 15$). The validation criteria were that the interassay precisions should be ≤15%. At the lowest concentration (10 ng/ml for sucralose; 1 ng/ml for the others) an intraassay precision of ≤25% was accepted since this concentration was close to LLOD and LOQ.

Cross-talk between the artificial sweeteners in MRM monitoring was investigated by analyzing pure individual stocks and compared with the extracted retention time of the artificial sweeteners in a mixture of standards.

The extraction efficiency (bias, %) was determined by analysis of 25 spiked plasma samples (five stocks at five levels) for each of the four artificial sweeteners, when IS was added before vs. after hydrolysis.

To test stability under different conditions, three stocks with acesulfame, cyclamate and saccharin in different concentrations (7.5, 30 and 50 ng/ml) were used. For sucralose, the stock concentrations were 20, 40 and 50 ng/ml. Freeze–thaw stability was evaluated after three freeze (−20°C) and thaw (room temperature) cycles. Short-term stability was assessed after storage at room temperature for 3 h. Long-term stability was investigated after storage at −20°C for 42 days. For all stability tests, the acceptance criteria were a relative standard deviation (SD) and relative deviation from target (percentage deviation) within ±15% based on triplicate measurements.

2.8 | Method for application in pregnancy-related biological matrices and assessment of accuracy and recovery

Samples of plasma, umbilical cord blood and amniotic fluid collected from three pregnant women scheduled for cesarean section were used to test the method's application to pregnancy-related human samples. A pool of breast milk divided into three stocks was used to test the method in this matrix. The samples were spiked with the four artificial sweeteners (25 ng/ml). The measured mean concentrations of the four artificial sweeteners in the different matrices from the three women/stocks of breast milk (analyzed in triplicates) were determined. The acceptance criterion was that the recovery should be within ±20% of the nominal spiked-in concentration, and that the interassay precision should be ≤15%. For comparison, aliquots of the samples without any additives were also analyzed.

3 | RESULTS AND DISCUSSION

3.1 | Method development

3.1.1 | Optimization of mass spectrometric parameters

The mass spectrometric method was screened and optimized by a systematic approach. Positive and negative ionization modes were both investigated to detect each analyte. All analytes showed sufficient ionization efficiency using negative ionization. The precursor and product ions were determined, and the highest ion abundance was achieved by optimizing source/gas and compound parameters. The optimal ionization conditions are presented in Table 1.

Internal standards (IS) were used for relative quantification, thus reducing the error owing to differences in injection volume, chromatography retention time variance and ionization differences between calibrators and samples. We tested the extraction efficiency of adding IS before vs. after protein precipitation, and found the extraction bias to be minor (<6% for acesulfame, cyclamate and saccharin; 10% for

TABLE 2 Linearity as judged from equations of calibration curves of the artificial sweeteners in plasma, amniotic fluid and breast milk[a]

Matrix	$y = ax + b$
Plasma	
Acesulfame	$y = 0.0086x - 0.00156$
Cyclamate	$y = 0.0060x - 0.00063$
Saccharin	$y = 0.0051x + 0.00370$
Sucralose	$y = 0.0023x + 0.00004$
Amniotic fluid	
Acesulfame	$y = 0.0135x + 0.01150$
Cyclamate	$y = 0.0304x + 0.00649$
Saccharin	$y = 0.0090x + 0.00118$
Sucralose	$y = 0.0013x - 0.00519$
Breast milk	
Acesulfame	$y = 0.0081x + 0.000219$
Cyclamate	$y = 0.0087x + 0.000192$
Saccharin	$y = 0.0048x + 0.003780$
Sucralose	$y = 0.0026x - 0.000560$

[a]For acesulfame, cyclamate and saccharin, the calibration range was 1–50 ng/ml (plus blank). For sucralose, the calibration range was 10–50 ng/ml (plus blank). All standards were within ±15% of the nominal concentration in each analytical run, and the correlation coefficient (R^2) was >0.98.

sucralose) and within the interassay precision accepted for this method.

The acquisition method for sucralose did not perform consistently in the lower concentration range (<10 ng/ml). For this reason, separate standard calibration samples and QC samples for sucralose were prepared and used in addition to the main calibration stocks used for the other three artificial sweeteners.

3.2 | Method validation

3.2.1 | Linearity, dilution integrity, carryover and cross-talk

In each validation batch, five calibration standards were analyzed, and the calibration curves showed a linear response in the concentration

TABLE 3 Dilution integrity as judged from dilution series in stock solution

		Nominal concentration					
Analyte	Item	5% 25 ng/ml	10% 50 ng/ml	25% 125 ng/ml	50% 250 ng/ml	75% 375 ng/ml	100% 500 ng/ml
Acesulfame $R^2 = 0.99$[d]	Mean[a]	26	51	124	250	383	510
	CV (%)[b]	0.7	0.4	0.7	0.2	0.3	0.7
	RE (%)[c]	3.7	2.3	−1.0	0	2.2	2.1
Cyclamate $R^2 = 0.99$[d]	Mean[a]	26	51	119	233	355	464
	CV (%)[b]	0.5	1.1	1.8	1.3	0.3	0.6
	RE (%)[c]	4.9	1.6	−4.9	−6.7	−5.4	−7.2
Saccharin $R^2 = 0.99$[d]	Mean[a]	26	52	125	258	412	521
	CV (%)[b]	0.3	0.5	3.3	2.9	0.5	3.4
	RE (%)[c]	3.6	3.3	0	3.0	9.8	4.2
Sucralose $R^2 = 0.99$[d]	Mean[a]	25	51	125	249	387	514
	CV (%)[b]	10	3.3	2.3	6.5	1.9	0.7
	RE (%)[c]	0	0.9	0	−0.4	3.1	2.8

[a]Mean (mean of three replicates).
[b]CV (%) = coefficient of variation: (SD/mean) × 100 (acceptance criterion, CV ≤15%).
[c]RE (%) = relative error expressed as [(mean observed concentrations − nominal concentration)/(nominal concentration)] × 100) (acceptance criterion, RE ≤15%).
[d]Correlation coefficients of the plotted dilution series.

TABLE 4 Interassay precision in plasma

		Nominal concentration			
Analyte	Item	1 ng/ml ($n = 15$)	10 ng/ml ($n = 18$)	34 ng/ml ($n = 18$)	500 ng/ml ($n = 15$)
Acesulfame	Mean	0.97	10.5	34.1	455
	CV (%)[a]	17	4	4	6
	RE (%)[b]	−2.7	5.2	0.3	−9.1
Cyclamate	Mean	1.10	9.9	33.0	444
	CV (%)[a]	9	6	8	10
	RE (%)[b]	9.7	−1.3	−2.9	−11
Saccharin	Mean	0.95	9.6	35.3	450
	CV (%)[a]	21	7	4	10
	RE (%)[b]	−4.8	−3.6	3.8	−10
		10 ng/ml ($n = 15$)	20 ng/ml ($n = 18$)	40 ng/ml ($n = 18$)	500 ng/ml ($n = 15$)
Sucralose	Mean	10.3	22.7	42.0	479
	CV (%)[a]	25	15	14	8
	RE (%)[b]	2.5	13	5.1	−4.2

[a]CV (%) = coefficient of variation: (SD/mean) × 100 (acceptance criterion, CV ≤15%) (in the lower end of the measuring range (1 ng/ml for acesulfame, cyclamate and saccharin; and 10 ng/ml for sucralose), the acceptance criteria was CV ≤25%).
[b]RE (%) = relative error expressed as [(mean observed concentrations − nominal concentration)/(nominal concentration)] × 100 (acceptance criterion, RE ≤15%).

range of 1–50 ng/ml for acesulfame, cyclamate and saccharin, and 10–50 ng/ml for sucralose (Table 2). The correlation coefficients (R^2) of all of the calibration curves were >0.98, which demonstrated a good linearity within the concentration range. Dilution integrities by back-calculated concentrations of the analytes from 500 ng/ml are shown in Table 3. Mean precision (CV) of ≤15% and deviation from the nominal concentration (relative error, RE) of ≤15% were accepted, but the methods performed much better, as shown in Table 3. The plotted replicates demonstrated satisfactory linearity (R^2 > 0.98, data not shown). Collectively, the results indicate that samples can be

TABLE 5 Stability assessment of four artificial sweeteners in plasma

Analyte	Stability type	Nominal concentration, ng/ml	Mean, ng/ml	SD, ng/ml[d]	Percentage deviation[e]
Acesulfame ($n = 3$)	Freeze–thaw[a]	7.5	7.7	0.1	2.3
		30	30	0.4	0.6
		50	50	0.9	0.4
	Short-term[b]	7.5	7.5	0.2	0.4
		30	30	0.3	−0.7
		50	50	0.3	−0.7
	Long-term[c]	7.5	7.6	0.1	1.6
		30	31	0.1	1.6
		50	50	0.6	0.6
Cyclamate ($n = 3$)	Freeze–thaw[a]	7.5	7.3	0.2	−2.1
		30	30	0.6	0.2
		50	50	1.5	−0.1
	Short-term[b]	7.5	7.5	0.1	−0.4
		30	30	0.0	−1.1
		50	50	0.8	−0.4
	Long-term[c]	7.5	7.6	0.1	0.8
		30	30	0.6	−0.1
		50	50	0.4	−0.2
Saccharin ($n = 3$)	Freeze–thaw[a]	7.5	7.5	0.1	0.6
		30	31	0.6	3.3
		50	51	1.3	1.8
	Short-term[b]	7.5	7.4	0.3	−1.9
		30	30	0.2	0.6
		50	51	0.5	2.7
	Long-term[c]	7.5	7.7	0.2	2.5
		30	31	0.2	3.4
		50	51	0.3	1.4
Sucralose ($n = 3$)	Freeze–thaw[a]	20	17	1.4	−15
		40	37	3.0	−8.8
		50	49	1.9	−2.5
	Short-term[b]	20	21	2.3	4.5
		40	42	3.3	6.0
		50	55	4.3	10.3
	Long-term[c]	20	17	0.3	−16
		40	39	0.9	−3.8
		50	47	3.5	−6.8

[a]Freeze–thaw (three cycles).
[b]Short-term (room temperature, 3 h).
[c]Long-term (−20°C, 42 days).
[d]Relative standard deviation (SD).
[e]Relative deviation from the nominal concentration (percentage deviation).

diluted up to 10-fold when their concentrations are higher than the upper calibration point (50 ng/ml). This finding expands the quantification range up to 500 ng/ml. No carryover was found at 500 ng/ml for any of the analytes (data not shown). No cross-talk between the artificial sweeteners in the MRM monitoring was found based on comparison of the retention time for each compound in pure solution vs. in a mixture of all sweeteners, and from the finding that pure individual stocks only caused a peak for the right compound without any background or interference for the other sweeteners (data not shown).

3.2.2 | Precision

The interassay precision for the determination of the four artificial sweeteners in plasma is summarized in Table 4. The validation criteria were that the CV should be ≤15%. In plasma, the interassay precisions were all ≤10% for acesulfame, cyclamate and saccharin, and ≤15% for sucralose (Table 4). Likewise, in breast milk, the interassay precisions were all ≤7% for acesulfame, cyclamate and saccharin, and ≤ 9% for sucralose. At the lower end of the measuring range (10 ng/ml for sucralose; 1 ng/ml for the others), a CV of ≤25% was accepted. However, it should be noted that measures of especially saccharin and sucralose in this concentration level should be interpreted with caution owing to the relative high imprecision (Table 4).

The LLODs calculated as described in Section 2.7. were 0.86 ng/ml for acesulfame, 0.79 ng/ml for cyclamate, 0.82 ng/ml for saccharin and 6.9 ng/ml for sucralose. The LOQs were evaluated as a signal-to-noise ratio of 10 and were <10 ng/ml for sucralose and <1 ng/ml for the other sweeteners (evaluated in all four matrices).

3.2.3 | Stability

The results of the freeze–thaw, short-term and long-term stability are shown in Table 5. All four artificial sweeteners had satisfactory relative deviation from the nominal concentration (percentage deviation) within ±15% for all three types of stability studies at all three concentrations levels. The only exception was sucralose, which deviated by 16% from the nominal concentration after 42 days of storage at −20°C at concentration level of 20 ng/ml (Table 5).

3.3 | Method application in breast milk and pregnancy-related biological matrices and assessment of accuracy and recovery

To demonstrate the usability of the methods in human matrices related to pregnancy and lactation, samples of plasma, umbilical cord blood and amniotic fluid from three pregnant women were spiked with 25 ng/ml artificial sweeteners and analyzed. The same was carried out with a pool of breast milk. The results are shown in Table 6. All of the blank samples provided analyte concentrations below the detection limit, and the spiking concentrations had recoveries (%)

TABLE 6 Recoveries and CVs of artificial sweeteners in human plasma, umbilical cord blood, amniotic fluid and breast milk

Analyte	Matrix	Spiked[a] 25 ng/ml Mean ± SD	Recovery (%)[b]	CV (%)[c]
Acesulfame	Plasma	25 ± 0.2	101	0.9%
	Umbilical cord	28 ± 0.3	111	0.9%
	Amniotic fluid	28 ± 0.2	111	0.8%
	Breast milk	23 ± 0.7	92	3.1%
Cyclamate	Plasma	28 ± 0.6	113	2.2%
	Umbilical cord	30 ± 0.5	119	1.8%
	Amniotic fluid	29 ± 0.3	115	1.2%
	Breast milk	22 ± 0.9	90	4.3%
Saccharin	Plasma	26 ± 0.5	104	1.7%
	Umbilical cord	29 ± 0.4	114	1.5%
	Amniotic fluid	27 ± 0.7	110	2.4%
	Breast milk	22 ± 0.4	90	1.9%
Sucralose	Plasma	24 ± 2.0	95	8.4%
	Umbilical cord	24 ± 1.3	94	5.6%
	Amniotic fluid	25 ± 2.2	98	8.8%
	Breast milk	23 ± 3.3	91	14.3

[a]Results from three women analyzed in triplicate are given as means ± SD.
[b]Recovery (%) is expressed as [(mean observed concentrations − nominal concentration)/(nominal concentration)] × 100) (acceptance criterion, ±20%).
[c]CV, (SD/mean) × 100 (acceptance criterion, CV ≤15%).

within ±20%. For all spiked samples, the CV calculated on the repeated measures was within the acceptance criterion of CV ≤15%. Chromatograms from the analysis of the spiked samples are shown in Figure 1.

Overall, the findings demonstrate a sufficient accuracy and recovery and that the methods can be used for the measurement of the four artificial sweeteners in plasma, umbilical cord blood, amniotic fluid and breast milk.

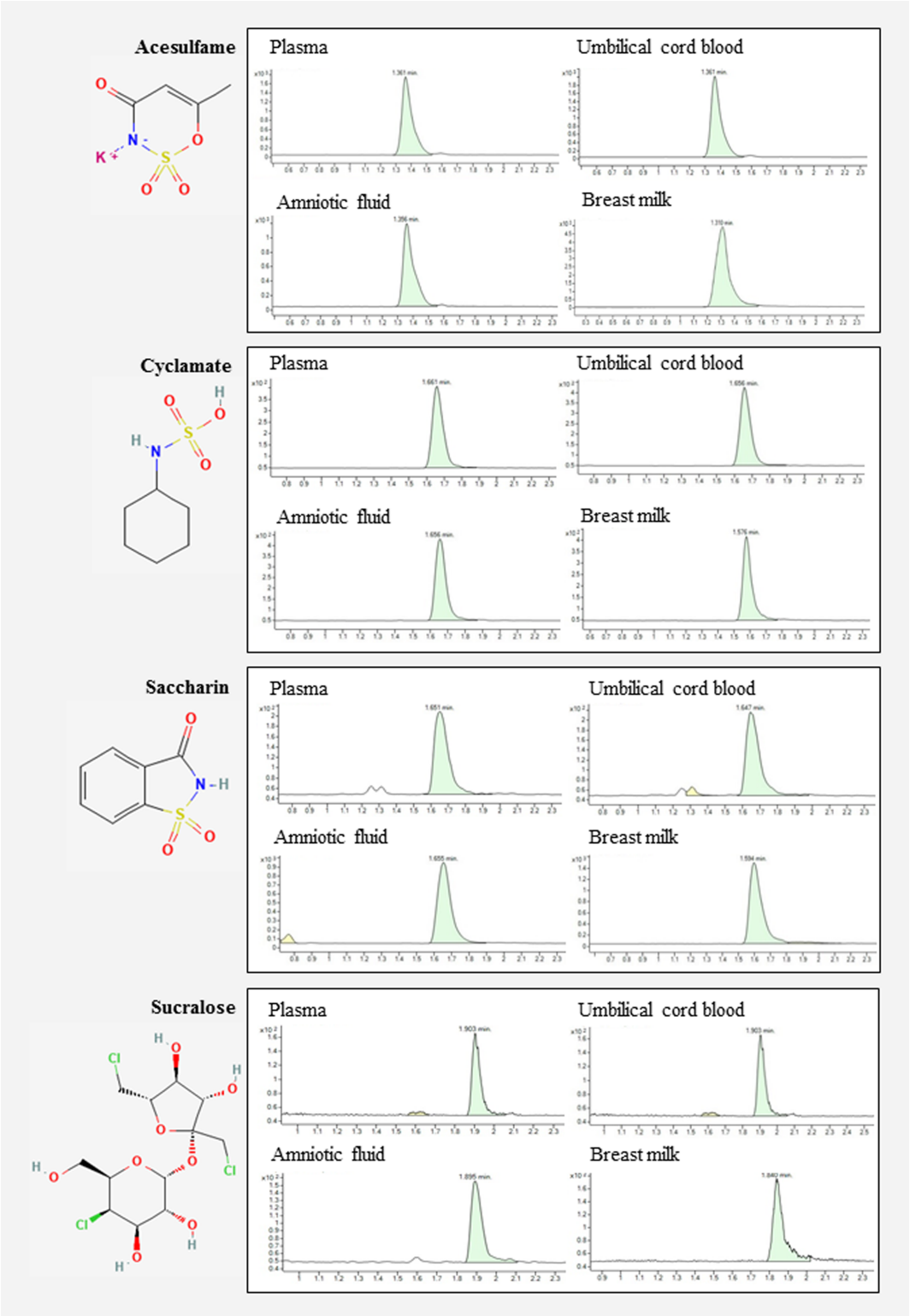

FIGURE 1 Typical multiple reaction monitoring chromatograms for the four sweeteners in the different matrices at a spiked concentration of 25 ng/ml. The structures are from PubChem

4 | CONCLUSIONS

We have developed an overall LC–MS/MS method for the simultaneous quantification of acesulfame, cyclamate, saccharin and sucralose in human plasma, umbilical cord blood, amniotic fluid and breast milk. The results were successfully validated with the analysis of the four artificial sweeteners in spiked human samples from pregnant women and in breast milk. To the best of our knowledge, this is the first published validated assay for simultaneous quantification of these compounds in human pregnancy-related matrices and breast milk. The accuracy, precision, freeze–thaw and long-term stability of the samples met the validation criteria. Overall, the method provided a simple, fast and accurate way of quantitatively analyzing acesulfame, cyclamate, saccharin and sucralose, and is judged to be suitable for use in clinical studies, including during pregnancy and lactation.

ACKNOWLEDGEMENTS

We thank Margrethe Sønderskov Christensen and Jette Birk Larsen, Department of Clinical Biochemistry, Aarhus University Hospital, Denmark, for excellent technical assistance.

The research was funded by the Department of Clinical Biochemistry at Aarhus University Hospital, The Novo Nordisk Foundation, P. Carl Petersens Foundation, A.P. Møllers Foundation, and Helga and Peter Kornings Foundation. The artificial sweeteners were a gift from Arla, Denmark.

CONFLICT OF INTEREST

There are no conflicts to declare.

DATA AVAILABILITY STATEMENT

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

ORCID

Eva Greibe https://orcid.org/0000-0002-0417-1195

REFERENCES

Azad, M. B., Sharma, A. K., de Souza, R. J., Dolinsky, V. W., Becker, A. B., Mandhane, P. J., Turvey, S. E., Subbarao, P., Lefebvre, D. L., & Sears, M. R. (2016). Association between artificially sweetened beverage consumption during pregnancy and infant body mass index. *JAMA Pediatrics*, *170*, 662–670. https://doi.org/10.1001/jamapediatrics.2016.0301

Chang, C.-S., & Yeh, S. (2014). Detection of 10 sweeteners in various foods by liquid chromatography/tandem mass spectrometry. *Journal of Food and Drug Analysis*, *22*, 318–328. https://doi.org/10.1016/j.jfda.2014.01.024

Lange, F. T., Scheurer, M., & Brauch, H. J. (2012). Artificial sweeteners—A recently recognized class of emerging environmental contaminants: A review. *Analytical and Bioanalytical Chemistry*, *403*, 2503–2518. https://doi.org/10.1007/s00216-012-5892-z

Mejia, E., & Pearlman, M. (2019). Natural alternative sweeteners and diabetes management. *Current Diabetes Reports*, *19*, 142. https://doi.org/10.1007/s11892-019-1273-8

Rother, K. I., Sylvetsky, A. C., Walter, P. J., Garraffo, H. M., & Fields, D. A. (2018). Pharmacokinetics of sucralose and acesulfame-potassium in breast milk following ingestion of diet soda. *Journal of Pediatric Gastroenterology and Nutrition*, *66*, 466–470. https://doi.org/10.1097/MPG.0000000000001817

Schiffman, S. S., & Rother, K. I. (2013). Sucralose, a synthetic organochlorine sweetener: Overview of biological issues. *Journal of Toxicology and Environmental Health. Part B, Critical Reviews*, *16*, 399–451. https://doi.org/10.1080/10937404.2013.842523

Sylvetsky, A. C., Bauman, V., Blau, J. E., Garraffo, H. M., Walter, P. J., & Rother, K. I. (2017). Plasma concentrations of sucralose in children and adults. *Toxicological and Environmental Chemistry*, *99*, 535–542. https://doi.org/10.1080/02772248.2016.1234754

Zhang, T., Gan, Z., Gao, C., Ma, L., Li, Y., Li, X., & Sun, H. (2016). Occurrence of artificial sweeteners in human liver and paired blood and urine samples from adults in Tianjin, China and their implications for human exposure. *Environmental Science. Processes & Impacts*, *18*, 1169–1176. https://doi.org/10.1039/C6EM00130K

Zhu, Y., Olsen, S. F., Mendola, P., Halldorsson, T. I., Rawal, S., Hinkle, S. N., Yeung, E. H., Chavarro, J. E., Grunnet, L. G., Granström, C., Bjerregaard, A. A., Hu, F. B., & Zhang, C. (2017). Maternal consumption of artificially sweetened beverages during pregnancy, and offspring growth through 7 years of age: A prospective cohort study. *International Journal of Epidemiology*, *46*, 1499–1508. https://doi.org/10.1093/ije/dyx095

How to cite this article: Greibe, E., Leth-Møller, M., Stampe, S., Ovesen, P., Pedersen, M., & Hoffmann-Lücke, E. (2022). Development and validation of an LC–MS/MS method for the quantification of artificial sweeteners in human matrices. *Biomedical Chromatography*, *36*(6), e5350. https://doi.org/10.1002/bmc.5350