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Gas chromatographic properties of common cholesterol and phytosterol oxidation products

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Abstract

The most common cholesterol and phytosterol oxidation products found in foodstuffs or biological matrices are the 7α- and 7β-hydroxysterol, 7-ketosterol, 5α,6α- and 5β,6β-epoxysterol, and triol derivatives of sterols. This study focused on the preparation and purification of such products derived from campesterol, stigmasterol and β-sitosterol. The identity of the substances was confirmed by mass spectroscopic analysis. The elution order of a complex mixture composed of the 7α- and 7β-hydroxysterol, 7-ketosterol, 5α,6α- and 5β,6β-epoxysterol, and triol derivatives of cholesterol, campesterol, stigmasterol and β-sitosterol was recorded on an apolar as well as a medium-polarity capillary column in relation to two commonly used internal standards, i.e. α-cholestane and 19-hydroxy cholesterol. Flame-ionization detector as well as mass spectrometry response factors were derived from a gravimetrically prepared mixture of commercially available cholesterol oxide standards. It was proven that the ionization efficiency of cholesterol and phytosterol oxides are very similar and that response factors obtained for cholesterol oxidation products are also valid for quantitative work regarding phytosterol oxidation products.

Keywords: Cholesterol oxidation products; Phytosterol oxidation products; Detector response factors; Gas–liquid chromatography; Mass spectrometry

1. Introduction

Sterols are an important part of the unsaponifiable matter of fats and oils. By virtue of their unsaturated character, they are vulnerable towards free-radical mediated oxidation [1]. Cholesterol oxidation products (COPs) have been found in foods of animal origin; 7α- and 7β-hydroxycholesterol, 7-ketocholesterol (7-ketochol), 5α,6α- and 5β,6β-epoxycholesterol, and cholestane-3β,5,6β-triol (chol-triol) being the major products of cholesterol (chol) oxidation [2–9]. COPs are reported to exert several negative biological effects, such as cytotoxicity, carcinogenicity, mutagenicity and teratogenicity [10] and may play an important role in the pathogenesis of atherosclerosis [11–13].

Phytosterols are key components of the unsaponifiable matter of vegetable oils and fats. Due to their structural similarities, phytosterols form oxidation products that are similar to cholesterol using analogous oxidative pathways (Fig. 1). Some in vitro studies [14–16] have shown that phytosterol oxidation products (POPs) trigger cytotoxic effects comparable to COPs, although damage by POPs seems to be less severe. POPs were found to be absorbed by the intestine in rats [17] and small amounts of such compounds were identified in the plasma of healthy human volunteers [18].

In contrast to cholesterol oxidation there are only a few reports on the formation and analysis of POPs [19–31]. For the determination of POPs, similar procedures are in use as for COPs analysis. The applied methodology normally includes the following steps: (i) hydrolysis of esterified sterols by saponification or transesterification, (ii) enrichment and

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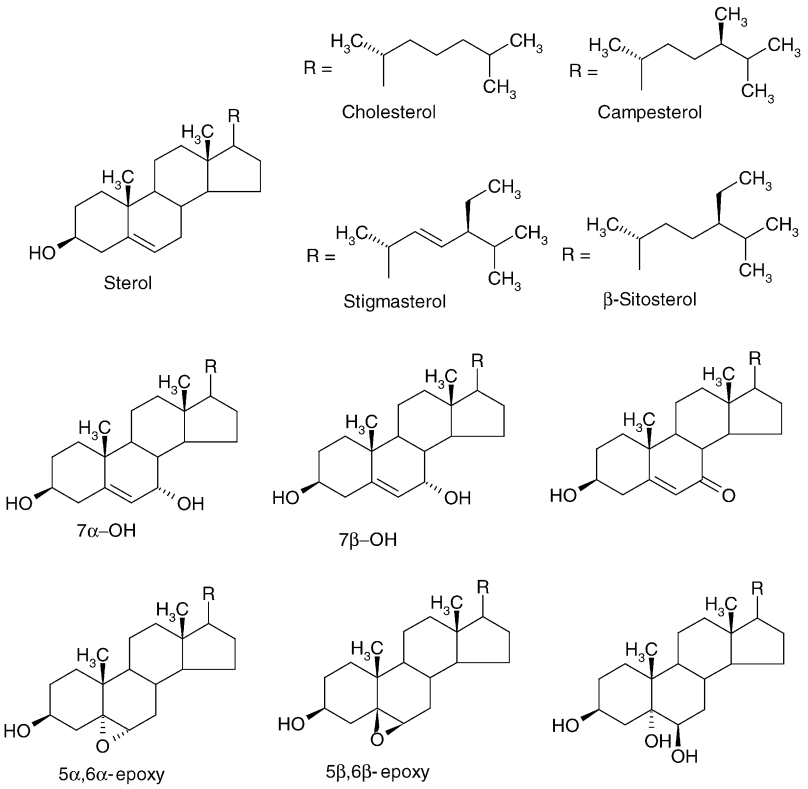

Fig. 1. Chemical structures of the most common cholesterol, campesterol, stigmasterol and β-sitosterol oxidation products.

purification by some form of chromatography and (iii) separation and quantification by gas–liquid chromatography (GLC) or high-performance liquid chromatography (HPLC). GLC is the preferred method as it is more precise and sensitive. Frequently, the GLC system is interfaced to a mass spectrometer (MS) for identification purposes.

Fused silica capillary columns coated with non-polar stationary phases (100% dimethylpolysiloxane or 5% phenyl–95% dimethylpolysiloxane) of standard dimensions (0.25–0.32 mm i.d. and 25–30 m length) are mostly used to separate COPs by GLC (see [32] for review). The major products of cholesterol oxidation (as trimethylsilyl ether derivates) elute on 100% dimethylpolysiloxane columns in the order [32]: cholesterol, 7α-hydroxycholestersol (7α-OH-chol), 19-hydroxycholesterol (19-OH-chol), 5β,6β-epoxycholesterol (β-epoxychol), 5α,6α-epoxycholesterol (α-epoxychol), 7β-hydroxycholesterol (7β-OH-chol), cholestane-3β,5,6β-triol (chol-triol), 7-ketocholesterol (7-ketochol) and 25-hydroxycholesterol (25-OH-chol), while on 5% phenyl–95% dimethylpolysiloxane the elution order is: 7α-OH-chol, chol, 19-OH-chol, 7β-OH-chol, β-epoxychol, α-epoxychol, chol-triol, 25-OH-chol, and 7-ketochol. Chol-triol and 7-ketochol tend to partially overlap on 100% dimethylpolysiloxane, while 25-OH-chol and 7-ketochol form a critical pair on 5% phenyl–95% dimethylpolysiloxane columns. 19-OH-chol, which is not a product of cholesterol autoxidation, is included here because it is often used as internal standard for quantification.

A few studies made use of 50% phenyl–50% dimethylpolysiloxane stationary phases [33–35]; the sequence of COPs elution was: 7α-OH-chol, 19-OH-chol, chol, 7β-OH-chol, β-epoxychol, chol-triol, α-epoxychol, 25-OH-chol and 7-ketochol. Although those medium-polar columns do not offer a real advantage for the separation of the major products of cholesterol oxidation in comparison to non-polar columns, they may be employed for confirmatory purposes.

Quantification of COPs by GLC or GLC–MS is usually done using the internal standard (IS) method. A number of IS compounds were proposed, 19-OH-chol being the most widely used one. The 5α-cholestane (5α-chol) is often added after sample clean up to serve as a secondary recovery standard for the primary IS. Due to their structural similarity, flame-ionization detection (FID) response factors of COPs should be of the same magnitude. Therefore, when another steroidal compound (19-OH-chol, 5α-chol, etc.) is used as IS, response factors of major COPs should deviate from unity only within experimental error. In general, this assumption has been experimentally substantiated [25,36–38], although larger deviations from unity response were also reported [33,39,40].

A major challenge for POP analysis is that vegetable fats and oils contain a number of sterols (campesterol, stigmasterol, β-sitosterol, etc.); thus, numerous oxidation products can be formed which have to be separated and quantified. Analysis will become even more complicated if foodstuffs

contain both COPs and POPs (cumulatively designated as sterol oxidation products (SOPs)). The situation is worsened by the fact that reference substances for POPs, which are a prerequisite for developing analytical methods, are not available commercially. This might be a reason why reports regarding POPs are scarcely found in the literature.

The aim of this work was to prepare POPs of sufficient purity and to define GLC conditions to separate the major products of sterol oxidation (COPs plus POPs) in a single GLC run. Furthermore, quantitative aspects such as FID and MS response factors for SOPs were included in the study.

2. Experimental

2.1. Chemicals and materials

All solvents were of analytical grade; *n*-hexane and *n*-heptane were purchased from Fluka (Vienna, Austria), 2-propanol, ethyl acetate, ethanol, pyridine, from Merck (Vienna, Austria), Sylon BFT [bis(trimethyl)triflouracetamide + trimethylchlorosilane, 99:1] from Supelco (Vienna, Austria), acetic acid from Roth (Vienna, Austria), diethyl ether and toluene from Riedel-de Haën (Vienna, Austria).

5α-Cholestane (5α-chol), 5-cholesten-3β-ol (cholesterol, chol), cholestane-5α,6α-epoxy-3β-ol (5α,6α-epoxy cholesterol, α-epoxychol), cholestane-5β,6β-epoxy-3β-ol (5β,6β-epoxy cholesterol, β-epoxychol), cholestane-3β,5,6β-triol (chol-triol), 5-cholesten-3β-ol-7-one (7-ketocholesterol, 7-ketochol) and 5-cholesten-3β,25-diol (25-hydroxycholesterol, 25-OH-chol) were provided by Sigma–Aldrich (Vienna, Austria); 5-cholesten-3β,19-diol (19-hydroxycholesterol, 19-OH-chol), 5-cholesten-3β,7α-diol (7α-hydroxycholesterol, 7α-OH-chol) and 5-cholesten-3β,7β-diol (7β-hydroxycholesterol, 7β-OH-chol) were purchased from Steraloids (Newport, Rhode Island, USA). A plant sterol mixture (Aldrich), which contained ca. 54% β-sitosterol, 22% stigmasterol and 24% campesterol, and stigmasterol (purity 95%) from Fluka was used for the sterol oxidation experiments.

Silica gel for column chromatography (0.2–0.5 mm), silica gel 60 G for thin layer chromatography, $NaHCO_3$, anhydrous Na_2SO_4, HCl (32%, *m*/*m*) and 2′,7′-dichlorofluoresceine were from Merck, *m*-chloroperbenzoic acid was from Fluka.

2.2. Synthesis and purification of phytosterol oxidation products

α-Epoxy-, β-epoxy-, 7-keto-, 7α-OH- and 7β-OH-derivates of stigmasterol, campesterol, and β-sitosterol were prepared by thermal oxidation. The plant sterol mixture, which was fortified with stigmasterol, was thermo-oxidized by heating to 130 °C in a ventilated oven for 24 h (100 mg as a thin layer in a Petri dish with a diameter of 14 cm). Thereafter, the solid material was dissolved in 5 ml of *n*-hexane-diethyl ether (95:5, v/v). Column chromatography was used for initial clean up of oxidized phytosterols. Silica gel was dry packed into a glass column (18 mm diameter) to a height of 14 cm and pre-wetted with 25 ml *n*-hexane; excess solvent was drained and the sample applied to the column. Non-oxidized sterols were eluted with a step-wise gradient of diethyl ether in *n*-hexane (50 ml each of 10:90, 20:80 and 40:60, v/v). Finally, POPs were eluted with 50 ml acetone. The acetone fraction was evaporated to dryness and the residue dissolved in 0.5 ml diethyl ether. Aliquots of 100 μl were applied as a band to laboratory-prepared silica gel G 60 thin-layer chromatography plates (0.5 mm layer thickness). On both edges of the plate a COPs reference solution containing chol, 7α-OH-chol, 7-ketochol, α-epoxychol and choltriol was spotted and the plate developed with toluene–ethyl acetate–acetic acid (60:40:1, v/v/v) [41]. Substances were visualized by spraying with 2′,7′-dichlorofluoresceine (0.1% in ethanol) and viewing under UV light. Bands corresponding to epoxy-, 7-keto-, and 7-OH-derivatives of phytosterols were located using the COPs solution as a reference, and were scraped off. Substances were recovered from the gel by vortexing with diethyl ether (3 × 5 ml).

Triol derivatives were laboratory-prepared according to Dzeletovic et al. [42]. In brief, 200 mg of phytosterols were dissolved in 3 ml of dichloromethane, 95 mg of *m*-chloroperbenzoic acid added and stirred for 2 h. The solvent was removed and the epoxysterols extracted with 10 ml diethyl ether. The organic phase was washed with 10% aqueous sodium bicarbonate, then with water and dried over Na_2SO_4. After removal of the solvent triol derivates were prepared by refluxing in 2 ml of methanol–6 M HCl (5:1, v/v) for 2 h, and extracting them with diethyl ether. The ether extract was evaporated to dryness and purified from non-oxidized sterols and other interfering compounds by column and thin layer chromatography (see above).

The concentrations of the purified standards were estimated by GLC–FID using the IS method. Therefore, the purified SOPs were evaporated to dryness and dissolved in 5 ml of *n*-hexane. To 100 μl of this solution 1 ml IS (0.02 mg 5α-cholestane/ml *n*-heptane) was added and subjected to GLC analysis. For quantification unity response factors were used.

Finally, purified standards were dissolved in *n*-hexane to a concentration of approximately 0.02 mg/ml.

2.3. Trimethylsilyl (TMS) ether derivatisation

One milliliter of SOP standards and 1 ml of external standard solutions (5α-cholestane and 19-hydroxycholesterol, 0.02 mg each/ml *n*-heptane) were pipetted into autosampler vials, the solvent evaporated under a stream of nitrogen and the residue was dissolved in 100 μl of pyridine at mildly elevated temperature (37 °C). For derivatisation 100 μl of Sylon BFT were added and heated in an oven to 60 °C for 30 min. The reagents were removed under a stream of nitrogen, and the residue was dissolved in 100 μl of *n*-heptane. The conditions chosen converted the triols to *bis*-TMS derivatives.

2.4. GLC–FID analysis

For GLC–FID analysis, a Carlo Erba HRGC 5300 Mega Series gas chromatograph (Thermo Finnigan, Rodano, Italy) equipped with an on-column injector and a flame ionisation detector was used. The fused silica capillary columns tested were: (a) HP-5 (5% phenyl–95% dimethylpolysiloxane), 30 m × 0.25 mm i.d., 0.25 μm film thickness and (b) DB-17 HT (50% phenyl–50% dimethylpolysiloxane), 30 m × 0.25 mm i.d., 0.15 μm film thickness (both obtained from Agilent, Vienna). To each column a 1.0 m × 0.32 mm deactivated fused silica pre-column was connected via a press-fit connector (Supelco, Vienna). The oven temperature program was as follows: 90 °C held for 1 min, then heated to 270 °C using a rate of 30 °C/min, held for 1 min, and finally raised to 300 °C with a rate of 3 °C/min and held there for 12 min. The detector temperature was set to 310 °C. Hydrogen was used as the carrier gas at 0.1 MPa head-pressure. A volume of 0.5 μl standard solution (around 50 ng per component) was injected. Peaks were integrated with ChromCard Ver. 1.19 software (Thermo Finnigan, Rodano, Italy).

2.5. GLC–MS analysis

For GLC–MS analysis a Fisons Instruments GC 8000 series gas chromatograph equipped with an on-column injector and coupled to a Fisons Instruments MD 800 mass spectrometer (Thermo Finnigan) was used. The same conditions as for the GLC–FID experiments were applied, except that helium was the carrier gas (0.1 MPa head pressure). The mass spectrometer was operated in electron impact ionisation (70 eV), full scan (50–650 *m*/*z*) mode. The MS parameters were: scan time, 0.7 s; inter scan, 0.1 s; source temperature, 220 °C; interface temperature, 250 °C. Integration was performed with MassLab Ver. 1.3 software (Thermo Finnigan).

2.6. Determination of response factors

For the determination of response factors, three calibration mixtures were prepared by gravimetry. The mixtures contained known amounts of α-epoxy-, β-epoxy-, 7-keto-, 7α-OH- and 7β-OH-cholesterol and two internal standard substances (5α-cholestane and 19-OH-chol). The mixtures were converted to TMS-derivatives before injection. Response factors were calculated according to:

$$\mathrm{RF_i} = \frac{\mathrm{area_{IS}} \times \mathrm{conc_{COP_i}}}{\mathrm{area_{COP_i}} \times \mathrm{conc_{IS}}}$$

where $\mathrm{RF_i}$ is the response factor of $\mathrm{COP_i}$, $\mathrm{area_{IS}}$ the peak area of the internal standard compound, $\mathrm{area_{COP_i}}$ the peak area of $\mathrm{COP_i}$, $\mathrm{conc_{IS}}$ the concentration of the internal standard compound in the calibration mix and $\mathrm{conc_{COP_i}}$ is the concentration of $\mathrm{COP_i}$ in the calibration mix.

In the GC–MS experiments, the total ion current was used for peak area estimation.

3. Results and discussion

3.1. Preparation and purification of phytosterol oxidation products

Reference substances for phytosterol oxidation products are currently not commercially available and have to be laboratory-prepared, either by thermo-oxidation of sterols in an aqueous dispersion [21], or in the solid state [25], or by chemical synthesis [17,24,43,44]. Heating of a mixture of campesterol, stigmasterol and β-sitosterol in air at 130 °C for 24 h resulted in the formation of 7α-OH- and 7β-OH-sterols, 7-ketosterols, and α- and β-epoxysterols with yields of 0.35–0.65%. For stigmasterol, much higher rates of formation of oxidation products were observed when exposed to 180 °C [25]. Triol derivatives could not be obtained by heat treatment, as they are not stable at high temperatures. Therefore, they were synthesized by oxidation of phytosterols with *m*-chloroperbenzoic acid (yield 5%).

The synthesized POPs were contaminated either with non-oxidized sterols or other unknown by-products. Column chromatography with silica gel allowed the separation of SOPs from the majority of non-oxidized sterols. Preparative TLC on silica gel plates, which were developed in toluene–ethyl acetate–acetic acid (60:40:1, v/v/v), was used to fractionate the oxidized phytosterols. Triol derivatives of campesterol, stigmasterol and β-sitosterol showed the least mobility (R_F 0.04), followed by 7-OH-sterols (R_F 0.15); 7-ketosterols and epoxysterols eluted as one band with an R_F value of 0.27. The epimeric forms of the 7-OH-sterols were only partially separated and were therefore eluted together in order to avoid discriminatory effects. In other TLC systems, and in particular with HPTLC plates, the epimeric 7-OH-sterols as well as the 7-keto- and the epoxysterols can be separated completely [45].

3.2. Gas chromatographic properties of sterol oxidation product–TMS ethers on HP-5 and DB-17 HT capillary columns

GLC retention data for POPs are scant, in particular if mixtures containing all major oxidation products of plant sterols as well as cholesterol are considered. Such data are useful for studying the occurrence of SOPs in total diets. To aid chromatogram interpretation, we fractionated the POP mixture by TLC and each recovered fraction was silylated and subjected to GLC–FID and GLC–MS to record retention times relative to two commonly used IS (5α-cholestane and 19-OH-chol). Sterol oxidation product–TMS ethers were identified by comparing their mass spectral data (Table 1) to published spectra [21,25,27,28,43,44].

GLC conditions (column temperature program and injection technique) were optimized with regard to retention and separation of the individual compounds and the repeatability in terms of relative standard deviation (R.S.D.) of the peak areas obtained. The final temperature of the temperature pro-

Table 1
Mass spectral data of TMS ether derivatives of oxidized sterols

Sterol oxide TMS ethers	Diagnostic ions	*m/z*			
		Cholesterol	Campesterol	Stigmasterol	β-Sitosterol
7α-/7β-OH	$[M-90]^+$	456 (bp)	470 (bp)	482 (bp)	484 (bp)
α-/β-Epoxy	M^+, $[M-90]^+$	474, 384 (bp)	488, 398 (bp)	500, 410 (bp)	502, 412 (bp)
Triol	$[M-18-90]^+$, $[M-18-2\times90]^+$		470, 360 (bp)	482, 372 (bp)	484, 374 (bp)
7-Keto	M^+, others	472, 367 (bp)	486, 381 (bp)	498, 359 (bp)	500, 395 (bp)

M^+, molecular ion; $[M-90]$, loss of trimethyl silanol group; $[M-18-90]$, loss of hydroxyl and trimethyl silanol group; $[M-18-2\times90]$, loss of hydroxyl and two trimethyl silanol groups; bp, base peak.

gram had practically no influence on the retention behavior within the experimental range (290–310 °C); a final temperature of 300 °C was selected for further experiments. Likewise the type of carrier gas (H_2 for GLC–FID, He for GLC–MS) did not influence the separation efficiency of the columns tested.

Relative retention times (RRTs) of the TMS ethers of sterols and SOPs in relation to 5α-cholestane and 19-OH-chol for the HP-5 and the DB-17 HT columns using H_2 (GLC–FID) and He (GLC–MS) as carrier gas are given in Table 2. Chromatograms of a mixed standard solution are depicted in Fig. 2 for the DB-17HT and in Fig. 3 for the HP-5 column. In SOP work, it is common practice to use two ISs. One is added at the very beginning of the sample preparation procedure, a second one is added right before the GC analysis to check for recovery of the primary IS. As 19-OH-cholesterol, which is not formed by auto- or enzymatic oxidation, is often used as primary standard and 5α-cholestane as the secondary standard, both were included in this study and used to calculate relative retention times.

The order of elution was similar for both capillary columns used in this study and the one on HP-5 matched perfectly the

Table 2
Relative retention times of sterol oxidation product-TMS ethers obtained with HP-5 (30 m × 0.25 mm, 0.25 μm) and DB-17 HT (30 m × 0.25 mm, 0.25 μm) capillary columns

TMS ethers	HP-5				DB-17 HT			
	He		H_2		He		H_2	
	5α-Chol	19-OH	5α-Chol	19-OH	5α-Chol	19-OH	5α-Chol	19-OH
5α-Chol	–	0.745	–	0.766	–	0.859	–	0.832
7α-OH-chol	1.249	0.930	1.222	0.936	1.098	0.940	1.121	0.932
Chol	1.276	0.951	1.253	0.960	1.182	1.013	1.211	1.007
19-OH-chol	1.342	–	1.305	–	1.171	–	1.202	–
7α-OH-camp	1.351	1.010	1.314	1.012	1.178	1.008	nd	nd
7α-OH-stigma	1.374	1.024	1.330	1.022	*1.202*	*1.029*	nd	nd
7β-OH-chol	1.387	1.034	1.342	1.028	*1.206*	*1.032*	1.242	1.033
Camp	1.390	1.036	1.357	1.065	1.283	1.096	nd	nd
Stigma	1.397	1.045	1.383	1.063	1.311	1.119	nd	nd
β-Epoxychol	1.414	1.054	1.372	1.051	1.335	1.142	1.371	1.141
α-Epoxychol	*1.438*	*1.071*	1.392	1.067	*1.361*	*1.164*	1.398	1.163
7α-OH-sito	*1.442*	*1.077*	1.397	1.069	1.245	1.066	nd	nd
Sito	1.504	1.121	1.440	1.122	1.361	1.164	nd	nd
7β-OH-camp	1.513	1.129	1.447	1.115	1.298	1.111	nd	nd
7β-OH-stigma	1.526	1.137	1.457	1.119	1.325	1.134	nd	nd
Chol-triol	*1.560*	*1.162*	1.489	1.141	*1.361*	*1.165*	1.410	1.173
β-Epoxycamp	*1.567*	*1.166*	1.490	1.141	1.452	1.231	nd	nd
α-Epoxycamp	1.591	1.185	1.517	1.162	*1.468*	*1.256*	nd	nd
β-Epoxystigma	1.604	1.192	1.527	1.173	*1.469*	*1.275*	nd	nd
α-Epoxystigma	1.624	1.210	1.551	1.192	1.501	1.284	nd	nd
7β-OH-sito	1.627	1.212	1.547	1.184	1.372	1.174	nd	nd
7-Ketochol	1.642	1.223	1.563	1.197	1.541	1.319	1.598	1.329
β-Epoxysito	1.701	1.265	1.620	1.239	1.561	1.308	nd	nd
Camp-triol	*1.722*	*1.283*	1.639	1.249	1.595	1.365	nd	nd
α-Epoxysito	*1.724*	*1.284*	1.641	1.256	1.581	1.356	nd	nd
Stigma-triol	1.775	1.323	1.671	1.284	1.624	1.390	nd	nd
7-Ketocamp	1.822	1.357	1.723	1.318	1.666	1.426	nd	nd
7-Ketostigma	*1.888*	*1.407*	1.773	1.363	1.706	1.460	nd	nd
Sito-triol	*1.892*	*1.409*	1.773	1.358	1.693	1.449	nd	nd
7-Ketosito	2.013	1.499	1.881	1.442	1.764	1.510	nd	nd

Figures in italics denote co-elution; nd, not determined. For GC–MS separation, He was used as carrier gas, while H_2 was used for GC–FID.

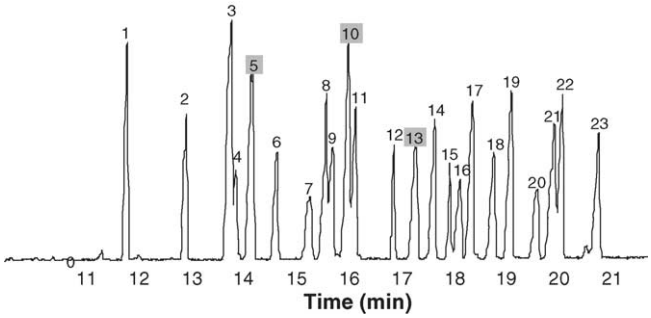

Fig. 2. GC–MS separation of the six major TMS ether derivatives of oxidation products from cholesterol, campesterol, stigmasterol and β-sitosterol on a medium polarity capillary column DB-17 HT, 30 m × 0.25 mm, 0.15 μm, column temperature 90 °C (1 min) at 30 °C/min to 270 °C (1 min) at 3 °C/min to 300 °C (12 min). (1) 5α-Cholestane, (2) 7α-OH-chol, (3) 19-OH-chol, (4) 7α-OH-camp, (5) 7α-OH-stig + 7β-OH-chol, (6) 7α-OH-sito, (7) 7β-OH-camp, (8) 7β-OH-stig, (9) β-epoxychol, (10) α-epoxychol + chol-triol, (11) 7β-OH-sito, (12) β-epoxycamp, (13) α-epoxycamp + β-epoxystig, (14) α-epoxystig, (15) β-epoxysito, (16) 7-ketochol, (17) α-epoxysito, (18) camp-triol, (19) stig-triol, (20) 7-ketocamp, (21) sito-triol, (22) 7-ketostig and (23) 7-ketosito.

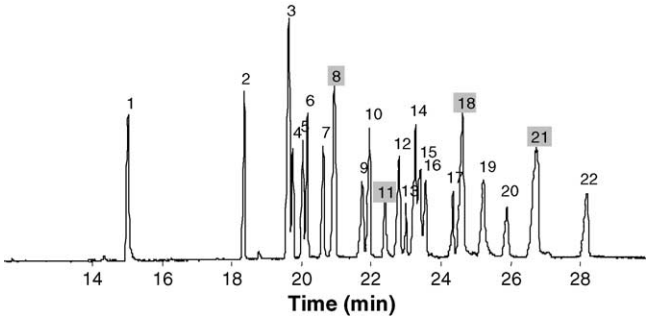

Fig. 3. GC–MS separation of the six major TMS ether derivates of oxidation products from cholesterol, campesterol, stigmasterol and β-sitosterol on a non-polar capillary column HP-5, 30 m × 0.25 mm, 0.25 μm, column temperature 90 °C (1 min) at 30 °C/min to 270 °C (1 min) at 3 °C/min to 300 °C (12 min). (1) 5α-Chol, (2) 7α-OH-chol, (3) 19-OH-chol, (4) 7α-OH-camp, (5) 7α-OH-stig (6) 7β-OH-chol, (7) β-epoxychol, (8) α-epoxychol + 7α-OH-sito, (9) 7β-OH-camp, (10) 7β-OH-stig, (11) chol-triol + β-epoxycamp, (12) α-epoxycamp, (13) β-epoxystig, (14) 7β-OH-sito, (15) α-epoxystig, (16) 7-ketochol, (17) β-epoxysito, (18) α-epoxysito + camp-triol, (19) stig-triol, (20) 7-ketocamp, (21) 7-ketostig + sito-triol and (22) 7-ketosito.

order of elution of POPs as described recently by Johnsson et al. for a 30 m × 0.25 mm DB-5 MS column [28]. The only difference noted was the elution of 7-ketostigmasterol and sitosterol–triol in reversed order. In an earlier study by the same principal author [26], who then used a longer capillary column but of the same stationary phase type (50 m × 0.22 mm HP-5 MS), the elution order was different for quite a number of compounds. Compared to one of the other few studies regarding GLC separation of phytosterol oxides [24] even more pronounced differences were seen, although a capillary column with very similar separation characteristics (RTx5MS, Restek) was used. Lampi et al. [25] also used an RTx5MS column for the separation of thermo-oxidized rapeseed oil sterols. Their elution pattern is in general agreement with this study, although they were not able to identify and/or separate oxidation products derived from brassicasterol and stigmasterol.

Fig. 4 presents the elution order of oxidation products of individual sterols in a simplified form. The basic elution pattern starting with the α-OH-derivative followed by the parent sterol, the 7β-OH-, β-epoxy-, α-epoxy-, triol- and 7-keto-derivative was preserved and can be made out in form of individual clusters for the different sterols in Fig. 4. The observed complex elution pattern is the result of shifting the individual clusters of the POPs towards longer retention times.

Only a few differences were noticed between the HP-5 and the DB-17 HT columns: on HP-5 cholesterol eluted earlier than 19-OH-chol, whereas on DB-17 HT they changed position. Chol-triol eluted after α-epoxychol, which is in agreement with the elution order of COPs observed by Schmarr et al. [34], but in contrast to the findings of Rodriguez-Estrada et al. [35]. In the chromatogram provided by the latter authors chol-triol eluted just before α-epoxychol and was only partially resolved. This might be due to the fact that the Chrompack TAP column (65% phenyl–35% dimethylpolysiloxane) they used is even more polar than DB-17 HT.

The SOP separation on DB-17 HT was only performed by GLC–MS and He as carrier gas, except for COPs. Thus no results are shown for SOP separation with H_2 as carrier gas (GLC–FID). 7α-OH-stig and 7β-OH-chol, α-epoxychol and chol-triol, and α-epoxycamp and β-epoxystig co-eluted on this column. Particularly the first and the last overlaps may lead to problems in identification and quantification since 7-OH-sterols as well as epoxysterols may occur in higher amounts in common foodstuffs. Also the internal standard 19-OH-chol eluted closely before 7α-OH-camp. If a sample contained a higher amount of 7α-OH-camp, it could probably co-elute with 19-OH-chol.

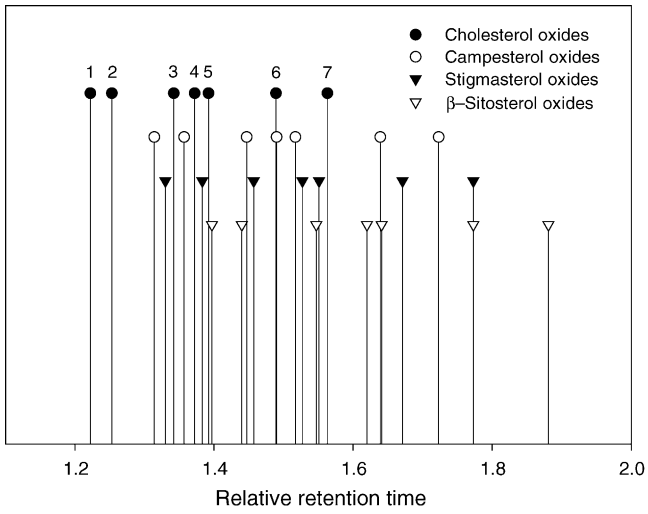

Fig. 4. Schematic presentation of the elution order of sterol oxidation product–TMS on HP-5. GC-FID and H_2 as carrier gas was used: (1) 7α-OH, (2) sterol, (3) 7β-OH, (4) β-epoxy, (5) α-epoxy, (6) triol and (7) 7-keto.

Table 3
GLC–FID and GLC–MS response factors of common cholesterol oxide TMS ethers in relation to 5α-cholestane or 19-OH-cholesterol

TMS ether	FID				MS			
	5α-chol		19-OH		5α-chol		19-OH	
	Mean	R.S.D. (%)	Mean	R.S.D. (%)	Mean	R.S.D. (%)	Mean	R.S.D. (%)
5α-Chol	–	–	1.028	1.4	–	–	1.094	1.1
19-OH-chol	0.973	1.3	–	–	0.914	1.1	–	–
Chol	1.003	2.3	1.031	1.0	0.963	2.5	1.054	2.0
7α-OH-chol	1.037	0.8	1.066	1.5	1.055	1.7	1.153	1.6
7β-OH-chol	0.966	0.7	0.993	1.0	1.043	2.3	1.14	1.8
α-Epoxychol	1.177	0.9	1.209	1.1	1.155	2.0	1.263	2.2
β-Epoxychol	1.204	1.8	1.238	2.7	1.156	3.6	1.264	3.2
Chol-triol	1.253	2.0	1.288	1.6	1.196	1.7	1.308	1.4
7-Ketochol	1.250	1.8	1.284	1.6	1.221	1.6	1.336	1.6

An HP-5 column was (30 m × 0.25 mm, 0.25 μm) was used. Mean, arithmetic mean value of nine replicate injections; R.S.D., relative standard deviation.

On HP-5, even four peaks were not resolved: α-epoxychol and 7α-OH-sito, chol-triol and β-epoxycamp, α-epoxysito and camp-triol, and 7-ketostig and sito-triol co-eluted (Fig. 3). In contrast to the separation on DB-17 HT only the co-elution of α-epoxychol and 7α-OH-sito may cause a problem, as triol derivates are only trace components in common foods, thus they would not disturb quantification. Their identification could still be performed by mass spectrometric detection in selective ion chromatography mode. The separation of the internal standard 19-OH-chol and 7α-OH-camp was still not ideal, but better as on DB-17 HT. However, in case of high 7α-OH-camp concentrations, the two peaks could overlap.

3.3. Quantification of sterol oxidation products

Mass spectrometry, most commonly in the single ion monitoring mode, is widely used for quantification of SOPs. Calibration is straightforward in case of COPs, where high-purity reference substance are commercially available. Since this is not the case for POPs, quantitative data for those compounds in biological samples are scarce.

We utilized the strong structural similarities of COPs and POPs and therefore their similar FID system response to estimate the quantities of the synthesized POPs by calibrating the FID system with a gravimetrically prepared mixture of commercially available COP standards. Provided that the assumption that COPs and POPs have the same FID response holds true a GLC–MS system can be calibrated for quantitative POPs work. A comparison of full scan mode MS response factors obtained by using the gravimetrically prepared COPs mixture and the FID calibrated POP mixture should allow to validate the underlying assumption.

For this purpose, three gravimetrically prepared COP standard mixtures including 5α-chol and 19-OH-chol as IS were derivatized to TMS ethers, and each mixture analysed in triplicate by GLC–FID and GLC–MS (Table 3). Differences between the FID and MS response factors were within experimental error. Regardless whether they were refered to 5α-chol or 19-OH-chol, the response factors ranged between 1.0 and 1.3, which is in good agreement with published data [25,32,39,40].

A mixture of TMS-derivatized POPs with unknown composition was chromatographed and quantified using the response factors obtained for COPs. Next this mixture was analysed by GLC–MS and the quantitative composition, known form the previous GLC–FID experiment, was used to calculate MS response factors for POPs (MS was operated in full scan mode). The resulting response factors for POPs agreed within experimental error with the ones determined for the gravimetrically prepared COPs calibration mixture (Table 4). Obviously, COPs and POPs are ionized with the same efficiency in electron impact MS. Therefore, we speculate that laboratory-prepared POP reference standards can be quantified by GLC–FID and later on be used for calibration of a GLC–MS system, both in full scan as well as in selected ion monitoring mode. Alternatively, POP standards of sufficient purity have to be synthesized in such quantities which would allow preparation of calibration

Table 4
GLC–MS response factors of common phytosterol oxide TMS ethers in relation to 5α-cholestane or 19-OH-cholesterol

TMS ether	5α-Chol		19-OH-chol	
	Mean	R.S.D. (%)	Mean	R.S.D. (%)
7α-OH-camp	1.110	1.8	1.166	1.7
7β-OH-camp	1.090	1.1	1.145	0.8
α-Epoxycamp	1.137	2.8	1.194	2.9
Camp-triol	1.205	1.7	1.276	2.9
7-Ketocamp	1.258	3.7	1.322	3.8
7α-OH-stig	1.037	1.9	1.09	1.8
7β-OH-stig	1.019	1.1	1.145	0.8
α-Epoxystig	1.137	2.8	1.071	1.0
Stig-triol	1.198	3.3	1.259	3.2
7-Ketostig	1.261	2.5	1.324	2.2
7α-OH-sito	1.102	2.2	1.158	2.0
7β-OH-sito	1.098	1.1	1.154	0.9
α-Epoxysito	1.139	2.6	1.196	2.3
Sito-triol	1.206	1.1	1.277	0.7
7-Ketosito	1.282	1.3	1.347	1.7

An HP-5 column (30 m × 0.25 mm, 0.25 μm) was used. Mean, arithmetic mean value of four replicate injections; R.S.D., relative standard deviation.

solutions by gravimetry. This would indeed be the more suitable way to guarantee metrological traceability of results, but at much higher costs and efforts.

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References

[1] G.P. Savage, P.C. Dutta, M.T. Rodriguez-Estrada, Asia Pac. J. Clin. Nutr. 11 (2002) 72.
[2] F. Guardiola, R. Codony, D. Miskin, M. Rafecas, J. Boatella, J. Agric. Food Chem. 43 (1995) 1903.
[3] S.-M. Lai, J.I. Gray, D.J. Buckley, P.M. Kelly, J. Agric. Food Chem. 43 (1995) 1127.
[4] C. Maraschiello, E. Esteve, J.A.G. Regueiro, Lipids 33 (1998) 705.
[5] E. Novelli, E. Zanardi, G.P. Ghiretti, G. Campanini, G. Dazzi, G. Madarena, R. Chizzolini, Meat Sci. 48 (1998) 29.
[6] F. Dionisi, P.A. Golay, J.M. Aeschlimann, L.B. Fay, J. Agric. Food Chem. 46 (1998) 2227.
[7] J.H. Nielsen, C.E. Olsen, C. Duedahl, L.H. Skibsted, J. Dairy Res. 62 (1995) 101.
[8] S.X. Li, G. Cherian, D.U. Ahn, R.T. Hardin, J.S. Sim, J. Agric. Food Chem. 44 (1996) 3830.
[9] S.W. Park, P.B. Addis, J. Food Sci. 51 (1986) 1380.
[10] G.J. Schroepfer Jr., Physiol. Rev. 80 (2000) 361.
[11] F. Guardiola, R. Codony, P.B. Addis, M. Rafecas, J. Boatella, Food Chem. Toxicol. 34 (1996) 193.
[12] A.J. Brown, W. Jessup, Atherosclerosis 142 (1999) 1.
[13] G. Leonarduzzi, B. Sottero, G. Poli, J. Nutr. Biochem. 13 (2002) 700.
[14] W. Meyer, H. Jungnickel, M. Jandke, K. Dettner, G. Spiteller, Phytochemistry 47 (1998) 789.
[15] C. Adcox, L. Boyd, L. Oehrl, J. Allen, G. Fenner, J. Agric. Food Chem. 49 (2001) 2090.
[16] L. Maguire, M. Konoplyannikov, A. Ford, A.R. Maguire, N.M. O'Brien, Br. J. Nutr. 90 (2003) 767.
[17] A. Grandgirard, J.-P. Sergiel, M. Nour, J. Demaison-Meloche, C. Giniès, Lipids 34 (1999) 563.
[18] A. Grandgirard, L. Martine, L. Demaison, C. Cordelet, C. Joffre, O. Berdeaux, E. Semon, Br. J. Nutr. 91 (2004) 101.
[19] K. Lee, A.M. Herian, N.A. Higley, J. Food Prot. 48 (1985) 158.
[20] J. Nourooz-Zadeh, L.-A. Appelqvist, J. Am. Oil Chem. Soc. 69 (1992) 288.
[21] P.C. Dutta, L.-A. Appelqvist, J. Am. Oil Chem. Soc. 74 (1997) 647.
[22] P.C. Dutta, J. Am. Oil Chem. Soc. 74 (1997) 659.
[23] L.L. Oehrl, A.P. Hansen, C.A. Rohrer, G.P. Fenner, L.C. Boyd, J. Am. Oil Chem. Soc. 78 (2001) 1073.
[24] J. Plat, H. Brzezinka, D. Lütjohann, R.P. Mensink, K. von Bergmann, J. Lipid Res. (2001) 2030.
[25] A.-M. Lampi, L. Juntunen, J. Toivo, V. Piironen, J. Chromatogr. B 777 (2002) 83.
[26] P.C. Dutta, in: F. Guardiola, P.C. Dutta, R. Codony, G.P. Savage (Eds.), Cholesterol and Phytosterol Oxidation Products: Analysis, Occurrence, and Biological Effects, AOCS Press, Champaign, IL, 2002, p. 335.
[27] L. Johnsson, P. Dutta, J. Am. Oil Chem. Soc. 80 (2003) 767.
[28] L. Johnsson, R.E. Andersson, P. Dutta, J. Am. Oil Chem. Soc. 80 (2003) 777.
[29] R. Bortolomeazzi, F. Cordaro, L. Pizzale, L.S. Conte, J. Agric. Food Chem. 51 (2003) 2394.
[30] S. Säynäjoki, S. Sundberg, L. Soupas, A.-M. Lampi, V. Piironen, Food Chem. 80 (2003) 415.
[31] P. Lambelet, A. Grandgirard, S. Gregoire, P. Juaneda, J.-L. Sebedio, C. Bertoli, J. Agric. Food Chem. 51 (2003) 4284.
[32] F. Guardiola, J. Boatella, R. Codony, in: F. Guardiola, P.C. Dutta, R. Codony, G.P. Savage (Eds.), Cholesterol and Phytosterol Oxidation Products: Analysis, Occurrence, and Biological Effects, AOCS Press, Champaign, IL, 2002, p. 50.
[33] J.N. Morgan, D.J. Armstrong, J. Food Sci. 54 (1989) 427.
[34] H.-G. Schmarr, H.B. Gross, T. Shibamoto, J. Agric. Food Chem. 44 (1996) 512.
[35] M.T. Rodriguez-Estrada, M.F. Caboni, A. Costa, G. Lercker, J. High Resolut. Chromatogr. 21 (1998) 509.
[36] J.E. Pie, K. Spahis, C. Seillan, J. Agric. Food Chem. 38 (1990) 973.
[37] A. Angulo, J.M. Romera, M. Ramirez, A. Gil, J. Agric. Food Chem. 45 (1997) 4318.
[38] F. Guardiola, R. Codony, M. Rafecas, J. Boatella, J. Chromatogr. A 705 (1995) 289.
[39] G. Maerker, K.C. Jones, J. Am. Oil Chem. Soc. 70 (1993) 255.
[40] S.M. Lai, J.I. Gray, M.E. Zabik, J. Agric. Food Chem. 43 (1995) 1122.
[41] G. Maerker, F.J. Bunick, J. Am. Oil Chem. Soc. 63 (1986) 771.
[42] S. Dzeletovic, O. Breuer, E. Lund, U. Diczfalusy, Anal. Biochem. 225 (1995) 73.
[43] R. Bortolomeazzi, M. De Zan, L. Piazzale, L.S. Conte, J. Agric. Food Chem. 47 (1999) 3069.
[44] C. Johannes, R.L. Lorenz, Anal. Biochem. 325 (2004) 107.
[45] H. Shan, J. Pang, S. Li, T.B. Chiang, W.K. Wilson, G.J. Schroepfer Jr., Steroids 68 (2003) 221.