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Spectroscopic characterisation of dimeric oxidation products of phytosterols

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ABSTRACT

Sterol dimers are the main oxidation products formed during sterols degradation at elevated temperatures. An investigation was carried out to decipher the structure of dimers differing in polarity, formed during β-sitosterol thermo-oxidation. The oxidation products were fractionated using silica gel into non-polar (NP), mid-polar (MP) and polar fractions (P). Oligomers were further separated by size-exclusion chromatography (SEC). Tentative chemical structures of non-polar, mid-polar and polar dimers were identified using Ag^+/CIS–MS and APCI–MS procedures after on-line RP-HPLC separation. Further structures were verified by NMR and FT-IR spectroscopies.

1. Introduction

β-Sitosterol is the most abundant plant sterol found in food products, such as plant oils, nuts, seeds or fruits (Moreau, Whitaker, & Hicks, 2002). Due to the ability of plant sterols and stanols to decrease amounts of low density lipoprotein cholesterol in blood, the variety of phytosterol-enriched foods is still growing (de Jong, Ros, Ocke, & Verhagen, 2008). Phytosterols are susceptible to auto- and/or enzymatic oxidation, and their oxidative stability is affected by the presence of unsaturation sites, conditions of oxidation (i.e., temperature, time, oxygen availability) and the matrix composition (Dutta & Savage, 2002; Rudzinska, Uchman, & Wasowicz, 2005; Winkler, Warner, & Glynn, 2007). Recently, Rudzinska, Przybylski and Wąsowicz (2009) published a holistic approach to phytosterols' thermo-oxidation, reporting the percentage of all groups of compounds formed during thermo-oxidative degradation of β-sitosterol standard at different temperatures (at 60, 120 and 180 °C), including volatiles, fragmented sterols, oxysterols and oligomers. Amongst sterol oligomers, dimers are the most abundant, however higher-order oligomers are also formed (Lampi, Kemmo, Mäkelä, Heikkinen, & Piironen, 2009; Rudzinska, Przybylski, Zhao, & Curtis, 2010; Rudzinska et al., 2009). Recently, formation of oligomers during fatty acid cholesteryl esters oxidation at 100 and 140 °C was reported (Lehtonen, Lampi, Agalga, Struijs, & Piironen, 2011).

During oxidation, sterols are expected to form similar dimers as those observed for fatty acids, which are bound by ether (C—O—C) and peroxy (C—O—O—C) linkages when oxygen is in excess, or direct carbon–carbon (C—C) linkages in oxygen starvation conditions (Christopoulou & Perkins, 1989; Muizebelt & Nielen, 1996). In fact our previous studies proved formation of sterol dimers during thermo-oxidation through ether bridge, as 3β,3β′-sitosteryl ether (Fig. 3a) was identified as the predominant non-polar dimer formed during β-sitosterol oxidative degradation at 180 °C for 24 h (Sosińska, Przybylski, Hazendonk, Zhao, & Curtis, 2013). Disteryl ethers were mainly investigated as by-products of industrial bleaching and were found in vegetable oils and table margarine

Abbreviations: SEC, size-exclusion chromatography; APCI–MS, atmospheric pressure chemical ionisation–mass spectrometry; CIS–MS, coordination-ion spray – mass spectrometry; FT-IR, Fourier transform infrared; ELSD, evaporative light scattering detector; DEPT, distortionless enhancement by polarization transfer; COSY, two-dimensional proton correlation spectroscopy; NOESY, nuclear Overhauser enhancement spectroscopy; HSQC, heteronuclear single quantum coherence; HMBC, heteronuclear multiple bond correlation; BDE, bond dissociation enthalpy.

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(Kaufman, Vennekel, & Hamza, 1970; Schulte & Weber, 1987). At frying conditions polymerisation of lipids occurs resulting in changes of properties of the frying oil; moreover digestibility of frying fats has been suggested to decrease along with the polymerisation (Billek, 2000). Polymerisation of sterols could affect their health-promoting properties, especially in the case of cholesterol-lowering plant sterol-fortified food products. No adverse effects of disteryl ethers were observed towards mice and rats (Kaufman et al., 1970; Weber, Benning, & Schulte, 1988); however, this dimer has to be considered a dimer of two non-oxidized units. Further consideration has to be addressed for possible adverse effects of sterol polymers when they consist of oxidised monomer units.

During thermo-oxidation a wide variety of higher order molecules is formed; this work focuses on the structural identification of dimeric phytosterol oxidation products with differing polarity, applying different techniques, such as Ag^+/CIS–MS and APCI–MS after on-line RP-HPLC separation; along with NMR and FT-IR spectroscopies.

2. Materials and methods

2.1. Chemicals

β-Sitosterol (78.3% purity, containing campesterol and β-sitostanol), deuterated chloroform ($CDCl_3$, 99.8% D), tetramethylsilane (TMS) and $AgBF_4$ were purchased from Sigma–Aldrich (St. Louis, MO, USA). HPLC and LC–MS grade solvents, along with ACS grade silica gel (60 Å, 70–230 mesh) were purchased from Fisher Scientific Company (Ottawa, ON, Canada) and VWR (Mississauga, ON, Canada).

2.2. Heating and pre-fractionation

Heating procedure was as described in Sosińska et al. (2013). Briefly, sitosterol standard was heated in the presence of oxygen at 180 °C for 24 h. Heated sample, dissolved in toluene (25 mg) was placed on a silica gel column (1.3 g), and the non-polar (NP), mid-polar (MP) and polar (P) fractions were eluted with hexanes/diisopropyl ether (88:12, v/v), diisopropyl ether, and acetone, respectively. After evaporation of solvents, collected fractions were dissolved in dichloromethane prior to separation on SEC columns. The heating experiments and the pre-cleaning procedure were repeated in order to obtain enough material for the separation of dimers.

2.3. Isolation of dimers on HPSEC columns

Dimer fractions were isolated using high-performance SEC (HPSEC) on a Finnigan Surveyor liquid chromatograph (Thermo Electron, Waltham, MA, USA). The non-polar, mid-polar and polar fractions were injected on two Phenogel columns connected in series (500 and 100 Å, 5 μm, 300 × 7.80 mm with guard column; Phenomenex, Torrance, CA, USA) and kept at 25 °C. Dichloromethane was used as mobile phase at a flow rate of 1.0 mL/min. The non-polar, mid-polar and polar dimer fractions were collected using a Gilson (Middleton, WI, USA) FC 203B fraction collector. Components were detected by evaporative light scattering detector (Sedex 75; Sedere, Alfortville, France) operated at 30 °C with purified air at a pressure of 2.5 bar.

2.4. RP-HPLC/ELSD

Dimer fractions collected from HPSEC were separated on a Kinetex core–shell C18 column (2.6 μm; 150 × 3 mm; Phenomenex), using a Finnigan Surveyor liquid chromatograph (Thermo Electron) with a Sedex 75 evaporative light scattering detector (ELSD) operated at 30 °C with purified air at a pressure of 2.5 bar. Analyses were performed at 35 °C with gradient elution of acetonitrile and dichloromethane (B) at a flow rate 0.4 mL/min (0–1 min isocratic at 15% B, 1–6 min linear gradient to 35% B, 6–22 min linear gradient to 40% B, 22–26 min linear gradient to 50% B, 26–30 min linear gradient to 100% B, after 10 min isocratic at 100% B column was equilibrated under initial conditions).

2.5. RP-HPLC/UV and RP-HPLC/APCI–MS

The analyses were performed on an Accela HPLC coupled to an Accela PDA detector and an Exactive Orbitrap MS (Thermo Fisher Scientific) using the same column and gradient elution as described above (Section 2.4). Components were monitored at 243 and 280 nm. The mass spectrometer was equipped with an APCI ion source, and the following conditions were used: positive ion mode; spray current 4.8 μA; capillary and heater temperatures 300 and 350 °C; capillary, tube lens and skimmer voltage 50, 150 and 25 V. Nitrogen was used as a sheath and auxiliary gas with values set at 35 and 5 arbitrary units, respectively. Spectra of all-ions fragmentation preformed in an HCD cell (high energy collisional dissociation) at 25 eV were also recorded. Mass spectra were recorded from m/z 150 to 2400. Xcalibur 2.1.0 software was used for data acquisition and analysis. The mass spectrometer was calibrated within the range 195–1822 Da using caffeine, MRFA and Ultramark 1621 (Sigma–Aldrich).

2.6. RP-HPLC/Ag^+ CIS–MS

The analyses were performed on an Accela HPLC coupled to an Exactive Orbitrap MS (Thermo Fisher Scientific) using the same column and gradient elution as described above. 50 μM $AgBF_4$ in isopropanol, delivered by Mighty Mini pump (Scientific Systems Inc., SSI, State College, PA, USA) at rate of 0.08 mL/min, was mixed post-column via a T-piece with the HPLC flow. The mass spectrometer was equipped with an ESI ion source, and the following conditions were used: positive ion mode; spray voltage 5.2 kV; capillary temperature 400 °C; capillary, tube lens and skimmer voltage 140, 250 and 50 V. Nitrogen was used as a sheath and auxiliary gas with values set at 45 and 5 arbitrary units, respectively. Mass spectra were recorded from m/z 300 to 1800. Xcalibur 2.1.0 software was used for data acquisition and analysis. The mass spectrometer was calibrated within the range 195–1822 Da using caffeine, MRFA and Ultramark 1621 (Sigma–Aldrich).

2.7. NMR and IR spectroscopy

Solution state NMR spectra in $CDCl_3$ were recorded on a Bruker Avance II 300 spectrometer, using 5 mm HX BB probe. The spectrometer was operated at 300.13 MHz for ^1H and 75.47 MHz for ^{13}C. ^1H, ^{13}C, DEPT 135, ^1H–^1H COSY, ^1H–^1H NOESY, ^1H–^{13}C HSQC and ^1H–^{13}C HMBC NMR spectra were recorded using tetramethylsilane (TMS) as an internal standard and chemical shifts are given in ppm (δ). Applied acquisition and processing parameters were as described in Sosińska et al. (2013).

IR spectra were recorded on a Bruker ALPHA-S equipped with DTGS detector, and platinum ATR module with diamond crystal plate and KBr as a beam splitter.

2.7.1. IR-FT and ^1H NMR spectral data

NP2 fraction: IR (KBr) ν_{max} (cm^{-1}): 2955, 2935, 2869, 1732, 1722, 1684, 1681, 1654, 1633, 1464, 1378, 1340, 1253, 1173, 1137, 1092, 1069, 1015, 959. ^1H NMR ($CDCl_3$) δ values (ppm):