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Highly sensitive, quick and simple quantification method for mono and disaccharides in aqueous media using liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry (LC–APCI–MS)

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ABSTRACT

A highly sensitive, rapid LC–APCI–MS method for identification and quantification of mono and disaccharides in simple or complex aqueous phase has been developed. This original method is easy to use, no derivation and no post-column injection are needed. The separation is performed with a hydrophilic amino interaction (HILIC) column allowing high-throughput analysis with analysis times of 15 min for monosaccharides to 22 min for disaccharides. The development of the method carried out with 9 standard saccharides allowed to point out a dynamic range from 0.1–25.6 to 1–256 $\mu g\,mL^{-1}$ depending on the considered sugar. Next, the method was validated on saccharides at known concentrations in water and on 2 real samples: orange juice and aqueous phase obtained after enzymatic hydrolysis of sunflower seeds.

1. Introduction

The fractionation hydrolysis of plant biomass in general and lignocellulosic in particular is an important research topic today. In the near future, projects developed by this way can increase the production yields of bio-ethyl alcohol [1–4], or can obtain proteins or peptides with biological activities of interest for the pharmaceutical or agro-industrial domain among various applications [5]. The biological hydrolysis of the biomass can be realized on the whole seed with an insufficient control of the hydrolysis, by using micro-organisms acting directly on the substrate. Or, it can be specific and controlled, with the use of purified or partially purified enzymes, in order to control the degradation of cell walls. These processes still need to be optimized and improved. Research in the field of enzyme processes is based on two strategies: reducing the cost of enzymatic hydrolysis with innovative enzymatic systems and improving the bioconversion of glucose in ethanol. Researchers working on enzymatic systems often consider mono or disaccharide production as a good way to evaluate the degree of biomass hydrolysis [6]. Depending on the project purposes, different sorts of analysis can be realized from biochemical methods for the total sugars or reducing sugars content [7,8] to separative methods for individual sugar quantification. The analysis of monosaccharides has always presented a double challenge: to find the appropriate column and detector. On the one hand, the traditional separation on reverse phase (RP) is not suitable for this analysis. Indeed C_{18} columns, which are practical and easy to use, are not able to separate each monosaccharide. Moreover, no simple separative method is reported to be highly reliable and robust for all the saccharides studied. On the other hand, as monosaccharides are devoid of chromophores it is impossible to use UV–vis detector which is the ideal detector in terms of sensitivity, linearity and reproducibility. These two constraints have necessitated the development of new strategies. GC/FID methods provide a good separation of monosaccharides and a good sensitivity, but require prior steps of reduction and derivation which are very time consuming (>100 min). However, this solution does not resolve all of the problems: the alditol acetate derivation does not separate glucose from fructose and TMS derivation forms 4 compounds for each monosaccharide in the solution [9,10]. For 20 years, LC-methods have been developed and have provided real improvements for quickness and user convenience. This has been made possible by advances in the domain of the separation phases (ionic, HILIC) and detectors, which are increasingly sensitive. Few methods have been developed to perform sugar analysis without derivation. LC equipped with refractometric detector and separation based on amino interactions provide a good linearity of the response, but only for high concentrations (from 0.01 to 70 $g\,mL^{-1}$). Moreover, this method has a poor sensitivity, is inconsistent with separation methods using gradients and is highly sensitive to the flow rate variation and the universality of the detector does not work on complex matrices [11,12]. Anionic separation coupled with a

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pulsed amperometric detector (PAD) improves the sensitivity (until 10 μg L^{-1}) and the specificity because only the oxidable compounds at a given potential can be observed. Unfortunately, this method often requires post-column addition of NaOH at high concentration leading to the formation of anionic compounds in order to improve sensitivity (column can not perform at pH > 9) [13,14]. Using the very sensitive and semi-universal evaporative light scattering detector (ELSD) and charge aerosol detector (CAD) associated with HILIC separation has provided more flexibility and simplicity of implementation since no derivation is needed [15]. It is interesting to note that methods requiring derivation lead to good results with the use of RP separation combined with UV–vis detector. These methods employ 2-aminopyridine (AP), 4-aminobenzoicacid ethyl ester (ABEE) and 1-phenyl-3-methyl-5-pyrazolone (PMP) as well as its methoxy analogue (PMPMP) [16–19] for derivation.

Over the past decade, the development of LC/MS methods dedicated to the analysis of sugars and monosaccharides in particular, has led to significant advances in terms of sensitivity and specificity while maintaining speed and simplicity of implementation. Sugar ionization in atmospheric pressure ionization (API) type sources represented the main challenge because of their low efficiency of ionization in negative mode directly related to their low acidic character. For example, we can notice a LC/MS–MS method in which three monosaccharides were studied by following their pseudo-molecular ion $[M-H]^-$ [20]. The strategy of using adducts has been extensively studied to increase the ionization rate, and many techniques are also reported in the literature. Often used with a HILIC separation which mobile phases (water and acetonitrile) are fully compatible with API ionization, the various LC/MS existing methods can be classified by type of formed adduct, polarity and ion mode. We can find numerous methods using electrospray ionization (ESI) operating in negative ion mode in the way to follow adducts formed with acetate [21], halogens (Cl^-, Br^-, I^-) [22–25] and also nitrate, sulfate or phosphate ions [24]. Operating in positive ion mode, ESI can be used to follow sugar adducts formed with metallic ions (Li^+, Na^+, K^+, Cs^+) [26] or ammonium ions [27]. The atmospheric pressure chemical ionization (APCI) is sometimes used in negative ion mode, these methods mainly follow chlorine adducts [28,29].

Note that most LC/MS methods reported here require post-column addition of a suitable solution to obtain the wanted adducts with the exception of the ammonium adduct in ESI positive [27] and chlorine adduct used in APCI negative [28,29].

In this paper, we describe and analyze a HPLC/MS method able to give to researchers a quick and powerful method, for the qualitative and quantitative analysis of a large pool of mono and disaccharides in complex matrices. The method presented requires no derivation step and no post-column addition thus allowing analysis of a large scale of samples through a rapid and simple procedure.

2. Experimental

2.1. Chemicals

The standard sugar samples used for the assays were rhamnose, galactose, fructose and inositol acquired from Merck (Darmstadt, DE), arabinose and xylose acquired from Acros Organics (Geel, BE) and fucose (from Sigma, Lyon, FR), mannose (from Extrasynthèse, Genay, FR), glucose (from VWR, Fontenay-sous-Bois, FR), cellobiose (from Serva, Heidelberg, DE) and sucrose (from Fisher Scientific Labosi, Elancourt, FR). Standard sugar samples were prepared at 1 g L^{-1} in deionized water. All stock solutions and calibration standards were prepared in water and kept at −18 °C until use. Courtonne Reagent is a lead (II) acetate trihydrate (purchased from Sigma, Lyon, FR) solution at 300 g L^{-1} used for partial purification of samples. Acetonitrile and chloroform were provided from Carlo Erba Réactifs (Val de Reuil, FR).

2.2. Samples preparation

Three types of real samples were used for testing this method. The first is a commercial orange juice (low number of saccharides in a simple matrix), the second is a solution of sugars to a known concentration, and the third is an aqueous phase obtained after an enzymatic hydrolysis of sunflower seeds (high number of saccharides in a complex matrix). The enzymatic hydrolysis was performed on crushed seeds, with a seeds/water ratio of 1/1.5 (w/v), an enzyme/seed ratio of 5% (v/w) over 4 h at 50 °C. After reaction, the mixture was centrifuged (15,000 × *g*, 20 °C) in order to obtain the aqueous phase. The enzymatic cocktail used was a multiactivity mixture of cellulases and hemicellulases (Depol 40, Biocatalysts, UK). Both activities are able to produce monomers and oligomers of sugars. After the hydrolysis step, a rapid purification was realized with Courtonne reagent able to precipitate soluble proteins in order to preserve the life time of the column. 1.5 mL of reagent and 5 mL of the sample are mixed and placed in an ice bath for 5 min. Then, Na_2HPO_4 is added to eliminate the excess of lead acetate. The sample is centrifuged at 9000 rpm during 10 min at 20 °C. Supernatants were adjusted to 25 mL with distilled water. An aliquot was filtered through 0.22 μm syringe filter into auto sample vials and loaded into the Thermo Electron auto injector.

2.3. Instrumentation and chromatographic conditions

The mobile phases used for the separation consisted of 100% distilled water (pump A) and 99% acetonitrile with 1% of chloroform (pump B). The samples were analyzed in isocratic mode with a sample injection volume constant at 20 μL and the mobile phase flow rate at 1 mL min^{-1} with 75% acetonitrile over 30 min.

The analytical column is a YMC-Pack Polyamine II (250 mm × 4.6 mm) composed of a polymeric support (silica base) with mixed secondary and tertiary amino derivative (particles size are 5 μm and pores size are 12 nm). The pre-column is a 7.5 mm × 4.6 mm cartridge. Both were obtained from YMC Europe GmbH (Dinslaken, DE).

The HPLC/MS system is composed of the quaternary solvent delivery pump and linear ion trap mass spectrometer (LTQ-MS, Thermo Electron Corporation, Waltham, MA, USA). LTQ is equipped with an atmospheric pressure chemical ionization (APCI) interface operating in negative ion mode. Xcalibur 2.0 SR2 software is used for computer control and data process. The operational parameters of the MS were: discharge current = 4 μA; temperature of the heated capillary: 225 °C, APCI vaporizer 400 °C; flow rate of sheath gas, auxiliary gas and sweep gas were set to 50, 12, 20 in arbitrary units respectively; capillary voltage was −13 V. The mass setup for the sugars detection were performed in order to follow two isotopes $[M+Cl]^-$ ions ($[M+35]^-$ and $[M+37]^-$): for rhamnose and fucose: $[M+35]^-$ = 199 and $[M+37]^-$ = 201; for xylose and arabinose: $[M+35]^-$ = 185 and $[M+37]^-$ = 187; fructose, mannose, glucose, galactose and inositol: $[M+35]^-$ = 215 and $[M+37]^-$ = 217; sucrose and cellobiose: $[M+35]^-$ = 377 and $[M+37]^-$ = 379. For this study, the detection was performed in full scan mode (with *m*/*z* measured from 100 to 500) and in SIM mode.

2.4. External calibration

External calibrations were developed by analyzing each saccharide at various concentrations depending on the responses of each sugars (Table 1). Nine concentrations were tested. They allowed us to determine the limits of detection and quantification of each

Table 1
Concentration of sugars ($\mu g\,mL^{-1}$) used for the external and internal calibrations.

Dilution	Rha[a]	Xyl	Fru	Glc	Cel	Fuc	Ara	Man	Gal	Suc
C1[b]	0.4	1.0	0.1	0.2	1.0	0.2	1.0	1.0	1.0	0.2
C2	0.8	2.0	0.2	0.4	2.0	0.4	2.0	2.0	2.0	0.4
C3	1.6	4.0	0.4	0.8	4.0	0.8	4.0	4.0	4.0	0.8
C4	3.2	8.0	0.8	1.6	8.0	1.6	8.0	8.0	8.0	1.6
C5	6.4	16.0	1.6	3.2	16.0	3.2	16.0	16.0	16.0	3.2
C6	12.8	32.0	3.2	6.4	32.0	6.4	32.0	32.0	32.0	6.4
C7	25.6	64.0	6.4	12.8	64.0	12.8	64.0	64.0	64.0	12.8
C8	51.2	128.0	12.8	25.6	128.0	25.6	128.0	128.0	128.0	25.6
C9	102.4	256.0	25.6	51.2	256.0	51.2	256.0	256.0	256.0	51.2

[a] Rha, rhamnose; Xyl, xylose; Fru, fructose; Glc, glucose; Cel, cellobiose; Fuc, fucose; Ara, arabinose; Man, mannose; Gal, galactose; Suc, sucrose.
[b] C1 is based on the LOD of each saccharide ($\mu g\,mL^{-1}$).

mono or disaccharide. Calibration curves were obtained by plotting area versus detected concentrations in the linearity range.

2.5. Internal calibration

Internal calibration was realized with inositol at 0.01 mg mL^{-1} as internal standard for each sample tested. All sugars samples were quantified daily with inositol. Reproducibility is measured with daily variations of inositol area response. Repeatability is measured with the daily variation of the average of area variation. The linearity of the response was observed by plotting monosaccharide concentrations versus the monosaccharide/inositol ratio.

3. Results and discussion

3.1. Separation and MS method optimization

3.1.1. Separation

The HPLC method was developed so as to achieve one of the best possible separation of various sugars of interest i.e. eight monosaccharides (rhamnose, fucose, xylose, arabinose, fructose, mannose, galactose, glucose) and two disaccharides (sucrose, cellobiose).

At this stage, compound detection was realized through an evaporative light scattering detector (ELSD) which represents a good alternative in the case of compounds that do not respond in the UV–vis. This detector provides high degree of sensitivity. In comparison, the detection by refractometry (RI) conventionally used for the analysis of sugars did not seem appropriate because of the weak sensitivities obtained and the constraint to perform the analysis in isocratic mode.

We selected columns acting with hydrophilic amino interaction (HILIC) as they are thought to be the best option on the market to solve our problem. We focused on the relative selectivity for the ten studied sugars. Moreover, the mobile phases water and acetonitrile used in this type of chromatography are fully compatible with both DEDL and MS detection, which is a prerequisite for the subsequent development of the LC/MS method.

In so doing, we wanted to test the robustness of the HILIC columns tested. We know that the column grafted with amines are likely to generate, during any analysis, the formation of Schiff bases by nucleophilic attack of an amine on the carbonyl group of sugar. Amine groups engaged in this type of covalent bond being no more able to interact with the free sugars, we can expect a fairly rapid deterioration of the chromatographic conditions. So ELSD, and its high sensitivity, has been a major contribution to our study because it has led, via the injection of very small amounts of sugar, to maintain the performance of the column by minimizing the formation of Schiff bases. In this way, we expect to maintain the performance of the separation as long as possible.

Due to the complexity of separating several stereo and geo isomer groups (rhamnose/fucose, xylose/arabinose, mannose/glucose/galactose), the maintenance of selectivity over a long period was an important condition to select an efficient column.

A first test was done on a silica base column with a classical grafting of primary amines. Correct selectivity was first observed as only two sugars among the ten standards we tested were not separable (rhamnose and fucose). Unfortunately, we have observed a quite rapid degradation of sensitivity and of separative performances. We immediately related these results with the presupposed problem of the formation of Schiff bases. We had to face the fact that this type of column with an amine I grafting was not adapted to our study at all.

At this step, it is necessary to use other types of columns. A Zwitteranionic column (ZIC-HILIC) could be an alternative. This column can performed sugars analysis in the same conditions, and avoid the formation of Schiff bases. The selectivity obtained with this column seems to be efficient in separating mono- di- and tri-saccharides. Unfortunately it is not sufficiently effective to guarantee a reliable selectivity for a large number of monosaccharides, which is one of the main goals of the method we were developing.

For our second test, we chose a silica-based column – whose specificity comes from the stationary phase-covered by a covalently grafted polymer film containing secondary and tertiary amine groups. That is why we chose a specific column that was only grafted with amine groups II and III (less nucleophile) in order to avoid or to minimize the formation of Schiff bases and to maintain the same separation with constant sensitivity. Moreover the retention time of monosaccharides gives a good selectivity [30,31].

The Polyamine II column (YMC) has provided a good separation of most standards of sugars while retaining a good stability.

As shown in Fig. 1, all standards, except glucose and galactose, are well separated. Retention times (Rt) vary from 6.50 min for rhamnose to 19.00 min for cellobiose. First, rhamnose and fucose are detected with m/z = 199 and 201, at 6.50 and 7.20 min respectively, just before xylose (8.00 min) and arabinose (8.40 min) with m/z = 185 and 187. Next, followed with m/z = 215 and 217, fructose, mannose and glucose (Rt 9.85, 10.35 and 11.45 min respectively), inositol (m/z = 215 and 217) is detected between sucrose and cellobiose standard (m/z = 377 and 379) at 17.45 min. Sucrose RT is 15.50 min and cellobiose RT is 19.00 min. The stereochemical isomerization of glucose and galactose (position of –OH group in C4) does not fully explain their co-elution: xylose and arabinose are differentiated by the same stereochemical isomerization and they are separated enough to allow qualitative and quantitative study.

3.1.2. MS method

For the development of the MS method, we decided to work in negative ion mode because of the larger specificity expected in comparison with the positive ion mode (in relation with the complexity of our sample matrix). We first tested the efficiency of acetate adducts via the introduction of a solution of 0.2 M ammonium acetate with a post-column addition at a flow

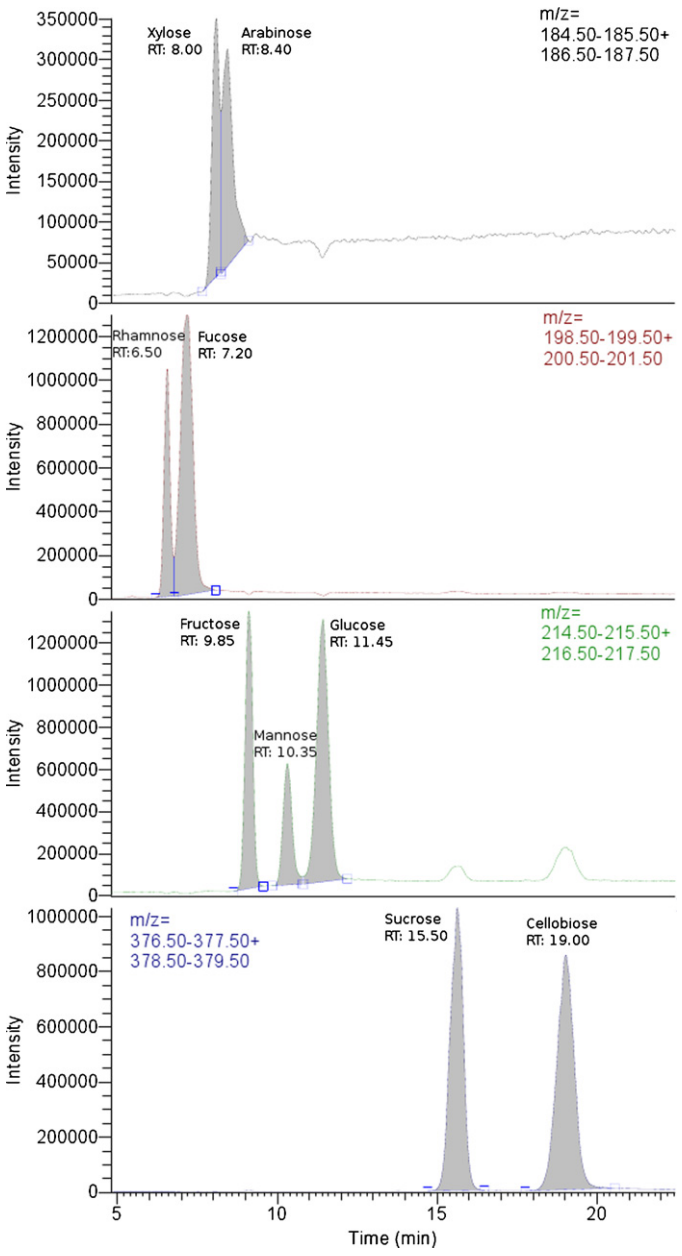

Fig. 1. Representative separation chromatogram obtained for standard sugars concentrations at 1 g L^{-1} with a volume of injection of 20 μL. Separation is performed on a YMC-Pack Polyamine II (250 mm × 4.6 mm), chromatograms are obtained by following chlorine adducts $[M+35]^-$ and $[M+37]^-$.

rate of 3 μL min^{-1} in order to maintain a constant concentration $C = 0.001$ M in the API source. This technique allows a good qualitative analysis but two major drawbacks appear. A lot of characteristic ions are obtained in addition to the acetate adduct preferentially expected i.e. the pseudo molecular ion $(M-H)^-$, the TFA adduct $(M+113)^-$, but also chlorinated adducts $(M+35)^-$ and $(M+37)^-$ and other unidentified adducts. TFA adducts can be easily explained, since trifluoroacetic acid, which is widely used as an additive in many HPLC methods, is very persistent in the LC device. Regarding the chlorine adducts, we can assert that the chlorine ions come from the column itself and not from the LCMS system because this phenomenon has only been seen under these specific conditions. The presence of these undesired Cl^- ions was probably the cause of an undesirable effects materialized by salt deposition (NaCl) at the entrance of the mass detector, which leads to a partial jamming of the capillary, hindering useful quantitative results. In order to increase the predominance of acetate adducts among the characteristic ions, the concentration of ammonium acetate solution was gradually increased. Unfortunately, no significant results were observed and therefore we kept the initial concentration of 0.2 M in the injection syringe.

The LC–ESI–MS method that was initially developed has provided good results concerning the sensitivity of the sugars of interest (LOD range), which first enabled us to have a qualitative approach to our samples. From a quantitative point of view, the method has not been effectively exploited because we noticed a poor linearity and also a very poor repeatability of the measured areas in the external range. The use of an internal calibration standard (inositol) did not significantly improve the results. Besides, the internal dynamic range is far too small, and associated with a poor repeatability of the response.

At this stage we have decided to turn around the previously described problem (unwanted presence of chloride ions in the system) by exploiting the chlorinated adducts $[M+35]^-$ and $[M+37]^-$ previously observed. To do this, we first determined the concentration of chlorine ions by doping acetonitrile phase with chlorine additive, and also opted for APCI ionization in order to avoid the salt deposit, at the entrance of the capillary, observed in ESI. In practice we used chloroform as an additive and its concentration in phase B (1%) was optimized to obtain the best possible response in APCI operating in negative ion mode ($[M+35]^-$ and $[37+M]^-$) while maintaining a low concentration in order to maintain the column quality. However, the amount of ion added is sufficient to overcome the artifacts created by the release of Cl^- ions from the column. A noticeable improvement in reproducibility and repeatability of responses was observed. Meanwhile a good degree of sensitivity was maintained with no salt deposit at the top end of the capillary.

3.2. Limit of detection and limit of quantification (LOD and LOQ)

LOD is determined for each carbohydrate reference compound. A sugar standard compound is considered detectable when the peak signal is 3 times higher than the average noise level. LOD and LOQ have been determined for the scan mode and the SIM mode. Results obtained in SIM mode are shown in Table 2. SIM mode provided the best results, due to highest sensitivity of the signal/noise ratio (the signal increase due to the detection window centered on the ion of interest, and the noise decrease due to the non detection of others ions), but the quality of the results obtained in scan mode allows us to identify unknown saccharides without loss of important information. In this study, best LOD varied from 0.03 μg mL^{-1} for fucose and fructose to 1.50 μg mL^{-1} for mannose and galactose. The LOD were tested in triplicate over 15 days and did not vary at levels of concentrations tested. LOQ is defined with the signal/noise ratio equal to 10. LOQ are contained between 0.09 μg mL^{-1} for fucose and fructose, to 5.00 μg mL^{-1} for mannose and galactose. The saccharides constituting the couples xylose/arabinose and rhamnose/fucose are easy to identify. The difference between the retention are respectively 40 and 30 s. However, to obtain a single peak which is useful for a good quantification, it is sometimes necessary to achieve a dilution of the sample. And because these couples are formed by isomers, LC/MS–MS methods, often used in case of co-elution, are not able to differentiate the studied saccharides.

3.3. External calibration

The responses observed for external calibration are linear in the concentration range tested (Table 3) except for sucrose and cellobiose where square terms are needed for the correlation curve. Indeed both disaccharides can be split into the monomer during ionization: a peak at the same RT was detected for mass $m/z = 215$

Table 2
Dynamic range, interday residual standard deviation, limit of detection and limit of quantification obtained in SIM mode for cellobiose, glucose, sucrose, fucose, fructose, mannose, xylose, galactose, rhamnose and arabinose. Results presented are produced with internal and external calibrations.

Internal calibration	Linearity range ($\mu g\,mL^{-1}$)	Interday RSD	LOD ($\mu g\,mL^{-1}$)	LOQ ($\mu g\,mL^{-1}$)
Cellobiose	1.7–256	0.095	0.50	1.66
Glucose	0.3–51.2	0.073	0.08	0.25
Sucrose	1.0–51.2	0.096	0.30	0.99
Fucose	0.1–51.2	0.048	0.03	0.09
Fructose	0.1–25.6	0.086	0.03	0.10
Mannose	5.0–256	0.105	1.50	5.00
Xylose	1.7–256	0.083	0.50	1.67
Galactose	5.0–256	0.087	1.50	5.00
Rhamnose	0.3–102.4	0.081	0.10	0.34
Arabinose	0.7–256	0.101	0.20	0.67
External calibration	**Linearity range ($\mu g\,mL^{-1}$)**	**Interday RSD**	**LOD ($\mu g\,mL^{-1}$)**	**LOQ ($\mu g\,mL^{-1}$)**
Cellobiose	1.7–256	0.090	0.50	1.66
Glucose	0.3–51.2	0.270	0.08	0.25
Sucrose	1.0–51.2	0.114	0.30	0.99
Fucose	0.1–51.2	0.153	0.03	0.09
Fructose	0.1–25.6	0.347	0.03	0.10
Mannose	5.0–256	0.251	1.50	5.00
Xylose	1.7–256	0.205	0.50	1.67
Galactose	5.0–256	0.253	1.50	5.00
Rhamnose	0.3–102.4	0.125	0.10	0.34
Arabinose	0.7–256	0.279	0.20	0.67

and 217 corresponding to C6 (glucose and fructose) constituents of sucrose and cellobiose (Fig. 1). Good coefficients of correlation are obtained (in scan and SIM mode).

3.4. Internal calibration

The internal curves of standards obtained by the LC–APCI(−) method are linear in the concentration ratio interval tested with correlation coefficient of 1.0000 for glucose, 0.9999 for rhamnose, xylose, fructose and fucose, 0.9998 for galactose, 0.9997 for mannose and 0.9987 for arabinose. For disaccharide curves (sucrose and cellobiose) a square term is needed for a good correlation. Replicate analyses realized the same day show a variation of the response from 1.6 to 2.1% of the peak area of inositol. These results show a good reproducibility of the method. However, series of analysis spread out several days show a variation of the average of the inositol area detected of 7.5%. This value, using for reproducibility of the method, depends on the performance of the instrument. Good coefficients of correlation are obtained in SCAN and SIM mode (Table 4).

3.5. Uncertainty of the method

RSD has been evaluated for each monosaccharide, in SCAN and SIM mode. All monosaccharides were analyzed for each concentration once a week over 4 weeks. Results obtained in SCAN mode showed a high variability with a RSD of 0.09–0.48 depending on the monosaccharides and its concentration (data not shown). Reproducibility is better in SIM mode, with a RSD varying from 0.09 for cellobiose to 0.35 for fructose using an external calibration. The best results are obtained in SIM mode using internal calibration: the RSD varying from 0.05 for fucose to 0.10 for sucrose, mannose and arabinose (Table 2).

3.6. Method validations

The developed LC/MS methodology was validated on 2 different samples: orange juice containing a small number of sugars in a simple matrix and an aqueous sample obtained by enzymatic hydrolysis with various sugars in a complex matrix.

3.6.1. Quantitative and qualitative analysis of sugars in orange juice

Orange juice mainly contains sucrose, glucose and fructose. The analysis performed (Fig. 2) was able to detect and quantify these 3 sugars, and measured values are close to those obtained with other methods with 8.4 g of sugars for 100 mL (glucose 26.6 $g\,L^{-1}$, fructose 22.9 $g\,L^{-1}$ and sucrose 35.5 $g\,L^{-1}$). This result showed that the developed method is able to determine the amount of sugars contained in a simple aqueous matrix. The orange juice was diluted 1000-fold. The matrix effect of each analyte in orange juice had been evaluated by adding all saccharides at C6 concentration into matrix

Table 3
Results for individual monosaccharide standard curves from LC–APCI analysis uses for the external. Analysis were done in triplicate at 9 concentration levels. (corr. c.: correlation coefficient).

Peak name	Slope*X	Slope*X2	Offset	corr. c.
Rhamnose	818713	0	0	0.9998
Xylose	472129	0	0	0.9996
Fructose	4276520	0	0	0.9997
Glucose	2619730	0	0	0.9995
Cellobiose	335703	761.28	601634	0.9998
Fucose	793486	0	0	1
Arabinose	47483	0	0	0.9965
Mannose	1464190	0	0	0.9988
Galactose	1157670	0	0	0.9997
Sucrose	1104413	3645	583757	0.9996

Table 4
Results for internal calibration curves from LC–APCI analysis with inositol as internal standard ($mg\,mL^{-1}$). Analyses were done in triplicate at 9 concentration levels (corr. c.: correlation coefficient).

Peak name	Slope*X	Slope*X2	Offset	corr. c.
Rhamnose	0.02	0	0	0.9999
Xylose	0.01	0	0	0.9999
Fructose	0.10	0	0	0.9999
Glucose	0.06	0	0	1
Cellobiose	0.017	0.000027	−0.035	0.9982
Fucose	0.20	0	0	0.9999
Arabinose	0	0	0	0.9987
Mannose	0.03	0	0	0.9997
Galactose	0.02	0	0	0.9998
Sucrose	0.059	0.000220	−0.040	0.9996

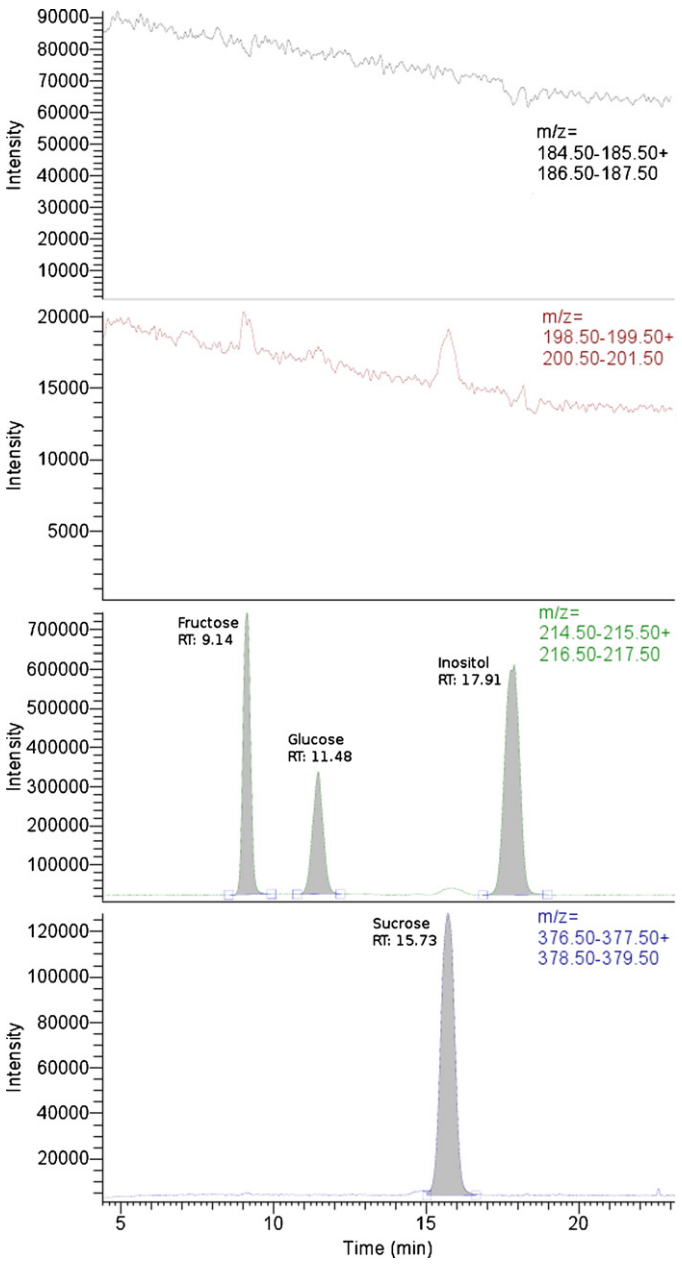

Fig. 2. Chromatogram of an orange juice partially purified and diluted by a factor of 1000. Separation is performed on a YMC-Pack Polyamine II (250 mm × 4.6 mm), volume of injection 20 μL, mobile phase flow rate 1 mL min^{-1} with 75% acetonitrile and 1% chloroform. Chromatograms are obtained by following chlorine adducts $[M+35]^-$ and $[M+37]^-$.

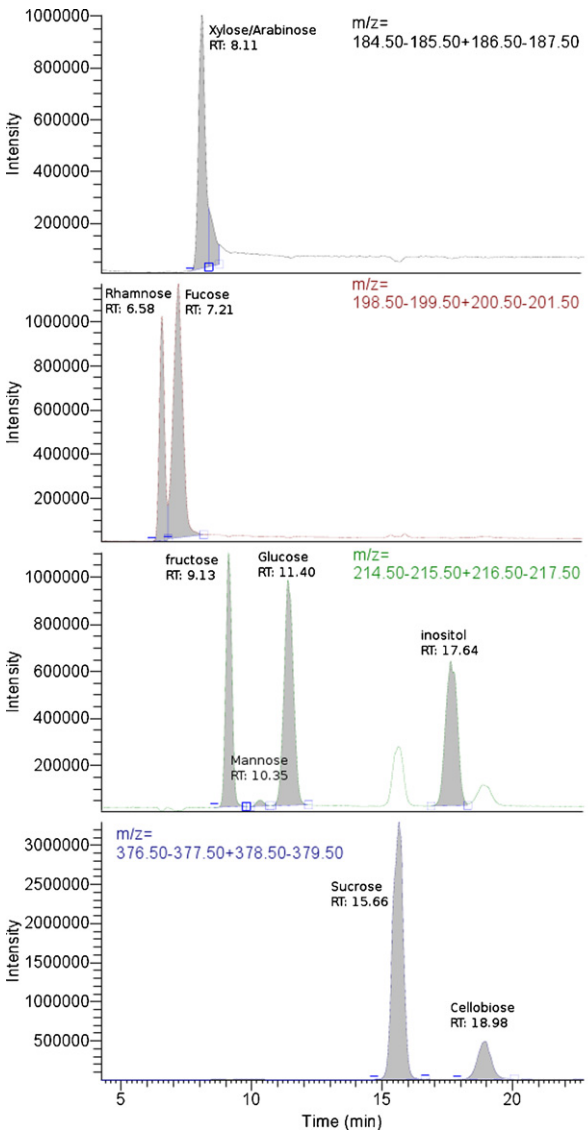

Fig. 3. Chromatogram of a saccharides mixture at known concentrations. Separation is performed on a YMC-Pack Polyamine II (250 mm × 4.6 mm), volume of injection 20 μL, mobile phase flow rate 1 mL min^{-1} with 75% acetonitrile and 1% chloroform. Chromatograms are obtained by following chlorine adducts $[M+35]^-$ and $[M+37]^-$.

and compared with the same analysis in distilled water. Except for glucose, fructose and sucrose which are naturally present in orange juice at high amounts, all of the concentrations found are in the uncertainty of the method. The results varying from 0.03% of error made for fucose, to 10% for cellobiose. The difficulty to integrate the cellobiose peak, explained by the high amount of sucrose in orange juice matrix, is responsible of the error made. So, because the results obtained in orange juice matrix are contained in the uncertainty of the method, we can assert that we have observed no matrix effect in these conditions of analysis.

3.6.2. Quantitative and qualitative analysis of sugars in a known solution

A mixture of all standard sugars (excepted galactose) at a known concentration was prepared for method validation. To test the separation of xylose and arabinose, concentrations of these two saccharides are deliberately high (respectively 75 and 125 μg mL^{-1}). The chromatogram obtained (Fig. 3) show a good separation of each saccharide tested, except, as expected, for xylose and arabinose. Quantitative results reported in Table 5 are the average of results obtained by triplicate analysis. The method test provides good results; the error deviation varies from 1 to 7% depending on the sugars. Due to the bad separation at high concentration of xylose and arabinose, the error made on quantitative results are around 10%.

3.6.3. Quantitative and qualitative analysis of sugars of enzymatic hydrolysis of sunflower seeds

In the same way, analyses have been realized on aqueous sample (diluted 100-fold) obtained after an enzymatic hydrolysis of sunflower seeds (Fig. 4). The enzymes have released monosaccharides from cellulose and hemicelluloses. The following sugars have been identified and quantified: rhamnose, xylose, fucose, arabinose, fructose, mannose, glucose and/or galactose, cellobiose and sucrose. In this mixture, xylose and arabinose are at low concen-

Table 5
Known concentration, average of results done in triplicate obtained from LC–APCI analysis with inositol as internal standard and error made obtained on a saccharides mixture at known concentration.

Sugar	Rhamnose	Xylose	Fructose	Glucose	Cellobiose	Fucose	Arabinose	Mannose	Sucrose
Concentration of sugar in solution ($\mu g\,mL^{-1}$)	40	75	10	20	50	50	125	10	75
Results ($\mu g\,mL^{-1}$) (average of 3 replicates)	41.39	81.40	9.30	19.46	53.85	48.34	112.59	10.10	73.01
Error (%)	3.48	8.53	7.00	2.70	7.70	3.32	9.93	1.00	2.65

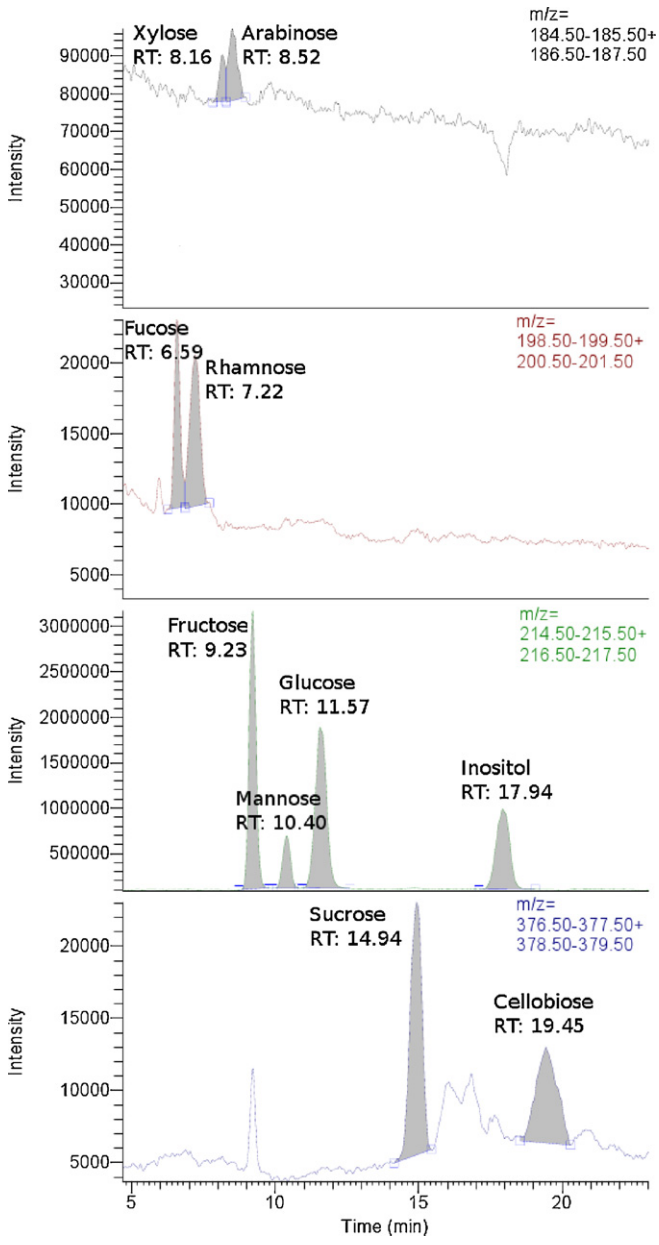

Fig. 4. Chromatogram of an aqueous phase obtained after enzymatic hydrolysis of sunflower seeds and diluted 100-fold. Separation is performed on a YMC-Pack Polyamine II (250 mm × 4.6 mm), volume of injection 20 μL, mobile phase flow rate 1 mL min^{-1} with 75% acetonitrile and 1% chloroform. Chromatograms are obtained by following chlorine adducts $[M+35]^-$ and $[M+37]^-$.

tration, near to the LOD. Once again, resolutions obtained allowed us to quantify the different sugars tested. Total sugar content found with this method is 73.8 g L^{-1}. The same sample tested with Nelson–Somogyi biochemical method shows a concentration of reducing sugars of 72.3 g L^{-1}. It is important to note that xylose (Rt = 8.16) and arabinose (Rt = 8.51) even in a small quantity, are close, but quantification always remains possible.

4. Conclusion

The method developed using an aqueous HILIC separation followed by APCI(−) MS detection allowed us to meet all of our goals. The sample preparation is fast, without any derivation needed. The method itself, with a total run time of 22 min is fast and robust. With the use of the external calibration and without the disaccharides quantification, the run time can be reduced to 15 min. It is possible to perform this analysis on a large scale and with various types of samples. This constitutes a real advantage comparatively to the traditional methods. The present method has been validated on two different real samples, and is expected to work for other sorts of aqueous sample containing sugars.

The results obtained have shown the possibility of working with inositol as an internal standard (at a concentration of 0.01 g L^{-1} during this test). Indeed, the linearity range of the sugars/inositol ratios is obtained for a concentration range varying from 0.1 to 25.6 $\mu g\,mL^{-1}$ for fructose, from 5 to 256 $\mu g\,mL^{-1}$ for mannose and galactose. Moreover it is also possible to work with inositol as external standard with one test at the beginning of the run and one at the end of the run. The slope of external standard curves may vary from day to day depending on the performance of the instrument. During the test period, a slope variation of 10% was observed. Quantification by the external calibration is therefore advisable only if a standard curve is obtained before and after every series of analysis. It is important to note that no matrix effect has been observed during these tests when inositol is used alone or in combination.

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