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Validation of a gas chromatography–mass spectrometry method for the analysis of sterol oxidation products in serum

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Abstract

A validated gas chromatography–mass spectrometry (GC–MS) detection method for the quantitative analysis of sterol oxidation products (SOPs) in serum is described. After a lipid extraction procedure with chloroform–methanol, a cold saponification and purification by solid phase extraction, oxysterols were derivatizated to form trimethyl-sylil-ethers which were subjected to GC–MS analysis. Calibration curves for cholesterol oxidation products showed determination coefficient (R^2) of 1.0, with low values for the coefficient of variation of the response factors (<1%). Detection and quantification limits were below 5 ng/mL and 10 ng/mL, respectively. Recovery data were between 77.65% and 110.29% (CV < 10% for all compounds). Good results were obtained for within- and between-day repeatability, with values below 10%. In conclusion, the method performed is suitable for the determination and quantification of SOPs in serum.

Keywords: Serum; Sterols; COPs; SOPs; Linearity; Recovery; Repeatability

1. Introduction

Sterols are unsaponificable lipids susceptible to oxidation by reactive oxygen species, light, UV light, ionizing radiation, chemical catalysts, hydroxyperoxides lipids and enzymatic reactions, leading to the formation of sterol oxidation products (SOPs) both of animal and vegetal origin: cholesterol oxidation products (COPs) and phytosterol oxidation products (POPs), respectively [1–5].

Studies carried out in humans point out that COPs could be absorbed from the diet [6,7]. POPs have been detected to be absorbed in experiments carried out with rats [8–10] and they have also been found in human healthy volunteers [11,12]. The toxicological effects of COPs have been well documented because of their wide range of adverse biological effects related to citotoxicity, apoptosis, mutagenesis and carcinogenesis; being specially important the fact that the presence of COPs in human macrophages/foam cells and atherosclerotic plaques has been suggested to induce the development of atherosclerosis [13–17]. 7β-Hydroxycholesterol concentration in plasma was identified as the strongest predictor of a rapid process of carotid atherosclerosis in humans [18]. For these reasons, it is very convenient to know the content of COPs in foods, especially those which have suffered technological or cooking processes, whose heating treatments are known to generate oxidation processes. Several studies about COPs formation and content in different types of foods have been carried out [2,19–22].

Due to the structural similarity between phytosterols and cholesterol, it has been postulated that POPs could cause health damages comparable to COPs. There are only a few reports regarding the cytotoxic effects of POPs, which seem to be less severe [23–25]. Some research works attending to find out a proper methodology for the identification and quantification of POPs in vegetable oils, potato chips and French fries [26–28], milks [29], enriched spreads [30,31] have been performed. Recently, it has been tried to optimize the analytical method for the determination of POPs in foods using a method based on saponification and transesterification of the lipid extract, in combination with solid phase extraction (SPE) [32]. The commercialization of sterol-enriched foods as functional foods due to their hypocholesterolemic effects could lead to an increase in the organism sterol levels. These sterols could be oxidized by the same ways of cholesterol giving rise to POPs formation.

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Concerning the methods used for the quantitation of SOPs, there is a lack of uniformity and also a large variation of data in products or tissues of very similar nature when analyzed in different laboratories [33]. A validated GC–MS detection method for the quantitative analysis of SOPs has great importance in order to calculate the daily intake and their formation in the organism. There are not POPs standards commercially available, so the analytical methods for POPs have been carried out using commercial COPs as model substances, assuming that the ionization efficiency of both types of molecules and their behaviour is very similar, as they have similar structures. Response factors obtained for COPs are also valid for quantitative work regarding POPs [34–36].

The structural similarity of SOPs forces to develop a highly selective method for their proper identification and quantification. The development of accurate and sensitive methods for qualitative and quantitative analysis of oxysterol in biological samples has become a new challenge for basic investigations in agricultural science and nutritional and clinical research [37]. The purpose of this study was to develop and validate a method to evaluate the presence of SOPs in a specific matrix (human serum) by using a suitable gas chromatography–mass spectrometry method that enables the quantification of SOPs (COPs and POPs).

2. Material and methods

2.1. Chemicals and samples

7α-Hydroxycholesterol, 7β-hydroxycholesterol, 5,6β-epoxycholesterol, 5,6α-epoxycholesterol, cholestanetriol, 25-hydroxycholesterol, 7-ketocholesterol and 19-hydroxycholesterol were purchased from Steraloids (Wilton, NH, U.S.A.). The commercial mixture of stigmasterol and β-sitosterol was from Fluka (Buchs, Switzerland) (purity: 60% β-sitosterol and 30% campesterol). Tri-Sil reagent was obtained from Pierce (Rockford, IL, U.S.A.). Acetone, chloroform, diethyl ether, methanol, hexane, sodium sulphate anhydrous and potassium hydroxide were obtained from Panreac (Barcelona, Spain). Hexane for gas chromatography and butylhydroxytoluene (BHT) were from Merck & Co. Inc. (Whitehouse Station, NJ, U.S.A.). Sep-pack Vac 6cc silica 1g cartridges were obtained from Waters (Milford, Massachussets, U.S.A.).

Blood samples were obtained from 9 healthy volunteers from the Department of Food Science, Nutrition, Physiology and Toxicology of the University of Navarra. Volunteers did not receive any special diet. Serum was obtained by the centrifugation of blood during 15 min at 3500 rpm using an Eppendorf Centrifuge 5804R (Hamburg, Germany) and samples were stored at −80 °C until their analysis.

2.2. Preparation of stock and standard solutions of SOPs

Three stock solutions of internal standard were prepared by dissolving 2 mg of 19-hydroxycholesterol in 100 mL of a mixture of hexane and 2-propanol (3:2, v/v). The internal standard variability was calculated to assure its validity. Each solution was silylated as indicated below, analyzed by GC–MS in triplicate and total coefficient of variation of the areas obtained was 2.3%, ensuring that any of the internal standard solutions prepared was correct. Three stock solutions of a mixture of COPs were made. In each stock solution, approximately 4 mg of each component was weighed and diluted to 100 mL using a mixture of hexane and 2-propanol (3:2, v/v) giving rise to 40 μg mL^{-1} approximately for each oxysterol-standard. With each stock solution, 13 different amounts ranging from 3.2 μL to 12.5 mL were taken and diluted to 25 mL in order to obtain the corresponding series of standard solutions, whose concentrations ranged from 0.05 μg mL^{-1} to 20 μg mL^{-1}.

2.3. Preparation of POPs

As no phytosterol oxidation products are commercially available, preparation of POPs was carried out for further identification purposes. Phytosterol oxidation products were obtained by thermo-oxidation and chemical synthesis following the method described by Conchillo et al. [31]. Briefly, 8 mL of the commercial mixture β-sitosterol-campesterol (6:3, w/w) (1 mg mL^{-1}) were placed in a 200 mL open vial and the solvent was evaporated under nitrogen. Afterwards, it was thermo-oxidized by heating at 150 °C for 2 h in an oven in order to form 7α-hydroxy, 7β-hydroxy, 5,6β-epoxy, 5,6α-epoxy, 25-hydroxy, 7-keto derivates. Finally, they were dried under nitrogen stream. The resulting thermo-oxidized mixture was dissolved in 8 mL of *n*-hexane-isopropanol (3:2, v/v). The same process was repeated with stigmasterol. Triol derivates were also synthesized as suggested by Dzeletovic et al. [38]. Phytosterol standards were stirred for 2 h with *m*-chloroperbenzoic acid to form the epoxy derivates, which were extracted with diethyl ether and purified by SPE. The epoxy derivates were then refluxed with methanol:HCl 6 M (5:1, v/v) for 2 h, giving rise to the triol derivates.

2.4. Preparation of sample solutions of SOPs

Lipids were extracted from serum according to a modified version of the method described by Folch et al. [39] using chloroform–methanol (2:1, v/v). 1 mL of serum was introduced into a centrifuge tube, and 6 mL of chloroform and 3 mL of methanol were added. After shaking on vortex for 1 min, samples were then centrifuged at 4000 rpm for 15 min to facilitate phase separation using a Hermle Z320 centrifuge (Apeldoorn, The Netherlands). The chloroform (lower) layer was transferred to a test tube taking care of not transferring any solid material which remains at the interface between the upper and lower phases. 10 mL of 1N KOH solution in methanol and 1 mL of stock solution of the internal standard (19-hydroxycholesterol 20 μg mL^{-1}) were then added to perform a cold saponification at room temperature for 20 h, in darkness and under continuous agitation in an orbital shaker (Rotaterm P; Selecta, Barcelona, Spain) at 100 rpm. 1 mL of BHT (100 ng mL^{-1}) was added as antioxidant in order to avoid SOPs artifact formation during the saponification. The unsaponifiable material was extracted with

diethyl ether and purified by SPE according to the procedures described in detail in Guardiola et al. [40], as follows. The sample was transferred to a separating funnel with 10 mL of distilled water and 10 mL of diethyl ether. After shaking, the upper layer (organic phase) was recovered and the aqueous phase was re-extracted twice with 2 portions of 10 mL of diethyl ether. The organic phases were transferred to the same funnel. The diethyl ether fraction was washed in the funnel, first in 5 mL of 0.5 M aqueous KOH solution, and then in 2 portions of 5 mL of distilled water. The wasted organic extract is dried with anhydrous sodium sulphate and recovered in a round-bottomed flask, from which the solvent was evaporated to dryness using a rotavapor at 30 °C. The non-saponifiable extract was redissolved in 5 mL of hexane and applied to a SPE silica cartridge, previously equilibrated with 5 mL of hexane. The cartridge was subsequently treated with 10 mL of hexane:diethyl ether (95:5, v/v), 30 mL of hexane:diethyl ether (90:10, v/v) and finally with 10 mL of hexane:diethyl ether (80:20, v/v). SOPs were finally eluted from the SPE cartridge with 10 mL of a mixture of acetone/methanol (60:20, v/v).

2.5. Derivatization

Either samples' solutions of SOPs or standard solutions of COPs (1 mL) were derivatized to trimethylsilyl (TMS) ethers according to a slight adaptation of the method described by Dutta and Appelqvist [26]. The adaptation affected only the amount of derivatizating solution and final sample volume. In brief, the procedure was as follows: after drying the solvent from the sample or standard solution, 400 μL of Tri-Sil reagent was added and the tubes were kept at 60 °C for 45 min. The solvent was evaporated under a stream of nitrogen and the TMS-ether derivates were dissolved in 400 μL of hexane. Dissolved samples were filtrated using a syringe driven filter unit Millex®-HV (0.45 μm × 13 mm) (Millipore Corporation, Bedford, MA 01730, U.S.A.) prior to GC–MS analysis in order to avoid the damage of the capillary column.

2.6. Gas chromatography–mass spectrometry analysis

Gas chromatography–mass spectrometry analysis was performed on a GC 6890N Hewlett Packard coupled to a 5975 Mass Selective Detector (Agilent Technologies Inc., CA, U.S.A.). The TMS-ethers derivates of sterol oxides were separated on a capillary column Varian VF-5ms CP8947 (50 m × 250 μm × 0.25 μm film thickness) (Varian, France). Oven temperature conditions were optimized in order to achieve a proper separation of the individual compounds, taking into account both cholesterol and phytosterol oxidation products. After testing different ramps, oven was finally programmed with an initial temperature of 75 °C, heated to 250 °C at a rate of 30 °C min^{-1}, raised to 290 °C at rate of 8 °C min^{-1}, and finally, it was raised to 292 °C at a rate of 0.05 °C min^{-1}.

High purity helium was used as a carrier gas at a flow rate of 1 mL min^{-1}. The inlet pressure used was 13.40 psi. The injector temperature was 250 °C and the transfer line to detector at 280 °C. The samples were injected in a splitless mode with an injection volume of 1 μL. The mass spectrometer was operated in electron impact ionization (70 eV). Identification of the peaks was made by the characteristic ion fragmentation of the standard substances and the quantification was made using selected ion monitoring (SIM) analysis. For that purpose, 4 different time programs were performed, one for each family of oxysterols studied monitoring the characteristic ion of each SOPs, indicated in bold in Table 1. Integration was performed with Agilent G1701DA GC/MSD ChemStation (Agilent Technologies Inc., CA, U.S.A.).

2.7. Method validation

The validation was done according to the International Conference on Harmonisation guidance for the validation of analytic methods [41]. It was based on the following criteria: selectivity, linearity, limit of detection and limit of quantification, precision (within- and between-day variability) and recovery. Criteria used to verify linearity were: determination coefficient ($R^2 > 0.99$), coefficient of variation between response factors <5%, slope interval not having to include zero and intercept interval having to include zero. These data were obtained through the application of a linear regression test that also includes an ANOVA test that allows concluding about the linear dependence between areas and concentrations of the assayed samples.

Limit of detection (DL) and quantification (QL) were determined with the following formulas, by using the calibration curve method for the analysis of the standard deviation of the response:

$$\mathrm{DL} = \frac{(3 \times Sbl) + Ybl}{b\sqrt{n}}$$

$$\mathrm{QL} = \frac{(10 \times Sbl) + Ybl}{b\sqrt{n}}$$

where *Ybl* and *b* are the respective intercept and slope of a curve made representing the ratio of the area for each concentration level/area internal standard versus the ratio concentration oxysterol/concentration internal standard after analyzing three serum samples fortified with the following concentrations: 0.01 μg mL^{-1}, 0.021 μg mL^{-1} and 0.042 μg mL^{-1}; *Sbl* the intercept of another curve obtained representing the standard deviation for each concentration level versus the concentration; *n* is the number of replicates made in order to improve the accuracy.

Repeatability or within-day variability was assessed using 9 determinations (3 concentrations/3 replicates each) for all the compounds studied covering the specified range for the assay. To assess intermediate precision or the between-day variability, the previous procedure was carried out but in different days. These precision tests were performed injecting standard compounds solutions of the following concentrations: 10 ng mL^{-1}, 20 μg mL^{-1} and 40 μg mL^{-1}.

Recovery was based on fortification of samples of the control serum with a known mixture of standard compounds at 3 different concentrations: (1) approximately the double quantity as the determined from analysis of control serum; (2) the same

Table 1
Characteristic ions of SOPs and retention times obtained in a Varian VF-5ms CP8947 column

Oxysterol	Characteristic ions (*m/z*)	Retention times (min)	Relative retention times
7α-Hydroxycholesterol	456 **457** 458 546	21.577	0.889
7α-Hydroxycampesterol	470 **471** 472	24.418	1.008
7α-Hydroxystigmasterol	482 **483** 484	24.605	1.015
7β-hydroxycholesterol	456 457 **458** 546	25.497	1.050
7α-Hydroxysitosterol	484 **485** 486	26.787	1.106
5,6β-Epoxycholesterol	356 384 445 **474**	26.994	1.112
5,6α-Epoxycholesterol	**366** 384 459 474	27.397	1.128
7β-Hydroxycampesterol	470 471 **472**	28.943	1.195
7β-Hydroxystigmasterol	482 483 **484**	29.194	1.205
Cholestanetriol	**403** 404 456 546	30.176	1.243
5,6β-Epoxycampesterol	370 383 398 **488**	30.956	1.278
5,6β-Epoxystigmasterol	253 382 410 **500**	31.645	1.306
7β-Hydroxysitosterol	484 485 **486**	31.850	1.314
5,6α-Epoxycampesterol	398 **380** 488	31.886	1.315
25-Hydroxycholesterol	131 **271** 327 456	32.253	1.328
5,6α-Epoxystigmasterol	253 **392** 410 500	32.464	1.340
7-Ketocholesterol	131 367 **472** 514	33.185	1.387
Campestanetriol	**417** 418 470 560	34.805	1.436
5,6β-Epoxysitosterol	384 394 412 **502**	35.850	1.438
Stigmastanetriol	**429** 253 482 572	35.109	1.449
25-Hydroxycampesterol	**131** 470 545 560	35.264	1.455
5,6α-Epoxysitosterol	**394** 397 412 502	35.886	1.481
25-Hydroxystigmasterol	**131** 482 557 572	37.337	1.541
7-Ketocampesterol	**486** 381 487 396	38.033	1.570
Sitostanetriol	**431** 432 484 574	38.573	1.592
7-Ketostigmasterol	357 359 **498** 347	40.069	1.654
25-Hydroxysitosterol	**131** 484 559 574	40.979	1.691
7-Ketositoserol	395 **500** 501 410	43.652	1.801

Note: ions in bold are used for quantification purposes.

quantity; (3) a quantity above the limit of quantification. The recovery was calculated according to

$$\%R = \frac{\mathrm{Mfc} - \mathrm{Mc}}{\mathrm{Mf}} \times 100$$

where $\%R$ is percent recovery, Mfc is the raw amount in μg of compound determined in the fortified sample, Mc the raw amount in μg of compound in the unfortified material and Mf is the fortification amount in μg.

2.8. Statistical analysis

Mean, standard deviation and coefficient of variation of data are shown in tables. Software used for statistical analysis of the data was the SPSS 15.0 program (SPSS Inc., Chicago, IL, U.S.A.).

3. Results and discussion

3.1. Selectivity

Mass spectra data of the TMS ethers derivates of SOPs are presented in Table 1 pointing in bolds those used for the quantification in the SIM spectra. The ions identified for each SOPs were compared to previously published spectra [26,32]. Retention times (RT) and relative retention times (RRT) in relation to 19-hydroxycholesterol are also shown in Table 1. The order of elution of SOPs followed the sequence 7α-hydroxy, 7β-hydroxy, 5,6β-epoxy, 5,6α-epoxy, triol, 25-hydroxy, 7-keto oxyderivates for every compound. Along 45 min the oxyderivates from cholesterol, campesterol, stigmasterol, and sitosterol eluted in the order shown in Fig. 1, showing slight differences in the pattern compared to oxysterol separations performed in other works with different columns. Apprich and Ulberth [35] pointed out the importance of the type of stationary phase of columns but also the length of the capillary column for the order of the elution of sterol oxidation products. The most difficult part of the chromatogram to be resolved was that comprising RRT from 1.436 to 1.455, which included 4 compounds. In particular, the coelu-

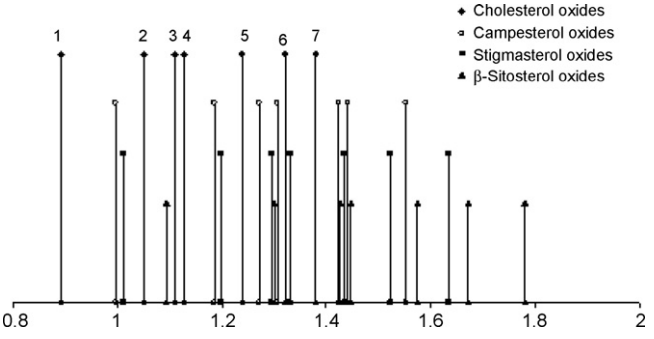

Fig. 1. Representation of the relative retention times of TMS-oxysterol in relation to 19-hydroxycholesterol on VF-5ms (Varian). GC–MS and He as a carrier gas were used. The elution order of TMS-oxysterols was: (1) 7α-hydroxy, (2) 7β-hydroxy, (3) 5,6β-epoxy, (4) 5,6α-epoxy, (5) triol, (6) 25-hydroxy and (7) 7-keto.

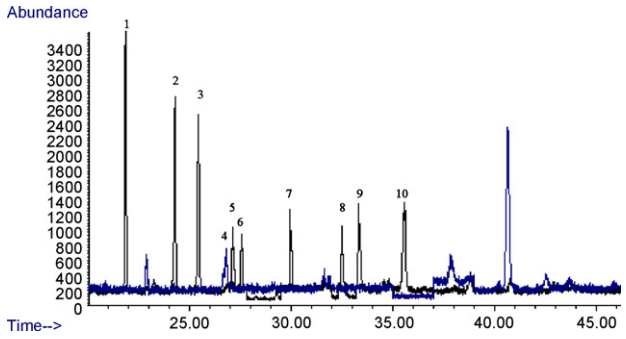

Fig. 2. Serum sample chromatograms on VF-5ms (Varian). GC–MS and He as a carrier gas were used. The elution order of TMS-oxysterols was: (1) 7α-hydroxycholesterol, (2) 19-hydroxycholesterol, (3) 7β-hydroxycholesterol, (4) 7α-hydroxysitosterol, (5) 5,6β-epoxycholesterol, (6) 5,6α-epoxycholesterol, (7) cholestanetriol, (8) 7β-hydroxysitosterol, (9) 25-hydroxycholesterol and (10) 7-ketocholesterol.

tion of campestanetriol and 5,6β-epoxysitosterol was detected. However, no problems were found to carry out a proper identification and quantification of these compounds, as ions used for all of them were different and SIM evaluation of the chromatogram enabled good selectivity among compounds.

Problems with the presence of non-oxidized sterols were found neither. As no phytosterol oxidation products are commercially available, a previous synthesis of POPs was done according to the procedure described by Conchillo et al. [31], with a thermo-oxidation of sterol standards and a chemical synthesis for obtaining the triol derivates. Purification by SPE completely eliminated the non-oxidized sterols, if this is performed at a proper filtration speed, avoiding overlapping of sterols with their oxides.

The results of the analysis of a sample from one of the volunteers are represented in Fig. 2. Two overlapped chromatograms showing the oxidation products from the two families detected in the sample, cholesterol and sitosterol, are shown. No oxidation products from campesterol and stigmasterol were detected in any serum samples. All TMS-ether derivates of COPs were identified. Only 2 sitosterol oxides, 7α-hydroxysitosterol and 7β-hydroxysitosterol, were quantified in the serum samples. They were detected in every analyzed serum sample. Using the calibration curves of their corresponding COPs, the mean value obtained for both compounds in the 9 volunteers were 47.3 ng mL^{-1} and 49.0 ng mL^{-1}. In some studies, it was found out that these compounds could be absorbed from the diet [10]. Plat el al. [11] found oxyphytosterols in serum from a sitosterolemic patient, while the concentrations of serum oxyphytosterols in healthy control subjects were below the limit of detection. Grandgirard et al. [12] identified however noticeable quantities of β-epoxysitosterol and sitostanetriol in healthy human volunteers. Some authors maintained that oxyphytosterol in healthy subjects could derive from the oxidation catalysed by UV light of skin phytosterols absorbed from cosmetic products [42]. This fact could explain the different types and proportion of oxyphytosterols in healthy serum samples. Oxyderivates from campesterol or stigmasterol were not found in this work. It would be interesting to analyze the presence of SOPs in subjects consuming sterol-enriched foods for long periods.

3.2. Linearity

The linearity was evaluated by analyzing the three batches of stock and corresponding standard solutions containing the COPs at concentration levels in a range between 0.01 μg mL^{-1} and 40 μg mL^{-1} for each studied compound (7α-hydroxycholesterol, 7β-hydroxycholesterol, 5,6β-epoxycholesterol, 5,6α-epoxycholesterol, cholestanetriol, 25-hydroxycholesterol and 7-ketocholesterol). Three replicates of each standard solution were analyzed. For each oxysterol, calibration lines were plotted representing the ratio of area of selected ion/area of internal standard versus the ratio of their respective concentrations. No significant differences were detected for the slope and the intercept among the 3 calibration curves obtained with the 3 different batches of solutions ($p < 0.05$), so one of them was finally chosen for each compound (Table 2). These curves were linear within the concentration ranges studied, with determination coefficients (R^2) of 1.0 for each compound. The coefficients of variation of the response factors were also calculated, obtaining values below 1%, which indicated a very low dispersion of data. The slope interval for every oxysterol did not include zero, and the intercept interval did (data not shown). ANOVA data arisen from the linear regression test confirmed that area values for every COP were linearly dependant on the concentration (data not shown). It can be concluded that linearity parameters achieved adequate values for validation purposes.

3.3. Limit of detection and limit of quantification

The limit of detection is the lowest amount of SOPs in a serum sample which can be detected but not necessarily quantitated as

Table 2
Linearity of the plot of area response ratio vs. concentration ratio; limit of detection and limit of quantification

Oxycholesterol	Calibration curve	Determination coefficient (R^2)	CV of response factors	LD (ng mL^{-1})	LQ (ng mL^{-1})
7α-Hydroxycholesterol	$y = 1.6134x + 0.00000001$	1.000	0.01	1.23	3.68
7β-Hydroxycholesterol	$y = 1.517x + 0.00000006$	1.000	0.02	2.38	6.29
5,6β-Epoxycholesterol	$y = 0.6141x + 0.00000002$	1.000	0.94	3.22	8.06
5,6α-Epoxycholesterol	$y = 0.5289x - 0.000009$	1.000	0.16	4.14	9.76
Cholestanetriol	$y = 1.3454x + 0.00000009$	1.000	0.36	1.84	5.52
25-Hydroxycholesterol	$y = 0.8423x + 0.0000009$	1.000	0.14	2.83	9.29
7-Ketocholesterol	$y = 0.5031x + 0.00000008$	1.000	0.15	3.09	9.00

A Varian VF-5ms CP8947 column (50 m × 250 μm × 0.25 μm) was used.

Table 3
Precision within-day and between-day (repeatability and intermediate precision)

Oxycholesterol	Theoretical concentration ($\mu g\,mL^{-1}$)	Within-day variability measured concentration ($\mu g\,mL^{-1}$), CV(%) ($n=9$)	Between-day variability measured concentration ($\mu g\,mL^{-1}$), CV(%) ($n=9$)
7α-Hydroxycholesterol	40.50	41.21; 0.07	40.57; 0.13
	20.25	20.29; 0.74	19.99; 0.08
	0.01	0.0075; 2.80	0.007; 0.93
7β-Hydroxycholesterol	41.50	40.69; 0.24	38.07; 1.00
	20.75	21.94; 2.66	18.87; 4.62
	0.01	0.007; 5.06	0.007; 2.77
5,6β-Epoxycholesterol	40.00	38.04; 1.34	39.89; 0.28
	20.00	21.35; 4.86	19.93; 0.38
	0.01	0.009; 2.62	0.007; 4.09
5,6α-Epoxycholesterol	40.30	39.21; 1.34	38.74; 0.72
	20.15	20.40; 2.73	9.36; 1.60
	0.01	0.011; 3.08	0.009; 6.08
Cholestanetriol	40.20	39.55; 0.36	39.80; 0.09
	20.10	19.08; 1.91	19.56; 0.29
	0.01	0.009; 5.06	0.008; 1.36
25-Hydroxycholesterol	41.30	41.25; 1.76	40.86; 1.45
	20.65	21.54; 0.60	21.44; 4.35
	0.01	0.007; 5.76	0.007; 3.37
7-Ketocholesterol	39.90	42.16; 3.99	40.46; 0.29
	19.95	20.17; 2.64	20.69; 0.91
	0.01	0.010; 4.34	0.007; 1.95

A Varian VF-5ms CP8947 column (50 m × 250 μm × 0.25 μm) was used.

Table 4
Recovery data obtained for the three spiking levels and mean recovery values with the corresponding coefficients of variation

Oxycholesterol	Sample ($\mu g\,mL^{-1}$)	Spiked sample (0.01 $\mu g\,mL^{-1}$)		Spiked sample (20 $\mu g\,mL^{-1}$)		Spiked sample (40 $\mu g\,mL^{-1}$)		Recovery mean (%)	CV(%)
		Data ($\mu g\,mL^{-1}$)	% Recovery	Data ($\mu g\,mL^{-1}$)	% Recovery	Data ($\mu g\,mL^{-1}$)	% Recovery		
7α-Hydroxycholesterol	0.087	0.089	98.77	17.77	88.12	33.49	88.05	89.98	8.92
	0.080	0.090		17.70		34.34			
	0.073	0.090		17.64		32.07			
7β-Hydroxycholesterol	0.076	0.082	80.27	15.39	77.68	30.61	74.99	77.65	3.40
	0.068	0.081		14.87		29.77			
	0.076	0.081		16.47		29.83			
5,6β-Epoxycholesterol	0.050	0.056	88.66	20.66	102.65	33.10	104.12	93.33	8.74
	0.042	0.055		20.55		37.02			
	0.046	0.055		20.34		36.00			
5,6α-Epoxycholesterol	0.055	0.064	103.16	20.29	101.20	35.12	87.51	97.29	8.76
	0.060	0.071		19.94		35.83			
	0.052	0.063		20.06		33.22			
Cholestanetriol	0.057	0.069	98.89	19.67	98.13	33.96	84.48	93.83	8.64
	0.063	0.068		19.89		33.97			
	0.055	0.068		19.70		33.97			
25-Hydroxycholesterol	0.053	0.060	91.18	19.00	95.85	33.57	84.21	91.75	7.12
	0.052	0.063		18.86		33.51			
	0.052	0.063		19.69		33.69			
7-Ketocholesterol	0.087	0.097	111.32	23.05	102.66	42.88	116.89	110.29	6.50
	0.083	0.106		23.63		41.71			
	0.088	0.088		23.95		39.16			

A Varian VF-5ms CP8947 column (50 m × 250 μm × 0.25 μm) was used.

an exact value. The limit of quantification is the lowest amount of SOPs which can be exactly quantitated.

Table 2 shows the LD and the LQ for each oxysterol. The method enabled quantitation of all oxysterols at concentrations below 10 ng mL^{-1}. The greatest sensibility was found for 7β-hydroxycholesterol (3.68 ng mL^{-1}), whereas the lowest was found for 5,6α-epoxycholesterol (9.76 ng mL^{-1}). Detection of both epoxydes was more difficult than for the rest of compounds. After testing different ions for that purpose, *m/z* 474 (molecular ion) was selected for 5,6β-epoxycholesterol and *m/z* 366 for the 5,6α-epoxycholesterol. It has to be pointed out that 5,6α-epoxycholesterol was the compound with the lowest ratio between the areas of the quantification ion, *m/z* 366 corresponding to M+−18−90 (loss of hydroxyl and trimethyl silanol group), to the total area which might be the reason for this higher LD.

Dzeletovic et al. [38], using isotope dilution-mass spectrometry determination of COPs in plasma, estimated by the method signal to noise, detection limits ranging from 0.3 ng mL^{-1} to 6 ng mL^{-1}, which are in accordance with our study. Other studies including validation parameters in foods showed detection limits within the range of 0.5–3 μg mL^{-1} for sitosterol oxidation products [43]. Grandgirard et al. [12] demonstrated that oxyphytosterols are present in serum samples of healthy human subjects from 4.8 ng mL^{-1} to 57.2 ng mL^{-1}. This finding points out the importance of the analyzed matrix when validation studies are performed.

3.4. Precision

Precision of the method was studied testing the within-day (repeatability) and the between-day (intermediate precision) variability at three different concentrations of oxysterols standards (10 ng mL^{-1}, 20 μg mL^{-1} and 40 μg mL^{-1}). The repeatability was calculated as within-day coefficient of variation of areas obtained using 9 determinations (3 from each concentration) analyzed in the same day and the same analyst. Intermediate precision was evaluated by the analysis of 9 determinations in 3 different days and by the same analyst. Results were also expressed as CV of the peaks' areas (Table 3).

Results for repeatability showed good precision of the method with coefficient of variation values below 10%. A narrow dispersion of values was also observed for intermediate precision, with coefficients of variation between 0.08% and 6.08%.

3.5. Recovery

Recovery should ideally be carried out using reference materials supplied by standard organizations, but there are no commercially available serum samples with known SOPs concentration. In this study spiked serum samples have been used for recovery evaluation, testing 3 different spiking amounts for each oxysterol: 0.01 μg, 20 μg and 40 μg. Both the serum and the spiked samples were subjected to the whole sample preparation procedure and the quantified serum COPs concentrations were introduced to the calculated regression model for each oxycholesterol. Results are presented in Table 4. Serum samples used in this study showed quantifiable values for all the compounds studied. The recoveries for the lowest spiking level (0.01 μg mL^{-1}) ranged between 80% and 111% for 7 β-hydroxycholesterol and 7-ketocholesterol, respectively, achieving good values despite the low addition level. For the 2 higher spiking levels, the recoveries reported ranged from 75% to 117%. Regarding the mean recovery values, the lowest values were detected for both isomers of 7-hydroxycholesterol, with a mean value of 77.65% for the 7β-hydroxycholesterol and 89.98% for 7α-hydroxycholesterol. Both epoxycholesterols, cholestanetriol and 25-hydroxycholesterol showed good mean recoveries, values ranging from 91.75% to 97.29%.

The highest recovery mean value corresponded to 7-ketocholesterol (110.29%). Calvo et al. [44] also pointed out higher recovery data for 7-ketocholesterol comparing to others, when evaluating the determination of COPs in milk products. A cold saponification was performed due to the fact that 7-ketocholesterol can be converted into cholest-3,5-dien-7-one even under room temperature reaching to a destruction of 60–89% of the compound [45]. Recoveries higher than 100% are also reported for the sterol oxidation products possibly due to matrix effects or other analytical difficulties [32,34]. Nevertheless, coefficients of variation among values obtained at the 3 different tested amounts were lower than 10%, which was considered acceptable for validation purposes according to the followed ICH guidelines. No influence of a wide range of concentrations of SOPs in serum affected recovery data. These recovery values will be taken into account for quantification of SOPs in this matrix.

In summary, the proposed method using COPs as model substances assuming a similar behaviour to POPs can be considered an adequate analytical instrument to determine sterol oxidation products (cholesterol and phytosterol oxyderivates) in serum, meanwhile no pure POPs standards were commercial by available.

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