Carbohydrate Research 344 (2009) 120–126

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

Study on the maltooligosaccharide composition of mucilage samples collected along the northern Adriatic coast

Nunzio Penna [a,*], Samuela Capellacci [b], Fabio Ricci [b], Marco Giorgi [b], Antonella Penna [b], Giorgio Famiglini [c], Elisabetta Pierini [c], Helga Trufelli [c], Achille Cappiello [c]

[a] Chemical Oceanography, University of Urbino, Loc. Crocicchia, 61029 Urbino (PU), Italy
[b] Center of Environmental Biology, University of Urbino, V.le Trieste, 296-61100 Pesaro (PU), Italy
[c] Istituto di Scienze Chimiche 'F. Bruner', Piazza Rinascimento 6, 61029, Urbino (PU), Italy

ARTICLE INFO

Article history:
Received 5 May 2008
Received in revised form 7 October 2008
Accepted 9 October 2008
Available online 17 October 2008

Keywords:
Mucilage
Adriatic sea
Maltooligosaccharides
Carbohydrates
HPLC–RI
ESIMS/MS

ABSTRACT

The mucilage phenomenon, a sporadic but massive accumulation of gelatinous material, can cause serious damage to the tourism and fishing industries along the Adriatic coast. Mucilage is presently thought to be the result of the aggregation of dissolved organic matter (DOM) into particulate organic matter (POM). Three principal classes of compounds have been identified in organic matter by spectrometric determination: carbohydrates, proteins and lipids. Carbohydrates are suspected to play a role in the first steps of DOM aggregation. Despite its importance in understanding the processes leading to mucilage formation, our present knowledge of the composition of the mucilage carbohydrate fraction is incomplete. Due to its high sensitivity and specificity, liquid chromatography coupled with electrospray-ionization tandem mass spectrometry (LC–ESIMS/MS) is gaining an increasing importance as a powerful technique for carbohydrate purification and characterization in complex samples. In this work, LC–ESIMS/MS is proposed as a useful method for the investigation of the oligosaccharide content in mucilage samples. The approach was applied using 3–7 unit maltooligosaccharides as reference compounds. The composition of the investigated mucilage sample was further investigated combining LC–ESIMS/MS with classic approaches, such as spectroscopic techniques and liquid chromatography coupled with the refractory index LC–RI.

1. Introduction

The first record of the presence of mucilaginous material in the Adriatic Sea dates back to the year 1729.[1] Several records have been registered in various periods from the year 1800 up to the present date. Recent episodes occurring from 1997 to 2004 have been of much lesser intensity than preceding events occurring during 1989 and 1991, when considering the extension of area, as well as the period of duration.[2–7] One of the most likely hypotheses regarding mucilage formation is based on the composition of algal populations: diatoms as well as dinoflagellates, under unbalanced nutritional conditions (N/P ratio), can produce exudates responsible for the formation of aggregates.[8–10]

Additional climatic conditions such as a calm sea, stability along the water column and high water temperature are necessary as well. Bacteria also play an important role in the formation of mucilaginous material since it is produced as result of interactions among these and organic substances.[11] Another recent hypothesis concerns the role of marine viruses.[12,13] This paper mainly focuses on the characterization of the maltooligosaccharide content of the water-soluble fraction of mucilage. The identification of these maltooligosaccharides is therefore important to obtain more information about the trigger mechanisms leading to the conversion of dissolved organic matter (DOM) into particulate organic matter (POM).[14,15]

In our previous work[34] we used liquid chromatography coupled to a refractive index detector (HPLC–RI) to qualitatively investigate the oligosaccharide content of mucilage. Considering the low selectivity of the RI detection, in this work the oligosaccharides investigation has been carried out using liquid chromatography coupled to electrospray-ionization tandem mass spectrometry (HPLC–ESIMS/MS).

Nowadays, several advantages make this approach one of the methods of choice for carbohydrate purification and characterization in the field of glycobiology.[16] Unlike GC–MS, maltooligosaccharides can be analyzed in their native form without hydrolysis and derivatization, and the preliminary chromatographic separation allows the purification of the carbohydrates from the complex mucilage matrix. In addition ESIMS/MS detection can be exploited to obtain structural information regarding the eluted maltooligosaccharides. Based on these assumptions, a two-stage ion-trap

* Corresponding author. Tel.: +39 0721 423526; fax: +39 0721 423522.
E-mail address: cebiam@uniurb.it (N. Penna).

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doi:10.1016/j.carres.2008.10.008

mass spectrometer, equipped with an ESI ion source was employed to qualitatively investigate the maltooligosaccharide composition of three macroaggregate samples collected in various locations of the northern Adriatic Sea during the summers of 2002, 2003 and 2004.

2. Results

As reported in Section 1, the main goal of this study is to provide useful information regarding the maltooligosaccharide content of the samples under investigation. With this aim, the soluble mucilage water fraction, in which maltooligosaccharides are suspected to be present, was firstly analyzed by HPLC–RI. As an example, the comparison between the chromatograms displayed in Figure 1A (standard) and Figure 1B (sample) shows that maltotetraose and maltopentaose are likely present in sample B. A confirmation of peak identity was tempted by adding a known amount of the standard to the real sample; the enhancement of the peaks of interest (Fig. 1C and D) seems to confirm the presence of the two maltooligosaccharides. Following this approach, the preliminary screening by HPLC–RI revealed the presence of maltotriose and maltopentaose in mucilage sample A, while the presence of maltopentaose can be hypothesized in sample C.

The preliminary evaluation of the electrospray-ionization behaviour of the standard maltooligosaccharides revealed that these compounds are mainly ionized in the positive-ion mode through the formation of intense mono-sodiated molecular ions, $[M+Na]^+$, with an excellent signal-to-noise ratio as reported in the ESIMS spectrum of maltopentaose (Fig. 2). This ionization behaviour is typical of neutral maltooligosaccharides, as these are strong ligands for alkali metal ions.[16–18]

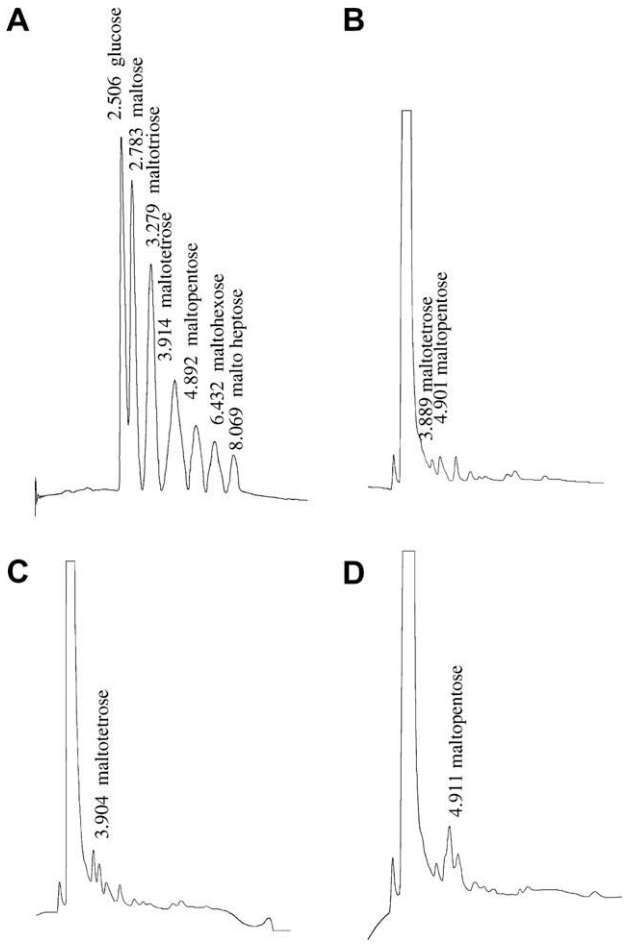

Figure 1. HPLC–RI chromatograms of (A) standard 100 mg/L, (B) sample B, (C) sample B spiked with tetraose (3 mg/L) and (D) sample B spiked with pentaose (3 mg/L).

MS/MS experiments were carried out to investigate the fragmentation patterns of the standard maltooligosaccharides. The CID spectra obtained for their sodiated molecular ions are reported in Figure 2. The nomenclature for oligosaccharide fragmentation was first described by Domon and Costello.[19] The fragmentation process of the underivatized alkali-cationized ions of maltooligosaccharides may lead to glycosidic cleavage with charge retention at the reducing end, which leads to Y_n or Z_n fragments, or with charge retention at the nonreducing end, which leads to B_n or C_n fragment ions (subscripts refer to the number of rings retained). A_n and X_n fragment ions, produced by cleavage across the glycosidic ring, may also be formed and are labelled by assigning each ring bond a number, and counting clockwise. According to this scheme of fragmentation, the CID spectrum of the maltopentaose sodium adduct ion (*m/z* 851) is characterized by the presence of both C/Y- (*m/z* 527 and 689) and B/Z-type ions (*m/z* 509 and 671) (Fig. 2C). The most abundant fragment at *m/z* 791 may be attributed to the presence of a $^{0,2}A_5$ type fragment ion, probably through a loss of $C_2H_4O_2$, as suggested by Spengler et al.[20] and Cancilla et al.[21] This fragmentation pathway is recognizable in the CID spectra of all the other standard maltooligosaccharides investigated (Fig. 2A, B, D, E) and corresponds well with the mechanisms discussed in the literature for cationized α-(1→4)-linked oligohexoses.[20–24]

Chromatographic experiments on the mucilage samples were first carried out in the SIM mode, monitoring the ions with an *m/z* value correspondend to that of the sodium adduct ions of the selected maltooligosaccharides. A first identification was attempted by comparing the retention times of the selected sample ion profiles with those of the standards. As an example, Figure 3 displays the total ion chromatogram (TIC) of a 1 mg/L standard maltooligosaccharide solution (Fig. 3A) and the extracted ion chromatograms (EICs) of the mucilage sample collected offshore the coast of Pesaro at the beginning of the summer of 2003 (Fig. 3B–F). The comparison between the retention times shows an interesting correlation between the standard and the real mucilage sample. However, to obtain accurate structural information, the chromatographic separations were repeated by performing tandem mass spectrometry experiments. Every compound of interest that eluted from the column was fragmented in the ion-trap analyzer, and its CID spectrum was compared with that of its reference standard. As an example, the fragmentation pathways of the compounds eluting at the retention time of peak 2 in Figure 3C and peak 3 in Figure 3D (Fig. 4B and C) showed a high similarity with those of maltotetraose and maltopentaose (Fig. 2B and C), both in fragment type and abundance. The presence of a complete series of C/Y- and B/Z-type ions suggests that these compounds may be linear oligohexoses composed of four and five units, respectively. In addition, the presence of a dominant loss of $C_2H_4O_2$ (−60 amu, $^{0,2}A_n$ type fragment) in both spectra demonstrates that the hexose units are linked by a (1→4)-glycosidic bond. This assumption is supported by results found in the literature.[18,20–24] CID spectra of α,β-(1→4)-linked oligohexose are characterized by a dominant loss of 60 amu accompanied by cleavages at both extremities of the glycosidic bond, while fragments originating from other common losses, such as $C_3H_6O_3$ (−90 amu) and $C_4H_8O_4$ (−120 amu), are absent.[18,22–24] The ion-trap CID spectra of an oligohexose linked by other kinds of glycosidic bonds are characterized by different dominant losses. For example, The CID spectra of the oligohexoses with the α,β-(1→6)-linkage exhibit abundant fragments at −60, −90 and −120 amu, while the α,β-(1→3)-oligohexoses are subject to a dominant loss of $C_3H_6O_3$.[18,20–24] On the contrary, the two main

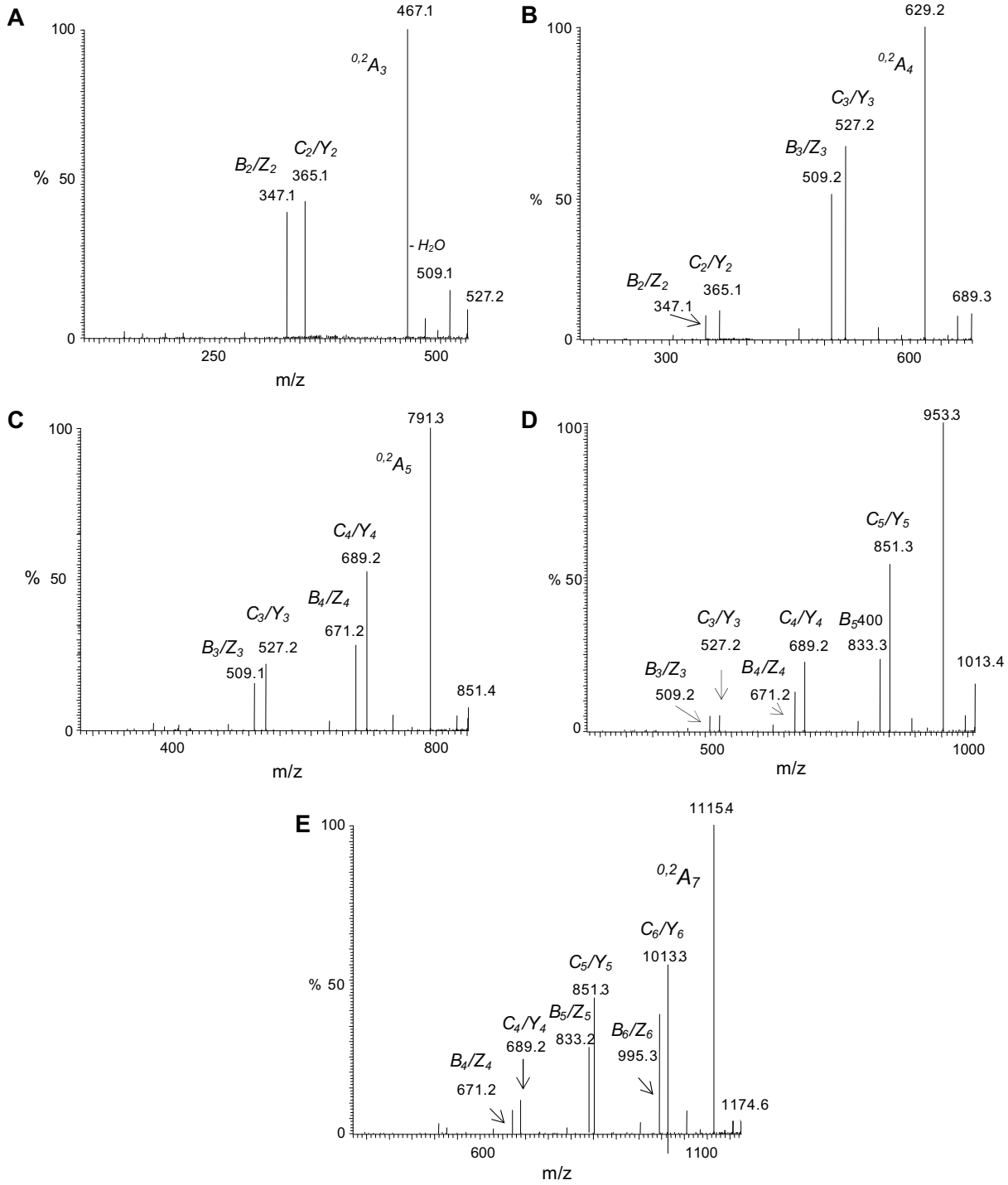

Figure 2. Sodium adduct ions CID spectra of (A) maltotriose (*m/z* 527), (B) maltotetraose (*m/z* 689), (C) maltopentaose (*m/z* 851), (D) maltohexaose (*m/z* 1013) and (E) maltoheptaose (*m/z* 1175).

dissociation pathways for Na-cationized (1→3)-linked oligosaccharides are represented by the loss of H_2O and $C_3H_6O_3$, while the (1→1)- and (1→2)-linked oligohexoses do not show any ring fragmentation.[20,23,24] These MS/MS data together with the retention time information allowed us to assert that the signals relative to peaks 2 and 3 may be correlated to the presence of maltotetraose and maltopentaose in sample B, as previously hypothesized by the HPLC–RI approach. The interpretation of the CID spectra relative to the peaks 1 (Fig. 4A), 4 (Fig. 4D) and 5 (Fig. 4E) revealed that also 3-, 6- and 7-unit maltooligosaccharides were present in this sample. In fact, their fragmentation pathways were matchable with those of the reference standards (Fig. 2A, D, and E). These data, together with the retention time information, clearly demonstrate that maltotetraose, maltohexaose and maltoheptaose were present in sample B as well. Table 2 briefly summarizes the results obtained in the other macroaggregates investigated. These data clearly demonstrate that 3–7 unit linear maltooligosaccharides were found using HPLC–ESIMS/MS, in all the samples investigated. To further reinforce the results of this study, we report results obtained from the spectrophotometric analyses of the organic

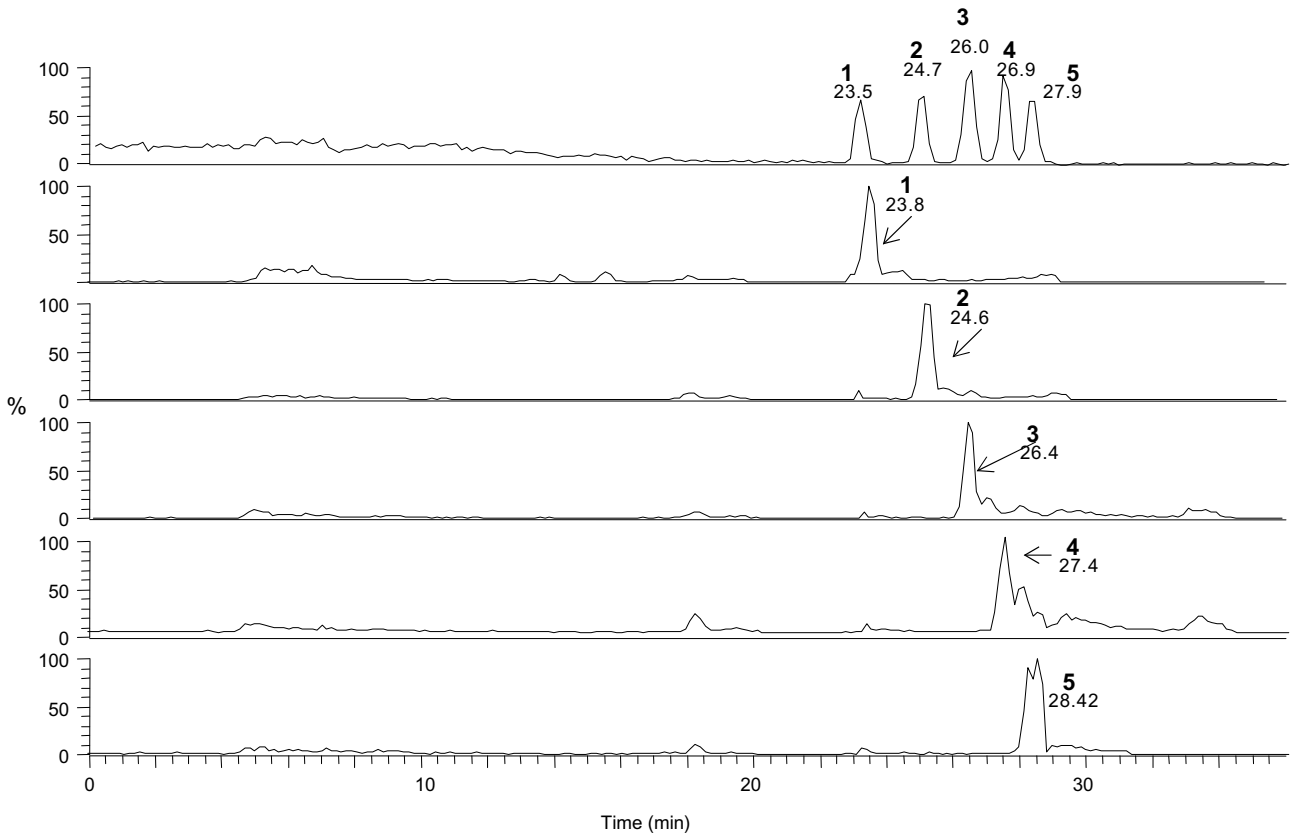

Figure 3. SIM chromatograms for the HPLC–ESIMS analysis of (A) TIC of 1 mg/L the standard solution; (B) EIC of *m/z* 527 in sample B; (C) EIC of *m/z* 689 in sample B; (D) EIC of *m/z* 851 in sample B; (E) EIC of *m/z* 1013 in sample B and (F) EIC of *m/z* 1175 in sample B. Identified peaks: (1) maltotriose; (2) maltotetraose; (3) maltopentaose; (4) maltohexaose and (5) maltoheptaose.

fraction, which was constituted mainly of carbohydrates, and, in smaller quantities, proteins and lipids (Table 1). The percentage of organic matter averaged 30% as dry weight, the percentage of proteins and lipids as organic matter was 11% and 8%, respectively, while the total carbohydrate content varied between 26% and 29%. These results correlate well with results found in the literature.[25–28]

3. Discussion

Both the soluble polysaccharides and the lighter fraction, such as that constituted by oligosaccharides, are part of the constituents of DOM and its derivative POM.[15,30]

Revelant studies have demonstrated that polysaccharides can spontaneously assemble to form polymer microgels,[29] which can aggregate and anneal to each other allowing the formation of transparent exopolymeric particles (TEPs).[14,31,32] These TEPs support the coagulation processes allowing the formation of aggregates, such as mucilage.[30,33] We can also stress that the maltooligosaccharides are present in the composition of the mucilage.

Data obtained in the present study can be used to illustrate some considerations:

(a) All mucilage samples collected in different periods along the Marche Region coast near Pesaro reveal a similar composition in terms of carbohydrate, protein and lipid contents in their organic matter, as statistical analysis (the one-way ANOVA test gives results which are not significant for organic matter, carbohydrates, proteins and lipids).

(b) The presence of maltooligosaccharides is significant because they appear together with more complex polysaccharides in all mucilage samples collected in different periods and in different locations.

(c) These data clearly demonstrate that 3–7 unit linear maltodextrines were found in all the samples investigated, using HPLC–ESIMS/MS. These results also point out the low sensitivity of RI detection, which allowed us to identify no more than two maltooligosaccharides for each sample. RI detection, when compared to ESIMS, has the advantage of being a highly diffused, low-cost system of detection. In this context, efforts will be addressed to the development of suitable sample preconcentration methods, with the future goal of applying RI as a rapid screening method for the investigation of maltooligosaccharides in mucilage samples.

4. Experimental

4.1. General methods

4.1.1. Reagents and chemicals

Ultra-pure water and LC grade acetonitrile and methanol were purchased from Sigma–Aldrich Chemie (Steinheim, Germany) and degassed before use. Standard solutions of glucose (CAS 50-99-7, MW 180 Da), maltose (CAS 6368-53-7, MW 360.32), maltotriose (CAS 1109-28-0, MW 504 Da), maltotetraose (CAS 34612-38-9, MW 666 Da), maltopentaose (34620-76-3, MW 828 Da), maltohexaose (CAS 34620-77-4, MW 990 Da) and maltoheptaose (CAS 34620-78-5, MW 1152) were prepared with dialyzed sea

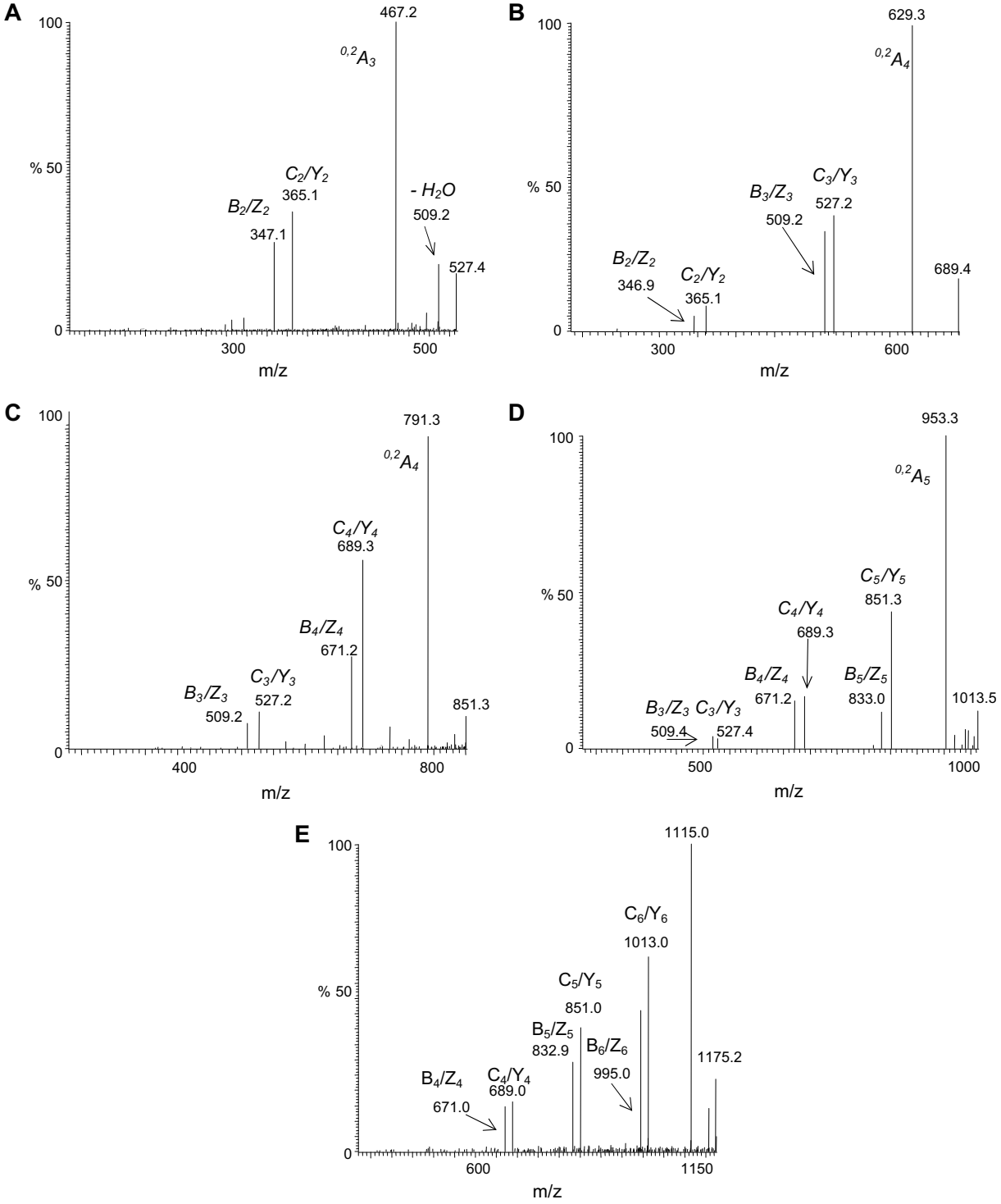

Figure 4. CID spectra of the oligosaccharides detected in sample B: (A) peak 1 in B; (B) peak 2 in C; (C) peak 3 in D; (D) peak 4 in E and (E) peak 5 in F.

water. All the standard maltooligosaccharides were purchased from Supelco (Bellefonte, PA, USA).

4.1.2. Sample collection and preparation

Mucilaginous samples were collected in surface water (creamy surface layer) along the Marche coast of Pesaro, Pesaro (43°55′.50 N, 12°54′.50 E), Fano (43°50′.15 N, 13°03′.08 E) and Marotta (43°45′ 06 N, 13°11′ 30 E), in polyethylene bottles in different periods over the summers of 2002, 2003 and 2004 (Table 1). The samples were stored at −20 °C before the analysis. After defrosting, the mucilage was centrifuged at 4000 rpm for 20 min. Subsequently, the mucilage samples were dialyzed using Spectra-Pore molecular membrane tubing (MWCO: 12,000–14,000).

A first freeze-dried fraction of the dialyzed mucilage was incinerated in a muffle furnace at 500 °C for 1 h, to evaluate the content in organic matter. A second fraction of the dialyzed samples was analyzed by HPLC–RI and HPLC–ESIMS/MS techniques. Before the chromatographic analysis, 120 mg samples of the freeze-dried

Table 1
Chemical composition of mucilage collected along the coast of Pesaro in 2003, of Fano in 2002 and of Marotta in 2004

Mucilage sample	Sampling location (collection date)	Organic matter(%)	Total carbohydrates (%)	Proteins (%)	Lipids (%)
A (16/06/2003)	Pesaro N 43°55′.50″ E 12°54′.50″	31.50 ± 0.17	27.33 ± 0.09	12.70 ± 0.07	10.60 ± 0.05
B (31/07/2002)	Fano N 43°50′.15″ E 13°03′.08″	31.53 ± 0.19	26.84 ± 0.15	11.54 ± 0.06	7.07 ± 0.06
C (01/07/2004)	Marotta N 43°45′.06″ E 13°11′.30″	31.01 ± 0.16	29.42 ± 0.09	10.65 ± 0.06	8.37 ± 0.08
p-Value		0.986	0.583	0.349	0.055

Mean composition of mucilage in terms of organic matter (percentage of dry weight), total carbohydrates, proteins and lipids (percentage of organic matter).

Table 2
Detected oligosaccharides in the water-soluble fraction of the mucilage samples under investigation

Sample	Sampling location	Collection date	Detected oligosaccharides by RI	Detected oligosaccharides by ESIMS/MS
Sample A	Pesaro	16/06/2003	Maltotetraose, maltopentaose	Maltotriose, maltotetraose maltopentaose, maltohexaose, maltoheptaose
Sample B	Fano	31/07/2002	Maltotriose, maltopentaose	Maltotriose, maltotetraose maltopentaose, maltohexaose, maltoheptaose
Sample C	Marotta	01/07/2004	Maltopentaose	Maltotriose, maltotetraose maltopentaose, maltohexaose, maltoheptaose

material were each dissolved in 2 mL of Mili-Q water and shaken for 4 h to stimulate the environmental conditions as much as possible. The aqueous phase was then separated by centrifugation at 4000 rpm (ALC centrifuge PK 130) for 20 min. No acid hydrolysis was carried out on the samples because this treatment destroys the greater part of the maltooligosaccharide chains. The supernatant, containing the water-soluble fraction of the mucilage, was used to analyze the maltooligosaccharide content.

4.2. Spectrophotometric methods

Quantitative determination of total carbohydrate was performed with the Pakulski–Benner extraction method[35] by using 12 M H_2SO_4 Analyses of dissolved monomeric carbohydrate were conducted using the MBTH method of Johnson and Sieburth[36] and Johnson et al.[37] For instrumental calibration standards, aqueous solutions of glucose ranging from 1 to 10 mg/L were prepared, and the absorbtion was measured in a 10-mm glass cell against a spectrophotometric blank consisting of 1.7 mL of acetone and 1.0 mL of doubly distilled water.

Total protein of the mucilage samples was extracted according to Rausch.[38] The quantification of total protein in the extract was performed by using the Setchel modification[39] of the Coomassie Brilliant Blue dye-binding method originally described by Bradford[40] with standard aqueous solutions of Bovine Serum Albumin (BSA, Bio-Chemika) ranging from 2 to 8 mg/L for instrumental calibration. Absorbance of the samples was measured at 595 nm on a dual-beam Shimadzu spectrophotometer (PharmaSpec UV-1700).

Total lipid content was determined gravimetrically with the extraction procedure of Folch[41] with 2:1 chloroform–methanol. After dispersion, the whole mixture was agitated for 15–20 min in an orbital shaker at room temperature. The homogenate was centrifuged to recover the liquid phase. Then the solvent was washed with 0.2 vol of 0.9% NaCl solution. After vortexing, the mixture was centrifuged at low speed (2000 rpm) to separate the two phases. After siphoning off the upper phase, the lower chloroform phase containing lipids was evaporated under vacuum and subsequently weighed on an analytical scale (OHAUS Mod. Explorer). The measurements were done in triplicate for each sample.

4.3. HPLC–RI conditions

The analyses were performed using a Shimadzu LC-10 AT liquid chromatograph (Shimadzu Italia S.r.l. Italia, Milano) coupled with a Varian Star 9040 refractometer (Varian, Palo Alto, CA) set at 30 °C. Data were acquired using Shimadzu CROMATOPLUS software. Chromatographic separations were carried out using a 250 mm × 4.6 mm Varian Nucleosil column packed with 5-μm C_{18} stationary phase. Mili-Q water was used as the mobile phase at a flow rate of 800 μL/min. The injection volume was 50 μL. A 100 mg/L aqueous solution containing glucose and six maltooligosaccharides (maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose) was used as reference solution.

4.4. HPLC–ESIMS/MS conditions

The ionization behaviour of the selected maltooligosaccharides was first investigated in both positive- and negative-ion mode by infusion. Ionization conditions were then optimized by flow injection analysis (FIA) using a SpectraSystem P2000 binary gradient pump (ThermoElectron Corporation, San Jose, California, USA). Each maltooligosaccharide was introduced into the ESI source at a concentration of 1 mg/L, using a flow rate of 150 μL/min and an injection volume of 10 μL. The mobile phase was composed of methanol and water in equal quantities. Methanol was preferred to acetonitrile because it improves signal response in electrospray-ionization MS.[42] During the acquisition of the full-scan spectra, experimental parameters such as spray voltage, pressure of sheath and auxiliary gasses, heated capillary voltage and temperature were carefully evaluated to enhance signal intensity. Chromatographic separations were performed at a flow rate of 250 L/min, using a 250 mm × 2.1 mm Alltech Alltima Amino column packed with a 5-μm aminosilica stationary phase (Alletch Associates, Deerfield, Illinois, USA). The solvent gradient was composed of water (phase A) and methanol (phase B). During the analyses, the LC solvent composition varied from 10% to 70% water in 40 min. The injection volume was 10 L. The column was connected to an ESI-quadrupole ion-trap mass spectrometer for MS/MS analysis (LCQ^{DUO}, ThermoElectron Corporation, San Jose, California, USA). Mass spectra of maltooligosaccharide sodium adduct ions were acquired in positive-ion mode using the following settings: spray voltage 4.5 kV; sheath gas pressure 50 psi; auxiliary gas pressure 20 psi; heated capillary voltage and temperature 46 V and 200 °C, respectively. During selected ion monitoring (SIM) experiments, ions were accumulated in the trap for 200 ms, while three microscans were summed to provide the final signal. Tandem mass spectra were obtained by collision-induced dissociation (CID), using helium as the damping gas and varying the collision energy between 30% and 35%. The maximum injection time was set at 200 ms and the five microscans were summed.

5. Conclusions

In this study, the composition of the organic fraction of marine mucilage was investigated with particular emphasis on its content in maltooligosaccharides. The proposed method combined classical analytical approaches, such as the HPLC–RI technique with HPLC–ESIMS/MS. The spectrophotometric analyses allowed us to quantify the total carbohydrate, protein and lipid contents. The results obtained revealed that mucilage samples exhibited a similar chemical composition in carbohydrates, proteins and lipids.

Both HPLC–RI and HPLC–ESIMS/MS were applied for the investigation of the maltooligosaccharides present in the water-soluble fraction of mucilage. The analysis of three different northern Adriatic Sea mucilage samples revealed the presence of these carbohydrates belonging to the class of maltooligosaccharides.

Although the HPLC–ESIMS/MS investigation has been addressed to a restricted set of maltooligosaccharides, the results obtained demonstrate the applicability of HPLC–MS techniques for the characterization of marine mucilage carbohydrate composition.

Acknowledgements

This research was financially supported by EU Seed Project GOCE-CT-003875-2005, and the authors thank Professor M.B.L. Rocchi for statistical analysis.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2008.10.008.

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