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Testing Clean-Up Methods for the Quantification of Monosaccharides and Uronic Acids

Irene Bargagli [1,2,3], Francesca Sabatini [1,2,*], Francesca Modugno [1] and Jeannette Jacqueline Łucejko [1,*]

[1] Department of Chemistry and Industrial Chemistry, University of Pisa, Via Moruzzi 13, 56124 Pisa, Italy
[2] Institute of Chemical Science and Technologies "G. Natta" (CNR-SCITEC), Via Elce di Sotto, 8, 06123 Perugia, Italy
[3] Department of Chemistry, Biology and Biotechnology, University of Perugia, Via dell' Elce di Sotto, 8, 06123 Perugia, Italy
* Correspondence: f.sabatini4@gmail.com (F.S.); jeannette.lucejko@unipi.it (J.J.Ł.)

Abstract: The determination of carbohydrate composition is extremely important for quality control in food and beverages, in material science, in pharmaceutics, and in the field of cultural heritage. Considering the complexity and the heterogeneity of the matrices, the optimization of extraction and purification steps aiming at maximizing the saccharide recovery from the matrix and effectively removing interferences is mandatory. The presence of inorganic components, besides being detrimental to the analytical instrumentation, can catalyze the isomerization of some sugars causing an alteration to their quantitative and qualitative profiles. In the present paper, protocols for suppressing the interference of inorganic ions in the quantification of monosaccharides and uronic acids by Gas Chromatography–Mass Spectrometry (GC-MS) are proposed. Two clean-up methods based on ion exchange resins (Amberlite MB-6113 and Amberlite IRN-150) and one making use of solid-phase extraction with a polypropylene Solid-Phase Extraction (SPE) column were tested on a standard carbohydrate solution, and the elution conditions optimized. The best purification conditions, in terms of higher recovery yield values for seven monosaccharides and two uronic acids, were obtained using SPE. Furthermore, the optimized SPE method was validated on a sample of mural painting rich in saccharides and inorganic material.

Keywords: monosaccharides; purification; solid-phase extraction (SPE); ion exchange resins; GC-MS

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1. Introduction

Carbohydrates are the most abundant compounds in nature. They are synthesized by green plants during photosynthesis and can be classified into two categories: simple and complex carbohydrates. Simple sugars, called monosaccharides, are carbohydrates that cannot be transformed into smaller molecules by hydrolysis contrarily to complex saccharides, commonly known also as oligosaccharides or polysaccharides, formed by hundreds or thousands of monosaccharide units.

The determination and quantification of free monosaccharides and of saccharides constituting polysaccharides are of considerable importance in various fields. As regards the food industry, the determination of the glucose/fructose ratio in honey has allowed possible adulterations with sugars from cheaper sources such as corn, sugar cane or beet to be sought out [1–4]. Numerous studies are also devoted to the analysis of monosaccharides in vegetables [5,6] and in confectionery products [7]. Attention has also been paid to the study of the antioxidant properties [8,9] and immunomodulating effects [10] of monosaccharides in pharmaceuticals. Polysaccharide materials such as plant gums have also been widely employed since remote antiquity to produce artistic objects, e.g., used as binders for painting [11], in writing inks [12], in illuminated manuscripts [13], in portrait miniatures [14], and as adhesives in metal leaf decorations [15]. Therefore, the

determination of monosaccharides in artistic samples after hydrolysis provides important information on the provenience, nature of the binder, and thus on the artistic technique.

The quantitative analysis of mixtures of monosaccharides is highly challenging considering their structural similarity, and consequently their chemical one. Thus, chromatography constitutes the election method to separate and determine the content of single saccharides. High-Performance Liquid Chromatography (HPLC) has been efficiently employed thanks to the high solubility of these analytes in water [16–18], but also gas chromatography preceded by proper derivatization steps has been successfully used exploiting monosaccharides high polarity [19,20].

The analysis of saccharide materials from a complex matrix often requires clean-up steps to eliminate interferences such as inorganic compounds [15]. Some inorganic materials seem to act as catalysts for the isomerization of some sugars, producing a modification of the quantitative and qualitative profiles of the saccharides [21]. Furthermore, their presence may adversely affect the analytical instrumentation, such as in the case of sulfates, which are detrimental for Anionic Exchange Liquid Chromatography (HPAEC) setups [22] and for mass spectrometric detectors, since the ionization efficiency is reduced and ionic suppression favored, affecting the detection of the analytes and the instrumentation maintenance [23].

The high level of inorganic ions may be contained in a wide variety of samples. Those from cultural heritage, as paint samples, represent a proper example considering that pigments, fillers, and additives of painting surfaces are rich in inorganic materials [24]. The analytical methods allowing the targeting and profiling of saccharides in oligosaccharides and in complex polysaccharides require their hydrolysis. A hydrolysis method commonly used involves the use of sulfuric acid [22], making the purification of the hydrolyzed sample mandatory in order to be injected into a chromatographic system. Another aspect to consider is that widely spread and exploited saccharide matrices, such as sugar cane and beetroot, contain further classes of compounds such as phenolic acids, flavonoids, and phenolic compounds (responsible for the antioxidant activity of the relative vegetable extracts [25]) that could affect the determination of saccharides. In particular, the fluoresce of phenols may interfere with saccharide detection when HPLC coupled with a fluorescence detector is used; in addition, the presence of compounds with hydroxyl groups may undergo derivatization reactions applied for analysis of the saccharide by Gas Chromatography–Mass Spectrometry (GC-MS) complicating the resulting chromatographic profiles.

Saccharide purification can occur by physical or chromatographic separation as well as by chemical precipitation [26]. Chromatographic separation is the most common approach, with ion-exchange columns being the most widely used. The stationary phase of these columns is made up of resins classified as “weak” or “strong” exchangers. These terms are not related to the strength of ion binding, but to the extent that the ionization state of the functional groups of exchangers varies with pH. A “weak” exchanger is ionized over only a limited pH range, while a “strong” one shows no variation in ion exchange capacity with changes in pH [23].

In the literature, different applications of cationic exchange resins have been reported, such as Dowex® 50W X8 for removing interferents as inorganic ions in the analysis of monosaccharides deriving from glycoproteins and glycopeptides [27] and of saccharides in paint binders of ancient wall paintings [16]. Amberlyst® has been used for the purification of sugars hydrolysates from wheat bran [28]. Another cationic exchange resin, Amberlite 732, and the anion exchange resin, Amberlite FPA53, have been tested for the purification of the xylo-oligosaccharides originating from bamboo [29]. Ion exchange resins with an affinity for both anions and cations have also been frequently employed in the purification of mixtures of monosaccharides derived from the hydrolysis of polysaccharides, such as in the case of Zeolit DMF [30] and Amberlite MB 6113 [24] for the purification of saccharides in painting matrices.

The aim of the present paper is to evaluate the best clean-up method, in terms of analyte recoveries, for the quantification of monosaccharides and uronic acids. Thus, two

ion exchange resins and a Solid-Phase Extraction (SPE) have been tested. The purified extracts have been derivatized and then analyzed by gas chromatography (GC-MS) to determine the recovery of the purification steps. The derivatization reaction applied is widely used in the literature for the determination of monosaccharides in complex matrices [30] and involves the mercaptalation of the analytes on the carbonylic function, followed by two silanization steps involving all the hydroxylic groups. The presence of a high level of inorganic materials can severely affect the GC-MS analyses and interfere with the derivatization reaction, lowering its recovery and therefore affecting the reproducibility and sensitivity of the method [30]. To confirm the results obtained, the efficacy of the best purification method was validated by analyzing the preparation layer of a mural painting sample containing saccharides in the presence of inorganic material.

2. Materials and Methods

2.1. Standards, Chemicals, and Materials

The standard materials used for preparing sugar solutions were all purchased from Sigma-Aldrich (St. Louis, MO, USA): D-(+)-galactose, L-(−)-fucose, L-(+)-arabinose, L-(−)-rhamnose, L-(−)-mannose, D-(+)-xylose, D-(+)-glucose, D-glucuronic acid, D-galacturonic acid monohydrate, and mannitol.

The following chemicals were used: trifluoroacetic acid (TFA, 99% purity, Fluka), acetonitrile (ACN, 99.9% purity, Sigma-Aldrich), pyridine anhydrous (Py, Fluka, Milan, Italy), ethanethiol (EtSH, 99.5% purity, Sigma-Aldrich), N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and N,O-Bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (BSTFA 1%TMCS) (Sigma-Aldrich), and n-hexane (Sigma-Aldrich).

For the purification with ion exchange resins, glass Pasteur pipettes (7 mm diameter, length 230 mm) were packaged with two types of Amberlite resins (Amberlite MB-6113 (Merck) and Amberlite IRN-150 (DuPont™)) characterized by an exchanger bed composed by strongly acid and strongly basic ion resins and color indicator. The chemical-physical properties of these resins are summarized in Table 1.

Table 1. Chemical–physical properties of Amberlite MB-6113 and Amberlite IRN-150.

	Amberlite MB-6113		**Amberlite IRN-150**	
Type	Cation Resin	Anion Resin	Cation Resin	Anion Resin
Copolymer	Styrene-Divinylbenzene	Styrene-Divinylbenzene	Styrene-Divinylbenzene	Styrene-Divinylbenzene
Matrix	gel	gel	gel	gel
Functional group	Sulfonic acid	Trimethylammonium	Sulfonic acid	Trimethylammonium
Ionic ratio	37–46%	63–54%	50%	50%
Temperature stability	5–60 °C		5–100 °C	
pH stability	-		0–14	

For the solid-phase extraction, Macherey-Nagel™ Chromabond™ NH2 Polypropylene SPE columns were used. These columns are characterized by an aminopropyl-modified silica phase acting as a weak anion exchanger (base material silica, pore size 60 Å, particle size 45 µm, specific surface 500 m^2/g, pH stability 2–8, carbon content 3.5 %).

2.2. Sample for Validation

A sample (3.7 mg), collected from the preparation layer of ancient African mural painting and dated approximately to 1500 B.C., was selected. The sample comes from the east-central region of Ethiopia, which has the highest concentration of ancient rock painting samples in the nation. It was analyzed by Attenuated Total Reflectance Fourier Transform Infrared spectroscopy (ATR-FT-IR) in order to verify the inorganic content and by GC-MS, after the application of a validated procedure entailing acidic hydrolysis [30] and of the optimized SPE purification, for assessing the type of plant gum present.

2.3. Apparatus

2.3.1. GC-MS

A gas chromatograph GC 6890N coupled to a single quadrupole mass spectrometer with a 5975 selective mass detector (Agilent Technologies) was used. The injector was PTV type, used in splitless mode at a temperature of 300 °C. The column was a fused silica capillary column HP-5MS (J&W Scientific, Agilent Technologies, USA), 30 m × 0.2 mm ID (5% phenyl-95% methylolysiloxane, film thickness 0.25 µm, J&W Scientific, Agilent Technologies, Palo Alto, CA, USA) with a quartz press fit. Pure helium at 99.995% with a flow of 1.2 mL/min was used as carrier gas. The programmed temperature was as follows: 80 °C kept constant for 1 min, increased up to 190 °C with a ramp of 2.5 °C/min, kept constant for 15 min, increased up to 280 °C with a ramp of 20 °C/min, kept constant for 10 min. The injection volume was 2 µL.

The mass spectrometer uses electron-impact ionization (70 eV); the ion source temperature was set to 230 °C while the interface temperature to 280 °C. The mass spectra were registered in Total Ion Current (TIC) mode (*m*/*z* 40–700) and in Selected Ion Monitoring (SIM) mode. For the acquisition in SIM mode, the following ions were selected: *m/z* 249 and *m/z* 319 for xylose and arabinose; *m/z* 249 and *m/z* 333 for rhamnose and fucose; *m/z* 135, *m/z* 305, and *m/z* 319 for galacturonic and glucuronic acids; *m/z* 307, *m/z* 319 and *m/z* 331 for glucose, mannose, and galactose, respectively; while for mannitol *m/z* 307 and *m/z* 319.

2.3.2. ATR-FT-IR

ATR-FT-IR spectra were collected using a Thermo-Scientific™ Nicolet™ iS50 FTIR spectrometer equipped with an iS50 ATR multi-range, diamond sampling station. The spectra were recorded as 16 scans at 4 cm^{-1} spectral resolution in the range of 450–4000 cm^{-1}.

2.4. Analytical Procedure

2.4.1. Preparation of Standard Solutions

For all clean-up tests, a mixture solution in bi-distilled water containing 7 monosaccharides and 2 uronic acids at 100 ppm was used. Mannitol was used as an internal standard for GC-MS quantification; 5 µL of a 0.1 mg/g (100 ppm) solution of mannitol in bi-distilled water was added to each sample before the derivatization phase.

2.4.2. Purification with Ion Exchange Resins

The ionic exchange resins selected in this work for the purification step have been already used in the literature [24]. The procedure involves the following steps: (i) conditioning of the resin: 3 mL of bi-distilled water was used; (ii) sample loading: 100 µL of monosaccharide standard solution at 5 ppm (5 µL of mix standard solution at 100 ppm in 95 µL of bi-distilled water) was loaded on the column; (iii) elution: monosaccharides were eluted with bi-distilled water and collected. Different volumes and working pH of the eluent phase were tested as reported in Table 2.

Table 2. Recoveries obtained for a standard solution of 7 monosaccharides and 2 uronic acids (each at 10 ppm) with Amberlite MB-6113 and IRN-150 resins under different elution conditions. For the percentage recoveries, the relative confidence interval is reported. Each experiment was conducted in triplicate.

	Amberlite MB-6113		**Amberlite IRN-150**			
	1 mL H_2O	**3 mL H_2O**	**3 mL H_2O**	**3 mL TFA 2 M (pH < 0)**	**3 mL TFA 10^{-4} M (pH = 4)**	**3 mL NH_3 6 × 10^{-6} M (pH = 9)**
				Recoveries (%)		
Xylose	2.1 ± 0.1	2.6 ± 1.2	6.8 ± 0.5	16.6 ± 0.7	2.6 ± 0.1	4.0 ± 1.2
Arabinose	2.2 ± 0.1	3.6 ± 1.8	8.9 ± 1.9	19.2 ± 0.6	3.0 ± 1.0	4.9 ± 1.1
Rhamnose	2.1 ± 1.1	2.6 ± 0.9	8.7 ± 0.7	13.6 ± 8.3	2.4 ± 0.4	4.7 ± 0.2

Table 2. *Cont.*

	Amberlite MB-6113		Amberlite IRN-150			
	1 mL H_2O	**3 mL H_2O**	**3 mL H_2O**	**3 mL TFA 2 M (pH < 0)**	**3 mL TFA 10^{-4} M (pH = 4)**	**3 mL NH_3 6 × 10^{-6} M (pH = 9)**
				Recoveries (%)		
Fucose	3.0 ± 0.1	6.7 ± 3.1	17.1 ± 3.4	25.1 ± 3.8	6.2 ± 0.4	10.5 ± 0.2
Galacturonic acid	1.5 ± 0.1	1.4 ± 1.2	4.8 ± 1.5	7.5 ± 0.3	1.0 ± 0.6	2.2 ± 0.5
Glucuronic acid	1.2 ± 0.1	1.5 ± 1.0	4.0 ± 0.5	9.7 ± 0.4	1.1 ± 0.8	2.0 ± 0.1
Glucose	7.3 ± 4.3	11.4 ± 0.7	40.0 ± 19.6	51.7 ± 37.2	46.8 ± 46.3	15.3 ± 2.1
Mannose	2.8 ± 0.7	2.7 ± 1.2	9.2 ± 1.5	14.0 ± 7.8	2.3 ± 0.9	5.0 ± 0.2
Galactose	2.3 ± 0.3	3.2 ± 1.4	9.7 ± 2.1	13.7 ± 4.9	2.9 ± 0.1	4.9 ± 0.1

2.4.3. Solid-Phase Extraction

Macherey-Nagel™ Chromabond™ NH2 Polypropylene SPE columns have been already used in a previous work [5]. Given the excellent performance in terms of recovery of monosaccharides, the same SPE columns were tested to evaluate the recovery of different saccharides than those investigated in the paper [5].

A volume of 5 µL standard solution containing 7 monosaccharides and 2 uronic acids (each at a concentration of 100 ppm) was dried under nitrogen flow and solubilized in 200 µL of an aqueous solution 40% in ACN, to be extracted on the SPE cartridge.

The same step above mentioned for the ion exchange resins was also applied for the solid-phase extraction; in this case, the conditioning of the column was performed with 1 mL of 100% ACN and then with 1 mL of 40% ACN. Only elution was optimized, while for the other steps the conditions described in the literature were adopted [5].

2.4.4. Derivatization

An amount of 5 µL of mannitol solution (100 ppm), as Internal Standard (SI), was added to the purified standard solution of monosaccharides and uronic acids, and then dried under nitrogen flow. The addition of an Internal Standard enabled the correction of the derivatization yield.

The derivatization used, followed a validated procedure [30] based on the formation of linear derivatives of sugars. This involved mercaptalation by adding 25 µL of EtSH: TFA (2:1 v/v) mixture to the sample. After 10 min at room temperature, 100 µL of BSTFA was added as a derivatizing agent. The solution was placed in a water bath at 60 °C for 15 min. After drying under nitrogen flow, 50 µL of BSTFA with 1% TMCS and 100 µL of pyridine were added. The solution was heated in a water bath at 60 °C for 45 min. Finally, the solvent was evaporated and 50 µL of n-hexane was added, and then 2 µL of the derivatized sample was injected in the GC-MS.

2.4.5. Analysis of the Validation Sample

The GC-MS procedure for the analysis of vegetable gums in paint samples adopted [30] is integrated into a multi-step procedure for analyzing glycerolipids, terpenoid resins, natural waxes, proteinaceous, and polysaccharide materials.

To 3.7 mg of the wall paint sample (described in Section 2.2), 500 µL of TFA 2M was added and the microwave oven hydrolysis (120 °C, 800 W) was conducted in 45 mL of double-distilled water. The acidic hydrolysis produced free monosaccharides and uronic acids from the polysaccharides present in the sample. The hydrolysate was then filtered on a PTFE filter, first conditioned with 50 µL of TFA (2M), and then vial and filter rinsed with 100 µL of bi-distilled water to maximize the recoveries.

Filtering eliminates insoluble materials present in the hydrolysate. The sample is then dried under a stream of nitrogen, subjected to the procedure of clean-up, derivatized, and finally injected in GC-MS.

3. Results

In order to find the most selective and sensitive stationary phase for the seven monosaccharides and two uronic acids in terms of recovery, the results collected using the two commercial ionic exchange resins were compared and discussed, drawing consideration on the advantages of the application of SPE to the profiling of saccharides in complex matrices containing polysaccharides.

The results are expressed as percentage recoveries, calculated as a percentage ratio between the concentration determined by GC-MS in standard solution subjected to a clean-up procedure with the ion exchange resins or the SPE, and the concentration in the standard solution without a clean-up step. The concentration of the analytes eluted from the ion exchange resins or the SPE cartridge was obtained, integrating the areas in the SIM chromatogram for each analyte and using calibration curves. Each experiment was conducted in triplicate and average values are provided. Figure 1 shows the GC-MS chromatogram in SIM mode collected for a standard sugar solution of seven monosaccharides and two uronic acids.

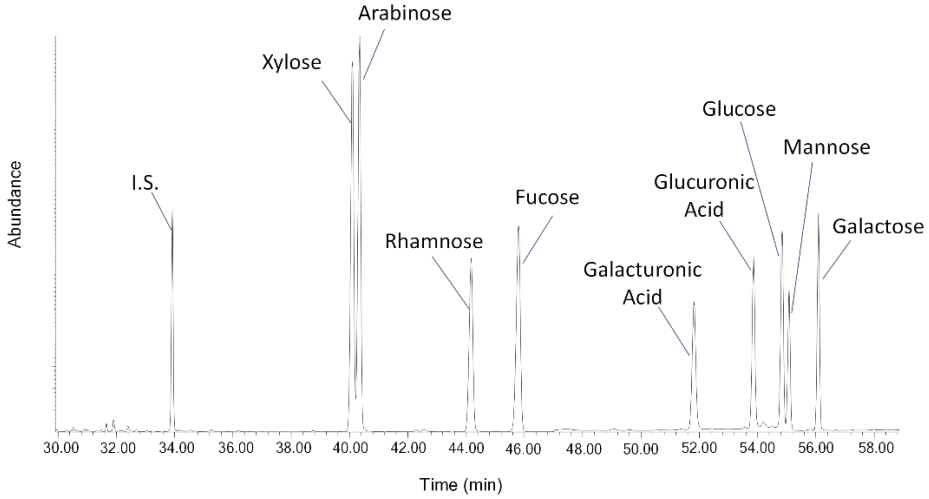

Figure 1. GC-MS chromatogram in SIM mode of a standard sugar solution of 7 monosaccharides and 2 uronic acids (each at 10 ppm), where I.S internal standard—Mannitol (10 ppm).

Different conditions were tested, and the working plan was adapted step by step on the basis of the recoveries determined. The results of the tests carried out with the two types of Amberlite ionic exchange resins are summarized in Table 2 and carefully explained following.

Elution with 1 mL and 3 mL of water from Amberlite MB-6113 resin provided low recoveries and poor reproducibility for each analyte. The first experiment with Amberlite IRN-150 involved the elution of the analytes with 3 mL of bi-distilled water to compare the recoveries to those obtained by Amberlite MB-6113. The recoveries collected by IRN-150 are higher than those achieved by MB-6113, but not yet satisfactory (Table 2). The elution in strongly acidic conditions, using a TFA 2 M solution (pH < 0), shows a significant improvement in monosaccharides recoveries. To maximize the recoveries, further tests were performed by eluting the analytes at pH 4 and 9, since Amberlite IRN-150 resin provides optimal performance in this pH range [31]. In particular, the elution with 3 mL of 10^{-4} M TFA solution (pH = 4) and with 3 mL NH_3 $6{\cdot}10^{-6}$ M (pH = 9) was tested. In both cases, there was no improvement in monosaccharide recoveries compared to those obtained by the elution with a TFA 2 M solution.

Given the results obtained with two Amberlite resins, the purification with ion exchange resin with solid-phase extraction was replaced with an aminopropyl-modified silica phase. The potentiality of this type of column to also remove phenolics compounds that could affect derivatization can be a useful advantage when we deal with specific matrices [5]. In the literature, the elution of monosaccharides from this type of column is carried

out with 1 mL of 40% ACN solution [5]; thus, these conditions were initially adopted. The recoveries in the first, second, and third milliliter of the elution with a 40% ACN solution, collecting and analyzing the eluates separately, are summarized in Table 3 (Experiment A). The results, based on three replicates, are reported as average recovery, and confidence intervals are defined by the product between the t student value (95% confidence) and the standard deviation divided by the root of the number of measurements. The histograms relative to monosaccharides recoveries in each fraction, depicted in Figure 2, show that 1 mL of acetonitrile solution recovers almost the total amount of saccharides, leading to a nearly better performance than those of both ion exchange resins. Nevertheless, uronic acids are poorly recovered by the aminopropyl-modified phase and, in particular, for galacturonic acid, the recovery tends to zero.

Table 3. Results obtained for a clean-up test using a Macherey-Nagel™ Chromabond™ NH2 Polypropylene SPE column applied to a standard solution of 7 monosaccharides and 2 uronic acids (each at 10 ppm). For the percentage recoveries, the relative confidence interval is reported. The measurements were conducted in triplicate. Experiment A and B refers to the optimization of the volume and the pH of elution respectively while experiment C represent the best results.

	Recoveries %				
Experiment	**A**			**B**	**C**
Elution Condition	**1° mL ACN 40%**	**2° mL ACN 40%**	**3° mL ACN 40%**	**1 mL TFA 400 mM**	**1 mL ACN 40% 400 mM TFA**
Xylose	68.5 ± 1.3	3.3 ± 0.3	3.6 ± 0.2	1.5 ± 0.4	80.1 ± 0.6
Arabinose	40.4 ± 3.8	5.0 ± 0.1	5.1 ± 1.4	2.0 ± 0.9	75.9 ± 1.8
Rhamnose	55.6 ± 1.4	1.6 ± 1.7	4.4 ± 0.6	0.8 ± 0.2	55.0 ± 3.5
Fucose	53.1 ± 0.5	1.6 ± 1.2	2.9 ± 2.1	0.7 ± 0.2	66.4 ± 1.7
Galacturonic acid	0.4 ± 0.1	0.4 ± 0.1	1.4 ± 0.2	41.6 ± 2.7	62.6 ± 4.2
Glucuronic acid	13.9 ± 3.5	1.8 ± 0.2	3.6 ± 0.8	77.3 ± 1.2	90.3 ± 2.3
Glucose	72.9 ± 1.9	1.5 ± 0.2	10.0 ± 0.6	1.2 ± 0.03	74.8 ±1.8
Mannose	53.4 ± 1.9	5.0 ± 0.7	6.0 ± 1.7	1.9 ± 0.1	57.8 ± 6.1
Galactose	49.6 ± 2.4	4.0 ± 0.3	4.9 ± 0.1	2.7 ± 0.1	52.1 ± 0.7

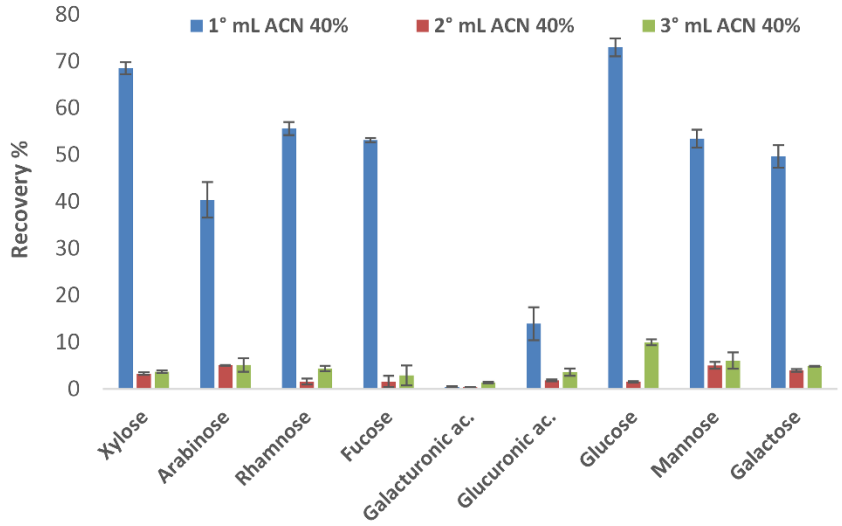

Figure 2. Recoveries, expressed as a percentage with respect to the concentration of starting solution, reported for each fraction of eluate analyzed, relative to the clean-up test results (Experiment A) using an SPE column applied to a standard solution of 7 monosaccharides and 2 uronic acids (each at 10 ppm). The experiment was conducted in triplicate and the error bars represent the confidence interval.

The poor recovery of uronic acids is due to the need of acidic conditions to shift the equilibrium towards their undissociated form to achieve their elution from the stationary phase. To improve their recoveries, after the elution with 1 mL of 40% ACN, a further elution with 1 mL of a slightly acidic aqueous solution of TFA 400 mM (pH 0.4) was tested. The recoveries (Experiment B, Table 3) and the histograms (Figure 3) highlight that the elution with TFA solution drastically increases the uronic acid recoveries (100 times more for galacturonic acid, 6 times for glucuronic acid).

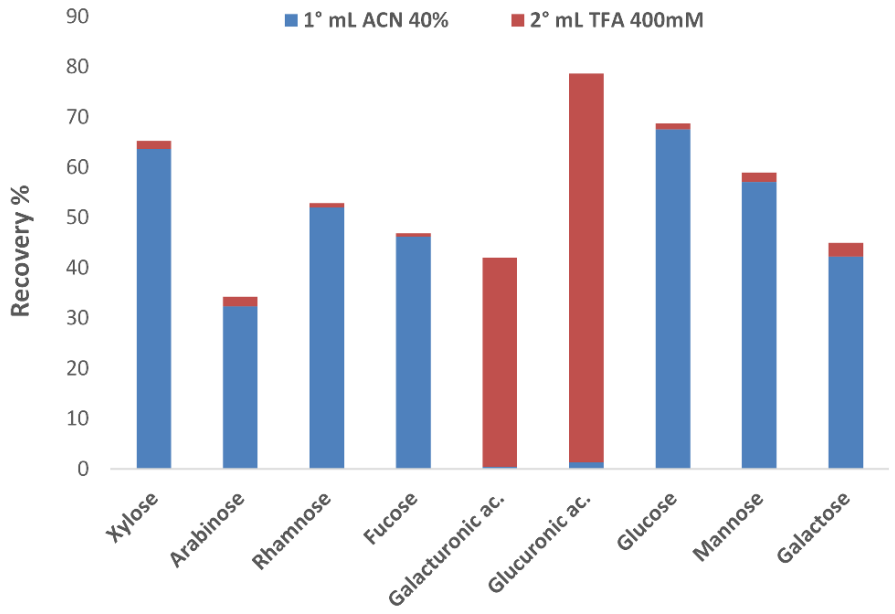

Figure 3. Recoveries, expressed as a percentage with respect to the concentration of starting solution, reported for each fraction of eluate analyzed, relative to the clean-up test results (Experiment B) using a Macherey-Nagel™ Chromabond™ NH2 Polypropylene SPE column applied to a standard solution of 7 monosaccharides and 2 uronic acids (each at 10 ppm). The experiment was conducted in triplicate and the error bars represent the confidence interval.

Finally, an elution with 1 mL of trifluoroacetic acid 400 mM in a 40% acetonitrile solution was tested in order to recover all the analytes in a single step. Eluting all analytes in a single step is considerably advantageous allowing us to shorten the analytical procedure (dry the aqueous solution under the nitrogen stream) and to collect the analytes in a lower volume, more suitable for analytical purposes. The results (Figure 4) show that all analytes were recovered with a single milliliter of elution. These latter conditions outperform all previous solid-phase extraction experiments conducted. Recoveries of more than 70% were obtained for xylose, arabinose, glucuronic acid, and glucose, while recoveries for the remaining analytes are typically greater than 50%. The recoveries achieved highlight the higher efficiency of the SPE methods for saccharide and uronic acid extraction with respect to results obtained for both the tested Amberlite resins.

The solid-phase extraction using a Macherey-Nagel™ Chromabond™ NH2 Polypropylene SPE column under the optimized conditions was applied to the GC-MS analysis of a validation sample, rich in saccharides and inorganics and collected from the preparation layer of a mural painting. The ATR-FT-IR analysis allowed us to confirm the expected high level of inorganic material in the sample and to determine it. In particular, the presence of a clay mineral, kaolinite ($Al_2Si_2O_5(OH)_4$), was identified by the characteristic bands in the mid-IR range of 3686–3619 cm^{-1} as well as at 1029, 1007, 915, 784, and 750 cm^{-1} (Figure 5) [32,33]. The presence of gypsum ($CaSO_4 \cdot 2H_2O$) was also assessed on the basis of typical bands at 595, 664, 1618, 3398, and 3529 cm^{-1}.

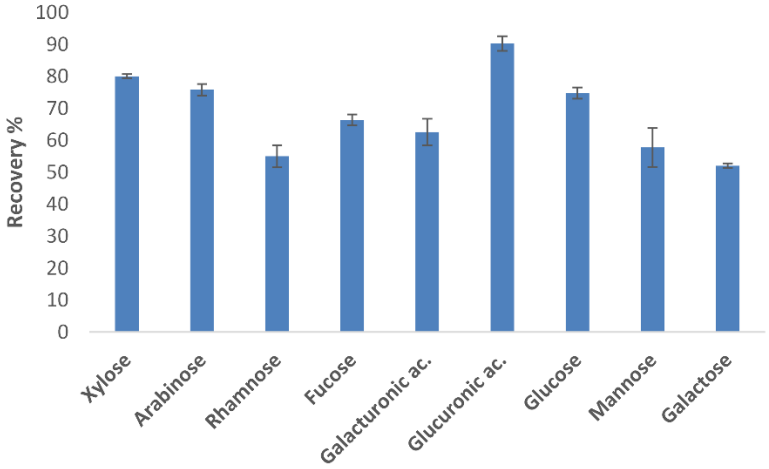

Figure 4. Recoveries (expressed as a percentage with respect to the concentration of starting solution) are reported for each fraction of eluate collected from the analysis of a standard solution of 7 monosaccharides and 2 uronic acids (each at 10 ppm). The clean-up test selected, makes use of a Macherey-Nagel™ Chromabond™ NH2 Polypropylene SPE column (Experiment C). The experiment was conducted in triplicate and the error bars represent the confidence intervals.

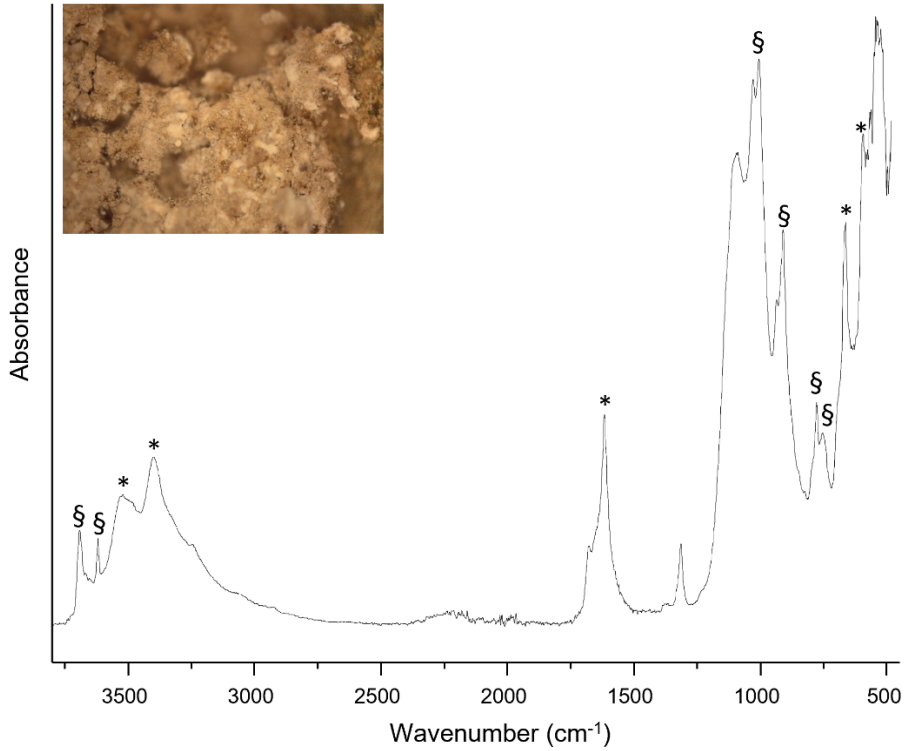

Figure 5. ATR-FT-IR spectrum of the preparation layer of a mural painting sample, recorded with 16 scans at 4 cm^{-1} spectral resolution. The bands associated with the presence of kaolinite (750, 784, 915, 1007, 1029, 3616, and 3686 cm^{-1}) are highlighted with the asterisk (*), while those due to gypsum (595, 664, 1618, 3398, and 3529 cm^{-1}) are labeled with the symbol (§). The optical microscope image of the sample is depicted on the top.

Given the high content of inorganic fraction, the sample is a suitable candidate to test the effectiveness of the optimized purification method. The GC-MS chromatogram in SIM mode of the mural paint sample after the purification step on a Macherey-Nagel™ Chromabond™ NH2 Polypropylene SPE column is shown in Figure 6, and the concentration of monosaccharide and uronic acids present is reported in Table 4.

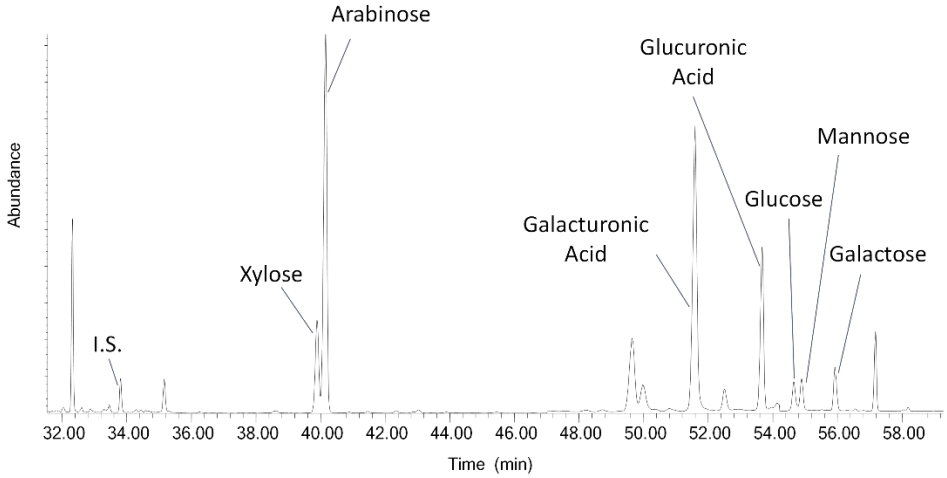

Figure 6. GC-MS chromatogram in SIM mode of the preparation layer of the archaeological sample subjected to the SPE column clean up under optimum elution conditions (experiment C). The peaks in the chromatogram with no assignment were not identified. I.S.: internal standard.

Table 4. Concentrations (ppm) of monosaccharides and uronic acids determined in a sample collected from the preparation layer of an ancient mural painting (3.7 mg).

Analyte	Concentration (ppm)
Xylose	240
Arabinose	895
Rhamnose	0
Fucose	0
Galacturonic acid	957
Glucuronic acid	1145
Glucose	124
Mannose	427
Galactose	222

Since the presence of inorganic ions seems to have a considerable influence on the derivatization reaction [30], the purification effectiveness was evaluated for the archaeological sample. The recovery of mannitol, the internal standard of derivatization (I.S.), was calculated as the ratio between the initial I.S. concentration and that obtained from the integration of the chromatographic peak of the derivatized I.S. The I.S. recovery for the paint sample rich in the inorganic sample was 0.82, demonstrating the achievement of good efficiency of derivatization, and the effective removal of the interferents.

On the basis of the literature, the evaluation of the relative content of saccharides and uronic acids can provide information on the vegetal gum employed [11]. The most abundant saccharides present in the hydrolysate sample are arabinose and the two uronic acids. Given the absence of fucose and rhamnose, the profile appears to be comparable to that of a fruit tree vegetable gum.

4. Discussion

In this paper, two distinct methods for suppressing inorganic ion interference in the quantification of monosaccharides and uronic acids by GC-MS were investigated, employing two ion exchange resins and a solid-phase extraction. The use of the two different ion exchange resins, Amberlite MB-6113 and IRN-150, did not give satisfactory results, even though the Amberlite IRN-150 resin performed better under acid elution conditions using 3 mL of TFA 2 M. The very low recoveries and the lack of reproducibility can be also due to the non-efficient packing of the Pasteur column (Eddy-diffusion parameter (A) of the Van Deemeter equation) and to the variable amount of resin introduced.

The aim of the paper was addressed by the determination of the best clean-up method. Thus, the best results were obtained with an SPE using a Macherey-Nagel™ Chromabond™ NH2 Polypropylene SPE column eluting with 1 mL of trifluoroacetic acid 400 mM in a 40% acetonitrile solution, allowing the simultaneous elution of both monosaccharides and uronic acids. Indeed, the acidic elution enables good recoveries of uronic acids, which, in acidic pH, are present in their undissociated form. These uronic acids are thus efficiently eluted (recoveries higher than 60%) from the stationary phase of the SPE column. Under these conditions, recoveries of more than 70% were obtained for xylose, arabinose, glucuronic acid, and glucose. For the other analytes, recoveries are typically greater than 50%.

Finally, the preparation layer of a wall paint sample with a high content of inorganic fraction, assessed by preliminary ATR-FT-IR analysis, was successfully purified and analyzed by GC-MS allowing confirmation of the effectiveness of the method in the removal of inorganic ions and to ascribe the saccharide profile of the hydrolyzed sample to that of a fruit tree gum.

In conclusion, this paper provides a first insight and comparison of clean-up procedures for quantifying monosaccharides and uronic acids in mural paintings. These results fill a gap in the literature related to the quantitative assessment of the removal of inorganic interferences from complex matrices containing saccharides such as paintings, where the presence of inorganic pigments is well known. Nevertheless, future studies need to be carried out to test the efficiency of the optimized method on other matrices not only concerning artistic fields but also foodstuff and writing inks. Moreover, our next steps will be focused on developing and optimizing protocols for the removal of other types of interferences, along with testing SPE columns with different stationary phases and exchanger properties, assessing the most detrimental for the profiling of polysaccharides.

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