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Improving on AOAC 2001.02: GOS Determination in Foods Using HPAEC–PAD

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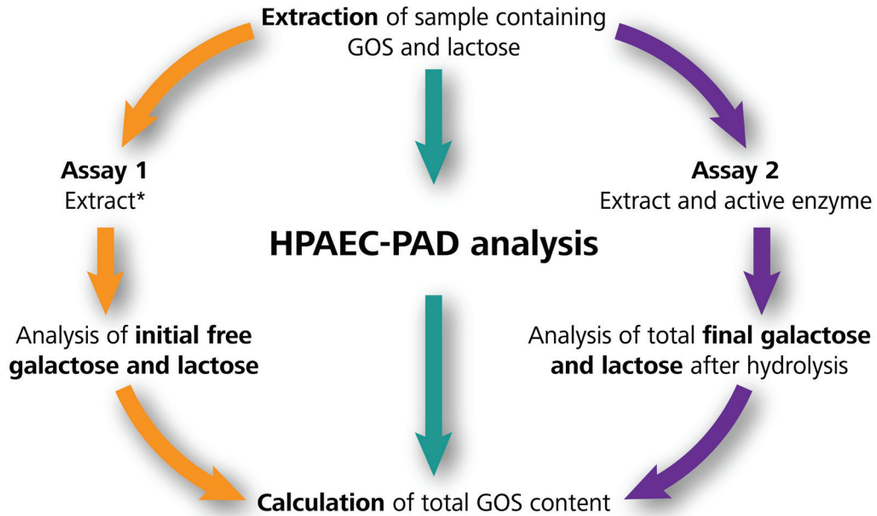
Our diet is critical for our health. Interest has increased in food additives and dietary supplements such as prebiotics like galactooligosaccharides (GOSs). The determination of total GOS contents in food and supplements is essential to fulfil strict food labelling and safety requirements. The most widely used method for total GOS determination is based on enzymatic hydrolysis to break down the complex molecules into simple carbohydrates prior to their chromatographic analysis. This article outlines the advantage of using an improvement to method AOAC(2001.02) using high performance anion exchange chromatography with pulsed amperometric detection (HPAEC–PAD) and full sample automation after enzymatic hydrolysis.

The β-galactooligosaccharides (GOSs) are oligosaccharides composed of a chain of galactose units and an optional terminal glucose unit. GOSs are a natural component of human and animal colostrum. At 5–12 g/L GOS, they are a major constituent of human breast milk and increasingly added as a supplement to infant formulas to achieve similar benefits (1,2). GOSs are also present in small amounts in various other kinds of foods and beverages, and are very resistant to hydrolysis by our digestive process (1). They reach the colon and are selectively consumed by *bifidobacteria* and *lactobacilli*, supporting the growth of these non-pathogenic bacteria (3,4). Substrates

Figure 1: Schematic stepwise procedure for the determination of total GOS content. Contrary to AOAC, the improved method uses the extract directly for measuring initial galactose and lactose concentrations, without the deactivated enzyme treatment. Analysis is done after anion separation by pulsed amperometric detection, as described in the Experimental section. The total GOS content is calculated from the galactose and lactose contents in the initial and hydrolyzed (final) solutions.



*AOAC(2001.02): Extract plus deactivated enzyme

with such properties are well known as prebiotics.

Prebiotics are reported to result in some health benefits (5–7). According to Global Market Insights, Inc., the global prebiotic market is expected to surpass 8.5 billion USD by 2024 (8). GOS supplements are available either raw, or as powders or syrups with concentrations of 50–70%, and are subsequently used by food manufacturers to enrich consumer products or sold as supplements (1,9).

Legislation of GOSs

Studies about the health effects of GOSs or fructooligosaccharides (FOSs) recommend maximum doses of 15–25 g/day (10), also supported by the World Health Organization. For example, the daily dose of the supplement Bimuno contains 2.9 g GOS. In the United States, several GOS products are generally recognized as safe (GRAS) by the Food and Drug Administration (FDA) (11). As infant formulas fall under dietary foods, they must meet stricter criteria and thus the use of GOSs and FOSs in such products is regulated, for example, in Switzerland and the European Union. The maximal allowance is 0.8 g/100 mL, as a combination of 90% GOS and 10% FOS (11–13). Otherwise, no limits regarding the GOS amount in food or as nutritional supplements are given.

Table 1: Tested samples with their total GOS content and other given relevant ingredients according to the supplier

Sample	GOS %	Other information
Aptamil "HA 1"	5.1	51.8% total sugars (lactose only)
Bimuno Daily	79.7	15.6% other sugars
FOS Powder	>95	total fructose, sucrose, glucose content is ~5%
GOS Powder	min. 70	max. 30% lactose and monosaccharide content; max. 3.5% water content
Customer samples	23–25	8–10.5% lactose
Vivinal GOS Powder	28.5	0.5% galactose; 36% lactose; all values given on dry matter
Vivinal GOS Syrup	59	1.4% galactose; 19% lactose; all values given on dry matter

Standard Determination Method for Total GOS Content

Currently, the most widely used method for the determination of total GOS content in food products is the AOAC Official Method 2001.02 (14). This method is based on the extraction of GOSs from a sample followed by enzymatic hydrolysis of the oligosaccharides into monosaccharides and their subsequent analyses with high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD).

The working principle behind AOAC(2001.02) is the comparison of a control solution (treated with the deactivated enzyme) with one which has been treated and hydrolyzed with the active enzyme β-galactosidase from *Aspergillus oryzae*. The enzyme catalyzes the splitting of glycosidic bonds and hydrolyzes GOS and lactose into glucose and galactose. The concentration differences of free galactose and lactose determined in these two solutions is used to calculate the total GOS concentration (Figure 1).

The AOAC(2001.02) method is a fully validated standard procedure. Nevertheless, the sample preparation is rather complex and could profit from improvements. One shortcoming

Figure 2: Overlaid chromatograms of Bimuno, untreated (black) and treated with enzyme (orange).

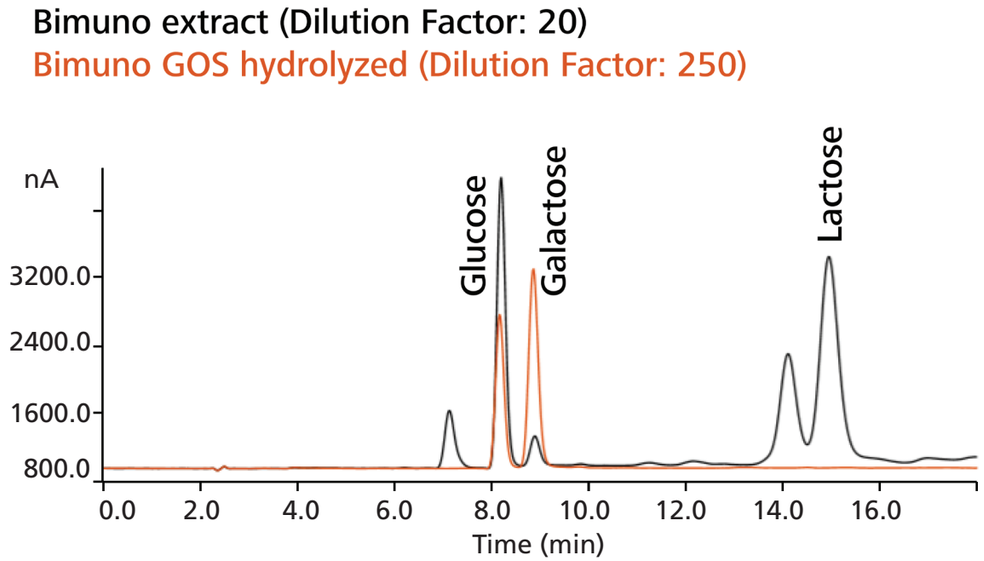
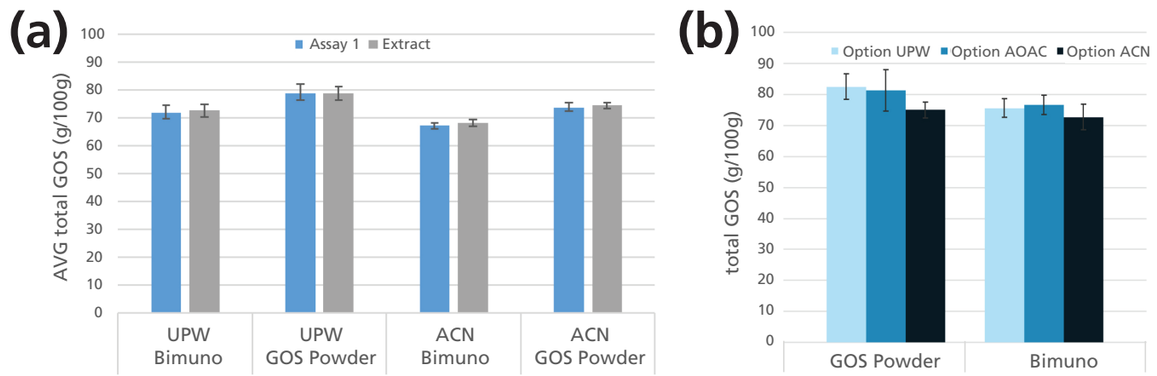


Figure 3: (a) Comparison of average total GOS contents determined with either Assay 1 (blue, with deactivated enzyme) or the extract (grey) with error bars indicating standard deviation. For both samples, no significant difference between the total GOS calculation are visible. This finding is true for dilutions in UPW and in ACN. (b) Reproducibility of results for samples of GOS Powder and Bimuno, showing the average total GOS content obtained with the three different options regarding calibration and sample dilution medium (Option UPW n=7, Options AOAC and ACN n=4 for GOS Powder and n=6 for Bimuno).



is the incubation of the control solution with the deactivated enzyme, a rather expensive substrate, to determine the initial sugar concentrations (Figure 1) rather than using the pure extract. The second critical point after thorough evaluation is the sample dilution procedure. According to AOAC(2001.02) this is done in acetonitrile, while standards are based on ultrapure water.

The present study focused on simplifying the entire procedure to increase the ease of use and the overall efficiency of the method.

Experimental

Samples were prepared according to AOAC(2001.02), however they were not stirred during incubation but manually mixed in defined time steps. After extraction and incubation, samples were centrifuged (Sigma 2-16KL centrifuge) and diluted without prior filtration. Sample dilution was performed in 20% acetonitrile (ACN, puriss. p.a., ≥99.5%, Sigma Aldrich) (AOAC procedure) or in ultrapure water (UPW, resistivity >18 MΩ·cm [25 °C], type I grade) supplied by a Milli-Q water purification system (Merck), as a modification.

Measurements were performed on a 940 Professional IC Vario TWO/SeS/PP equipped with a 858 Professional Sample Processor (Metrohm). The Metrosep Carb 2 - 250/4.0 separation column was used in combination with a 945 Professional Detector Vario – Amperometry (Metrohm) equipped with a Thin-Layer cell (50 µm spacer, 3 mm gold [Au] working and palladium [Pd] reference electrodes). As mobile phase, a thoroughly degassed sodium hydroxide eluent (NaOH, prepared from 50–52% sodium hydroxide, Sigma Aldrich) with sodium acetate (anhydrous, ≥99.0%, Sigma Aldrich) was used. Inline dialysis with a Low Volume dialysis cell (Metrohm) was utilized as a fully automated step within the chromatographic run. This step was used to further purify the sample before injection into the chromatographic system to remove proteins and larger molecules from the sample matrix, thus protecting the column and increasing its lifetime. All data was collected and automatically processed with MagIC Net 3.3 Professional software (Metrohm).

Standards and Samples: Calibration standards were prepared from sugar powders purchased from Sigma Aldrich and UPW (Merck): glucose (≥99.5%), and galactose (≥98%) in a concentration range of 2–32 mg/L, and lactose monohydrate (≥99.5%) in a range of 3.6–57 mg/L.

Commercially available standards and food products were used as analytical

Table 2: Total GOS content determined with the modified AOAC(2001.02) method for five different samples with relevant statistical data

Sample	Total GOS target (g/100 g)	Total GOS (n) (g/100 g)	Recovery (%)	AVG Spike 1 (g/100 g) (Recovery %)	AVG Spike 2 (g/100 g) (Recovery %)	Repeatability day-to-day Total GOS (g/100 g)	RSD (%)
Bimuno	79.7	75.7 ± 3.0 (n=7)	95	36.8 ± 1.4 (98%)	88.4 ± 12.7 (96%)	76.8 ± 0.6	0.6
GOS Powder	>70	82.6 ± 4.1 (n=7)	–	–	–	86.2 ± 1.1	1.2
Vivinal Powder	28.5	27.8 ± 0.5 (n=4)	98	37.8 ± 0.1 (91%)	48.6 ± 0.1 (91%)	28.5 ± 0.3	1.0
Aptamil	5.1	8.4 ± 2.0 (n=7)	–	2.6 ± 0.1 (108%)	–	9.1 ± 0.3	3.6
Customer sample	22	23.5 ± 0.1 (n=4)	107	11.4 ± 0.1 (101%)	21.7 ± 0.1 (101%)	–	–

samples. GOS powder was obtained from Carbosynth Ltd. (Compton, UK). FOS powder from chicory root was purchased from Megazyme Ltd. (Bray, Ireland). The infant nutritional formula Aptamil "HA 1" was bought in a Swiss supermarket. Bimuno Daily was directly purchased from Clasado Biosciences (Reading, UK). Vivinal GOS Powder and Vivinal GOS Syrup were provided by FrieslandCampina Domo (Amersfoort, the Netherlands). Additional samples were provided by European customers. The samples and their GOS content according to the suppliers are listed in Table 1.

Results and Discussion

Determination of the galactose and lactose concentration is crucial for the calculation of the total GOS content. Therefore good separation as well as verification that any coelution with other compounds can be excluded is mandatory for proper total GOS quantification. Separation is achieved in 18 min using the Metrosep Carb 2 - 250/4.0 column at 30 °C with a 250 mM NaOH eluent at a flow rate of 0.8 mL/min (Figure 2). A high-pressure gradient after 18 min with 500 mM NaOAc ensures a clean column within a total measurement time of 33 min.

Improvements on AOAC(2001.02)

Enzyme usage: Tests were performed to check if using the extract directly as a control solution instead of using extract treated with the deactivated β-galactosidase is sufficient. The deactivated enzyme in the control sample was found to have no effect on the final results (Figure 3), rendering it an unnecessary and somewhat costly addition. Significant time is saved by eliminating the enzyme deactivation procedure, using the diluted extract in Figure 1 instead.

Acetonitrile: In the official AOAC method, calibration standards are prepared in UPW but samples are diluted in 20% acetonitrile. If the galactose and lactose content from dilutions of samples in UPW are similar to those which were diluted in 20% acetonitrile, this reagent could be removed. A control experiment was performed and the results compared:

- Dilutions in UPW evaluated with UPW calibration ("UPW option")
- Dilutions in acetonitrile evaluated with UPW calibration (AOAC Official Method procedure)
- Dilutions in acetonitrile evaluated with acetonitrile calibration ("ACN option").

Regarding calibration, standards prepared with 20% acetonitrile exhibited higher sensitivity compared to standards in UPW, but acetonitrile standards also had a higher standard deviation. The reproducibility of the total GOS content was compared among the three different options (Figure 3b).

For the GOS powder test sample, the highest average of total GOS (82.55 g/100 g) was obtained with the UPW option, which also lies in the range of the AOAC option. As no reference values of the total GOS content are given by the manufacturer, a clear statement regarding the better option cannot be made. The UPW and AOAC preparation options exhibit similar results, but the ACN option results in lower total GOS contents than the others. To conclude, the UPW and AOAC options provide similar results and therefore, it is legitimate to use the UPW option for sample preparation.

Tests were performed to determine if the acetonitrile had a stabilizing effect on samples, but this proved not to be the case. This supports the improvement of the AOAC method by performing sample dilutions with UPW instead of acetonitrile.

Automation: Automation possibilities introduced in this method also save time and money for analysts. Manually intensive, time-consuming preparation and cleaning steps are eliminated, and valuable assets such as the column are protected with, for example, Inline Dialysis.

Validation: For the modified method using

the Metrosep Carb 2 - 250/4.0, a validation according to AOAC(2001.02) Appendices L and F (15,16) was performed, that is, running replicate standards to determine variability within a day, running samples for several days (day-to-day variability), as well as spiking with the GOS powder for spike recovery tests. Overall validation criteria (15,16) were reached (Table 2) and showed the successful application of the modified method.

Conclusion

The market of GOS-containing products is continuously growing and with it, the need for suitable analytical detection techniques. With the newly developed and validated HPAEC-PAD method, an improved alternative to the overall accepted AOAC Official Method 2001.02 is available. The optimized method is suitable to determine total GOS in selected products by analyzing the galactose and lactose concentration before and after enzymatic hydrolysis.

This improved method benefits users with simplified sample preparation, which saves time and reagents, and lowers the cost per sample. Aside from infant formulas, all samples exhibited high reproducibility as well as short-term repeatability. Generally, the method is reliable for samples containing high levels of GOSs, while for infant formulas (with a low GOS-to-lactose ratio) the determination of total GOS content turned out to be challenging, which is also a problem for AOAC(2001.02).

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Carmen Ziegler joined the Metrohm family in autumn 2019 to complete her Master's thesis under the guidance of the Competence Center Ion Chromatography about the method development for GOS analysis. After successfully obtaining her Master's degree in Chemistry from ETH Zürich, Switzerland, she began working in the Systems Engineering department at Metrohm AG where she focused on software testing. She now works at Solvias AG in Kaiseraugst, Switzerland. Elke Süss is currently an application specialist in the Metrohm Competence Center for Ion Chromatography. With an M.Sc. in Geoecology, she received a Ph.D. in Environmental Geochemistry in 2011 from Bayreuth University, Germany. After a position as laboratory manager at Bayreuth University, she worked as a Research Assistant and then PostDoc at ETH Zürich and Eawag focusing on trace elemental speciation in various environmental matrices. In 2018, she joined Metrohm AG as a member of the Ion Chromatography team.

Alyson Lanciki is currently the Scientific Editor of Metrohm AG. In 2010, she received her Ph.D. in Analytical and Environmental Chemistry from South Dakota State University, USA. After a post-doctoral position in France, Alyson joined the Metrohm family in 2013 as an ion chromatography application specialist in the Netherlands at Metrohm Applikon, where she eventually became the global marketing manager for the Metrohm Process Analytics brand of instruments.

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