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**ANALYSIS OF SOLUBLE SUGARS IN PLANT-BASED
PRODUCTS: FROM SAMPLE PRETREATMENT TO HPLC
ANALYSIS**

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ABSTRACT

Sugars influence the sensory, nutritional, and technological properties of food products, making their reliable analysis essential for product development. Previous studies suggest that optimal extraction conditions depend on the sample matrix and the degree of polymerization of the sugars. Plant-based products are often complex matrices due to the presence of interfering compounds such as polysaccharides, lipids, and proteins. Consequently, understanding how different pretreatment methods affect the extraction efficiency of sugars in diverse plant-based matrices is essential for the development of reliable analytical methods.

The aim of this research was to develop a reliable pretreatment method for the analysis of free mono- and disaccharides and raffinose family oligosaccharides (RFOs) (soluble sugars) in plant-based products using hydrophilic interaction liquid chromatography with an evaporative light scattering detector (HILIC-ELSD), while considering the requirements of routine analysis in the Valio laboratory. In addition, identification and separation of polyols were considered. A Box–Behnken response surface design was employed to determine the most suitable extraction solvent, time, and temperature. Furthermore, the applicability of Carrez clarification and ultra centrifugal filtration for the removal of interfering compounds was evaluated, and the chromatographic performance of HILIC-ELSD was compared with that of high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD).

This study demonstrated that water extraction at 25°C for 20 min was the most broadly applicable method across different plant-based matrices and sugars with varying degrees of polymerization. Although Carrez clarification and ultra centrifugal filtration yielded comparable results, only ultra centrifugal filtration enabled efficient removal of polysaccharides, thereby improving chromatographic performance and extending column lifetime. Overall, this study provided valuable insights into the optimization of soluble sugar extraction in diverse plant-based products.

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TIIVISTELMÄ

Sokerit vaikuttavat elintarvikkeiden aistinvaraisiin, ravitsemuksellisiin ja teknologisiin ominaisuuksiin, minkä vuoksi niiden luotettava määrittäminen on keskeistä tuotekehityksessä. Aiemmat tutkimukset viittaavat siihen, että optimaaliset olosuhteet sokerien uuttamiseen riippuvat näytematriisista sekä sokerien polymeroitumisasteesta. Kasvipohjaiset tuotteet sisältävät usein määrittäystä häiritseviä yhdisteitä, kuten polysakkarideja, lipidejä ja proteiineja, minkä vuoksi on olennaista ymmärtää, miten erilaiset esikäsittelymenetelmät vaikuttavat sokerien uuttotehokkuuteen erilaisissa kasvipohjaisissa matriiseissa luotettavien määrittäysmenetelmien kehittämiseksi.

Tämän tutkimuksen tavoitteena oli kehittää luotettava esikäsittelymenetelmä kasvipohjaisten tuotteiden vapaiden mono- ja disakkaridien sekä raffinoosiryhmän oligosakkaridien (RFO) (liukoiset sokerit) määrittelyyn hyödyntäen hydrofiilistä vuorovaikutuskromatografiaa ja haihduttavaa valonsirontailmaisinta (HILIC-ELSD), huomioiden rutiinityöskentelyn vaatimukset Valion laboratoriossa. Tutkimuksessa käsiteltiin myös sokerialkoholien tunnistamista ja kromatografista erotusta. Box-Behnken-vastepintamallia hyödynnettiin sopivan uuttoliuottimen, -lämpötilan ja -ajan määrittämiseksi. Lisäksi tutkittiin määrittäystä häiritsevien yhdisteiden poistamista Carrez-saostuksella ja ultrasentrifugointisuodatuksella sekä verrattiin HILIC-ELSD-menetelmän kromatografista suorituskkyä korkean erotuskkyyn anioninvaihtokromatografiaan pulssiamperometrisellä ilmaisimella (HPAEC-PAD).

Vedellä uuttaminen 25 °C:ssa 20 minuutin ajan oli laajimmin sovellettavissa oleva menetelmä eri kasvipohjaisiin matriiseihin ja eri polymeroitumisasteen sokereihin. Vaikka Carrez-saostus ja ultrasentrifugointisuodatus tuottivat keskenään vertailukelpoisia tuloksia, ainoastaan ultrasentrifugointisuodatus mahdollisti polysakkaridien tehokkaan poistamisen, mikä paransi kromatografista suorituskkyä ja pidensi kolonnin käyttöikää. Tämä tutkimus tarjosi tärkeää tietoa liukoisten sokerien uuton optimoinnista erilaisissa kasvipohjaisissa tuotteissa.

PREFACE

This master's thesis was conducted as a collaboration between the Department of Food and Nutrition at University of Helsinki, and Valio Ltd. The work was carried out as a part of the Valio Food 2.0 C-project and funded by Valio Ltd. The thesis was supervised by Associate Professor Ndegwa Maina, Docent Velimatti Ollilainen, ETM Research scientist Sanna Kainulainen, and ETT Manager research analytics Tuuli Koivumäki. I wish to express my gratitude to all my supervisors for their guidance and expertise throughout this project.

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1 INTRODUCTION

1.1 Background of the topic

Sustainable food systems are a major research topic currently and new plant-based food innovations have been ascendant. Plant-based raw materials such as cereals and legumes are particularly valuable due to their lower greenhouse gas emissions, reduced water consumption, and decreased contribution to deforestation (Semba et al. 2021). In plant-based products, carbohydrates influence the sensory, nutritional, and technological properties, and consequently impact product formulation and development. Reliable analytical methods are essential in order to develop products with desired characteristics. This study focused on the analysis of mono- and disaccharides, raffinose family oligosaccharides (RFOs), and polyols from plant-based products.

Different terms are used to classify carbohydrates. According to the definition made by Food and Agriculture Organization (FAO) and World Health Organization (WHO), sugars are monosaccharides, disaccharides, and polyols with a degree of polymerisation (DP) of 1–2 (FAO 1998). In the European Union, polyols are excluded from the definition (EU 2011). Despite of this classification, many authors have included also oligosaccharides (DP 3–9) in the analysis of soluble sugars (Berrios et al. 2010; Giannoccaro et al. 2006; Goyoaga et al. 2011; Kotha et al. 2020; Shanmugavelan et al. 2013). For a consistent expression, the term soluble sugars is also used in this thesis to describe mono-, di-, and oligosaccharides and polyols.

Raffinose family oligosaccharides (RFOs) are one of the main soluble sugars in legumes and cereals (Biesiekierski et al. 2011; Fan et al. 2015; Ispiryan et al. 2020; Marmouzi et al. 2016). The structure is composed of sucrose and a varying number of $\alpha(1\rightarrow6)$ linked galactosyl units (Figure 1). RFOs are classified as FODMAPs, an acronym for fermentable oligo-, di-, and monosaccharides and polyols (Gibson and Shepherd 2005). The main physiological feature of FODMAPs is that they are poorly absorbed in the small intestine, are osmotically active, and undergo rapid microbial fermentation in the colon, causing abdominal discomfort in some individuals (Gibson and Shepherd 2010). This has increased research interest in the analysis of RFOs in order to develop food products with desired characteristics.

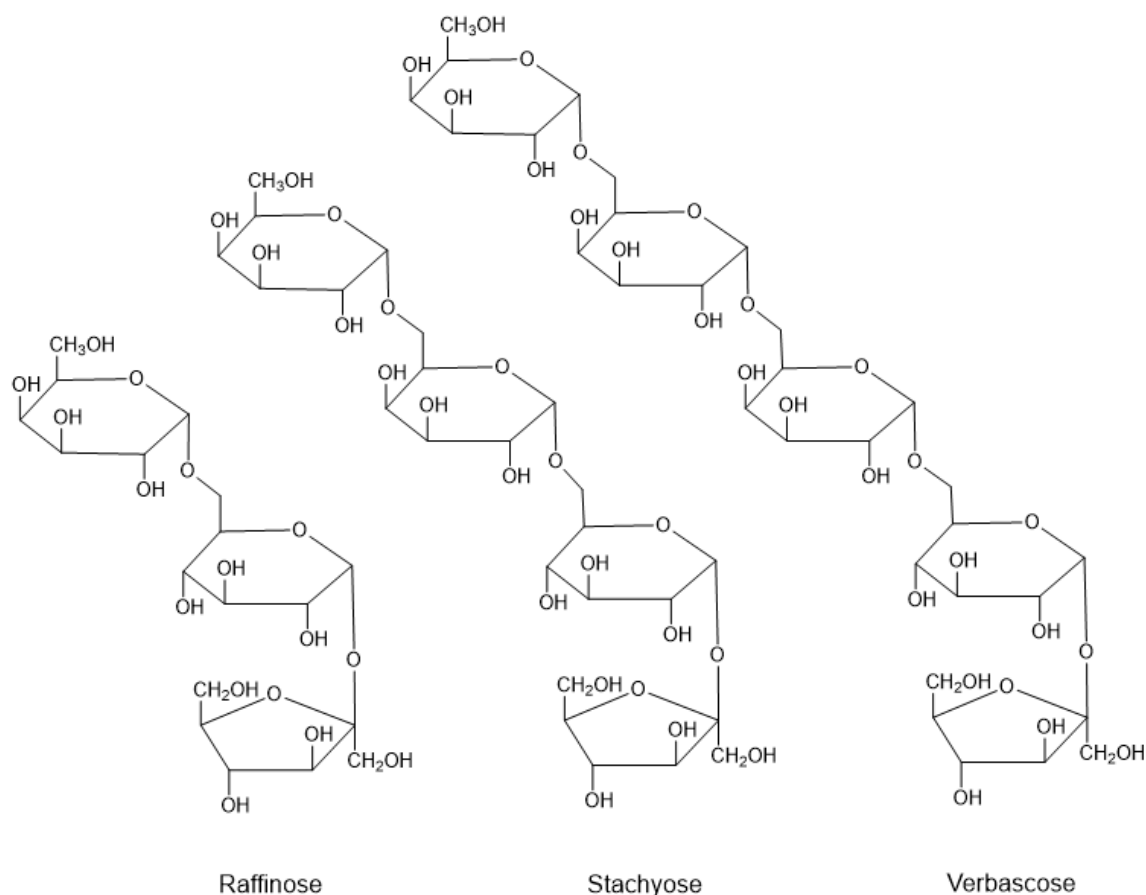


Figure 1. Structures of the common raffinose family oligosaccharides in plant-based raw materials: raffinose, stachyose, and verbascose.

1.2 Extraction of soluble sugars from plant-based materials

Extraction procedures are critical during quantitative analysis. Especially with complex matrices, comprehensive solubilization of analytes from a sample matrix to the extraction solvent is challenging. For example, polysaccharides or proteins can interfere extraction by entrapping sugars inside of their structures (Hoehnel et al. 2022; Xiaoli et al. 2008). The use of aqueous ethanol as an extraction solvent can reduce interferences caused by co-extraction of polysaccharides, but at the same time it may simultaneously impair the solubility of the target analytes. To date, extraction condition optimization has largely been conducted for raw materials rather than for processed products (Ekvall et al. 2007; Giannoccaro et al. 2006; Johansen et al. 1996; Jovanovic-Malinovska et al. 2015; Kim et al. 2003; Kotha et al. 2020; Xiaoli et al. 2008). Previous studies suggest that the optimal extraction conditions depend on the sample matrix and the DP of the sugars. This complexity highlights the need to adapt extraction conditions specifically for plant-based products.

Existing official standard methods are not directly applicable for simultaneous analysis of different soluble sugars from plant-based products with HPLC (AOAC International 2018; China National Standards 2023). For example, AOAC 2018.16 method covers the analysis of mono- and disaccharides, but it excludes oligosaccharides and polyols from its scope. Correspondingly, GB 5009.289-2023 standard method covers the analysis of galactooligosaccharides (GOS), but excludes samples that contain other oligomeric compounds.

1.3 High-performance liquid chromatography analysis of soluble sugars

Multiple analytical methods have been utilized in the carbohydrate analysis (Jie et al. 2021). Since sugars are non-volatile compounds, gas chromatography (GC) is not a suitable method without derivatization. The additional pretreatment step for GC analysis increases the workload and may interfere with quantitative analysis, if derivatization is not efficient and selective (Ikegami et al. 2008). This limitation can be overcome using high-performance liquid chromatography (HPLC). Commonly used HPLC instruments in soluble sugar analysis are high-performance anion-exchange chromatography (HPAEC) coupled with pulsed amperometric detector (PAD) and hydrophilic interaction liquid chromatography (HILIC) coupled with either evaporative light scattering detector (ELSD), refractive index detector (RID) or mass spectrometer (MS).

1.3.1 HILIC-ELSD

HILIC has become increasingly used in sugar analysis as a result of advances in column technology (Crha et al. 2024). HILIC system consists of a polar stationary phase coupled with a mobile phase containing high concentration of organic solvent, typically a concentration of 60–80% acetonitrile in water has been employed in sugar analyses (Jovanovic-Malinovska et al. 2015; Koh et al. 2018; Ma et al. 2014; Muir et al. 2009; Pedrosa et al. 2012; Pismennõi et al. 2021; Shanmugavelan et al. 2013; Xiaoli et al. 2008). Water in the mobile phase forms a water-enriched layer on the polar stationary phase and analytes partition between acetonitrile and water according to their polarity. When water content is low, analytes will partition close to the stationary phase, which enhances the retention. Correspondingly, elution of polar analytes is promoted by increasing the water content in the mobile phase. The retention mechanism is complex and still not fully understood (Cavazzini et al. 2023). Since partitioning alone does not fully explain selectivity of HILIC columns, other interactions such as hydrogen bonding and electrostatic forces between analytes and the stationary phase are also significant. The stationary phase can be for example, modified silica gel containing amide, diol or amino groups. These chemical modifications can be used to increase the selectivity of HILIC.

The operating principle of ELSD is a three-step process, which includes nebulization, evaporation of the mobile phase, and detection of the scattered light from the analytes (Dvořáčková et al. 2014). First the mobile phase is nebulized into small aerosols by nebulizer gas, typically air or nitrogen. In the second stage, mobile phase droplets are evaporated in the heated drift tube. The evaporation efficiency depends on the mobile phase composition, eluent flow rate, nebulizer flow rate, and analytes. For example, when the mobile phase contains more water or the flow rate is increased, higher drift tube temperature is required. However, the signal response can decline or analyte loss occur when using too high drift tube temperature (Luo et al. 2024). In the final phase, the non-volatile analytes are detected by measuring the scattered light from the analytes. The response is not completely linear, which causes some measurement error during accurate quantification. However, with an applicable standard curve, the linear range can be observed and reliable quantification performed (Luo et al. 2024). HILIC-ELSD has a stable signal response over the time, so quantification can be made easily with external calibration method. Additional advantage in HILIC-ELSD over HPAEC-PAD is its tolerance to ethanol, which enables its use as an extraction solvent without a need to evaporate the solvent prior to analysis.

1.3.2 HPAEC-PAD

The separation mechanism of the HPAEC system is based on electrostatic interactions between the analytes and the stationary phase, as well as polarizability of the analyte, and its ionic radius (Nesterenko 2023). Since sugars are weak acids they possess a negative charge in a strong alkaline environment ($\text{pH} > 12$), thus they can be separated with a positively charged anion-exchange column and a mobile phase containing water with typically either sodium or potassium hydroxide (Jie et al. 2021).

PAD is one of the most common electrochemical detectors (Nesterenko 2023). It utilizes an applied potential to create a redox reaction between the electrode (typically gold electrode) and the analyte (Corradini et al. 2012). The current resulting from the redox reaction can then be detected and is proportional to the analyte concentration. In PAD, the potential is applied in pulses and the detection of analytes occur during the constant potential (Figure 2). Both positive and negative pulses are used to first remove the analytes from the electrode and subsequently reactivate the electrode. HPAEC-PAD method is more sensitive than HILIC-ELSD, in which case it is preferred in trace level analysis. The aqueous mobile phase used in HPAEC-PAD prevents possible precipitation of carbohydrates, whereas in HILIC-ELSD larger carbohydrates can precipitate as a result of high concentration of organic solvent (Pöhl et al. 2017). The drawback of HPAEC-PAD is that the signal response in PAD reduces during the run. Hence, the reduction needs to be corrected e.g., with an internal standard, which makes quantification more laborious compared to HILIC-ELSD.

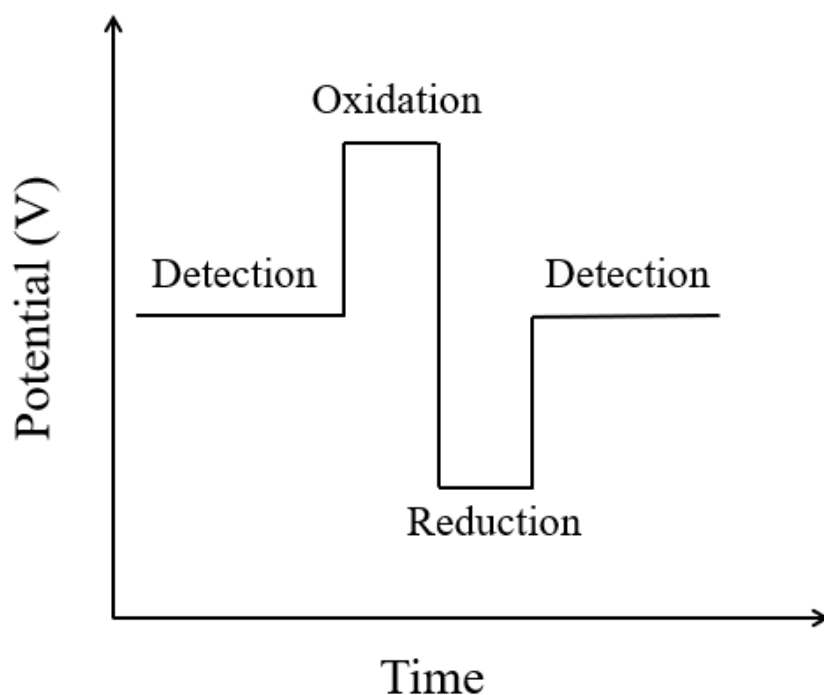


Figure 2. The operating principle of the pulsed amperometric detector (PAD), where positive potential is applied to remove analytes from the electrode surface, followed by electrode reactivation during the reduction phase.

1.4 Experimental designs

Experimental designs are used to investigate how different factors impact the response variable (NIST/SEMATECH 2012). There are different types of designs depending on the objectives, number of factors, and available resources. For example, if the objective is screening, full factorial design, fractional factorial design, or Plackett-Burman design can be used. In this study, the objective was to investigate interactions between factors and identify local maximums. Therefore, a response surface design was utilized. Box-Behnken and central composite design are the two commonly used response surface designs (NIST/SEMATECH 2012). In Box-Behnken, each factor is analysed at three different levels. One factor at a time is set into the middle level, while the other factors are either in the high or low levels (Figure 3). In addition, centre points (typically 3), where all the variables are set in the middle level, are analysed (NIST/SEMATECH 2012). This decreases the variance difference of the centre and edge points and stabilises the variance during the analysis.

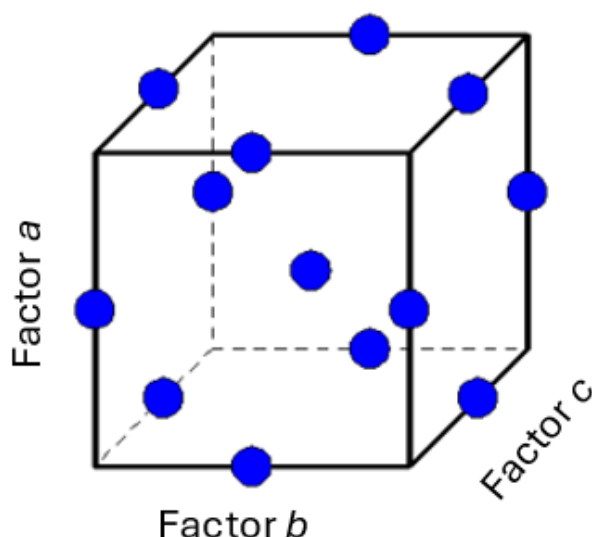


Figure 3. Selection of the measurement points according to the Box-Behnken principle. Figure revised from NIST/SEMATECH (2012).

The benefit of the Box-Behnken method is that the measurement points are selected in a way that extreme conditions can be avoided. In central composite design, measurement points are located at extreme values, like in factorial design. While central composite design is more flexible since sequential experiments are possible, Box-Behnken was selected in this thesis, because the measurement points were at the levels of interest and reliable estimation could be obtained with fewer measurements.

1.5 Method validation

The absolute true value of a measurement cannot be known, because results always include uncertainties. Validation is a process where the method performance is studied and the extent of uncertainty is established. Measurement uncertainty comprises both systematic and random errors (Magnusson et al. 2017). Systematic error is the difference between a measured value and an assigned value. It is determined using standard reference materials and proficiency tests, and it is reported as trueness (Cantwell H. 2025). If no reference material is available, recovery analyses can be used to assess the trueness (Hägg 2016). Random error describes the variation observed in replicate analyses of the same sample, and it is expressed as precision. Depending on the set of conditions, precision can be characterized as repeatability, reproducibility, or within-laboratory reproducibility (Magnusson et al. 2017). Repeatability refers to the variation observed when analyses are conducted in the same laboratory, using the same method and instrument, and by the same person over a short period of time. Reproducibility describes the degree of variation that occurs when a laboratory, instrument, and analyst is changed, but the sample and analytical method remains the same. Within-laboratory reproducibility, or intermediate precision, describes the level of variation when the laboratory is the same but sources of variation such as reagents, personnel, and instrument maintenance are considered.

Besides estimating the measurement uncertainty, validation provides the framework where quantification can be reliably performed. For example, resolution, limit of detection (LOD), limit of quantification (LOQ), linearity, and measurement range are important parameters to determine during validation. LOD describes the lowest analyte concentration that can be reliably distinguished from background noise, while LOQ is the smallest concentration that can be accurately quantified (Thompson et al. 2002). LOD and LOQ are determined by analysing the standard deviation of either a blank or a low concentration sample and multiplying it by a factor corresponding to the desired relative standard deviation (RSD). The multiplier recommended by IUPAC for calculating LOQ is 10, which corresponds to a RSD of 10% (Currie 1995).

In-house quality control (QC) samples are essential for monitoring method performance. Control charts, such as X-charts, are commonly used for this purpose. They are created by plotting independent QC-sample measurements on the chart and defining the expected range of variation. This enables the detection of atypical measurement levels. In X-charts, the warning and action limits are typically set at two and three times the standard deviation from the average value, respectively (Magnusson et al. 2017). This means there is approximately a 5% probability that a result exceeds the warning limit even when the method is functioning normally. Correspondingly, only 0.3% of all results would be false positives when the action limit is set at three times the standard deviation.

1.6 The aim of this thesis

The aim of this thesis was to develop a reliable pretreatment method for the analysis of free mono- and disaccharides and raffinose family oligosaccharides (RFOs) (soluble sugars) in plant-based products using HILIC-ELSD, while considering the requirements of a routine analysis in the Valio laboratory. In addition, separation and identification of polyols were considered. A Box–Behnken response surface experimental design was applied to determine the most suitable extraction solvent, time, and temperature. Furthermore, the applicability of Carrez clarification and ultra centrifugal filtration for the removal of interfering compounds was compared, and the chromatographic performance of HILIC-ELSD was evaluated against the HPAEC-PAD. The hypothesis of this thesis was that polysaccharides, lipids, and proteins present in plant-based products affect the extraction efficiency of soluble sugars, and the optimal extraction conditions depend on the sample matrix and the degree of polymerization (DP) of the sugars.

2 MATERIALS AND METHODS

2.1 Materials

Three different plant-based products were used in this study. A plant-based gurt and a meat alternative were the test samples and a plant-based drink was used as an in-house QC-sample. The samples were obtained from Valio's product development and selected for this study based on their representativeness for the method development. In practice, these samples contained adequate amount of different soluble sugars for reliable quantification. The samples covered different raw materials, and polysaccharides, proteins, and lipids as interfering compounds. The plant-based gurt represented a liquid sample and the meat alternative represented a solid matrix. Specific details of the sample materials are excluded from the thesis, due to confidentiality. The meat alternative was homogenized and frozen and the plant-based gurt was divided into several batches and frozen.

2.2 Instruments

The HILIC-ELSD system used in this study was Shimadzu Nexera XR 700 bar with ELSD-LT III detector and Phenomenex Luna Omega 3 μm SUGAR 100 Å, 250 x 4.6 mm column. The HPAEC-PAD system was Waters e2695 with Waters 2465 electrochemical detector and Dionex CarboPac PA 20 column. Leco TGM800 thermogravimetric moisture determinator was used for the residual moisture content analysis. The water bath for the extraction phase was Julabo, EcoTemp TW 20 coupled with Magnetic e motion 2 mag, MIX control 20. The Scale used in the study was Mettler Toledo AX204. Ultra Centrifugal filters were Amicon Ultra 0.5 ml 10 kDa purchased from Sigma-Aldrich.

2.3 Reagents and standards

Pure ethanol (ETAX AaS, Anora Group Oyj), LC-MS grade acetonitrile (J.T. Baker), and Carrez reagents; $\text{K}_4\text{Fe}(\text{CN})_6 \cdot 3\text{H}_2\text{O}$ and $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ were used in this study. Standards were: D-Glucose ($\geq 99.5\%$ Sigma-Aldrich), Sucrose ($\geq 99.5\%$ Sigma-Aldrich), D-(+)-Raffinose pentahydrate ($\geq 98.0\%$ Sigma-Aldrich), Stachyose Hydrate ($> 98.0\%$, TCI), Verbascose ($> 95\%$, Megazyme), Maltose monohydrate ($\geq 99\%$, Sigma-Aldrich), Maltotriose hydrate ($\geq 95\%$, Sigma-Aldrich), D-Fructose ($\geq 99\%$, Sigma-Aldrich), Myo-inositol ($\geq 99\%$, Sigma-Aldrich), Cellobiose (GBI), Melibiose ($> 98.0\%$, Sigma-Aldrich), D-pinitol (95% , Sigma-Aldrich), and D-mannitol ($\geq 99\%$, Sigma-Aldrich).

2.4 Box-Behnken experimental design

The effect of different extraction conditions was investigated by quantifying the three most common sugars in the samples with HILIC-ELSD. Glucose, sucrose, and stachyose were quantified from the gurt, while sucrose, stachyose, and verbascose were quantified from the meat alternative. This selection of analytes enabled examination of the matrix effect and how different extraction conditions influenced the extraction yield of the analysed sugars with different degrees of polymerization.

Box-Behnken response surface method was used for establishing the most suitable extraction conditions. Minitab Statistical Software was used in the analysis. Extraction solvent, time, and temperature varied between the different methods (Table 1). The methods were coded as A–M. The middle level of the extraction solvent was set to 50% ethanol, for practical reasons and to emphasize conditions that could be applied in routine analyses in the future. According to the Box-Behnken principles, 13 different pretreatment combinations were analysed. All analyses were performed in triplicate and repeated two times to increase reliability. The number of centre point measurements was set to three, leading to 18 total measurements of the M-method (centre point). The order of analyses was randomized.

Table 1. Extraction conditions selected according to the Box–Behnken design (Figure 3). Method codes indicate the extraction conditions under which each measurement was performed. n = number of replicates.

Method code	n	Ethanol (%) in water	Temperature (°C)	Time (min)
A	6	50	25	10
B	6	50	65	10
C	6	50	25	30
D	6	50	65	30
E	6	0	25	20
F	6	0	65	20
G	6	80	65	20
H	6	80	25	20
I	6	0	45	30
J	6	80	45	30
K	6	80	45	10
L	6	0	45	10
M	18	50	45	20

2.5 Pretreatments

Three pretreatment methods (thesis method, Valio method, University method) were utilized in the study to compare the effect of pretreatment, including extraction conditions and procedures for removing interfering compounds, into the soluble sugar yield. The thesis method was developed based on parameters commonly used in the literature for the extraction of soluble sugars in plant-based materials. In all thesis method variants A-M (see Table 1), a portion of 3 g of the thawed sample was extracted with 25 ml of different water and ethanol concentrations under continuous stirring. A water bath was used to keep a constant temperature during the extraction time. After the specific extraction time, samples were cooled rapidly and the volumetric flask (50 ml) was filled to the mark. After thorough shaking, 1.5 ml of the extract was transferred into a centrifuge tube and centrifuged at 13 000 g for 5 min. The supernatant was filtered with 10 kDa ultra centrifugal filter at 13 000 g for 15 min. Finally, the filtrate was diluted in acetonitrile 1:2 (0.5 ml acetonitrile and 0.5 ml the sample) prior to the HILIC-ELSD analysis.

Valio method consisted of water extraction at room temperature and Carrez clarification for removing proteins and lipids prior the HILIC-ELSD analysis. The pretreatment protocol was according to Valio protocol and is excluded due to confidentiality.

The pretreatment method used at the University of Helsinki is designed for dried legume samples. Therefore, the meat alternative was freeze-dried prior the HPAEC-PAD analysis according to Valio protocol. A 100 mg portion of the freeze-dried sample was extracted with 5 ml cold MilliQ water. After vortex, the sample was centrifuged at 10 000 rpm for 15 min at 4 °C. The supernatant was collected and filtrated with ultra centrifugal filter (10 kDa) at 13 000 g for 10 min. Subsequently, 300 µl of the filtrate was transferred into HPLC vial together with 20 µl of the internal standard (2-deoxy-D-galactose).

2.6 Quantitative analysis with HILIC-ELSD

Quantification with HILIC-ELSD was performed using an external calibration method. Calibration was carried out with a six-point calibration curve at concentrations of 1 g/l, 0.7 g/l, 0.4 g/l, 0.2 g/l, 0.1 g/l, and 0.05 g/l. All six calibration points were utilized in the quantification. The full calibration set was analysed in the beginning of every sample run. LabSolutions software was used in the data processing. The chromatographic program required optimization for separating all the target standard compounds with HILIC-ELSD. Resolution was improved by changing the proportion of acetonitrile in the mobile phase, gradient program, column temperature, and flow rate. The aim was to ensure baseline separation for each analyte.

A plant-based drink was used as a QC-sample to verify daily instrument performance. Glucose and sucrose were quantified in the QC-sample and the results were monitored using X-charts. The QC-sample was always analysed at the beginning and end of each sequence to ensure stable instrument performance throughout the analysis.

2.7 Quantitative analysis with HPAEC-PAD

Quantification with HPAEC-PAD was conducted with an external calibration method coupled with an internal standard (2-deoxy-D-galactose). A six point calibration curve was used with concentrations of 0.2 g/l, 0.15 g/l, 0.1 g/l, 0.075 g/l, 0.05 g/l, and 0.02 g/l. Concentration of the internal standard was 0.05 g/l. Standard curves were constructed by plotting the peak height ratio of the analyte to the internal standard against the analyte concentration. In this way, the reduction in PAD response during the run was corrected.

A gradient elution using 200 mM NaOH (A), water (B), and 1 M sodium acetate in 200 mM NaOH (C) as the mobile phase was applied in the HPAEC-PAD analysis (Figure 2). The flow rate was set to 0.4 ml/min.

Table 2. The gradient program in the HPAEC-PAD system used at the University of Helsinki. Solvent A refers to 200 mM NaOH, B is water, and C is 1 M sodium acetate in 200 mM NaOH.

Time (min)	A(%)	B(%)	C(%)
15	5	95	0
30	100	0	0
31	85	0	15
39	85	0	15
40	100	0	0
55	100	0	0
56	5	95	0
66	5	95	0

The analyte concentration in the fresh meat alternative (g/100 g) was obtained according to Equation (1), where C denotes the analyte concentration in the vial, F is the dilution factor, V is the extraction volume, m is the sample weight, D is the dry matter content, and W is the residual moisture content. The same equation was applied to the gurt sample, but without the conversion factors of D and W .

$$\text{g/100 g (f.m.)} = \left(\frac{C \text{ (mg/ml)} \cdot V \text{ (ml)} \cdot F}{m \text{ (mg)} \cdot (100\% - W(\%))} \cdot 100 \right) \cdot D(\%) \quad (1)$$

2.8 Determination of dry matter and residual moisture content

Dry matter content of the meat alternative and the residual moisture content of the freeze-dried meat alternative were analysed to enable comparison of results obtained using different methods on a fresh matter (f.m.) basis. Dry matter content $D(\%)$ was analysed with a gravimetric method according to protocol used at Valio. The detailed description of the method is excluded due to confidentiality. The average value of three replicates were used in the calculations. The residual moisture content of the freeze-dried sample was analysed with Leco TGM800 thermogravimetric moisture determinator. Average moisture content $W(\%)$ was calculated from four replicates.

2.9 Dixon's Q test for removing possible outliers

Dixon's Q test was utilized for removing possible outliers from the data before further statistical analyses (Rorabacher 1991). Dixon's test statistics Q^- and Q^+ are given by Equations (2) and (3), respectively. In the Equations, $z_1 \leq z_2 \leq \dots \leq z_N$ denotes the dataset in ascending order. The test statistic Q^- indicates whether the smallest value is a potential outlier, while Q^+ indicates if largest value is a potential outlier. If the test statistic was over the critical value presented at Rorabacher (1991), the measurement was considered as an outlier. Outliers were determined with 95% confidence level. Correct equation and critical values were selected based on the number of replicates (n).

$$Q^- = \begin{cases} \frac{z_2 - z_1}{z_N - z_1} & \text{if } 3 \leq n \leq 7 \\ \frac{z_2 - z_1}{z_{(N-2)} - z_1} & \text{if } 8 \leq n \leq 12 \\ \frac{z_3 - z_1}{z_{(N-2)} - z_1} & \text{if } n \geq 13 \end{cases} \quad (2)$$

$$Q^+ = \begin{cases} \frac{z_N - z_{N-1}}{z_N - z_1} & \text{if } 3 \leq n \leq 7 \\ \frac{z_N - z_{(N-1)}}{z_N - z_2} & \text{if } 8 \leq n \leq 12 \\ \frac{z_N - z_{N-1}}{z_N - z_3} & \text{if } n \geq 13 \end{cases} \quad (3)$$

2.10 Method comparison with variance analysis

Minitab Statistical Software was used in the variance analyses. Since equal variances could not be assumed (Levene's test), Welch's test was used in place of the standard ANOVA. Statistical differences were evaluated at the 95% confidence level. Overlapping confidence intervals indicated that no statistically significant difference between the methods was observed.

2.11 Evaluation of the method performance

The method performance of the most promising thesis method was evaluated. A complete method validation would have required a more versatile sample set, longer analysis period, and proficiency test or reference material. Limit of detection (LOD) and limit of quantification (LOQ) were determined by diluting the lowest standard solution by 1:2 and calculating the standard deviation from five replicate measurements. LOD and LOQ was calculated according to the guidelines presented in Cantwell H. (2025). In Equation (4) and (5), SD denotes the standard deviation and n is the number of replicates. Since the standard solution did not go through the whole pretreatment procedure, results represented only the instrument performance.

$$\text{LOD} = 3 \cdot \frac{\text{SD}}{\sqrt{n}} \quad (4)$$

$$\text{LOQ} = 10 \cdot \frac{\text{SD}}{\sqrt{n}} \quad (5)$$

Repeatability was determined by analysing the test samples on four separate analysis dates between 16.05.2025 and 24.7.2025. Three replicates were analysed each time to estimate within-day variation. Each replicate was individually prepared. Because analyses were performed during a relatively short period of time and by a same analyst, intermediate precision could not be determined. Therefore, it was only a preliminary estimation of the total variation of the method.

For calculating repeatability, guidelines from Cantwell H. (2025) Annex C was utilized. Within-day (s_r) and between days (s_{between}) repeatability standard deviations were calculated according to Equations (6) and (7), respectively. In the Equations, MS_w and MS_b denote the mean squares within-day and between days, respectively. The number of replicates (n) was three. Total variation was determined by combining the terms s_r and s_{between} . The relative total variation was calculated according to Equation (8) to allow comparison of analytes with different concentrations.

$$s_r = \sqrt{MS_w} \quad (6)$$

$$s_{\text{between}} = \sqrt{\frac{MS_b - MS_w}{n}} \quad (7)$$

$$s_{\text{total}} = \frac{\sqrt{s_r^2 + s_{\text{between}}^2}}{\text{Average analyte concentration (g/100 g)}} \cdot 100 \quad (8)$$

Trueness was evaluated by recovery analyses because no reference material or proficiency tests were available. Recovery analyses were performed in three level standard additions. The additions (spikes) were performed such that the total analyte concentration remained within the calibration range and increased gradually (Tables 3 and 4). The gurt sample was spiked with glucose, sucrose, and stachyose. The meat alternative was spiked with sucrose and stachyose. Analyses were conducted as triplicates and repeated two times. Standards were spiked directly to the sample as a stock solution and the samples went through the whole pretreatment procedure.

Table 3. Recovery analysis of the gurt sample using three different standard spiking levels. Total concentrations are expressed as g/100 g fresh matter.

Analyte	Original (g/100 g)	Spike 1 (g/100 g)	Spike 2 (g/100 g)	Spike 3 (g/100 g)
Glucose	1.6	2.0	2.6	3.2
Sucrose	1.1	1.4	2.0	2.7
Stachyose	0.5	0.7	1.0	1.4

Table 4. Recovery analysis of the meat alternative sample using three different standard spiking levels. Total concentrations are expressed as g/100 g fresh matter.

Analyte	Original (g/100 g)	Spike 1 (g/100 g)	Spike 2 (g/100 g)	Spike 3 (g/100 g)
Sucrose	0.5	0.8	1.1	1.6
Stachyose	0.3	0.4	0.6	0.9

Recovery (%) was calculated according to Equation (9), where B denotes the analyte concentration in the spiked sample, A is the analyte concentration in non-spiked sample, and C signifies the calculated concentration of the added analyte. The results were reported as an average value of the replicates and different spiking levels ($n=18$).

$$\text{Recovery \%} = 100 \cdot \frac{B - A}{C} \quad (9)$$

Only a preliminary measurement uncertainty could be estimated in such a short period of time, with only two test samples and without a reference material or proficiency tests. Measurement uncertainty was calculated separately for the gurt and meat alternative samples, because determining a single universal measurement uncertainty would have required a more diverse set of samples. In addition, results from different analytes were combined, since different concentration ranges of each analyte would have been needed to obtain analyte specific measurement uncertainties. Random error $U(R_w)$ was established by repeatability analyses. In particular, $U(R_w)$ was obtained as the average of the total repeatabilities (s_{total}) of different analytes. Systematic error was estimated by recovery analyses and the average bias $U(\text{recovery})$ was calculated according to Equation (10). Expanded measurement uncertainty was established by combining the uncertainty factors $U(R_w)$ and $U(\text{recovery})$ as seen in Equation (11). Expanded measurement uncertainty U was given at 95 % confidence level, thus the coverage factor of two (2) was used.

$$U(\text{recovery}) = |\text{Average recovery\%} - 100\%| \quad (10)$$

$$U = 2 \cdot \sqrt{U(R_w)^2 + U(\text{recovery})^2} \quad (11)$$

2.12 Schematic representation of the thesis

A schematic representation of the thesis is illustrated in Figure 4. Three pretreatment methods were utilized in the study: extraction of with water and ethanol at different ratios and ultra centrifugal filtration (thesis method, see Table 1), pretreatment with Carrez clarification and water extraction (Valio method), and extraction with cold water and ultra centrifugal filtration (University method). The same samples were analysed in Valio using HILIC-ELSD and the University of Helsinki, using HPAEC-PAD, for the comparison of chromatographic performances.

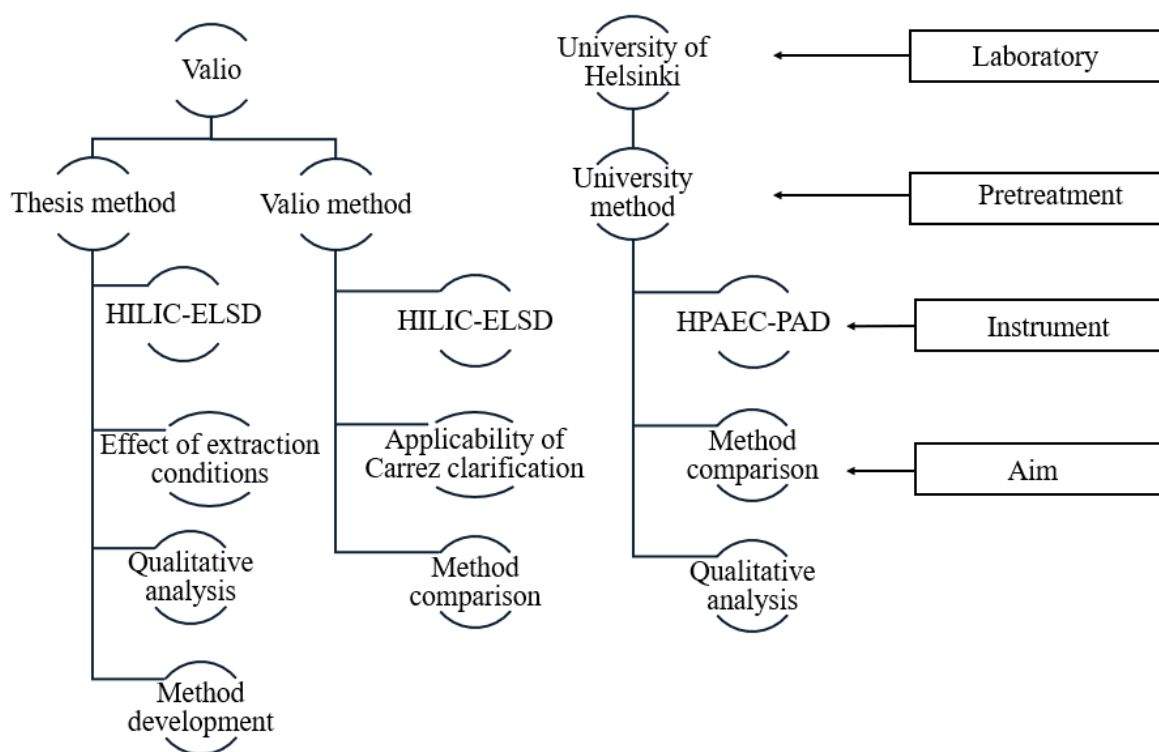


Figure 4. A schematic representation of the research. Analyses were conducted in two different laboratories, with three different pretreatment methods, and with two chromatographic instruments.

3 RESULTS

3.1 Separation of sugar analytes with HILIC-ELSD

Separation of the standard compounds required optimization in the HILIC-ELSD system. Especially challenging was the separation of maltose and myo-inositol, and maltotriose and raffinose. Also, pinitol and mannitol were eluting close to fructose and glucose, respectively. Since the test samples did not contain these sugar alcohols, pinitol and mannitol were excluded from the standard solution.

All the other standard compounds were baseline separated ($R > 1.5$) except maltose and myo-inositol, and maltotriose and raffinose. Since these compounds were not quantified in this study, further optimization was not conducted, but this co-elution should be noted in the future. The most sufficient separation of the standards was achieved with a column temperature of 25°C, a flow rate of 1 ml/min, and by combining isocratic and gradient elution (Figure 5). Isocratic elution was applied first 35 minutes (80% acetonitrile in MilliQ water), after which gradient elution was applied until reaching 50% acetonitrile content. The benefit of the gradient elution can be seen in the peak shapes of stachyose and verbascose, which are more narrow compared to analytes during the isocratic elution (Figure 5). The total run time was 60 min.

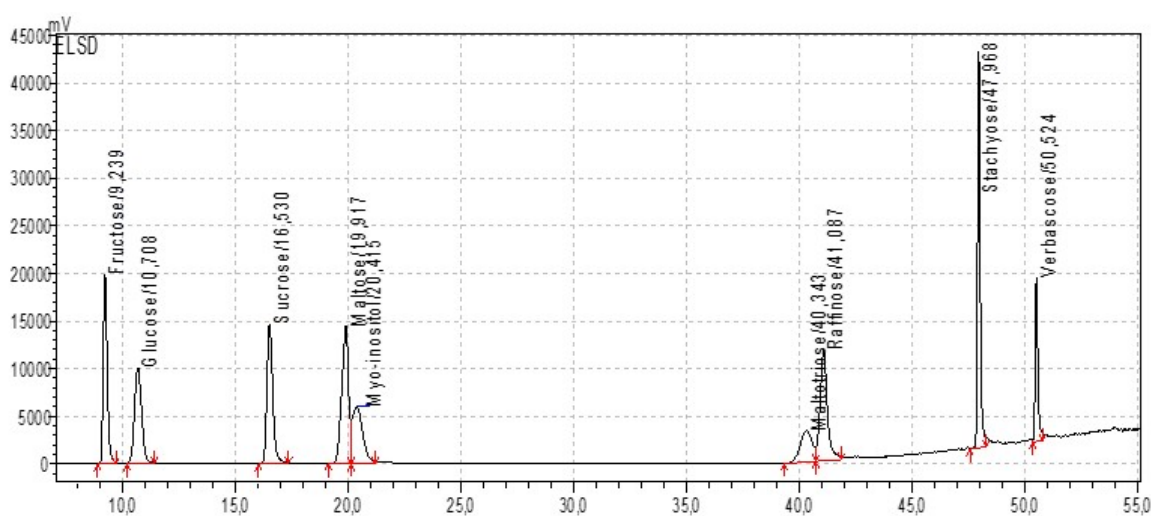


Figure 5. The chromatogram showing separation of soluble sugars in the standard solution with HILIC-ELSD.

3.2 Separation of sugar analytes with HPAEC-PAD

The separation of the sugar analytes expected to be present in plant-based products is presented in Figure 6. Since the CarboPac PA20 column is not applicable for the separation of sugar alcohols, only mono-, di-, and oligosaccharides were analysed using HPAEC-PAD. The elution order of the standards differed from that observed with HILIC-ELSD (Figure 5). For example, maltose and maltotriose eluted after RFOs, whereas in the HILIC-ELSD system the elution order was reversed. Cellobiose and verbascose, as well as glucose and sucrose, were not baseline-separated, however, the overall resolution was sufficient for all analytes.

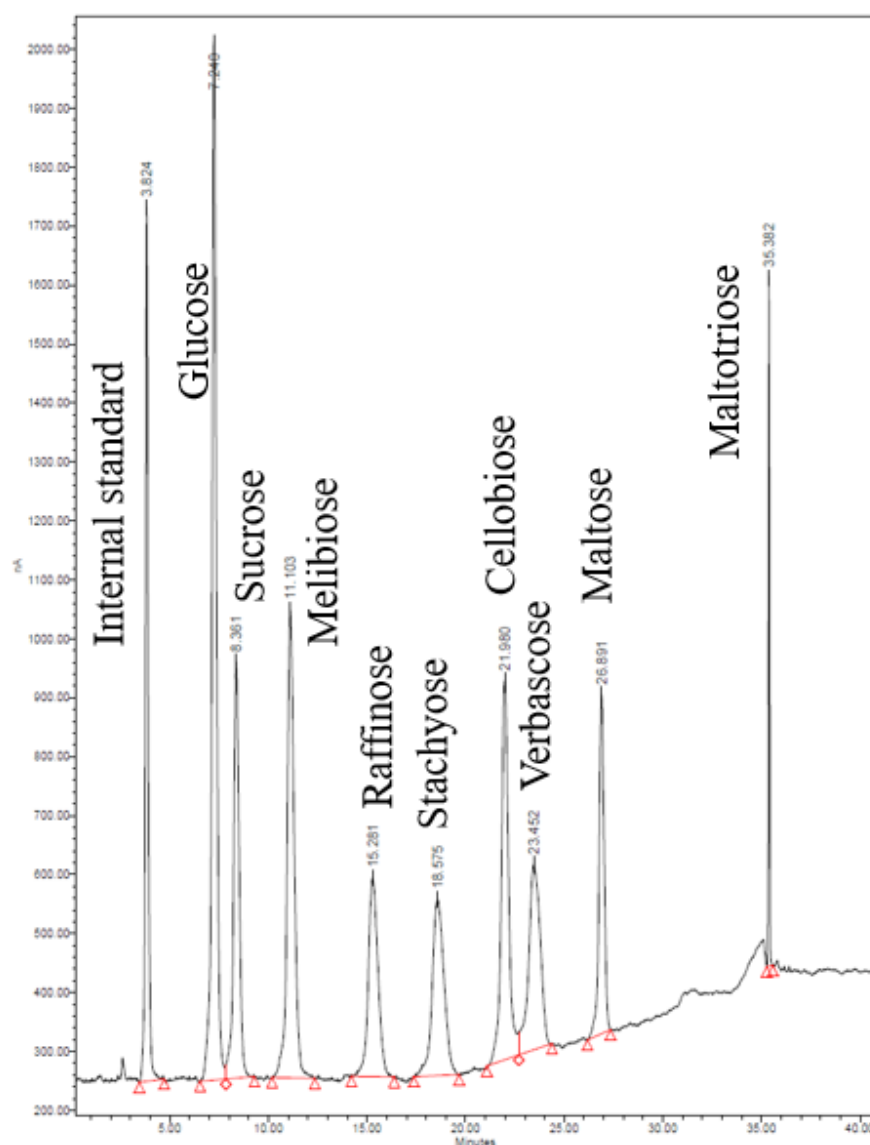


Figure 6. The chromatogram showing separation of soluble sugars in the standard solution with HPAEC-PAD.

3.3 Qualitative analysis with HILIC-ELSD and HPAEC-PAD

The chromatograms of the samples are not shown, due to confidentiality.

Glucose, fructose, sucrose, and stachyose were identified in the gurt sample. The meat alternative had a more versatile sugar profile compared to the gurt, containing glucose, myo-inositol, sucrose, maltose, maltotriose, raffinose, stachyose and verbascose. Maltotriose was identified only with the HPAEC-PAD, as the sensitivity of the HILIC-ELSD was not sufficient to detect such a low concentration.

Some unknown compounds were detected in the both samples using both chromatographic systems, however, they did not interfere with the analysis of soluble sugars. The number of unknown compounds was the same in both chromatographic systems, indicating that they were not contaminants.

3.4 Quantitative analysis with HILIC-ELSD

The accuracy of the calibration curves with HILIC-ELSD is presented in Table 5. The accuracy slightly decreased in the middle of the calibration curves, and the lowest calibration points were clearly inaccurate for glucose and sucrose. While the analyte concentration had an effect on accuracy, the fit of the calibration curves was still sufficient for every analyte. The smallest observed determination coefficient (R^2) was 0.990 for glucose, 0.992 for sucrose, 0.998 for stachyose, and 0.997 for verbascose.

Table 5. Accuracy of the calibration curves with HILIC-ELSD, established by calculating the difference between a measured concentration and the actual concentration (%) $\pm$ standard deviation. Nine independent measurements were used to assess the variation between the runs.

Level (g/l)	Glucose (%)	Sucrose (%)	Stachyose (%)	Verbascope (%)
0.05	125 $\pm$ 3	123 $\pm$ 4	107 $\pm$ 4	106 $\pm$ 6
0.1	93 $\pm$ 1	93 $\pm$ 1	97 $\pm$ 2	100 $\pm$ 5
0.2	85 $\pm$ 2	87 $\pm$ 3	96 $\pm$ 3	94 $\pm$ 2
0.4	92 $\pm$ 2	92 $\pm$ 3	98 $\pm$ 2	98 $\pm$ 2
0.7	99 $\pm$ 1	100 $\pm$ 1	100 $\pm$ 1	99 $\pm$ 1
1	106 $\pm$ 1	105 $\pm$ 2	102 $\pm$ 1	102 $\pm$ 1

Tables 6 and 7 present the HILIC-ELSD measurements obtained using the thesis method under different extraction conditions for the gurt and the meat alternative samples, respectively. A total of three outliers were removed from the dataset according to Equation (3). Glucose was the most abundant sugar in the gurt sample, whereas sucrose and verbascose were the two most abundant sugar analytes in the meat alternative.

Table 6. Measurements data for the gurt sample obtained using the thesis method under different extraction conditions with HILIC-ELSD. Concentrations are expressed as g/100 g fresh matter $\pm$ standard deviation. One outlier was removed from the glucose measurements analysed using method D according to Equation (3) and the critical values reported by Rorabacher (1991). n = number of replicates.

Method	Glucose g/100 g		Sucrose g/100 g		Stachyose g/100 g	
	Avg.	n	Avg.	n	Avg.	n
A	1.45 $\pm$ 0.16	6	0.99 $\pm$ 0.05	6	0.45 $\pm$ 0.04	6
B	1.47 $\pm$ 0.04	6	1.10 $\pm$ 0.04	6	0.47 $\pm$ 0.01	6
C	1.37 $\pm$ 0.06	6	1.10 $\pm$ 0.15	6	0.45 $\pm$ 0.02	6
D	1.41 $\pm$ 0.02	5	1.15 $\pm$ 0.13	6	0.47 $\pm$ 0.03	6
E	1.62 $\pm$ 0.10	6	1.12 $\pm$ 0.05	6	0.52 $\pm$ 0.02	6
F	1.42 $\pm$ 0.15	6	1.03 $\pm$ 0.09	6	0.46 $\pm$ 0.04	6
G	1.52 $\pm$ 0.16	6	1.10 $\pm$ 0.11	6	0.42 $\pm$ 0.05	6
H	1.41 $\pm$ 0.09	6	1.02 $\pm$ 0.07	6	0.40 $\pm$ 0.02	6
I	1.50 $\pm$ 0.07	6	1.06 $\pm$ 0.08	6	0.50 $\pm$ 0.02	6
J	1.43 $\pm$ 0.06	6	1.03 $\pm$ 0.07	6	0.39 $\pm$ 0.02	6
K	1.41 $\pm$ 0.11	6	0.99 $\pm$ 0.06	6	0.42 $\pm$ 0.03	6
L	1.35 $\pm$ 0.16	6	1.07 $\pm$ 0.16	6	0.44 $\pm$ 0.03	6
M	1.47 $\pm$ 0.12	18	1.08 $\pm$ 0.08	18	0.46 $\pm$ 0.03	18
Valio	1.56 $\pm$ 0.09	9	1.15 $\pm$ 0.10	9	0.50 $\pm$ 0.02	9

Table 7. Measurements data for the meat alternative sample obtained using the thesis method under different extraction conditions with HILIC-ELSD. Concentrations are expressed as g/100 g fresh matter $\pm$ standard deviation. Two outliers were removed from the verbascose measurements analysed using methods G and J according to Equation (3) and critical values reported by Rorabacher (1991). n = number of replicates.

Method	Sucrose (g/100 g)		Stachyose (g/100 g)		Verbascose (g/100 g)	
	Avg.	n	Avg.	n	Avg.	n
A	0.48 $\pm$ 0.03	6	0.22 $\pm$ 0.01	6	0.39 $\pm$ 0.01	6
B	0.48 $\pm$ 0.02	6	0.23 $\pm$ 0.01	6	0.44 $\pm$ 0.01	6
C	0.46 $\pm$ 0.02	6	0.22 $\pm$ 0.01	6	0.43 $\pm$ 0.04	6
D	0.48 $\pm$ 0.01	6	0.23 $\pm$ 0.01	6	0.43 $\pm$ 0.03	6
E	0.48 $\pm$ 0.04	6	0.27 $\pm$ 0.02	6	0.53 $\pm$ 0.02	6
F	0.54 $\pm$ 0.05	6	0.27 $\pm$ 0.01	6	0.52 $\pm$ 0.03	6
G	0.45 $\pm$ 0.04	6	0.17 $\pm$ 0.01	6	0.17 $\pm$ 0.01	5
H	0.38 $\pm$ 0.02	6	0.13 $\pm$ 0.01	6	0.13 $\pm$ 0.01	6
I	0.48 $\pm$ 0.02	6	0.27 $\pm$ 0.01	6	0.53 $\pm$ 0.03	6
J	0.48 $\pm$ 0.03	6	0.16 $\pm$ 0.01	6	0.15 $\pm$ 0.00	5
K	0.43 $\pm$ 0.05	6	0.14 $\pm$ 0.01	6	0.14 $\pm$ 0.02	6
L	0.48 $\pm$ 0.02	6	0.27 $\pm$ 0.02	6	0.53 $\pm$ 0.05	6
M	0.47 $\pm$ 0.02	18	0.22 $\pm$ 0.01	18	0.41 $\pm$ 0.03	18
Valio	0.51 $\pm$ 0.01	9	0.29 $\pm$ 0.01	9	0.60 $\pm$ 0.02	9

The in-house QC-sample substantiated the instrument performance in each run. The results did not change between the beginning and the end of the run, indicating that the instrument response remained stable throughout the analysis (Appendix 1). The X-charts established that the measurements were distributed on both side of the average value, thus no systematic trends were observed.

The centre point method (M) was used to assess the variation in the test samples throughout the study. Similarly to the QC-sample, measurements of the meat alternative did not exhibit a systematic trend or exceed the warning limit more than once (Appendix 2). In contrast, the X-charts of the gurt sample showed a decrease in measured levels over time, and a consistent trend was observed for all analytes (Appendix 3). As a similar trend was not observed for the QC-sample or the meat alternative, it was suggested that the gurt sample was not stable under frozen storage conditions. All measurement points were nevertheless retained in the dataset, however, this effect was identified as a source of variation.

3.5 Quantitative analysis with HPAEC-PAD

Analytes were well separated in the HPAEC-PAD system, thus no chromatographic optimization was required. The determination coefficients (R^2) of the standard curves were 0.999 for glucose, 0.988 for sucrose, 0.992 for stachyose, and 0.991 for verbascose. Hence, the linearity of the calibration curves was similar for both chromatographic systems. The measurement results of the HPAEC-PAD analyses are presented in Table 8. Replicate measurements were repeatable and no outliers were observed.

Table 8. HPAEC-PAD measurements of the gurt and meat alternative samples, calculated according to Equation (1) and reported as g/100 g fresh matter $\pm$ standard deviation. n = number of replicates, n.d. = not detected.

Sample	n	Glucose (g/100 g)	Sucrose (g/100 g)	Stachyose (g/100 g)	Verbascope (g/100 g)
Gurt	3	1.40 $\pm$ 0.03	1.07 $\pm$ 0.05	0.57 $\pm$ 0.01	n.d
Meat alternative	2	n.d	0.45	0.19	0.43

3.6 Dry matter and residual moisture content

The dry matter content of the fresh meat alternative and the residual moisture content of the freeze-dried meat alternative were used to convert analyte concentrations to a fresh matter basis, respectively. The dry matter content (D) of the fresh meat alternative was, on average 49.8%, with a standard deviation of 0.1%. The residual moisture content (W) of the freeze-dried meat alternative was, on average 1.3%, ranging from 1.23% to 1.37%.

3.7 The effect of extraction conditions

The response surface method demonstrated how different extraction conditions affected the extraction yield of soluble sugars. The regression data can be seen in Appendix 4. The statistical significance of the solvent clearly increased when the DP increased. For example, solvent was not a significant factor when extracting glucose (p-value = 0.358) and sucrose (p-value = 0.224), but for stachyose (p-value <0.001), increased ethanol concentration significantly decreased the yield in the gurt sample. In the meat alternative, solvent was a significant factor with every analyte (p-value <0.001), and the magnitude of its effect was increased in a relation to analyte's size.

Extraction time was not independently a significant factor, but some minor interactions were observed. For example, temperature and time interaction was statistically significant when extracting verbascose from the meat alternative (p-value = 0.040). However, the effect was almost 20-times less compared to the effect of solvent (Coef -0.011 vs -0.190). Solvent and time interaction was also statistically significant during the analysis of stachyose from the gurt sample (p-value <0.001).

Response surface plots presented in Figures 7 and 8 illustrate how the solvent and temperature interaction appeared in the gurt and the meat alternative samples. Glucose and sucrose from the gurt sample had saddle shaped response surfaces, whereas all the other analytes had quadratic or planar shaped surfaces. Solvent and temperature interaction was statistically significant for every analyte, except for sucrose from the meat alternative. That is, the effect of temperature on extraction yield varied depending on the solvent.

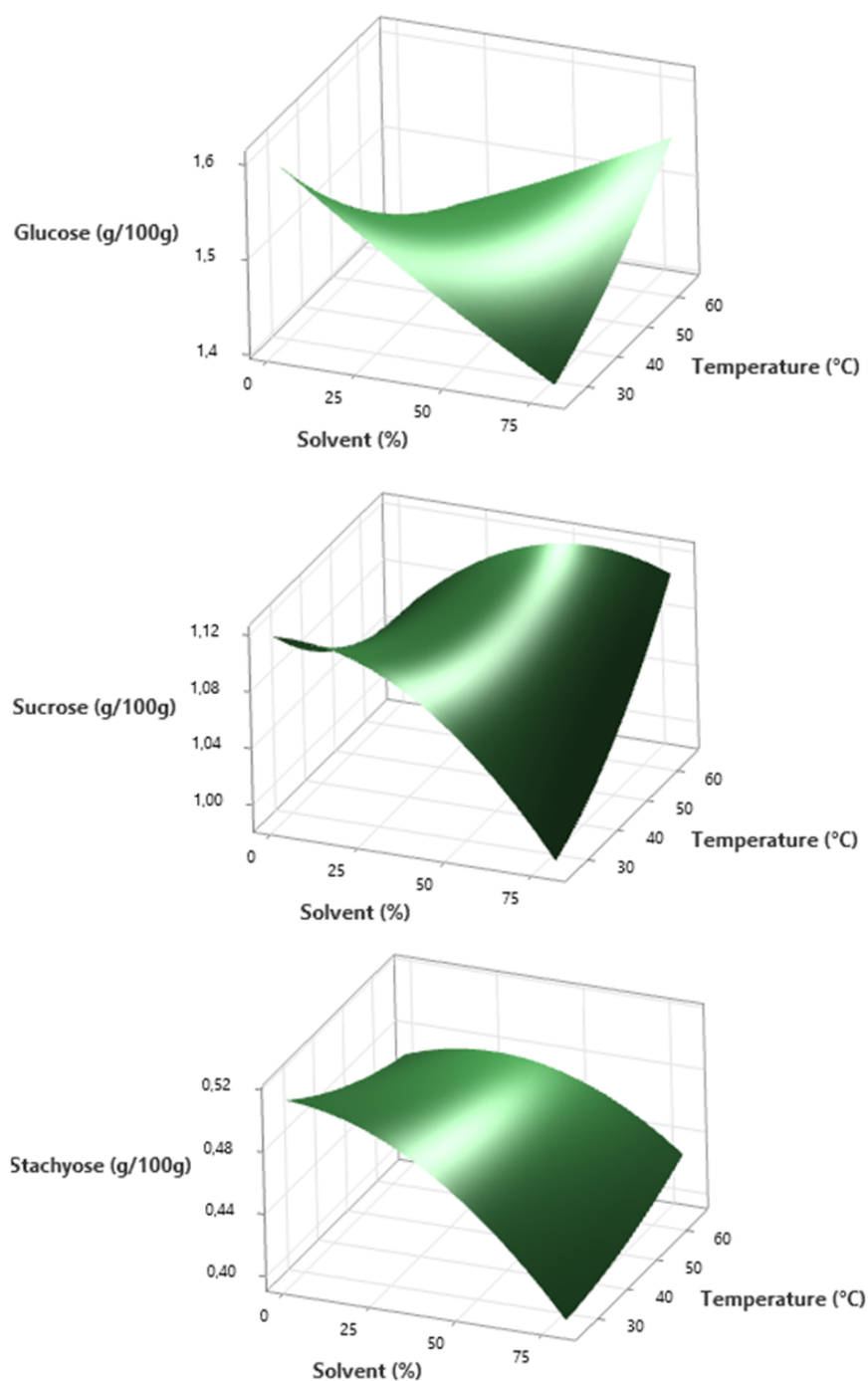


Figure 7. Effect of temperature and solvent on the concentration of a measured analyte in the gurt sample. Solvent (%) indicates the proportion of ethanol in MilliQ water (from 100% water to 80% ethanol).

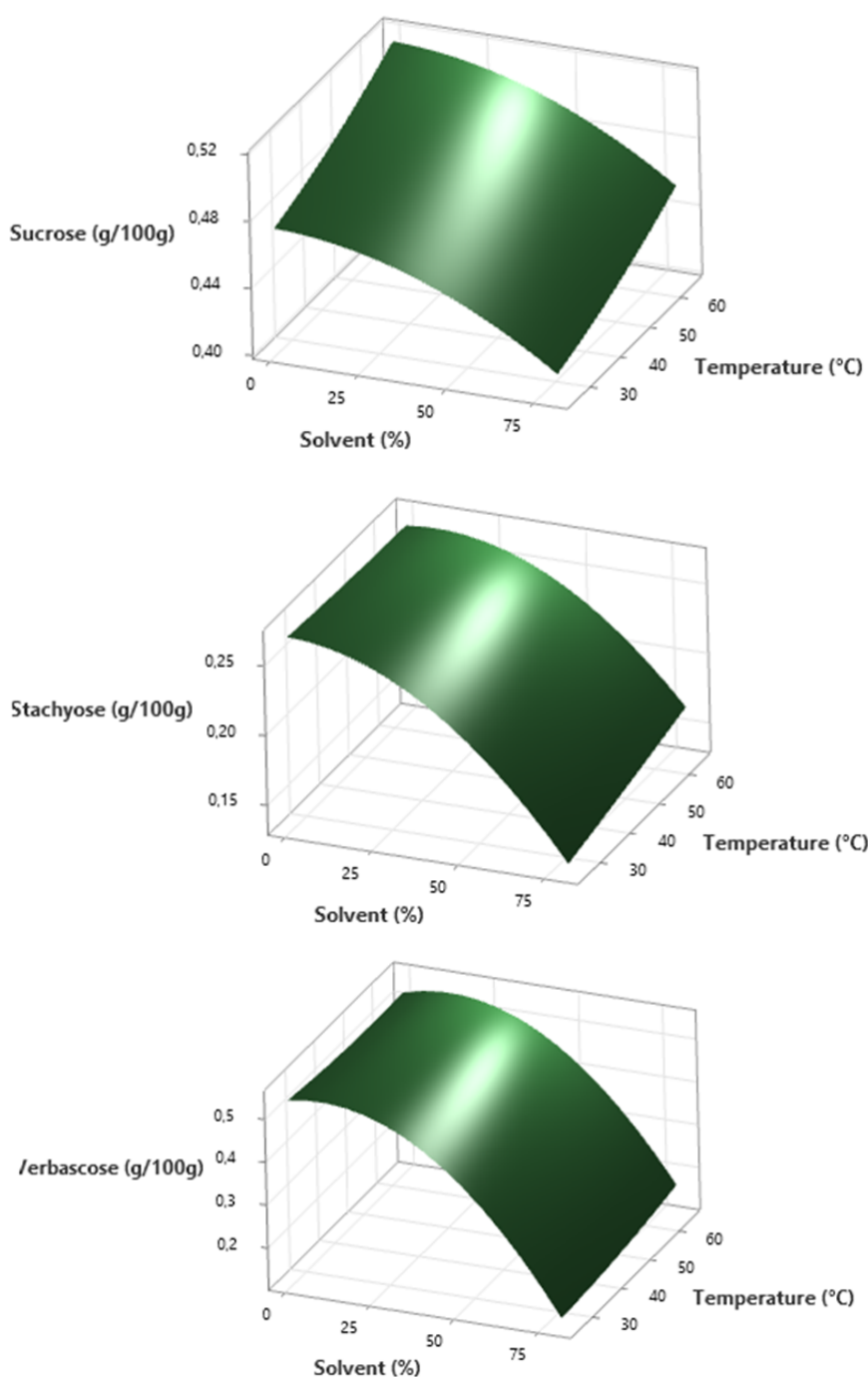


Figure 8. Effect of temperature and solvent on the concentration of measured analyte from the meat alternative sample. Solvent (%) indicated the proportion of ethanol in MilliQ water (from 100% water to 80% ethanol).

Extraction with 80% ethanol at 25°C resulted in the lowest yields for every analyte, regardless of the matrix. Increasing the extraction temperature to 65°C markedly improved the extraction efficiency of mono- and disaccharides (Figure 7). In fact, the extraction yields of glucose and sucrose obtained using 80% ethanol at 65°C were comparable to those achieved with water extraction at 25°C. This positive temperature effect during aqueous ethanol extraction was not observed for oligosaccharides. The temperature effect during water extraction was matrix-dependent; higher extraction temperatures decreased the yield in the gurt

sample, whereas in the meat alternative it resulted in similar or increased yields of soluble sugars.

Besides investigating the changes in the extraction yield, samples were also visually observed. When water was used as the extraction solvent, a distinct lipid layer was observed in the meat alternative sample after the centrifugation step. During ethanol extraction, lipids appeared to be considerably more soluble. In the gurt sample, substantial precipitation was observed when extraction was performed with 80% ethanol, whereas similar precipitation was not observed in the meat alternative sample.

3.8 Method comparison

Because equal variances could not be assumed (Levene's test), Welch's test was used to investigate the differences between the methods. The results from the variance analyses are presented in Figures 9 and 10. The confidence intervals are presented at the 95% confidence level. Non overlapping intervals indicate that the methods are statistically significantly different (p-value <0.05). It should be noted that the number of replicates differed between the methods, which affected the size of the confidence intervals shown in the graphs. The number of replicates for each method are presented in Tables 1 and 8. The differences of means, and p-values from the variance analyses are listed in Appendices 5–10.

The most clear difference between the methods was observed in the analysis of stachyose and verbascose from the meat alternative. Thesis methods that utilized 80% ethanol as an extraction solvent (G, H, J, K) clearly produced the lowest yields. A similar “W-shaped” pattern was also seen in the analysis of stachyose from the gurt sample, with the difference that the methods using water at elevated temperatures (L, F) resulted also lower yields.

Comparable results were obtained using water extraction and Carrez clarification (Valio method) and method E (water extraction at 25°C with ultra centrifugal filtration), suggesting that Carrez clarification did not significantly affect the extraction yield. Method E and the University method (UH) employed water as an extraction solvent with ultra centrifugal filtration. There was no statistically significant differences between the methods in the analysis of the gurt sample, but differences were observed in the analysis of RFOs from the meat alternative. One major difference between the methods was that fresh sample was used in method E, whereas the meat alternative was freeze-dried in the University method. Therefore, some error might be caused by the fresh matter weight conversion calculations required by the University method. Overall, extraction directly from the fresh sample was discovered to be repeatable and efficient.

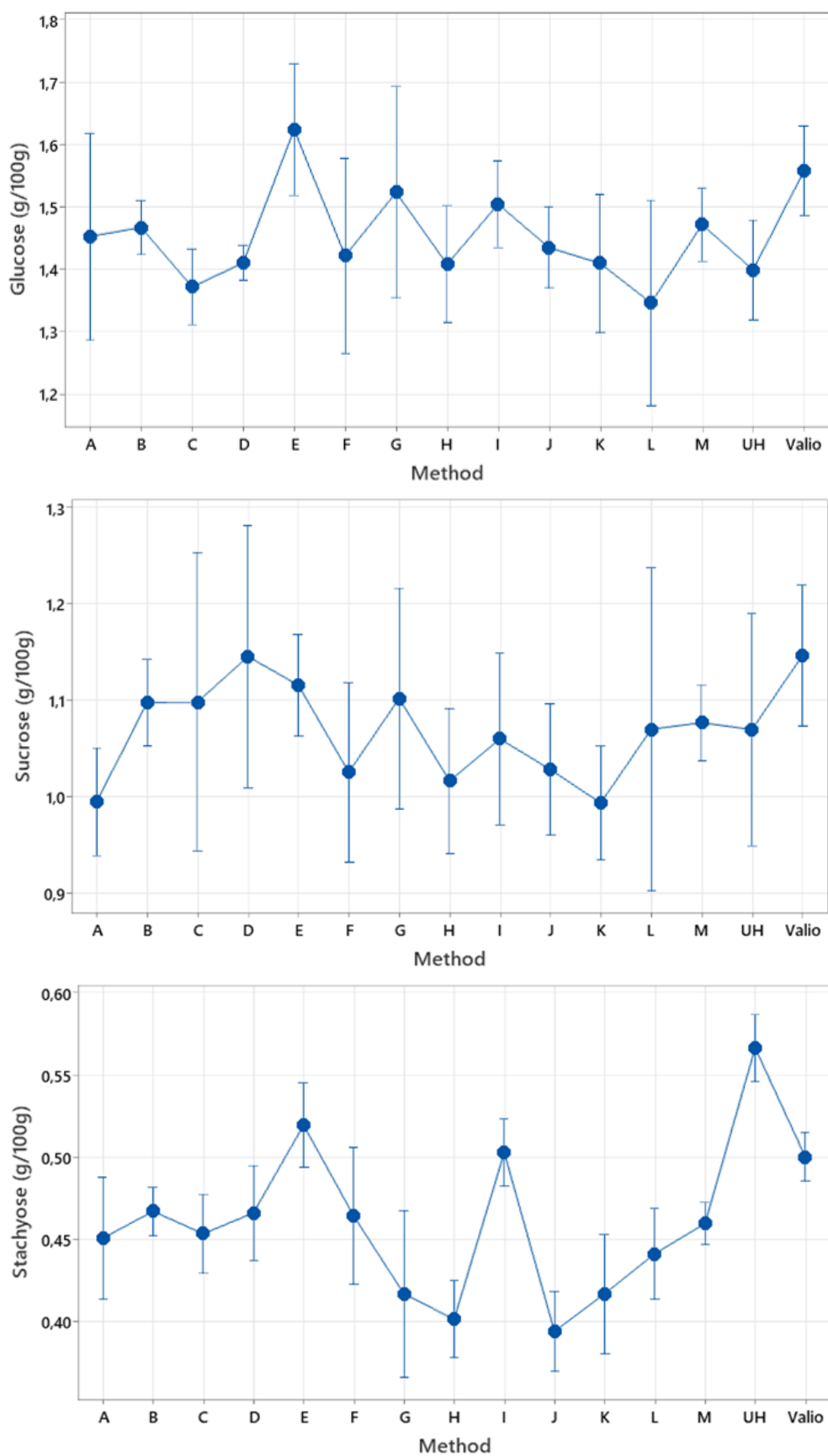


Figure 9. Variance analysis (Welch's test) of different pretreatment methods for the gurt sample. Confidence intervals are shown at the 95% confidence level. Non overlapping intervals indicate statistically significant differences (p-value <0.05).

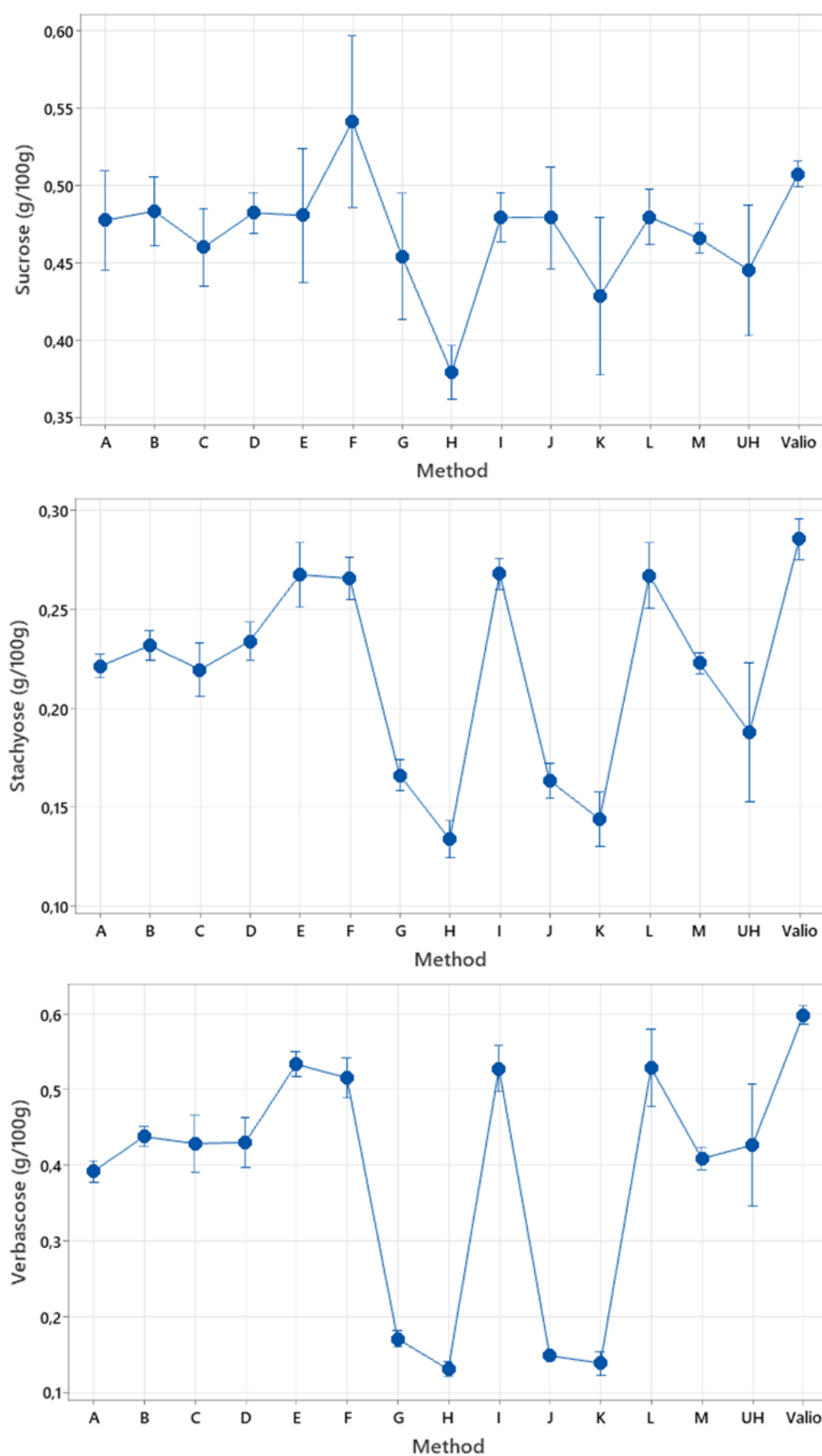


Figure 10. Variance analysis (Welch's test) of different pretreatment methods for the meat alternative sample. Confidence intervals are shown at the 95% confidence level. Non overlapping intervals indicate statistically significant differences (p-value < 0.05).

3.9 Method performance of the developed method

Based on the results presented in sections 3.7 and 3.8, and practical considerations of the routine analysis, method E was selected for the most promising thesis method, therefore its method performance was evaluated. This method comprised of water extraction at 25°C with 20 min extraction time.

The limit of detection (LOD) and limit of quantification (LOQ) for each analyte is presented in Table 9. Analysis of stachyose resulted in the most variation, causing slightly higher LOD and LOQ values. These results indicated that the sensitivity of the HILIC-ELSD was sufficient for quantifying the three most common soluble sugars from the test samples.

Table 9. Limit of detection (LOD) and limit of quantification (LOQ) for individual analytes analysed by HILIC-ELSD. The results were calculated according to Equations (4) and (5).

Analyte	LOD (g/100 g)	LOQ (g/100 g)
Glucose	0.004	0.013
Sucrose	0.005	0.018
Stachyose	0.014	0.048
Verbascose	0.006	0.020

Table 10 presents the variation within a day (s_r), between days (s_{between}), and the total repeatability (s_{total}) for each analyte and sample matrix. The analysis of glucose from the gurt sample clearly contained the most variation. Sucrose and stachyose were analysed in the both samples, and no clear matrix effect was observed. The most repeatable was the analysis of verbascose from the meat alternative.

Table 10. Within-day repeatability (s_r), between-day repeatability (s_{between}), and relative total repeatability (s_{total}) for each analyte determined in the gurt and meat alternative samples, calculated according to Equations (6), (7), and (8). n.d. = not detected.

Analyte	Gurt			Meat alternative		
	s_r (g/100 g)	s_{between} (g/100 g)	s_{total} (%)	s_r (g/100 g)	s_{between} (g/100 g)	s_{total} (%)
Glucose	0.04	0.17	10.9	n.d.	n.d.	n.d.
Sucrose	0.03	0.07	6.7	0.02	0.04	8.2
Stachyose	0.01	0.04	8.2	0.01	0.01	5.7
Verbascose	n.d.	n.d.	n.d.	0.02	0.03	5.6

Recovery analyses indicated that no significant analyte loss occur during the pretreatment procedure nor during the HILIC-ELSD analysis (Table 11). The recovery (%) was reported as an average recovery of the three addition levels, utilizing 18 replicates. Recoveries of the gurt sample were slightly higher compared to the meat alternative, but the level of variation was similar with both samples.

Table 11. Recovery (%) values for the gurt and meat alternative samples, calculated according to Equation (9). The results represent the average recovery values of the three spiking levels $\pm$ standard deviation (n=18). n.d. = not detected.

Analyte	Gurt (%)	Meat alternative (%)
Glucose	114 $\pm$ 8	n.d.
Sucrose	118 $\pm$ 11	104 $\pm$ 8
Stachyose	110 $\pm$ 12	98 $\pm$ 9
Average	114	101

The uncertainty caused by random error, $U(R_w)$, was on average of 9% for the gurt sample and 7% for the meat alternative. The bias determined by recovery analyses, $U(\text{recovery})$, were on averages of 14% and 1% for the gurt and meat alternative sample, respectively. Therefore, the expanded measurement uncertainty, (U), was 33% for the gurt sample and 13% for the meat alternative.

4 DISCUSSION

4.1 Soluble sugars in the test samples

Based on the raw material composition, the expected RFOs were identified in the test samples. Stachyose was the only RFO found in the gurt sample, whereas the meat alternative sample exhibited a more diverse RFO profile, containing raffinose, stachyose, and verbascose. Myo-inositol was the only sugar alcohol identified in the test samples using HILIC-ELSD. Previous studies have also confirmed the presence of myo-inositol in various legumes (Ekvall et al. 2006; Hou et al. 2009; Ispiryan et al. 2020). The physiological impact of myo-inositol is not yet well defined, but it has been suggested that it may have similar effects in human gut as other FODMAPs (Ispiryan et al. 2020; Martínez-Villaluenga et al. 2008). In plants, myo-inositol is, for example, released from galactinol during RFO synthesis (Martínez-Villaluenga et al. 2008).

Appropriate chromatographic conditions should be established based on the required resolution and the practicality of the analysis time. In this study, the resolution achieved for mono-, di-, and oligosaccharides using HPAEC–PAD was slightly better than that obtained with HILIC–ELSD. The CarboPac PA20 column used in HPAEC was not suitable for sugar alcohol analysis, and an alternative column would be required in the sugar alcohol analysis. In contrast, HILIC with the Luna Omega Sugar column could potentially be applicable for sugar alcohol analysis after chromatographic optimization.

Plant-based products typically contain a variety of raw materials, and the applied food processing methods, for example, enzyme treatment can alter the final sugar profile. This may result in complex chromatograms, where identification of soluble sugars based on the retention times is not accurate and may be time consuming. Mass spectrometry (MS) would provide a more selective and accurate method than ELSD or PAD and therefore, future studies should aim to identify these compounds using MS. Another challenge in the soluble sugar analysis of plant-based products is that the sugars can easily co-elute in the chromatographic system. This challenge has been acknowledged by other authors as well. Muir et al. (2009) resolved co-elution challenges by utilizing two different columns and mobile phase combinations to separate different FODMAP compounds from vegetables and fruits. However, this approach would not be practical for routine analyses.

4.2 Factors affecting the extraction efficiency

Extraction parameters influenced the extraction yield differently depending on the matrix and DP of the sugars. These results clarify why there is no consensus of the optimal extraction conditions in the literature (Ekvall et al. 2007; Hoehnel et al. 2022; Jovanovic-Malinovska et al. 2015; Kim et al. 2003; Xiaoli et al. 2008).

The extraction yield of RFOs decreased significantly when aqueous ethanol was used as an extraction solvent compared to water, and this was consistent with both samples. The extraction efficiency of ethanol was clearly insufficient for sugar analytes with a DP greater than 4. In contrast, Jovanovic-Malinovska et al. (2015) reported that the extraction yield of RFOs increased up to an ethanol concentration of 60%, after which it decreased. One notable difference was that Jovanovic-Malinovska et al. (2015) utilized ultrasound-assisted extraction, whereas magnetic stirrer was used in this study. Ultrasound-assisted extraction may enhance extraction efficiency and thereby enable the use of aqueous ethanol as an extraction solvent. Additionally, Xiaoli et al. (2008) reported that 50% ethanol was the most effective solvent for extracting RFOs from chickpea. In their study, three extraction cycles were applied, which may also enhance the extraction due to the increased solvent volume. These findings suggest that when aqueous ethanol is used as an extraction solvent, more effective extraction procedure such as ultrasound-assisted extraction or multiple extraction cycles may be required to achieve efficient solubilization of RFOs. The advantage of using aqueous ethanol as an extraction solvent is that polysaccharides are not efficiently solubilized, whereas during water extraction, additional procedures for removal of polysaccharides need to be considered.

The matrix effect was particularly evident during water extraction at elevated temperatures. In the gurt sample, the extraction yield reduced when hot water was used, whereas in the meat alternative the yield either remained comparable or increased with increasing temperature. This suggests that the matrix components such as polysaccharides and proteins present in the samples influenced the extraction process differently. Although both samples contained polysaccharides their structural characteristics differed, which may have resulted in different interactions with water under elevated temperatures. These observations highlight the importance of understanding the sample matrix and how potential interfering compounds react during the extraction process.

Proteins can entrap sugars inside of their network structures, thereby reducing the extraction yield (Kim et al. 2003; Xiaoli et al. 2008). In the gurt sample, clear precipitation was observed during ethanol extraction. However, this did not affect the extraction yields of glucose or sucrose at elevated temperatures. This suggests that, under the conditions studied, solvent extraction efficiency was more significant than sugar entrapment resulting from protein denaturation and/or polysaccharide precipitation.

During the extraction of soluble sugars, it is important to maximise the extraction yield while preventing enzymatic hydrolysis. In addition, the extraction procedure should be robust. Georgelis et al. (2018) and Hoehnel et al. (2022) observed enzymatic degradation in potato tuber, strawberry fruit, cereals, and legumes during water extraction, resulting in overestimation of soluble sugars. Aqueous ethanol extraction (60% and 80%, respectively) inactivated the enzymes and therefore, aqueous ethanol extraction was concluded as the most suitable extraction solvent. In processed products, enzyme activity is no longer a concern, and no indications of sugar degradation during water extraction were observed in this thesis. Georgelis et al. (2018) reported that extracting with 60% ethanol at 80 °C produced also more repeatable results than water extraction at cold temperature. Such systematic trend was not observed in this thesis.

Extraction directly from the fresh sample is a convenient approach for routine analyses. However, sample representativeness can be an issue, for example in the case of meat alternatives. This thesis demonstrated that repeatable results was obtained directly from the fresh sample after homogenization step.

4.3 Removal of interferences

The advantage of using Carrez clarification over the ultra centrifugal filtration was that lipids were more efficiently removed. The results of this thesis indicated that Carrez clarification did not cause analyte losses or substantially increase the chromatographic baseline. Carrez clarification has also been used in the pretreatments of plant-based samples by other authors (Huang et al. 2023; Ispiryan et al. 2019; Stöckl et al. 2024). In contrast, Solé-Enrech et al. (2023) removed interfering lipids using high-speed centrifugation. In this study, a distinct lipid layer was observed after centrifugation, however, centrifugation alone was not sufficient, as a large lipid-derived peak was detected in the chromatogram. The lipid peak eluted before any analyte, thus it did not affect the quantification of soluble sugars.

Ultra centrifugal filtration provided a slightly more stable baseline and better peak shape for oligosaccharides compared to the Carrez method currently used at Valio. This may result from more efficient removal of polysaccharides. Although polysaccharides were not sufficiently removed with the method currently used at Valio, this did not affect the extraction efficiency of soluble sugars. However, injecting polysaccharides into the system can influence the column lifetime. Therefore, if only the Carrez clarification method is used for samples containing high amounts of polysaccharides, additional pretreatment procedures for their removal should be considered. Further studies should verify the compatibility of Carrez clarification together with ultra centrifugal filtration. Another potential approach for removing polysaccharides could be precipitation with acetonitrile, the eluent used in HILIC-ELSD. For HPAEC-PAD analysis, ultra-centrifugal filtration would be a more practical method, since water is used as the eluent.

4.4 Applicability of the developed pretreatment method for plant-based products

Water extraction at 25°C for 20 min (method E) was selected as the most applicable thesis method for soluble sugar analysis of various plant-based products. The method currently used at Valio was also producing statistically similar results, indicating that Carrez clarification would also be applicable for plant-based products.

The performance of method E was evaluated against the values reported by a well-established outsourcing laboratory, which applies an accredited in-house HPAEC–PAD method for RFOs and an HPAEC–PAD method for glucose and sucrose in accordance with AOAC Official Method 982.14. This comparison of method performance is presented in Table 12. For the meat alternative sample, method E showed high comparability with the corresponding methods used by the outsourcing laboratory, indicating the applicability of the developed method. For the gurt sample, the measurement uncertainty of method E was higher than that of the reference methods, demonstrating that further verification is required for gurt-type samples.

Table 12. Method performance comparison between method E with HILIC–ELSD and reference methods with HPAEC–PAD used by a well-established outsourcing laboratory. The outsourcing laboratory employed separate methods for raffinose family oligosaccharides (RFOs) and glucose (Glu) and sucrose (Suc).

Method performance	Method E		Outsourcing laboratory	
	Gurt	Meat alternative	RFOs	Glu, Suc
Recovery (%)	114	101	90–110	not known
Measurement uncertainty (%)	33	13	7–12	15 (>0.4 g/100 g) 25 (<0.4 g/100 g)

The recovery analyses of the gurt sample contributed the most to the poor measurement uncertainty. Recovery analyses do not directly establish the extraction efficiency of the native analyte but rather it substantiates if some analyte loss occurs during the pretreatment. Therefore, it is used to estimate measurement uncertainty. Some bias can be caused by the fact that the spiked analytes are more easily extracted, since they are not bound to the matrix like the native analytes (Cantwell H. 2025). According to AOAC guidelines, recovery should be 97–103% for analyte concentration of 1% and 95–105% for the analyte concentration of 0.1% (AOAC International 2016). The recovery values in this thesis were higher than those in the guidelines. The bias could be due to the suboptimal calibration curves, since the curves were optimized to the analyte level in the initial sample material and not to the level with a standard addition.

LOD and LOQ values in this study represented only the HILIC-ELSD performance. The entire pretreatment procedure should have been included in the analysis, to obtain a more comprehensive LOD and LOQ. However, the sensitivity of HILIC-ELSD was clearly sufficient for the analyte concentrations determined in this thesis.

5 CONCLUSION

This study confirmed that the optimal extraction conditions for soluble sugars were dependent on the sample matrix and DP of the sugar analytes. During water extraction at high temperatures compared to low temperatures, extraction yields decreased in the gurt sample, but remained comparable or were moderately higher in the meat alternative. This difference likely results from variations in the polysaccharide composition of the samples. For sugars with a higher degree of polymerization ($DP \geq 4$), yields decreased significantly when aqueous ethanol was used as the extraction solvent compared to water, and this effect was consistent in both matrices. Statistically significant interaction between the extraction solvent and temperature was observed for every analyte, except for sucrose in the meat alternative. In practice, the positive effect of higher temperature in ethanol extraction (80% ethanol at 65°C) was particularly significant in mono- and disaccharide analysis, where it resulted in yields comparable to those obtained with water extraction at 25°C. For RFOs, the interaction effect was not sufficiently large to make ethanol extraction feasible.

It was observed that interfering compounds affected both the extraction yield of soluble sugars and chromatographic performance, highlighting the importance of proper pretreatment. A key advantage of the ultra centrifugal filtration was its efficient removal of polysaccharides, which was not achieved with the Carrez method. Nevertheless, this study indicated the suitability of Carrez clarification (Valio method), as the results were comparable with those obtained with ultra centrifugal filtration under optimized extraction conditions. However, additional procedures for removing polysaccharides should be considered when Carrez clarification alone is applied, in order to obtain cleaner chromatograms and to extend the column lifetime.

Water extraction at 25°C for 20 min (method E) was concluded to be the generally most applicable extraction condition across a wide range of sample matrices and sugars with varying DP values. Method E demonstrated good method performance for the meat alternative. The response surface analysis suggests the applicability of the method for gurt-type matrices as well, but further verification is required to decrease the measurement uncertainty. Samples should also be analysed fresh to prevent changes that may occur during freezing.

Both chromatographic methods were suitable for the analysis of mono- and disaccharides and RFOs from the test samples. The resolution was slightly better in HPAEC-PAD with the CarboPac PA20 column. An advantage of HILIC-ELSD with the Luna Omega Sugar column is its potential applicability for the analysis of sugar alcohols. For example, the presence of myo-inositol in the meat alternative sample was detected in this study using HILIC-ELSD, but further chromatographic optimization is required to enable adequate separation of other sugar alcohols.

DECLARATION OF THE USE OF AI

Copilot Microsoft 365 was utilized in this thesis only for the final language revision. AI did not generate any ideas, text or analyses for this thesis.

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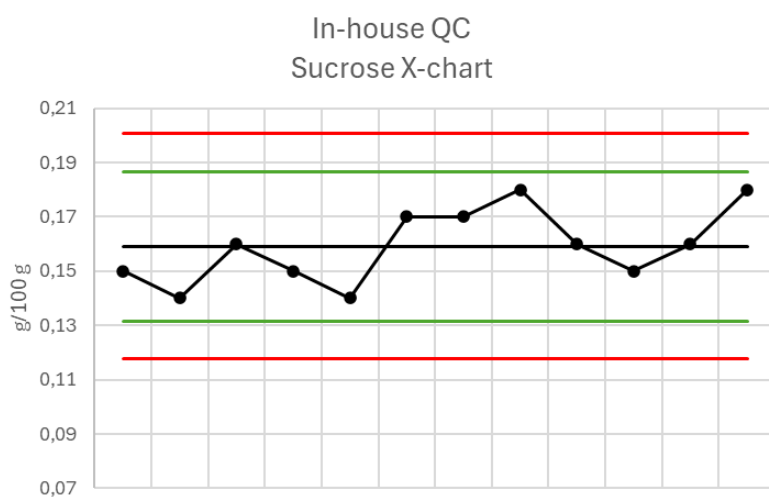
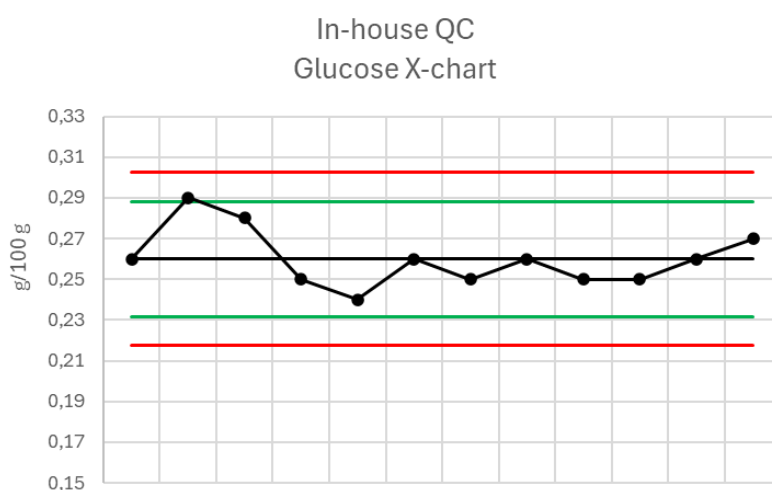
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APPENDICES

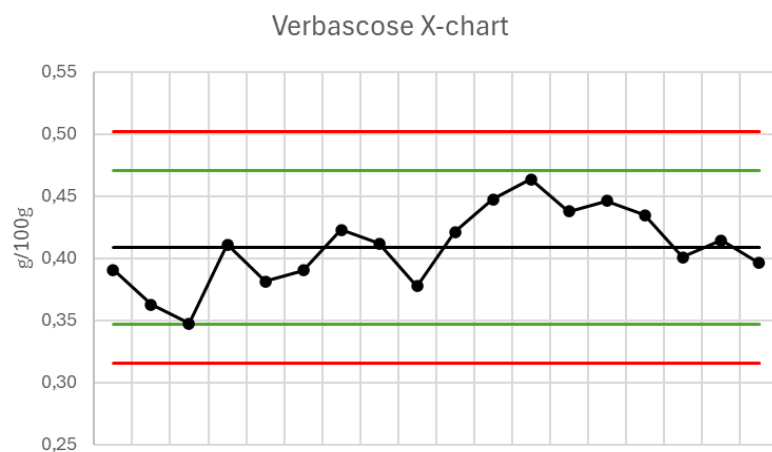
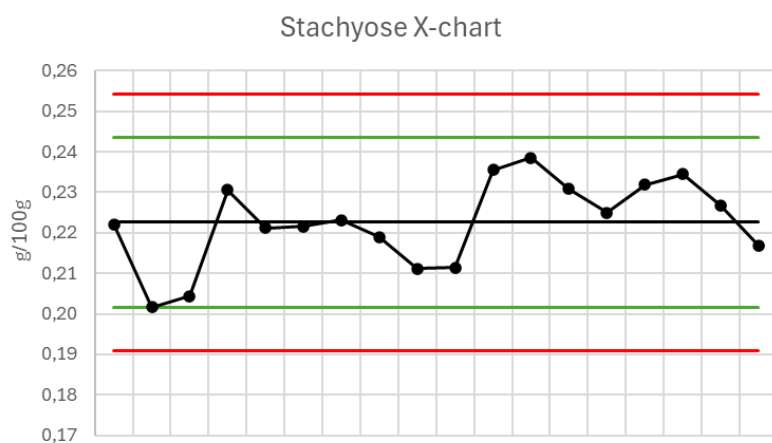
Appendix 1. X-charts, QC sample

X-charts of the QC-sample, analysed with Valio method. The black line represent the average concentration, the green line is the warning limit (two times the standard deviation from the average value), and the red line is the action limit (three times the standard deviation from the average value). Only the first QC-sample of the run was reported into the X-chart, while other replicates were used to confirm that the level of results remained similar throughout the run.



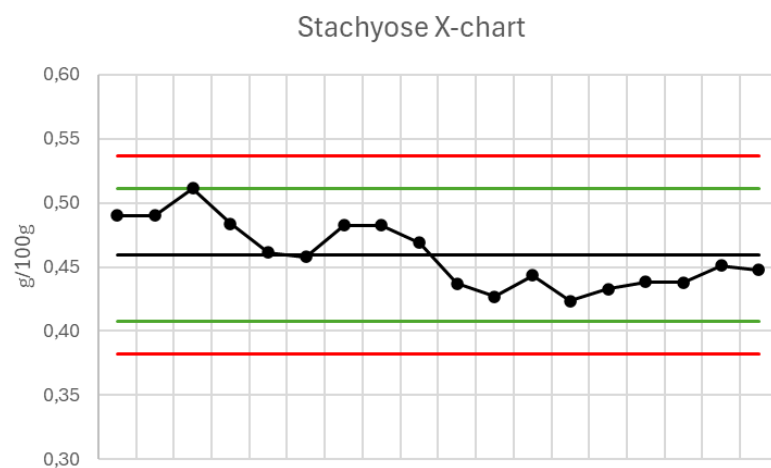
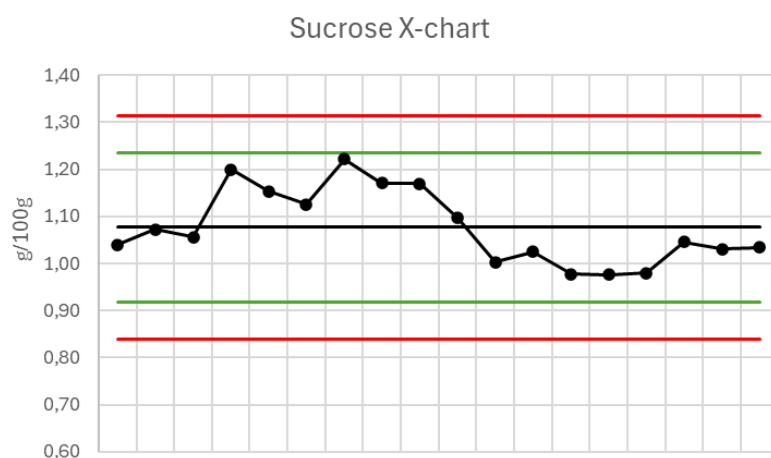
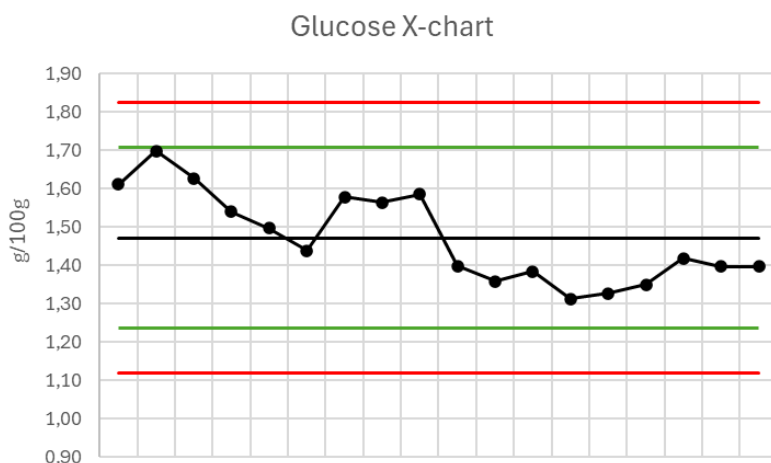
Appendix 2. X-charts, meat alternative sample

X-charts of the meat alternative sample, analysed with the thesis method using the centre point extraction conditions (method M). The black line represent the average concentration, the green line is the warning limit (two times the standard deviation from the average value), and the red line is the action limit (three times the standard deviation from the average value). Three consecutive measurement points were analysed in the same run. All measurements were analysed during 28.5.2025–3.7.2025.



Appendix 3. X-charts, gurt sample

X-charts of the gurt sample, analysed with the thesis method using the centre point extraction conditions (method M). The black line represent the average concentration, the green line is the warning limit (two times the standard deviation from the average value), and the red line is the action limit (three times the standard deviation from the average value). Three consecutive measurement points were analysed in the same run. All measurements were analysed during 16.5.2025–3.7.2025.



Appendix 4. Regression data from the Box-Behnken response surface method

Gurt sample, factors	Glucose		Sucrose		Stachyose	
	Coef	p-value	Coef	p-value	Coef	p-value
Constant	1.474	0.000	1.083	0.000	0.470	0.000
Solvent	-0.015	0.358	-0.016	0.224	-0.037	0.000
Temperature	-0.014	0.413	0.012	0.371	-0.004	0.377
Time	0.012	0.489	0.020	0.141	0.008	0.071
Solvent x Solvent	0.006	0.810	-0.035	0.101	-0.026	0.000
Temperature x Temperature	0.013	1.01	0.017	0.386	0.006	0.353
Time x Time	-0.058	0.019	-0.010	0.618	-0.006	0.333
Solvent x Temperature	0.081	0.001	0.047	0.013	0.019	0.001
Solvent x Time	-0.042	0.069	0.015	0.432	-0.022	0.000
Temperature x Time	0.007	0.758	-0.014	0.464	-0.001	0.880

Meat alternative sample, factors	Sucrose		Stachyose		Verbascose	
	Coef	p-value	Coef	p-value	Coef	p-value
Constant	0.474	0.000	0.239	0.000	0.464	0.000
Solvent	-0.030	0.000	-0.058	0.000	-0.190	0.000
Temperature	0.020	0.000	0.006	0.000	0.007	0.081
Time	0.003	0.592	0.002	0.183	0.004	0.249
Solvent x Solvent	-0.014	0.084	-0.032	0.000	-0.135	0.000
Temperature x Temperature	0.004	0.629	0.001	0.740	0.007	0.191
Time x Time	0.006	0.377	0.003	0.176	0.006	0.292
Solvent x Temperature	0.000	0.975	0.008	0.000	0.015	0.007
Solvent x Time	0.010	0.146	0.004	0.073	0.003	0.527
Temperature x Time	0.004	0.567	0.001	0.656	-0.011	0.040

Appendix 5. Variance data of glucose analysis from the gurt

Method	Difference of means	p-value	Method	Difference of means	p-value
B - A	0.0146	1.000	I - E	-0.1193	0.542
C - A	-0.0807	0.987	J - E	-0.1889	0.102
D - A	-0.0423	1.000	K - E	-0.2139	0.132
E - A	0.1712	0.636	L - E	-0.2775	0.142
F - A	-0.0310	1.000	M - E	-0.1519	0.263
G - A	0.0706	1.000	UH - E	-0.0246	1.000
H - A	-0.0446	1.000	Valio - E	-0.0659	0.983
I - A	0.0519	1.000	G - F	0.1015	0.994
J - A	-0.0178	1.000	H - F	-0.0137	1.000
K - A	-0.0428	1.000	I - F	0.0829	0.982
L - A	-0.1063	0.992	J - F	0.0132	1.000
M - A	0.0193	1.000	K - F	-0.0118	1.000
UH - A	0.1466	0.675	L - F	-0.0754	1.000
Valio - A	0.1053	0.946	M - F	0.0502	1.000
C - B	-0.0953	0.207	UH - F	0.1775	0.422
D - B	-0.0568	0.350	Valio - F	0.1363	0.760
E - B	0.1566	0.191	H - G	-0.1152	0.935
F - B	-0.0455	1.000	I - G	-0.0187	1.000
G - B	0.0560	0.999	J - G	-0.0883	0.980
H - B	-0.0592	0.945	K - G	-0.1133	0.958
I - B	0.0373	0.991	L - G	-0.1769	0.800
J - B	-0.0323	0.996	M - G	-0.0513	1.000
K - B	-0.0573	0.982	UH - G	0.0760	0.992
L - B	-0.1209	0.828	Valio - G	0.0347	1.000
M - B	0.0047	1.000	I - H	0.0965	0.695
UH - B	0.1320	0.078	J - H	0.0269	1.000
Valio - B	0.0907	0.462	K - H	0.0019	1.000
D - C	0.0385	0.934	L - H	-0.0617	1.000
E - C	0.2519	0.021	M - H	0.0639	0.971
F - C	0.0498	1.000	UH - H	0.1912	0.062
G - C	0.1513	0.680	Valio - H	0.1499	0.230
H - C	0.0361	1.000	J - I	-0.0696	0.819
I - C	0.1326	0.116	K - I	-0.0947	0.822
J - C	0.0630	0.843	L - I	-0.1582	0.628
K - C	0.0380	1.000	M - I	-0.0326	1.000
L - C	-0.0256	1.000	UH - I	0.0947	0.402
M - C	0.1000	0.344	Valio - I	0.0534	0.984
UH - C	0.2273	0.007	K - J	-0.0250	1.000
Valio - C	0.1860	0.016	L - J	-0.0886	0.977
E - D	0.2134	0.055	M - J	0.0370	0.999
F - D	0.0113	1.000	UH - J	0.1643	0.043
G - D	0.1128	0.873	Valio - J	0.1231	0.239
H - D	-0.0024	1.000	L - K	-0.0636	1.000
I - D	0.0942	0.258	M - K	0.0620	0.989
J - D	0.0245	0.999	UH - K	0.1893	0.122
K - D	-0.0005	1.000	Valio - K	0.1481	0.369
L - D	-0.0641	0.996	M - L	0.1256	0.844
M - D	0.0615	0.727	UH - L	0.2529	0.168
UH - D	0.1888	0.038	Valio - L	0.2116	0.329
Valio - D	0.1476	0.040	UH - M	0.1273	0.106
F - E	-0.2021	0.393	Valio - M	0.0860	0.738
G - E	-0.1006	0.979	Valio - UH	-0.0413	0.995
H - E	-0.2158	0.084			

Appendix 6. Variance data of sucrose analysis from the gurt

Method	Difference of means	p-value	Method	Difference of means	p-value
B - A	0.1027	0.123	I - E	-0.0559	0.966
C - A	0.1034	0.906	J - E	-0.0876	0.453
D - A	0.1506	0.469	K - E	-0.1223	0.080
E - A	0.1209	0.072	L - E	-0.0458	1.000
F - A	0.0305	1.000	M - E	-0.0390	0.972
G - A	0.1071	0.684	UH - E	-0.0462	0.960
H - A	0.0215	1.000	Valio - E	0.0307	1.000
I - A	0.0651	0.921	G - F	0.0766	0.977
J - A	0.0333	0.998	H - F	-0.0090	1.000
K - A	-0.0013	1.000	I - F	0.0345	1.000
L - A	0.0751	0.993	J - F	0.0028	1.000
M - A	0.0819	0.322	K - F	-0.0318	1.000
UH - A	0.0748	0.706	L - F	0.0446	1.000
Valio - A	0.1517	0.066	M - F	0.0514	0.983
C - B	0.0007	1.000	UH - F	0.0442	0.998
D - B	0.0478	0.999	Valio - F	0.1212	0.496
E - B	0.0182	1.000	H - G	-0.0855	0.919
F - B	-0.0722	0.848	I - G	-0.0420	1.000
G - B	0.0044	1.000	J - G	-0.0738	0.961
H - B	-0.0812	0.572	K - G	-0.1084	0.681
I - B	-0.0376	0.998	L - G	-0.0320	1.000
J - B	-0.0694	0.671	M - G	-0.0252	1.000
K - B	-0.1040	0.137	UH - G	-0.0323	1.000
L - B	-0.0276	1.000	Valio - G	0.0446	1.000
M - B	-0.0208	1.000	I - H	0.0435	0.999
UH - B	-0.0280	0.998	J - H	0.0118	1.000
Valio - B	0.0490	0.978	K - H	-0.0228	1.000
D - C	0.0471	1.000	L - H	0.0536	1.000
E - C	0.0175	1.000	M - H	0.0604	0.878
F - C	-0.0729	0.997	UH - H	0.0532	0.971
G - C	0.0036	1.000	Valio - H	0.1302	0.265
H - C	-0.0819	0.985	J - I	-0.0318	1.000
I - C	-0.0384	1.000	K - I	-0.0664	0.920
J - C	-0.0701	0.995	L - I	0.0100	1.000
K - C	-0.1048	0.903	M - I	0.0168	1.000
L - C	-0.0283	1.000	UH - I	0.0097	1.000
M - C	-0.0215	1.000	Valio - I	0.0866	0.842
UH - C	-0.0287	1.000	K - J	-0.0346	0.998
Valio - C	0.0482	1.000	L - J	0.0418	1.000
E - D	-0.0296	1.000	M - J	0.0486	0.951
F - D	-0.1200	0.822	UH - J	0.0414	0.993
G - D	-0.0435	1.000	Valio - J	0.1184	0.324
H - D	-0.1290	0.697	L - K	0.0764	0.992
I - D	-0.0855	0.973	M - K	0.0832	0.342
J - D	-0.1172	0.769	UH - K	0.0761	0.708
K - D	-0.1519	0.466	Valio - K	0.1530	0.068
L - D	-0.0754	0.999	M - L	0.0068	1.000
M - D	-0.0686	0.983	UH - L	-0.0004	1.000
UH - D	-0.0758	0.980	Valio - L	0.0766	0.996
Valio - D	0.0011	1.000	UH - M	-0.0071	1.000
F - E	-0.0904	0.678	Valio - M	0.0698	0.824
G - E	-0.0139	1.000	Valio - UH	0.0769	0.840
H - E	-0.0994	0.382			

Appendix 7. Variance data of stachyose analysis from the gurt

Method	Difference of means	p-value	Method	Difference of means	p-value
B - A	0.0163	0.995	I - E	-0.0167	0.980
C - A	0.0026	1.000	J - E	-0.1255	0.000
D - A	0.0153	1.000	K - E	-0.1026	0.008
E - A	0.0687	0.097	L - E	-0.0782	0.013
F - A	0.0134	1.000	M - E	-0.0599	0.021
G - A	-0.0339	0.968	UH - E	0.0470	0.093
H - A	-0.0494	0.341	Valio - E	-0.0193	0.916
I - A	0.0521	0.261	G - F	-0.0474	0.832
J - A	-0.0568	0.216	H - F	-0.0629	0.206
K - A	-0.0339	0.900	I - F	0.0387	0.691
L - A	-0.0095	1.000	J - F	-0.0702	0.134
M - A	0.0088	1.000	K - F	-0.0473	0.663
UH - A	0.1157	0.007	L - F	-0.0229	0.991
Valio - A	0.0495	0.284	M - F	-0.0046	1.000
C - B	-0.0137	0.985	UH - F	0.1023	0.023
D - B	-0.0009	1.000	Valio - F	0.0361	0.729
E - B	0.0525	0.052	H - G	-0.0155	1.000
F - B	-0.0029	1.000	I - G	0.0860	0.116
G - B	-0.0502	0.556	J - G	-0.0228	0.996
H - B	-0.0657	0.008	K - G	0.0001	1.000
I - B	0.03580	0.128	L - G	0.0244	0.995
J - B	-0.0731	0.005	M - G	0.0427	0.725
K - B	-0.0502	0.248	UH - G	0.1497	0.010
L - B	-0.0258	0.704	Valio - G	0.0834	0.129
M - B	-0.00749	1.000	I - H	0.1015	0.000
UH - B	0.09944	0.000	J - H	-0.0073	1.000
Valio - B	0.03318	0.074	K - H	0.0156	0.999
D - C	0.0127	1.000	L - H	0.0399	0.358
E - C	0.0661	0.026	M - H	0.0582	0.014
F - C	0.0108	1.000	UH - H	0.1652	0.000
G - C	-0.0366	0.892	Valio - H	0.0989	0.000
H - C	-0.0521	0.081	J - I	-0.1089	0.000
I - C	0.0495	0.077	K - I	-0.0860	0.023
J - C	-0.0594	0.042	L - I	-0.0616	0.039
K - C	-0.0365	0.688	M - I	-0.04328	0.039
L - C	-0.0121	1.000	UH - I	0.06364	0.007
M - C	0.0062	1.000	Valio - I	-0.0026	1.000
UH - C	0.1131	0.001	K - J	0.0229	0.974
Valio - C	0.0468	0.072	L - J	0.0473	0.203
E - D	0.0534	0.143	M - J	0.0656	0.008
F - D	-0.0019	1.000	UH - J	0.1725	0.000
G - D	-0.0493	0.678	Valio - J	0.1063	0.000
H - D	-0.0648	0.043	L - K	0.0244	0.972
I - D	0.0367	0.425	M - K	0.0427	0.407
J - D	-0.0721	0.024	UH - K	0.1496	0.001
K - D	-0.0492	0.401	Valio - K	0.0833	0.027
L - D	-0.0249	0.925	M - L	0.0183	0.950
M - D	-0.0065	1.000	UH - L	0.1252	0.001
UH - D	0.1004	0.003	Valio - L	0.0590	0.040
Valio - D	0.0341	0.451	UH - M	0.10692	0.000
F - E	-0.0553	0.342	Valio - M	0.04067	0.010
G - E	-0.1027	0.053	Valio - UH	-0.06625	0.001
H - E	-0.1182	0.000			

Appendix 8. Variance data of sucrose analysis from the meat alternative

Method	Difference of means	p-value	Method	Difference of means	p-value
B - A	0.0060	1.000	I - E	-0.0010	1.000
C - A	-0.0173	0.995	J - E	-0.0015	1.000
D - A	0.0049	1.000	K - E	-0.0520	0.762
E - A	0.0032	1.000	L - E	-0.0010	1.000
F - A	0.0639	0.485	M - E	-0.0149	0.999
G - A	-0.0232	0.993	UH - E	-0.0353	0.729
H - A	-0.0983	0.005	Valio - E	0.0268	0.916
I - A	0.0022	1.000	G - F	-0.0872	0.219
J - A	0.0017	1.000	H - F	-0.1622	0.009
K - A	-0.0487	0.718	I - F	-0.0617	0.432
L - A	0.0023	1.000	J - F	-0.0622	0.523
M - A	-0.0116	0.999	K - F	-0.1127	0.095
UH - A	-0.0320	0.540	L - F	-0.0617	0.441
Valio - A	0.0301	0.612	M - F	-0.0756	0.242
C - B	-0.0234	0.857	UH - F	-0.0960	0.108
D - B	-0.0011	1.000	Valio - F	-0.0339	0.919
E - B	-0.0028	1.000	H - G	-0.0751	0.081
F - B	0.0579	0.529	I - G	0.0254	0.939
G - B	-0.0293	0.909	J - G	0.0249	0.988
H - B	-0.1043	0.000	K - G	-0.0255	0.998
I - B	-0.0038	1.000	L - G	0.0255	0.946
J - B	-0.0044	1.000	M - G	0.0116	1.000
K - B	-0.0548	0.503	UH - G	-0.0088	1.000
L - B	-0.0038	1.000	Valio - G	0.0533	0.275
M - B	-0.01768	0.836	I - H	0.10048	0.000
UH - B	-0.03808	0.118	J - H	0.1000	0.005
Valio - B	0.02401	0.493	K - H	0.0495	0.583
D - C	0.0222	0.756	L - H	0.10055	0.000
E - C	0.0206	0.996	M - H	0.08664	0.000
F - C	0.0813	0.209	UH - H	0.06624	0.003
G - C	-0.0059	1.000	Valio - H	0.12834	0.000
H - C	-0.0810	0.003	J - I	-0.0005	1.000
I - C	0.0195	0.892	K - I	-0.0509	0.546
J - C	0.0190	0.991	L - I	0.00007	1.000
K - C	-0.0314	0.957	M - I	-0.01384	0.848
L - C	0.0196	0.912	UH - I	-0.03424	0.060
M - C	0.0057	1.000	Valio - I	0.02786	0.108
UH - C	-0.0147	0.951	K - J	-0.0504	0.690
Valio - C	0.0474	0.073	L - J	0.0006	1.000
E - D	-0.0017	1.000	M - J	-0.0133	0.997
F - D	0.0590	0.470	UH - J	-0.0337	0.511
G - D	-0.0281	0.881	Valio - J	0.0284	0.694
H - D	-0.10318	0.000	L - K	0.0510	0.555
I - D	-0.00270	1.000	M - K	0.0371	0.822
J - D	-0.0032	1.000	UH - K	0.0167	0.999
K - D	-0.0536	0.480	Valio - K	0.0788	0.157
L - D	-0.00263	1.000	M - L	-0.01391	0.896
M - D	-0.01654	0.534	UH - L	-0.03431	0.087
UH - D	-0.03693	0.028	Valio - L	0.02779	0.176
Valio - D	0.02516	0.084	UH - M	-0.02040	0.164
F - E	0.0607	0.652	Valio - M	0.04170	0.000
G - E	-0.0265	0.993	Valio - UH	0.06209	0.003
H - E	-0.1015	0.025			

Appendix 9. Variance data of stachyose analysis from the meat alternative

Method	Difference of means	p-value	Method	Difference of means	p-value
B - A	0.01046	0.368	I - E	0.00044	1.000
C - A	-0.00170	1.000	J - E	-0.10391	0.000
D - A	0.01269	0.344	K - E	-0.12339	0.000
E - A	0.04625	0.009	L - E	-0.00039	1.000
F - A	0.04439	0.001	M - E	-0.04475	0.010
G - A	-0.05495	0.000	UH - E	-0.07958	0.001
H - A	-0.08739	0.000	Valio - E	0.01799	0.585
I - A	0.04668	0.000	G - F	-0.09934	0.000
J - A	-0.05767	0.000	H - F	-0.13178	0.000
K - A	-0.07714	0.000	I - F	0.00230	1.000
L - A	0.04586	0.011	J - F	-0.10205	0.000
M - A	0.00150	1.000	K - F	-0.12153	0.000
UH - A	-0.03333	0.038	L - F	0.00147	1.000
Valio - A	0.06424	0.000	M - F	-0.04288	0.000
C - B	-0.01216	0.750	UH - F	-0.07772	0.000
D - B	0.00223	1.000	Valio - F	0.01985	0.186
E - B	0.03579	0.032	H - G	-0.03244	0.002
F - B	0.03393	0.003	I - G	0.10163	0.000
G - B	-0.06541	0.000	J - G	-0.00272	1.000
H - B	-0.09785	0.000	K - G	-0.02219	0.163
I - B	0.03622	0.000	L - G	0.10081	0.000
J - B	-0.06813	0.000	M - G	0.05645	0.000
K - B	-0.08760	0.000	UH - G	0.02162	0.091
L - B	0.03540	0.038	Valio - G	0.11919	0.000
M - B	-0.00896	0.581	I - H	0.13407	0.000
UH - B	-0.04379	0.009	J - H	0.02973	0.006
Valio - B	0.05378	0.000	K - H	0.01025	0.927
D - C	0.01438	0.646	L - H	0.13325	0.000
E - C	0.04794	0.007	M - H	0.08889	0.000
F - C	0.04608	0.002	UH - H	0.05406	0.002
G - C	-0.05325	0.001	Valio - H	0.15163	0.000
H - C	-0.08569	0.000	J - I	-0.10435	0.000
I - C	0.04838	0.002	K - I	-0.12382	0.000
J - C	-0.05597	0.001	L - I	-0.00083	1.000
K - C	-0.07544	0.000	M - I	-0.04518	0.000
L - C	0.04755	0.008	UH - I	-0.08001	0.001
M - C	0.00320	1.000	Valio - I	0.01756	0.188
UH - C	-0.03163	0.041	K - J	-0.01947	0.290
Valio - C	0.06594	0.000	L - J	0.10352	0.000
E - D	0.03356	0.046	M - J	0.05917	0.000
F - D	0.03170	0.008	UH - J	0.02434	0.061
G - D	-0.06764	0.000	Valio - J	0.12191	0.000
H - D	-0.10008	0.000	L - K	0.12300	0.000
I - D	0.03400	0.002	M - K	0.07864	0.000
J - D	-0.07035	0.000	UH - K	0.04381	0.009
K - D	-0.08983	0.000	Valio - K	0.14138	0.000
L - D	0.03317	0.055	M - L	-0.04436	0.012
M - D	-0.01118	0.503	UH - L	-0.07919	0.001
UH - D	-0.04602	0.004	Valio - L	0.01838	0.579
Valio - D	0.05155	0.000	UH - M	-0.03483	0.023
F - E	-0.00186	1.000	Valio - M	0.06274	0.000
G - E	-0.10120	0.000	Valio - UH	0.09757	0.000
H - E	-0.13364	0.000			

Appendix 10. Variance data of verbascope analysis from the meat alternative

Method	Difference of means	p-value	Method	Difference of means	p-value
B - A	0.04594	0.005	G - E	-0.36293	0.000
C - A	0.0368	0.613	H - E	-0.40319	0.000
D - A	0.0382	0.427	I - E	-0.0058	1.000
E - A	0.14169	0.000	J - E	-0.38527	0.000
F - A	0.1235	0.000	K - E	-0.39508	0.000
G - A	-0.22125	0.000	L - E	-0.0050	1.000
H - A	-0.26150	0.000	M - E	-0.12476	0.000
I - A	0.1359	0.001	UH - E	-0.10690	0.009
J - A	-0.24359	0.000	Valio - E	0.06476	0.000
K - A	-0.25339	0.000	G - F	-0.3448	0.000
L - A	0.1367	0.015	H - F	-0.3850	0.000
M - A	0.01692	0.854	I - F	0.0123	1.000
UH - A	0.03479	0.239	J - F	-0.3671	0.000
Valio - A	0.20645	0.000	K - F	-0.3769	0.000
C - B	-0.0092	1.000	L - F	0.0131	1.000
D - B	-0.0077	1.000	M - F	-0.1066	0.000
E - B	0.09575	0.000	UH - F	-0.0888	0.011
F - B	0.0776	0.007	Valio - F	0.0829	0.004
G - B	-0.26719	0.000	H - G	-0.04026	0.002
H - B	-0.30744	0.000	I - G	0.3571	0.000
I - B	0.0899	0.008	J - G	-0.02234	0.048
J - B	-0.28953	0.000	K - G	-0.03214	0.058
K - B	-0.29933	0.000	L - G	0.3579	0.000
L - B	0.0907	0.095	M - G	0.23817	0.000
M - B	-0.02902	0.156	Valio - G	0.42770	0.000
UH - B	-0.01115	0.940	I - H	0.3974	0.000
Valio - B	0.16051	0.000	J - H	0.01792	0.111
D - C	0.0014	1.000	K - H	0.00811	0.994
E - C	0.1049	0.011	L - H	0.3982	0.000
F - C	0.0868	0.033	M - H	0.27843	0.000
G - C	-0.2580	0.000	Valio - H	0.46795	0.000
H - C	-0.2983	0.000	J - I	-0.3795	0.000
I - C	0.0991	0.017	K - I	-0.3893	0.000
J - C	-0.2804	0.000	L - I	0.0008	1.000
K - C	-0.2902	0.000	M - I	-0.1190	0.001
L - C	0.0999	0.082	UH - I	-0.1011	0.008
M - C	-0.0199	0.988	Valio - I	0.0706	0.028
UH - C	-0.0020	1.000	K - J	-0.00980	0.932
Valio - C	0.1697	0.001	L - J	0.3803	0.000
E - D	0.1035	0.004	M - J	0.26051	0.000
F - D	0.0853	0.018	Valio - J	0.45004	0.000
G - D	-0.2595	0.000	L - K	0.3901	0.000
H - D	-0.2997	0.000	M - K	0.27031	0.000
I - D	0.0976	0.010	UH - K	0.28818	0.001
J - D	-0.2818	0.000	Valio - K	0.45984	0.000
K - D	-0.2916	0.000	M - L	-0.1197	0.025
L - D	0.0984	0.075	UH - L	-0.1019	0.062
M - D	-0.0213	0.958	Valio - L	0.0698	0.243
UH - D	-0.0034	1.000	UH - M	0.01786	0.816
Valio - D	0.1682	0.000	Valio - M	0.18952	0.000
F - E	-0.0181	0.949	Valio - UH	0.17166	0.005
UH - G	0.2560	0.000	UH - J	0.2784	0.000
UH - H	0.2963	0.000			