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Evaluating raffinose family oligosaccharides and their decomposition products by ion chromatography – a method development and advanced repeatability study

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ABSTRACT

During the germination of legumes, raffinose family oligosaccharides (RFOs) are decomposed into mono- and disaccharides. As legumes are a traditional part of the human nutrition, storage carbohydrates are generally incorporated in the daily meals and can cause flatulence or even abdominal pain. However, their decomposition products can be metabolized without any difficulty. To date, no validated method is available to quantify the main decomposition products, glucose, and galactose simultaneously with the RFOs and the starch decomposition product maltose in a single measurement without derivatization. To provide a suitable method and fill the existing knowledge gap, a highly sensitive ion chromatography method was established. By using an optimized gradient, the separation of the RFOs, namely raffinose, stachyose, and verbascose, from their decomposition products, including glucose, galactose, fructose, and saccharose was accomplished. An advanced repeatability study proved that the method could be used for up to 4 days until the sample or eluent degradation made further measurement unfeasible. To improve the repeatability while avoiding to overload the chromatogram with several internal standards, a mathematical approach was presented to cope with different decomposition characteristics of the analytes and the internal standard. This study presents a modified and validated method allowing the measurement of all relevant carbohydrates in the germination of legumes. By using only a single method, a high number of samples can be processed. Therefore, a deeper and more detailed insight into the changes in the carbohydrate spectrum during the germination of legumes becomes feasible.

Introduction

During legume germination, the spectrum of carbohydrates changes profoundly. The raw seeds mainly contain saccharose and the RFOs raffinose, stachyose, and verbascose [4,8,21,29]. However, at the beginning of the germination, the RFOs are decomposed into the mono- and disaccharides galactose, saccharose, glucose, and fructose [20], which are further metabolized by the germ during growth ([7]). As many legumes p. e. faba beans also contain starch [4,12], maltose should also be considered as an additional degradation product. The simultaneous measurement of the sugar spectrum enables the assessment of the impact of different durations of germination. Moreover, it allows to optimize the degradation of RFOs while avoiding substantial loss of mono- and disaccharides due to germ metabolism. Possible applications of an optimized degradation of RFOs are the production of food and beverages. There, the RFOs as flatulence causing agents should be reduced to a minimum, while losses in biomass due to a prolonged germination need to be prevented. Furthermore, the preservation of mono- and disaccharides might be important as carbon source, easily available for microorganism in a subsequent microbial fermentation. A different aspect is the research and better understanding of the germ metabolism itself, by simultaneous monitoring of the RFOs and their decomposition products. A new approach was required as various methods are described in the literature, but none facilitates the measurement of RFOs and the relevant decomposition products as well as maltose without derivatization. Oligo-, mono-, and disaccharides were separated by high performance liquid chromatography with refractive index detector (HPLC-RI) [3,7,10,15,16,18,19,22,25], by ion chromatography [1,2,7–11,13,17,23,26,30], by size exclusion chromatography with refractive index detection (HPSEC-RI) [11], and by liquid chromatography with mass spectrometry (LC-MS) [6]. Although RFOs could be separated by conventional methods, no validated method could

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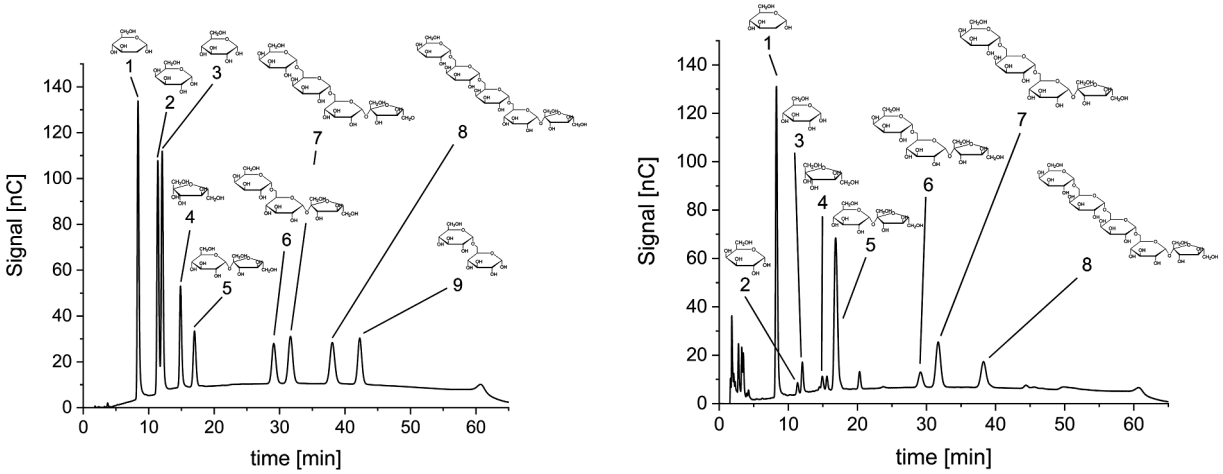

Fig. 1. Chromatograms showing analytical standards (left) and lupines, germinated for 3 days (right). Indicated are 2-deoxy-D-glucose (ISTD) (1), galactose (2), glucose (3), fructose (4), saccharose (5), raffinose (6), stachyose (7), verbascose (8), and maltose (9).

Table 1
Results of the analytical validation.

Carbohydrate	linear range [µg/mL]	coefficient of determination r^2 [-]	LOD [µg/mL]	LOQ [µg/mL]	recovery
Galactose	12.5–562.5	0.997	0.74	2.48	88%
Glucose	12.5–562.5	0.997	0.72	2.40	86%
Fructose	12.5–562.5	0.997	1.70	5.67	88%
Saccharose	12.5–562.5	0.996	3.15	10.49	109%
Raffinose	12.5–562.5	0.997	4.36	14.53	85%
Stachyose	12.5–562.5	0.994	3.86	12.85	85%
Verbascose	12.5–500	0.993	4.46	14.88	87%
Maltose	12.5–500	0.986	4.08	13.61	87%

distinguish between galactose and glucose while also measuring the other carbohydrates relevant in the germination of legumes. Therefore, most methods quantify either galactose or glucose or the sum of both. Nevertheless, it could be assumed that galactose is metabolized primarily while glucose accumulates in the first days of the germination until RFOs and galactose are depleted. Hence, simultaneous monitoring of galactose and glucose along with RFOs would allow a deeper insight into the metabolism of legumes instead of merely evaluating RFO degradation. As a different approach, gas chromatography (GC) was successfully used to quantify glucose and galactose among other monosaccharides and RFOs in legumes [24,26,27,32]. However, time-consuming and laborious derivatization of the samples is required to measure the carbohydrates by GC [26]. To date, no method has been able to determine all described RFOs and their degradation products without derivatization.

In germination experiments, various samples are taken from multiple biological replicates over an experimental duration of several days to a few weeks. Therefore, a simple, stable, and reliable method is required to analyze RFOs and their degradation products over a broad range of concentrations.

This study presents the first method to determine the RFOs (raffinose, stachyose, verbascose) and their individual degradation products (galactose, glucose, saccharose, fructose) along with maltose using ion chromatography with pulsed amperometric detection (HPAEC-PAD). Furthermore, an advanced repeatability study proved that a high number of samples could be measured in a single setup by applying a mathematical adjustment to compensate the degradation of the analytes.

Material and methods

Chemicals

The analytical standards galactose (purity ≥ 99%; CAS 59-23-4), glucose (purity ≥ 99.5%; CAS 50-99-7), saccharose (purity ≥ 99.5%; CAS 57-50-1), fructose (purity ≥ 99%; CAS 57-48-7), raffinose (purity ≥ 98%; CAS 17,629-30-0), stachyose (purity ≥ 98%; CAS 54,261-98-2), verbascose (purity ≥ 97%; CAS 546-62-3), maltose (purity ≥ 99%; CAS 6363-53-7), and 2-deoxy-D-glucose (purity ≥ 98%; CAS 154-17-6) were purchased from Sigma-Aldrich (Steinheim, Germany). 50% sodium hydroxide solution (CAS 1310-73-2) for the eluent was acquired from VWR International (Darmstadt, Germany). Methanol (purity ≥ 99.8%; CAS 67-56-1) for the extraction of carbohydrates from lupine samples was obtained from VWR International (Darmstadt, Germany). Lupine seeds (*Lupinus angustifolius* BOREGIN) were received from Saatzucht Steinach (Steinach, Germany). Water used for dilution and buffers was membrane filtrated with a micropore water purification system (Thermo Fisher Scientific Inc., Waltham, USA).

Analytical device

The HPAEC system consisted of an ICS AS/AP autosampler (non-cooled), an ICS 5000 DP pump module, and an ICS 5000 DC column compartment (all from Thermo Fisher Scientific Inc., Waltham, USA). The analytical column was a Dionex CarboPac PA100 column (2 × 250 mm), and the guard column was a Dionex CarboPac PA100 column (2 × 50 mm) (both Thermo Fisher Scientific Inc., Waltham, USA). The electrochemical detector cell comprised of a titanium cell body, a disposable gold working electrode, and a pH-Ag/AgCl reference electrode. PAD was used throughout the measurements as per the following settings: 0.1 V at 0.00 s; 0.1 V at 0.40 s; −2.0 V at 0.41 s; −2.0 V at 0.42 s; 0.6 V at 0.43 s; −0.1 V at 0.44 s; −0.1 V at 0.50 s. Data processing was performed with Chromeleon 7.2 software from Thermo Fisher Scientific Inc. (Waltham, Germany).

Chromatographic optimization

The mobile phase consisted of 0.145 M sodium hydroxide (A) and HPLC grade water (B). The final gradient was 10% A at 0 min, 25% A at 10 min, 25% A at 12 min, 95% A at 52 min, 95% A at 56 min, 10% A at 57 min, followed by 8 min of equilibration at 10% A. A concave curve (curve 6 in Chromeleon 7.2) was applied for the changes in the eluent ratio. The injection volume was 1 µL and the flow rate was 0.25 mL/min. The total run time of the gradient was 65 min. Considering a lag time of

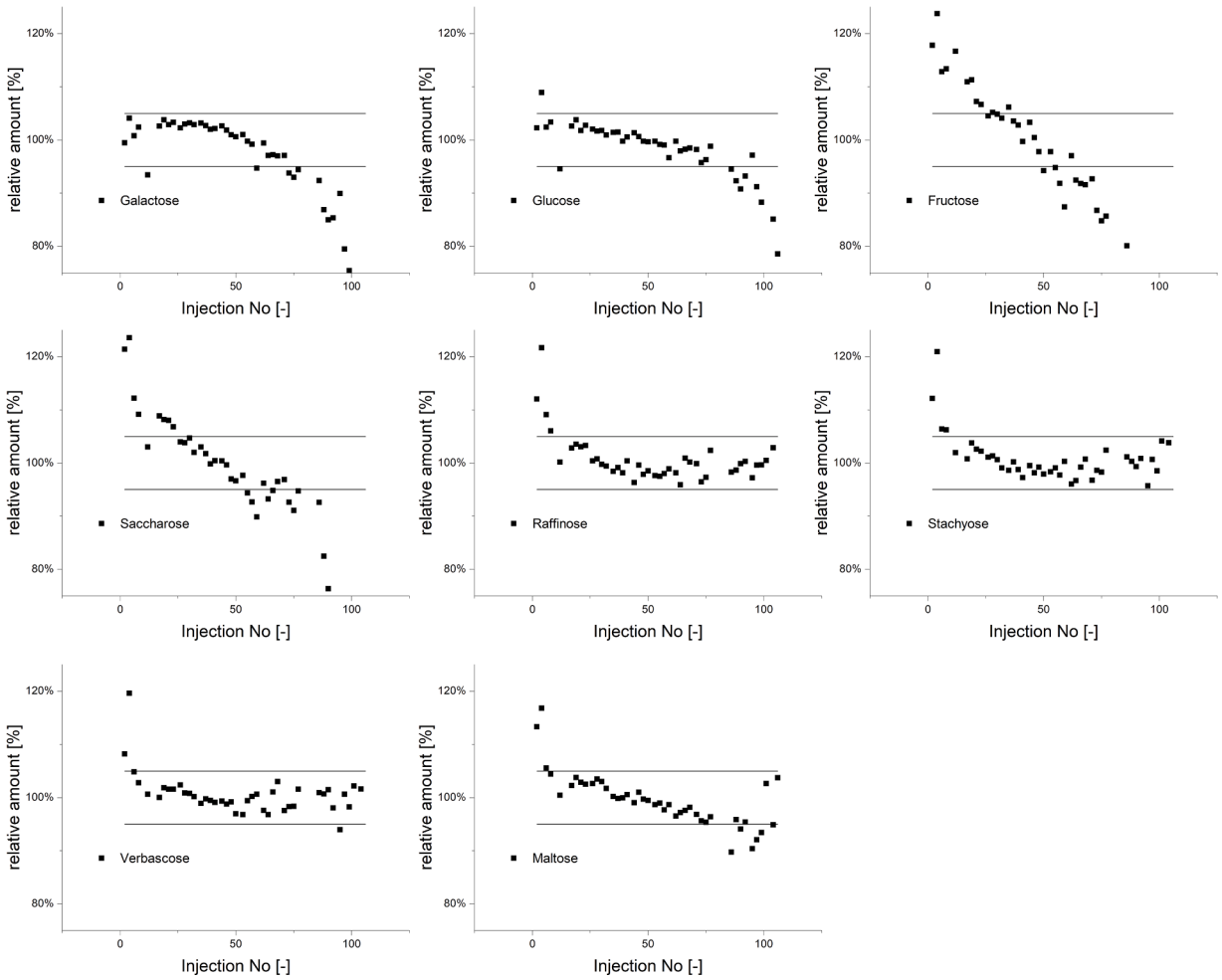

Fig. 2. Relative amounts of RFOs and their degradation products during 106 injections. The continuous lines represent 95% and 105% of the respective mean values.

3–8 min between the injections for flushing the autosampler, injection loop etc., a total run time of 68–73 min per injection was required.

Sample preparation

The impact of the legume matrix on the measurement was identified by the recovery analysis. Therefore, lupines were germinated isothermally at 20 °C for 5 days. The seeds were initially soaked for 3.5 h, and for another 10 min at the three following days at 20 °C, respectively. After soaking, the seeds were placed in a temperature regulated germinator on wet fleece paper. The ratio of germinating to not-germinating seeds was calculated from a representative sample of a minimum of 85 seeds and reached 91% at day 5. Samples for the recovery analysis were frozen at −20 °C, freeze-dried, and ground to pass through a 0.5 mm mesh. 1 g of the resulting flour was mixed with 30 mL of 50% methanol, extracted for 30 min at ambient temperature on a shaker, and consecutively centrifuged at ambient temperature for 10 min at 4400 g. An extraction time of 30 min was chosen as no differences were observed for longer extraction times [5,31]. Aqueous 50% methanol was considered as applicable solvent, as it shows a high hydrophilic character and inhibits enzymatic activity during sample extraction and storage [14]. Regarding the extraction temperature, Xiaoli et al. [31] stated that elevated temperatures up to 50 °C improve the extractability of oligosaccharides with 50% (v/v) ethanol, while Johansen et al. [14] observed no improvement between 20 °C and 50 °C in the extraction of mono-, di-, and oligosaccharides in pea with 50% (v/v) methanol. The supernatant was stored at −20 °C until the analysis and filtered through syringe filters (0.45 μm pore size) prior to injection.

Analytical validation

The limit of detection (LOD) and the limit of quantification (LOQ) were calculated from the fluctuations of the baseline and the slope of the calibration curve (Eqs. (1) and (2)). The fluctuation was determined as the mean value based on three different chromatograms. In each chromatogram, 24 baseline fluctuations were evaluated between peaks at different times.

$$LOD_n = 3 \cdot \frac{mean\ fluctuation\ of\ baseline}{slope\ of\ calibration\ curve_n} \quad (1)$$

$$LOQ_n = 10 \cdot \frac{mean\ fluctuation\ of\ baseline}{slope\ of\ calibration\ curve_n} \quad (2)$$

To evaluate the range of linearity, mixtures with 12.5, 31.3, 62.5, 125.0, 250.0, 375.0, 500.0, and 562.5 μg/mL concentrations of each analytical standard were prepared and injected.

For the recovery analysis, analytical standards were added to the lupine extract to obtain added concentrations of 0,56.25,112.5,168.75,225.0, and 281.3 μg/mL. Due to high concentrations of saccharose in the lupine extract, additional samples were prepared by diluting the lupine extract with HPLC grade water to 1:4 v/v before the standards were added. The six additional samples were used solely for the recovery of saccharose. The recovery analysis was performed in triplicate.

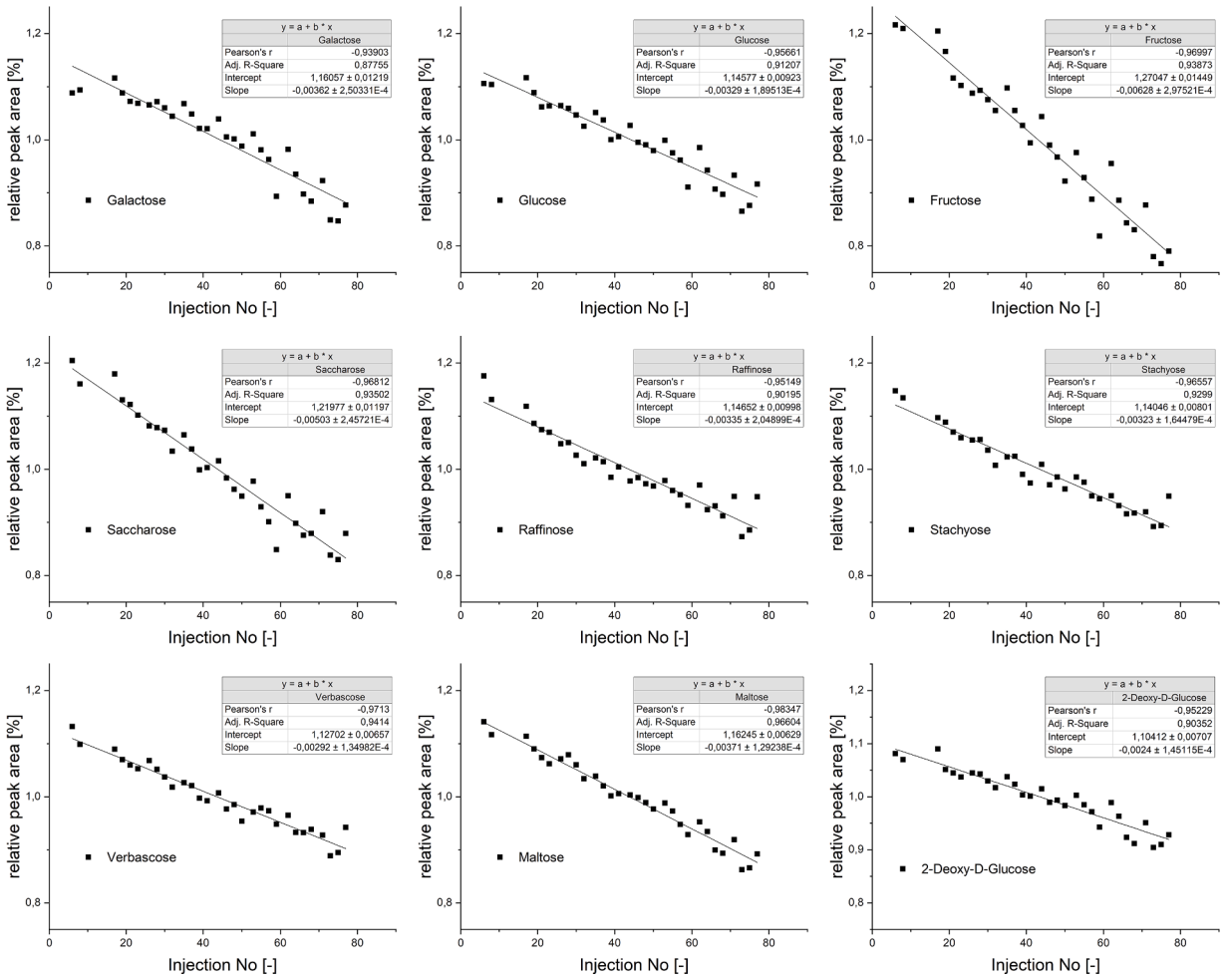

Fig. 3. Decreasing trend of the individual sugars from the 6th to 77th injection.

Advanced repeatability study

The repeatability of the method was evaluated by measuring a mixture of respective carbohydrates (250 µg/mL each) multiple times over five days. During the whole run (106 injections), this standard mix was injected every 2nd time and after 6 injections, a blank was injected to evaluate the carry over effects. Based on the results, a mathematical adjustment was done to compensate for the degradation of the analytes.

Results and discussion

Chromatographic optimization

A slightly concave gradient improved the spacing among the later-eluting peaks [28]. The introduction of a plateau in the gradient between 10 and 12 min enabled an improved distinction between glucose and galactose in the chromatogram for over 100 injections. Fig. 1 shows representative chromatograms of standards and a sample. With the exception of galactose and glucose, all mono-, di-, and oligosaccharides were distinguished clearly by baseline separation. Difficulties to distinguish the peaks of interests from matrix peaks and to differentiate raffinose and stachyose were reported for HPAEC-PAD separation [26]. By introducing a gradient with increasing sodium hydroxide concentration this inadequacy was solved and it was proofed that HPAEC-PAD is applicable for RFOs and their decomposition products. Due to its chemical similarity to glucose and galactose, 2-deoxy-D-glucose was chosen as the internal standard.

Analytical validation

The linearity between the signal response and the concentration of the respective analytical standard in the injected sample was proved for all carbohydrates in this study (see Table 1). The coefficient of determination was always greater than 0.99, except for maltose.

LOD and LOQ were lower for monosaccharides than those for RFO, saccharose, and maltose. This could be because for monosaccharides, more molecules were injected as compared to di- and oligosaccharides at the same concentration. Moreover, monosaccharides resulted in a more intense signal response as they are generally more prone to oxidation. In the lupine matrix, the recoveries for all carbohydrates were between 85 and 88% with an exception of saccharose whose recovery was 109%.

Advanced repeatability and mathematical adjustment

The measured amounts of individual sugars of the same standard mix are represented in Fig. 2. The given values are relative to their, respective mean values. With an exception of galactose, the values of the first runs were above the tolerance range of 5% around the mean value. From the 6th injection onwards, measurements resulted in acceptable values until the 75th injection for galactose, glucose, and maltose, and until the end of measurement for RFOs. The relative amounts of galactose, glucose, and maltose indicated a slightly decreasing trend. Nevertheless, for saccharose and fructose, a strongly decreasing trend was perceived as intolerable for adequate measurements. This leads to the assumption that the degradation of the internal standard 2-deoxy-D-glucose is not directly comparable with the degradation of the analytes.

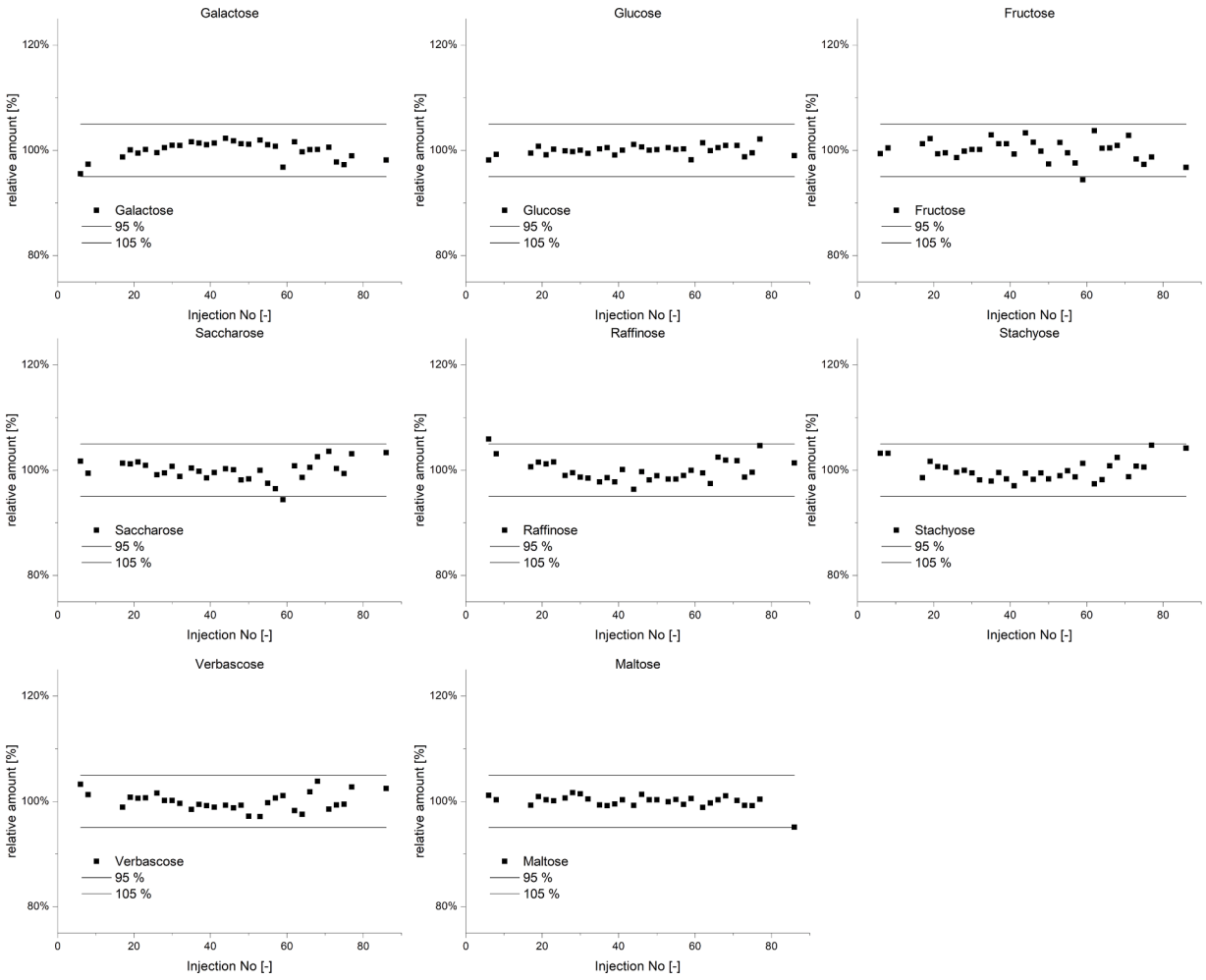

Fig. 4. Relative amounts of RFOs and their degradation products after the mathematical adjustment. The continuous lines represent values of 95% and 105% of the, respective mean values.

A mathematical adjustment was applied to avoid overlapping peaks in the chromatogram by introducing further internal standards.

To study the differences in the decomposition of the internal standard and the analytes, the changes in the relative peak areas from injection 6 to 77 were compared. Fig. 3 shows that the decomposition followed a linear trend. By using the slope of the linear decline, the proceeding degradation could be compared. With the exceptions of saccharose (slope −0.0050) and fructose (slope −0.0063), all measured sugars (slope −0.0029 to −0.0037) decomposed only slightly faster than the internal standard (slope −0.0024). This indicates that saccharose and fructose decomposed at least twice as fast as the internal standard.

To introduce a mathematical adjustment in addition to the internal standard, the measured concentrations of the analytes were plotted against the number of injections to gain the gradient of the linear decrease of individual analytes. The absolute value f_i of the individual gradient was multiplied with the number of injections and added to the measured concentration (see Eq. (3)). The absolute values f_i used for the correction were $4\bullet10^{-4}$ (galactose), $3\bullet10^{-4}$ (glucose), $8\bullet10^{-4}$ (fructose), $7\bullet10^{-4}$ (saccharose), $2\bullet10^{-4}$ (raffinose), $2\bullet10^{-4}$ (stachyose), $1\bullet10^{-4}$ (verbascose), and $3\bullet10^{-4}$ (maltose).

$$concentration_{adjusted} = concentration_{measured} + f_i \cdot n_{injection} \quad (3)$$

With this adjustment, long-term measurements up to at least 80 injections were measured adequately after the method reached stability starting with injection 6. Therefore, it was proved that the same concentrations for the measured analytes were obtained repeatedly for an aplicable run time of approx. 95 h or 4 days.

The comparison of Figs. 2 and 4 shows that the mathematical adjustment not only corrected the unacceptable results of saccharose and fructose but also decreased the fluctuations in the resulting concentrations of all sugars in this study.

Conclusion

In this study, a modified and validated method was presented, enabling the separation of glucose and galactose along with the measurement of RFOs and the starch decomposition product maltose via HPAEC-PAD. Especially for germination studies of legumes, this allows a more detailed assessment of the changes in the carbohydrate spectrum as all the relevant mono-, di-, and oligosaccharides can be measured via a single method. To our best knowledge, no long-term repeatability study with a mathematical adjustment for the degradation of the carbohydrates was reported so far. Including such a study into the validation process of new methods enables a more detailed knowledge about the performance of the method. Moreover, it avoids reporting inadequate results due to long lasting measurements.

Before an adequate measurement is performed, the system needs to be stabilized within the first 3–5 h. Therefore, 3 to 5 blanks should be injected initially. The evaluation of the long-term repeatability proved that stable and adequate measurements are possible for up to 4 days, without interrupting the system or renewing the mobile phase. Nevertheless, the origin of the degradation, whether it is a chemical or

physical effect was not identified. Additional investigations are required to unveil the phenomenon.

The benefit of this novel method goes beyond the measurements of germinating legumes. In the growing market of meat alternatives, plants are used to substitute ingredients of animal origin. While legumes are well known for their RFO content, other plants like parsnips, leek, or wheat bran contain considerable amounts of these sugars, too. Generally, these plant ingredients bring RFOs into the meat alternatives and can trigger flatulence or abdominal pain. As the present method can be adapted easily toward these products, RFOs can be monitored and optimal strategies can be implemented for their removal.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Stefan Ritter: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft, Visualization. **Arndt Nobis:** Conceptualization, Validation, Writing – original draft. **Martina Gastl:** Writing – review & editing, Supervision. **Thomas Becker:** Resources, Supervision, Writing – review & editing.

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