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High-performance thin-layer chromatography combined with pattern recognition techniques as tool to distinguish thickening agents

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Abstract

A simple, rapid, and accurate high-performance thin-layer chromatography (HPTLC) method was applied in combination with powerful pattern recognition techniques for differentiating thickening agents, which are mainly based on polysaccharides or biopolymers. After methanolysis, the monomeric units of the thickeners were separated by HPTLC and detected using derivatization with the aniline diphenylamine $\alpha$-phosphoric acid reagent. According to their resulting fingerprint and chemical pattern, the thickening agents studied have been classified by principal component analysis and by hierarchic cluster analysis in several groups. This newly combined approach using HPTLC fingerprints and pattern recognition techniques differentiated high similarity thickeners. Monomeric units responsible for the classification of the investigated thickener have been identified. The results showed that the HPTLC technique in combination with chemometrics can be a very reliable technique for authentication of high similarity thickening agents and can be used for a quick screening of additives in foodstuffs.

Keywords

High-performance thin-layer chromatography; HPTLC fingerprint; Pattern recognition; Thickeners; Polysaccharides; Biopolymers
1. Introduction

Biopolymers are mainly based on polysaccharides or proteins. Plant biopolymers are widely distributed in seaweeds and in terrestrial plant materials like in seeds, roots, rhizomes, tubers, hulls, piths and exudates of trees. Other sources are microorganisms (e.g., producing the thickener xanthan) and faunal biopolymers like chitin and its derivative chitosan, glycogen, gelatin and casein. Plant biopolymers possess structural properties, but they are also involved in gelling, providing viscosity, stabilizing properties and storage of energy and water. In the food, feed, cosmetics, pharmaceutical and medicine industry, polysaccharide-based biopolymers were widely used as thickening or gelling agent, stabilizer or vegetable gum. For instance, agar and pectin are added to provide a firm texture to food preparations, as for jams, puddings, soups and sauces. As hydrocolloids, polysaccharide-based biopolymers build stable gels and are used to stabilize emulsions and suspensions (Benjamin, 2012).

Regulatory authorities strictly control the approval of food additives. Chemical modifications are generally not allowed, with the exception of approved and permitted derivatives of starch, cellulose and alginate. Polysaccharide-based thickening or gelling agents usually have a similar chemical composition, and thus, reliable and fast analytical methods are required to distinguish between these additives (Benjamin, 2012; Morlock, & Gamlich, 2012). Authentication of food additives at all steps of the food production process is important for the consumer and producing industry. Recently, separation techniques such as capillary electrophoresis (Volpi, Maccari, & Linhardt, 2008), gas chromatography and high performance liquid chromatography (HPLC; Wang, & Fang, 2004) as well as structure elucidation techniques such as mass spectrometry and nuclear magnetic resonance (Dong, 2003) have been successfully applied for determination and identification of polysaccharides. Structure elucidation techniques for polysaccharide analysis are time-consuming, expensive and not suited for widespread routine application in the food industry.

With regard to the analytical methods combined in this study, i.e., high-performance thin-layer chromatography (HPTLC) and chemometrics, there exist only few reports on the use of the single techniques, but none in combination. For identification of polysaccharides using analytical methods combined with chemometrics, the polysaccharide profile from *Ganoderma* was analyzed by HPLC and unsupervised chemometrics techniques (Sun et al., 2014). Fourier-transform infrared spectroscopy (FTIR) was used in combination with a pattern recognition
technique for the analysis of thickening agents (Černá et al., 2003). Seven analytical parameters such as specific optical rotation, intrinsic viscosity, content of nitrogen, arabinose, rhamnose, galactose and uronic acids were used as variables for chemometric characterization of exudate gums and the identification of adulterated ones (Mocak et al., 1998). The first thin-layer chromatography (TLC) paper about detection and identification of sugar components was reported by Günther & Schweiger in 1968. Though TLC was recognized as simple, fast, robust, and low cost technique for the investigation of different types of polysaccharides based on their monomeric pattern, only few papers have been reported so far. The HPTLC fingerprint of hydrolyzed extracts of polysaccharides was investigated from the fruiting bodies and spores of Lingzhi (Di, Chan, Leung, & Huie, 2003). A HPTLC method has been developed to distinguish polysaccharides present in six traditional Chinese herbs after acidic hydrolysis (Yang, Guan, Zhang, & Li, 2010). Also, the HPTLC fingerprint of several industrial polysaccharides was determined on a Si 50000 stationary phase (Wards, et al., 2001). In our previous paper (Morlock, & Gamlich, 2012), a HPTLC method was developed for characterization and profiling of biopolymers used as food thickening agents, based on their monomeric pattern after extraction and methanolysis. This HPTLC method was also applied for investigation of antidiabetic polysaccharides of Ocimum basilicum seeds (Yili et al., 2014) and Apocynum venetum leaves (Shi et al., 2015). Further, HPLC, GC-MS, capillary electrophoresis and FTIR were applied for analysis of gums/hydrocolloids and modified starches in food samples such as chocolate products, cacao, fruit products, ice creams, frozen desserts as well as mayonnaise (Eliasson, 2006).

Despite of the increasing use of polysaccharide-based thickening agents in the food industry, there has been a limited number of studies regarding the determination of their authenticity so far. Thus, this study laid focus on the classification of the HPTLC fingerprints (methylated monomeric profiles) of thickeners and hydrocolloids. To the best of our knowledge, this is the first report of the combination of HPTLC fingerprints of biopolymers and pattern recognition techniques. For classifying the thickening agents according to their monomeric units, PCA and hierarchic cluster analysis (HCA) were used. The potential of this fast, low-cost and simple HPTLC method combined with chemometrics was explored for classification and identification of biopolymers, and consequently, as proof of their authenticity.
2. Materials and methods

2.1. Chemicals and materials

Ultrapure water (18 MΩ cm) was produced by Synergy System (Millipore, Schwalbach, Germany). Ethyl acetate and methanol were of technical grade (BASF, Ludwigshafen, Germany) and distilled prior to use. i-Propyl acetate, o-phosphoric acid (85%), hydrochloric acid (37%), diphenylamine (≥98%), sodium hydroxide pellets, magnesium chloride, phenolphthalein indicator (all analytical grade), D(-)-fructose (Fru, >99%), D(+)–glucose-1-hydrate (Glc, DAB), D(+)-galactose (Gal, ≥98%), D(+)–mannose (Man), L(+)–rhamnose (Rha, >99%), D(+)–xylose (Xyl, >99%), and D(+)–galacturonic acid monohydrate (GalA) and HPTLC plates silica gel 60 (20 x 10 cm) were obtained from Merck, Darmstadt, Germany. L(–)-Fucose (Fuc, >99%), D-glucuronic acid (GlcA, >97%) and acetyl chloride (> 98%) were from Fluka, Buchs, Switzerland. Aniline (≥99.9%) was purchased from Fisher Scientific, Schwerte, Germany, pyridine (≥99%) from Sigma Aldrich, St. Louis, USA, and L(+)–Arabinose (Ara, ≥99%) from Acros Organics, Geel, Belgium.

2.2. Sample preparation and standard solutions

The commercially available thickening agents used and their sample preparation were described in detail elsewhere (Morlock, & Gamlich, 2012). Sample preparation was performed according to § 64 LFGB standard method L 00.00-13 (Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinärmedizin (BgVV), 1986). Each thickener sample (10 mg) as well as sugars or uronic acids (10 mg each, 3 mg for Fuc) were dissolved in 1 mL methanolic hydrochloric acid (2 mol/L; for agar agar and carrageenan 0.5 mol/L). After methanolysis at 100 °C for 4 h, 50 µL pyridine were added for neutralization. Samples were centrifuged (3 min, 10000 x g, Biofuge, Heraeus, Thermo Fisher Scientific, Waltham, USA) if required. The supernatant was diluted 1:1 with methanol and shaken for 5 s using the vortex (step 8, ca. 3000 rpm, Vortex-Genie 2, Scientific Industries, New York, USA). For the two standard mixtures (150 ng/µL; 450 ng/µL for Fru), 30 µL (90 µL for methylated Fuc and Fru) of the respective solutions were diluted in 2 mL methanol (mixture 1: Fru, GalA, Rha, Xyl and Gal; mixture 2: GlcA, Fuc, Ara, Man and Glc).

2.3. HPTLC method
Sample volumes of 1 to 7 µL and 2, 5, 10 and 15 µL of each standard mixture were sprayed as 8-mm bands with a 8-mm distance from lower edge, 10-mm distance from the left side and 9-mm track distance using the Automatic TLC Sampler 4. Drying of the application zones (30 s), plate activity adjustment (5 min with a saturated aqueous magnesium chloride solution), development with a mixture of i-propyl acetate, ethyl acetate, methanol and water 5:4:1:0.1 (V/V/V/V) and plate drying (2 min) were performed in the Automatic Developing Chamber 2 up to a migration distance of 60 mm (from the lower plate edge). The chromatogram was automatically dipped in an aniline diphenylamine o-phosphoric acid reagent (1:1 mixture of diphenylamine and aniline solutions, both 2 % in acetone, and 10 % addition of a 85 % o-phosphoric acid) using the TLC Immersion Device (immersion time 1 s; immersion speed 3.5 cm/s) and heated at 110 °C for 5 min (TLC Plate Heater). Documentation was performed under white light illumination (transmission and reflection mode; TLC Visualizer) using winCATS software. Instrumentation used was from CAMAG, Muttenz, Switzerland.

2.4. Data acquisition and multivariate analysis

The chromatogram images were exported from winCATS software to ImageJ (1.48c version, Wayne Rasband, National Institute of Health, Bethesda, MD, USA). The image analysis procedure was described by Ristivojević et al., 2014. Data pre-treatment procedures were denoising, normalization, followed by warping/registering. Denoising of the images was done using a 3-pixels median filter. The standard normal variate procedure was performed by scaling each sample to the sum of intensity. Peak alignment was employed to correct the inter- and intra-plate peak shift due to variations in experimental conditions such as mobile phase composition, humidity, temperature, operator handling and instrumental instability. The chromatograms were warped to the reference by deleting or adding baseline segments near the selected signals using Correlation Optimized Warping (COW) to equalize the hRf values (Ristivojević et al., 2014; Wong, Razmovski-Naumovski, Li, Kong, Li, George, & Chan, 2014; Tang, et al., 2014). The data were additionally pre-processed using mean centering scaling. Each sample track was transformed by ImageJ. PCA and hierarchic cluster analysis (HCA) were performed by PLS ToolBox, v.6.2.1, for MATLAB 7.12.0 (R2011a), MathWorks, Natick, MA, USA. PCA was carried out as an exploratory data analysis by using a singular value decomposition algorithm and a 0.95 confidence level for Q and T² Hotelling limits for outliers.

3. Results and discussion
3.1. Fingerprints of thickening agents

In our previous paper (Morlock, & Gamlich, 2012), a HPTLC method was developed for profiling and distinguishing of thickening agents based on their methylated monomeric units (Table 1). Therein, the HPTLC fingerprints of plant biopolymers were described in detail. Visual examination of the HPTLC chromatograms of thickening agents after methanolysis and derivatization (Fig. 1 and Table S-1) revealed a reliable differentiation in the chemical composition between the different groups of thickening agents. These were rich in monosaccharides and some like pectins in respective sugar acids. The HPTLC pattern was dominated by gray, brown and green bands due to the selective derivatization with the aniline diphenylamine o-phosphoric acid reagent. Hydroxypropylmethylcellulose showed the most complex monomer profile, if compared to other thickening agents. Also alginates as well as gummis traganth, arabicum and karaya had a rich profile and clearly different from other samples. In contrast, guaran and carubin were only based on Man, Gal and Ara units or starch on glucose (detected as two bands due to the methylation). Though the differentiation between most thickening agent classes was clear, differences within a group were apparent. For example, two sorts of pectins were apparent. Pectin A contained GalA, Gal, while pectin formulations with a content of only 20% pectin consisted of GalA, Rha and Ara.

For a statistically supported classification and an automated differentiation of the thickening agents, the potential of multivariate data analysis was explored. ImageJ was employed, which is a Java-based freeware for digital picture manipulation such as filtering, background subtraction, and grayscale conversion. The track profile plots of the HPTLC chromatograms of the two standard mixtures (Fig. 2) and of the samples were generated. The grayscale image was chosen because of the similarity of the colors. The multivariate results obtained for the grayscale intensity showed the best separation.
3.2. Application of PCA

PCA, a commonly used multivariate technique, was employed for clustering of the thickening agents. It visualized the data based on their similarities and dissimilarity, reduced the number of dimensions into 2 or 3 and determined the most important variables responsible for differentiation between the thickening agent classes. PCA established the relation between objects (thickening agents) and variables ($h_{RF}$ values). It transformed the original data set obtained from the ImageJ software, into a new set of variables known as principal components (PCs), which were linear combinations of the original variables (Koley et al., 2014; Lazarević, Andrić, Trifković, Tešić, & Milojković-Opsenica, 2012).

In this study, PCA was performed on the data set of 48 thickening agents. The first four components described 73.99% of the total variability. The first principal component (PC1) described 43.08% of the total variability, while PC2 specified 12.80% of the total variability (Fig. 3, A). According to this 2D PC score, there were several groups of thickener according to the chemical similarity or dissimilarity. Alginic acid and its sodium, potassium, and ammonium salts formed one cluster on the lower right side of the PC score (Fig. 3, A). Sodium and potassium alginate shared the same chemical composition, which can vary in the ratio of $\beta$-D-mannuronic acid and $\alpha$-L-guluronic acid. Propylen glycol alginate (Fig. 1, track 11) as chemically modified thickener contained organic rests of propylene glycol, and thus, was positioned on the lower left side of the PC score (Fig. 3, A). One sodium alginate sample seemed to be a mixture with propylen glycol alginate (Fig. 1, track 6), though labelled as sodium alginate. This mixed sample was located between propylene glycol on the lower left side and the clustered group on the lower right side of the PC score. Agar agar and carrageen contained Gal and 3,6-anhydroGal as monomeric units, and formed mutually clusters on the upper right side of the PC score (Fig. 3, A).

In case of integrating PC4 (Fig. 3, B), the 3D score plot of the three principal components PC1, PC2, and PC4 visually showed a differentiation between xanthan, guaran and carubin, although guaran and carubin contained the same monomeric units (Man, Gal and Ara) and showed almost the same HPTLC pattern. Further, in the case of guaran, the two lower bands are similar in intensity because guaran contains one Man molecule at every second Gal moiety, whereas carubin contains one Man molecule on every fourth Gal moiety.
Starch and derivatives of cellulose formed one cluster in the left, lower middle, except for hydroxypropylmethylcellulose, which had the complex fingerprint and formed a subgroup on the upper middle of the 3D PC score (Fig. 3, B). There was a good separation between two sorts of pectin along the PC3 direction; one sort of pectin was composed of Gal A and Gal, while the second was of Gal A, Rha and Ara. The three gummis (gummi karaya, arabicum and tragant) showed a different pattern each (due to the different monomers such as GalA, Rha, Fuc, Ara, Xyl and Gal) and thus were positioned separately, more on the centre and left middle on the PC score (Fig. 3, B).

The loading plot revealed the most influential monomeric units, discriminating best between the thickening agents. Gal was the substantial one which led to the separation of alginic acid and its salts from other samples, since it showed a high positive impact alongside the PC1 direction. Our results recommended Gal as markers for the differentiation between alginic acid/alginate and other thickening agents. PC1 was negatively contributed by Rha, GlcA and Fru (Fig. 3, C). These variables are potential markers to distinguish thickening agents positioned on the left side of the PC score (Fig. 3, A). Further, Gal, GlcA and Fru had the highest positive impact along the PC2 direction, while Rha, Ara and monosacharides with hR value 6 had a negative impact along the PC2 direction (Fig. 3, D). These variables were suggested as the most influential in distinguishing pectin, xanthan, guaran and carubin from carrageen, agar agar and alginates. Monosaccharides such as Gal, Man, Fru, Xyl, Rha and GlcA significantly contributed to the differentiation along the PC4 direction (Fig. 3, E). These variables were recognized as discrimination factor for starch and cellulose from other samples. Also, Man as a monomeric unit of guaran, carubin and xanthan could be a potential marker for discrimination between these samples and pectin, alginate, starch and cellulose-based thickening agents.

### 3.3 Application of HCA

The HCA is another commonly used pattern recognition technique. Initially, the HCA method considers each sample as an independent group, i.e. there are n groups. Then, the two closest points merge into a new group. The distance between the new group and the other n 2 groups (samples) is then calculated as previously; the closest two groups are merged into another new group. The process continues until all observations are clustered into one group. Finally, the results are displayed as a dendrogram. Then, a decision rule is used to determine the number of
clusters and subclusters. There are several methods for hierarchical clustering, such as the single and complete linkage methods. In this paper, the Euclidean distance was chosen as the measure of similarity, and the Ward method was applied for the clustering algorithm (Morlock, Ristivojević, & Chernetsova, 2014; Roshan et al., 2013). At a 60% similarity level, there are two clusters (Fig. 4). One cluster contained alginate and alginic acid, guaran and carubin as well as derivates of cellulose. The second cluster was formed by the other thickening agents, such as pectin, carrageen and agar agar. The results obtained by HCA (Fig. 4) were in accordance with the results obtained by PCA (Fig. 3). At a 50% similarity level, the first subcluster consisted of alginate and alginic acid due to the same monomer units (ManA and GulA), which was also evident from PCA. Guaran and carubin consisted of Gal, Man and Ara, and formed the second subcluster, while glucose polymers (starch, microcrystalline cellulose and Na-carboxymethylcellulose) formed one subcluster. Hydroxypropylmethylcellulose, chemically different from natural cellulose, formed one separated subcluster (Fig. 4). The third cluster of derivates of Gal and 3,6-anhydroGal, showed a good separation between agar agar and carrageen despite their very similar chemical composition. Pectin samples were quite similar because they contained the same monomeric units. Interestingly, one of the three xanthan samples was separately grouped near to starch, which however, showed a very similar pattern to xanthan (Fig. 1, track 30 versus 44), most likely due to small variations in the hRf value or signal intensity. Hence, despite the increasing extent of automatic processes, the reflection of the analyst is still needed, especially for such special cases.

4. Conclusions
HPTLC in combination with pattern recognition techniques as a relatively new approach showed potential for a fast, simple, comprehensive and effective determination of the authenticity and quality of thickening agents. Pattern recognition techniques, such as PCA and HCA, showed a good discrimination between structurally similar thickening agents. Gal was recognized as marker for differentiation between alginate and other thickening agents, whereas Rha, GlcA, and Fru were potential markers to distinguish xanthan and gummi tragant from other thickening agents. Ara, Rha, Gal, GlcA and Fru were found most influential in distinguishing Na-carboxymethylcellulose, pectin A and gummi tragant from other thickening agents. Man was recognized as potential marker for distinguishing guaran, carubin, and xanthan from pectin,
alginates, starch and cellulose-based thickening agents. HCA allowed to distinguish thickening agents with the same chemical composition such as agar agar and carrageen. This confirmed the potential of HPTLC fingerprints in combination with multivariate tools to support the classification and authentication of thickening agents and the identification of adulterants of biopolymers. The described technique is also capable for determination of the authenticity of thickening agents in complex food products, which is focus of another study.

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Appendix A. Supplementary data

Supplementary data associated with this article (Table S-1) can be found in the online version at http://dx.doi.org/...
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Table 1
Overview on the $hR_F$ values of the methylated monosaccharides and sugar acids in both standard mixtures (mix 1 and mix 2).

<table>
<thead>
<tr>
<th>No.</th>
<th>Methylated monomeric unit</th>
<th>$hR_F$ values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mix 1</td>
</tr>
<tr>
<td>1</td>
<td>Galactose (Gal)</td>
<td>13</td>
</tr>
<tr>
<td>2</td>
<td>Glucose (Glc)</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>Mannose (Man)</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>Arabinose (Ara)</td>
<td>25</td>
</tr>
<tr>
<td>5</td>
<td>Fucose (Fuc)</td>
<td>31</td>
</tr>
<tr>
<td>6</td>
<td>Xylose (Xyl)</td>
<td>34</td>
</tr>
<tr>
<td>7</td>
<td>Rhamnose (Rha)</td>
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</tr>
<tr>
<td>8</td>
<td>Galacturonic acid (GalA)</td>
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</tr>
<tr>
<td>9</td>
<td>Glucuronic acid (GlcA)</td>
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</tr>
<tr>
<td>10</td>
<td>Fructose (Fru)</td>
<td>95</td>
</tr>
</tbody>
</table>
Highlights

- Rapid and reliable classification of different thickening agents
- Potential markers identified for distinguishing of thickening agents
- Characteristic HPTLC fingerprints of thickening agents analyzed by chemometrics
- Planar chromatographic profiling combined with pattern recognition techniques
- HPTLC separation and derivatization of the methylated monomeric units