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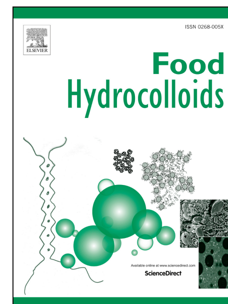

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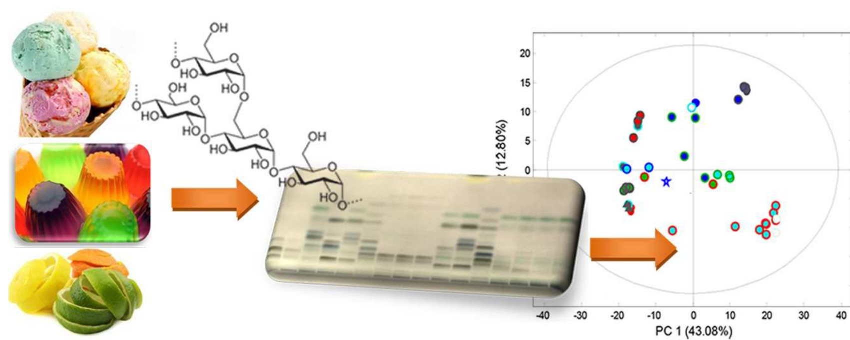

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High-performance thin-layer chromatography combined with pattern recognition techniques as tool to distinguish thickening agents

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24 **Abstract**

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

37

38 **Keywords**

39 High-performance thin-layer chromatography; HPTLC fingerprint; Pattern recognition;
40 Thickeners; Polysaccharides; Biopolymers

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41 # 1. Introduction

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

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

66 With regard to the analytical methods combined in this study, *i. e.* high-performance thin-layer
67 chromatography (HPTLC) and chemometrics, there exist only few reports on the use of the
68 single techniques, but none in combination. For identification of polysaccharides using analytical
69 methods combined with chemometrics, the polysaccharide profile from *Ganoderma* was
70 analyzed by HPLC and unsupervised chemometrics techniques (Sun *et al.*, 2014). Fourier-
71 transform infrared spectroscopy (FTIR) was used in combination with a pattern recognition

72 technique for the analysis of thickening agents (Černá *et al.*, 2003). Seven analytical parameters
73 such as specific optical rotation, intrinsic viscosity, content of nitrogen, arabinose, rhamnose,
74 galactose and uronic acids were used as variables for chemometric characterization of exudate
75 gums and the identification of adulterated ones (Mocak *et al.*, 1998). The first thin-layer
76 chromatography (TLC) paper about detection and identification of sugar components was
77 reported by Günther & Schweiger in 1968. Though TLC was recognized as simple, fast, robust,
78 and low cost technique for the investigation of different types of polysaccharides based on their
79 monomeric pattern, only few papers have been reported so far. The HPTLC fingerprint of
80 hydrolyzed extracts of polysaccharides was investigated from the fruiting bodies and spores of
81 Lingzhi (Di, Chan, Leung, & Huie, 2003). A HPTLC method has been developed to distinguish
82 polysaccharides present in six traditional Chinese herbs after acidic hydrolysis (Yang, Guan,
83 Zhang, & Li, 2010). Also, the HPTLC fingerprint of several industrial polysaccharides was
84 determined on a Si 50000 stationary phase (Wards, *et al.*, 2001). In our previous paper (Morlock,
85 & Gamlich, 2012), a HPTLC method was developed for characterization and profiling of
86 biopolymers used as food thickening agents, based on their monomeric pattern after extraction
87 and methanolysis. This HPTLC method was also applied for investigation of antidiabetic
88 polysaccharides of *Ocimum basilicum* seeds (Yili *et al.*, 2014) and *Apocynum venetum* leaves
89 (Shi *et al.*, 2015). Further, HPLC, GC-MS, capillary electrophoresis and FTIR were applied for
90 analysis of gums/hydrocolloids and modified starches in food samples such as chocolate
91 products, cacao, fruit products, ice creams, frozen desserts as well as mayonnaise (Eliasson,
92 2006).
93 Despite of the increasing use of polysaccharide-based thickening agents in the food industry,
94 there has been a limited number of studies regarding the determination of their authenticity so
95 far. Thus, this study laid focus on the classification of the HPTLC fingerprints (methylated
96 monomeric profiles) of thickeners and hydrocolloids. To the best of our knowledge, this is the
97 first report of the combination of HPTLC fingerprints of biopolymers and pattern recognition
98 techniques. For classifying the thickening agents according to their monomeric units, PCA and
99 hierarchic cluster analysis (HCA) were used. The potential of this fast, low-cost and simple
100 HPTLC method combined with chemometrics was explored for classification and identification
101 of biopolymers, and consequently, as proof of their authenticity.
102

103 # 2. Materials and methods

104 ## 2.1. Chemicals and materials

105 Ultrapure water (18 MΩ cm) was produced by Synergy System (Millipore, Schwalbach,
106 Germany). Ethyl acetate and methanol were of technical grade (BASF, Ludwigshafen, Germany)
107 and distilled prior to use. *i*-Propyl acetate, *o*-phosphoric acid (85%), hydrochloric acid (37%),
108 diphenylamine (≥98%), sodium hydroxide pellets, magnesium chloride, phenolphthalein
109 indicator (all analytical grade), D(–)-fructose (Fru, >99%), D(+)-glucose-1-hydrate (Glc, DAB),
110 D(+)-galactose (Gal, ≥98%), D(+)-mannose (Man), L(+)-rhamnose (Rha, >99%), D(+)-xylose
111 (Xyl, >99%), and D(+)-galacturonic acid monohydrate (GalA) and HPTLC plates silica gel 60
112 (20 x 10 cm) were obtained from Merck, Darmstadt, Germany. L(–)-Fucose (Fuc, >99%), D-
113 glucuronic acid (GlcA, >97%) and acetyl chloride (>98%) were from Fluka, Buchs, Switzerland.
114 Aniline (≥99.9%) was purchased from Fisher Scientific, Schwerte, Germany, pyridine (≥99%)
115 from Sigma Aldrich, St. Louis, USA, and L(+)-Arabinose (Ara, ≥99%) from Acros Organics,
116 Geel, Belgium.

117

118 ## 2.2. Sample preparation and standard solutions

119 The commercially available thickening agents used and their sample preparation were described
120 in detail elsewhere (Morlock, & Gamlich, 2012). Sample preparation was performed according
121 to § 64 LFGB standard method L 00.00-13 (Bundesinstitut für gesundheitlichen
122 Verbraucherschutz und Veterinärmedizin (BgVV), 1986). Each thickener sample (10 mg) as well
123 as sugars or uronic acids (10 mg each, 3 mg for Fuc) were dissolved in 1 mL methanolic
124 hydrochloric acid (2 mol/L; for agar agar and carrageenan 0.5 mol/L). After methanolysis at 100
125 °C for 4 h, 50 µL pyridine were added for neutralization. Samples were centrifuged (3 min,
126 10000 x g, Biofuge, Heraeus, Thermo Fisher Scientific, Waltham, USA) if required. The
127 supernatant was diluted 1:1 with methanol and shaken for 5 s using the vortex (step 8, ca. 3000
128 rpm, Vortex-Genie 2, Scientific Industries, New York, USA). For the two standard mixtures (150
129 ng/µL; 450 ng/µL for Fru), 30 µL (90 µL for methylated Fuc and Fru) of the respective solutions
130 were diluted in 2 mL methanol (mixture 1: Fru, GalA, Rha, Xyl and Gal; mixture 2: GlcA, Fuc,
131 Ara, Man and Glc).

132

133 ## 2.3. HPTLC method

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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 hR_F 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 T^2 Hotelling limits for outliers.

3. Results and discussion

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166 **3.1. Fingerprints of thickening agents**

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

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

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191 **3.2. Application of PCA**

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

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

215 In case of integrating *PC*4 (Fig. 3, B), the 3D score plot of the three principal components *PC*1,
216 *PC*2, and *PC*4 visually showed a differentiation between xanthan, guaran and carubin, although
217 guaran and carubin contained the same monomeric units (Man, Gal and Ara) and showed almost
218 the same HPTLC pattern. Further, in the case of guaran, the two lower bands are similar in
219 intensity because guaran contains one Man molecule at every second Gal moiety, whereas
220 carubin contains one Man molecule on every fourth Gal moiety.

221 Starch and derivatives of cellulose formed one cluster in the left, lower middle, except for
222 hydroxypropylmethylcellulose, which had the complex fingerprint and formed a subgroup on the
223 upper middle of the 3D PC score (Fig. 3, B). There was a good separation between two sorts of
224 pectin along the *PC*3 direction; one sort of pectin was composed of Gal A and Gal, while the
225 second was of Gal A, Rha and Ara. The three gummis (gummi karaya, arabicum and traganth)
226 showed a different pattern each (due to the different monomers such as GalA, Rha, Fuc, Ara, Xyl
227 and Gal) and thus were positioned seperately, more on the centre and left middle on the PC score
228 (Fig. 3, B).
229 The loading plot revealed the most influential monomeric units, discriminating best between the
230 thickening agents. Gal was the substantial one which led to the separation of alginic acid and its
231 salts from other samples, since it showed a high positive impact alongside the *PC*1 direction. Our
232 results recommended Gal as markers for the differentiation between alginic acid/aliginate and
233 other thickening agents. *PC*1 was negatively contributed by Rha, GlcA and Fru (Fig. 3, C).
234 These variables are potential markers to distinguish thickening agents positioned on the left side
235 of the PC score (Fig. 3, A). Further, Gal, GlcA and Fru had the highest positive impact along the
236 *PC*2 direction, while Rha, Ara and monosacharides with hR_F value 6 had a negative impact along
237 the *PC*2 direction (Fig. 3, D). These variables were suggested as the most influential in
238 distinguishing pectin, xanthan, guaran and carubin from carrageen, agar agar and alginates.
239 Monosaccharides such as Gal, Man, Fru, Xyl, Rha and GlcA significantly contributed to the
240 differentiation along the *PC*4 direction (Fig. 3, E). These variables were recognized as
241 discrimination factor for starch and cellulose from other samples. Also, Man as a monomeric unit
242 of guaran, carubin and xanthan could be a potential marker for discrimination between these
243 samples and pectin, alginate, starch and cellulose-based thickening agents.
244

245 **3.3 Application of HCA**

246 The HCA is another commonly used pattern recognition technique. Initially, the HCA method
247 considers each sample as an independent group, *i. e.* there are n groups. Then, the two closest
248 points merge into a new group. The distance between the new group and the other n 2 groups
249 (samples) is then calculated as previously; the closest two groups are merged into another new
250 group. The process continues until all observations are clustered into one group. Finally, the
251 results are displayed as a dendrogram. Then, a decision rule is used to determine the number of

252 clusters and subclusters. There are several methods for hierarchical clustering, such as the single
253 and complete linkage methods. In this paper, the Euclidean distance was chosen as the measure
254 of similarity, and the Ward method was applied for the clustering algorithm (Morlock,
255 Ristivojević, & Chernetsova, 2014; Roshan *et al.*, 2013). At a 60% similarity level, there are two
256 clusters (Fig. 4). One cluster contained alginate and alginic acid, guaran and carubin as well as
257 derivates of cellulose. The second cluster was formed by the other thickening agents, such as
258 pectin, carrageen and agar agar. The results obtained by HCA (Fig. 4) were in accordance with
259 the results obtained by PCA (Fig. 3). At a 50% similarity level, the first subcluster consisted of
260 alginate and alginic acid due to the same monomer units (ManA and GulA), which was also
261 evident from PCA. Guaran and carubin consisted of Gal, Man and Ara, and formed the second
262 subcluster, while glucose polymers (starch, microcrystalline cellulose and Na-
263 carboxymethylcellulose) formed one subcluster. Hydroxypropylmethylcellulose, chemically
264 different from natural cellulose, formed one separated subcluster (Fig. 4). The third cluster of
265 derivates of Gal and 3,6-anhydroGal, showed a good separation between agar agar and carrageen
266 despite their very similar chemical composition. Pectin samples were quite similar because they
267 contained the same monomeric units. Interestingly, one of the three xanthan samples was
268 separately grouped near to starch, which however, showed a very similar pattern to xanthan (Fig.
269 1, track 30 *versus* 44), most likely due to small variations in the hR_F value or signal intensity.
270 Hence, despite the increasing extent of automatic processes, the reflection of the analyst is still
271 needed, especially for such special cases.
272

273 ## 4. Conclusions

274 HPTLC in combination with pattern recognition techniques as a relatively new approach showed
275 potential for a fast, simple, comprehensive and effective determination of the authenticity and
276 quality of thickening agents. Pattern recognition techniques, such as PCA and HCA, showed a
277 good discrimination between structurally similar thickening agents. Gal was recognized as
278 marker for differentiation between aliginate and other thickening agents, whereas Rha, GlcA,
279 and Fru were potential markers to distinguish xanthan and gummi traganth from other thickening
280 agents. Ara, Rha, Gal, GlcA and Fru were found most influential in distinguishing Na-
281 carboxymethylcellulose, pectin A and gummi traganth from other thickening agents. Man was
282 recognized as potential marker for distinguishing guaran, carubin, and xanthan from pectin,

283 aliginate, starch and cellulose-based thickening agents. HCA allowed to distinguish thickening
284 agents with the same chemical composition such as agar agar and carrageen. This confirmed the
285 potential of HPTLC fingerprints in combination with multivariate tools to support the
286 classification and authentication of thickening agents and the identification of adulterants of
287 biopolymers. The described technique is also capable for determination of the authenticity of
288 thickening agents in complex food products, which is focus of another study.

289

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293

294 **Appendix A. Supplementary data**
295 Supplementary data associated with this article (Table S-1) can be found in the online version at
296 http://dx.doi.org/...

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368 **Table 1**

369 Overview on the hR_F values of the methylated monosaccharides and sugar acids in both standard

370 mixtures (mix 1 and mix 2).

No.	Methylated monomeric unit	hR_F values	
		Mix 1	Mix 2
1	Galactose (Gal)	13	
2	Glucose (Glc)		15
3	Mannose (Man)		20
4	Arabinose (Ara)		25
5	Fucose (Fuc)		31
6	Xylose (Xyl)	34	
7	Rhamnose (Rha)	51	
8	Galacturonic acid (GalA)	60	
9	Glucuronic acid (GlcA)		67
10	Fructose (Fru)	95	

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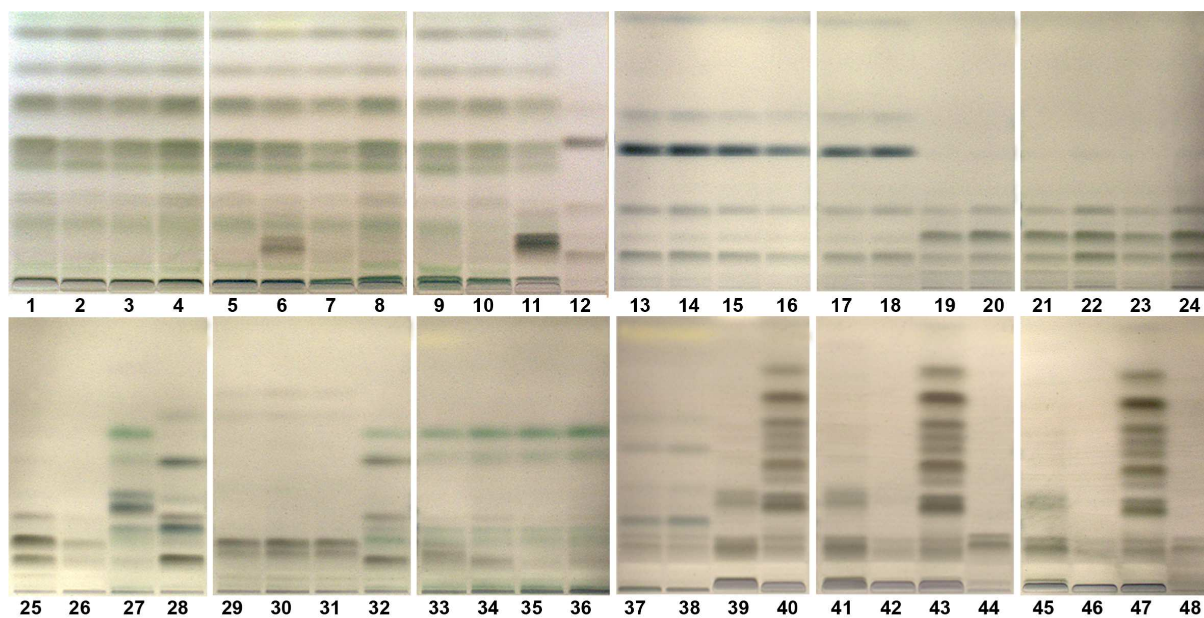

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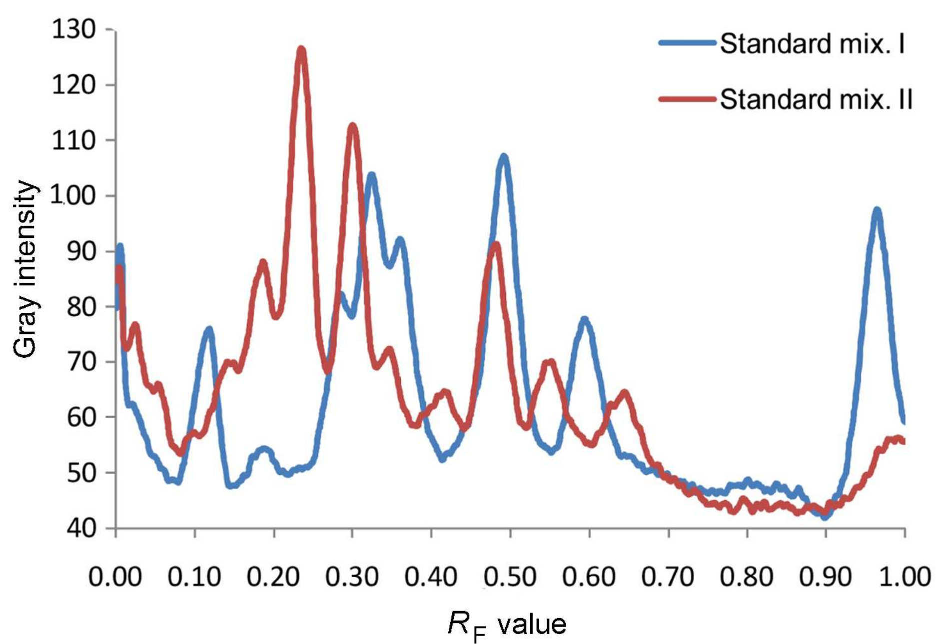

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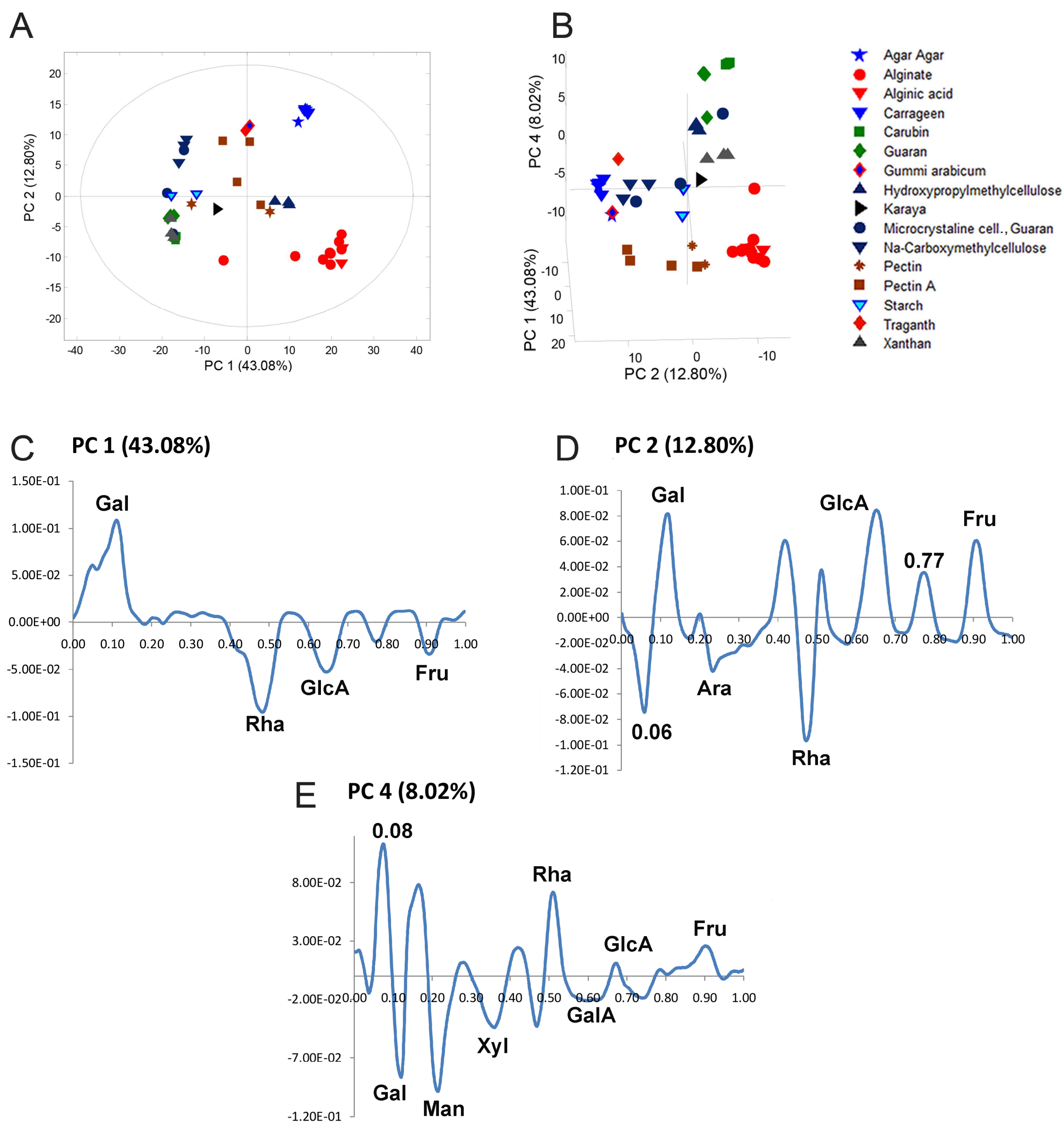

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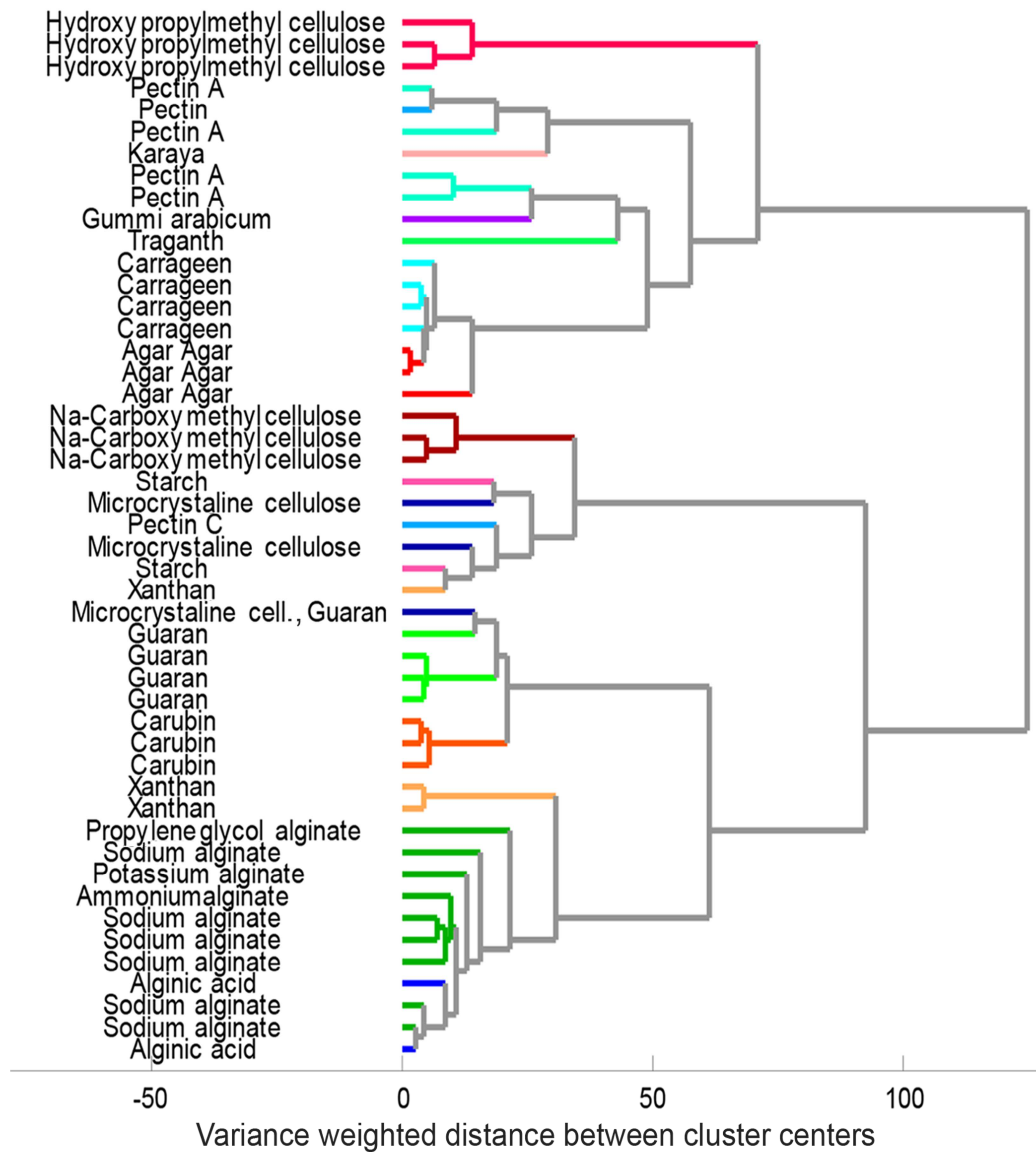

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Highlights

- 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

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