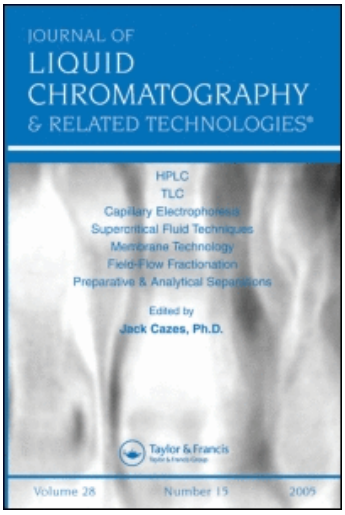

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Solid Phase Extraction of Oligo- and Polysaccharides; Application to Maltodextrins and Honey Qualitative Analysis

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Abstract: Oligosaccharides and polysaccharides are frequently found in complex matrices such as food products, foodstuffs, cosmetics or pharmaceutical preparations. Prior sugar analysis, such samples require selective extraction and/or purification.

Two methods are proposed and compared using test mixtures such as maltodextrins and commercial sugar syrup. Procedures include solid phase extraction and liquid chromatography with an evaporative light scattering detector. The choice of the system is highly dependent on the degree of polymerisation of the analytes. Aminopropyl bonded phases were the best choice for solid phase extraction and chromatography of oligosaccharides having a degree of polymerization higher than 4. Besides, apolar phases used for extraction, as well as for chromatography, were revealed to be well adapted for polysaccharides analyses. Both methods, having recoveries up to 90%, were applied to acacia honey and it was shown that oligosaccharides detection can be easily performed. Besides, trace analysis of polysaccharides in honey was also possible using a high enrichment factor. Polysaccharides presence was confirmed by Maldi-TOF mass spectrometry.

Keywords: Column liquid chromatography, Solid phase extraction, Carbohydrates, Sugar syrup, Maltodextrins and honey

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INTRODUCTION

Liquid chromatography (LC) is a method of choice for sugars analysis.[1–4] High performance hydrophilic interaction chromatography (HILIC)[2] and reversed phase LC[3] are mainly used with the evaporative light scattering detector (ELSD). Hydrophilic interaction systems operate well with amino-propyl bonded phase and hydro-organic mobile phase. High selectivities and resolutions can be obtained. However, column life time is short when amino bonded silica is used.[5] This is due to phase hydrolysis. Amino bonded polymeric phase was revealed to be much more stable.[6] Reversed-phase systems have more chemical stability than amino bonded silica, but anomer resolution of sugars occurs. Comparison of these chromatographic systems for sugar analysis was discussed previously.[1,6,7] However, both systems having different retention mechanisms can be potentially also used in solid phase extraction (SPE) procedures. The presence of interferences leads to elaborate sample preparation such as SPE before analysis. For sugar analysis, SPE was mainly used in cleanup mode using apolar[8,9] or ion exchange phases[10] to remove interferences. In these conditions, concentration of solutes (mono-, di-, and trisaccharides) is not possible, these solutes having too low retention. Graphitised carbon was also used as stationary phase for fractionation and purification of sugars (dextrans) with the degree of polymerisation (DP) up to 20.[11] Only one work reported the use of some amino phases for sugars retention in a SPE method.[12] The major monosaccharides and sucrose in tobacco were successfully isolated with recovery values from 40 to 91% in the $g \cdot kg^{-1}$ range using home made bonded silica. The main objective of this work was to compare hydrophilic interaction and reversed-phase SPE in the trace enrichment mode for some oligosaccharides and polysaccharides in commercial test mixtures, such as sugar syrups or maltodextrins. These compounds are frequently found in food products, foodstuffs, cosmetics or pharmaceutical preparations and can also be used as a low cost product for adulteration in high quality products. In the last part of this work, the SPE methods will be applied to a complex sample such as honey in which such solutes were not really studied before. Actually, only low DP oligosaccharides were studied in previous works[13,14] and the maximum DP value found in honey was 4.

Besides, the analytical systems will also be first and briefly considered, since SPE results will be based on LC qualitative and quantitative analyses, especially SPE recoveries.

EXPERIMENTAL

The solutes including glucose, fructose, maltose, maltotriose, maltoheptaose (all used for peak identification) and maltodextrin mixture (DP 13–17) were purchased from Sigma-Aldrich (St Quentin Fallavier, France) and

used as received. Sugar syrup was an industrial sample of corn syrup. Solvents were of HPLC grade (Baker, Phillipsburg, NJ, USA).

The analyses were made using an HP1050 system from Agilent Technologies (Walbronn, Germany) and an ELSD (Sedere, Vitry/Seine, France). ELSD settings were temperature 45°C and pressure 2.5 bars.

Sample Preparation

Solutes and saccharides mixtures were dissolved in water (Table 1) or hydro-organic mixture as described in Tables 2–4, and stocked refrigerated. Prior to injection, samples were filtered on 0.45 μm syringe filters. SPE was performed using a 12 port vacuum manifold; flow rate was approximately 3 $mL \cdot min^{-1}$. The cartridges packed with amino phase were: Strata NH_2 (500 mg/6 mL) (Phenomenex, LePecq, France), Bond Elut NH_2 (500 mg/3 mL) (Varian, Walnut Creek, CA USA), Isolute NH_2 (500 mg/6 mL) (Interchim, Montluçon, France). The cartridges packed with apolar phase were - 3 bonded silicas - Lichrolut C_{18} (500 mg/3 mL) (Merck, Darmstadt, Germany), DSC C_{18} (500 mg/3 mL) (Supelco, Belfonte, USA), Bond Elut C_{18} (500 mg/3 mL) (Varian), - 3 polymeric phases - ENVI-ChromP (500 mg/6 mL) (Supelco), Strata X (500 mg/6 mL) (Phenomenex), Atoll (500 mg/6 mL) (Interchim) – and a graphitized carbon - ENVI-Carb (500 mg/6 mL) (Supelco).

Reversed-Phase LC

The column was Omnispher C_{18} (Varian) (250 × 4.6 mm, 5 μm). The mobile phase gradient was from water-methanol 1% and methanol. The gradient profile was: $t = 0–15$ min 0% methanol, $t = 15–45$ min linear increase to 100% methanol. The flow rate was 1 $mL \cdot min^{-1}$ and the injection volume 200 μL.

HILIC

The column was Luna NH_2 (Phenomenex) (150 × 2 mm, 3 μm). The mobile phase was from water and acetonitrile. The gradient profile was: $t = 0–3$ min 20% water, $t = 3–7$ min linear increase to 35% water, $t = 7–45$ min 35% water, $t = 45–70$ min linear increase to 80% water. The flow rate was 0.2 $mL \cdot min^{-1}$ and the injection volume 3 or 5 μL.

Mass Spectrometry

MALDI-TOF was performed using Voyager-DE STR (Perseptive). The Sample matrix was sinapinic acid. The Laser was N_2, 337 nm.

Table 1. Calibration for recovery estimation in SPE. Calibration curves were made using log-log coordinates: $\log Area = slope \log C + intercept$ where *Area* is the peak area of solutes of interest and *C* is the total concentration of the mixture

LC system	Mixture and oncentration (*C*) range ($g \cdot L^{-1}$)	Solute of interest[a]	Calibration coefficients			Expected concentration after SPE for 100% recovery[c] ($g \cdot L^{-1}$)
			Slope	*Intercept*	R^2	
HILIC	Sugar syrup 10–20	Maltose	2.36 ± 0.04	1.43 ± 0.05	0.9981	16.7
		Maltotriose	2.05 ± 0.03	1.18 ± 0.04	0.9982	16.7
		Oligosaccharides[b] $\left(\sum \text{DP } 4\text{–}12\right)$	2.71 ± 0.05	0.03 ± 0.06	0.9976	16.7
	Maltodextrin 1–10	Polysaccharides	0.512 ± 0.005	3.719 ±0.004	0.9993	5.0
Reversed phase	Sugar syrup 1–10	Polysaccharides	1.75 ± 0.02	2.36 ± 0.01	0.9993	6.33
	Maltodextrin 10–75	Polysaccharides	1.71 ± 0.02	1.31 ± 0.03	0.9994	50.0

[a]The *Area* ($\mu V \cdot s$) was measured from peak of solute of interest.

[b]*Area* was the sum of peaks corresponding to DP 4–12.

[c]Expected value of extract concentration if SPE recovery of solute of interest is 100% using concentrations and protocols in Tables 2–4.

Table 2. Protocol for hydrophilic interaction SPE with acetonitrile-water used for oligosaccharides extraction

Step	Solvents
Condition/equilibrate	5 mL water then 5 mL acetonitrile-water (80:20)
Sample loading	100 mL sample (sugar syrup, honey 0.5 $g \cdot L^{-1}$) in acetonitrile-water (80:20) mixture (50 mg loaded)
Wash	3 mL acetonitrile-water (80:20)
Elution	3 mL water (for honey: evaporation to dryness and dissolution in 0.3 mL water)

Table 3. Protocol for hydrophilic interaction SPE with methanol-water used for polysaccharides extraction

Step	Solvents
Condition/equilibrate	5 mL water then 5 mL methanol-water (80:20)
Sample loading	300 mL sample (maltodextrins 0.05 $g \cdot L^{-1}$) in methanol-water (80:20) mixture (15 mg loaded)
Wash	3 mL methanol-water (80:20)
Elution	3 mL water

SPE Recovery Calculation

Because pure standards of most of oligo- and polysaccharides are not commercially available, or very expensive, recoveries were estimated using raw samples at various concentrations (Table 1). Because of the concentration factor in SPE, higher concentrations of oligo- or polysaccharides were required

Table 4. Protocol for reversed-phase SPE used for polysaccharides extraction

Step	Solvents
Condition/equilibrate	5 mL methanol then 5 mL water
Sample loading	Maltodextrins: 20 mL sample (10 or 0.24 $g \cdot L^{-1}$) in water (200 mg or 4.8 mg loaded) Sugar syrup: 25 mL sample (0.76 $g \cdot L^{-1}$) in water (19 mg loaded) Honey: 250 mL sample (40 $g \cdot L^{-1}$) in water (10 000 mg loaded)
Wash	9 mL water, allow to dry few minutes, then 5 mL water
Elution	6 mL methanol-water (30:70) Evaporation to dryness and dissolution in 4 mL (maltodextrins 10 $g \cdot L^{-1}$) or 3 mL (others) water

for calibration in comparison with extracted samples. The expected concentrations of SPE extract (assuming that recovery is 100%) were within the studied range concentration. Calibration curve coefficients are reported in Table 1. Because of the detector used, calibration curves were made using log-log coordinates.[6,7] The repeatability of area for both SPE and LC analyses was lower than 5%.

RESULTS AND DISCUSSION

Maltodextrins and sugar syrup were used as test mixtures and LC analysis will first be considered to characterise these raw samples. Their qualitative composition is well known and they both contain oligo- and polysaccharides, which monomer is mainly $\alpha(1-4)$ D-glucose. The maltodextrin mixture was chosen because it contains mainly polysaccharides and traces of oligosaccharides, since the opposite is true for sugar syrup mixture. The determination of such solute behaviours and trace enrichment is important before their analyses in complex sample such as honey.

LC Analysis Using ELSD

HILIC (Figure 1a) and reversed-phase (Figure 2a) analyses of the same maltodextrin mixture were performed using ELSD. Polysaccharides resolution in

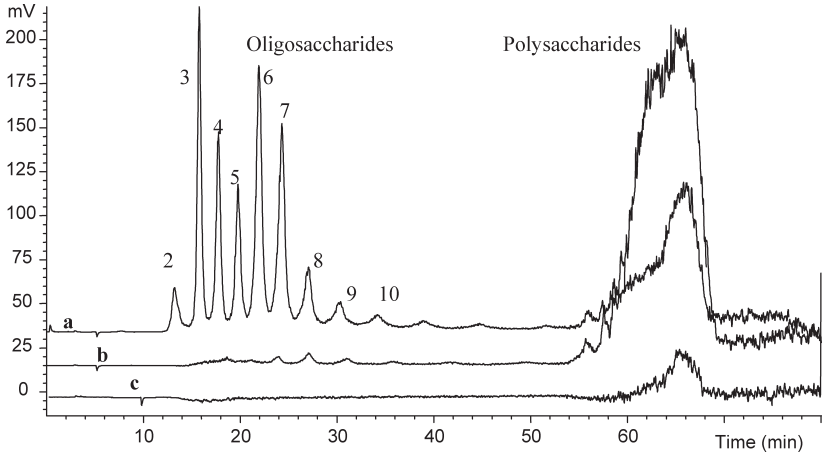

Figure 1. HILIC chromatograms of the maltodextrin mixture : raw sample ($10\ g \cdot L^{-1}$) (a); SPE extract using Envi-ChromP cartridge and methanol-water (sample $10\ g \cdot L^{-1}$, Table 4) (b); and SPE extract using Strata NH_2 cartridge and methanol-water (sample $0.05\ g \cdot L^{-1}$, Table 3) (c); LC column Luna NH_2; other conditions: see Experimental. Oligosaccharides peak labels are DP values as in other figures.

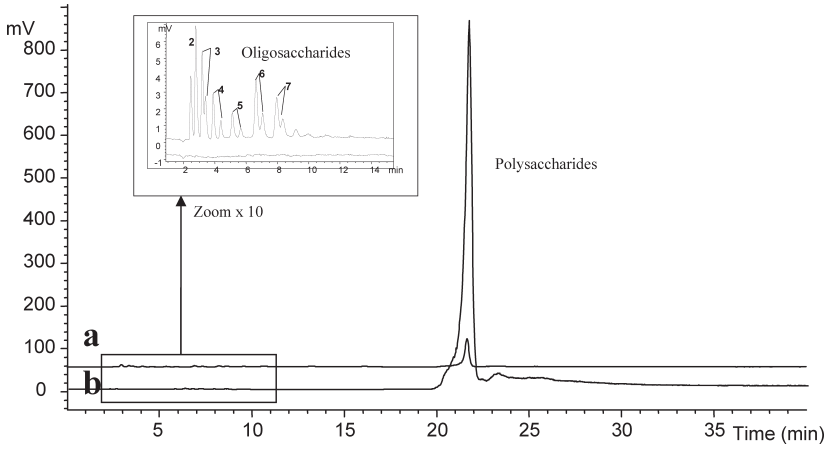

Figure 2. Reversed-phase LC of maltodextrin mixture ($0.24\ g \cdot L^{-1}$): raw sample (a) and SPE extract (b); SPE was made using Envi-ChromP cartridge (Table 4). LC column Omnispher C_{18}; other conditions: see Experimental.

individual DP was not required and they were eluted as a single and large peak. This allows simple area integration.

Using standard amino bonded silica, short column life times are noted because of the phase hydrolysis.[1] This phenomenon occurs especially for polysaccharides analysis because a high water content in the mobile phase is required[5] and fatal baseline drift and noise appeared. However, as a result, high quality silica bonded phase such as Luna NH_2, recently introduced, can be much more stable in the presence of pure water mobile phase. No or low baseline drift is observed using gradient elution and ELSD, compared to using other costly polymeric amino phases. Only a moderate increase in the noise level is observed (Figures 1a and 3a) with very high water content in the mobile phase in comparison with standard bonded amino silica. Repeatability on retention time was better than 2%, but it can be also noted that during a long period of use (1 year), a shift in retention time can be noted (Figures 1 and 3). However, separation and detection of oligomeric and/or polymeric sugar mixture are possible using gradient elution with no shift during a few weeks.

The commercial maltodextrin sample used was claimed to have an average DP of 13–17. Obviously, these DP values seem to be the minor components of the mixture and two distributions are obtained (Figure 1a). Since for these oligomeric compounds, the logarithm of retention factor is proportional to DP, peak identification was easy, especially in the isocratic window, e.g., from 7 to 45 min in Figure 1. The first distribution corresponds to DP 2–8 and the second one to polysaccharides (DP $>$ 12). Clearly, mean DP is not convenient to characterise such samples.

Chromatography fingerprints can be potentially used for distribution determination. This is especially true if a mass detector such as ELSD is

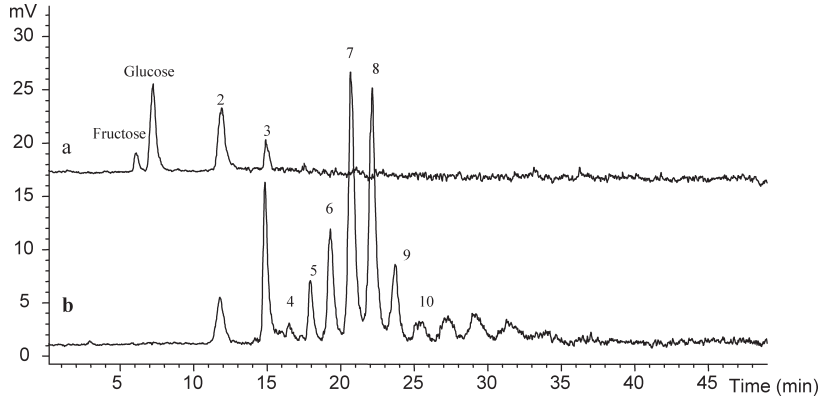

Figure 3. HILIC chromatograms of sugar syrup ($0.5\ g \cdot L^{-1}$): raw sample (a); and SPE extract (b); SPE was made using Strata NH_2 cartridge with acetonitrile-water (Table 2). LC column: Luna NH_2; other conditions: see Experimental.

used. Under isocratic elution, ELSD response in logarithmic scale is quite linear as a function of injected mass for a family of solutes such as neutral sugars.[7] Despite many authors claims that calibration is not necessary, this is often not true under gradient elution. The same maltodextrin sample was injected onto the HILIC and reversed-phase LC (Figures 1a and 2a). As a result, if we compare relative intensities of oligo- (DP $<$ 12) and polysaccharide peaks under gradient elution, the former is much higher in HILIC system (Figure 1a) and the latter is higher in reversed-phase LC (Figure 2a). For example, the maltotriose response was 36 fold higher in HILIC than in reversed-phase LC for the same injected quantity in the conditions used. It can be noted, that there is no direct correlation between the two LC systems as far as quantitative results are concerned, because retention and peak width are quite different. Variations in the relative response of various solutes are due especially to nebulisation yield variation of ELSD, which is more important with organic solvent than with water. Besides, nebulisation is also dependent on many parameters, such as density or viscosity of the mobile phase which affect droplet formation.[7] Using maltodextrins standards, the slope of calibration curves has been claimed[6] to be quite similar for DP6 (slope 1.5247) in comparison with DP 10 (slope 1.5034) for example. Much more variations are observed using other detections, such as refractometry, pulsed amperometry or mass spectrometry.[7] However, LC fingerprints can not be used for real distribution characterisation using LC-ELSD for a large distribution, such as mixtures containing both oligo- and polysaccharides. Consequently, quantitative analyses of maltodextrins and sugar syrup were not performed in this work.

On one hand, HILIC system is well adapted for oligosaccharide analysis for which enhanced detection level is obtained in comparison with

polysaccharides response and, besides, each oligosaccharide is eluted as a single peak. On the other hand, unfortunately reversed-phase LC provides anomers resolution (each oligosaccharide is eluted as a doublet, Figure 2a). This last system is the best choice for polysaccharide analysis, since the ELSD response is higher for this class of solute. However, both hydrophilic interaction and reversed-phase systems will be tested for SPE.

Hydrophilic Interaction SPE Using Acetonitrile-Water and Methanol-Water

Amino bonded SPE phase was tested in acetonitrile-water media using sugar syrup in order to extract minor oligosaccharides from major monosaccharides (glucose and fructose). Acetonitrile-water was first chosen because it operates well in LC. Acetonitrile/water, 80/20, was the optimum composition for sample loading, an increase of acetonitrile content provides better recovery (especially DP 2–3) but lower solubility of sugar syrup. Maltodextrin mixture was not tested for extraction in such conditions, mainly because the solubility of polysaccharides in acetonitrile-water was too low. The SPE method was applied to sugar syrup (Table 2) and chromatograms are reported in Figures 3a and 3b. The three brands of SPE stationary phase (see experimental) gave similar results in term of oligosaccharides recovery.

Clearly, monosaccharides are not extracted; di- and trisaccharides are partially extracted. DP 2 and DP 3 recoveries were 9 and 25%, respectively. Oligosaccharides (DP 4–12) are well retained on the amino phase, since recovery was approximately 100%. This protocol can be applied to complex samples to obtain trace enrichment of oligosaccharides.

Methanol-water was used as an alternative to acetonitrile-water in SPE to overcome the solubility problem of polysaccharides in the maltodextrin mixture. Using the protocol as in Table 3, trace enrichment of polysaccharides was more effective than in acetonitrile-water media. In such conditions, monosaccharides and oligosaccharides (DP < 12) were not retained (Figure 1c) since recovery of polysaccharides was approximately 25%.

Because sample loading can be made using pure water, reversed-phase SPE will be more appropriate to polysaccharide extraction in term of solute solubility.

Reversed-Phase SPE Using Methanol-Water

Various stationary phases were tested for sugar extraction using the protocol as in Table 4. Results were highly dependent on the sorbent. Recoveries of polysaccharides in maltodextrins were from 12 to 65% using various octadecyl bonded silica (Lichrolut C_{18}: $12 \pm 1\%$, DSC C_{18}: $65 \pm 3\%$, Bond Elut C_{18}: $56 \pm 2\%$), from 15 to 95% using various polymeric phase (ENVI-ChromP: $95 \pm 4\%$, Strata X: $83 \pm 4\%$, Atoll: $15 \pm 2\%$), and 3% using graphitic

carbon. No correlation is obtained between recovery and category of the SPE stationary phase. Better results were obtained onto Envi-ChromP in terms of recovery value (Figure 2). Besides, oligosaccharides from sugar syrup or maltodextrins having DP lower than 7 were not retained; since those having $7 > DP > 12$ were slightly retained (Figure 1b). It can be noted that the polysaccharide peak is higher in the extract (Figure 1b) than in the raw sample (Figure 1a) because of the enrichment factor, which was 5 (Table 4).

As far as sugar syrup is concerned, a moderate enrichment factor equal to 8 (Table 4), allowed the easy detection of the polysaccharides fraction (Figure 4). Recovery was approximately 91%. Polysaccharide detection was possible only if reversed-phase LC was used; indeed polysaccharides in the EnviChromP extract were not detected using the HILIC system. This was due to the lower response of the ELSD as discussed previously.

Application to Honey Analysis

Many works concerned the analysis of major sugars in honeys using liquid chromatography coupled to a refractometric detector,[15,16] ion chromatography,[17–19] gas chromatography coupled to a flame-ionization,[20,21] or a mass spectrometer.[22,23] Only the presence of monosaccharides, disaccharides, and rarely some oligosaccharides of low DP ($DP \leq 4$), were considered in honey.

However, sugar syrup can be used for honey adulteration. The presence of the oligosaccharides ($DP > 4$) naturally present in natural honey or/and coming from addition of syrup was not studied up till today. It will be now possible using SPE on amino phase and HILIC. Using conditions in Table 2, acacia honey analysis is reported in Figure 5a. Clearly, the presence of DP3, DP4, and probably DP5, can be detected in the sample using comparison

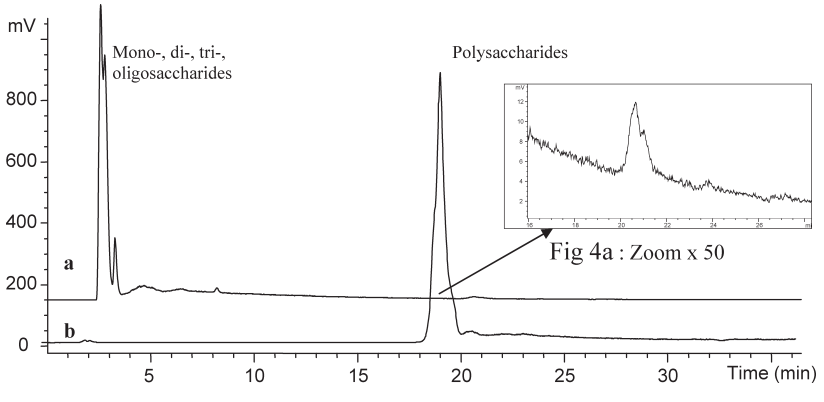

Figure 4. Reversed-phase LC of sugar syrup (0.76 g · L^{-1}): raw sample (a) and SPE extract (b); SPE was made using Envi-ChromP cartridge (Table 4). LC column Omnispher C_{18}; other conditions: see Experimental.

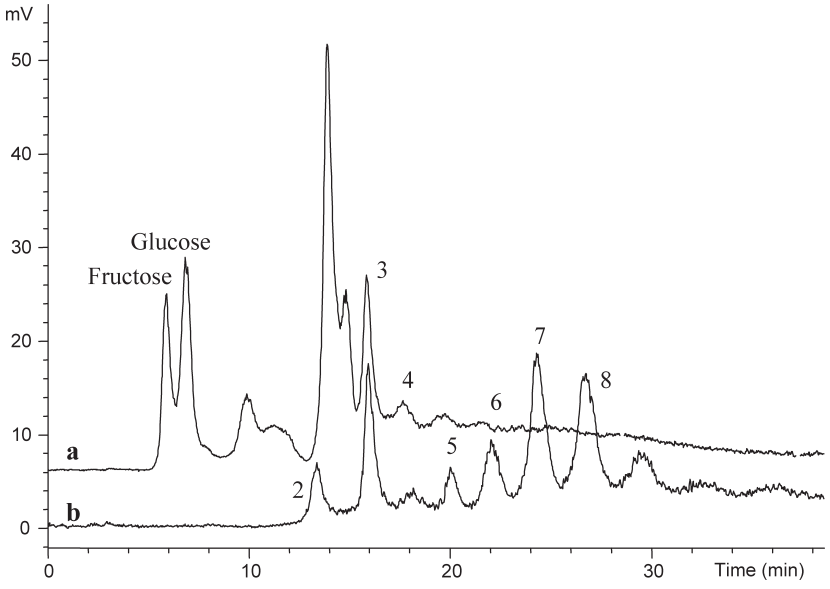

Figure 5. HILIC chromatogram of SPE extract acacia honey (a); and sugar syrup extract (b); SPE was made using Strata NH_2 cartridge and acetonitrile-water (Table 2). LC column Luna NH_2; other conditions: see Experimental.

with sugar syrup analysis (Figure 5b). These oligosaccharides could be from endogenous or exogenous (adulteration) origins. More studies have to be made. Besides, quantitative analysis has to be studied and the occurrence of endogenous oligosaccharides having DP $\geq$ 4 has to be also considered as a function of the floral origins.

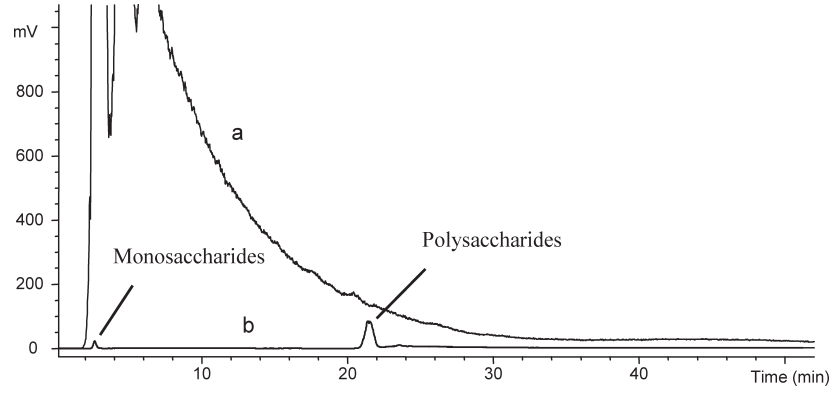

Figure 6. Reversed-phase LC of acacia honey: raw sample (a); and SPE extract (b); SPE was made using Envi-ChromP cartridge (Table 4). LC column: Omnispher C_{18}; other conditions : see Experimental.

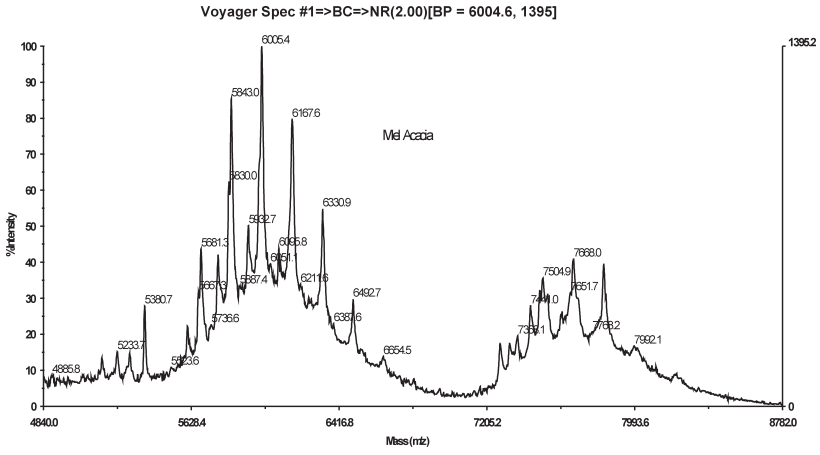

Figure 7. MALDI-TOF spectra of acacia honey extract. SPE was made using Envi-ChromP cartridge as in Figure 6b and Table 4.

Indeed, honey characterisation, e.g., by floral species is also an important challenge. To the best of our knowledge, no previous studies were focused on polysaccharides (DP $>$ 10) in honey. Figure 6 shows the analysis of 40 $g \cdot L^{-1}$ acacia honey and an extract obtained using reversed-phase SPE and LC. It corresponds to the analysis of water-methanol extract using SPE on ENVI-Chrom P phase (Table 4). High quantities of honey (10 g) can be loaded because of the high glucose and fructose concentration (more than 85% of the dry matter) which are not retained on the cartridge. Besides, because of the important quantity of sample loaded on the SPE cartridge, low levels of monosaccharides still remain in the extract (Figure 6b). Clearly, polysaccharides were not detected without SPE (Figure 6a) but the enrichment factor up to 80 allows their detection in the SPE extract (Figure 6b). This was confirmed by mass spectrometry analysis with a MALDI-TOF system of the honey extract (Figure 7). In fact, the spectrum highlights peak differences of 162 mass units, which are characteristic of the saccharidic sequence. Polysaccharides can be present in honey; concentration should be in the $mg \cdot kg^{-1}$ range. However, further studies have to be conducted to better understand their occurrences and origins and to provide quantitative results.

CONCLUSIONS

Thanks to SPE concentration, oligosaccharides and polysaccharides detections as minor components offer new potentialities for some food samples analysis. The proposed method uses only SPE and simple LC-ELSD apparatus commonly available in laboratories.

The study carried out on various commercial SPE cartridges allows determination of the most appropriate sorbent for sugars retention according to their degree of polymerization. This was highly relevant in reversed-phase SPE. An extraction recovery of the oligo- or polysaccharides over 90% can be obtained. Hydrophilic interaction systems for both SPE and LC are suitable for analysis of oligosaccharides. Since much lower restriction in solubility occurs, reversed-phase SPE and reversed-phase LC offer an efficient method for trace analysis of polysaccharides. Moreover, ELSD response is higher using the HILIC system for oligosaccharides, since it is higher in reversed-phase LC for polysaccharides.

The analysis of an acacia honey allows highlighting the presence of polysaccharides. Honey is a natural product of high quality and is the target of many frauds. For example, low cost sugar syrups, which can be difficult to detect at low concentration, can be deliberately added. The combination of SPE and LC-ELSD can bring new solutions for qualitative and quantitative analyses of oligo- and polysaccharides and new prospects in the quality control of honeys.

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