Cleaning materials: A compositional multi-analytical characterization of commercial agar powders

CLEANING MATERIALS: A COMPOSITIONAL MULTI-ANALYTICAL CHARACTERIZATION OF COMMERCIAL AGAR POWDERS

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ABSTRACT

Effectiveness and selectivity are the main requirements for a proper cleaning of artworks. The complexity of the soiling and the limits of traditional cleaning methods make it necessary to increase the knowledge on the chemical and physical mechanisms involved in cleaning methods. Agar gels have been successfully used in the restoration practise, but it is not yet clarified if their cleaning capability is due only to the solvent action or also to the polysaccharide structure. A multi-analytical procedure including elemental analyses, vibrational spectroscopies, thermogravimetric analysis and pyrolysis-gas chromatography/mass spectrometry is proposed to characterize agar powders in terms of morphology, composition and thermal stability, and to differentiate agar products intended for different applications, i.e. conservation of Cultural Heritage, biology and food industry. The multi-analytical approach proposed allows differentiating agar samples with slight compositional differences. In particular, agar products can be rapidly differentiated by a semi-quantitative analysis of pyrolysis-gas chromatography/mass spectrometry data. The results presented show that agar powders used for cleaning treatments contain impurities probably derived from cellulosic residues.

KEYWORDS: Agar; Cleaning materials; Cultural Heritage; Py-GC/MS

1. Introduction

Cleaning of artworks consists in removing from their surface unwanted materials, such as discoloured varnishes, efflorescences, films, black crusts or metal staining (e.g. in the case of bases of bronze monuments). The compositional and micro-structural complexity of the materials to be removed call for the design of new highly selective cleaning methods. In this context, gels have been proposed and developed as cleaning materials because they offer several advantages over free solvents. In particular, gels allow for a better selectivity of the cleaning method and for a gentle removal of extraneous materials which could alter the proper readability of the surface underneath. In fact, the use of gel systems ensures a fine control of liquid transport phenomena, thus limiting the penetration in the artwork of free solvents which could lead to swelling and leaching of the organic components in paintings or to mobilization of soluble salts inside the porosity of stone materials. Gels also allow for a better control of the evaporation of the solvent and, more in general, they ensure a higher control of the cleaning over the area to be treated. To be used as cleaning materials, gels must not leave residues and should be easily removed after their application, thus minimizing mechanical stresses on the surface of the artwork. Although gels are already used by restorers and in several other fields (e.g. food, pharmaceutical and cosmetic industries), the relationship among structure, properties and performances are not yet fully understood within conservation.

Gels are composed of interconnected long polymer chains dispersed into a liquid (water and/or other solvents), forming a three-dimensional network which is swelled by the solvent. This kind of structure is able to limit the solvent action to the very surface of the material to be cleaned, reducing risks due to a deep solvent penetration. Polymer gels are usually classified into chemical and physical gels according to the nature of the cross-links connecting polymer chains in a 3D network. Chemical gels are characterized by strong cohesive forces (i.e. covalent bonds) and are irreversible systems, meaning that their cross-linked structure is stable and insoluble; on the other hand, physical gels present weaker interactions (i.e. van der Waals or hydrogen bonds) compared to typical covalent bonds and therefore these systems are usually reversible [1]. The latter class of gels represents a potential improvement in the range of options available as cleaning tools, and agar, being thermo-reversible, belongs to this one.

Agar is a polysaccharide extracted from different species of *Gelidiales* and *Gracilariales* red seaweed [2]. It is a natural polymer used as a biological culture medium for growing fungi or bacteria and it is extensively employed in the food industry as a thickener. It has a

sugar skeleton consisting of two alternating polysaccharides: agarose and agaropectin (Figure 1) [3,4]. The first one is a linear polymer consisting of alternating β-D-galactose and 3,6-anhydro-L-galactose units linked by glycosidic bonds and forms the agar gelling fraction. Agaropectin, the other main agar component, is a heterogeneous agarose heavily modified by substitution of various hydroxyls with sulphate, methoxy and pyruvate groups, that reduce the gelling properties. Depending on the seaweed source, the agarose and agaropectin contents may vary, affecting physicochemical, mechanical and rheological properties of agar gels [5,6]. In particular, as higher is the amount of anhydrous units as stronger is the gel.

Agar forms a hydrophilic and thermoreversible semi-rigid gel after heating and a following cooling process. Indeed, during the cooling, the polysaccharidic chains arrange in a double-helix ordered structure by hydrogen bonding and generate a three-dimensional network containing water [7]. This gel seems to have an intrinsic capacity to be used as a cleaning tool and not only as a thickener of a liquid phase [2,8,9,10]. However, a review of the literature highlights that it is still not clear if the cleaning capability of agar gel is due to the action of solvents (water or organic solvents) entrapped into the polysaccharidic network or also to the polysaccharide structure in itself [11,12]. To clarify this point, the role of agar composition, of minor components (mono and disaccharides or inorganic compounds) and the different performances of various commercial agar samples should be better investigated. Thus, it is of great interest to determine and possibly to correlate compositional differences of agar samples to the specific functional properties of the corresponding gels. Starting from the pioneering works of Araki [2,9] and Duckworth [3] to more recent studies, many attempts have been undertaken to elucidate the chemical composition of agar. The reductive hydrolysis method of Stevenson and Forneaux or its variants are usually used in conjunction with gas chromatography-mass spectrometry (GC/MS) to determine and quantify the monosaccharide composition of polysaccharides from red seaweeds [13,14,15]. ^{1}H and ^{13}C NMR have proved to be powerful techniques for the characterization of agar and recently also Raman and infrared spectroscopies have been used to study in detail the chemical composition of agar, carrageenan and other polysaccharides from red seaweeds [16,17,18,19].

Although these techniques, or a combination of them, have been proposed to differentiate agar from other polysaccharides extracted from red seaweeds, results are sometimes contradictory. Even more challenging is the possibility to discriminate agar samples of various origins.

In this work, two commercial agar powders used by restores for cleaning treatments, an agar sample commonly used for biological applications and an agar product intended for the food industry have been selected and characterized by a multi-analytical approach including: (i) the observation of raw samples at high magnification by scanning electron microscopy; (ii) elemental analyses and vibrational spectroscopy, to identify the organic and inorganic moieties in agar samples; (iii) thermogravimetric analysis, to investigate thermal stability and to quantify the inorganic components possibly present in agar samples; (iv) thermally assisted hydrolysis and methylation (THM) in combination with pyrolysis-gas chromatography/mass spectrometry (Py-GC/MS), to get additional and detailed information on the chemical composition of the polysaccharide chains and minor organic components. THM is a well-known reaction that combines analytical pyrolysis with an alkaline reagent, tetramethylammonium hydroxide (TMAH), to obtain simultaneous hydrolysis and methylation of the products [20].

In the past, analytical pyrolysis was successfully applied to carbohydrate analysis in several studies [21, 22, 23, 24]. In particular, Fabbri *et al.* pyrolysed agar in the presence of hexamethyldisilazane (HMDS) [21]. They identified several markers of the galactose units and especially the trimethylsilylether of 2-furyl hydroxymethyl ketone, considered a characteristic pyrolysis product of agar. The same marker had been identified also in pyrograms of agarose and agar obtained without derivatization and it is thought to be a major pyrolysis product of the 3,6-anhydro-L-galactose units [25].

Other few studies reporting on the use of Py-GC/MS and thermogravimetric analysis to characterize polysaccharides from seaweeds were addressed to investigate the thermal conversion of such polysaccharides for the production of fuels and chemicals, more than to elucidate their chemical structure and composition [26].

In this work, THM has been applied to derivatize polar moieties and to get detailed information on the monosaccharide composition of agar and especially on the type of glycosidic bond between the different repeating units.

The final aim of this study is to assess if it is possible to easily and rapidly differentiate agar samples of different provenance and to correlate compositional differences with specific properties and performances of agar gels used as cleaning materials. In addition, the present work aims at providing a general frame in order to better understand the gel working mechanisms, allowing conservators to optimize their use as to the soiling to remove.

2. Experimental

2.1. Materials

Four different agar powders with different provenance were selected and characterized: AgarArt (CTS, Italy) and Agar Purissimo (Bresciani, Italy) are among the most used gelling materials in the field of conservation; a third agar sample, mostly used for biological applications, was purchased from Sigma Aldrich (A7002_CAS:9002-18-0) and the last agar powder, used in the food industry, was imported from the United Kingdom and in the following is named Agar Food.

The following standard carbohydrates were purchased from Sigma Aldrich and were analysed by Py-GC/MS to be compared with the pyrograms of agar powders: agarose (CAS:9012-36-6), D-(+)-galactose (CAS:59-23-4), 1,6-anhydro-D-glucose (CAS:498-07-7) and D-(+)-glucose (CAS:50-99-7).

The derivatizant used for Py-GC/MS analyses was tetramethylammonium hydroxide (TMAH) in aqueous solution at concentration of 25 % in weight (Sigma-Aldrich, Italy).

2.2. Multi-analytical procedure

2.2.1. Morphological characterization and elemental analyses

Preliminary morphological characterization was carried out by Leitz Wild M420 stereomicroscope with different magnifications. Scanning electron microscopy (SEM) analyses were carried out by JEOL 5910LV microscopy equipped with EDS probe for elemental analyses. The observations were performed directly on raw agar particles, covered by graphite film using back scattered and secondary electrons (BES-SEI) and EDS probe for elemental analyses. The specimen chamber was maintained in high vacuum (HV) and accelerating voltage was 20 kV. Analyses were repeated on five particles for each type of agar.

Additional elemental analyses were obtained by x-ray fluorescence investigations. Analyses were carried out directly on the powder by a portable spectrometer Micro-EDXRF Bruker Artax 200 equipped with x-ray tube (MO anode with adjustable anodic potential from 0 to 50 kV) with a beam collimated to 0,65 mm in diameter. The working condition was 30 kV and 1500 µA without helium flux to avoid sample loss.

2.2.2. Thermogravimetric analyses (TGA)

TGA was carried out by a TA Q500 model from TA Instruments by heating samples contained in alumina pans at a rate of 10 °C/min from 25 to 800 °C in air.

2.2.3. Fourier Transform Infrared Spectroscopy (FTIR)

Infrared spectroscopic characterization was performed with a FTIR spectrometer Thermo Nicolet Nexus equipped with an Olympus Continuμm IR microscope and a mercury cadmium telluride detector (MCT/A) cooled with liquid nitrogen with a spectral range of 4000-650 cm^{-1}. Samples were mounted in diamond anvil cell placed under the microscope objective. The spectral resolution used was 2 cm^{-1}, with accumulations of 256 scans.

2.2.4. Fourier Transform Raman Spectroscopy (FT-Raman)

Raman spectroscopic analyses were carried out with a Nicolet NXR 9650 FT-Raman spectrometer equipped with an InGaAs detector, a diode pumped Nd:YAG laser (wavelength 1064 nm, laser power 200 mW). Spectra with a resolution of 4 cm^{-1} and 256 scans were recorded using a Thermo Scientific MicroStage FT-Raman microscope where powdered samples can be analysed.

2.2.5. Pyrolysis-gas chromatography/mass spectrometry (Py-GC/MS)

For Py-GC/MS analyses agar powders were derivatized with the Thermally Assisted Hydrolysis and Methylation method (THM) using TMAH. Samples were prepared by dissolving 0.1 mg of powder in 50 µL of TMAH solution in an oil bath at 90 °C for about 30 minutes, then 8 µL of solution was spread into quartz wool inserted in a quartz tube for pyrolysis.

A CDS Pyroprobe 1500 (Analytical Inc., USA) filament pyrolyzer directly connected to a GC/MS system was used. The GC was a 6890N Network GC System (Agilent Technologies, USA) gas chromatograph with a methylphenyl-polysiloxane cross-linked 5% phenyl methyl silicone (30 m, 0.25 mm i.d., 0.25 µm film thickness) capillary column. The temperature of pyrolysis is 600 °C, the temperature of pyrolysis interface is 300 °C and the temperature of the injector is kept at 280 °C. The carrier gas was helium (1.0 mL/min) and split ratio was 1/20 of the total flow. The mass spectrometer coupled to the GC apparatus was a 5973 Network Mass Selective Detector (Agilent Technologies, USA). Mass spectra were recorded under electron impact at 70 eV, scan range 40-600 m/z. The interface was kept at 280 °C, ion source at 230 °C and quadrupole mass analyzer at 150 °C. All instruments were controlled by Enhanced Chem Station (ver. 9.00.00.38) software. The mass spectra assignment was done with the Wiley 138 and NIST1992 libraries and by comparison with literature data [21].

3. Results and discussion

3.1. Morphological characterization and elemental analyses

Morphology of raw agar particles is considered an interesting issue because it could influence the solubility process, the homogeneity of the gel structure and its strength. Agar particles, analysed as obtained by suppliers and without any preliminary treatment, seem to have no particular and peculiar differences in terms of morphological aspect. Stereomicroscope images of the different samples show particles with irregular shape and variable dimensions, ranging from few microns to hundreds of microns. The heterogeneity of the analysed powders was also confirmed by scanning electron microscopy which clearly shows particles with rounded and pointy forms in each of the agar samples. EDS analysis (Figure 2) and x-ray fluorescence allowed to classify the raw materials in two classes: one group, composed of Agar Purissimo and AgarArt, shows an important contribution of alkaline metals, such as Na and K, a contribution of Ca, a low content of S, but a significant amount of P, which on the contrary is not recorded in Sigma and Food agar powders. These latter are characterised by the presence of Ca, Na and Fe, a lower amount of K and an interesting contribution of Br. Moreover, all powders except AgarArt show a Cl contribution; this last result could be due to frequent washings and purification treatments of the red seaweed extract before drying.

Elemental analyses, repeated on five particles for each type of agar, gave similar results. Overall, these results prove that the different agar powders contain a variety of ionic impurities. The network-surface interactions are strictly dependent on the substrate polarity, therefore the presence of ionic impurities is a fundamental indication for the restorer, especially with reference to the unsuitable practice of using agar gel plugs for the measurements of surface conductivity of artworks before and after cleaning.

3.2. Thermogravimetric analysis

Thermogravimetric analyses of agar samples provided information on both thermal stability and chemical composition. The oxidative atmosphere of the TGA chamber induced the complete degradation of any organic substance and allowed to determine the amount of inorganic components, which is the residue at the end of the analysis. In general, dehydration and depolymerization are considered the two main processes associated with the degradation mechanism of polysaccharides. Thermal degradation of polysaccharides occurs at first by release of volatiles, such as H_2O, CO_2 and CO, and scission of glucose rings. Then at higher temperature depolymerisation reactions completely degrade the polysaccharidic chains.

TGA curves of agar samples in Figure 3 show a first weight loss at 100-130 ºC due to the release of physisorbed water. The second weight loss occurs at a different temperature depending on the sample and points to a different thermal stability of the agar powders considered for this study. While Agar Purissimo and Agar Food begin to degrade soon after dehydration, at approximately 140 °C, Agar Sigma and AgarArt show a higher stability, undergoing a fast and steep weight loss at temperatures higher than 200 °C. The third weight loss occurring at over 400 °C leads to a complete degradation of the organic components. At the end of the analysis all powders gave a remarkable residue which is in the range of 3-7% of the total weight depending on the agar sample. According to XRF and EDS analyses, the inorganic residue consists of alkaline sulphates and phosphates, in particular with Mn traces in AgarArt and Agar Purissimo residues and Zn and Ti in Food and Sigma ones.

3.3. Vibrational Spectroscopies

Infrared and Raman spectroscopies have been used to characterize qualitatively the chemical composition of four agar samples. All FTIR spectra recorded show very similar absorption features. In Figure 4a the FTIR spectra of the different samples are reported. On the contrary, FT-Raman spectra recorded in different points of the samples show some peculiarities (Supplementary Material, Figure S2). At first glance all spectra present the same common scattering pattern represented by the spectra of Agar Sigma and Agar Food samples. However in the spectra of Agar Purissimo and Agar Food it is possible to identify some extra features. The representative FT-Raman spectra are reported in Figure 4b (the spectrum shown for the Agar Purissimo sample is that with the most evident extra features). The origin of these new features will be discussed later.

The common patterns for the IR spectra and all the FT-Raman spectra, as expected, can be assigned to the characteristic absorption and scattering bands of polysaccharides from red seaweed in accordance with the literature data [13,17,27].

The strong and broad band in the range 3400-3300 cm^{-1} of IR spectra is assigned to the stretching vibration of OH groups. In the same region Raman spectra show weak signals of hydroxyl band. At lower wavenumber (region 2900-2800 cm^{-1}) the strong Raman signals and the weak IR absorptions are assigned to CH stretching modes. The peaks observed in the region between 1430 and 1200 cm^{-1} (both in IR and Raman spectra) can be assigned to sulphates and C-O-H bending vibrations of hydroxyl groups. In particular, the Raman peaks occurring in the regions 1370-1320 cm^{-1} and 1280-1240 cm^{-1} are characteristic of symmetric and asymmetric stretching of organic sulphates [28]. The most prominent band

in IR and Raman spectra of agar powders at 1074 cm^{-1} and 1080 cm^{-1} respectively, can be assigned to the C-O stretching of 3,6-anhydro-L-galactose, with the contribution of the C-C-O out-of-phase stretching of primary and secondary alcohols of saccharide rings. Peaks at approximately 930 and 890 cm^{-1} can be assigned to 3,6-anhydro-L-galactose and galactose units respectively [13,18,27]. FTIR and Raman spectra also show several other weak features that can be assigned to sulphate side-groups on D-galactose: indeed the weak signal at 870 cm^{-1}, which appears in both FTIR and Raman spectra, is assigned to the C-O-SO_3^- vibration of the sulphate group in position C6 of D-galactose, while the Raman peak of medium intensity at 845 cm^{-1} is assigned to sulphate groups in position C4. Moreover, according to Souza [13] the shoulder present at 820 cm^{-1} in FTIR spectra (marked with an asterisk in Figure 4) is related to the presence of the sulphate group linked to the C6 of D-galactose units, which are converted to 3,6-anhydrogalactose by the alkaline treatment used during the extractive procedure. The region of the spectra between 770 and 740 cm^{-1} presents other characteristic bands of agar assigned to the skeletal bending of pyranose rings.

The extra features observed in the FT-Raman spectra of Agar Purissimo and Agar Food can be highlighted by a spectral subtraction between spectra recorded in different points of the samples (Supplementary Material, Figures S2, S3 and S4). Both samples show the same series of extra peaks located at 2994, 2890, 1457, 1343, 1269, 1117, 1071, 914, 841, 770, 540, and 404 cm^{-1}. All these peaks well correlate with the Raman peaks of crystalline glucose [29]. This evidence proves that Raman spectra can be used to identify the presence of glucose in agar samples.

3.4. Pyrolysis-Gas Cromatography/Mass Spectrometry

Py-GC/MS analyses were performed in order to better elucidate structure and composition of the polysaccharides present in agar powders. Previous studies demonstrate that by THM is possible to identify characteristic markers of both mono- and polysaccharides [21,23]. Under the alkaline condition provided by the presence of TMAH, reducing sugars react producing the so called saccharinic acids. The alkaline degradation of carbohydrates is known as Nef-Isbell mechanism [30] and during the complex reaction pathway isomerization of an anionic intermediate takes place, resulting in an equilibrium mixture of intermediate anions and two isomers as final products. An important consequence of the anion formation is the destruction of chirality at the C2 position. The reprotonation of the anion results in the formation of a C2 epimer (for example, if this mechanism is applied to glucose, mannose is obtained). As a consequence of this rearrangement carbohydrates

which differ in the stereochemistry of position 2 and 3 only, yield the same pairs of isomers [21, 22, 30]. In THM there are two competing reactions occurring simultaneously, the pyrolytic cleavage of the polymeric structure already methylated and the alkaline degradation followed by methylation. Also, several side reactions may occur, depending on pyrolysis temperature, concentration of the reagent and state of the sample [31].
In a previous study by some of the authors [23] we found that preparing the sample by dissolution in a TMAH aqueous solution prior to the analysis is better than adding TMAH to the solid sample. Applying this procedure cleaner and more readable pyrograms were obtained, side-reactions were reduced and the degree of methylation was improved.
Free monosaccharides and 3-O-substituted monosaccharides (or 1,3 linked disaccharides/polysaccharides) give the same reaction products, generically named 3-deoxy aldonic acids (or saccharinic acids). Furthermore, sugars which only differ in the stereochemistry of position C2 or C3 (for example, glucose and fructose) yield the same pair of deoxy aldonic acids. 4-O substituted monosaccharides and in general 1,4 linked polysaccharides (i.e cellulose) react in slightly different ways, giving isosaccharinic acids as characteristic markers [30]. On the contrary the 1,6-glycosidic bond is not detectable because these carbon atoms are not sensitive to alkaline degradation [32]. The identity of the saccharinic acids produced upon alkaline degradation can thus be used to provide distinction between 3-O and 4-O substituted sugars.
Pyrograms of the four agar samples (Figure 5) highlight an interesting and tricky composition. The main compounds identified in the pyrograms are listed in Table 1. They were identified using a combination of mass spectral databases and retention data for standard components. Assignments of the saccharinic acids are interchangeable, due to the identical mass spectra of the isomers. Also, the assignment of saccharinic acids to a specific sugar only on the basis of THM results is usually not possible, due to the rearrangement reactions previously detailed. In the present study the assignment is also based on the fact that we have a prior knowledge of the polymer, knowing that the base monomer is galactose. Furthermore, galactose, being a epimer on C4, gives its own specific couple of isomers. All agar powders confirm the presence of galactose, according to peaks **10**, **12**,**15**, **16**, which correspond to the permethylated 3-deoxy-hexonic acid methyl ester (**12**, **16**) and partially methylated one (**10**, **15**), as confirmed by the analyses carried out on the galactose standard (not reported here). These markers are assigned to the D-galactose linked on C3 present in agarose and in agaropectin. Galactose derived from agaropectin can be derivatized on C6 with different substituents, but with THM the substitution on C6 is not detectable, because these carbon atoms are not sensitive to

alkaline degradation [32]. Permethylated (**9**, **14**) and partially methylated (**8**, **13**) saccharinic acids are assumed to be derivatives of glucose. This assignment is supported by the previously discussed spectroscopic results (see Section 3.3), that show evidences of the presence of free glucose.

Agar Purissimo and AgarArt show an important difference compared to the other two samples: in their pyrograms three additional peaks, which are completely absent in the other two samples, were found and assigned on the basis of previous studies [21,23,33] to the partially methylated (**6**, **7**) and permethylated (**11**) isosaccharinic acid derived from 4-O-substituted glucose. The isomer of the permethylated compound (**11**) was not found in the pyrogram, probably because of its co-elution with other analytes. All the carbohydrate markers have in fact similar mass spectra and often identical molecular mass can cause co-elution or minimal differences in retention time, as shown in Figure 5, where the carbohydrate markers elute in a range of about 1 minute.

These compounds, typical of cellulose, are probably indicative of the presence of algal residues in Agar Purissimo and AgarArt, due to incomplete product purification. The absence of cellulosic derivatives in the agarose standard (not shown here) and in agar from Sigma-Aldrich, which is supposed to be a pure product, confirms this hypothesis. Moreover, the presence in all of the four powders of peaks **8** and **14**, due to the reaction of free or 3-O-substitued glucose, should be indicative of the presence of glucose, not chemically bonded to the polysaccharide chains of agar.

Some minor common products were also identified in the first part of the pyrograms of the four samples, but none of them can be considered diagnostic of agar (see full pyrograms reported in Figure S5, Supplementary Material). Product **5** (i.e. 1,2,4-trimethoxybenzene) is a typical THM product from mono- and polysaccharides whose formation from thermochemolysis of polysaccharides was reported in the literature [21,22,33,34]. Smaller molecules deriving from degradation of polysaccharide chains and THM main products (peaks **1-3**) were identified at low retention time. Peak **4**, detected in all the samples, is unknown, but this product was also identified in previous works [30], in particular in the pyrograms of cellobiose, cellulose and cellulose acetate, and may be considered as an additional marker of the presence of 1-4 glycosidic bonds. It is noteworthy that 2-furyl hydroxymethyl ketone, that was reported to be a marker of the 3,6-anhydrogalactose units [21,25], was not detected, possibly because the strong alkaline environment created by TMAH prevents formation and detection of this pyrolysis product.

1,6-Anhydro-β-glucopyranose (or levoglucosan) was also analyzed and was used as a general reference for anhydrosugars, because the anhydrous derivative of galactose is not

commercially available. The pyrogram of levoglucosan shows a simple pattern, in which its permethylated compound and peaks 9 and 13 of glucose derivatives are detected. On the other hand, the pyrograms of the four agar powders and of standard agarose do not show any anhydrosugar peak. This can be explained considering that in agar the anhydrous derivative of galactose is linked on C4 with galactose to form the agarose chain, thus its reactivity may be different from that of the unlinked form. Therefore THM of agarose and of the different agar powders can only confirm the presence of galactose, but it is not able to detect the anhydrous part of the polymer. This lack of information is important, because it is known from the literature [3] that the anhydrous part of the polymer is responsible of the strength of the gel and it is also affected by the alkaline treatment applied to extract agar from red seaweeds.

Despite this limit, the high reproducibility of the Py-GC/MS analyses in the presence of TMAH permitted a semi-quantitative assessment of the composition of the different agar samples. This was done by determining the content percentage of the main constituents of agar by integration of their chromatographic peaks. Only peaks at higher retentions times, reported in Figure 5, were used in the quantification process, while pyrolysis products eluted at shorter retention time were not used because not relevant from the quantitative point of view. The percentage data reported in Table 2 have been obtained by dividing the area of the pyrolysis markers of the main components with the total area of all the pyrolysis markers. In particular, the amount of galactose is indicative of the purity of the sample, because the polysaccharides of agar are composed mainly of galactose. Table 2 confirms that the analysed agar samples differ in their purity. Sigma and Food show higher purities with 82-87% of galactose and only 2-7% of free or 3-O-sobstitued glucose, respectively. On the other hand, the obtained semi-quantitative results confirm that Agar Purissimo and AgarArt show a more heterogeneous chemical composition in accordance with the qualitative data provided by Py-GC/MS. The amount of galactose in AgarArt is considerably higher than in Agar Purissimo, with percentage values of 73% and 56%, respectively, thus suggesting a better efficacy of the purification process. Moreover, the compositional differences are even more evident if the ratio between free/3-O-substitued glucose and 4-O-substituted glucose is considered. Agar Purissimo shows an important amount of 4-O-substituted glucose (*ca.* 23%) with also a moderate amount of free/3-O-substitued one (11%), on the contrary AgarArt exhibits only 3% and 9% of free/3-O-substitued and 4-O-substituted glucose, respectively. This finding is confirmed by Raman spectra where peaks due to crystalline glucose were detected in Agar Purissimo, but not in AgarArt. Possibly, these differences in composition between Agar Purissimo and AgarArt

could be related to differences in the performance of the corresponding gels during cleaning treatments. A detailed characterization of the performance of agar gels aimed at addressing this question is currently ongoing.

Overall, Py-GC/MS proves to be a valuable technique to differentiate agar powders of different origin and to identify impurities: in Agar Purissimo and AgarArt all possible pyrolysis products of galactose (**11**, **12**, **15**, **16** peaks) were identified, together with some additional peaks (**6**, **7**, **9**) that can be associated to the expression of (1→4)-linked units of glucose. For this reason, Agar Purissimo and AgarArt seem to be multicomponent products composed by different types of polysaccharides, probably derived from cellulosic residues. On the contrary Agar Food and Sigma are purer in composition. In this respect it is noteworthy that the purity of Agar Sigma is not properly indicated on the label of the product; the only hint is about the ash residue, indicated as 2.0-4.5%. The purity of the other three powders, and by extension the performance of their corresponding gels, may change overtime as the companies change their suppliers.

4. Conclusions

Four different agar products commercialized for different applications, such as conservation of Cultural Heritage, food industry and biological applications, have been characterized. The multi-analytical approach proposed provides qualitative and semi-quantitative compositional data that allow differentiating agar samples with slight compositional differences. The four agar powders are very similar both as for the particle morphology and composition. Elemental analyses show some differences in the content of P, S and metals.

FTIR spectral patterns are too similar to discriminate among the four agar samples. The principal signals have been assigned to the vibrations of specific functional groups of the polysaccharide structure and some minor peaks have been assigned to sulphates, possibly due to the agaropectin component. FT-Raman spectral patterns confirm the agarose and agaropectin chemical composition of the samples and clearly show the presence of crystalline glucose in Agar Purissimo and Agar Food samples. This feature is also in accordance with the semi-quantitative analysis of the composition of the four agar samples performed by Py-GC/MS, proving that these two powders contain the higher amount of glucose. Importantly, TGA curves show that Agar Purissimo and Agar Food start to lose weight at lower temperature than the other agar samples, which may be due

to the volatilization of free glucose. TGA curves also show a variable and significant content of inorganic compounds.

Agar products intended for conservation treatments can be easily distinguished from the other agar samples by Py-GC/MS, because of the presence of partially methylated and permethylated isosaccharinic acids derived from 4-O-substituted glucose. In addition, the two agar samples used as cleaning materials can be differentiated by a semi-quantitative analysis of Py-GC/MS data. Overall, Py-GC/MS data show that agar powders used for cleaning treatments contain impurities probably derived from cellulosic residues.

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Figure captions

Figure 1. Chemical structure of agarose and of possible repeating disaccharide units of agaropectin with different substituents (i.e. hydroxy, methoxy, sulphate, ketal pyruvate).

Figure 2. EDS analysis of a Agar Art (a) and Agar Food (b). Insets show SEM images of typical particles of powdery agar samples.

Figure 3. TGA curves of agar samples: Agar Art (red, circles), Agar Purissimo (blue, squares), Sigma (black, asterisks) and Food (green, diamonds).

Figure 4. FTIR (left) and FT-Raman (right) spectra of agar samples: Agar Art (a), Agar Purissimo (b), Agar Sigma (c) and Agar Food (d).

Figure 5. Pyrograms of agar samples: Agar Art (a), Agar Purissimo (b), Agar Sigma (c) and Agar Food (d).

Table 1. Characteristic pyrolysis products of agar samples derivatized with TMAH.

Peak	Rt (min)	Assignment*	Tentative sugar assignments	m/z	MM
1	3.15	*2-hydroxypropanoic acid, methyl ester*	-	45	104
2	3.44	*Methoxyacetic acid, methyl ester*	-	45	104
3	3.80	*2-methoxypropionic acid, methyl ester*	-	59	118
4	10.20	*Unknown (m/z 144, 131, 113, 99, 71)*	-	71	-
5	12.88	*1,2,4-trimethoxybenzene*	-	168	168
6	13.77	*tri-O-methyl-3-deoxy-2-methoxymethyl-D-erythro-pentonic acid, methyl ester*	**GLU 4-O substituted**	173	236
7	14.16	*tri-O-methyl-3-deoxy-2-methoxymethyl-D-threo-pentonic acid, methyl ester*	**GLU 4-O substituted**	173	236
8	14.20	*tri-O-methyl-3-deoxy-D-arabino-hexonic acid, methyl ester*	**GLU**	129	206
9	14.24	*tetra-O-methyl-3-deoxy-D-arabino-hexonic acid, methyl ester*	**GLU**	129	220
10	14.37	*tri-O-methyl-3-deoxy-D-xylo-hexonic acid, methyl ester*	**GAL**	129	236
11	14.40	*tetra-O-methyl-3-deoxy-2-methoxymethyl-D-erythro/threo-pentonic acid, methyl ester*	**GLU 4-O substituted**	129	264
12	14.46	*tetra-O-methyl-3-deoxy-D-xylo-hexonic acid, methyl ester*	**GAL**	129	250
13	14.51	*tri-O-methyl-3-deoxy-D-ribo-hexonic acid, methyl ester*	**GLU**	129	206
14	14.61	*tetra-O-methyl-3-deoxy-D-ribo-hexonic acid, methyl ester*	**GLU**	129	220
15	14.65	*tri-O-methyl-3-deoxy-D-lyxo-hexonic acid, methyl ester*	**GAL**	129	236
16	14.76	*tetra-O-methyl-3-deoxy-D-lyxo-hexonic acid, methyl ester*	**GAL**	129	250

*Assignments of saccharinc acid isomers are interchangeable

Table 2. Content percentage of the three main constituents of agar as identified by Py-GC/MS analyses.

	Agar Purissimo	**Agar Art**	**Agar Sigma**	**Agar Food**
GAL (%)	56 ± 7	73 ± 1	82 ± 7	87 ± 8
GLU (%)	11 ± 3	3 ± 2	2 ± 0.5	7 ± 2
GLU-4-substituted (%)	23 ± 3	9 ± 7	-	-

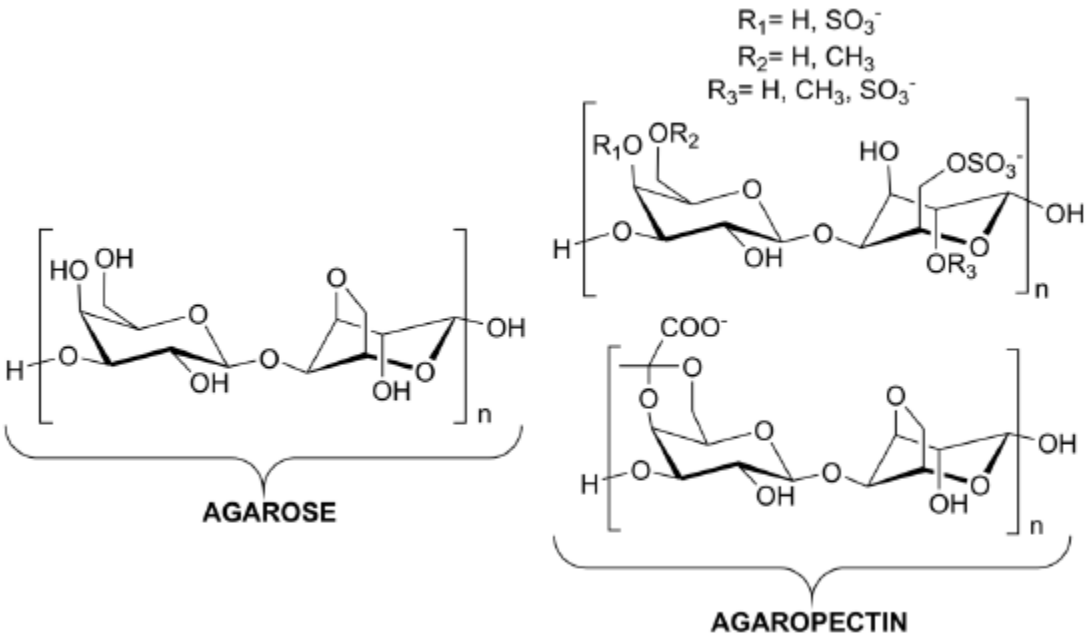
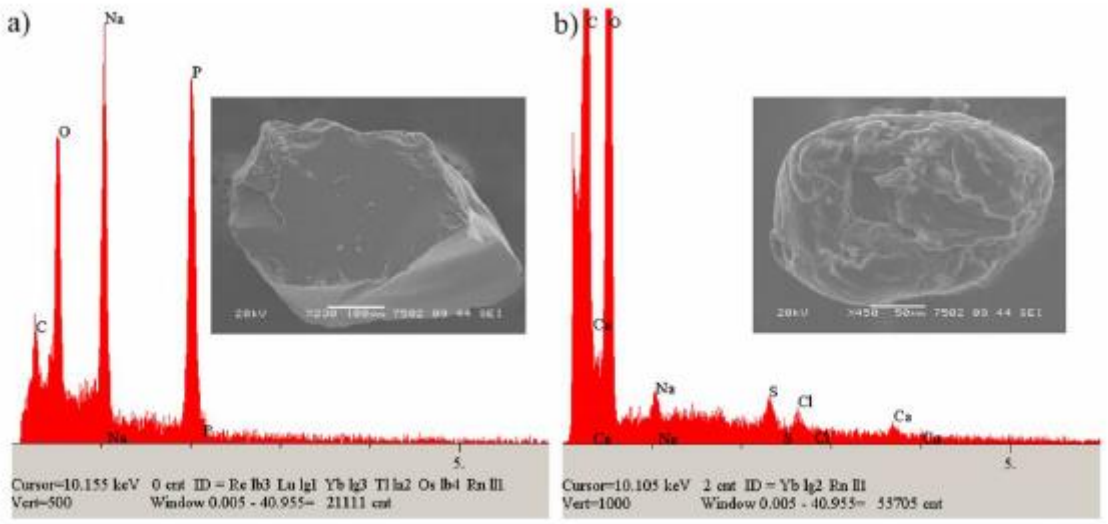


Figure 1


a)
Na
P
O
C
Na
P
5.
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Vert=500 Window 0.005 - 40.955= 21111 cnt
b)
C
O
Ca
Na
S
Cl
Ca
Ca
Na
Cl
Ca
5.
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Figure 2

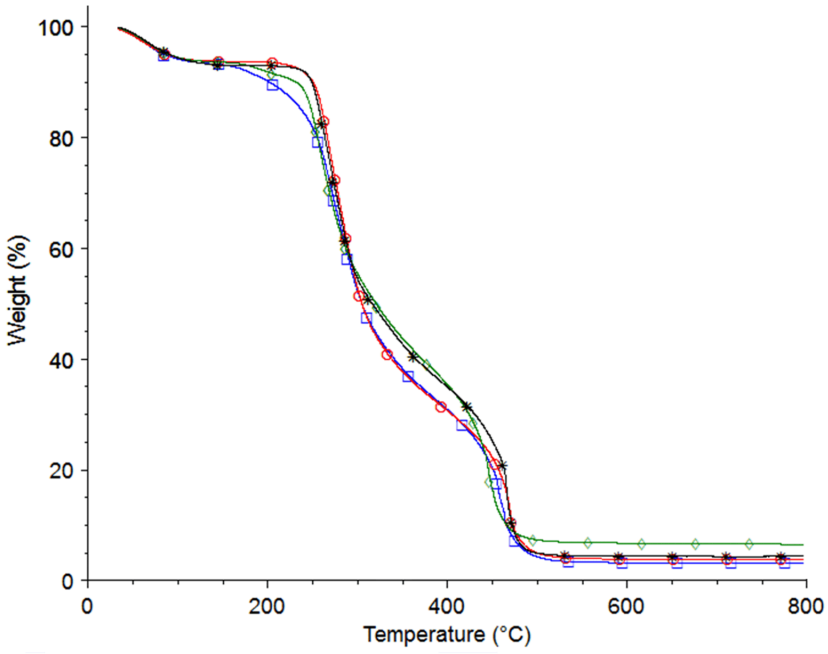

Figure 3

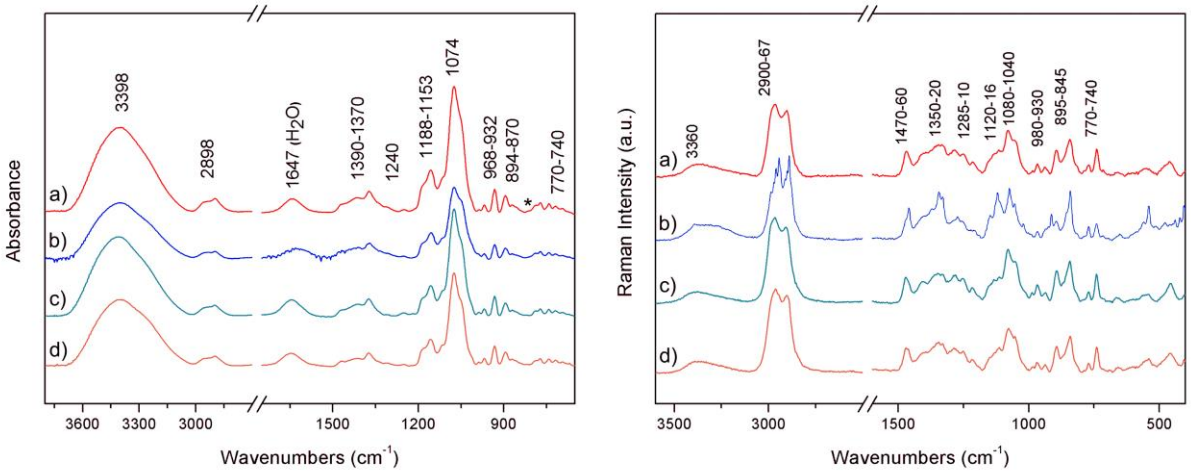
Absorbance
3398
2898
1647 (H2O)
1390-1370
1240
1188-1153
1074
968-932
894-870
770-740
a)
b)
c)
d)
3600
3300
3000
1500
1200
900
Wavenumbers (cm-1)
Raman Intensity (a.u.)
3360
2900-67
1470-60
1350-20
1285-10
1120-16
1080-1040
980-930
895-845
770-740
a)
b)
c)
d)
3500
3000
1500
1000
500
Wavenumbers (cm-1)

Figure 4

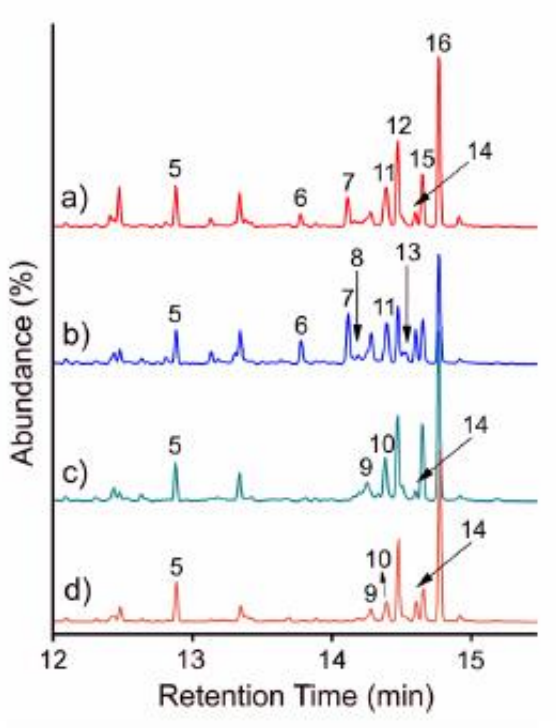
a)
b)
c)
d)
5
6
7
8
9
10
11
12
13
14
15
16
Abundance (%)
12
13
14
15
Retention Time (min)

Figure 5

Supplementary Material

Cleaning materials: a compositional multi-analytical characterization of commercial agar powders

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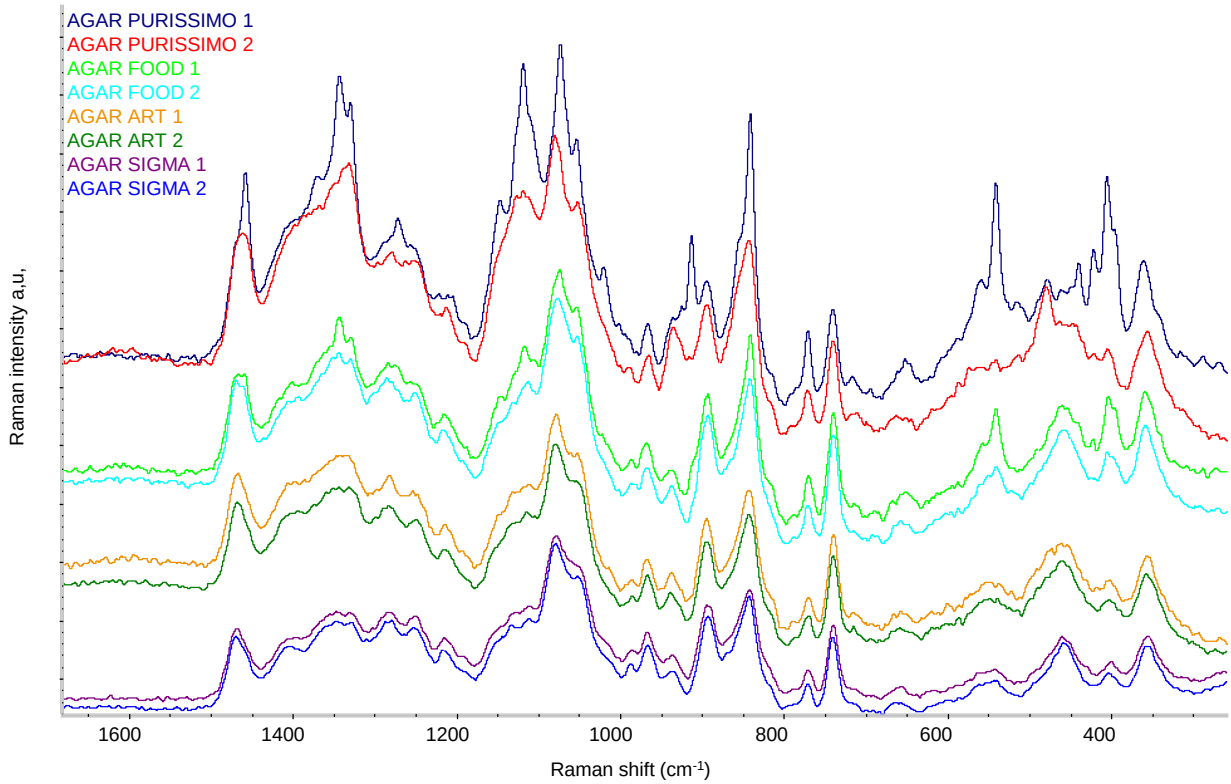

Figure S1. FT-Raman spectra of Agar samples in the region 1600 - 300 cm^{-1}. For every sample two different spectra are reported. Agar Purissimo and Agar Food show some extra Raman lines floating on the characteristic spectral pattern of agar represented by the spectra of Agar Sigma and Agar Art samples.

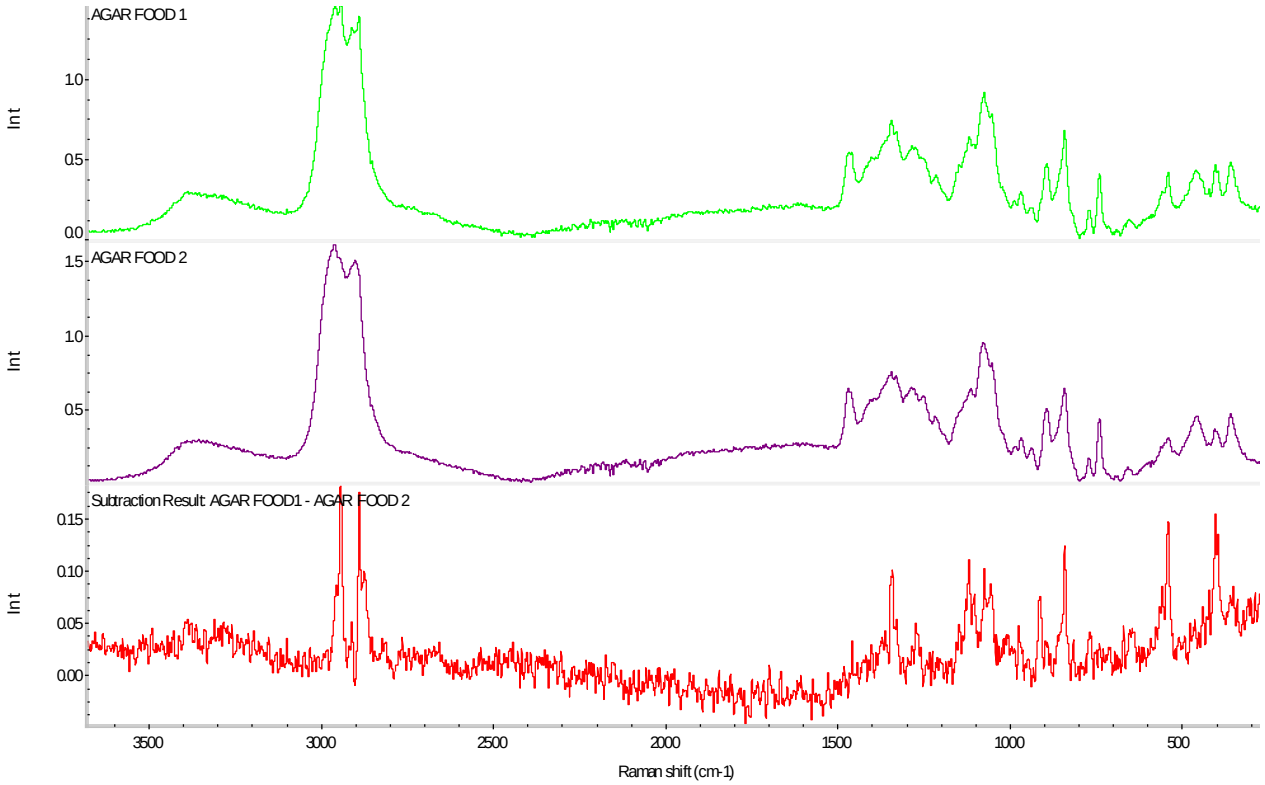

Figure S2. FT-Raman spectra of Agar Food recorded in two different points of the sample and their spectral subtraction (red line).

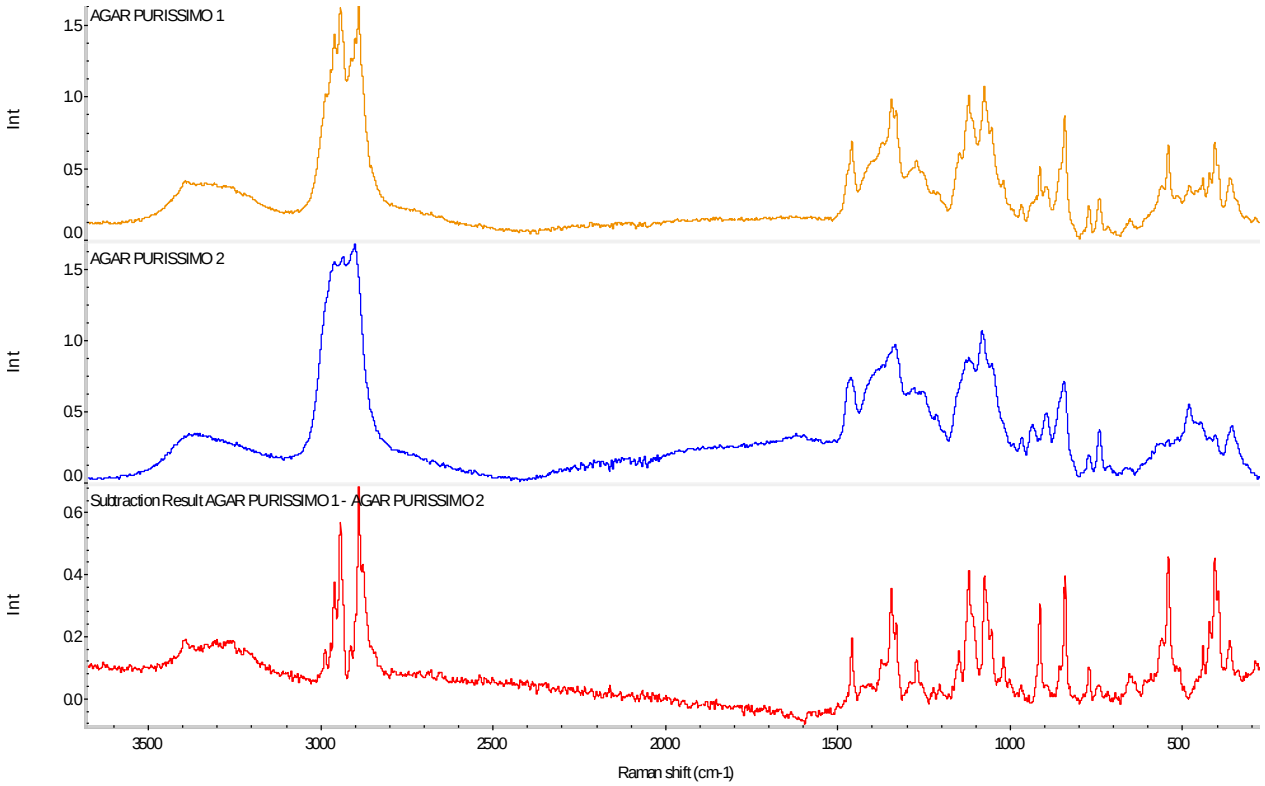

Figure S3. FT-Raman spectra of Agar Purissimo recorded in two different points of the sample and their spectral subtraction (red line).

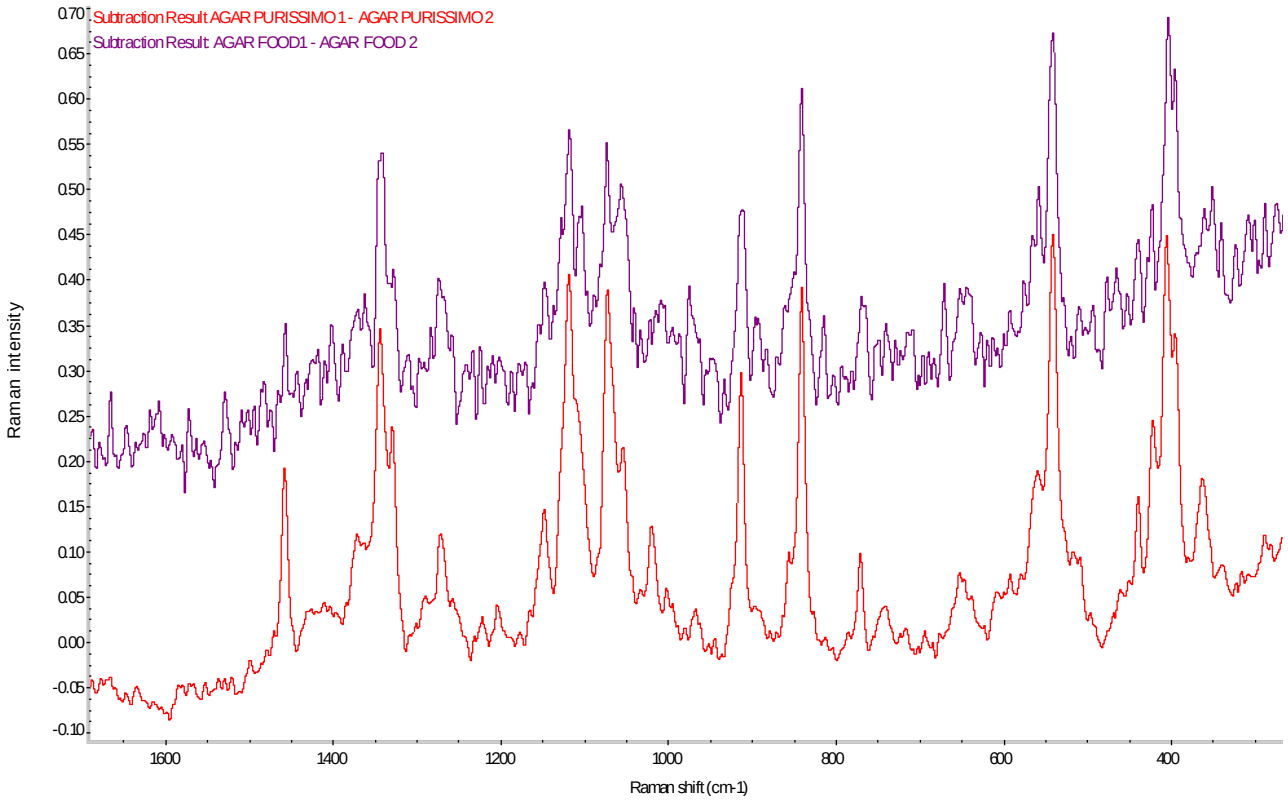

Figure S4. Comparison of the Agar Food and Agar Purissimo spectral subtractions in the region 1600 - 300 cm^{-1}. The peaks of the two samples are superimposable and are located at 1457, 1343, 1269, 1117, 1071, 914, 841, 770, 540, and 404 cm^{-1} revealing the presence of crystalline glucose.

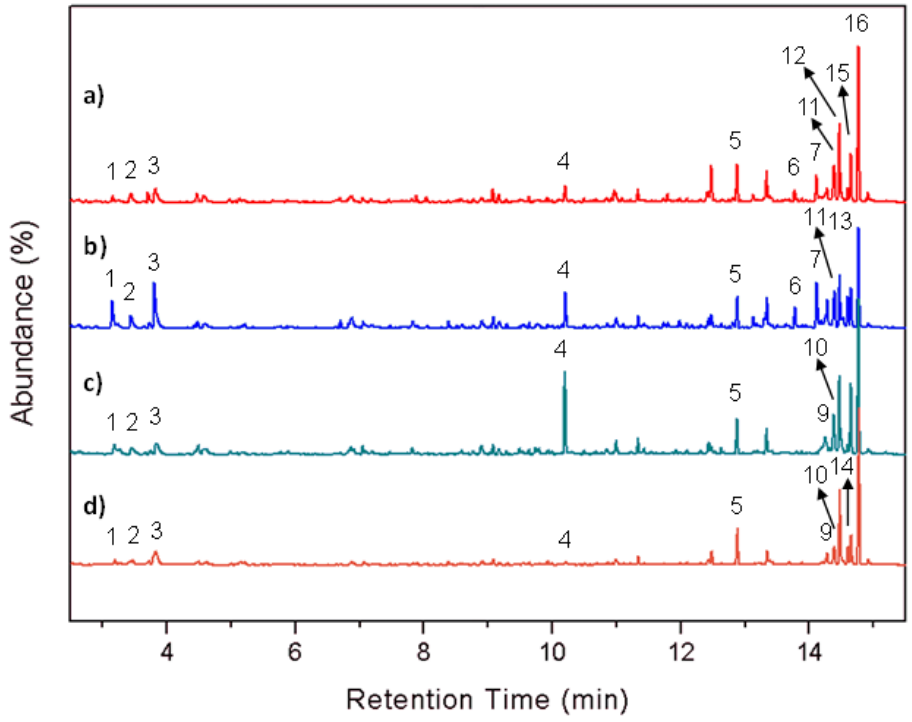

Figure S5. Full pyrograms of agar samples: Agar Art (a), Agar Purissimo (b), Agar Sigma (c) and Agar Food (d).