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RESEARCH ARTICLE

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Application of accelerated heteronuclear single quantum coherence experiments to the rapid quantification of monosaccharides and disaccharides in dairy products

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Abstract

Monosaccharides and disaccharides are important dietary components, but if insufficiently metabolized by some population subgroups, they are also linked to disease patterns. Thus, the correct analytical identification, quantification, and labeling of these food components are crucial to inform and potentially protect consumers. Enzymatic assays and high-performance anion-exchange chromatography with pulsed amperometric detection are established methods for the quantification of monosaccharides and disaccharides that, however, require long measuring times (60–180 min). Accelerated methods for the identification and quantification of the nutritionally relevant monosaccharides and disaccharides D-glucose, D-galactose, D-fructose, sucrose, lactose, and maltose were therefore developed. To realize this goal, the NMR experiments HSQC (heteronuclear single quantum coherence) and acceleration by sharing adjacent polarization (ASAP)-HSQC were applied. Measurement times were reduced to 27 and 6 min, respectively, by optimizing the interscan delay and applying non-uniform sampling. The optimized methods were used to quantify D-glucose, D-galactose, D-fructose, sucrose, and lactose in various dairy products. Results of the HSQC and ASAP-HSQC methods are equivalent to the results of the reference methods in terms of both precision and accuracy, demonstrating that these methods can be used to correctly analyze nutritionally relevant monosaccharides and disaccharides in short times.

KEYWORDS

ASAP-HSQC, carbohydrates, dairy products, disaccharides, HSQC, monosaccharides, NMR, non-uniform sampling, quantification

1 | INTRODUCTION

Monosaccharides and disaccharides are important food components and are often found in large quantities in both processed and unprocessed food products. Dairy products often contain lactose (milk sugar) and, especially in Europe, added sucrose or, especially in the United States, fructose and/or glucose. Lactose-free alternatives also contain glucose and galactose from enzymatic hydrolysis of lactose into its monomers. Added

fruits may also increase sucrose, fructose, and glucose contents.

Monosaccharides and disaccharides have a substantial impact on the human metabolism. Although they are certainly important nutrients, excessively high consumption of monosaccharides and disaccharides has been correlated with weight gain and consequently with the development of type-2 diabetes.[1,2] In addition, specific metabolism-compromised population subgroups suffer from diseases/conditions, such as fructosuria, hereditary fructose intolerance, galactosemia, or lactose intolerance, that are based on the consumption of certain monosaccharides and disaccharides.[3,4] All mentioned clinical indications have in common that the respective sugars must be consistently avoided. It is therefore important for these vulnerable populations that sugars are accurately identified, quantitated, and labeled in food products. Correct labeling needs to be controlled, as must the (un) intended contamination with, or addition of, (for the mentioned vulnerable subgroups) potentially harmful sugars. Also, the addition of inexpensive sugars instead of more expensive ones may contribute to food fraud.

In order to support food label verification, rapid analytical methods that are able to correctly identify and quantitate monosaccharides and disaccharides in food products are required. To date, rather complex and lengthy methods, such as those established as official methods in Germany, are most often used by the food inspection agencies. Enzymatic methods are used, for example, to quantify lactose and galactose in milk and milk products as well as their lactose-free alternatives, or to quantify sucrose and glucose in milk products and ice cream.[5–7] However, enzymatic assays are only able to quantify a maximum of three analytes at the same time and are also more difficult to automate. This increases the analysis time to several hours if many saccharides are present in the food product. In addition to enzymatic assays, chromatographic methods can be used to quantify monosaccharides and disaccharides. High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) is an excellent option, suitable for quantifying a large number of monosaccharides and disaccharides simultaneously. However, the chromatographic runs take up to 90 min per sample.[8,9] Gas chromatographic methods can also be used to quantify sugar components. However, in addition to the runtime of the chromatographic separation, the analytes must be derivatized, which increases the analysis time.[10] None of the currently established methods is able to successfully quantify a large number of analytes in short measuring times.

Proton NMR spectroscopic methods are characterized by their rapidity. Quantitative ^{1}H-NMR measurements have already been used for the quantification of nutritionally relevant monosaccharides and disaccharides. They are often coupled with multivariate evaluation strategies because their resolution is usually not sufficient to separate and quantify a large number of chemically equivalent analytes.[11,12] Two-dimensional experiments such as the HSQC (heteronuclear single quantum coherence) experiment can adequately resolve signals of more analytes because extra dispersion is gained due to the introduction of the second dimension, a carbon dimension that has greater resolution. Because sugar carbons are all protonated, sugars can successfully be detected using HSQC pulse sequences.[13] On the downside, even with the marked improvement in sensitivity from proton-detected ('inverse') pulse-programs HSQC experiment times are usually comparably long and therefore do not satisfy the requirements of fast methods. However, measures to reduce the experimental time, such as optimizing the interscan delay and using non-uniform sampling (NUS), help to speed up these methods.

A crucial factor for proper quantification is a sufficient interscan delay, which together with the acquisition time describes the recovery time. The recovery time between two scans should be sufficiently long to ensure a complete return of all contained spins to the initial state. Only with complete relaxation of all spins can maximum signal intensity and thus correct quantification be achieved. Because the acquisition time depends on the spectral width and can therefore usually not be set flexibly, complete relaxation can be ensured via the setting of the interscan delay. With an interscan delay of five times the length of the longitudinal relaxation time, it can be assumed that 99% of the nuclei have returned to the initial state.[14,15] Reducing the interscan delay may probably negatively affect analyte integrals due to insufficient relaxation between scans, so the interscan delay cannot be minimized arbitrarily.

By using NUS, only a fraction of randomly distributed data points is acquired and remaining data points are added via reconstruction. The use of NUS and the resulting lower number of recorded data points can reduce the measurement time of the methods while maintaining the same resolution.[16] When using NUS, increased formation of spectral artifacts may occur due to the violation of the Nyquist theorem. NUS sampling, that is, the selection of data points to be measured, is therefore of great importance. In addition to a random distribution, exponentially weighted or other weighting functions can be used.[16,17] One of the basic theorems of the CS algorithm relates the number of sampling points for a good reconstruction to the number of significant points in a spectrum. As an approximation to this rule, the specification of the NUS level in percent is often used,

which is also used in the context of this work.[18] NUS-coupled spectra have already been used for quantification purposes; however, quantification may be limited especially when low-intensity signals are encountered.[19] A suitable working range as well as a suitable NUS level therefore needs to be determined for the quantification of the monosaccharides and disaccharides.

Time-optimized HSQC pulse sequences such as the acceleration by sharing adjacent polarization (ASAP)-HSQC pulse sequence are perfectly suited to obtaining HSQC spectra in a short measurement time.[20] The method can also be coupled with NUS, which further minimizes the experiment time. The extent to which the ASAP-HSQC experiment also provides quantitative results was investigated in the present work.

Here, different time-optimized HSQC methods for the analysis of D-glucose, D-galactose, D-fructose, sucrose, lactose, and maltose were optimized to allow for a rapid identification and quantification of typical sugars in dairy products.

2 | EXPERIMENTAL

2.1 | Reference material and food samples

D-Glucose (≥99.5%), D-galactose (≥99%), D-fructose (≥99%), lactose monohydrate (≥99.5%), and maltose monohydrate (≥99%) were purchased from Sigma Aldrich (St. Louis, USA), D-allose (>99%) from Carbosynth (Compton, UK) and sucrose (≥99.5%) from Carl Roth (Karlsruhe, Germany). All food samples were bought at a local grocery store (Karlsruhe, Germany).

2.2 | Enzymatic assays

The following enzymatic assay kits (Roche Diagnostics, Basel, Switzerland) were used: D-glucose/ D-fructose, sucrose/ D-glucose/ D-fructose, maltose/sucrose/D-glucose, lactose/D-galactose, lactose/D-glucose. Assays were carried out according to the manufacturer's specifications.

2.3 | HPAEC-PAD

D-Glucose, D-galactose, D-fructose, and sucrose were analyzed on an ICS-5000 system (Dionex, detector CS-5000 DC, Thermo Fisher Scientific, Waltham, USA) equipped with a CarboPacPA20 column (150 mm × 3 mm i.d., 6 μm particle size, Dionex, Thermo Fisher Scientific, Waltham, USA). A gradient of (A) bidistilled water, (B) 0.1 M sodium hydroxide (NaOH, Sigma Aldrich, St. Louis, USA), and (C) 0.1 M NaOH with 0.2 M sodium acetate (>97%, Sigma Aldrich, St. Louis, USA) was used with a flow rate of 0.4 ml/min at 25°C. The column was rinsed before every run with 100% B for 10 min and 96.5% A and 3.5% B for another 10 min. After injection, the portion of A was increased to 98% within 0.5 min, and this composition was held for 23.5 min. From minute 24 to minute 28, the portion of B was increased to 40% (60% A). Following 2 min, the portion of B was increased linearly to 100% and held for 3 min. The portion of C was increased to 100% from minute 40 to minute 42 and held for another 11 min. The gradient has been optimized for this application.

Lactose and maltose were analyzed on a CarboPacPA200 column (250 mm × 3 mm i.d., 6 μm particle size, Dionex, Thermo Fisher Scientific, Waltham, USA). The following gradient composed of (A) bidistilled water, (B) 0.1 M NaOH, and (C) 0.1 M NaOH with 0.5 M sodium acetate was used: rinsing with 100% B for 10 min and then 90% A and 10% B for 4.1 min. After injection, the following gradient was applied: 0.1–1.5 min, linear to 97% A and 3% B, holding for 7.5 min, 8.0–8.1 min, linear to 99% A and 1% B, isocratic for 13.9 min, 22–32 min, linear to 100% B, isocratic for 4 min, 36.0–36.1 min, linear to 30% B and 70% C, holding for 3.9 min followed by rinsing the column with 100% C for 10 min. The gradient has been optimized for this application.

Calibration was carried out using clarified (see Section 2.5, sample preparation) mixtures of the analytes D-glucose, D-galactose, D-fructose, sucrose, lactose, and maltose of the concentrations 1.0, 2.5, 5.0, 7.5, and 10.0 mg/L, each containing D-allose as internal reference (5.0 mg/L).

2.4 | HSQC-experiments

NMR spectroscopy was carried out on an Ascend 500 MHz spectrometer (Bruker Biospin, Ettlingen, Germany) equipped with a Prodigy cryoprobe. Calibration used clarified (see Section 2.5) mixtures of the analytes D-glucose, D-galactose, D-fructose, sucrose, lactose, and maltose, concentrations 1.0, 2.5, 5.0, 7.5, and 10.0 g/L, each containing D-allose as internal reference (5.0 g/L). To account for potential losses due to the addition of Carrez-reagents also not pre-treated mixtures of the above-mentioned analytes were used for calibration.

Solutions were mixed with 10% deuterium oxide (99.9%, Deutero, Kastellaun, Germany) and 0.5 μL acetone (≥99.8%, VWR, Radnor, USA) for spectral calibration (^{1}H = 2.22 ppm, ^{13}C = 30.89 ppm[21]). All spectra

were recorded at 25°C. HSQC pulse programs were standard Bruker sequences. The ASAP-HSQC pulse program (asap_hsqc_sp_bruker) was provided by the working group of Prof. B. Luy (Karlsruhe Institute of Technology, KIT, Karlsruhe, Germany; further information to pulse program see Schulze-Sünninghausen et al.[20]). In the ASAP-HSQC measurements, the interscan delay (D_1) could be reduced to 0.05 s. A spectral width of 4.50 ppm acquiring 1024 data points (for an acquisition time, AQ, of 0.227 s) in the ^{1}H dimension and 100.00 ppm using 1024 data points (for an AQ of 0.041 s) in the ^{13}C dimension was used for all measurements. Further acquisition parameters are presented in Section 3.1. Linear prediction and zero filling (two times the recorded time dimension, i.e., 2048 data points) were performed for all spectra in both dimensions. A cosine bell apodization was performed as a weighting function. All spectra were also subjected to manual phase correction and automatic baseline correction. When using NUS, an unweighted sampling was applied. We also tested exponentially weighted sampling functions, which resulted in worse calibration functions as compared to using unweighted sampling (data not shown). The reconstruction algorithm IST was used as compressed sensing method. Hilbert transformation in the indirect dimension is applied to allow phase adjustment. We used the processing parameters listed above, especially also linear prediction in the indirect dimension, because no degraded resolution (as postulated in Mobli and Hoch,[22] among others) was observed.

The following $^{1}H/^{13}C$ correlation signals were selected for quantification (Figure 1): D-glucose 3.47/76.47 ppm; D-galactose 4.58/97.14 ppm; D-fructose 4.10/75.05 ppm; D-allose 3.63/67.52 ppm; sucrose 5.40/92.78 ppm; lactose 3.65/78.90 ppm; maltose 5.39/100.14 ppm. These signals were selected because they do not overlap with any other food-related sugar signals or other signals from the matrices and have suitable resolution. 2D-volume integrals were determined by manual integration using TopSpin version 4.0.2 (Bruker Biospin, Ettlingen, Germany).

The interday precision of the method "HSQC D_1=1.5 s, 50% NUS" was first analyzed by triplicate determination of five concentration levels (1.0, 2.5, 5.0, 7.5, and 10.0 g/L) on the same day. To determine the intraday precision, measurements were carried out in triplicate on six different days or on three different days for the method "ASAP-HSQC with 50% NUS". Measurements were carried out on the identical spectrometer, and the above-described processing was carried out by the same

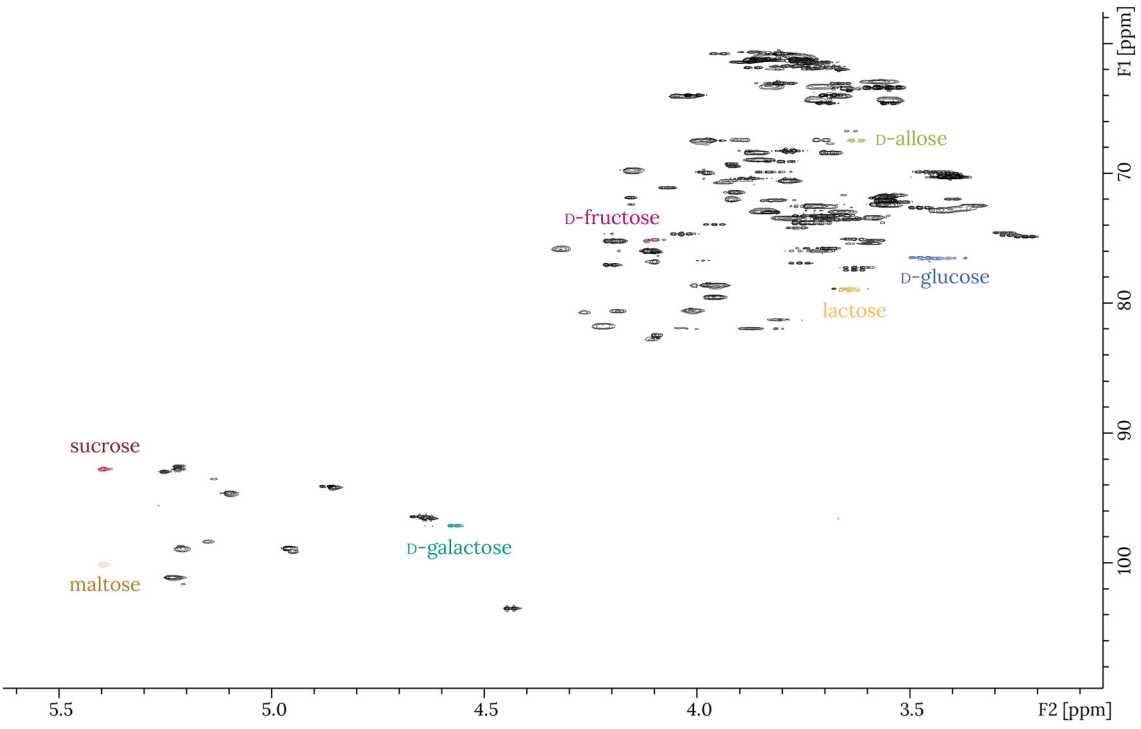

FIGURE 1 HSQC-spectrum of an aqueous solution of the sugars D-glucose, D-galactose, D-fructose, D-allose, sucrose, lactose, and maltose. Chemical shifts of the selected signals for quantification are quoted in Section 2.4. The measurement was carried out with the following acquisition parameters: pulse sequence "hsqcetgp," two scans, size of time domain 1024 in both dimensions, $D_1 = 1.5$ s: for more acquisition and processing parameters see Section 2.4. HSQC: heteronuclear single quantum coherence

operator. Limits of quantification (LOQ) were determined according to DIN 32645 using the calibration line method.[23]

Longitudinal relaxation times were acquired with the standard Bruker pulse program t1ir (inversion recovery experiment) with 16 scans, a spectral width of 19.99 ppm and an AQ of 0.819 s. We used aqueous solutions of the individual analyte ($c = 5.0$ g/L), mixed with 10% deuterium oxide (99.9%, Deutero, Kastellaun, Germany).

2.5 | Sample preparation

Milk-based cocoa drink (15 ml), strawberry flavored mixed milk drink (15 ml), lactose-free milk-based cocoa drink (12.5 ml), or mango flavored buttermilk (7.5 ml) were clarified by successive addition of (A) 4 ml of aqueous potassium hexacyanoferrate (II) solution (150 g/L, ≥99% Carl Roth, Karlsruhe, Germany) and (B) 4 ml of aqueous zinc sulfate solution (296 g/L, ≥97%, Sigma-Aldrich, St. Louis, USA). The pH was adjusted to 7.0 with sodium hydroxide solution. The supernatant was removed after centrifugation (9392 g, 10 min), the residue was washed with water, and the supernatants were combined. The volume was made up to 50 ml in a volumetric flask. Before measurement, an aliquot was filtered through a syringe filter (Teflon, 0.45 μm) and diluted according to the working range of the method used.

2.6 | Recovery experiments

To perform recovery experiments, the samples described above were spiked with the sugars that were previously identified in these samples. For these experiments, half the sample volume was used, and the analytes were added as an aqueous solution. The final analyte concentrations matched their contents in the total sample volume. In addition, analytes that were not found in the samples were added in amounts resulting in concentrations in the middle of the working range of the method used. Subsequently, the samples were prepared according to Section 2.5 and diluted corresponding to the method used.

2.7 | Statistics

For the statistical analysis of data generated by the different methods, a one-factor analysis of variance was performed in combination with a post hoc Tukey test in Origin 2019.

3 | RESULTS AND DISCUSSION

3.1 | Optimization of acquisition parameters

In order to reliably quantify D-glucose, D-galactose, D-fructose, sucrose, lactose, and maltose by using HSQC pulse sequences, the acquisition parameters had to be optimized, ensuring sufficient separation of the signals to be used for quantification (see Section 2.4 and Figure 1) as well as high precision and accuracy of the quantitative data. Different calibration strategies are possible for quantitative purposes. First, quantification was carried out using an external calibration. However, data showed only moderate precision (data not shown). Consequently, D-allose was used as internal standard for the NMR measurement. Analytes were quantified by determining integral ratios (analyte/internal standard) resulting in improved precision of the calibration data. Therefore, integral ratios of analytes to those from D-allose were used for quantification in the preferred method (Figure S1). Because the internal standard was not used to double-check the performance of the Carrez clarification but to improve reproducibility of the NMR data it was added after Carrez treatment.

Applied HSQC pulse programs and acquisition parameters that were used to optimize each pulse sequence (measurement of aqueous standard compounds with 10% D_2O) are shown in Table 1. For the selected

TABLE 1 Optimized acquisition parameters of the HSQC-methods

Acquisition parameter	Tested options	Selected option(s)
Pulse program	hsqcetgp hsqcetgpsp hsdqedetgp	hsqcetgp
Number of scans	2, 4, 8, 16	2
Size of time domain (^{1}H, ^{13}C)	1024, 256 1024, 512 1024, 1024	1,024, 1,024
Interscan delay (D_1) [s]	1.0, 1.5, 3.0, 4.5, 6.0, 7.5, 9.0	1.5, 3.0
Non-uniform sampling (NUS)		
NUS-sampling	unweighted exponentially weighted	unweighted
NUS-level [%]	10, 20, 30, 40, 50, 60, 70, 80, 90	50

Note: HSQC: heteronuclear single quantum coherence.

concentration range, signal-to-noise ratios ≥10 were determined for all analytes when two scans per increment were acquired. In order to maximize the resolution in the ^{13}C dimension the size of time domain was set to 1,024 allowing for a maximal separation of analyte signals. The influence of different interscan delays on the HSQC integrals of the analytes was tested in a series of measurements. As a starting point, the longitudinal relaxation times of the analytes were determined (Table S1) and, based on this, possible interscan delays were selected (Table 1). Because the integrals did not show any significant changes between $D_1 = 4.5$ s and $D_1 = 9.0$ s, the measurement of larger interscan delays was not performed. Integrals of the HSQC experiments with $D_1 = 3.0$ s did not show significantly smaller values than integrals that were determined from HSQC experiments with longer interscan delays. However, integrals obtained from HSQC experiments with an interscan delay of 1.5 s partially deviated from integrals of HSQC experiments with longer interscan delays. Therefore, both interscan delays (3.0 s and 1.5 s) were tested for measurements in dairy matrices (Section 3.2). The NUS-amount was set to 50% because at lower NUS-levels an increased formation of spectral artifacts was observed, which affect analyte quantification. The use of NUS therefore reduced the HSQC experiment time by 50%.

Interday and intraday precisions were analyzed as measures for the repeatability of the approaches. For this purpose, the individual concentrations of each analyte were considered. The NUS-HSQC method with an interscan delay of 1.5 s and a NUS-level of 50% was characterized by an interday precision ≤5.5% when all analytes (D-glucose, D-galactose, D-fructose, sucrose, lactose, and maltose) in concentrations of 2.5 to 10.0 g/L were considered. For the lowest concentration tested (1.0 g/L), an interday precision of max. 12.7% (D-fructose) was determined. The intraday precision of the four higher concentrations was generally ≤5.3%; for the concentration 1.0 g/L a maximum intraday precision of 9.4% was determined. Thus, by applying the optimized parameters the method can be performed precisely with, however, less precise measurements at the lower end of the working range. Consequently, the precision at a concentration level 1.0 g/L is not always fully satisfying because the measurements for a series of individual sugars showed higher standard deviations of the integrals than others. Hence, quantification is recommended at levels above 2.5 g/L, although (less precise) analyses between 1.0 and 2.5 g/L are still possible.

LOQs of the individual analytes as determined for the optimized methods in an aqueous solution are between 0.02 and 1.59 g/L (Table S2). However, as mentioned above, quantitative determination of concentrations below 1.0 g/L are not generally recommended due to the slightly impaired precision of the data. The LOQs determined for maltose are significantly higher than those of the other sugars, which cannot be explained at present. Because none of the products studied contains maltose natively, this is not a limitation of the methods.

In addition to HSQC methods, a time-optimized ASAP-HSQC pulse sequence was used.[20] Parameters that were optimized for the HSQC methods were applied to the ASAP-HSQC experiment (number of scans 2, time domain 1,024 data points, 50% NUS) and their suitability was confirmed by revaluation of parameters such as signal-to-noise ratio and artifact formation. The interscan delay could be reduced to 0.05 s. The NUS-coupled ASAP-HSQC method showed interday precisions of up to 55.7% when the lowest calibration concentration of 1.0 g/L was analyzed. Consequently, the calibration range was adjusted to concentrations between 2.5 and 10.0 g/L. In general, the ASAP-HSQC data were less precise as compared to those obtained from the HSQC method (interday precision ≤11.6% and intraday precision ≤11.9% at the concentration range 2.5 to 10.0 g/L). LOQs were between 0.05 and 1.01 g/L (Table S2). Again, due to the low precision at lower concentrations, quantification of concentrations below 2.5 g/L should be avoided.

The optimized methods take significantly less time as compared to the enzymatic and chromatographic reference methods (Table 2). The ASAP-HSQC pulse sequence coupled with NUS requires only 6 min to precisely measure all sugars in concentrations above 2.5 g/L. Next to using the ASAP pulse sequence the application of NUS

TABLE 2 Experiment times (t_{Exp}) for the quantification of D-glucose, D-galactose, D-fructose, sucrose, lactose, and maltose with the described methods

Method description	t_{Exp} [min]
Enzymatic assays (three different assays)	~60 min per assay
HPAEC-PAD (separation on two different columns necessary)	73 min and 60 min (see 2.3)
HSQC, $D_1 = 1.5$ s	53 min
HSQC, $D_1 = 1.5$ s, 50% NUS	27 min
HSQC, $D_1 = 3.0$ s	104 min
HSQC, $D_1 = 3.0$ s, 50% NUS	53 min
ASAP-HSQC	12 min
ASAP-HSQC, 50% NUS	6 min

Notes: All methods were performed with the same sample pretreatment, which was not included in the reported analysis times. HPAEC-PAD: high-performance anion-exchange chromatography with pulsed amperometric detection, HSQC: heteronuclear single quantum coherence, NUS: non-uniform sampling, ASAP: acceleration by sharing adjacent polarization.

largely contributes to the reduction of analysis times. Application of 50% NUS did not negatively affect calibrations in aqueous solutions. In order to investigate a potential negative impact of the application of NUS on the quantification in dairy matrices, in the following, all methods were also carried out without NUS.

3.2 | Application of the optimized methods to dairy products

The optimized HSQC methods (Tables 1 and 2) were used to quantify monosaccharides and disaccharides in dairy products (Figure 2). Due to matrix components such as proteins and lipids, a Carrez-based clarification of the samples was required. An alternative clarification strategy based on polymer precipitation by using ethanol and lipid extraction with hexane was less suitable than the standard Carrez-based clarification, which is also used in most reference methods (data not shown).

Besides applying the optimized HSQC and ASAP-HSQC based approaches, all dairy products were also analyzed by using the enzymatic- and the HPAEC-PAD-based reference methods. None of the analyzed dairy beverages contained maltose, and only mango-flavored buttermilk contained D-fructose. No differences in analyte

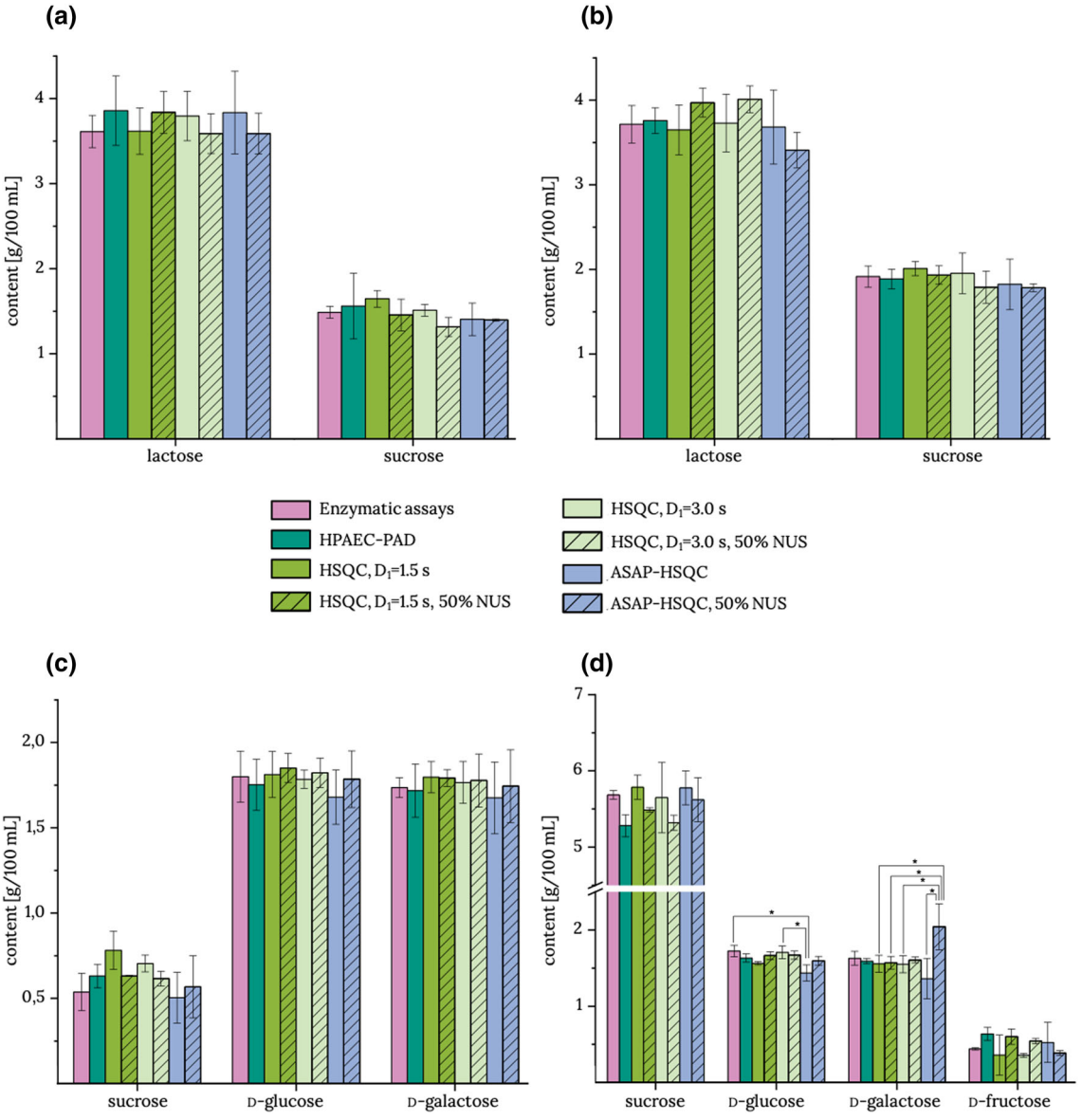

FIGURE 2 Contents of monosaccharides and disaccharides in the following products: (a) milk-based cocoa drink, (b) strawberry flavored mixed milk drink, (c) lactose-free milk-based cocoa drink; (d) mango flavored buttermilk. HPAEC-PAD: high-performance anion-exchange chromatography with pulsed amperometric detection, HSQC: heteronuclear single quantum coherence, NUS: non-uniform sampling, ASAP: acceleration by sharing adjacent polarization. * indicates statistically significantly different mean values (one-factor analysis of variance, $\alpha = 0.05$, post hoc Tukey test)

concentrations were found in three out of the four dairy beverages, regardless of the method used (Figure 2a–c). However, the sensitivity of the methods is not sufficient to quantify lactose in the lactose-free variant.

The use of the ASAP-HSQC method to quantify D-glucose and D-galactose in mango-flavored buttermilk resulted in significant differences compared to some of the other methods, whereas no differences were found among sucrose or D-fructose values (Figure 2d). However, the standard deviations of the determined fructose contents are relatively high, which can be explained by the fact that measurements are taken close to the LOQs. When determined via ASAP-HSQC without 50% NUS, the D-glucose concentration was significantly lower than the enzymatically determined concentration. The galactose concentration determined using ASAP-HSQC with 50% NUS was significantly higher compared to the value obtained with the other NMR-based methods. HSQC methods not based on the ASAP pulse sequence provided equivalent results to the reference methods for all sugars present, including D-glucose and D-galactose. Moreover, standard deviations were higher when values were obtained via ASAP-HSQC, especially when no NUS-coupling was used. Thus, NUS-coupling not only reduces experiment times, but may also increase precision and should therefore be included in HSQC methods for sugar analysis in dairy beverages.

The results of all other methods were judged to be sufficiently precise, and concentrations that were analyzed by using HSQC methods did generally not differ from those obtained by the application of the reference methods. This also held true for the fastest HSQC method ($D_1 = 1.5$ s, 50% NUS) with an analysis time of 27 min, demonstrating that a reduction of the interscan delay from 3.0 to 1.5 s appeared to be feasible without compromising accuracy (see also Section 3.1). The greatest time saving was achieved by using the ASAP-HSQC method with NUS, which quantifies the above-mentioned analytes equivalently to the reference methods in only 6 min. However, as mentioned before ASAP-HSQC data appeared to be less precise as compared to the normal HSQC data.

In addition to comparing HSQC and HSQC-ASAP data to those of reference methods, recovery rates were determined in order to judge accuracy. Recovery rates as obtained by the application of the HSQC and ASAP-HSQC approaches were generally acceptable and in the same range as the recovery rates of the reference methods (Table 3). Therefore, any analyte losses were probably caused by the sample preparation procedure, which was identical for all methods used. We investigated the influence of the Carrez clarification on analyte quantification and were able to determine that

TABLE 3 Recovery rates determined in the following dairy products: A: milk-based cocoa drink, B: strawberry flavored mixed milk drink, C: lactose-free milk-based cocoa drink; D: mango flavored buttermilk

	Recovery [%] ± standard deviation [%]									
	A		B		C			D		
	Lactose	Sucrose	Lactose	Sucrose	Sucrose	D-Glc	D-Gal	Sucrose	D-Glc	D-Gal	D-Fru
Enzymatic assay	88.5 ± 7.1	100.0 ± 7.2	80.7 ± 7.8	82.9 ± 6.9	97.4 ± 2.3	94.3 ± 3.0	93.1 ± 1.8	95.7 ± 2.5	90.7 ± 1.1	103.9 ± 1.3	85.4 ± 1.5
HPAEC-PAD	88.1 ± 5.9	88.4 ± 6.0	84.5 ± 11.4	82.2 ± 6.1	89.9 ± 6.1	81.3 ± 6.8	80.1 ± 5.5	92.7 ± 9.7	84.2 ± 7.3	83.9 ± 8.2	75.1 ± 3.7
HSQC, $D_1 = 1.5$ s	89.3 ± 6.2	84.7 ± 7.0	82.6 ± 12.5	81.0 ± 9.1	84.0 ± 1.6	79.9 ± 2.8	80.2 ± 1.4	100.7 ± 2.8	95.1 ± 2.5	92.9 ± 1.3	95.7 ± 7.2
HSQC, $D_1 = 1.5$ s, 50% NUS	91.8 ± 0.3	93.6 ± 2.4	87.5 ± 14.9	91.5 ± 8.1	90.4 ± 1.3	82.5 ± 3.6	86.7 ± 5.6	93.3 ± 2.8	89.2 ± 3.0	86.5 ± 4.7	80.9 ± 6.6
HSQC, $D_1 = 3.0$ s	86.8 ± 2.6	85.9 ± 1.1	89.2 ± 15.4	84.2 ± 14.4	87.9 ± 4.0	82.7 ± 4.2	85.0 ± 3.4	97.6 ± 1.8	89.3 ± 1.3	88.1 ± 2.4	87.4 ± 2.5
HSQC, $D_1 = 3.0$ s, 50% NUS	94.3 ± 0.5	94.3 ± 0.5	87.4 ± 12.0	87.4 ± 11.9	82.9 ± 6.0	80.1 ± 4.3	84.0 + 3.4	95.6 ± 2.0	88.7 ± 2.6	85.5 ± 3.6	82.4 ± 3.8
ASAP-HSQC	94.8 ± 3.2	89.3 ± 1.6	82.5 ± 13.7	76.4 ± 14.5	92.9 ± 4.5	83.3 ± 1.1	86.9 ± 0.5	96.9 ± 4.9	92.0 ± 2.2	90.8 ± 7.1	81.4 ± 4.4
ASAP-HSQC, 50% NUS	95.6 ± 3.7	95.5 ± 2.3	81.4 ± 11.9	83.5 ± 9.0	97.7 ± 9.1	85.0 ± 2.3	79.4 ± 5.6	101.9 ± 3.9	94.7 ± 1.5	78.1 ± 14.1	84.2 ± 3.1

Notes: HPAEC-PAD: high-performance anion-exchange chromatography with pulsed amperometric detection, HSQC: heteronuclear single quantum coherence, NUS: non-uniform sampling, ASAP: acceleration by sharing adjacent polarization, D-Glc: D-glucose, D-Gal: D-galactose, D-Fru: D-fructose.

systematically occurring analyte losses are caused by the Carrez clarification (data not shown). As briefly mentioned above, an alternative clarification strategy (polymer precipitation by using ethanol, lipid extraction with hexane) did not improve the results. We therefore used the lowest practicable concentration of Carrez reagents to keep the influence as minimal as possible.

In a second step, sugars that were not identified in the analyzed dairy products were added to the matrices, and recovery rates were determined. Again, these recovery rates were in the same range as the recovery rates determined using the reference methods (Tables S3–S6). This demonstrated that the optimized HSQC methods determined all sugars (D-glucose, D-galactose, D-fructose, sucrose, lactose, and maltose) with the same accuracy as the reference methods used. Application of the ASAP-HSQC methods appeared to be feasible but less accurate data were obtained from the buttermilk matrix (D-glucose, D-galactose), and slightly worse precision data may not outweigh the advantage of saving about 20 min as compared to the fastest HSQC approach used here. In addition, due to the high energy input during the ASAP-HSQC sequence it is currently problematic to automate this pulse sequence; automation of this pulse sequence is not recommended because of the possibility of damaging the probe head. Thus, for routine applications the HSQC method ($D_1 = 1.5$ s, 50% NUS) appeared to be most suitable among the methods tested here.

4 | CONCLUSIONS

HSQC experiments provide the necessary resolution to analyze common monosaccharides and disaccharides in food products. By optimizing the interscan delay to 1.5 s and using non-uniform sampling (NUS), we were able to quantitate D-glucose, D-galactose, D-fructose, sucrose, lactose, and maltose in concentrations from 1.0 to 10.0 g/L in only 27 min. Data are precise, and accuracy tested for a range of dairy products did not deviate from other methods commonly used to quantitate sugars in food products (enzymatic analysis, HPAEC-PAD). Furthermore, it was possible to optimize an ASAP-HSQC method with 50% NUS for the quantitation of sugars in dairy products within 6 min. However, data were slightly less precise, and the working range of the method had to be reduced (2.5 and 10.0 g/L). Taking into account the lack of automation of the ASAP-HSQC method, the NUS-HSQC method has the most potential to be used as a standard method in routine laboratories. The methods can also be used to quantify the analytes in additional products if sufficient resolution of the signals to be quantified is ensured. An extension of the sugars to be analyzed is also quite possible under the occurrence of a suitable signal for quantification.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

L.F.: Conceptualization, methodology, investigation, laboratory work, writing and editing; M.B.: Conceptualization, methodology, writing, supervision.

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