Short application note

Food & beverage



Sugars in dairy products based on ISO 22184

Keywords

ALEXYS Carbohydrate Analyzer, HPAEC-PAD, DECADE Elite, SenCell, SweetSep™ AEX20, ISO 22184, IDF 244, galactose, glucose, fructose, sucrose, lactose, maltose, dairy products, nutritional labeling.

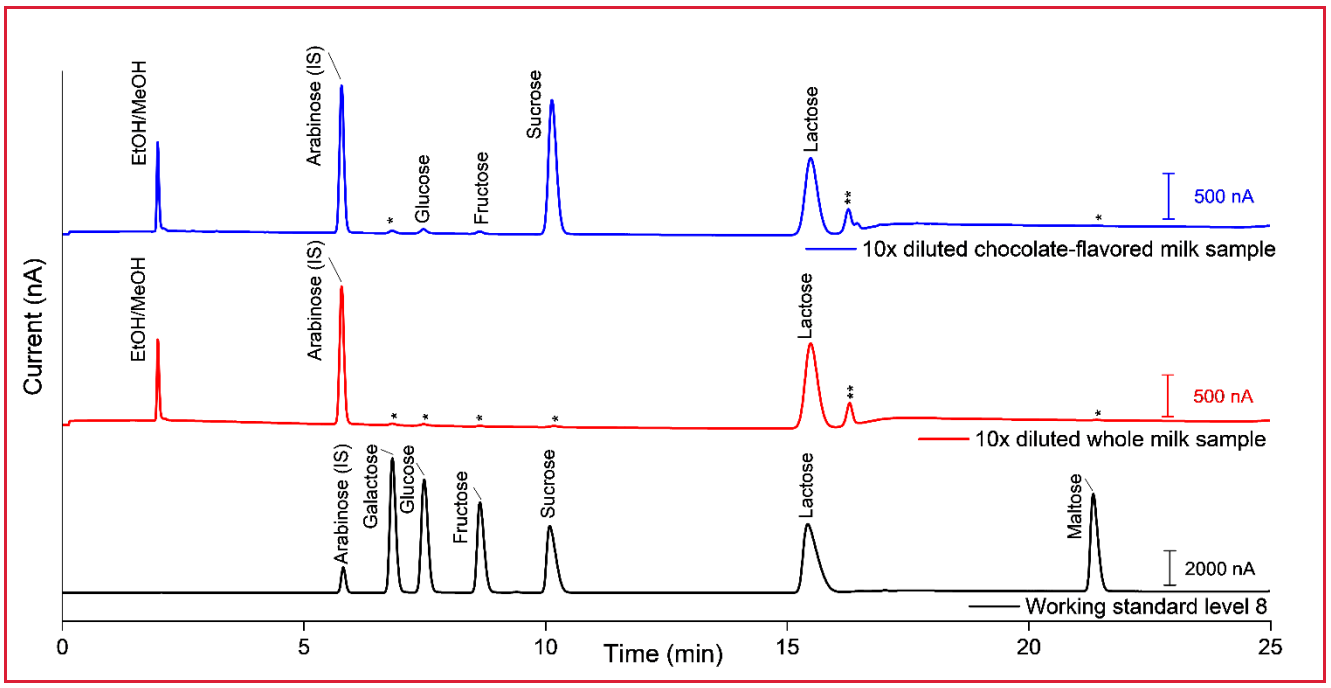


Fig. 1. Chromatogram of 1 µL injection of working standard level 8 (black trace, 260 mg/L galactose, glucose, fructose, and 400 mg/L sucrose, lactose, and maltose), 10× diluted whole milk sample (red trace), and 10× diluted chocolate-flavored milk sample (blue trace). EtOH/MeOH in the sample originates from the sample preparation described in ISO 22184, arabinose was added as an internal standard (IS), * = minor peak below the lowest calibration level, ** = unidentified peak from the matrix.

Introduction

Carbohydrates are present in nearly all dairy products. Lactose is the main sugar in natural milk, and smaller amounts of other mono- and disaccharides occur depending on the product and any added ingredients [1]. In the European Union, sugars are part of the mandatory nutrition labeling on food, including dairy products (Regulation (EU)



Fig. 2. ALEXYS Carbohydrate Analyzer.

Table 1. HPAEC-PAD conditions

HPAEC system	ALEXYS™ Carbohydrate Analyzer (Antec Scientific)
Columns	SweetSep™ AEX20, 2.1 × 50 mm precolumn, 5 µm SweetSep™ AEX20, 2.1 × 200 mm column, 5 µm Borate ion trap, 2.1 × 50 mm column, 10 µm (all columns Antec Scientific)
Mobile phases	A: DI water, B: 100 mM NaOH and C: 100 mM NaOH + 100 mM NaOAc Eluents blanketed with nitrogen 5.0
Flow rate	0.18 mL/min
Backpressure	About 225 bar
Injection volume	1 µL (partial loopfill, 2 µL loop volume)
Temperature	5°C for sample cooling (AS6.1L), 20°C for separation (CT2.1), 35°C for detection (DECADE Elite)
Flow cell	SenCell Au WE, HyREF Pd RE, AST setting 2
Potential waveform (4-step)	E1, E2, E3, E4: +0.1, -2.0, +0.6, -0.1 V ts, t1, t2, t3, t4: 0.2, 0.4, 0.02, 0.01, 0.07 s
Range	20 µA/V
I-cell	About 0.1 µA
ADF	0.5 Hz



Table 2. Gradient program

Time (min)	*Mobile phase*	*%A*	*%B*	*%C*	*Description*
0–12.5	10 mM NaOH	90	10	0	Elution & detection
12.5–21	100 mM NaOH	0	100	0	
21–26	100 mM NaOH, 100 mM NaOAc	0	0	100	Column clean-up and regeneration
26–31	100 mM NaOH	0	100	0	
31–45	10 mM NaOH	90	10	0	Equilibration, starting conditions

No 1169/2011) [2]. Therefore, producers and testing laboratories need a reliable analytical method for the mono- and disaccharides found in dairy products.

In 2021, the International Organization for Standardization (ISO) and International Dairy Federation (IDF) published ISO 22184 / IDF 244, a method that determines galactose, glucose, fructose, sucrose, lactose, and maltose in dairy products in one chromatographic run by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), using arabinose as an internal standard [1, 3]. The ISO 22184 method uses a post-column NaOH addition to improve sensitivity and stability of the PAD response [1], which requires a second pump.

This application note demonstrates an adaptation of the ISO 22184 method on the ALEXYS Carbohydrate Analyzer (Fig. 2) using the SweetSep AEX20 anion-exchange column, without post-column NaOH addition. The method shown in this application note meets all ISO 22184 resolution requirements (Table 3), and its detection limits are well below the lowest ISO 22184 calibration level.

Method

The analysis of the sugars in dairy based on ISO 22184 was performed on the ALEXYS Carbohydrate Analyzer. Adjustments to the gradient program were made to accommodate the different selectivity of the SweetSep AEX20 columns used in this application note. Additionally, the injection volume has been modified from 5 µL in ISO 22184, to 1 µL in this method to improve peak shapes and avoid column overloading, especially for highly concentrated standards/samples such as reported in the reference [4]. The experimental conditions and gradient program are described in Table 1 and Table 2, respectively.

Five dairy products (whole milk, buttermilk, chocolate-flavored milk, young Gouda cheese, and unsalted butter) were obtained from supermarkets in The Netherlands. The five dairy products are categorized as low sugar content samples (< 12 g/100 g product for galactose, glucose, fructose, and < 18 g / 100 g product for sucrose, lactose, and maltose) according to

Table 3. Chromatographic resolution requirements and results

Components	*Required resolution*	*Achieved resolution on working standard level 8*
Arabinose–galactose	> 1.5	4.8
Galactose–glucose	> 1.5	2.6
Glucose–sucrose**	> 1.5	8.2
Fructose–sucrose	> 1.5	4.5
Fructose–melibiose*	> 1.5	8.2
Glucose–fructose**	-	4.3

*Melibiose is not part of the working standard described in ISO 22184, but ISO 22184 requires R_s > 1.5. Therefore, a separate melibiose standard was injected, and it eluted at 11.4 min, between sucrose and lactose.
**The elution order of the carbohydrates is different than described in ISO 22184, therefore the resolution of glucose-sucrose pair was calculated and the resolution of the adjacent peaks glucose-fructose pair was included.

ISO 22184. These samples were processed according to the described ISO 22184 protocol, including an extraction step with ethanol/methanol in PIPES buffer, pH adjustments, and protein removal using Carrez I and Carrez II reagents.
Aliquot of the samples were also spiked with a working standard solution level 4 (35 mg/L arabinose, 26 mg/L galactose, glucose, fructose, and 40 mg/L sucrose, lactose, and maltose), using equal volumes of the aliquot and spiking solution, before the final 10× dilution in DI water. The spike was added to confirm peak identity. Additionally, the spike recovery values were evaluated to check for matrix effects on separation and detection. All samples were injected in triplicate, with four calibration standards injected after every eight sample injections to monitor system stability as prescribed by ISO 22184.

Method performance

ISO 22184 requires a chromatographic resolution for specific sugar pairs. Table 3 lists the chromatographic resolution requirements for each sugar pair and the resolution achieved with the highest working standard. All sugars are baseline separated, with the lowest resolution of galactose-glucose (R_s = 2.6) well above the required minimum resolution of 1.5.

Calibration was performed over the range of 0.52–260 mg/L for galactose, glucose, and fructose, and 0.80–400 mg/L for sucrose, lactose, and maltose over eight working standard levels [1]. The calibration curves were built using an average of triplicate standard measurements (bracketing) against arabinose response as the internal standard, and fitted with quadratic fit without forcing through the origin. As the calibration spans a 500-fold concentration difference from the smallest to the largest concentration, a 1/amount^2 weighting factor was applied to ensure accurate fitting of the lower calibration levels. This weighting factor reduced the relative



standard error (RSE) from 36–74% (calculated without the weighting factor) to 4–9% for all components across the full calibration range. All analytes gave a small quadratic term, indicating that the calibration curves are only slightly curved at level 8.

Repeatability was assessed from 10 consecutive injections of the highest calibration standard. Retention time RSD was 0.09% to 0.16% and peak area RSD was 1.19% to 1.36% across the six sugars.

The limits of detection (LOD) were calculated as the concentration at which the analyte response corresponds to 3 times the signal-to-noise (S/N) ratio, with noise measured in accordance with ASTM standards (average peak-to-peak baseline noise of segments of 0.5 minute). The noise was determined using a 5-minute section of the baseline of the lowest working standard injection between t = 40 min and 45 min. The limits of quantification (LOQ) were determined the same way as LOD, but using 10 times the S/N ratio instead of 3 times. The LODs for all sugars (except for arabinose) vary between 0.013 and 0.033 mg/L, while the LOQs vary between 0.04 and 0.11 mg/L, indicating a good sensitivity of the method. The lowest calibration level was used as the reporting limit for samples. This corresponds to about 0.03 g/100 g (for monosaccharides) or 0.04 g/100 g (for disaccharides) in the product.

Analysis of sugars in dairy products

Five commercial dairy products, whole milk, buttermilk, a chocolate-flavored milk, young Gouda cheese, and butter, were extracted following ISO 22184 procedure and analyzed in triplicate measurements. Fig. 1 shows representative chromatograms of the whole milk sample and a more complex chocolate-flavored milk sample. In these samples, an unidentified matrix peak (** in Fig. 1) elutes after lactose. Over the course of the triplicate measurements, retention time shifts and detector response decreases were observed. The detector responses of the internal standard in the first set of sample measurements were higher by about 10% compared to the last measurement. The first set of sample injections gave higher quantification results for the minor sugars than sets 2 and 3 (for instance, 0.08 g/100 g glucose in milk in set 1, and ≤ 0.005 g/100 g in sets 2 and 3), while the main sugars differed by less than 10%. Since the internal standard did not fully compensate for the response decrease at low concentrations, only the last two sample measurement sets were averaged and reported as results.

In the whole milk sample, only lactose was found above the lowest calibration level, consistent with its role as the principal sugar in natural milk. Buttermilk contained less lactose but more galactose than milk, in line with lactose fermentation during production. Chocolate-flavored milk contained mostly sucrose (about 6.2 g/100 g) and lactose (about 3.5 g/100 g), with about 0.05 g/100 g glucose and 0.03 g/100 g fructose. In butter, lactose was found (0.88 g/100 g). The young Gouda cheese sample has no sugars above the reporting limit, which is expected for a cheese in which lactose has largely been fermented.

Table 4 compares the total sugar content found (average value of duplicate measurements, based on the internal-standard-corrected result) with the nutritional label on the product packaging. The total sugar contents in all five samples are within the EU tolerances for nutritional labeling (± 2 g if below 10 g/100 g, or ± 20% if between 10–40 g/100 g) [5].

ISO 22184 states that the method is not applicable for samples with sugar concentration < 0.1 % weight and samples with low-lactose concentration (< 1 mg/g product). Additionally, ISO 22184 does not cover butter as a dairy product. Therefore, the results of the young Gouda cheese and butter are shown for information purposes. The Antec application notes 220_009 and 220_041 describe methods that are better suited for the analysis of trace amounts of sugars in dairy products [6, 7].

Recoveries of the spike in the samples are listed in Table 5. It should be noted that the spike was added after extraction, and therefore these values reflect matrix effects on separation and

Table 4. Total sugar content vs nutritional label

Sample	*Sugars above reporting limit*	*Total sugars* (n=2)*	*Nutritional label**
Whole milk	Lactose	4.33	4.6
Buttermilk	Galactose and lactose	3.38	2.3
Chocolate-flavored milk	Glucose, fructose, sucrose, lactose	9.78	12
Young Gouda cheese**	None	<0.10	0.0
Butter**	Lactose	0.88	1.0

*Sugar content is expressed as g/100 g product for solid samples or g/100 mL product for liquid samples
**Outside of the ISO 22184 sample scope: young Gouda cheese (total sugars < 0.1%), butter (matrix not covered), shown for information only

Table 5. Recovery (%) of the spike in the sample

Sample	*Gal*	*Glu*	*Fru*	*Suc*	*Lac*	*Mal*
Whole milk	101	101	96	90	97	96
Buttermilk	97	85	92	84	100	88
Chocolate-flavored milk	98	99	107	90	112	94
Young Gouda cheese**	90	83	89	61	83	86
Butter**	94	81	88	76	93	84

Gal = galactose, glu = glucose, fru = fructose, suc = sucrose, lac = lactose, and mal = maltose
**Outside of ISO 22184 sample scope: young Gouda cheese (total sugars < 0.1%), butter (matrix not covered), shown for information only



detection, and not the extraction step. The recoveries for all liquid samples (whole milk, buttermilk, and chocolate-flavored milk) are between 84–112%, while those for the solid samples (young Gouda cheese and butter) are slightly lower. Sucrose gave the lowest recoveries in all samples.

Finally, this application note adapted different measurement conditions, including column selection, gradient program, injection volume, calibration weighting, and the omission of the post-column hydroxide addition compared to the original ISO 22184 method. Laboratories should verify the performance for their own sample types before using the method for compliance purposes, including retention and response stability over the sequence and the separation of lactose from matrix peaks. Contact Antec for more information.

References

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Conclusion

A method based on ISO 22184 for the analysis of sugars in dairy was implemented on the ALEXYS™ Carbohydrate Analyzer with the SweetSep™ AEX20 column, using a single pump without post-column NaOH addition. All ISO 22184 resolution requirements were met (the lowest resolution of 2.6 between galactose–glucose) in a 45-minute cycle time (ISO method: 52 minutes). Detection limits of 0.013 to 0.033 mg/L for all 6 sugars (arabinose excluded) are well below the lowest calibration level. Sugar contents measured in all five samples ranging from whole milk to butter were within the tolerance range of the nutritional product labels. Spike recoveries were 84–112% in the liquid products, and lower in the solid products. These results demonstrate that the presented method offers a simpler, single-pump alternative for the ISO 22184 method for sugar analysis in dairy products.



Table 6. Ordering information

ALEXYS analyzer	
180.0057W	ALEXYS Carbohydrate Analyzer - gradient (quaternary LPG)
116.4321	SenCell 2 mm Au HyREF
186.ATC00	CT2.1 Column Thermostat
Columns	
260.0026	SweetSep™ AEX20, 2.1 × 50 mm precolumn, 5 µm
260.0021	SweetSep™ AEX20, 2.1 × 200 mm column, 5 µm
260.0031	Borate ion trap, 2.1 × 50 mm column, 10 µm
Software*	
195.0035	Clarity CDS single instr. incl. LC, AS module

*) The ALEXYS Carbohydrate Analyzer can also be controlled by Thermo Fisher Scientific Chromeleon™ CDS and Agilent OpenLab CDS. Please contact Antec Scientific for more details.

Table 7. Reagents, standards and sample prep accessories

NaOH (50% w/w, certified)	Fisher Scientific, pn* SS254-500
Sodium acetate trihydrate, HPLC grade	Fisher Scientific, pn 10122400
DI water 18.2 MΩ·cm, TOC < 5 ppb	Young In Chromass Aquapuri Essence+ 393
Fructose	Sigma-Aldrich, pn F0127
Glucose	Sigma-Aldrich, pn G8270
Sucrose	Sigma-Aldrich, pn S9378
Maltose monohydrate	Sigma-Aldrich, pn M5885
Galactose	Sigma-Aldrich, pn G0750
Lactose monohydrate	Sigma-Aldrich, pn L3625
Arabinose	Sigma-Aldrich, pn A3131
Melibiose	Sigma, pn M5500-5g
Acetonitrile	Fisher, pn A955-212
Ethanol	VWR, pn 83813.360
Methanol	Fisher, pn 10767665
Glacial acetic acid	Merck, pn 1.00056.2500
Piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES)	Sigma, pn P8203
Potassium hexacyanoferrate (II) trihydrate	Fluka, pn 60280
Zinc acetate dihydrate	Sigma, pn 96459
Syringe filter	0.22 µm PES (Polyethersulfone) 25 mm Ø FFL/MLS
Nitrogen 5.0 (purity 99.999%)	Messer Netherlands, pn 100542102

*pn = part number

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For research purposes only. The information shown in this short application note is solely to demonstrate the applicability of the ALEXYS system and DECADE Elite detector. The actual performance may be affected by factors beyond Antec's control and may be adjusted accordingly. Specifications mentioned are subject to change without further notice.

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