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Comparative study on phytosterol oxidation products determination in high oleic sunflower oil by using cholesterol oxidation products or purified phytosterol oxidation products as reference materials

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ABSTRACT

Phytosterols received great interest over the last decade due to their capacity to compete with cholesterol absorption during digestion. However, phytosterols can be oxidized resulting in oxidation products (phytosterol oxidation products, POPs), which could be associated with negative effects on human health. Thus, different studies were focused on their determination in foods and biological systems. Since no POPs commercial standards are available, except for 7-ketositosterol, the cholesterol oxidation products (COPs) are often used as reference material (RM) for analytical purposes. However, up to date, no data confirmed the suitability of that approach. In the present study, two different laboratories were enrolled, and commercial standard COPs with pure isolated POPs, obtained by a validated semi-preparative HPLC method, were compared to determine the content of POPs in refined high oleic sunflower oil. Two technologies were also considered LC-Orbitrap-HRMS (LC-MS) and GC-MS. Except for 7-keto derivatives, the use of COPs as RM led to an overestimation of the POPs content by both tested systems LC-MS and GC-MS. Again, the Orbitrap technique was more sensitive than GC-MS, and significant differences ($p<0.05$) in the quantitation were also found between LC-MS and GC-MS when identical stock solutions were used. The results demonstrated that the analysis of POPs is challenging, and the use of pure POPs leads to reduce the risk of a poor determination of POPs in vegetable oils and the use of COPs as RM for detecting and quantifying POPs should be reconsidered.

1. Introduction

The phytosterols represent one of the main fractions of unsaponifiable matter of vegetable fats and oils, and could be found in free form or as steryl-fatty acid esters (where the 3β-OH group is esterified) [1]. The main detected phytosterols in plants are β-sitosterol, brassicasterol, campesterol and stigmasterol, and vegetable oils such as sunflower oil, canola oil, and soybean oil represent a good source of phytosterols. In the last decade, phytosterols received great interest due to their ability to compete with cholesterol absorption during the digestion process, lowering the blood cholesterol level, in particular the low-density lipoproteins [2]. However, phytosterols can be oxidized through auto-oxidation or photo-induced oxidation, and in a similar way to cholesterol, they can generate hazardous compounds such as phytosterol oxidation products (POPs), of which the 7α-hydroxy, 7β-hydroxy, 5,6α-epoxy, 5,6β-epoxy, triol and 7-keto represent the main isomers

Abbreviations: 19-HC, 19-hydroxycholesterol; 7-KC, 7-ketocholesterol; 7-KCam, 7-ketocampesterol; 7-KS, 7- ketositosterol; 7-KStig, 7-ketostigmasterol; 7α-HC, 7α-hydroxycholesterol; 7α-HCam, 7α-hydroxycampesterol; 7α-HS, 7α-hydroxysitosterol; 7α-HStig, 7α-hydroxystigmasterol; 7β-HC, 7β-hydroxycholesterol; 7β-HCam, 7β-hydroxycampesterol; 7β-HS, 7β-hydroxysitosterol; 7β-HStig, 7β-hydroxystigmasterol; APCI, Atmospheric Pressure Chemical Ionization; COPs, Cholesterol Oxidation Products; FID, Flame Ionization Detector; HOSO, High Oleic Sunflower Oil; HRMS, High Resolution Mass Spectrometry; LOD, Limit of Detection; LOQ, Limit of Quantification; POPs, Phytosterol Oxidation Products; PRM, Parallel Reaction Monitoring; RM, Reference Material; RSD, Relative Standard Deviation; SIM, Single Ion Monitoring; TIC, Total Ion Current; α-EC, 5,6α-epoxycholesterol; α-ECam, 5,6α-epoxycampesterol; α-ES, 5,6α-epoxysitosterol; α-EStig, 5,6α-epoxystigmasterol; β-EC, 5,6β-epoxycholesterol; β-ECam, 5,6β-epoxycampesterol; β-ES, 5,6β-epoxysitosterol; β-EStig, 5,6β-epoxystigmasterol.

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determined for each phytosterol [3–6]. Authors suggested a significant correlation between the absorption of POPs and an increased risk of the formation of lesions [7]; again, cytotoxicity, as well as pro-atherogenic and pro-inflammatory effects of POPs were also reported [8]. Moreover, the significant frequency of POPs in phytosterol-enriched meals highlights the health hazards associated with the continuous use of these products [9–13]. Thus, a sensitive and reproducible analytical method able to better determine the content of POPs in foods or ingredients is highly required. It might be pointed out that since no commercial reference standards of POPs are available, except for 7-ketositosterol, up to now the quantitation of POPs is mostly carried out using the cholesterol oxidation products (COPs) as reference material [6]. Poudel and co-authors [2] determined POPs in vegetable oils and liposomal formulations by LC-MS/MS using COPs as reference standards for optimizing the ionization parameters and calibration curves. Gallina Toschi et al. [14] determined POPs in silverskin through GC-MS based on COPs calibration curves. Others determined POPs in oil-in-water emulsions by GC-MS using COPs response factors [15]. Again, the COPs response factors achieved by GC-MS were used to determine POPs as related to photo-induced oxidation of phytosterols [16]; and other authors applied the same approach in cooked and baked food products [17].

Considering the actual literature, it might be highlighted that few studies reported the use of chemically synthesized or purified POPs for quantitative purposes. For instance, Plat and co-authors chemically synthesized deuterated and non-deuterated POPs for determining POPs in human serum and lipid emulsions [18]. Others attempted to add chemical synthesis of triol to thermo-oxidize phytosterols [3]. Again, authors thermo-oxidized pure β-sitosterol, campesterol and stigmasterol to generate POPs, which were purified by semi-preparative HPLC and used to build calibration curves for determining POPs in different vegetable oils [4].

To our knowledge, even if different studies investigated POPs using COPs as reference material or someone tried purified POPs, no data about the comparison between the use of pure POPs and COPs as reference material for analytical purposes were published. The aim of the present work was to assess whether pure isolated POPs and commercial standard COPs exhibit comparable analytical behavior with regard to mass spectrometry detection (GC-MS and LC-Orbitrap-HRMS). The analysis of POPs is challenging, and the present study may contribute to overcoming the limitations associated with the unavailability of reference standards improving the knowledge about using COPs as reference material for POPs detection.

2. MATERIAL and METHODS

2.1. Chemicals and solvents

All chemicals and solvents were of analytical grade. Millipore membrane filters (0.45 μm and 0.20 μm), chloroform, n-hexane, methanol, water (≥99.8 %), and ethanol were purchased from Merck (Darmstadt, Germany). β-Sitosterol (purity: >95%; sitosterol), anhydrous pyridine (99.8%), and N,O-Bis(trimethylsilyl)trifluoroacetamide with trimethylchlorosilane (BSTFA:TMCS, 99:1, v/v) were purchased from Sigma (St. Louis, MO, USA). Campesterol (purity >95 %), stigmasterol (purity: >95 %), and 19-hydroxycholesterol (19-HC; purity: >99 %; internal standard (IS)) were purchased by Larodan (Larodan AB - Retzius, SE). Hyper grade solvent for LC/GC analysis, *n*-heptane, 2-propanol, and formic acid were purchased by VWR (VWR International - Radnor, US). Acetone, pyridine, hexamethyldisilazane and trimethylchlorosilane were purchased from Sigma-Aldrich (Sigma-Aldrich—St. Louis, US). The 7-ketocholesterol (7-KC; purity: 99%), 7α-hydroxycholesterol (7α-HC; purity: 90%), 7β-hydroxycholesterol (7β-HC; purity: 95%), 5,6α-epoxycholesterol (α-EC; purity: 87%), 5,6β-epoxycholesterol (β-EC; purity:80 %) were supplied by Steraloids (Wilton, NH, USA). Silica solid-phase extraction (SPE) cartridges (500 mg/3 mL) from Phenomenex (Bologna, Italy) were utilized for POPs purification.

2.2. Preparation of phytosterol oxidation products (POPs) reference material

Pure phytosterols oxidation products (POPs) were obtained according to our validated method [4]. In brief, 5 mg of pure β-sitosterol, campesterol and stigmasterol each dissolved in 5 mL of acetone were individually put in glass petri dishes, dried under a stream of nitrogen and thermo-oxidized in a static oven at 180 °C for 90 min. Then, the samples were dissolved in 4 mL of *n*-heptane/2-propanol (98:2, *v/v*) mixture and the different isomers of POPs were collected through injection into a semi-preparative liquid chromatography system LC 20AR (Shimadzu - Kyoto, Japan) equipped with shim-pack UC-Diol II Core Focus as preparative column (250 mm, 10 mm ID, 5 μm; Shimadzu, Kyoto, Japan). From pure phytosterols each single isomer POP was isolated, in particular 7α-hydroxycampesterol (7α-HCam), 7α-hydroxystigmasterol (7α-HStig), 7α-hydroxysitosterol (7α-HS), 7β-hydroxycampesterol (7β-HCam), 7β-hydroxystigmasterol (7β-HStig), 7β-hydroxysitosterol (7β-HS), 5,6α-epoxysitosterol (α-ES), 5,6β-epoxysitosterol (β-ES), 5,6α-epoxystigmasterol (α-EStig), 5,6β-epoxystigmasterol (β-EStig), 5,6α-epoxycampesterol (α-ECam), 5,6β-epoxycampesterol (β-ECam), 7-ketocampesterol (7-KCam), 7-ketostigmasterol (7-KStig), 7- ketositosterol (7-KS).

2.3. Quality control of POPs reference material

To ascertain the amount and purity of each collected POP, 100 μL each were added of 19-HC (12 μg dissolved in 50 μL of methanol) dried under nitrogen flow (40°C), silylated [19] and 1 μL of TMS-derivatives were injected (split mode, 1:30) into a GC-MS and GC-FID (Shimadzu GC 2010; Kyoto, Japan). A capillary column Restek RTX-5 MS (50 m, 0.25 mm I.D., 0.1 μm film thickness; Bellefonte, PA), coated with 95% dimethyl and 5% diphenyl-polysiloxane was considered. The injector and FID temperatures were set at 325°C and 340°C, respectively; while in the case of GC-MS a temperature of 325°C and 200°C was set for transfer line and ion source, respectively. Helium at a constant linear velocity of 37.7 cm/s was employed as the carrier gas. For qualitative purposes, the POPs were recognized by comparing the mass spectra with those reported in literature, whereas the FID was used to determine the purity of recognized POPs. Due to the linear response of FID, the purity of POPs in each collected fraction was obtained by comparing the FID response of each POP isomer to that of the internal standard (19-hydroxycholesterols, 19-HC).

2.4. Determination of POPs by LC-orbitrap-HRMS

The determination of POPs was carried out according to literature [4]. Using an HPLC Ultimate 3000 system coupled to a Orbitrap high-resolution mass spectrometer (HRMS) Exploris 120 (Thermoscientific, USA) equipped with APCI ion source, 10 μL of filtered sample (0.22 μm, nylon filter) were injected. The elution was carried out using *n*-heptane (A) and 2-propanol (B) with the following gradient: 0–15 min 2% B; at 35 min and up to 40 min 20% B; 40–85 min 2% B. The separation was carried out on a Shim-pack UC-Diol II (250 mm, 2.1 mm ID, 3 μm, 100 Å; Shimadzu, Kyoto) maintained at 40°C. The acquisition was in positive and full scan mode (190–450 *m/z*) with a resolution of 120,000 at 300°C. Parallel reaction monitoring (PRM) was used to do the MS^2 analysis.

2.5. Determination of POPs by GC-MS

The TMS derivatives of POPs were determined in agree with Gallina Toschi et al. [14] using a GC-MS (Shimadzu GC 2010; Kyoto, Japan) equipped with a capillary column Restek Rxi-5 MS (60 m, 0.25 mm I.D., 0.25 μm film thickness; Bellefonte, PA) coated with 95% dimethyl and

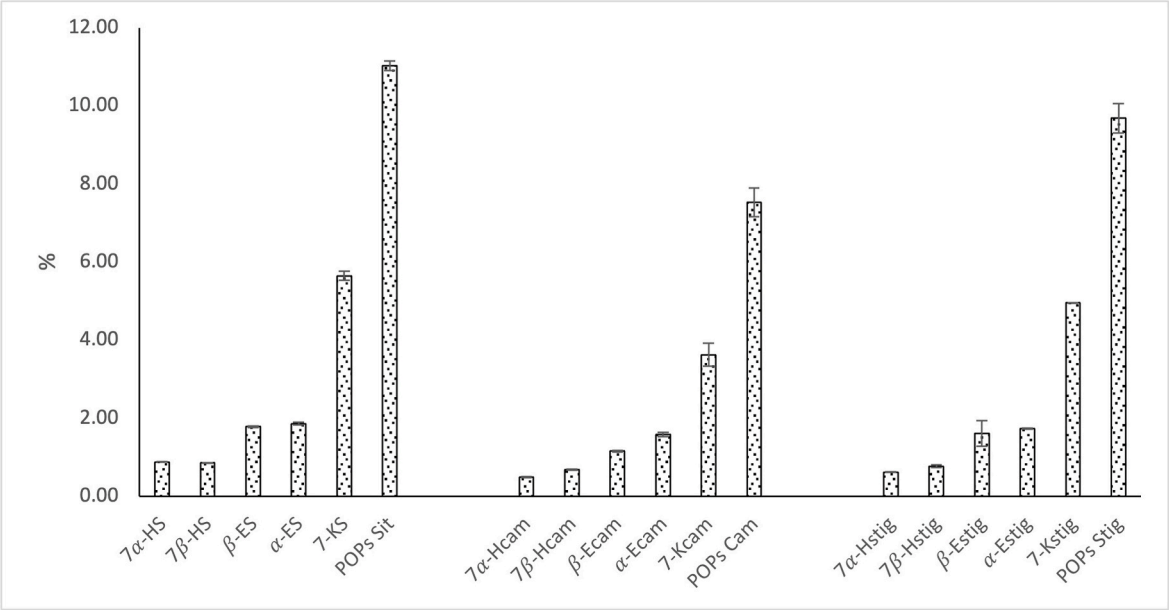

Fig. 1. Thermo-oxidative formation of POPs from pure phytosterols.

5% diphenyl-polysiloxane. The injector, transfer line and ion source temperatures were set at 325°C, 325°C and 200°C, respectively. The injection was carried out in splitless mode (2 min) and the separation of analytes was achieved by programming the oven temperature from 220°C to 330°C with a rate of 7°C/min using a 35.3 cm/s linear velocity of helium. A filament emission current of 70 eV was used to generate ions scanned at a rate of 1500 amu/s in a range from 50 to 600 *m/z*. For qualitative purposes the mass spectra were acquired in full scan (total ion current, TIC), whereas the characteristic and most abundant ions were used (single ion monitoring, SIM) for quantification: *m/z* 353 (19-HC); *m/z* 456 (7α/7β-HC); *m/z* 384 (α/β-EC); *m/z* 403 (triol); *m/z* 472 (7-KC); *m/z* 470 (7α/7β-HCam); *m/z* 482 (7α/7β-HStig); *m/z* 484 (7α/7β-HS); *m/z* 410 (α/β-EStig); *m/z* 398 (α/β-ECam); *m/z* 412 (α/β-ES); *m/z* 486 (7-KCam); *m/z* 498 (7-KStig); and *m/z* 500 (7-KS) [20].

2.6. Validation of GC-MS method

Since the validation of the LC-MS-orbitrap method was reported in our recently published study [4], in the present work the validation of the analytical method for determining POPs by GC-MS is described. Commercial COPs and purified POPs were used to validate the method considering specificity, linearity, limit of detection, precision and recovery. The linearity of the method was determined using commercial COPs or purified POPs in the range of 0.001 μg/mL – 5.060 μg/mL. The calibration curves were built at seven different concentration levels in triplicates by the internal standard method, where the POP or COP concentration/IS concentration ratio was plotted as related to POP or COP area/IS area ratio. The lowest concentrations that generated signals at least three times and ten times greater than the noise signal were used to determine the LOD and LOQ, respectively. Intraday and interday precision reported as relative standard deviation (%, RSD) was calculated by injecting (n=3) the identical sample on the same day for three consecutive days (n=3), respectively. Recovery was determined in refined high oleic sunflower oil (HOSO) by standard addition method at a concentration of 2.000 μg/g.

2.7. Determination of POPs in refined high oleic sunflower oil

In an amber flask containing 10 μL of IS (0.5 mg/mL) and 300 mg of refined high oleic sunflower oil were weighed and then 10 mL of 4N KOH in methanol containing BHT (5 mg/mL) were added. The cold saponification was carried out at room temperature (25°C) in the dark for 18h. Then, the unsaponifiable matter was extracted with diethyl ether and neutralized with double-distilled water until pH 7. After being filtered with anhydrous sodium sulfate (Na_2SO_4) the neutralized organic layer was dried at 38°C using a rotary evaporator.

To carry on the LC-Orbitrap-HRMS determination, the unsaponifiable matter was dissolved in 1 mL of *n*-heptane/2-propanol (98:2, v/v), filtered (0.22 μm, nylon filter) and 10 μL were injected and analyzed as reported above (section 2.4).

On the other hand, when GC-MS analysis was considered, the POPs fraction was isolated from unsaponifiable matter by SPE [21], silylated [20] and then the TMS derivatives were dissolved in 100 μL of *n*-hexane. One μL was injected into a GC and evaluated as reported in section 2.5.

2.8. Statistical analysis

In order to confirm if pure COPs can be used as reference material to quantify POPs, statistical analysis of results was carried out. The results are reported as the mean of at least three independent replicates (n=3) and standard deviation; a *t*-test with a 95% confidence level ($p<0.05$) was used to recognize differences between the two groups (COPs *vs* POPs) as related to the two laboratories involved in the study.

3. RESULTS and DISCUSSION

3.1. Isolation of POPs

The thermo-oxidative conditions tested led to the formation of 7-hydroxy, 5,6-epoxy and 7-keto oxidative derivatives of sitosterol, campesterol and stigmasterol. Fig. 1 reports the rate of formation estimated by GC-FID. The highest amount of POPs was obtained from β-sitosterol followed by stigmasterol and campesterol. In particular, under the tested conditions an oxidation rate of 11.03%, 9.68% and 7.53% was found for β-sitosterol, stigmasterol and campesterol, respectively. Those values are greater than that found for cholesterol where an oxidation rate of 6.0% was reported [22]. Under the tested conditions, the 7-keto derivatives were the most abundant isomers followed by epoxy-isomers and 7-hydroxy derivatives and the purity for each isolated compound

Table 1
Linearity of calibration curves (R^2), limit of detection (LOD), and limit of quantification (LOQ) of sterol oxidation products by GC-MS.

Sterol Oxidation Products	Calibration curve	R^2	LOD (μg/mL)	LOQ (μg/mL)
Cholesterol				
7α-HC	$y = 2.51\,x - 4.00^{-2}$	0.998	0.0132	0.0418
7β-HC	$y = 3.81\,x - 4.61^{-2}$	0.999	0.0128	0.0426
α-EC	$y = 0.22\,x - 6.33^{-3}$	0.997	0.0485	0.1615
β-EC	$y = 8.15\,x - 3.15^{-3}$	0.994	0.0512	0.1705
7-KC	$y = 0.79\,x - 2.09^{-2}$	0.998	0.0168	0.0560
β-Sitosterol				
7α-HS	$y = 3.43\,x - 5.87^{-2}$	0.995	0.0170	0.0547
7β-HS	$y = 3.47\,x - 6.00^{-2}$	0.992	0.0163	0.0543
α-ES	$y = 0.12\,x - 4.00^{-4}$	0.994	0.0310	0.1033
β-ES	$y = 0.10\,x - 4.00^{-4}$	0.990	0.0199	0.0663
7-KS	$y = 0.67\,x - 3.00^{-5}$	0.996	0.0415	0.1383
Campesterol				
7α-HCam	$y = 1.04\,x - 2.96^{-4}$	0.993	0.0261	0.0860
7β-HCam	$y = 1.06\,x - 3.00^{-4}$	0.993	0.0257	0.0857
α-ECam	$y = 9.00^{-5}\,x - 7.00^{-4}$	0.992	0.0212	0.0707
β-ECam	$y = 0.06\,x - 3.00^{-4}$	0.991	0.0941	0.3137
7-KCam	$y = 0.51\,x - 7.00^{-4}$	0.992	0.0392	0.1307
Stigmasterol				
7α-HStig	$y = 5.35\,x - 5.06^{-5}$	0.996	0.0641	0.2131
7β-HStig	$y = 5.32\,x - 5.00^{-5}$	0.991	0.0638	0.2127
α-EStig	$y = 0.07\,x - 1.00^{-4}$	0.994	0.0206	0.0687
β-EStig	$y = 0.08\,x - 2.00^{-4}$	0.993	0.0274	0.0913
7-KStig	$y = 0.50\,x - 1.20^{-3}$	0.990	0.1340	0.4467

was >90%. Authors [23] found that 7-KS and 7β-HStig were the main isomers detected when free stigmasterol was thermo-oxidized. Others [24] found the 5,6β-epoxy derivatives as the main POPs in heated rapeseed oil. Moreover, Rudzinska et al. [7] detected the 7-KStig, 7β-HStig and β-EStig as the main POPs when stigmasterol was stored at 60°C. However, as well reported by literature the different results could be ascribed to many different factors. In fact, the composition of the environment system where phytosterols are dissolved (such as bulk oils and emulsions), their form such as free or esters as well as the nature of esterified groups (e.g., fatty acids, phenols, glycoside, etc.) significantly impacts the kinetics of sterol oxidation [22,23,25,26].

3.2. GC-MS validation method

Table 1 reports the validation parameters of the GC-MS analytical method used to determine POPs. The method's sensitivity was determined by establishing the limits of detection (LOD) and quantification (LOQ). These parameters were determined for each compound to ensure accurate quantification across matrices. Linearity was evaluated by calculating the correlation coefficient (r^2) using the least squares method and confirming that its value was equal to or greater than 0.99 for all the analytes examined.

As reported in Table 1, all the isolated compounds displayed a good linearity (R^2>0.99) in the range considered. The LOD for COPs ranged between 0.0128 and 0.0512 μg/mL, while a range of 0.0163–0.0415 μg/mL, 0.0212–0.0941 μg/mL, and 0.0206–0.1340 μg/mL was assessed for sitosterol, campesterol and stigmasterol POPs derivatives, respectively. However, it might be pointed out that the highest sensitivity for 7β-HC was detected while the 7-KStig displayed the worst one. Thus, it might be hypothesized that considered POPs own different fragmentation behavior with respect to COPs when electronic ionization was considered. On the other hand, when Orbitrap® technology was considered the 7β-HCam exhibited the relatively lowest sensitivity [4].

That hypothesis was also established by the ionic abundance ratios calculated for each analyte (COP or purified POP) injected at the same concentration. The response factor as COP_{area}/IS_{area} or POP_{area}/IS_{area} ratio was estimated for both GC-MS and LC-MS-Orbitrap (Tables S1 and S2). The oxidative cholesterol derivatives (COPs) displayed a significant different (p<0.05) response with respect to the POPs for both techniques. When GC-MS was considered the α/β-EC and 7-KC displayed a greater response than relative phytosterol derivatives, whereas the 7α/7β-HStig exhibited the highest response (Table S1). However, it might be pointed out that no significant differences (p>0.05) were found between 7-KC, 7α/7β-HC and 7-KStig, 7α/7β-HStig, respectively. Again, COPs response determined by LC-MS-Orbitrap was significantly (p<0.05) lower than POPs and the β-ECam displayed the highest response (Table S2).

The recoveries were determined using both COPs and pure POPs as reference material. As reported in Table 2, using POPs as reference the recoveries ranged 90–97%, whereas when pure COPs were considered, the recoveries ranged from 86% to 102% displaying a larger variability. Again, the present study showed how the choice of reference material significantly (p<0.05) impacted the recoveries. The β-sitosterol derivatives showed better recovery values, ranging from 99.8% to 101.2% (based on COPs) and from 90.1% to 95.2% (based on purified POPs). On the other hand, campesterol displayed the worst performance in terms of recovery based on COPs (77.8–100.7%) while it significantly improved when pure POPs were considered (93.2–97.3%). The stigmasterol oxidizes also exhibited higher recoveries (92.1–102.2%) with COPs reference material than isolated pure POPs (91.7–94.1%). Those results agree with Menéndez-Carreño et al. [27] except for 7-KStig, which showed a recovery of 104%.

The intraday precision when COPs and POPs were used as reference material was lower than 6.3% and 5.3%, respectively. Again, the interday repeatability was lower than 4.9% and 5.3% as related to COPs and POPs, respectively. It might be pointed out, that no significant differences (p>0.05) were found on the repeatability as related to the reference material and results agree with literature [2,27]. Moreover, both intraday and interday confirmed an excellent precision of the method and β-sitosterol oxidized derivatives showed the best precision, with RSD values ranging from 2.1% to 4.8% (COPs based) and from 2.2% to 4.1% (POPs based) for intraday, and from 2.4% to 4.1% (COPs-based) and from 2.6% to 5.2% (POPs based) for interday precision.

3.3. Determination of POPs in refined high oleic sunflower oil

In order to compare the response of the two methods, both LC-MS-Orbitrap and GC-MS were compared using pure commercial COPs and isolated POPs as reference material for quantitation of POPs in refined high oleic sunflower oil (HOSO).

As reported in Table 3, the reference material significantly (p<0.05)

Table 2
Recovery (%), intraday and interday repeatability (%) determined on spiked refined high oleic sunflower oil by GC-MS as related to reference material (pure COPs (*C*); isolated POPs (*P*)).

Phytosterol Oxidation Products	Recovery *C*	Recovery *P*	*Sig. Recovery C x P*	Intraday *C*	Intraday *P*	*Sig. Intraday C x P*	Interday *C*	Interday *P*	*Sig. Interday C x P*
β-Sitosterol									
7α-HS	99.8	94.4	*	2.1	2.4	n.s.	2.5	3.3	n.s.
7β-HS	100.1	96.2	*	3.3	2.2	n.s.	2.4	3.8	n.s.
α-ES	101.2	95.2	*	4.2	3.5	n.s.	3.8	4.0	n.s.
β-ES	100.7	92.3	*	4.8	4.1	n.s.	3.4	2.6	n.s.
7-KS	86.4	90.1	*	2.6	3.5	n.s.	4.1	5.2	n.s.
Campesterol									
7α-HCam	77.8	96.7	*	3.7	2.6	n.s.	4.5	5.3	n.s.
7β-HCam	81.7	97.3	*	2.6	3.2	n.s.	3.3	3.6	n.s.
α-ECam	92.5	96.1	*	6.3	5.2	n.s.	3.4	5.1	n.s.
β-ECam	100.7	94.6	*	4.1	5.0	n.s.	4.2	5.2	n.s.
7-KCam	98.0	93.2	*	5.2	4.1	n.s.	4.9	3.5	n.s.
Stigmasterol									
7α-HStig	102.2	93.2	*	2.1	2.8	n.s.	2.5	3.1	n.s.
7β-HStig	92.1	94.1	*	3.6	2.6	n.s.	2.2	4.6	n.s.
α-EStig	98.6	92.5	*	4.1	3.3	n.s.	4.1	3.5	n.s.
β-EStig	99.7	93.6	*	3.4	5.3	n.s.	3.5	2.6	n.s.
7-KStig	100.5	91.7	*	2.1	3.8	n.s.	2.2	4.3	n.s.

Abbreviations: Sig., statistical significance; n.s., not significant; the asterisks denote the level of significance: *, $p<0.05$.

Table 3
Content of POPs in HOSO (mg/kg oil) determined by LC-Orbitrap-HRMS and GC-MS based on the different reference material (isolated POPs (*P*) and pure COPs (*C*)).

Phytosterol Oxidation Products	LC-Orbitrap-MS			GC-MS			LC-Orbitrap-MS (*P*) *x* GC-MS (*P*)
	Calibration P	*Calibration C*	*Sig.*	*Calibration P*	*Calibration C*	*Sig.*	*Sig.*
β-Sitosterol							
7α-HS	0.024 ± 0.003	0.126 ± 0.012	***	0.021 ± 0.001	0.115 ± 0.010	***	n.s
7β-HS	0.024 ± 0.003	0.136 ± 0.009	***	0.039 ± 0.005	0.132 ± 0.012	***	*
α-ES	0.647 ± 0.027	0.786 ± 0.035	**	0.542 ± 0.054	1.047 ± 0.094	***	*
β-ES	0.194 ± 0.034	7.175 ± 1.296	***	0.092 ± 0.004	1.475 ± 0.152	**	*
7-KS	0.946 ± 0.350	0.671 ± 0.226	n.s.	0.594 ± 0.072	0.515 ± 0.069	n.s.	*
Total	1.845 ± 0.590	8.894 ± 1.232	***	1.188 ± 0.192	3.284 ± 0.477	***	n.s.
Campesterol							
7α-HCam	0.004 ± 0.001	0.032 ± 0.002	***	nd	nd	–	
7β-HCam	0.018 ± 0.001	0.049 ± 0.002	***	0.008 ± 0.001	0.035 ± 0.004	***	*
α-ECam	0.068 ± 0.011	0.168 ± 0.027	**	0.037 ± 0.004	0.104 ± 0.009	**	*
β-ECam	0.004 ± 0.001	1.643 ± 0.242	***	nd	nd	–	–
7-KCam	0.187 ± 0.016	0.146 ± 0.019	n.s.	0.041 ± 0.005	0.053 ± 0.008	n.s.	*
Total	0.281 ± 0.042	2.038 ± 0.413	***	0.086 ± 0.014	0.192 ± 0.030	*	*
Stigmasterol							
7α-HStig	0.009 ± 0.001	0.032 ± 0.005	**	0.007 ± 0.000	0.029 ± 0.002	**	n.s.
7β-HStig	0.005 ± 0.002	0.085 ± 0.035	*	nd	nd	–	–
α-EStig	0.078 ± 0.003	0.199 ± 0.012	***	0.063 ± 0.005	0.177 ± 0.014	**	*
β-EStig	0.026 ± 0.007	1.522 ± 0.440	**	0.015 ± 0.002	0.837 ± 0.074	***	n.s.
7-KStig	0.118 ± 0.015	0.176 ± 0.023	n.s.	0.092 ± 0.007	0.139 ± 0.012	n.s.	n.s.
Total	0.236 ± 0.040	2.014 ± 0.728	***	0.177 ± 0.020	1.182 ± 0.144	***	n.s.
ΣTotal POPs	2.352 ± 0.672	12.946 ± 3.373	***	1.451 ± 0.226	4.658 ± 0.651	***	n.s.

Abbreviations: Sig., statistical significance; n.s., not significant; the asterisks denote the level of significance: *, $p<0.05$; **, $p<0.01$; ***$p<0.001$; nd, not detected.

affected the determination of POPs in HOSO. The observed differences between the results based on the two calibration curve systems highlight a potential issue in the quantification of POPs. That divergence raises questions about the consistency and reliability of the use of COPs for determining POPs. When pure COPs were used as reference material, a general overestimation was observed by both LC-MS and GC-MS, except for 7-keto derivatives, which displayed similar results. The total POPs determined by LC-MS based on isolated POPs in HOSO were from 1.88 to 2.83 mg/kg oil, while using COPs as standard a content ranged between 10.56 mg/kg oil and 15.33 mg/kg oil was found. On the other hand, by GC-MS the content was 1.29–1.61 mg/kg oil and 4.20–5.12 mg/kg oil as related to pure COPs and POPs as reference material, respectively. However, significant differences ($p>0.05$) were found in the content of total POPs between LC-MS-Orbitrap and GC-MS using POPs as chemical standards (Table 3). The β-sitosterol being the main sterol detected in HOSO generated the highest amount of POPs followed by campesterol and stigmasterol. Both LC-MS and GC-MS methods based on pure POPs confirmed the 7-KS and α-ES as the main oxidized sterols, whereas COPs led β-ES as the greatest POP. These results suggest that even if POPs and COPs could have similar pattern fragmentation [14,28,29] a different

ion abundance is generated as related to the ion source.

In the EI-MS, as reported for COPs, the characteristic ions of TMS ethers of POPs were $[M]^+$, $[M-90]^+$ or $[M-180]^+$, which corresponds to the loss of 1 or 2 TMSOH groups. As expected, $[M]^+$ was the base peak for the 7-keto derivatives; while the $[M-90]^+$ was the most abundant fragment for hydroxy derivatives. However, in the case of β-sitosterol epoxy derivates $[M]^+$ and $[M-90]^+$ was about 30% and 42%, respectively, of total ions; whereas about 20% and 14% for campesterol, 18% and 16% for stigmasterol were found. Considering the obtained results, the main differences observed in the quantification of POPs are related to the fragmentation of POPs. In fact, only when $[M]^+$ was the peak base and was used as quantifier ion not significant differences ($p>0.05$) were observed with respect to the reference material. As reported by literature [30] the different groups in the side chains of phytosterols (such as methyl, ethyl substituent, or double bound) could affect the abundance of characteristic ions; explaining the different responses.

When POPs are determined by LC-MS the derivatization procedure as well as SPE isolation can be avoided leading to a simplified analytical determination [4,6]. Considering the polarity of POPs, the APCI permits an efficient ionization of POPs; generally, the 7-keto derivatives generate ion $[M+H]^+$ whereas the 7-hydroxy and epoxy sterol derivatives produce characteristic ions $[M+H-H_2O]^+$ or $[M+H-2H_2O]^+$ due to the loss of one or two water molecules, respectively. Since the 7-keto sterol derivatives content did not display significant differences ($p>0.05$) as related to reference material, it can be hypothesized that different responses of unstable polar POPs could be ascribed to the energy required to lose water during the fragmentation. On the other hand, even though β-sitosterol, campesterol and stigmasterol share the same central sterol nucleus of cholesterol (four hydrocarbon rings, a hydroxyl group in C3, and a double bond in C5-C6) differences are in the side chain: β-sitosterol displays an ethyl substituent in C24; campesterol shows a methyl substituent in C24 and stigmasterol exhibits an ethyl substituent and additional double bound in C22-C23.

In the present work, two different laboratories (DISAFA - University of Turin; and R&D Fats and Oils Department, Soremartec srl) were enrolled for the determination of POPs in HOSO using the same standard solutions. Based on these results, for the first time, it was demonstrated that the use of COPs for determining POPs has to be reconsidered, in particular for quantitative scope. In fact, the present work confirms the suitability of 7-KC to be used as reference material for the determination of 7-keto phytosterol derivatives. However, it might be highlighted that significant differences ($p<0.05$) in the POPs content were observed as related to the considered equipment, since GC-MS displayed a lower value than those obtained by LC-Orbitrap-HRMS. Furthermore, a similar behavior was reported about COPs by Lutjohann et al. [31], where eleven laboratories were involved in the determination of COPs in lyophilized pooled sera using the same standard stock solutions. However, higher sensitivity was confirmed by LC-Orbitrap-HRMS, since 7α-HCam, β-ECam, and 7β-HStig (not detected by GC-MS) were at level <0.004 mg/kg of oil.

4. Conclusion

Over the past decade, there has been a significant increase in interest in phytosterol oxidation products due to their potential involvement in the development of various diseases. Accordingly, there is a pressing need for the development of a sensitive and reproducible analytical method that can more accurately determine the content of POPs in foods or ingredients. However, as no commercial pure standards for POPs are currently available, except for 7-ketositosterol, the use of COPs as reference material has been the prevailing applied approach. Recently, the isolation of pure POPs was reported through the use of a semi-preparative HPLC. Based on that, the primary purified POPs isomers (7-hydroxy; 5,6-epoxy and 7-keto derivatives of sitosterol, campesterol and stigmasterol) were employed to build calibration curves and utilized for the determination of POPs in refined high oleic sunflower oil. The results were then compared with those obtained using pure COPs. In the present study, liquid chromatography and gas chromatography were employed coupled with high-resolution mass spectrometry (Orbitrap® technology) and low-resolution mass spectrometry (single quadrupole), respectively. The use of COPs or POPs as reference material resulted in significant differences in the quantification of POPs. In particular, the use of COPs led to a general overestimation of POPs content, except for the 7-keto derivatives of sitosterol, campesterol, and stigmasterol. However, the LC-Orbitrap-HRMS displayed greater sensitivity than the GC-MS, as some isomers were not detected in HOSO. Once more, the results were significantly affected by the preparative procedure. Indeed, when GC-MS was considered, in addition to the saponification, also the isolation of POPs by SPE and their derivatization were carried out, which affected the final results. The present study for the first time compared the two approaches to quantify POPs. It demonstrated that the choice of the right reference material plays a crucial role in order to reach robust and reproducible results, and the use of pure COPs as chemical standards for POPs quantification in vegetable oil such as HOSO has to be reconsidered.

CRediT authorship contribution statement

Ambra Bonciolini: Writing – original draft, Visualization, Formal analysis. **Annalisa Vissio:** Formal analysis. **Raffaele Calaminici:** Writing – review & editing. **Emanuele Forte:** Writing – review & editing, Conceptualization. **Vladimiro Cardenia:** Writing – review & editing, Supervision, Resources, Conceptualization.

Ethics statement

No human or animal subjects were used in the course of the research for this study.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jafr.2025.101961.

Data availability

Data will be made available on request.

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