Strategy to identify and quantify polysaccharide gums in gelled food concentrates

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1. Introduction

Polysaccharides are regularly used for structuring food products, e.g. as thickeners in soups, sauces, and seasonings, stabilizers in ice cream and oil–water emulsions, and gelling agents in jams and jellies. The polysaccharides commonly used for structuring are, among others, starch, pectins, alginites, xanthan gum, and plant galactomannans such as guar gum and locust bean gum (LBG, also known as carob gum), and mixtures thereof. Especially the combination of xanthan gum with galactomannans was shown to form strong, thermoreversible gels (Copetti, Grassi, Lapasin, & Pricl, 1997), and is therefore widely utilized in food products.

The identification and quantification of polysaccharides used for food structuring is not necessarily straightforward. Whilst starch can be quantified according to internationally recognized methods (ISO 15914, 2004; Brunt, 2000; Brunt, Sanders, & Rozema, 1998; Casterline, Oles, & Ku, 1999), the selective identification and quantification of other structuring polysaccharides in complex food matrices is considerably more complicated. Most methods described in literature are limited to the identification and/or quantification of polysaccharide gums in bulk materials, mostly for authentication purposes. Anderson, Millar, and Weiping (1991), for instance, used 13C-NMR spectroscopy for the identification of gum Arabic. Capillary electrophoresis was used for the determination of adulteration of LBG with guar gum by measuring residual plant proteins (Flurer, 2000; Flurer, Crowe, & Wolnik, 2000; Ruiz-Angel, Simó-Alfonso, Mongay-Fernández, & Ramis-Ramos, 2002). For the direct discrimination of guar gum from LBG in bulk materials, Fourier Transform Infrared Spectroscopy is used by Prado, Kim, Ozen, and Mauer (2005), which method also allows for the individual identification and quantification of a number of other common polysaccharide gums, such as xanthan, tara, and fenugreek gums.

In processed food, the low levels of polysaccharide gums used for structuring, typically not exceeding 1%, and the complexity of the food matrix often complicates the identification and quantification of individual compounds. Meyer, Rosa, Hischenhuber, and Meyer (2001) developed a method based on polymerase chain reaction (PCR) of isolated plant DNA for the qualitative determination of guar gum and LBG in food products such as ice cream and cheese. This method has been further improved by Urdiain, Doménech-Sánchez, Alberti, Benedi, and Rosselló (2004, Urdiain, Doménech-Sánchez, Alberti, Benedi, and Rosselló (2005) and...
showed to be a very selective and sensitive procedure for the discrimination between guar gum and LBG in several food products. However, the method only proves indirectly the presence of distinct polysaccharide gums since it is based on the presence of residual plant DNA instead of the polysaccharide itself. Furthermore, the PCR methods provide qualitative data only. For the direct determination of polysaccharide gums, Pazur and Li (2004) used antibodies specific against gum Arabic, xanthan gum, or guar gum and applied those to determine the presence of the polysaccharide gums in ice creams, soups, dressings, and cheese. Although this technique is highly selective and can potentially be used for quantitative purposes, generating and purifying antibodies is a tedious task. Other research groups used monosaccharide analysis for identifying and quantifying polysaccharide gums after their isolation from the food matrix (Englyst, Wiggins, & Cummings, 1982; Glück & Their, 1980; Lawrence & Iyengar, 1985). This method is based on the total depolymerization of an isolated and purified polysaccharide fraction into monosaccharides and the consecutive identification and quantification of these products. This is still the most common method for quantifying carbohydrates since it is generally very accurate, is easy to perform, and does not require very sophisticated lab equipment. Still, the method lacks selectivity, especially when a mixture of polysaccharides with similar monosaccharide composition is present, and therefore, unambiguous identification and quantification of individual polysaccharides is usually not possible without additional analyses.

To enable the unambiguous identification and quantification of polysaccharide gums in complex food matrices, we here present a multi-angle strategy for the isolation, identification and selective quantification. The methods used within this approach include DNA detection by PCR, an isolation procedure for the polysaccharide gums from the food matrix, NMR spectroscopy, (quantitative) enzymatic fingerprinting, and quantitative monosaccharide analysis. This approach is tested on gelled food concentrates containing the commonly used polysaccharide gums xanthan gum and LBG.

2. Materials and methods

2.1. Materials

As gelled food concentrates the commercially available Knorr Wolowy Bulionetka (water, salt, flavour enhancer (MSG, IG), yeast extract, beef extract, vegetable fat, sugar, beef fat, carrots, aroma (contains milk), celeriac, thickeners (xanthan gum (0.51%), locust bean gum (0.22%)), chopped parsley, colorant (mixed carotenes) (Unilever, Warszawa, Poland) and the non-commercial model Wołowy Bulionetka (water, salt, flavour enhancer (MSG, IG), yeast extract, beef extract, vegetable fat, sugar, beef fat, carrots, aroma (contains milk), celeriac, thickeners (locust bean gum (0.22%), xanthan gum (0.45%), locust bean gum (0.31%)}, dried parsley, spices, vegetable fats and oils, acetic acid) (Unilever, Heilbronn, Germany) were used. Heat-stable α-amylase for total dietary fibre assay and protease (Subtilisin), both from Bacillus licheniformis, and 3-{trimethylsilyl}propionic-2,2,3,3-d4 acid (TSP) (sodium salt 98 atom% D) were purchased from Sigma–Aldrich Chemie B.V. (Zwijndrecht, Netherlands). Dialysis tubing was obtained from Medicell International Ltd. (London, UK), deuterated methanol (99.5 atom% D) from Acros Organics (Fisher Scientific, Landsmeer, The Netherlands), deuterated water (99.96 atom% D) from Cambridge Isotope Laboratories (Buchen B.V., Apeldoorn, The Netherlands), Qiagen Plasmid Maxi Kit from Qiagen (Venlo, The Netherlands), AmpliTaq Gold PCR Master Mix from Applied Biosystems (Nieuwerkerk a/d IJssel, The Netherlands), and the DNA ladder TrackIt™ from Invitrogen, Life Technologies Europe BV (Bleiswijk, The Netherlands).

endo-(1,4)-b-Mannanase from Aspergillus niger was purchased from Megazyme International Ireland (Wicklow, Ireland). Locust bean gum (Meyprodyn 200) was obtained from Danisco (Frankfurt/Main, Germany), guar gum from Sigma Aldrich, cassia gum from IMCD Deutschland GmbH & Co (Cologne, Germany), tara gum (HV grade) from Kalys S.A. (Bernin, France), and xanthan gum (FNCS) from Jungbunzlauer Suisse AG (Basel, Switzerland). All other reagents were of highest analytical grade available.

2.2. DNA isolation and amplification by polymerase chain reaction

Approximately 7–9 g of gelled food concentrate was dissolved at 80 °C in 40 ml of purified water, vortex-mixed for 1 min and centrifuged at 10,000 RPM for 15 min. DNA of Ceratonia siliqua (for determining LBG) and Xanthomonas campestris (for determining xanthan gum) was extracted using the Qiagen Plasmid Maxi Kit according to the manufacturer’s instructions. As positive controls 10 mg of commercial xanthan gum and LBG were dissolved in 10 ml of purified water, vortex-mixed and centrifuged at 10,000 RPM for 1 min. DNA was isolated from the supernatants as described for gelled food concentrates.

Ribosomal DNA amplification by PCR was performed according to a modification of the method described by Urdiaín, Doménech-Sánchez, Alberti, Benedi, and Rosselló (2004). The AmpliTaq Gold PCR Master Mix was used for PCR amplification. Primers (5′-GGCAAGCATGTAAGACCTGTC-3′) and (5′-CTTCAGAACATGGTATTAAGCTG-3′) were designed to amplify a 413-bp fragment specific for X. campestris (GenBank accession No. AFI95881.1).

Amplification comprises 35 cycles of denaturation at 94 °C for 90 s, annealing at 61 °C for 90 s, and extension at 72 °C for 90 s. The final products were extended at 72 °C for 5 min. Primers (5′-GCGAATTCGATACTTGGTGTGAATTCG-3′) and (5′-AGGATATGCTCGTTGAGCT-3′) were designed to amplify a 196-bp fragment specific for C. siliqua. Reaction mixes were pre-heated at 95 °C for 120 s. For the first PCR, amplification comprises 35 cycles of denaturation at 94 °C for 90 s, annealing at 61 °C for 90 s, and extension at 72 °C for 90 s. The nested PCR comprises 35 cycles of denaturation at 94 °C for 90 s, annealing at 58 °C for 90 s, and extension at 72 °C for 90 s. In both cases, a final extension at 72 °C for 5 min was included. PCR products (20 µl) were separated on a 2% agarose gel and stained with ethidium bromide. Positive/negative controls (xanthan gum, LBG, guar gum, and cassia gum) and a 50-bp DNA ladder were included.

2.3. Isolation of non-starch polysaccharides from gelled food concentrates

Approximately 25 g of gelled food concentrate was dissolved in 350 ml of hot (90 °C) water. The dissolved gel was mixed at 100 rpm at 80 °C for 30 min in a water bath and centrifuged while being hot using 500 ml Beckman polycarbonate bottles and a Beckman Avanti j-25 centrifuge in a pre-heated (80 °C) JA-10 rotor at 18,000×g for 5 min. The centrifuge was thermostatted at its maximum temperature (40 °C). To prevent polysaccharide gelling it was ensured that the supernatant stayed well above 70 °C. After centrifugation, the fat layer was removed by pipetting and the remaining supernatant was transferred into a clean bottle and was allowed to cool down.

The pH of the supernatant was adjusted to pH 6.0 by adding phosphate buffer (1 M). Starch or maltodextrins that are regularly present in gelled food concentrates either as added ingredients or as part of other ingredients were enzymatically degraded by incubating the solution with 4000 units of α-amylase at 60 °C for one hour. Sodium hydroxide (4 M) was then added to raise the pH to 7.0. Dissolved proteins were hydrolyzed by adding 0.5 units of protease and the mixture was incubated at 60 °C for two hours, after which the enzyme was inactivated upon boiling for 5 min.
Hydrolysis products, salt and other small molecules were removed by dialysis using 12 to 14 kDa cut-off (based on protein) dialysis tubing against demineralized water (three changes, total dialysis time was 20 h). The conductivity of the retentate was then below 500 µS/cm. The dialyzed sample was concentrated to approximately 10 ml by rotary evaporation at 45 °C and the sample was recovered by rinsing the evaporation flask four times with hot (95 °C) water. The final sample volume was 50 ml. Dissolved polysaccharides were precipitated by adding 150 ml of 96% ethanol. The mixture was allowed to stand for several hours at RT. The precipitate was recovered by centrifugation at 18,000×g for 5 min. The pellet was washed consecutively twice with 70% (v/v) aqueous ethanol and twice with 100% ethanol. To remove ethanol and residual fat, the pellet was then washed four times with acetone, twice with petroleum ether, and twice with heptane. During this last washing step, the sample was placed in an ultrasonic bath for 10 min. The sample was dried at 30 °C for 24 h. The resulting sample was named “polysaccharide fraction”.

2.4. NMR spectroscopy

Approximately 8 to 10 mg of homogenized and ground polysaccharide fraction or polysaccharide standards were wetted with 100 µl of deuterated methanol. Five ml of deuterated water and 0.5 ml of internal standard (0.2 mg/ml of TSP in D2O) was added. The mixture was heated until boiling upon homogenization using a magnetic stirrer. Then, the mixture was placed in an oven at 65–70 °C for 4–16 h. This procedure of stirring and heating was repeated in case the sample material was not fully dissolved. Finally, 650 µl of the solution was transferred into a pre-heated 5-mm NMR tube (65–70 °C).

1D 1H-NMR and 2D 1H-13C-HSQC experiments were carried out at 70 °C on a Bruker Avance III 600 NMR spectrometer, equipped with a 5-mm CryoProbe. The 1D 1H-NMR spectra were recorded with a NOESYGPR1D pulse sequence. Thirty-two scans were collected in 49 k data points with a recycle delay of 30 s, a mixing time of 150 ms, and an acquisition time of 3.5 s. Low power water suppression (16 Hz) was applied for 0.1 s.

The 2D 1H-13C-HSQC spectra were recorded with an HSQCETGSPSI pulse sequence. The spectral widths were 12 ppm (H dimension) and 150 ppm (13C dimension). Sixteen scans in the 1H dimension and 256 increments in the 13C dimension were collected. The recycle delay was 1.5 s.

The data were processed in TOPSPIN software version 3.1 (Bruker BioSpin GmbH, Rheinstetten, Germany). An exponential window function was applied to the free induction decay (FID) with a line-broadening factor of 0.3 Hz prior to the Fourier transformation. Manual phase correction and baseline correction was applied. All spectra were referenced to the methyl resonance of TSP (δ 0.0 ppm).

For targeted profiling, 1D 1H-NMR spectra were imported in Chenomx software (Chenomx NMR Suite Professional v7.11, Edmonton, Alberta, Canada). The compound models utilized in Chenomx were based on 0.1 g/l and 1 g/l solutions of polysaccharide standards in D2O (calibrated against internal standard TSP) and acquired under the same conditions as the sample solutions.

2.5. Enzymatic fingerprinting of galactomannans

The discrimination between different galactomannans by enzymatic fingerprinting was performed similar to Daas, Schols, and de Jongh (2000). The isolated polysaccharide fractions and polysaccharide standards were dissolved at 2 mg/ml sample in 50 mM sodium acetate buffer (pH 4.5). Endo-(1,4)-β-mannanase was added to a final concentration of 0.26 U/ml. Samples were incubated on a head-over-tail mixer at 30 °C for 24 h after which the enzyme was deactivated by heating at 99 °C for 30 min using a Thermomixer (Eppendorf AG, Hamburg). After cooling the samples to ambient temperature, insoluble material was removed by centrifugation at 15,000×g for 5 min prior to further sample preparation and analysis.

Enzymatic hydrolysis products were separated by high-performance anion-exchange chromatography coupled to pulsed amperometric detection (HPAEC-PAD). The HPAEC-PAD system consisted of a Dionex DX 500 apparatus equipped with a GP 40 gradient pump and an ED 40 electrochemical detector (Dionex Corporation, Sunnyvale, CA) equipped with a CarboPac PA1 guard column (4 × 50 mm) and a CarboPac PA1 separation column (4 × 250 mm, Dionex Corporation). Samples were diluted in 50 mM sodium acetate buffer to a final concentration of 0.4 mg/ml, whereas LBG, tara gum, and cassia gum standards were diluted to 0.1 mg/ml, and guar gum to 0.5 mg/ml. As internal standard arabinox was added to a level of 1 mg/l (20 µl of 0.1 g/l Ara in 2 ml sample). Separation of the enzymatic hydrolysis products was achieved by a 95-min gradient using 100 mM sodium hydroxide, 1 M sodium acetate in 100 mM sodium hydroxide, and MilliQ-purified water. A tertiary gradient was applied: The sodium hydroxide gradient was 0–20.0 min, 40 mM; 20.0–30.0 min, 40–100 mM; 30–75 min, 100 mM; 75–95 min, 40 mM. The simultaneous gradient of sodium acetate was 0.0–30.0 min, 0 mM; 30–65.0 min, 0–150 mM; 65–70.0 min, 1000 mM; 70–95 min, 0 mM. The flow rate was 1.0 ml/min and the injection volume was 20 µl.

For quantitative enzymatic fingerprinting, ten dilutions ranging from 100 to 1000 µg/ml in 50 mM sodium acetate buffer (pH 4.5) were prepared from a 2 mg/ml LBG suspension. The solutions were hydrolyzed with endo-(1,4)-β-mannanase as described above. After centrifugation at 15,000×g for 5 min, the hydrolysates were diluted ten times to obtain a ten-point calibration curve ranging from 10 to 10 µg/ml.

TotalChrom software version 6.3.2 (PerkinElmer, Inc., Waltham, MA) was used for data processing.

2.6. Monosaccharide analysis

Monosaccharide analysis by methanolysis followed by TFA hydrolysis was performed according to De Ruiter, Schols, Voragen, and Rombouts (1992). In short, polysaccharide fractions or polysaccharide standards (xanthan gum, LBG) were dissolved to a concentration of 1 mg/ml in water at 100 °C for 10 min, and 100 µl of the solution was taken for methanolysis/TFA hydrolysis. Hydrolysis products were dried and re-dissolved in 1 ml water. For HPAEC-PAD analysis of neutral monosaccharides, polysaccharide fractions and polysaccharide standards were diluted 3 and 5 times, respectively. For the analysis of uronic acids, polysaccharide fractions were analysed without further dilution, whereas polysaccharide standards were diluted 1 time.

A mixture of nine different monosaccharide standards (fucose, arabinose, rhamnose, galactose, glucose, xylose, mannose, galacturonic acid, and glucuronic acid) were included in the whole monosaccharide analysis procedure (in four fold) to enable the determination of recoveries and allow quantification based on these standards. Standards were diluted to a concentration of 10 µg/ml prior to injection for quantification of both neutral and acidic monosaccharides.

Hydrolysis products were separated and quantified by HPAEC-PAD on a Dionex ICS5000 system equipped with a CarboPac PA1 guard column (2 × 50 mm) and a CarboPac PA1 separation column (2 × 250 mm, Dionex Corporation) at a flow rate of 0.3 ml/min. Column temperature was maintained at 20 °C. The samples were stored in an autosampler at 10 °C. The injection volume was 10 µl. Separation of neutral monosaccharides was achieved according to Sengkhamparn, Verhoef, Schols,
Sajjaanantakul, and Voragen (2009), using MilliQ-purified water, 100 mM sodium hydroxide, and 1 M sodium acetate in 100 mM sodium hydroxide. The equilibrium time has been prolonged by 7 min resulting in an 80-min run. To enable optimal PAD detection at an eluent composition of 100% water, 0.5 M NaOH at 0.15 ml/min was introduced via post-column addition into the flow. Acidic monosaccharides were separated using a 7-min isocratic method consisting of 0.2 M NaOAc in 0.1 M NaOH, followed by regeneration of the column.

Chromeleon software version 7.1 (Dionex Corporation) was used for data processing.

3. Results and discussion

3.1. Fast and selective screening of polysaccharide gums in intact food products by PCR

Xanthan gum and locust bean gum (LBG) are polysaccharide gums from natural origin. Hence, during isolation and further refining of the gums, isolated nuclei and intact cells from the source organisms remain in the polysaccharide gums and thus end in the final food formulation. DNA sequences from the nuclear ribosomal spacers of X. campestris and C. siliqua were used to design PCR primers specific for each additive. PCR products were then separated by gel electrophoresis. The primer set for X. campestris should give a specific PCR fragment of 413 bp. In Fig. 1A the electrophoretic analysis of the PCR products is shown, demonstrating that PCR products of around 400 bp were found in the gelled food concentrate (lane 4) as well as in the xanthan gum standard (lane 4), LBG, guar gum, and cassia gum were used as negative controls and did not show a band (not shown).

Similarly, electrophoretic analysis of the PCR products from C. siliqua show bands at around 200 bp for the gelled food concentrate (lane 3) as well as in the xanthan gum standard (lane 4). LBG, guar gum, and cassia gum were used as negative controls and did not show a band (not shown).

Although, the PCR method is merely qualitative and is potentially susceptible to false negatives due to inhibitory effects by some polysaccharides (Demek & Adams, 1992) and due to processing conditions such as sterilization that in certain cases may degrade the DNA (Meyer et al., 2001), PCR allows for the fast and selective screening of polysaccharide gums that may be expected and can therefore be used as a first analytical screening tool to further define the analysis route.

3.2. Identification of polysaccharide gums

3.2.1. Isolation of non-starch polysaccharides from gelled food concentrates

To confirm the PCR results and to quantify the found polysaccharides, additional methods were selected for the analysis of polysaccharide gums present in gelled food concentrates. The gelled food concentrates, however, form a rather complex, heterogeneous matrix complicating the detailed analysis of polysaccharides. The polysaccharide gums of interest are generally present in concentrations of less than 1% (w/w) of the total gel, whereas numerous other compounds are present at high levels which potentially interfere with the identification and quantification of the gums. We therefore developed an isolation and purification method, which includes particle removal, enzymatic starch and protein degradation and removal, and thorough desalting. A concise isolation scheme is displayed in Fig. 2.

In the first step, gelled food concentrates were dissolved in hot water and particles and fat were removed by centrifugation. We discovered that the dissolved polysaccharides tend to gel when temperatures drop below a critical point, which was at approximately 60 °C, resulting in drastic reduction of polysaccharide yields. It was therefore ensured that the solution remained at >70 °C during centrifugation. Furthermore, to reduce heat transfer, polycarbonate centrifuge tubes are to be preferred over common glass tubes. We also pre-heated the centrifuge at 40 °C and pre-heated the centrifuge rotor at 80 °C to reduce cooling of the solution during centrifugation. Finally, centrifugation times were kept to a minimum.

The second critical step concerned the enzymatic starch/maltodextrins and protein removal. Initially, starch and/or maltodextrins were degraded using consecutively α-amylase and amyloglucosidase (AMG) according to ISO15914. When testing the enzymes on side activities, however, we detected considerable endo-(1,4)-β-mannanase activity in the AMG preparation used that also degraded galactomannans such as LBG. The use of AMG was therefore omitted. Starch/maltodextrins were nevertheless sufficiently removed, which was demonstrated by the presence of only low residual α-glucan levels in purified polysaccharide fractions as shown by NMR spectroscopy (Section 3.2.2). The presence of these low levels of α-glucan did not further impact the identification or quantification of the isolated polysaccharide gums, as shown in the following sections.

For removing water-soluble protein, an alkaline protease was selected since it was reported that alkaline proteases have superior properties over common proteases for the deproteinization of viscous, water-soluble polysaccharides (Wang, Yuan, Wang, Zhang, Yang & Xu, 2007). In addition, alkaline proteases have their optimum enzymatic activity at elevated temperatures, which helps in reducing the viscosity of the solution.

Efficient removal of small molecules such as enzymatic breakdown products and salt, but also added sucrose and maltodextrins showed to be critical in obtaining an end-product of sufficient purity. When dialysis was omitted and non-starch polysaccharides were recovered directly by ethanol precipitation, considerable levels of salt and other small molecules still remained in the polysaccharide fraction. With dialysis salt levels could be reduced to approximately 1% of initial levels. However, larger oligosaccharides such as starch and maltodextrin degradation products may be difficult to remove by dialysis. To remove remaining salt and oligosaccharides, the polysaccharide solution was concentrated and polysaccharides were precipitated by adding ethanol. To facilitate the solubility of salt and oligosaccharides, the precipitation was performed at room temperature. Extensive washing of the recovered ethanol precipitate with consecutively ethanol, acetone,
petroleum ether, and heptane finally removed most of the colour and of residual fat.

The purity of the isolated polysaccharide fraction was determined by NMR spectroscopy (Section 3.2.2) and monosaccharide analysis (Section 3.3.1).

3.2.2. Non-targeted polysaccharide characterization by NMR spectroscopy

The non-starch polysaccharide fraction isolated from the gelled food concentrate was measured by $^1$H-NMR and heteronuclear $^1$H-$^1$C-NMR spectroscopy (Ramesh, Yamaki, Ono, & Tsushida, 2001; Rinaudo, Milas, Lambert, & Vincendon, 1983; Vieira, Mendes, Gallao, & Sousa de Brito, 2007) and was compared to the spectra obtained from polysaccharide standards. The $^1$H-NMR spectra are displayed in Fig. 3. The isolated polysaccharide fraction gave numerous signals in the anomeric proton region between $\delta$ 4.7 and 5.3 ppm (Fig. 3A). Comparing these signals with polysaccharide standards, the signals could be assigned to galactomannan (between $\delta$ 4.7 and 5.1 ppm, see Fig. 3C-F) and yeast mannan (between $\delta$ 5.0 and 5.3 ppm). In addition, strong signals between $\delta$ 1.5 and 2.2 ppm are present, which could be assigned to acetyl and pyruvyl groups of xanthan (Fig. 3B). The assignments based on $^1$H-NMR were confirmed by heteronuclear $^1$H-$^1$C-NMR spectroscopy data (Supplementary information Figs. S1 and S2).

We furthermore explored whether 1D $^1$H-NMR spectroscopy is able to discriminate between different galactomannans. The most distinguishing signals are found at $\delta$ 4.13, 4.26, and 5.02 ppm, corresponding to H-2 and H-1 of $\alpha$-Manp-(1→), and H-1 of $\alpha$-Galp-(1→), respectively (Fig. 3C-F). These differences can be attributed to differences in galactosyl substitutions of the different

Fig. 2. Schematic representation of the isolation and purification of non-starch polysaccharides from gelled food concentrate.

Fig. 3. Identification of xanthan gum and LBG by 600 MHz $^1$H-NMR spectroscopy. $^1$H-NMR spectra of polysaccharide fraction from Knorr Wołowy (A) xanthan gum (B), LBG (C), guar gum (D), cassia gum (E), and tara gum (F). Xanthan gum is identified by the pyruvyl and acetyl signals at $\delta$ 1.47 and 2.17 ppm (as indicated by ◇) and the galactomannans by the signals found at $\delta$ 4.13, 4.76, and 5.05 ppm (as indicated by ▼).
galactomannans. Hence, the galactomannan present in the isolated polysaccharide fractions can be identified based on peak ratios of the respective NMR signals. Using the NMR data, it is evident that the Knorr Wołowy product contains LBG (compare Fig. 3A with Fig. 3C). Moreover, NMR targeted profiling procedures can be used to determine galactomannan ratios in case a mixture of two galactomannans is present. As an example, we mixed guar gum into a commercial, LBG-containing gelled food concentrate to obtain an LBG-to-guar gum ratio of 1:2. Using NMR curve fitting, we were able to determine this ratio in the polysaccharide fraction with 95% accuracy (Supplementary information S3).

Together, NMR spectroscopy enables the direct identification of the non-starch polysaccharides isolated from gelled food concentrates. In addition, ratios between mixtures of two different galactomannans can be accurately determined using NMR curve fitting procedures.

3.2.3. Discrimination of galactomannans by enzymatic fingerprinting

To confirm the data obtained by NMR spectroscopy, a second approach was chosen which is based on a method previously described by Daas et al. (2000). This method shows that galactomannans such as LBG, guar, cassia, and tara gums can be discriminated by enzymatic hydrolysis with an endo-(1,4)-b-mannanase followed by separation of the hydrolysis products by high-performance anion-exchange chromatography with pulsed-amperometric detection (HPAEC-PAD). We here apply this method to the polysaccharide fraction isolated from gelled food concentrates.

The chromatographic profiles of the 20–50 min range for the gelled food concentrate sample and four galactomannan standards are given in Fig. 4. This time range contains those chromatographic peaks that are specific for the individual galactomannans as already described by Daas et al. (2000). Specific peaks or regions are marked by arrows or dotted lines. The data demonstrate that the different galactomannans give specific enzymatic fingerprints for each of the four galactomannan standards (Fig. 4A-D). More specifically, tara gum, having a reported galactose-to-mannose ratio of 1:2.9, can be discriminated by its distinctive galactosylmannanotide peak eluting at 36.1 min and by its fingerprint pattern between 43.7 and 45.3 min (Fig. 4A, marked by arrow head and dotted line, respectively). Cassia gum (galactose-to-mannose ratio of 1:8.4) gives a high abundant mannotriose peak at 23.8 min in addition to the unique unidentified peaks at 34.8 and 39.2 min (Fig. 4B). Guar gum is the most densely substituted galactomannan in this series, with a galactose-to-mannose ratio of 1:1.5. Hence, peaks corresponding to unsubstituted or monosubstituted hydrolysis products are rather low compared to the complex, multisubstituted products. Therefore, guar gum can be discriminated by its distinctive rising baseline and complex fingerprint area between 42 and 48 min (Fig. 4C). Lastly, LBG (galactose-to-mannose ratio of 1:3.4) gives a typical fingerprint between 42 and 47 min, including a high digalactosylmannopentaose peak at 42.0 min (Fig. 4D).

The data further show that the profile obtained from the polysaccharide fraction isolated from the gelled food concentrate has the same characteristic fingerprint as the LBG standard (Fig. 4E, see in particular the region between 42 and 47 min) and is markedly different to the profiles of other galactomannan standards.

Together, the enzymatic fingerprinting method enables the discrimination between galactomannans in polysaccharide fractions isolated from complex food matrices.

3.3. Quantification of polysaccharide gums

3.3.1. Quantification and recoveries of xanthan gum

By using monosaccharide analysis we found that the isolated polysaccharide fraction contained predominantly mannone, galactose, glucose, and glucuronic acid, all of which are monosaccharide constituents that can be attributed to xanthan gum and/or LBG. In addition, galacturonic acid, arabinose, rhamnose, and xylose were detected (Table 1). The presence of rather high levels of galacturonic acid and only trace amounts of rhamnose and xylose is indicative for the presence of a homogalacturonan-type of pectin, which may be extracted from vegetable particles. Arabinose found indicates the presence of gum Arabic, which is regularly used as stabilizer for food flavours (Daquon & Abdullah, 2013). Together, the monosaccharide analysis indicates that the total carbohydrate content of the non-starch polysaccharide fractions isolated from the Knorr Wołowy product is 62%. Xanthan gum is a polysaccharide consisting of a pentasaccharide repeating unit composed of glucose, mannone, and glucuronic acid with a reported ratio of 2:2:1 (Valent, Darvill, McNeil, Roberts, & Albersheim, 1980). In contrast to glucose and mannone, glucuronic acid is a rather unique monosaccharide constituent and therefore, xanthan gum can be quantified by using the glucuronic acid content as determined by monosaccharide analysis. In addition to the carbohydrate backbone, xanthan gum also contains acetyl and pyruvyl substitution and cation counter ions (Kool, Gruppen, Sworn, & Schols, 2013; Valent et al., 1980) and therefore, the polysaccharide content of a xanthan gum standard was determined by monosaccharide analysis. In addition, incomplete methanolysis and hydrolysis, which also regularly occurs when subjecting xanthan gum to monosaccharide analysis, can be compensated by analysing such a xanthan gum standard. By comparing the calculated xanthan gum levels with theoretical data a xanthan gum recovery of on average 87 ± 16% was obtained (Table 2).

3.3.2. Development of a quantitative enzymatic fingerprinting method

LBG is a galactomannan consisting of a mannan backbone substituted by galactosyl residues in a ratio of 3.4:1. Since mannone is also present as a monosaccharide constituent in xanthan gum and NMR spectroscopy indicated the presence of yeast mannan, the galactose levels as determined by monosaccharide
analysis were taken for calculating LBG recoveries. However, an average recovery of 137% (Table 2) indicates that the polysaccharide fraction contains an additional galactose source leading to overestimation of LBG levels. By combining the data obtained by NMR spectroscopy and monosaccharide analysis, this source was identified as gum Arabic. Gum Arabic contains, in addition to arabinose, relatively large levels of galactose. Therefore, in contrast to xanthan gum quantification, monosaccharide analysis lacks the selectivity required for the correct quantification of galactomannans in complex food matrices.

For the selective quantification of LBG, we therefore took a different approach. We developed a quantitative enzymatic fingerprinting method, which is based on the method described for discriminating between different galactomannans (Daas et al., 2000). This approach enables the detection of specific hydrolysis products that are not formed from polysaccharides other than galactomannans. By constructing a calibration curve using the LBG standard, LBG can therefore be selectively quantified in gelled food concentrates, regardless of the presence of other polysaccharides such as xanthan gum, pectin, and gum Arabic.

Rather than calculating the areas of all peaks found in the enzymatic hydrolysis profile, we selected peaks which are specific for LBG, are chromatographically well resolved, and are of high intensity. We have identified four such hydrolysis products eluting in the range of 30–50 min, namely galactosylmannobiose, galactosylmannotriose, and digalactosylmannopentaose eluting at 31.7, 36.1, and 42.0 min, respectively, and an unidentified product eluting at 31.7, 36.1, and 42.0 min, respectively, and an unidentified product eluting at 44.1 min (Fig. 4). Four individual calibration curves were constructed, each showing good linearity ($r^2 = 0.996–0.999$). By calculating the LBG content in the polysaccharide fractions against each calibration curve, potential co-elution with interfering compounds is easily recognized. We found that when using the four calibration curves, the individual data were very comparable with relative standard deviations below 5%, demonstrating that each of the calibration curves can be used for quantifying LBG. By comparing the calculated data with theoretical values, recoveries of LBG from Knorr Wołowy were 70 ± 6% (Table 2).

To demonstrate the validity of our hypothesis, we prepared a non-commercial model system (EUR10153V1M1) that is very similar to the commercial gelled food concentrate. The commercial product, though, contains (meat) flavours high in gum Arabic, whereas the non-commercial product does not contain significant amounts of gum Arabic. When analysing the polysaccharide fraction obtained from the model system, monosaccharide analysis gave recoveries of on average 68%, whereas recoveries using quantitative enzymatic fingerprinting gave on average 64% (Table 2). Although recovery calculations show that some LBG is lost during the complex isolation procedure, the reproducibility of the procedure is good (<10%).

We further investigated whether LBG could still be correctly quantified in the presence of other galactomannans. When preparing 1-to-1 mixtures of LBG with the other galactomannans (tara, cassia, or guar gum), it was noted that the LBG/guar gum mixture resembled the pattern of pure LBG (Supplementary information S4). This can be explained by the fact that the absolute intensities of the guar gum peaks were much lower than those of LBG, which in turn is a direct effect from the higher galactose substitution of guar gum. Indeed, calibration curves constructed using a 1-to-1 mixture of LBG and guar gum were indistinguishable from those of pure LBG, and therefore, LBG can be correctly quantified in the presence of guar gum.

The galactomannans tara gum and cassia gum, however, are less densely galactosylated, and therefore the resulting chromatographic profiles and calibration curves of mixtures of LBG with either of these differ from those of pure LBG (Supplementary information S4). Although this has to be investigated further, we hypothesize that LBG can still be correctly quantified after determining LBG/cassia gum and LBG/tara gum ratios by NMR spectroscopy (as explained in Section 3.2.2) and preparing calibration curves of galactomannan mixtures using these ratios.

Together, we conclude that LBG can be selectively and accurately quantified in gelled food concentrates by quantitative enzymatic fingerprinting. The data further demonstrate that the isolation and analysis procedure is highly reproducible and can therefore be applied to unknown samples.

### 4. Conclusion

The current paper describes a strategy for the unambiguous identification and quantification of xanthan gum and locust bean gum from gelled food concentrates. DNA analysis by means of PCR enables very fast and selective screening of polysaccharides present and can be used to further define and specify the analysis strategy. An adequate isolation procedure giving good recoveries and reproducibilities for LBG and xanthan gum was developed.

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**Table 1**

Monosaccharide analysis of gelled food concentrate.

<table>
<thead>
<tr>
<th>Gal</th>
<th>Glc</th>
<th>Man</th>
<th>GalA</th>
<th>GlcA</th>
<th>Ara</th>
<th>Rha</th>
<th>Xyl</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.50 ± 0.04</td>
<td>1.62 ± 0.61</td>
<td>3.13 ± 0.60</td>
<td>0.10 ± 0.02</td>
<td>0.54 ± 0.10</td>
<td>0.15 ± 0.08</td>
<td>0.03 ± 0.02</td>
<td>0.09 ± 0.05</td>
</tr>
</tbody>
</table>

1 Abbreviations: Gal, galactose; Glc, glucose; Man, mannose; GalA, galacturonic acid; GlcA, glucuronic acid; Ara, arabinose; Rha, rhamnose; Xyl, xylose.

### Table 2

Recoveries and reproducibilities of xanthan gum and LBG from gelled food concentrate.1

<table>
<thead>
<tr>
<th>Xanthan gum1</th>
<th>Monosaccharide analysis2</th>
<th>Enzymatic fingerprinting</th>
</tr>
</thead>
<tbody>
<tr>
<td>Knorr Wołowy</td>
<td>Theoretical</td>
<td>0.51</td>
</tr>
<tr>
<td>EUR10153V1M1</td>
<td>Theoretical</td>
<td>0.22</td>
</tr>
</tbody>
</table>

1 Xanthan gum data were corrected for the purity of the xanthan gum standard.
2 Data represent averages ± standard deviations of four replicate isolations and are given as% (w/w). Recoveries are given in brackets.
NMR spectroscopy in combination with enzymatic fingerprinting gives direct and unambiguous identities of the polysaccharides present in the isolate. Selective and accurate quantification of LBG was obtained by quantitative enzymatic fingerprinting, whereas xanthan gum can be selectively and accurately quantified by monosaccharide analysis.

Although the method should be tested before application to other gelling systems, we hypothesize that the presented strategy in principle is suitable for the unambiguous identification and quantification of a broader range of polysaccharide gums in various food products.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.05.129.

References


