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Quantification of galacturonic acid in pectin-containing samples using a stable isotope dilution approach and LC-MS

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ABSTRACT

Reliable quantification of galacturonic acids (GalA) is essential for understanding the structural and functional properties of pectin-containing materials. However, conventional photometric methods often suffer from low reproducibility, limited sensitivity, and poorly understood reactions involved during analysis. Here, an LC-MS-based method for the sensitive and precise determination of total GalA contents in soluble and insoluble dietary fiber fractions of pectin containing samples is presented. The method is based on the degradation of GalA to the characteristic product 5-formyl-2-furancarboxylic acid (5FFA) in concentrated sulfuric acid under optimized conditions. The degradation product is extracted and quantified by UHPLC-ESI-MS. To compensate for degradation and extraction variability, the internal standard $^{13}C_6$-galacturonic acid is used. Quantification is achieved by single ion monitoring (SIM) of 5FFA and the equivalent ^{13}C-labeled degradation product. The validated method was successfully applied to various sample materials, including isolated galacturonic acid oligosaccharide standards with defined degrees of polymerization and plant-derived dietary fiber samples such as carrot, apple, and citrus pulp. Comparison to a widely used colorimetric assay demonstrated that the results of the two methods differ if applied to soluble fiber samples. Thus, the LC-MS approach represents a robust alternative to photometric assays, offering enhanced sensitivity, precision, and applicability for pectin analysis.

1. Introduction

Uronic acids (UA) are an essential component of various biopolymers that are widely utilized in the food and pharmaceutical industries. One of the most prominent examples is the complex cell wall polysaccharide pectin, which is predominantly found in the middle lamella and primary cell walls of higher plants (McNeil et al., 1984; Vogel, 2008). Pectin contents vary widely, with dicotyledonous plants containing up to 35 %, whereas monocotyledonous plants contain only up to 5–10 % (O'Neill et al., 1990; Vogel, 2008).

The widely accepted structural model by De Vries et al. (1982) describes pectin as a composite structure consisting of unbranched 'smooth regions' and branched 'hairy regions'. According to this model, the structure of the heteropolysaccharide pectin can be divided into two dominant substructures with galacturonic acid (GalA) being the main monosaccharide (Yapo, 2011). Homogalacturonan (uronic), a linear substructure consisting of α-1,4-linked D-GalA residues, may be further modified by acetyl groups in GalA positions *O*2 and/or *O*3 and methyl esters in position *O*6 (O'Neill et al., 1990). Another major substructure, rhamnogalacturonan-I, is composed of repeating disaccharide units of α-1,4-linked D-GalA and α-1,2-linked L-rhamnose. These units are often substituted with arabinans and/or (arabino)galactans at the *O*4 position of the rhamnose (Voragen et al., 2009). Plant cell walls also contain smaller amounts of rhamnogalacturonan-II and, depending on the plant material, xylogalacturonan polymers (Schols et al., 1995).

Industrially, pectin is mainly obtained via acid extraction from raw materials such as apple, sugar beet, and lemon pomace (Schmidt et al., 2015). However, the range of potential by-products suitable for pectin production is steadily increasing (Freitas et al., 2021). Due to its gel-forming, thickening, and stabilizing properties, pectin is used as a food additive. Popular applications include jams, jellies, drinks, and dairy products like yogurts (Laurent & Boulenguer, 2003; Schmidt et al., 2015). In the European Union, pectin is regulated as additive E440, with Regulation (EU) No. 231/2012 specifying that the UA content of pectin must be 'not less than 65% on the ash-free and anhydrous basis' (Commission Regulation (EU), 2012). Thus, the reliable determination of the UA content in pectin-rich samples is necessary for the structural characterization of plant cell wall samples as well as for the verification

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of legal regulations.

Traditionally, the UA content is analyzed photometrically following the removal of starch and protein in a total UA assay. The first approaches to analyzing UA were developed by Mann and Tollens (1896), who measured UA via the quantitative release of CO_2 from the carboxyl group during boiling in 12 % HCl. However, this method required large quantities of material and was subject to interference from other sample components. Tollens (1908) also published the first photometric determination of UA, the naphthoresorcinol assay, which, however, failed due to its low specificity. Other methods developed afterwards use concentrated H_2SO_4 and various aromatic reagents instead of heating in HCl to produce a color characteristic of UA. Bowness (1958) was the first to identify 5-formyl-2-furancarboxylic acid (5FFA) as the required acid degradation product, responsible for color formation when reacting with a chromogen. A common color reagent is carbazole, which was introduced by Dische (1947). This reagent exhibits limited sensitivity and specificity, requiring two hours for chromophore formation. Improved sensitivity was achieved using 3-phenylphenol (Blumenkrantz & Asboe-Hansen, 1973), harmine (Wardi et al., 1974), or 3,5-dimethylphenol (Scott, 1979). Inference from other sugars is one of the major challenges of these methods, which were sought to minimize through additives. The addition of borate to concentrated H_2SO_4 improved both color stability and sensitivity of the analysis (Bitter & Muir, 1962; Filisetti-Cozzi & Carpita, 1991; Gregory, 1960). Sulphamate has been shown to suppress color formation with neutral sugars, but generally leads to lower sensitivity and is poorly soluble, making its use challenging (Filisetti-Cozzi & Carpita, 1991; Galambos, 1967). Yapo (2012) highlights that the results of colorimetric assays are highly dependent on the reaction conditions. Slight variations, such as type and concentration of chromogen, H_2SO_4 concentration, heating temperature, various additives, and different pre-treatments, can lead to significant differences in the UA contents determined for the same sample material. The causes of these inaccuracies of colorimetric methods are hard to identify, mainly due to the lack of understanding of the underlying reaction mechanism, such as the structure and formation pathways of the chromophores, preventing targeted optimization. Disadvantages, such as self-condensation of the formed 5FFA, which also leads to colored products, have already been described by Rosenau et al. (2017).

To address these limitations, this study aims to establish a novel and robust analytical approach for the reliable and sensitive determination of total GalA content in pectin-rich samples. The proposed method uses the degradation products of GalA formed under acidic conditions as quantitative markers. These degradation products are analyzed by LC-MS to achieve accurate and reproducible quantification.

2. Material and methods

2.1. Chemicals

GalA monohydrate, D-glucuronic acid (GlcA), polygalacturonic acid sodium salt, 2-deoxy-D-glucose, 3-(Trimethylsilyl)propionate-d_4 sodium salt and furfural were purchased from Merck KGaA (Darmstadt, Germany), D-mannuronic acid (ManA) sodium salt and L-guluronic acid (GulA) sodium salt were obtained from Biosynth (Bratislava, Slovakia). D-[UL-$^{13}C_6$]GalA potassium salt was purchased from Omicron Biochemicals, Inc. (South Bend, IN, USA). 5FFA was purchased from BLD pharmatech GmbH (Reinbek, Germany), furan-2-carboxylic acid was obtained from Thermo Fisher Scientific Inc. (Darmstadt, Germany), and 3-phenylphenol was purchased from Apollo Scientific Ltd. (Manchester, UK). *Endo*-α-1,4-D-polygalacturonase (EC 3.2.1.15, from *T. reesei*) was kindly provided by Erbslöh Geisenheim GmbH (Geisenheim, Germany). Enzymes for preparative dietary fiber isolation, heat-stable α-amylase Termamyl 120 L (from *Bacillus licheniformis*), protease Alcalase 2.4 L (from *Bacillus licheniformis*), and amyloglucosidase AMG 300 L (from *Aspergillus niger*), were obtained from Merck KGaA (Darmstadt, Germany). All other chemicals and reagents were of analytical purity grade and purchased from VWR International GmbH (Darmstadt, Germany), Carl Roth GmbH + Co. KG (Karlsruhe, Germany), or Merck KGaA (Darmstadt, Germany).

2.2. Plant samples

Carrot pulp was obtained from Austria Juice (Allhartsberg, Austria), lemons were purchased from a local supermarket and peeled, and apple pomace was an industrial by-product with no further identification. Insoluble and soluble fiber (IDF, SDF) fractions were isolated as described in section 2.3.

2.3. Preparative dietary fiber isolation

IDF and SDF preparation was performed according to an upscaled version of AOAC method 985.29 (Official methods of analysis, 2005) with minor modifications (Keller et al., 2022). Dried, ground, and acetone-defatted plant material (15 g) was suspended in 200 mL of sodium phosphate buffer (0.08 M, pH 6.2) followed by enzymatic digestion with 1.5 mL of α-amylase (92 °C, 20 min). After cooling to room temperature, the pH was adjusted to 7.5 with 0.275 M NaOH, and 600 µL of protease was added. The samples were incubated at 60 °C for 30 min under continuous agitation. The pH was adjusted to 4.5 with 0.375 M HCl, followed by incubation with 700 µL of amyloglucosidase at 60 °C for 30 min. IDF was separated by centrifugation and washed with water, 96 % ethanol, and acetone. The supernatant and the wash water were combined, and SDF was precipitated with 4 volumes of 96 % ethanol overnight. The precipitate was recovered by centrifugation, washed with 80 % ethanol, 96 % ethanol, and acetone, and dried at 60 °C.

2.4. Structural characterization of the fiber samples

2.4.1. Monosaccharide composition

SDF and IDF (section 2.3) were hydrolyzed by combined methanolysis/trifluoroacetic acid hydrolysis using 1.25 M HCl in methanol at 80 °C for 16 h, followed by hydrolysis with 2 M trifluoroacetic acid at 121 °C for 1 h (De Ruiter et al., 1992). After evaporation, the obtained monosaccharides were dissolved in water and analyzed. IDF was additionally hydrolyzed using H_2SO_4 following Saeman's principle (Saeman et al., 1945). Samples were pretreated with 12 M H_2SO_4 under ice cooling for 10 min, followed by incubation at room temperature for 2 h. The acid concentration was adjusted to 1.6 M, and samples were hydrolyzed at 100 °C for 3 h. After hydrolysis, the samples were membrane filtered (PTFE syringe filter, 0.45 µm), and neutralized with 4 M NaOH. 2-Deoxy-D-glucose was added as an internal standard for quantification.

Monosaccharide analysis was performed by high performance anion exchange chromatography coupled with a pulsed amperometric detector (HPAEC-PAD, Alexys-HPLC-System with a DECADE Elite detector, Antec Scientific, Hoorn, NL) using a SweetSept AEX200 column (5 µm, 4 mm × 200 mm, Antec Scientific, Hoorn, NL). Separation was performed using a ternary, linear gradient, with deionized water (A), 10 mM NaOH (B), and 100 mM NaOH containing 100 mM sodium acetate (C). The following elution gradient was applied: 60 % A and 40 % B were hold for 12.5 min, followed by a linear change to 100 % C within 5.5 min. 100 % C was held for 6 min, and rinsing and equilibration steps followed the separation gradient. The flow rate was kept constant at 0.7 mL/min, the temperature at 30 °C. Results are shown as supplementary data (Figs. S1 - S3).

2.4.2. Degree of methylation and acetylation

The degree of methylation (DM) and the degree of acetylation (DAc) of pectin-containing samples were determined following the method of Müller-Maatsch et al. (2014). 3-(Trimethylsilyl)propionate-d_4 and 2 M NaOH in D_2O were added to the samples. After 2 h of sonication, the suspensions were filtered (PTFE, 0.45 µm) and centrifuged. The resulting supernatants were analyzed by ^{1}H NMR spectroscopy using an

Ascend 500 MHz NMR spectrometer (Bruker Biospin, Ettlingen, Germany) equipped with a Prodigy cryoprobe. DM and DAc were calculated by relating the amount of released methanol and acetic acid to the quantified galacturonic acid content according to section 2.8.5 and 2.9.

2.5. Production of galacturonic acid oligosaccharides

2.5.1. Enzymatic degradation of polygalacturonic acid

To prepare GalA-oligosaccharide standards with defined degrees of polymerization (DP), 0.5 g of polygalacturonic acid was dissolved in 100 mL of water and incubated with 9 U/g of *endo*-polygalacturonase at 35 °C for 1.5 h and 2 h in separate batches. Enzymes were inactivated by heating the hydrolysates at 95 °C for 10 min. The samples were combined, and high DP components were precipitated by adding 200 mL of acetonitrile (ACN). After centrifugation (7 min, 2683 *g*), the supernatant was evaporated and freeze-dried. The dried hydrolysate was redissolved in water for analysis.

2.5.2. Characterization and semipreparative isolation

In accordance with Leijdekkers et al. (2011), hydrophilic interaction liquid chromatography was used to identify the oligosaccharides in the hydrolysate. The analysis was carried out on a Nexera X2 UHPLC System (Shimadzu, Kyōto, Japan) coupled with an ESI-MS detector (LCMS 2020, Shimadzu, Kyōto, Japan). Chromatographic separation was performed on an Acquity UPLC BEH Amide column (1.7 µm, 2.1 mm × 150 mm) in combination with a VanGuard precolumn (1.7 µm, 2.1 mm × 5 mm; Waters Corporation, Milford, MA, USA). Elution was performed at a flow rate of 0.4 mL/min and a column oven temperature of 50 °C. The injection volume was set to 10 µL. The mobile phase consisted of 20:80 (*v*/v) (A) and 80:20 (v/v) (B) ACN:water, each containing 0.2 % formic acid and 10 mM ammonium formate. The following elution gradient was applied: 0–1 min, 100 % A; 1–31 min, linearly to 20 % A; 31–35 min, held at 20 % A; 35–36 min, linearly from 20 % A back to 100 % A; and 36–41 min, 100 % An under isocratic conditions. ESI–MS detection was performed in negative selected ion monitoring (SIM) mode (−4.5 kV) at a nebulization gas flow (nitrogen) of 1.5 L/min and 15 L/min drying gas. The interface temperature was set to 350 °C, the desolvation line temperature was 250 °C, and the heating block was operated at 200 °C. The different GalA-oligosaccharides with their DP dependent masses ((M = 194.14 u*DP − (18*(DP − 1))) were detected by monitoring their $[M\text{-}H]^-$ ions. For oligosaccharides with DP6 and higher, $[M\text{-}2H]^-$ ions were included in the SIM monitoring process.

The diluted hydrolysate containing the GalA-oligosaccharides was fractionated by using a semipreparative HPLC-System (Azura, KNAUER, Berlin, Germany), equipped with a XBridge BEH Amide column (5 µm, 10 mm × 250 mm; Waters Corporation, Milford, MA, USA). A flow rate of 4 mL/min was used, and the column temperature was set at 50 °C. Detection was carried out after the eluent was split 1/20, with the smaller fraction being directed to an evaporative light scattering detector (ELSD, Sedex 85, ERC GmbH, Riemerling, Germany), whereas the larger part was collected by using a multiposition valve (Azura V2.1S, KNAUER, Berlin, Germany). A gradient composed of 20:80 (*v*/v) (A) and 80:20 (v/v) (B) ACN:water with 0.2 % formic acid was used as follows: 0–1 min, 100 % A; 1–22 min, linearly to 45 % A; 22–40 min, linearly to 20 % A; 40–45 min, held at 20 % A; 45–50 min, linearly from 20 % A back to 100 % A; 50–65 min, equilibration with 100 % A. The isolated fractions were evaporated, freeze-dried, and weighed.

2.5.3. Determination of the purity

The purity of the standard compounds was determined by nuclear magnetic resonance (NMR) spectroscopy, following the principles described by Bharti and Roy (2012), and the UHPLC method described in section 2.5.2. The MS purity, which shows proportions of deviating DP, was proportionally calculated against the purity determined by NMR, which reflects the sugar content. NMR measurements were carried out on an Ascend 500 MHz NMR spectrometer (Bruker Biospin, Ettlingen, Germany) equipped with a Prodigy cryoprobe. Each standard was dissolved in 0.5 mL of a 0.5 mg/mL acetanilide solution in D_2O, and ^1H NMR experiments were performed using acetanilide for spectral calibration (δ_H: 2.17 ppm). Purity was calculated as follows:

$$P_X = \frac{I_X}{I_A} \times \frac{N_A}{N_X} \times \frac{M_x}{M_A} \times \frac{c_A}{c_X} \times 100$$

where, I is the integral area (δ_H: 2.17 ppm for acetanilide; δ_H: 5.4 ppm for the α-H atoms, except from the reducing end), N the number of nuclei, M the molar mass, c the concentration depending on the weight and P the purity of analyte (X) and acetanilide (A).

2.6. Pre-treatment of insoluble dietary fiber material

IDF samples require pre-hydrolysis prior to GalA quantification. Therefore, 200 µL of 12 M H_2SO_4 was added to 5 mg of IDF, mixed thoroughly and left on ice for 15 min. The mixture was shaken for another 10 min at room temperature. The sample was diluted with 3.5 mL of water and filtered through a PTFE syringe filter (0.45 µm).

2.7. Characterization of galacturonic acid degradation products

The degradation products of GalA in concentrated H_2SO_4 were identified and quantified using a UHPLC-UV method with various furfural derivatives as standard compounds. An aqueous solution containing 1–2 µmol of GalA was evaporated to dryness. Then, 0.5 mL of H_2SO_4 (or a borate-containing reagent, if appropriate) was added, and the mixture was cooled on ice for 3 min. The mixture was heated at 95 °C for 5 min and cooled on ice for 5 min. Water (0.5 mL) and 2 mL of 4 M NaOH were added while maintaining the mixture on ice. The residue was filtered through a PTFE syringe filter (0.45 µm), and the filtrate was analyzed undiluted. An external calibration with three furan derivatives (5FFA, furfural, and furan-2-carboxylic acid) were prepared from a stock solution (400 µM in 5 % ACN) in two concentration ranges: 10–90 µM and 50–250 µM. Analyses were performed on a Nexera LC-40D XS UHPLC system (Shimadzu, Kyōto, Japan) equipped with a UV/Vis detector (measuring at 260 nm). Separation was achieved on a Luna Omega Polar C18 column (150 × 2.1 mm, particle size 1.6 µm; Phenomenex, Torrance, CA, US) in combination with a polar C18 precolumn (Phenomenex, Torrance, CA, US) using water containing 0.2 % formic acid (A) and ACN (B) as eluents. The linear gradient was composed as follows: 0–12 min 100 % A; 12–20 min, linearly to 20 % A; 20–25 min held at 20 % A; 25–27 min, linearly back to 100 % A; 27–38 min, equilibration with 100 % A. The flow rate was set to 0.2 mL/min, with a column temperature of 40 °C and an injection volume of 25 µL.

2.8. Development of a LC-MS based approach to quantify galacturonic acid contents

2.8.1. Quantification of 5FFA by UHPLC-UV

To quantify the 5FFA-content, a GalA containing solution was heated in concentrated H_2SO_4 to produce 5FFA, which was extracted using an organic solvent (see section 2.8.2). Quantification of 5FFA was performed on a Nexera LC-40D XS UHPLC system (Shimadzu, Kyōto, Japan) equipped with a UV/Vis detector set at 280 nm. Samples were applied onto a Luna Omega Polar C18 column (150 × 2.1 mm, particle size 1.6 µm; Phenomenex, Torrance, CA, USA) in combination with a polar C18 precolumn Phenomenex, Torrance, CA, USA) using water containing 0.2 % formic acid (A) and ACN (B) as eluents. The linear gradient was composed as follows: 0–10 min 100 % A; 10–15 min, linearly to 20 % A; 15–28 min held at 20 % A; 18–23 min, linearly back to 100 % A; 23–25 min, equilibration with 100 % A. The flow rate was 0.4 mL/min. Column temperature and injection volume were set at 40 °C and 25 µL, respectively. For quantification, two calibration curves were prepared using 5FFA in 5 % aqueous ACN (10–90 µM; 50–250 µM).

2.8.2. Optimization of the extraction of 5FFA

In each experiment, 1.2 mL of sodium tetraborate in concentrated H_2SO_4 (12.5 mM) was added to 200 µL of an aqueous solution of 5FFA (100 µM). After cooling on ice for 3 min, the extraction of 5FFA was performed under two conditions: (a) following dilution of the H_2SO_4 with 0.5 mL of water and 3 mL of 4 M NaOH, using ethyl acetate, and (b) without dilution of the H_2SO_4, using dichloromethane (DCM). The volume of organic solvent (1–5 mL) and the number of extractions (1–3) were varied. The organic extract was washed twice with 1.5 mL of saturated sodium chloride solution and dried under nitrogen. To remove residual salts, the residue is reconstituted in ACN, which does not dissolve the residual NaCl. The ACN solution is transferred to a new container, dried again under nitrogen and finally dissolved in 5 % aqueous ACN for analysis. All experiments were performed in duplicate, and 5FFA was quantified as described in section 2.8.1.

2.8.3. Optimization of the heating step within the acidic degradation

The heating step was optimized using a design of experiment (DoE) approach. A full factorial design with two factors (temperature and time) at three levels each was employed, with all experiments performed in duplicate. For each experiment, 1.2 mL of sodium tetraborate in concentrated H_2SO_4 (12.5 mM) was added to 200 µL of an aqueous GalA solution (55 mg/L). The mixture was cooled on ice for 3 min and left at room temperature for an additional 5 min. The heating step was conducted in a water bath for different periods of time (5, 10, and 15 min) and temperatures (60 °C, 77.5 °C, and 95 °C). After cooling on ice for 5 min, the extraction procedure was carried out as described in section 2.8.2 using ethyl acetate (2 × 3 mL). Quantification of 5FFA was performed via UHPLC-UV as detailed in section 2.8.1. Statistical analysis of the results was conducted using OriginPro 2024 (OriginLab Corporation, Northampton, MA, USA).

2.8.4. Effect of the addition of borate

The impact of adding borate to concentrated H_2SO_4 on the formation of degradation products of four UA (GalA, GlcA, GulA, ManA; 55 mg/L) was investigated. Heating was performed at 95 °C for 5 min (section 2.8.3), using 1.2 mL of either sodium tetraborate in concentrated H_2SO_4 (12.5 mM) or simply H_2SO_4. Extraction with ethyl acetate (2 × 3 mL) followed section 2.8.2, and the degradation products were quantified by UHPLC-UV as described in section 2.8.1.

2.8.5. Stable isotope dilution approach using LC-MS

To quantify the GalA content in plant samples, an LC-MS stable isotope dilution approach was developed. For de-esterification prior to use, SDF was dissolved in 0.05 M NaOH and let rest for at least 5 min before analysis (McComb & McCready, 1952). IDF was pretreated as described in section 2.6. Samples were diluted with water to appropriate concentrations for analysis.

Sodium tetraborate in concentrated H_2SO_4 (1.2 mL, 12.5 mM) was added to 100 µL of an aqueous, suitably diluted GalA-containing solution, along with 100 µL of an aqueous D-[UL-$^{13}C_6$]GalA solution ($^{13}C_6$-GalA; 40 mg/L). The mixture was cooled on ice for 3 min and left at room temperature for an additional 5 min. Heating was performed in a water bath at 77.5 °C for 5 min. After cooling on ice for 5 min, the extraction procedure was carried out as described in section 2.8.2 using ethyl acetate (2 × 3 mL). The final extract was dissolved in 200 µL of 5 % aqueous ACN for analysis.

The GalA content was quantified via the determination of 5FFA using a Vanquish Flex Binary UHPLC system (Thermo Fisher Scientific, Waltham, MA, USA) coupled to a Q Exactive™ Plus Orbitrap-MS detector (Thermo Fisher Scientific, Waltham, MA, USA). Chromatographic separation was achieved using a Luna Omega Polar C18 column (150 × 2.1 mm, particle size 1.6 µm; Phenomenex, Torrance, CA, USA) in combination with a polar C18 precolumn (Phenomenex, Torrance, CA, USA). Water containing 0.2 % formic acid (A) and ACN (B) were used as eluents. The linear gradient was composed as follows: 0–10 min 100 % A; 10–15 min, linearly to 20 % A; 15–28 min held at 20 % A; 18–23 min, linearly back to 100 % A; 23–25 min, equilibration with 100 % A. The flow rate was 0.4 mL/min, and the column temperature was maintained at 40 °C with an injection volume of 25 µL. ESI-MS detection was performed in negative SIM mode with the following parameters: spray voltage of −2.5 kV and capillary temperature of 320 °C. Quantification was achieved by monitoring the $[M\text{-}H]^-$ ions of 5FFA (m/z 139.004) and $^{13}C_6$-labeled 5FFA (m/z 145.024). The relative peak areas of 5FFA to $^{13}C_6$-labeled 5FFA were used for quantification. Calibration was conducted using GalA-standards (10–85 mg/L), which were processed and analyzed under identical conditions as the samples.

The analysis was transferred to a Nexera X2 UHPLC System (Shimadzu, Kyōto, Japan) coupled with a single quadrupole mass analyzer (LCMS 2020, Shimadzu, Kyōto, Japan) to verify the transferability between different MS systems. Both the described sample preparation and the chromatographic conditions were adopted. ESI–MS detection was performed in negative selected ion monitoring (SIM) mode (−4.5 kV) at a nebulization gas flow (nitrogen) of 1.5 L/min and 15 L/min drying gas. The interface temperature was set to 350 °C, the desolvation line temperature was 250 °C, and the heating block was operated at 200 °C.

2.8.6. Method validation

Method validation data were processed using OriginPro 2024 (OriginLab Corporation, Northampton, MA, USA). The calibration curve was recorded in triplicate as described in Section 2.8.5 and results for each concentration were subsequently averaged. A Shapiro-Wilk test ($\alpha = 0.05$) was conducted to assess the normal distribution of data, based on ten replicate injections of the mid-range calibration point. The linear relationship was evaluated based on the coefficient of determination as well as by an F-test ($\alpha = 0.05$) in comparison to a quadratic fit. Homogeneity of variance between two sample groups was evaluated using an F-test ($\alpha = 0.05$), based on six replicate analyses of the highest and lowest calibration points (Kromidas, 2011).

As a measure of the repeatability of the LC-MS analysis, the instrumental coefficients of variation were determined from ten replicate injections of the same sample solution (thus, excluding the variation of the workup, i.e. acidic degradation and extraction), whereas coefficients of variation of the procedure were determined from six replicate analyses (thus, including the variation of the acidic degradation and extraction) of the highest and lowest calibration points. The coefficients of variations (expressed in %) were calculated from the quotient of the standard deviation and the analytical mean. The coefficients of variation of the procedure are then expressed as the arithmetic mean of the coefficients of variation at the highest and lowest calibration level. External recovery was determined by calculating the recovery rate of a GalA sample with a known concentration. To evaluate matrix effects, previously analyzed carrot pulp SDF was spiked with a known amount of GalA, and the recovery rate was subsequently calculated as recovery in matrix.

The limit of detection (LOD) and limit of quantification (LOQ) were determined according to (DIN 32645:2008–03, 2008). For this purpose, a calibration was performed in duplicate, covering a concentration range as close as possible to the LOQ or including it. Here, the calibration range was adjusted to 2–17 mg/L GalA. LOD and LOQ were calculated as follows:

$$LOD\left[\frac{mg}{L}\right] = \frac{s_y}{m} \times t \times \sqrt{\frac{1}{\widehat{N}} + \frac{1}{N} + \frac{\overline{x}^2}{\sum (x_i - \overline{x})^2}}$$

$$LOQ\left[\frac{mg}{L}\right] = k \times \frac{s_y}{m} \times t \times \sqrt{\frac{1}{\widehat{N}} + \frac{1}{N} + \frac{(k \times LOD - \overline{x})^2}{\sum (x_i - \overline{x})^2}}$$

where s_y, m, $\widehat{N}$, N, x and x_i are the residual standard deviation, the slope of the calibration curve, the number of parallel measurements, the total number of calibration solutions, the concentration of the center of the calibration range and an individual value of the independent variable x.

The t-value was selected based on a 95 % confidence level, with degrees of freedom $f = N - 2$. The critical factor k was set to 3.

2.9. Colorimetric assay

According to Blumenkrantz and Asboe-Hansen (1973) diluted pectic SDF or pretreated IDF (following section 2.6) were heated for 5 min at 95 °C in sodium tetraborate containing concentrated H_2SO_4 (12.5 mM). After cooling on ice for 5 min, 3-phenylphenol was added (0.15 % in 0.5 % aqueous NaOH), and absorption was measured at 520 nm after 20 min. A calibration (concentration range 10–85 mg/L) was performed using an aqueous GalA solution. Validation parameters were determined as described in 2.8.6.

3. Results and discussion

3.1. Isolation of GalA-oligosaccharides

In native sample materials, such as SDF or IDF of pectin-containing plant materials, GalA is found exclusively in its oligomeric or polymeric form. To evaluate the impact of increasing DP of HG on the analysis, standard compounds of defined DP are required. However, HG oligosaccharides (HGO) with a DP > 4 are rarely commercially available, so they have to be prepared in the laboratory.

Several methods for producing GalA-rich oligosaccharides have been reported in literature (Babbar, Dejonghe, et al., 2016; Bonnin et al., 2002; Vera-Guzman et al., 2017). Pectic oligosaccharides that are produced through enzymatic hydrolysis are frequently used for the purpose of structural elucidation, determination of health benefits, and for applications in the food industry (Babbar, Baldassarre, et al., 2016; Eichhofer et al., 2023; Vera-Guzman et al., 2017). Here, polygalacturonic acid was hydrolyzed using *endo*-polygalacturonase, an enzyme that catalyzes the random cleavage of 1,4-α-D-galactosiduronic linkages (Benen et al., 1999; Kant et al., 2013). By combining two hydrolysates that were incubated for 1.5 h and 2 h, respectively, oligosaccharides with wide ranges of DP were obtained in sufficient quantities. Fig. 1 shows the hydrophilic interaction liquid chromatogram of the combined hydrolysates. HGO with a DP up to 18 were identified by MS detection. However, considering the decreasing quantities of oligosaccharides with a DP >7, only HGO up to DP10 were semi-preparatively isolated for further analysis.

HGO with a DP of 3 to 10 were isolated in quantities exceeding 10 mg each. Identity was confirmed by MS analysis, and their purities were calculated based on NMR and the MS-peak areas for the different DP according to 2.5.3 and 2.5.2 (Table 1). A decline in purity was observed with increasing DP, ranging from 100 % for smaller DP oligosaccharides to 85 % for higher DP oligosaccharides. The semi-preparative isolation of the oligosaccharide with DP8 proved particularly challenging, resulting in a maximum purity of 80 %. Consequently, the DP8 standard was excluded from subsequent analyses to maintain the reliability of the results.

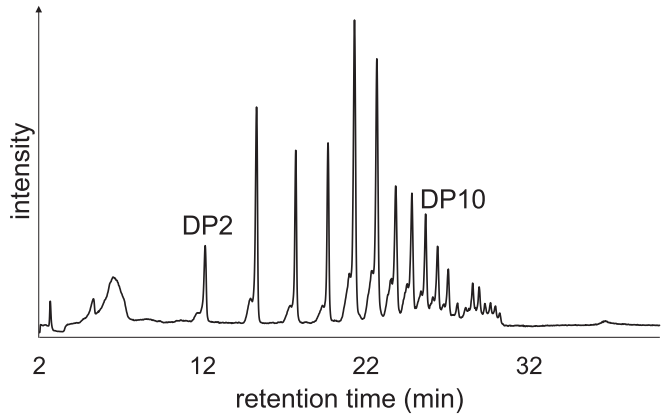

Fig. 1. UHPLC-MS chromatogram of galacturonic acid oligosaccharides with different degrees of polymerization (DP), obtained by enzyme digestion of polygalacturonic acid with *endo*-polygalacturonase.

Table 1
Purity of isolated galacturonic acid oligosaccharides with varying degree of polymerization (DP).

	Purity (%)
DP3	100
DP4	92
DP5	99
DP6	93
DP7	92
DP8	80
DP9	88
DP10	85

3.2. Acid degradation products of GalA

Under acidic conditions and heat, saccharides undergo degradation to form furan derivatives. For UA, dehydration in combination with decarboxylation yields furfural, whereas dehydration without decarboxylation results in the formation of 5FFA. Further degradation, such as formaldehyde elimination, can produce furan-2-carboxylic acid (Fig. 2). Additionally, polymerization of furans results in the formation of insoluble brown products, often referred to as humins (Bornik & Kroh, 2013; Li et al., 2007).

To determine the composition and yield of the furan derivatives formed during heating in concentrated H_2SO_4, degradation products were analyzed directly after diluting the acid, i.e. without extraction of the degradation products (Fig. 3). Spiking experiments confirmed the presence of 5FFA and furfural, whereas furan-2-carboxylic acid was not detected. Additional minor peaks at the beginning of the chromatogram remained unidentified. Quantification of the identified degradation products was performed using external calibration, demonstrating that 28 % of the initial GalA was converted to 5FFA and 7 % was recovered as furfural. The remaining portion was presumably lost by side reactions such as condensation. Indications of such condensation were observed visually, such as a brown coloration of the sample and the formation of insoluble precipitates, which were removed before analysis. Although not proven, it is likely that the soluble polymers elute as a broad signal after a retention time of 20 min.

It should be noted that these experiments required 400-fold higher concentrations of GalA in H_2SO_4 compared to the conditions used later in the developed method due to the lack of pre-concentration or extraction steps. This may have resulted in increased polymer formation compared to a strictly analytical approach. Nevertheless, 5FFA was clearly identified as the dominant low-molecular-weight degradation product and is therefore a promising analyte for the chromatographic determination of GalA. Also, its selective formation makes it suitable for the quantitative analysis of GalA without capturing neutral sugars as described in the subsequent sections.

3.3. Method development

Most photometric/colorimetric methods for determining total UA content also rely on the formation of 5FFA during the heating step. This compound reacts with a chromogen to produce a measurable chromophore (Blumenkrantz & Asboe-Hansen, 1973; Dische, 1947; Scott, 1979). However, the quantitative reliability of these methods is often questioned due to the incomplete characterization of the colored compounds formed in the reaction. Thus, the impact of other degradation products, also from neutral sugars, cannot reliably be estimated.

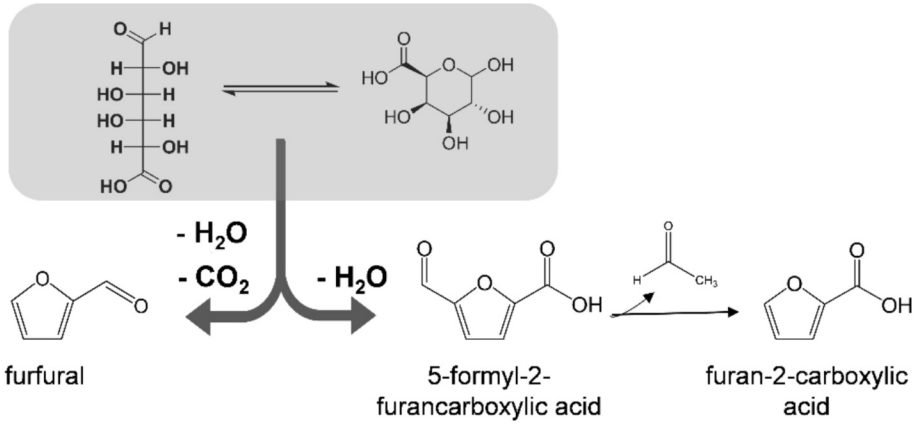

Fig. 2. Degradation products of galacturonic acid, which can be formed during heating in concentrated sulfuric acid based on decarboxylation and/or dehydration of the uronic acid.

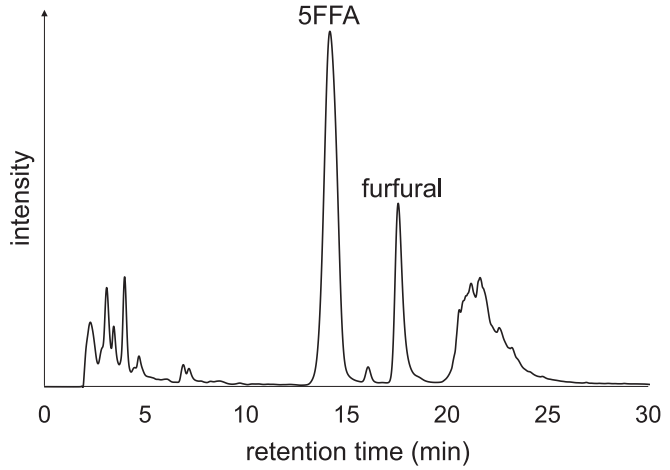

Fig. 3. Chromatographic separation of the acid degradation products of galacturonic acid, detected by UV spectroscopy at 260 nm. Quantification was performed using an external calibration according to section 2.7 (5FFA: 5-formyl-2-furancarboxylic acid).

Consequently, we sought to determine GalA contents through the chromatographic quantification of 5FFA produced during the heating of samples in concentrated acid. To increase sensitivity and specificity of our chromatographic approach, 5FFA was extracted from the acid matrix (see also section 3.3.1). The extracts were used to test the general workflow and develop a chromatographic separation on a reversed phase column. Fig. 4 shows three chromatograms of samples extracted and analyzed by UHPLC-UV. Following the degradation of both a GalA standard and a carrot SDF sample, extraction of the degradation products, and injection into the LC-system, 5FFA appears as a well-resolved peak at 6.5 min. Degradation products like furfural and 5-hydroxymethylfurfural, originating from other components of the carbohydrate matrix, are clearly separated and elute in bulk after 8 min. The method was further tested by analyzing a carrot sample spiked with a 60-fold excess of starch. Despite the complex carbohydrate matrix, the peak areas of 5FFA resulting from pure carrot SDF (panel b, Fig. 4) and SDF spiked with starch don't differ significantly and the GalA content of the spiked sample was quantified in triplicate with a low coefficient of variation of 4.5 %. To ensure complete elution of condensed compounds and maintain column performance, the chromatographic method was extended by the incorporation of a rinsing step. Next, both the extraction conditions and heating parameters were optimized to maximize 5FFA yield.

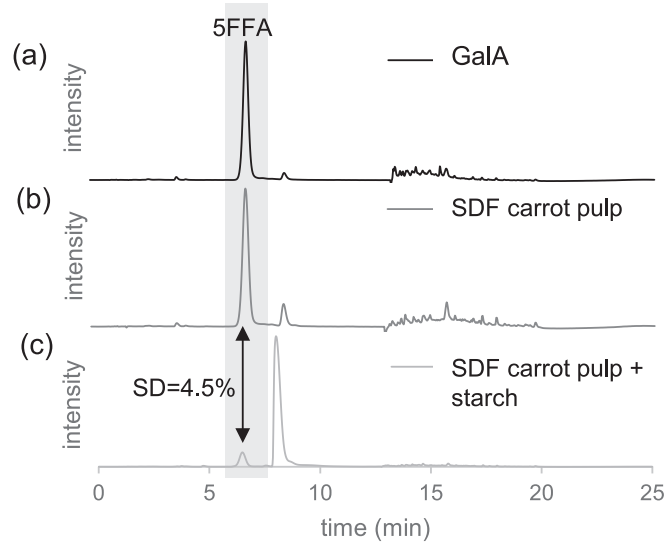

Fig. 4. Chromatographic separation of 5-formyl-2-furancarboxylic acid formed from a galacturonic acid (GalA) standard (a), from soluble dietary fiber (SDF) of carrot pomace (b) and from SDF of carrot pomace with 60-fold excess starch (c). Detection was carried out using a UV detector at 260 nm (SD: standard deviation).

3.3.1. *Optimization of the extraction of 5FFA*

The extraction was optimized evaluating the extraction solvent, number of extraction steps, and solvent volume, using recovery rates of 5FFA added directly into the concentrated acid. Suitable solvents for 5FFA extraction must be chemically inert to H_2SO_4, essentially immiscible with water, but sufficiently polar. Based on these criteria, DCM and ethyl acetate were tested. DCM is inert to H_2SO_4, which offers the advantage of direct extraction from concentrated acid but is less desirable due to its toxicity. Ethyl acetate, on the other hand, required partial neutralization of the acid with NaOH to prevent the formation of acetic acid. To remove remaining H_2SO_4 from the organic phase, washing is necessary. Since water is partially soluble in ethyl acetate, saturated NaCl solution was used to limit the water content remaining in the organic phase. Extractions with varying volumes of DCM (1–5 mL) showed negligible 5FFA recoveries, disqualifying DCM as a suitable solvent for this application. In contrast, ethyl acetate extraction achieved the highest recovery rate (75 %) if two successive extractions with 3 mL of organic solvent were performed, followed by two washing steps with 1.5 mL of saturated NaCl solution. Further repetitions of the extraction did not lead to any improvement in the recovery of 5FFA.

Approximately 25 % of the 5FFA was lost during extraction, probably due to condensation reactions of the furan derivatives, as previously described by Rosenau et al. (2017), and the low but partial solubility of ethyl acetate in water.

However, reproducibility of the ethyl acetate extraction step was confirmed over a relevant 5FFA concentration range (50 μM–250 μM). Recovery rates are presented in Table 2, demonstrating consistent performance across this concentration range.

The consistent loss of about 25 % and the good precision of the recovery rates across the tested concentration range confirm the reliability of the extraction. Based on these findings, all subsequent analyses used the optimized extraction protocol.

3.3.2. Optimization of the heating step

By adjusting heating temperature and time, the 5FFA yield can be maximized and, thus, the sensitivity increased. To identify the optimal parameters and establish the cause-effect relationships between these variables and the 5FFA output, a statistical DoE approach was applied. Specifically, a full factorial design was used, incorporating all possible combinations of the selected variables and levels (Jankovic et al., 2021). Three levels were chosen for both variables, temperature (60, 77.5, 95 °C) and time (5, 10, 15 min). This resulted in a total of 18 randomized experiments, each performed in duplicate. Comparable amounts of GalA were degraded, extracted, and quantified using HPLC-UV. The statistical analysis focused on maximizing 5FFA formation from GalA degradation, the results are shown in the Main Effects Plot (Fig. 5).

The plots show the independent effects of heating temperature and time on 5FFA yield. Both variables significantly influenced 5FFA concentration. However, the steeper slope between levels in the temperature plot compared to the time plot indicates that temperature had a more pronounced effect on 5FFA yield than heating time. The data revealed that the highest 5FFA yield was achieved at 77.5 °C after 5 min. Beyond this point, the yield decreased, likely due to condensation reactions of 5FFA (Rosenau et al., 2017). Thus, the optimal heating conditions for maximum 5FFA yield were identified as 77.5 °C for 5 min, which were adopted for subsequent analyses.

3.3.3. Effect of the addition of borate

Gregory (1960) was the first to introduce borate ions into the colorimetric carbazole reaction to quantify UA content. He observed a positive effect on the reaction's sensitivity. While a potential influence of borate on the degradation of UA to 5FFA in concentrated H_2SO_4 has been hypothesized, the underlying mechanism remains unclear. Previous studies reported that in the absence of borate, GalA produces the strongest color intensity, followed by GlcA, whereas ManA is undetectable without borate addition (Blumenkrantz & Asboe-Hansen, 1973; Filisetti-Cozzi & Carpita, 1991; Gregory, 1960; Scott, 1979).

To investigate this effect quantitatively, the 5FFA content formed during heating of various UA relevant to plant/algae research was analyzed with and without borate-containing H_2SO_4 (Fig. 6). For GalA, the presence of borate had no measurable impact on 5FFA yield, indicating that borate does not significantly influence its degradation. Differently, for other UA, borate significantly increased 5FFA concentrations. Notably, no 5FFA was detected if ManA was degraded in the absence of borate. These results are consistent with the results of Scott (1979), obtained from a photometric approach. According to this, the color intensity due to borate addition was only observed for UA with hydroxyl groups in the *threo* configuration at *C*3 and *C*4, enabling the formation of a 3,6-lactone. For the quantification of GalA, the addition of borate is unnecessary, given its negligible impact on 5FFA yield. However, considering potential future extensions of the method to other UA, borate-containing H_2SO_4 was used for the analyses to ensure broader applicability.

Table 2
Recoveries of 5-formyl-2-furancarboxylic acid (5FFA.), based on the use of a 5FFA standard in borate containing concentrated sulfuric acid, using extraction with ethyl acetate.

5FFA concentration (μM)	Recovery (%)
50	76.1 ± 0.43
150	75.4 ± 0.93
250	75.6 ± 1.17

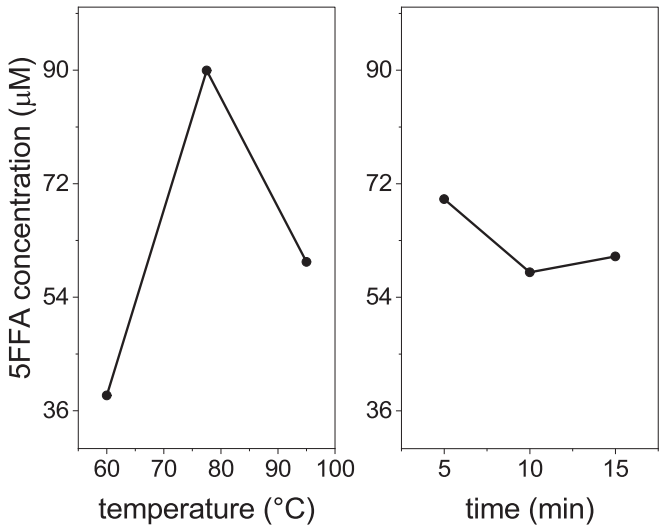

Fig. 5. Main Effects Plot from the Design of Experiment approach, illustrating the impact of heating temperature and time on the degradation of galacturonic acid to form 5-formyl-2-furancarboxylic acid (5FFA).

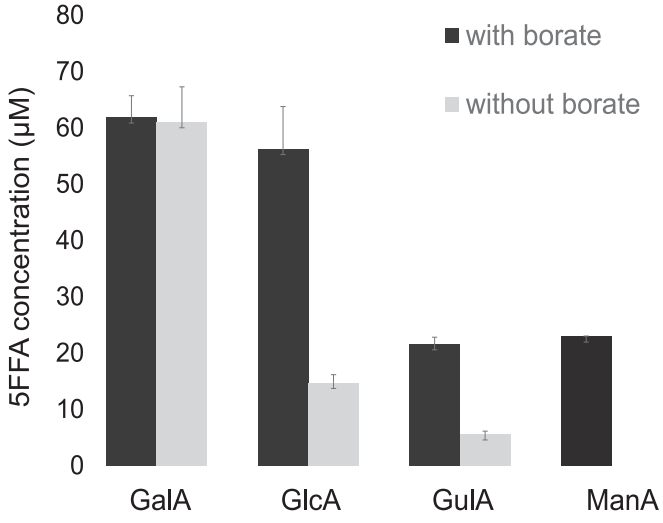

Fig. 6. 5-Formyl-2-furancarboxylic acid (5FFA) concentrations after heating galacturonic acid (GalA), glucuronic acid (GlcA), guluronic acid (GulA), and mannuronic acid (ManA) in concentrated sulfuric acid with and without 12.5 mM borate.

3.4. Sample analysis by LC-MS

3.4.1. Stable isotope dilution approach

The precision of the entire procedure using the UHPLC-UV method for final 5FFA analysis, determined from ten replicate analyses of a GalA standard solution according to section 2.8.6, was unsatisfactorily low with a coefficient of variation of the procedure of 71 %. After excluding three outliers, identified by a Dixon's Q test ($\alpha = 0.05$), from the dataset, the precision improved with a coefficient of variation of 30 %. However,

this value still indicates significant variability. Given the good reproducibility of both the extraction process (Table 2) and the chromatographic procedure (section 3.3), the variation is likely due to inconsistent degradation rates during the heating step. This issue aligns with observations by Yapo (2012), who provides an overview of various measurement results of comparable samples, analyzed photometrically by different working groups. To address this concern, the use of an internal standard is recommended to compensate for variations during the heating step. A fully ^{13}C-labeled, commercially available GalA ($^{13}C_6$-GalA) was chosen as the internal standard. The isotopologue was added at a constant concentration to the sample solution at the beginning of each experiment. MS detection is necessary, as the chromatographic separation of the resulting degradation products (5FFA and $^{13}C_6$-5FFA) is not possible. For quantification, the ratio of 5FFA and $^{13}C_6$-5FFA was formed.

3.4.2. Method validation

The method was validated according to section 2.8.6 using a GalA-standard solution. 5FFA and $^{13}C_6$-5FFA were separated and measured on a UHPLC-Orbitrap-MS system. The requirements for a normally distributed population and homogeneity of variances between the highest and lowest calibrated concentration are fulfilled, so that the LOD and LOQ can be determined using the calibration method listed in DIN32645. Within the selected calibration range, a linear relationship between the measured values and the concentration was confirmed. Table 3 summarizes the validation parameters and compares them with data of the photometric method which was validated using the same principles.

The validation parameters clearly demonstrate that LC-MS quantification is significantly more sensitive than the photometric method, with an approximately fivefold improvement in sensitivity achieved when comparing the LOD and LOQ of the two approaches. The incorporation of the stable isotope-labeled internal standard notably enhanced precision, with a coefficient of variation of the procedure of 1.93 %, a remarkable improvement over the photometric method. The high level of precision combined with excellent recovery rates, without matrix (external) and with matrix, confirms the method's reliability for quantifying GalA content. Differently, the photometric determination of GalA has been shown to result in overestimation. This discrepancy is likely – at least partially - attributable to the calibration range proposed by Blumenkrantz and Asboe-Hansen (1973), which falls below the statistical LOQ, thereby undermining reliable quantification.

To evaluate the benefit of the high-resolution Orbitrap mass analyzer compared to a simpler single quadrupole system, parts of the validation were repeated using a UHPLC coupled to a single quadrupole MS (Table 4).

Although LOD and LOQ values were slightly higher if a single quadrupole was used for detection, sensitivity in the SIM-mode was still roughly comparable with the LC-Orbitrap-MS method and superior to the colorimetric assay. However, the Orbitrap system performed better in terms of precision, particularly at low concentrations, where the single quadrupole exhibited high variability. Overall, the analysis can be successfully transferred to a single quadrupole MS system without significant loss of reliability, although the calibration range should be adjusted to account for the poorer precision at low concentrations.

Table 3
Validation parameters of the liquid chromatographic-Orbitrap-mass spectrometric (LC-Orbitrap-MS) galacturonic acid quantification in comparison with the photometric quantification (LOD: limit of detection; LOQ: limit of quantification; R^2: coefficient of determination).

	LC-Orbitrap-MS	Photometry
Calibration range (mg/L)	10–85 mg/L	
Regression equation	y = 0.02456x – 0.00383	y = 0.00574x + 0.15771
R^2	0.9991	0.9974
LOD (mg/L)	1.38	7.30
LOQ (mg/L)	4.75	24.96
Instrumental coefficient of variation (%)	1.01	4.26
Coefficient of variation of the procedure (%)	1.93	10.58
External recovery (%)	100.32 ± 1.47	122.10 ± 3.13
Recovery in matrix (%)	101.80 ± 2.99	116.74 ± 8.14

Table 4
Limit of detection (LOQ), limit of quantification (LOQ), and coefficients of variation of the procedure of galacturonic acid (GalA) analysis using the developed LC-MS method, but applying a single quadrupole mass spectrometer (Q).

		LC-Q-MS
LOD (mg/L)		1.71
LOQ (mg/L)		5.74
Coefficient of variation of the procedure (%)	low concentration (10 mg/L GalA)	20.25
	high concentration (85 mg/L GalA)	1.43

3.4.3. Application: analysis of GalA oligomers and real life samples

The method was validated using a GalA monosaccharide standard; however, the potential influence of the degree of polymerization (DP) of oligomeric or polymeric GalA on the results had not yet been addressed. To evaluate this, isolated GalA oligosaccharide standards (section 3.1) with varying DP were analyzed using the same method (LC-Orbitrap-MS) that was calibrated with the GalA monosaccharide equivalent, which was set as 100 % recovery (Table 5). The recoveries of all oligosaccharide standards were within the range of 100 ± 7 %. No correlation was observed between DP and the analysis results. Variations in recovery are likely attributable to differences in the purity of the oligosaccharide standards (Table 1). It can be concluded that the developed method can also be applied to soluble GalA oligo−/polymers without additional pre-treatment.

For insoluble samples such as IDF, however, pre-hydrolysis appears to be necessary to release GalA-containing polymers. A well-known, acid based pretreatment (Saeman et al. (1945)) was modified as such that both steps (treatments with 12 M H_2SO_4 and 1.6 M H_2SO_4) were performed at room temperature. Times for treatments with 12 M and 1.6 M H_2SO_4 were optimized by using carrot pulp IDF to release the maximum amount of degradable mono−/oligomeric GalA without forming 5FFA (data not shown), resulting in the procedure described in section 2.6.

McComb and McCready (1952) investigated the influence of methyl-esterified GalA using a methyl-esterified GalA monosaccharide standard in a photometric assay. They demonstrated that short-term heating for 5 min in concentrated H_2SO_4 acid was insufficient to fully hydrolyze the esters. However, after alkaline treatment, the esterified GalA was completely recovered. To evaluate the impact of DM on the GalA determination, the SDF from three raw materials with varying DM (Table 6) were dissolved either in water or 0.05 M NaOH and analyzed using the LC-MS approach. All samples exhibited higher GalA contents

Table 5
Recovery of galacturonic acid (GalA) in GalA-oligosaccharides with a defined degree of polymerization (DP), analyzed by the developed LC-Orbitrap-MS method.

DP of oligosaccharide	Recovery (% monosaccharide)
3	99.39 ± 2.28
4	93.05 ± 0.42
5	105.01 ± 0.25
6	100.48 ± 5.00
7	99.31 ± 0.37
9	105.57 ± 0.41
10	97.29 ± 0.60

Table 6
Comparison of the degrees of methylation (DM) and acetylation (DAc), and the uronic acid contents, analyzed as galacturonic acid (GalA), of three fruit and vegetable dietary fibers (IDF: insoluble dietary fiber, SDF: soluble dietary fiber) by the developed LC-MS method and the photometric method (RSD: percent relative standard deviation, $n = 3$).

		LC-Orbitrap-MS				Photometry			
		GalA (mg/100 mg)	RSD (%)	DM (%)	DAc (%)	GalA (mg/100 mg)	RSD (%)	DM (%)	DAc (%)
Carrot pulp	**IDF**	17.88 ± 0.29	1.60	33.79	35.60	18.87 ± 0.03	0.16	32.02	33.73
	SDF	38.90 ± 0.01	0.01	42.87	4.44	40.60 ± 6.83	16.81	41.08	4.24
Apple pulp	**IDF**	12.61 ± 0.40	3.20	54.42	73.80	13.57 ± 0.76	5.63	50.57	68.58
	SDF	65.49 ± 2.70	4.13	48.40	6.43	38.77 ± 1.43	6.68	81.76	10.86
Citrus peel	**IDF**	20.31 ± 0.22	1.10	59.45	19.28	21.17 ± 0.74	3.50	57.04	18.50
	SDF	16.83 ± 0.67	3.98	40.64	35.94	22.42 ± 7.17	31.96	30.51	26.98

after NaOH pretreatment, although the 4.63 % increase observed in carrot SDF was not significant. In contrast, SDF from apple pomace and lemon peel showed substantial increases of 27.71 % and 11.24 %, respectively (data shown in supplementary file Table S1). Acetyl groups are also hydrolyzed by alkali treatment. However, the influence of these groups on the resulting 5FFA is not relevant as the hydroxy groups at positions 2 and 3 are cleaved off as water or acetic acid during degradation. For IDF, alkaline treatment was not feasible due to the required pre-hydrolysis step to obtain a GalA containing solution of the materials. However, the applied acid treatments (pre-hydrolysis with 12 M H_2SO_4 for 2 h and degradation by heating for 5 min in concentrated H_2SO_4) are expected to sufficiently cleave the ester bonds.

Eventually, the final protocol of the method (section 2.8.5) was applied to both IDF and SDF fractions from three different sample materials: carrot pulp, apple pulp, and citrus peel (the monomer composition of the fiber samples is described in the supplementary data, Figs. S1-S3). The results were compared with those obtained from the photometric method (Table 6).

For IDF, the LC-MS results were comparable to those of the photometric method, with low standard deviations. In contrast, the GalA contents of SDF samples determined by the colorimetric assay are not comparable with the LC-MS results and generally show worse precision data. There is no obvious relationship between the discrepancies of the results of the two approaches and the DM and/or DAc levels of the samples determined by both methods.

Thus, the approach used affects the determined monomer composition of fiber samples but also has an influence on the calculated DM and DAc, which is consequently lower if higher GalA contents are determined. This can be particularly important for studies aiming to study structure-function relationships of various pectins. However, considering the unsatisfactory validation data of the photometric assay in section 3.4.2, the reliability of the method is questionable, and we suggest to use the novel, validated approach described here.

The method presented is specifically optimized for pectin-rich samples, such as those derived from terrestrial plants. This defines its current scope of application. Small amounts of GlcA, such as those present in rhamnogalacturonan type II (Schols et al., 1995), are tolerated. The addition of borate adjusts the degradation behavior of GlcA to approximate that of GalA, thereby minimizing analytical inaccuracies. On the other hand, the accurate determination of total uronic acids in algal samples containing GulA and ManA, as well as in samples consisting only of GlcA or in comparable ratios of GlcA and GalA, requires the development of modified analytical protocols, which are currently being established.

4. Conclusion

An accurate and precise LC-MS based method was successfully developed for the quantification of galacturonic acid contents in pectin-containing samples. The use of isotopically labeled galacturonic acid as an internal standard significantly improved the method's accuracy and precision. The influence of the degree of polymerization was investigated using galacturonic acid oligosaccharides, demonstrating that soluble galacturonic acid-containing samples can be directly analyzed after deesterification. Differently, insoluble sample materials require a pre-hydrolysis step. The application of the developed method to fiber samples and the comparison of the obtained results with data from the colorimetric assay revealed notable differences in galacturonic acid content, particularly in soluble dietary fiber materials. Thus, the LC-MS method shows clear advantages in terms of accuracy, precision, and sensitivity for the quantification of galacturonic acid.

CRediT authorship contribution statement

Johanna Braun: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Mirko Bunzel:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Johanna Braun reports financial support was provided by Fond der Chemischen Industrie (FCI). If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.carbpol.2025.123816.

Data availability

Data will be made available on request.

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