



Effects of gamma irradiation and periodate oxidation on the structure of dextrin assessed by mass spectrometry

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ABSTRACT

Sterilization of biomaterials by gamma irradiation should not change its structure, since this may affect also its bioactivity. In this work, dextrin and periodate-oxidized dextrin (ODEX), both irradiated and non-irradiated, were hydrolysed by α -amylase and the obtained oligosaccharides were fractionated by ligand-exchange/size exclusion chromatography and analyzed by matrix-assisted laser desorption/ionization (MALDI-MS) and electrospray ionization (ESI-MSⁿ) mass spectrometry. This allowed the identification of structures formed during partial periodate oxidation, which displayed aldehyde groups in different positions of dextrin backbone, according to the type of residue which was oxidized. Importantly, gamma irradiation did not structurally change either dextrin or ODEX, showing that it can be used as suitable terminal sterilization method of these materials for biomedical applications. To our best knowledge, this is the first report using MS-based techniques to evaluate the effects of partial periodate oxidation and of gamma irradiation on polysaccharides and, in particular, on dextrin and its oxidized form.

1. Introduction

Dextrins are low-molecular-weight carbohydrates produced by partial hydrolysis of glycogen or starch obtained under acidic and/or enzymatic conditions [1]. They are composed by a linear ($\alpha 1 \rightarrow 4$)-D-glucose residues backbone, branched with ($\alpha 1 \rightarrow 4,6$)-linked-D-glucose residues containing terminal or ($\alpha 1 \rightarrow 4$)-D-glucose oligomers. Some dextrins also present ($\alpha 1 \rightarrow 6$)-D-glucose residues in small percentage [2,3].

Dextrin is a low cost, broadly available raw material widely used in many industrial applications, such as adhesives in the manufacture of gummed tapes, textiles and paper, as moisturiser in cosmetics and accepted in food applications as a generally-recognized-as-safe (GRAS) ingredient [1,3]. Regarding biomedical applications, dextrin is still relatively unexplored, being clinically used as a peritoneal dialysis solution that can also perform as a drug delivery solution [4–6], and as wound dressing agent [7]. During last decade, due to its properties, which include the solubility in both water and DMSO, availability in medical grade, and availability of hydroxyl groups, dextrin has been

explored for the design and fabrication of (nano)hydrogels suitable for controlled release applications, tissue engineering scaffolds, excipient in tablets, bioadhesives or drug conjugates. Such applications have been extensively reviewed by Gonçalves et al. [1] and by Das and Pal [8].

Dextrin was used by our research group to develop a fully resorbable and injectable hydrogel. Dextrin was firstly oxidized (ODEX) with sodium periodate (NaIO_4) and then cross-linked with adipic acid dihydrazide, a non-toxic cross-linking molecule. The cross-linked ODEX is an *in situ* gelling hydrogel, able to incorporate nanogels, cells, biomolecules, and granular ceramics for bone regeneration applications [2,9,10].

Periodate oxidation (“glycol cleavage” oxidation) is a simple and effective method for introduction reactive groups in polysaccharide backbones. It is a catalysis-free aqueous reaction, where periodate ion (IO_4^-) attacks vicinal diols to cleave the carbon–carbon bond, leading to the formation of two aldehyde groups [11]. Initially, this method was routinely used in the characterization and elucidation of the polysaccharide structure through complete oxidation [11]. In later years, low and mild periodate oxidations have been used to functionalize

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polysaccharides: the aldehyde groups along the backbone serve as reactive chemical anchors for further reactions with nucleophilic molecules, allowing the immobilization/stabilization of drugs/biomolecules [12–14] and the formation of hydrogels [9,15,16].

Partial periodate oxidation has been extensively investigated in many polysaccharides, such as cellulose [17,18], alginate [16,19,20], hyaluronate [21], starch [22,23], pectin [24], chitosan [25], and dextran [15,26,27]. Such works have been mainly focused on the characterization of physico-chemical properties of the oxidized derivatives, such as molecular weight (M_w), radius of gyration (R_g), persistence length, intrinsic viscosity, rheology, thermal properties, crystallinity, and reaction kinetics. Fourier transform infrared (FTIR) spectroscopy, ^1H and/or ^{13}C nuclear magnetic resonance (NMR) and colorimetric titrations have been used to detect and/or quantify the aldehydes produced, which form hydrated, hemiacetals or hemiacetal structures [11,15,27], allowing to determine the oxidation degree of the polysaccharides. However, published data still lacks comprehensive characterization on the polysaccharides structural modifications during partial periodate oxidation.

Sterilization is a fundamental step for the manufacturing of biomaterials and implantable medical devices. However, many sterilization procedures have been shown to deeply affect polymer properties. Ionizing sterilization is the most popular procedure for medical devices sterilization [28–31]. No chemicals are needed, there is no additional processing and there is no waste. The main sources of radiation used in sterilization process are electron beam (e-beam) generated from electron accelerators, and gamma irradiation from radioactive sources, mainly ^{60}Co [30]. Gamma irradiation has been the most used sterilization technique, while e-beam is a newer and promising process for the sterilization of medical devices. Gamma irradiation has the advantages to display a higher penetration ability, inducing homogeneous dose delivery in thick products or large batches [30–32]. Nevertheless, ionizing irradiation may produce free radicals, resulting from the radiolysis of water molecules, which can react with the polysaccharide sugar residues and modify its chemical structure and molecular weight [31,33,34]. In this context, it is relevant performing detailed studies on chemical structure of the oxidized dextrin after irradiation.

The main goal of this work was to evaluate the effect of partial periodate oxidation followed by gamma irradiation on dextrin chemical structure for biomedical applications. The sugars and glycosidic-linkage composition were analysed before and after oxidation and irradiation. Mass spectrometry (MS), namely matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS), electrospray ionization mass spectrometry (ESI-MS) and tandem mass spectrometry (ESI-MS/MS) was used to identify and characterize the structural modifications induced by oxidation and irradiation processes. For this, the samples were hydrolysed with α -amylase and further fractionated by semi-preparative ligand-exchange/size exclusion chromatography (LEX/SEC). MS has been used for the structural characterization of polysaccharides and oligosaccharides [35–37], including the oxidation of oligosaccharides [38–40]. Biomolecules, and namely polysaccharides (cellulose, dextran, dextrin, etc), are being increasingly used in the field of biomedicine. Thus, the assessment of the effects of irradiation, commonly used to sterilize biomaterials, must be comprehensively carried out, in order to understand whether significant changes occur, potentially compromising functionality/bioactivity.

2. Material and methods

2.1. Materials

All reagents used were of laboratory grade from Sigma-Aldrich, unless stated otherwise. Dextrin was from Tackidex B 167 (Batch E 1445), generously provided by Roquette (Lestrem, France). Its structural characterization was done in a previous study [2].

2.2. Dextrin oxidation

Dextrin oxidation was performed as described by Molinos et al. [9], with some modifications. Briefly, aqueous solutions of dextrin (14 g; 2% w/v) were oxidized in the dark with 7.39 g of sodium *m*-periodate, to yield a theoretical degree of oxidation of 40%, at room temperature and with stirring. After 20 h, the oxidation reaction was stopped by adding drop wise 3.32 mL of diethylene glycol under stirring for 0.5h to reduce any unreacted periodate. Sodium *m*-periodate and diethylene glycol were removed by ultrafiltration, using a membrane with a molecular weight cut-off of 1000 Da (Merk Millipore, Billerica, MA, USA), and then the oxidized dextrin (ODEX) was lyophilized. The yield was about $69.5\% \pm 3.3$ of ODEX in each batch ($n = 12$ batches).

2.3. Determination of bioburden and sterilization dose

The initial bioburden (initial microbiological contamination) of non-sterilized ODEX was determined using conventional microbiological techniques and according to ISO 11737-1 guidelines [41]. Briefly, ODEX solutions using sterile phosphate buffered saline solution (PBS) with pH 7.4 were prepared at concentration 30% w/v, and allowed to stir for complete dissolution of ODEX and microorganisms' extraction (seven batches were used). Then, ODEX solution was plated on tryptic soy (TSA) medium (3 plates/batch) for growing of fastidious microorganisms and incubated at 30 °C. After 7 days, colony-forming units (CFU) were counted and the average bioburden determined. The obtained value was used to determine the irradiation dose necessary to obtain a sterility assurance level (SAL) of at least 10^{-6} , using for that the tabulated values depicted in ISO 11137-2 [42].

2.4. Gamma irradiation

Gamma irradiation was performed by IONISOS (Dagneux, France). Dextrin and ODEX were dissolved 30% w/v in PBS with pH 7.4 and irradiated using a ^{60}Co source, at 20 kGy (average dose rate of 2 kGy/h), at room temperature. The dextrin was also irradiated at 10 kGy (2 kGy/h) for comparison of the irradiation effects on the polysaccharidés structure.

2.5. Sterility test

The efficacy of gamma irradiation sterilization for ODEX samples was evaluated by a sterility test based on conventional microbiological techniques and described in ISO 11737-2 [43] with slight modifications. Briefly, after the sterilization processes, samples were transferred to test tubes containing tryptic soy broth (TSB) medium for growing of fastidious microorganisms and incubated under agitation, at 30 °C for 3 weeks. Culture medium alone served as negative control, whereas non-irradiated ODEX served as positive control. The turbidity of the broth indicated contamination and inefficient sterilization, while a clear, uncontaminated broth indicated efficient sterilization, producing a sterile solution. Sterility tests were carried out in triplicate.

2.6. Sugars and linkage analyses

Neutral sugars were determined by gas chromatography (GC) as alditol acetates [44]. The pre-hydrolysis was performed with H_2SO_4 72% at room temperature for 3 h followed by hydrolysis for 1 h in H_2SO_4 2 M at 120 °C. Monosaccharides were reduced with NaBH_4 (15% in NH_3 3 M) for 1 h at 30 °C and subsequently acetylated with acetic anhydride (3 mL) in the presence of 1-methylimidazole (450 μL) for 30 min at 30 °C. Alditol acetate derivatives were separated with dichloromethane and analysed by GC with a FID detector and equipped with a 30 m column DB-225 (J&W Scientific, Folsom, CA, USA) with i.d. and film thickness of 0.25 mm and 0.15 μm , respectively. The following oven temperature program used: the initial temperature was set at

200 °C and increased at a rate of 40 °C/min until 220 °C; then it was kept constant for 7 min and again increased at a rate of 20 °C/min until 230 °C; then, the temperature was kept constant for 1 min. The injector and detector temperatures were, respectively, 220 and 230 °C. The flow rate of the carrier gas (H₂) was set at 1.7 mL/min.

Uronic acids were determined colorimetrically according to a modification [44] of the method of Blumenkrantz and Asboe-Hansen [45]. Samples were prepared by pre-hydrolysis in 0.2 mL of 72% H₂SO₄ for 3 h at room temperature followed by hydrolysis for 1 h in H₂SO₄ 1 M at 100 °C. A calibration curve was made with D-galacturonic acid.

Linkage analysis was carried out by methylation as described by Ciucanu and Kerek [46] with modifications. Dextrin samples (1–2 mg) were dissolved in 1 mL of anhydrous dimethylsulfoxide (DMSO). Sodium hydroxide (NaOH) pellets were ground with a pestle in a dry mortar to get a fine powder in a N₂ atmosphere; then, powdered NaOH (about 40 mg) was added and the samples were methylated with CH₃I (80 µL) during 20 min. The samples were dissolved in 3 mL of chloroform:methanol (1:1, v/v) and dialyzed against water:ethanol (1:1, v/v) using a membrane with a molecular weight cut-off of 1000 Da. The methylated fractions were also carboxyl-reduced by a modification of the method described by Nunes et al. [37]. The partially methylated alditol acetates (PMAA) were separated and analyzed by gas chromatography with mass spectrometry detector (GC-MS) on a Shimadzu GC-MS-QP2010 Plus (Kyoto, Japan). The GC was equipped with a DB-1 (J&W Scientific, Folsom, CA, USA) capillary column (30 m length, 0.25 mm of internal diameter and 0.15 µm of film thickness). The samples were injected in split less mode (time of split less 5 min), with the injector operating at 220 °C, and using the following temperature program: 45 °C for 5 min with a linear increase of 10 °C/min up to 140 °C, and standing for 5 min at this temperature, followed by linear increase of 0.5 °C/min up to 170 °C, and standing for 1 min at this temperature, followed by linear increase of 15 °C/min up to 280 °C, with further 5 min at 280 °C. The carrier gas was helium with a flow rate of 1.7 mL/min and a column head pressure of 2.8 psi. The GC was connected to an Agilent 5973 mass quadrupole selective detector operating with an electron impact mode at 70 eV and scanning the range *m/z* 40–500 in a 1 s cycle in a full scan mode acquisition.

2.7. Size exclusion chromatography analysis

About 4–5 mg of ODEX was dissolved in 500 µL of 0.1 M NaNO₃ aqueous solution at room temperature, during 180 min reaching a sample concentration of ca. 1%. The size exclusion chromatography (SEC) analysis was carried out using two PL aquagel-OH MIXED 8 µm 300 × 7.5 mm columns protected by a PL aquagel-OH Guard 8 µm pre-column on a PL-GPC 110 system (Polymer Laboratories, UK). The columns, injector system, and the detector (RI) were maintained at 36 °C during the analysis. The eluent (0.1 M NaNO₃) was pumped at a flow rate of 0.9 mL/min. The injected volume was 100 µL. The columns were calibrated with pullulans (Polymer Laboratories, UK) in the range 0.7–48.0 kDa, allowing the determination of an estimated value of molecular weight (*M_w*), designated as apparent *M_w*.

2.8. Enzymatic hydrolysis and chromatographic separation

Dextrin and ODEX (2–3 mg) were hydrolyzed with an α-amylase (EC 3.2.1.1) from *Bacillus subtilis* (Fluka, 625 U/mg) in 1 mL ammonium hydrogen carbonate (5 mM, pH 7.0). The enzymatic hydrolysis was performed during 24 h, at 37 °C with continuous stirring.

The resulting oligosaccharides were separated by semi-preparative LEX/SEC on a high-performance liquid chromatograph equipped with a Shodex sugar KS 2002 column (300 mm of length and 20 mm of internal diameter) from Showa Denko K. K. (Tokyo, Japan). The column was maintained at 30 °C, the injected sample volume was 500 µL and ultrapure water was used as eluent at a flow rate of 2.80 mL/min. A refractive index detector (Knauer K-2401, Berlin, Germany) was used. All

collected fractions were dried, then neutral fractions were redissolved in 100 µL of ultrapure water, while acidic fractions were redissolved in 50 µL and kept frozen at -20 °C until analysis by MS. Before MS analysis, acidic fractions were decationized being incubated with AG 50 W-X8 cation exchange resin (Bio-Rad Laboratories, Hercules, CA) for 15 min at room temperature.

2.9. Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS)

Samples preparation for MALDI analysis was performed by mixing 4 µL of each sample, previously dissolved in water, to 4 µL of 2,5-dihydroxybenzoic acid matrix (10 mg/mL in methanol). From this mixture, 0.5 µL were deposited on the MALDI plate, allowed to dry at ambient conditions. MALDI-MS spectra were acquired using a MALDI-TOF/TOF Applied Biosystems 4800 Proteomics Analyzer (Applied Biosystems, Framingham, MA) instrument equipped with a nitrogen laser emitting at 337 nm and operating in a reflectron mode. Full-scan mass spectra ranging from *m/z* 500 to 4000 were acquired in the positive mode.

2.10. Electrospray ionization mass spectrometry (ESI-MS) and tandem mass spectrometry (ESI-MS/MS)

The ESI-MS and ESI-MS/MS experiments were carried out in the positive mode in a LXQ linear ion trap (LIT) mass spectrometer (ThermoFinnigan, San Jose, CA). Samples were diluted in methanol/water (1:1) containing formic acid 0.1% (v/v) and introduced into the electrospray source at a flow rate of 8 µL/min. The ESI conditions were as follows: nitrogen sheath gas 30 psi, spray voltage 5 kV, capillary temperature, 275 °C, capillary voltage 1 V, and the tube lens voltage, 40 V. Nitrogen was used as nebulizing and drying gas. ESI-MS spectra were acquired scanning the mass range from *m/z* 100 to 1500. In the MS/MS experiments, the collision energy used was set between 15 and 40 of normalized values. The data were processed using an Xcalibur data system (version 2.0).

3. Results and discussion

3.1. Determination of bioburden and sterility verification

The average bioburden (microbiological contamination) of ODEX before sterilization was 10.3 ± 7.5 CFU/g. A gamma irradiation dose of 20 kGy ensures the achievement of a SAL of at least 10⁻⁶ of the product. To confirm that such dose was enough, a sterility test after ODEX irradiation was performed. After 3 weeks of incubation in TSB, the samples were visually checked for turbidity. Sterile TSB, and irradiated ODEX were clear. At the same time, contamination evidenced by a cloudy aspect, was observed for the non-irradiated ODEX samples. Hence the results suggested that gamma irradiation at 20 kGy (2 kGy/h) effectively sterilizes ODEX solutions. The investigation of the eventual post-sterilization changes in the structure of polymers becomes fundamental, since such alterations can compromise their functionality/bioactivity.

3.2. Structural analysis of dextrin and ODEX

The dextrin used in this work, Tackidex, is a medical grade dextrin obtained by thermal and acidic hydrolysis of potato starch. A structural characterization of this material has been performed in a previously study [2], that is now extended to the oxidized and irradiated dextrin. Sugar analyses revealed that all samples of dextrin and ODEX are composed only by glucose, as confirmed by glycosidic-linkage analysis (Table 1). The oxidation process reduced the glucose amount by 44.4% (Table 1), while gamma irradiation of ODEX did not change its amount of glucose.

In the dextrin samples, glucose occurs mainly as (1 → 4)-linked

Table 1
Glycosidic-linkage analysis of irradiated (10 and 20 kGy) and non-irradiated (mol %) dextrin and oxidized dextrin (ODEX).

Linkages	Dextrin			ODEX	
	0 kGy	10 kGy	20 kGy	0 kGy	20 kGy
T-Glcp	15.8	16.6	16.4	28.1	27.0
4-Glcp	77.0	77.3	76.3	62.2	62.5
6-Glcp	3.2	3.2	3.3	4.8	4.7
4,6-Glcp	4.0	2.8	4.1	5.3	5.8
Glucose loss %	–	–	–	44.4	44.5

residues with small amounts of (1 → 4,6)- and (1 → 6)-linked residues (Table 1), as observed previously [2]. Similar results were obtained for the ODEX samples, with a slightly higher relative amount of branched, 6-Glcp and terminally linked residues, corresponding to a lower relative amount of 4-Glcp. Nevertheless, a high loss of glucose occurred in the oxidation process, as could be expected (Table 1). Shorter and oxidized residues likely formed during the oxidation reactions, such as glyoxal and D-erythrose [47], was not detected by methylation analysis due to the higher volatility of their alditol acetates. The linkage analysis showed that gamma irradiation and oxidation processes did not induce the formation of new glycosidic bonds. The formation of new glycosidic bonds during oxidation process was not expected, since periodate ion is a highly selective glycol-cleaving oxidant [11]. However, it is known that the aldehyde groups formed during oxidation react with nearby hydroxyl groups, forming hemiacetals or hemiacetals, in water. These transitory events could be either intra or inter-residue and, in certain situations, may interfere with the oxidation evolution, the polysaccharide stability/degradability, and with the determination of oxidation degree [11,27,48,49]. Irradiation seems not to change substantially the relative frequencies of the different linkages. It is known that gamma irradiation generates free radicals, in a dose dependent manner, which are capable of cleaving glycosidic bonds, leading to depolymerization of the polysaccharides, including starch and its derivatives [50–54]. The depolymerization reaction is dependent of many factors, such as atmosphere (e.g. presence or absence of O₂), temperature/phase and dose rate used during irradiation process [30,34]. Generally, higher irradiation dose levels and/or higher dose rates promote greater depolymerization [50–52,55–59]. The origin and type of polysaccharide are also relevant. For instance, it has been reported that alginates depolymerize even under lower doses of gamma irradiation (0.5–10 kGy) [60–63]. It is possible that the presence of carboxyl groups in alginate residues increases the production of radical species, leading to further depolymerization [64]. Moreover it has been demonstrated that guluronic acid/mannuronic acid ratio is an important factor for controlling the degradation rate of sodium alginate [62,63]. Concerning to dextrin, only one report was found [52]. In that work, dextrin (0.8–70 kDa) in solid state was irradiated at dose range between 5 and 100 kGy, at higher dose rate (6.316 kGy/h), and a reduction of dextrin viscosity was observed already for 5 kGy. In this work, a very small degradation was detected in dextrin solutions irradiated 20 kGy: according to linkage analysis, dextrin displayed an increase of terminally-linked Glc residues of only 0.6% (mol) (Table 1). The concentration of the solutions was 30% (w/v), while most works use dilute solutions (below 10%) to study the effect of gamma irradiation [30,65]. It has been reported that degradation decreases with increasing polysaccharide concentration (1–4% w/v) [57–59]. Additionally, SEC analysis were performed to confirm whether gamma irradiation affect the oxidized dextrin. The results (Table 2) revealed that the apparent *Mw* of ODEX before (3610 Da) and after (3790 Da) gamma irradiation were very similar, as well as the polydispersity (*Mw/Mn*) – 2.11 for ODEX non-irradiated and 2.23 for ODEX irradiated at 20 kGy. The SEC results, together with the linkages analysis, suggest that gamma irradiation did not alter the length of ODEX chains or its hydrodynamic volume. It may

Table 2
Results on SEC analysis of ODEX samples before and after the gamma irradiation.

Sample	<i>Mw</i> , Da	<i>Mn</i> , Da	<i>Mw/Mn</i>
ODEX	3610	1705	2.11
ODEX 20 kGy	3790	1700	2.23

be hypothesized that the low *Mw* of the dextrin used in this work (4.5 kDa) [2] makes it difficult the detection of depolymerization. Further studies should be performed, e.g. using MALLS or viscosity analysis, to evaluate the effect of the concentration of dextrin, as well as the impact of *Mw* on the depolymerisation reaction. Nevertheless, the evidences obtained point towards a very low depolymerisation under the conditions used in this work.

To our best knowledge, this is the first report that study the effect gamma irradiation on low molecular polysaccharides containing dialdehydes groups. The obtained results show that gamma irradiation can be used as a terminal sterilization process of dextrin and aldehyde-modified dextrin for biomedical applications, since it does not induce major structural modifications. Although gamma irradiation has been the most used sterilizing technique, e-beam irradiation is attracting more attention recently for the sterilization of polymeric-based medical devices [31]. Generally, e-beam irradiation causes less radiolysis than gamma irradiation, and less polymer degradation is the result of shorter processing times with higher irradiation dose rates. While gamma irradiation delivers a certain dose over a period of minutes to hours to a large volume of product, e-beam irradiation delivers the same dose in a few seconds but to a very small volume. Generally, similar radicals are formed when irradiating the materials by gamma irradiation and e-beam [32]. Therefore, it can be hypothesized that applying the same dose (20 kGy) of e-beam irradiation, no major structural modifications should occur. However, further studies should be performed to confirm this assumption.

3.3. Fractionation of oligosaccharides and modified oligosaccharides obtained by partial enzymatic hydrolysis using LEX/SEC chromatography

Dextrin and ODEX samples were selectively hydrolyzed by α -amylase, an endoglucanase which cleaves (α 1→4)-D-glucose linkages. This selective hydrolysis yields oligosaccharides of low molecular weight, allowing their separation by LEX/SEC and simplifying further MS spectra analyses. LEX/SEC provides an effective separation of acidic and neutral products, as well as the fractionation of neutral oligosaccharides according to their size [39,40,66].

The LEX/SEC chromatograms of dextrin and ODEX (irradiated and non-irradiated samples) are shown in Fig. 1. According to previous studies [38–40], the peak with the lowest elution time (10 min) was assigned to oligosaccharides bearing acidic residues, the one at 12 min was attributed to the enzyme, and the other peaks at 13–20 min were assigned to the neutral oligosaccharides.

The fractions eluted at 10 min (F10), 14 min (F14), 17 min (F17) and 19 min (F19) were selected to be analyzed by MALDI-MS, ESI-MS, and ESI-MS/MS. MS analyses allowed to assign F14, present in all samples, mainly to oligosaccharides with higher molecular weight, containing 4 to 9 residues, modified or not. The F17 observed only in dextrin samples (Fig. 1A–C), was assigned to oligosaccharides containing 2 and 5 residues. F19 corresponded mainly to mono-, di- and trisaccharides, also with or without modifications. The di- and trisaccharides thus eluted in fractions F17 and F19. In ODEX samples (Fig. 1D and E), the peak corresponding to F19 decreased significantly in relation to the other ones, probably due to the loss of glucose during the oxidation process. These results allowed to confirm that sodium periodate oxidation promoted structural modifications to dextrin. Comparing the chromatograms of the irradiated and non-irradiated

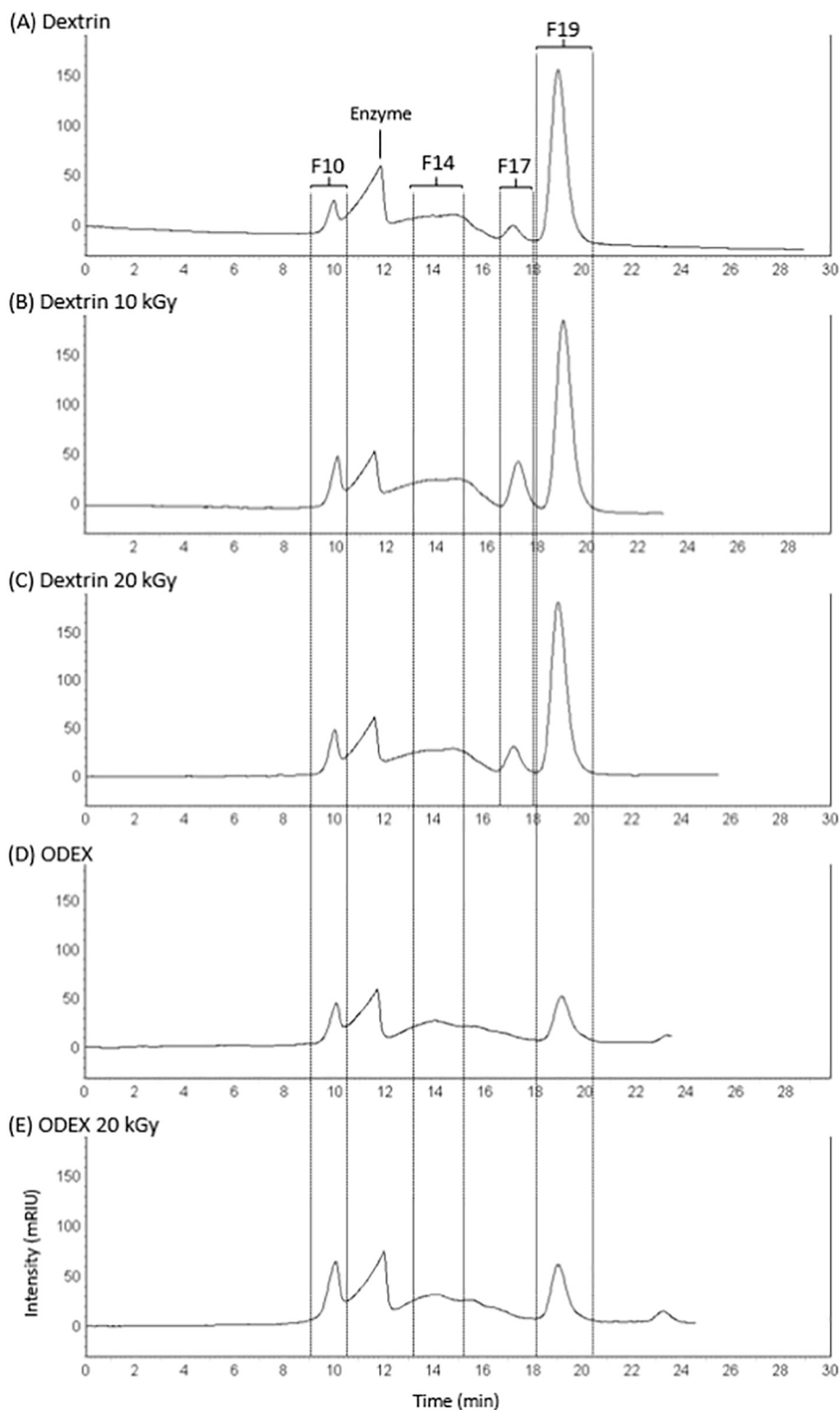


Fig. 1. LEX/SEC chromatograms of the oligosaccharides obtained after partial enzymatic hydrolysis with α -amylase of the dextrin non-irradiated (A), irradiated at 10 kGy (B) and at 20 kGy (C), and of the ODEX non-irradiated (D) and irradiated at 20 kGy (E).

samples, the LEX/SEC profile was similar, thus suggesting that gamma irradiation did not affect the structure of dextrin or ODEX, as observed by linkage analysis.

All samples displayed a peak at 10 min assigned to acidic oligosaccharides. However, it is possible that such peak does not correspond exclusively to acidic OS, since the analysis performed by *m*-phenylphenol, a colorimetric assay specific for uronic acids was negative for

all samples. In addition, ESI-MS and MALDI-MS spectra revealed very few acidic OS possessing carboxylic or carbonyl groups in hemiacetal form, present in trace amounts. It is known that the aldehyde groups formed during oxidation react with neighbour hydroxyl groups, forming hemiacetals, in water. These transitory events could be either intra or inter-residue [11,27,48,49]. As the dextrin and ODEX samples were dissolved in PBS buffer, such ions interacted with the LEX/SEC

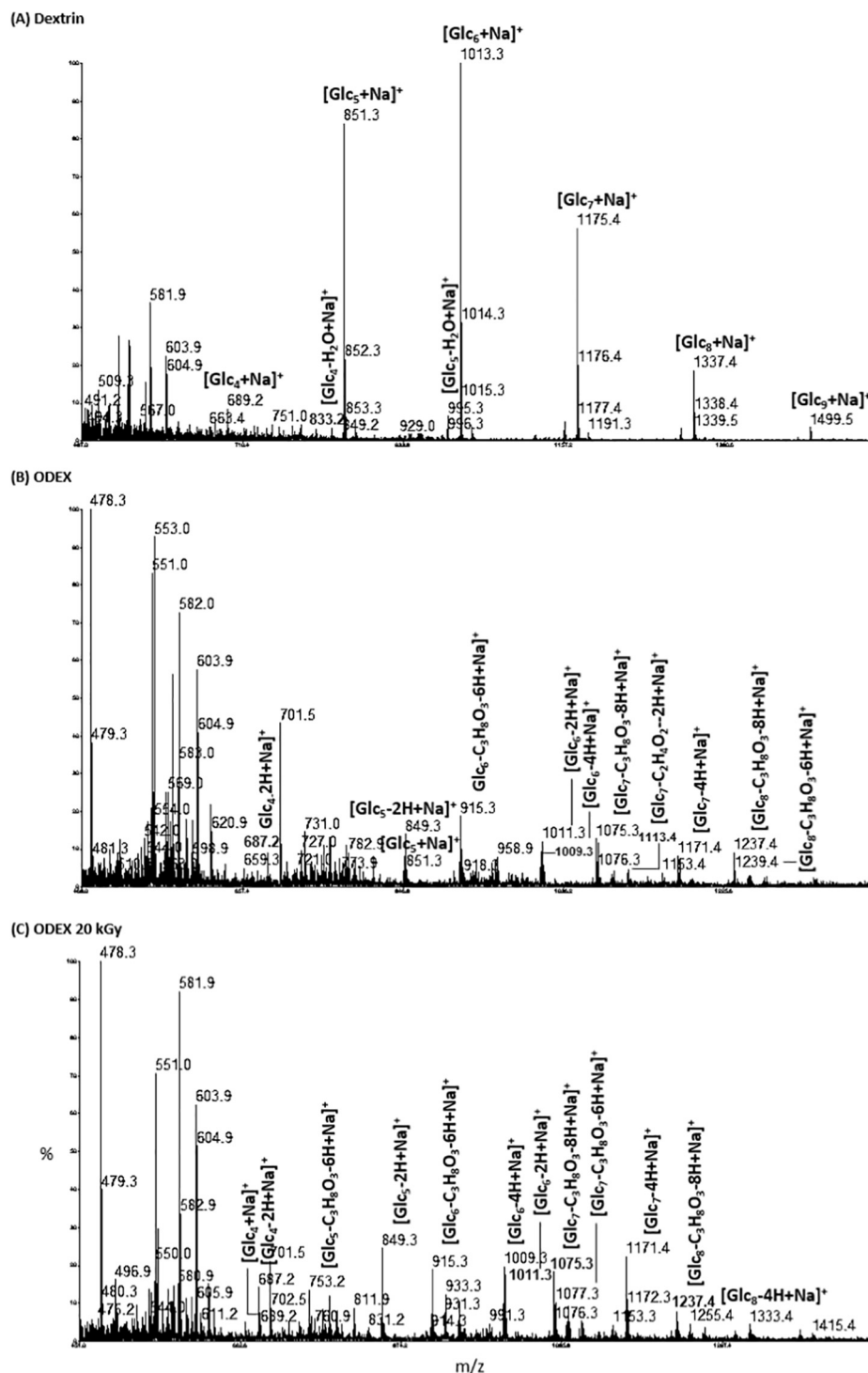


Fig. 2. MALDI-MS spectra of the fraction that eluted at retention time of 14 min (F14) of dextrin non-irradiated (A), ODEX non-irradiated (B), and irradiated at 20 kGy (C).

matrix, eluting at the same retention time as the acidic OS. Therefore, only the MS spectra of the neutral fraction will be discussed in detail.

3.4. Characterization of oligosaccharides from dextrin and ODEX samples by mass spectrometry

The structural changes of dextrin associated to partial periodate oxidation and gamma irradiation were studied by MS using different ionization techniques: ESI and MALDI-MS. Analyses of oligosaccharides

fractions with higher molecular weight were performed by MALDI-MS, since usually it generates only monocharged ions for larger molecules, unlike ESI-MS where multiple charged ions are commonly observed in this case [67]. Thus, the fractions F14 were analyzed by MALDI-MS. On the contrary, fractions F19 were analyzed only by ESI-MS and not by MALDI-MS due to the interference of the ions formed during the matrix ionization in the observation of low molecular weight oligosaccharides (DP lower than 3). Finally, fractions F17 were analyzed by MALDI- and ESI-MS. The oligosaccharides were observed in a positive mode as [M

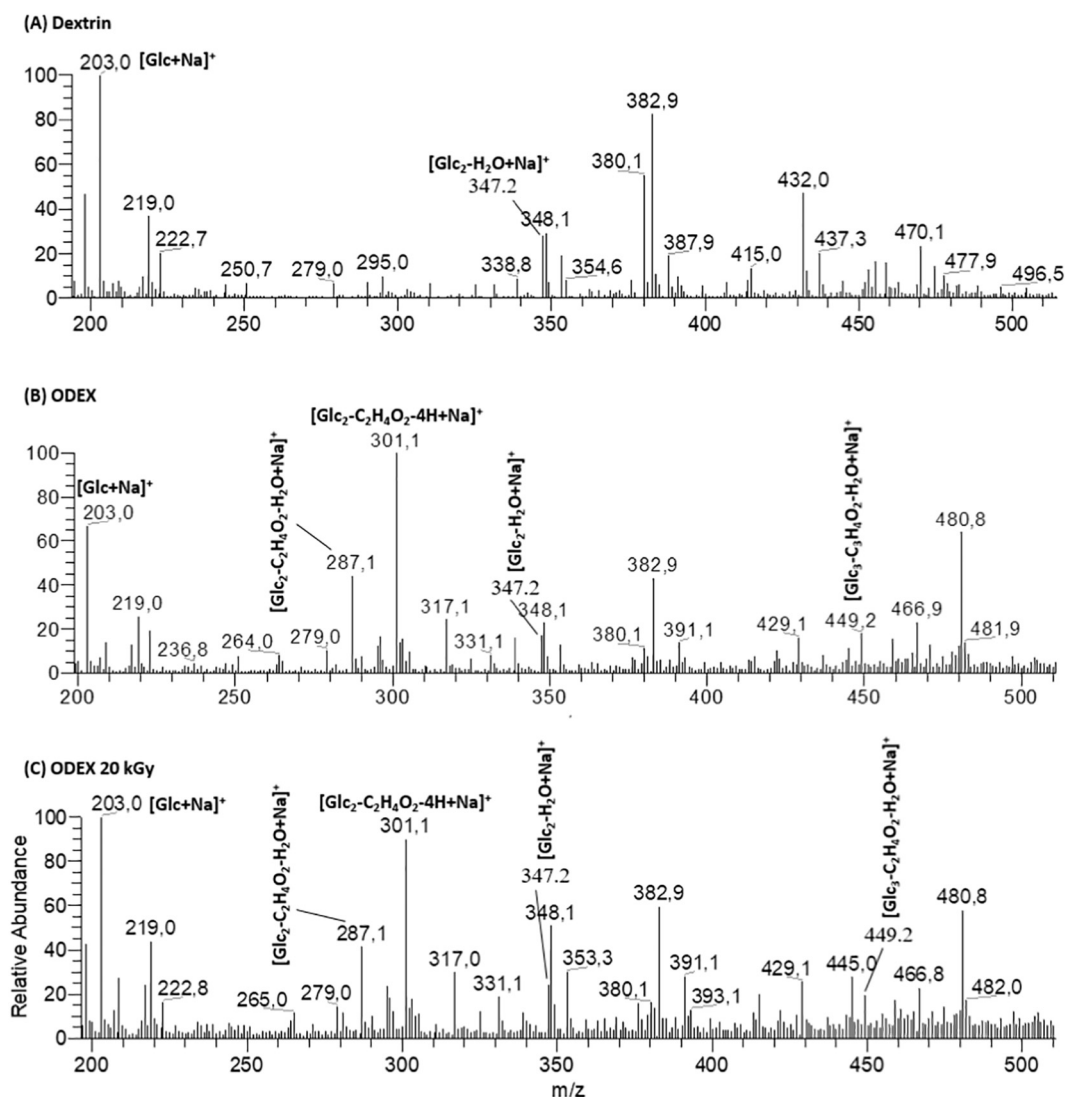


Fig. 3. ESI-MS spectra of the fraction eluted at 19 min (F19) of dextrin non-irradiated (A), ODEX non-irradiated (B), and irradiated at 20 kGy (C).

+Na)⁺ ions; as Glc was the only sugar detected by sugar and methylation analysis, the hexoses detected were all attributed to glucose.

3.4.1. Neutral fractions from dextrin samples

The selected fractions collected from LEX/SEC chromatography of dextrin non-irradiated and irradiated at 10 kGy and at 20 kGy were analyzed, for further comparison with the oxidized ones, as well as to study the impact of gamma irradiation. The neutral series of ions observed in the fractions F14, F17, and F19 of the dextrin samples were analysed. For each fraction, the MS spectra acquired were similar among all dextrin samples (irradiated and non-irradiated ones), confirming that gamma irradiation, under the tested conditions, did not affect the chemical structure of dextrin.

In all spectra of F14, F17, and F19, the major ions observed in dextrin (irradiated and non-irradiated samples) can be attributed to the sodium adducts of a series of hexose oligosaccharides (Glc_n), bearing from one to nine glucose residues ([Glc_n+Na]⁺, n = [1–9]). Small ions were also observed that may be attributed to Glc_n with less 18 Da, correspondent to dehydrated derivatives, [Glc_n-H₂O+Na]⁺ (n = [2–8]). A representative MALDI-MS spectrum for F14 is presented in Fig. 2A and a representative ESI-MS spectrum for F19 in Fig. 3A, both corresponding to non-irradiated dextrin.

The ions belonging to [Glc_n+Na]⁺ series are in accordance with that expected from the action of α-amylase on dextrin samples, and

corroborate the sugar and linkage analyses. The minor series observed, [Glc_n-H₂O+Na]⁺, as reported by Moreira et al. [67,68], which also observed dehydration products using MS-based techniques for carbohydrate analysis, may be attributed to the presence of the anomeric carbon at the reducing terminal.

3.4.2. Neutral fractions from ODEX samples

For the oxidized samples (ODEX), the fractions F14 and F19 were analyzed by MALDI-MS and ESI-MS, respectively. The neutral oxidation series of ions observed of both ODEX samples and the proposed assignments were summarized in Table 3. These fractions, mainly F14, allowed to identify chemical modifications resultant from partial periodate oxidation. The MS spectra of ODEX non-irradiated (Fig. 2B and 3B) were very similar to those of the irradiated ones (Fig. 2C and 3C), displaying the same series of ions, also corroborating that gamma irradiation did not promote additional chemical modifications on ODEX structure. These results are very relevant, since as aldehydes are very reactive groups, it was hypothesized that irradiation could promote the formation of oxygen reactive species that could originate new chemical species by reacting with ODEX. For instance, some studies performed in carbohydrates subjected to oxidation with HO[•] generated under Fenton reaction conditions (Fe²⁺/H₂O₂) demonstrated the formation of new chemical species resulted from insertion of oxygen, and the occurrence of oxidative ring scission [38–40]. However, the formations of such

Table 3

Neutral oligosaccharide $[M+Na]^+$ ions from ODEX non-irradiated and irradiated at 20 kGy observed in the MALDI-MS (F14 products) and ESI-MS (F19 products) spectra, with the identification of the m/z value and the proposed assignments.

<i>n</i>	(^o)	1	2	3	4	5	6	7	8	9
Glc _{<i>n</i>}	(0)	203[#]	365	–	689	851	1013	–	–	–
Glc _{<i>n</i>} -2H	(–2)	–	–	–	687	849	1011	1173	1335 ^a	–
Glc _{<i>n</i>} -4H	(–4)	–	–	–	685 ^b	847	1009	1171	1333 ^a	–
Glc _{<i>n</i>} -6H	(–6)	–	–	–	–	–	–	1169 ^a	1331 ^a	–
Glc _{<i>n</i>} -H ₂ O	(–18)	–	347	–	–	–	–	–	–	–
Glc _{<i>n</i>} -H ₂ O-2H	(–20)	–	–	–	669 ^a	831 ^a	993	1155	–	–
Glc _{<i>n</i>} -H ₂ O-4H	(–22)	–	–	–	–	829 ^a	991 ^a	1153	–	–
Glc _{<i>n</i>} -H ₂ O-6H	(–24)	–	–	–	–	–	–	1151 ^a	–	–
Glc _{<i>n</i>} -CH ₄ O-2H	(–34)	–	–	–	–	–	979	–	–	–
Glc _{<i>n</i>} -CH ₄ O-8H	(–40)	–	–	–	–	–	–	1135 ^a	–	–
Glc _{<i>n</i>} -CH ₄ O-10H	(–42)	–	–	–	–	–	–	1133 ^a	–	–
Glc _{<i>n</i>} -C ₂ H ₄ O ₂ -2H	(–62)	–	303	465	627	–	951	1113	1275	–
Glc _{<i>n</i>} -C ₂ H ₄ O ₂ -4H	(–64)	–	301	–	–	–	949	1111	1273	–
Glc _{<i>n</i>} -C ₂ H ₄ O ₂ -6H	(–66)	–	–	–	–	–	–	1009 ^a	1271	–
Glc _{<i>n</i>} -C ₂ H ₄ O ₂ -8H	(–68)	–	–	–	–	–	–	–	1269 ^b	–
Glc _{<i>n</i>} -C ₂ H ₄ O ₂ -H ₂ O	(–78)	–	287	449	611	–	–	1097 ^a	–	–
Glc _{<i>n</i>} -C ₂ H ₄ O ₂ -H ₂ O-2H	(–80)	–	–	–	609 ^a	771	933	1095	1257	–
Glc _{<i>n</i>} -C ₂ H ₄ O ₂ -H ₂ O-4H	(–82)	–	–	445	–	–	931 ^a	1093	1255	–
Glc _{<i>n</i>} -C ₂ H ₄ O ₂ -H ₂ O-6H	(–84)	–	–	443	–	–	–	1091	1253	–
Glc _{<i>n</i>} -C ₃ H ₈ O ₃ -4H	(–96)	–	–	–	593 ^b	755	917	1079	1241	–
Glc _{<i>n</i>} -C ₃ H ₈ O ₃ -6H	(–98)	–	–	–	–	753	915	1077	1239	1401 ^a
Glc _{<i>n</i>} -C ₃ H ₈ O ₃ -8H	(–100)	–	–	–	–	–	913	1075	1237	1399 ^a
Glc _{<i>n</i>} -C ₃ H ₈ O ₃ -10H	(–102)	–	–	–	–	–	–	–	1235 ^a	1397 ^a

* Values in brackets are the m/z value differences compared to the $[Glc_n+Na]^+$ ions.

The ions in bold were also observed in all dextrin samples.

^a Not observed in ODEX non-irradiated spectrum.

^b Not observed in ODEX irradiated at 20 kGy spectrum.

chemical species after ODEX irradiation was not observed in MS spectra, by comparison with ODEX non-irradiated spectra.

In the MALDI-MS spectra of the ODEX samples (Fig. 2B and C), it was possible to observe that the relative abundance of ions of unmodified oligosaccharides ($[Glc_n+Na]^+$ series) decreased significantly as compared to dextrin (Fig. 2A), and several new ions were observed. Relating to $[Glc_n-H_2O+Na]^+$ series in dextrin samples, only the ions at m/z 347 were observed in ESI-MS spectrum (Table 3), assigned as $[Glc_2-H_2O+Na]^+$. The new ions observed in MALDI- and ESI-MS could be attributed to oxidized oligosaccharides resulting from partial periodate oxidation process and will be described below.

The new ions observed in MALDI- and ESI-MS spectra of both ODEX samples allowed to identify ions belonging to the series with less 2 Da, 4 Da and 6 Da than the $[Glc_n+Na]^+$ series, assigned, respectively, as $[Glc_n-2H+Na]^+$, $[Glc_n-4H+Na]^+$ ($n = [4-8]$) and $[Glc_n-6H+Na]^+$ ($n = [7,8]$). These products must correspond to the oxidation of one, two or three residues of glucose of the oligosaccharides, respectively. Periodate oxidation occurs by the attack of periodate ion (IO_4^-) specifically to vicinal diols, breaking the carbon-carbon bond and yielding two aldehyde groups [11]. As dextrin is mainly composed by (1 → 4)-Glc, it only contains a vicinal diol at C2-C3, which in turn can be attacked by one periodate ion, forming aldehyde groups at C2 and C3 with loss of two hydrogens (–2H) per oxidized hexose (Supplementary Fig. S1-A). For instance, the product ions at m/z 1011 and 1009 present in the F14 of ODEX spectra (Fig. 2B and C) resulted from the formation of two ($[Glc_6-2H+Na]^+$) or four ($[Glc_6-4H+Na]^+$) aldehydes groups, corresponding to the oxidation of one and two (1 → 4)-linked residues of the oligosaccharides, respectively. It was also identified dehydrated oligosaccharides with loss of 2 Da ($[Glc_n-H_2O-2H+Na]^+$, $n = [4-7]$), 4 Da ($[Glc_n-H_2O-4H+Na]^+$, $n = [5-7]$), and one ion (m/z 1151) with loss of 6 Da, assigned as $[Glc_7-H_2O-6H+Na]^+$.

Other series of neutral oxidation products were identified as resulting from oxidative ring scissions reactions on terminal and branched residues. Non-reducing and reducing terminals, as well as, (1 → 4,6)-Glc residues, contain hydroxyl groups on each of three adjacent carbon atoms, which can be oxidized by 2 periodate ions, with the release of formic acid (HCOOH) [11]. Thus, the ions observed with less 60 Da than the $[Glc_n+Na]^+$ series, revealed by the ions at m/z 305, 629, 953 and 1115, were assigned to $[Glc_n-C_2H_4O_2+Na]^+$ ($n = 2, 4, 6-7$) and resulted from the oxidation of the reducing terminal of dextrin. At this terminal, C1-C2 and C2-C3 bonds could be attacked by two periodate ions, with the formation of an aldehyde at C3 position [11,69], leading to the formation of $(Glc_n-C_2H_4O_2)$ species (Supplementary Fig. S1-B). Oxidized oligosaccharides formed by carbon-carbon cleavage at reducing terminal can also display oxidative cleavages between C2-C3 at (1 → 4)-Glc ($[Glc_n-C_2H_4O_2-mH+Na]^+$; $n = [2-8]$; $m = [2-8]$). For example, the ion at m/z 1113, observed in the MALDI-MS spectra of ODEX, can be attributed to oligosaccharide modified at the reducing terminal (–C₂H₄O₂) and with other modification in another glucose residue (–2H), leading to $[Glc_7-C_2H_4O_2-2H+Na]^+$.

The ion series with less 32 Da than the $[Glc_n+Na]^+$ series were assigned to $[Glc_n-CH_4O+Na]^+$ ($n = 5-6$) and could be attributed to the oxidation of the non-reducing terminal or the (1 → 4,6)-Glc. At these residues, two periodate ions can attack the hydroxyl groups between C3-C4 and C2-C3 bonds. During these reactions occur the formation of aldehyde groups at C2 and C4 positions, and C3 is eliminated as formic acid [11,70], generating (Glc_n-CH_4O) species (Supplementary Fig. S1-C). For this series, the possibility of additional oxidation of other glucose residues was also observed, by the oxidative cleavage between C2-C3 of one (–2 Da), four (–8 Da) or five (–10 Da) residues, as revealed by the ions at m/z 979, 1135 and 1133, respectively.

An ion series with less 92 Da than the $[Glc_n+Na]^+$ was observed

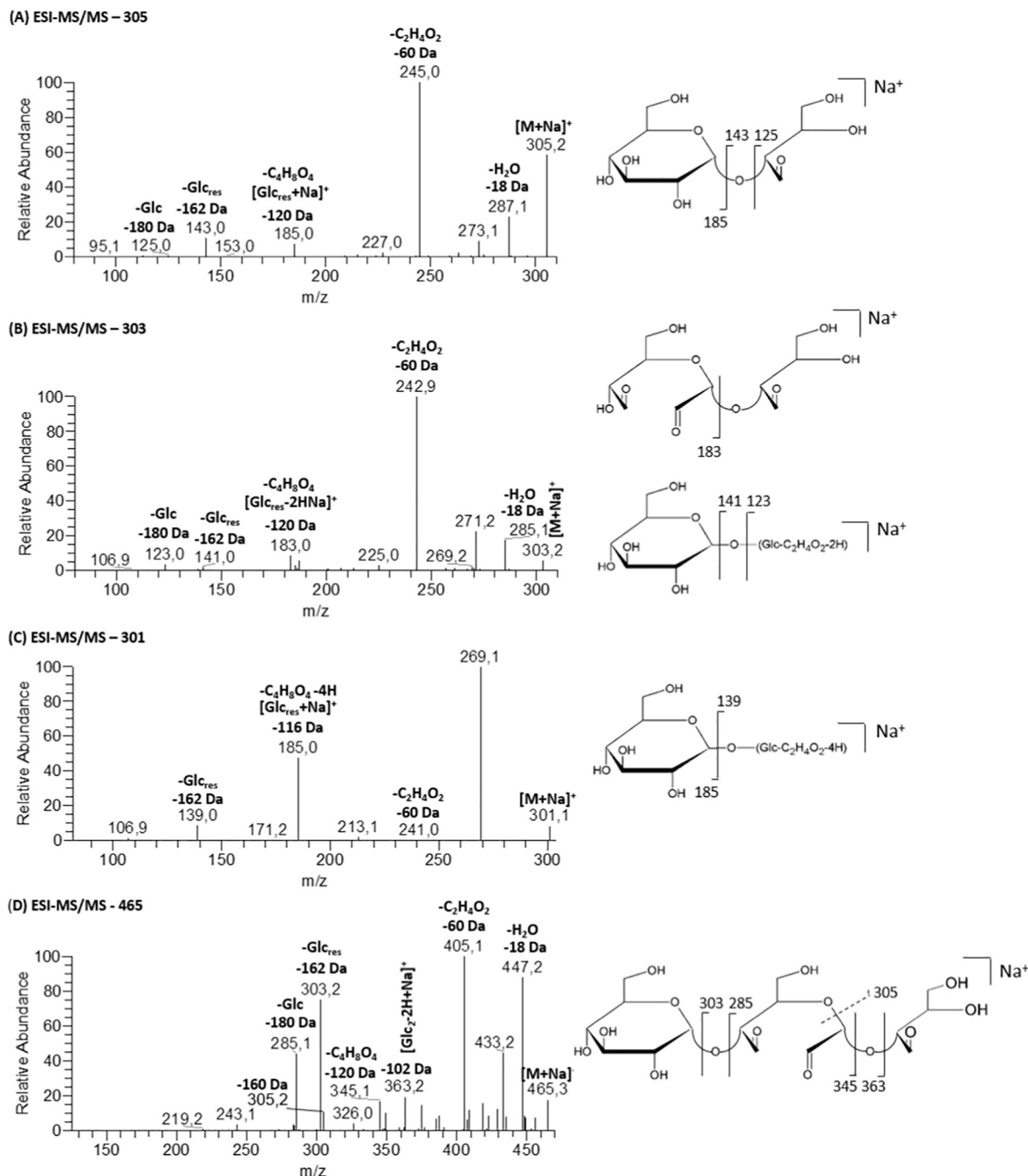


Fig. 4. ESI-MS/MS spectra and schematic fragmentation pathways of $[M + Na]^+$ ions identified after periodate oxidation of the dextrin: (A) $[Glc_2-C_2H_4O_2 + Na]^+$; (B) $[Glc_2-C_2H_4O_2-2H + Na]^+$; (C) $[Glc_2-C_2H_4O_2-4H + Na]^+$; (D) $[Glc_3-C_2H_4O_2-2H + Na]^+$.

and could be attributed to the oxidation of both terminal residues by periodate ions. For instance, the ion with m/z 597, observed in the MALDI-MS spectrum of ODEX irradiated at 20 kGy, can arise from oligosaccharides modified at the reducing ($-C_2H_4O_2$) and non-reducing ($-CH_4O$) terminals, leading to $[Glc_4-C_3H_8O_3 + Na]^+$. Oligosaccharides with both oxidized terminals can also present oxidative cleavages between C2-C3 at $(1 \rightarrow 4)\text{-Glc}$ ($[Glc_n-C_3H_8O_3-mH + Na]^+$; $n = [4-9]$; $m = [4-10]$).

3.4.3. Identification of structural features in oligosaccharides by tandem mass spectrometry

In order to confirm the structural features based on the MS spectra, all the oligosaccharides observed in ESI-MS from F19 were also analyzed by ESI-MS/MS. In this fraction, the only ions observed as a result

of partial periodate oxidation process are belonging to series $Glc_n-C_2H_4O_2$, $Glc_n-C_2H_4O_2-2H$ and $Glc_n-C_2H_4O_2-4H$, whose product ion spectra (ESI-MS/MS) will be described in detailed.

Previous studies performed on the fragmentation of $[M + Na]^+$ ions of hexose oligosaccharides under ESI-MS/MS conditions exhibited a characteristic predominant loss of water (-18 Da), and loss of a hexose residue (here represented as Glc_{res} , -162 Da), due to glycosidic bond cleavage [38,67]. Additionally, the cross-ring cleavages (cleavage of two bonds within the sugar ring) with loss of 60 Da ($-C_2H_4O_2$), typical for $(1 \rightarrow 4)$ -linked Glc units, were also detected [36].

The ion at m/z 305 observed in the ESI-MS/MS of ODEX samples (irradiated and non-irradiated), may correspond to $[Glc_2-C_2H_4O_2 + Na]^+$. The formation of this product can be attributed to the oxidation of the reducing terminal. The ESI-MS/MS spectrum (Fig. 4A)

showed the product ion at m/z 185, assigned to $[\text{Glc}_{\text{res}} + \text{Na}]^+$, formed due to the loss of 120 Da, indicating that this oligosaccharide bears an unmodified glucose and a tetrose ($\text{C}_4\text{H}_8\text{O}_4$). The presence of the tetrose resulted from the oxidative ring scission between C2 and C3 of the reducing terminal (loss of $\text{C}_2\text{H}_4\text{O}_2$), promoted by periodate. The product ions at m/z 125 and 143 are due to the loss of a Glc and Glc_{res} , respectively, confirming also that this oligosaccharide is composed by a glucose linked to a tetrose.

The product ion at m/z 303 ($[\text{Glc}_2\text{-C}_2\text{H}_4\text{O}_2\text{-2H} + \text{Na}]^+$) is present in small abundance in ESI-MS spectra of the ODEX samples (irradiated and non-irradiated). The ESI-MS/MS spectrum of this ion (Fig. 4B) showed the product ion at m/z 183, assigned to $[\text{Glc}_{\text{res}}\text{-2H} + \text{Na}]^+$ due to loss of 120 Da, indicating the presence of an oxidized hexose (-2H). The loss of 120 Da ($-\text{C}_4\text{H}_8\text{O}_4$) from the precursor ion allows to infer also that this oligosaccharide bears a tetrose at the reducing terminal, resulting from the oxidation ($-\text{C}_2\text{H}_4\text{O}_2$) of the initial glucose, promoted by periodate. However, the ions identified at m/z 123 ($-\text{Glc}$) and 141 ($-\text{Glc}_{\text{res}}$) indicate the presence of another isomer with one intact glucose, so that the loss of 2H may occur at the reducing terminal (tetrose-2H). The trace abundance of these ions can indicate that such isomer might be present in smaller abundance, and it is possible that the reducing terminal of dextrin was already oxidized before the periodate oxidation reaction performed.

The ion at m/z 301 is one of the most prominent ions in the ESI-MS spectra of the ODEX samples (irradiated and non-irradiated) and can be attributed to $[\text{Glc}_2\text{-C}_2\text{H}_4\text{O}_2\text{-4H} + \text{Na}]^+$. The ESI-MS/MS spectrum (Fig. 4C) showed the product ions at m/z 139 ($-\text{Glc}_{\text{res}}$) and 185 ($[\text{Glc} + \text{Na}]^+$) which confirm the presence of an intact glucose residue. The loss of 116 Da from the precursor ion suggests that this oligosaccharide bears a modified tetrose at the reducing terminal (tetrose-4H). However, the absence of other peaks hinders the identification of the exact locations of the loss of 4H.

The ion at m/z 465 is present in ESI-MS spectra of the ODEX samples (irradiated and non-irradiated) in residual abundance and was assigned to an oligosaccharide bearing a tetrose as a result of oxidation of the reducing terminal, and with one (1 \rightarrow 4)-Glc residue oxidized ($[\text{Glc}_3\text{-C}_2\text{H}_4\text{O}_2\text{-2H} + \text{Na}]^+$). The ESI-MS/MS spectrum (Fig. 4D) showed the ion at m/z 363, assigned to $[\text{Glc}_2\text{-2H} + \text{Na}]^+$, indicating that one glucose suffered an oxidative ring scission at C3-C4 bond. The ions at m/z 303 (-162 Da) and at m/z 285 (-180 Da), formed by loss of a Glc_{res} and Glc, respectively, indicate that the non-reducing terminal is composed by an unmodified glucose and the oxidative ring scission occurred in the middle of the oligosaccharide, originating aldehyde groups at C2 and C3, as confirmed by ion at m/z 305. The products ions at m/z 345 and 363, corresponding to the loss of 120 Da and 102 Da respectively, confirm that oligosaccharide contains a tetrose ($\text{C}_4\text{H}_8\text{O}_4$) at the reducing terminal caused by oxidative ring scission between C2 and C3 (loss of $\text{C}_2\text{H}_4\text{O}_2$) promoted by periodate.

4. Conclusions

In this study, different MS-based techniques (MALDI-MS, ESI-MS and ESI-MS/MS) were used, for the first time, for identification and characterization of dextrin structural modifications related to partial periodate oxidation. The results obtained confirmed that periodate ion is a glycol-cleaving oxidant highly selective, leading to formation of aldehydes groups. The results showed that during periodate oxidation reaction three major series of oxidation products are formed, according to the type of residue of the dextrin backbone which is attacked by periodate ion: i) the oxidation of (1 \rightarrow 4)-Glc residues leads to the formation of ($\text{Glc}_n\text{-mH}$; $n = [4\text{--}8]$; $m = [2\text{--}6]$) species; ii) the oxidation of reducing terminal residues, leads to the formation of ($\text{Glc}_n\text{-C}_2\text{H}_4\text{O}_2$; $n = [5\text{--}7]$) species, and iii) the oxidation of non-reducing terminal or (1 \rightarrow 4,6)-Glc residues produces ($\text{Glc}_n\text{-CH}_4\text{O}$; $n = [2\text{--}8]$) species. Oxidation products containing more than one type of residue oxidized were also identified.

Gamma irradiation is a commonly used method to sterilize biomaterials for biomedical applications. However, the impact of this sterilization procedure on the biomaterial structure/functionality has not been sufficiently addressed, in particular in the case of biological materials such as oxidized polysaccharides. In this study, it was demonstrated that gamma irradiation can be used as suitable terminal sterilization method for ODEX and dextrin related biomaterials. Indeed, sugars and glycosidic-linkage, as well as MS analysis showed that sterilizing gamma irradiation, under the tested conditions, did not promote changes on the chemical structure of dextrin or ODEX. It can be hypothesized that gamma irradiation will also not impact significantly the structure of other polysaccharides relevant in biomedicine, such as pullulan, alginate, cellulose and dextran.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.eurpolymj.2018.04.011>.

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