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Preanalytical and analytical challenges in gas chromatographic determination of cholesterol synthesis and absorption markers☆

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ABSTRACT

Introduction: Cholesterol homeostasis disruption contributes to the development of different pathologies. Non-cholesterol sterols (NCSs) serve as cholesterol synthesis markers (desmosterol and lathosterol), and cholesterol absorption surrogate markers (campesterol, stigmasterol and β-sitosterol). The study aimed to resolve certain new pre-analytical and analytical problems and ensure a reliable and validated method.

Materials and methods: Method optimization, validation and stability studies were executed in human serum and plasma. Freeze-thaw cycles were done with and without antioxidant. Gas chromatography-mass spectrometer (GC-MS) was used for NCSs confirmation and plasticizer identification, while GC-flame ionization detector (GC-FID) was used for NCSs quantitation.

Results: Intra- and inter-assay variabilities for all NCSs were 2.75–9.55% and 5.80–7.75% for plasma and 3.10–5.72% and 3.05–10.92% for serum, respectively. Recovery studies showed satisfactory percentage errors for all NCSs: 93.4–105.7% in plasma and 87.5–106.9 in serum. Derivatized samples were stable up to 7 days at − 20 °C and derivatization yield was affected by presence of plasticizers. Fatty acid amids were identified as interfering plastic leachates. Statistically different NCSs concentrations were observed after the 1st freeze-thaw cycle, in antioxidant-free samples, and after the 4th cycle in antioxidant-enriched samples.

Conclusions: All of the in-house procedures proved to be useful for minimizing the preanalytical and analytical variations, as proven by the validation results.

1. Introduction

Although the overall cholesterol metabolism is still not entirely clarified, it is known that the cholesterol homeostasis is maintained through the balance between cholesterol synthesis and absorption [1]. Non-cholesterol sterols (NCSs) can provide an important insight into the cholesterol synthesis and absorption pathways. Plasma concentrations of endogenous sterols, which basically represent cholesterol precursors, are indicators of cholesterol endogenous production, while exogenous phytosterols serve as surrogate markers of cholesterol gastrointestinal absorption [2]. Disturbance of cholesterol homeostasis, which occurs at synthesis or absorption level, can be associated with the development of hypercholesterolemia, atherosclerosis, obesity, metabolic syndrome, diabetes mellitus and cardiovascular, cerebrovascular as well as certain neurodegenerative diseases [1,3–6]. Results of recent studies indicate the importance of estimating the efficiency of cholesterol absorption and synthesis for better identification of individual differences in cholesterol metabolism, and the selection of the most appropriate therapeutic approach (lifestyle changes or drug therapy with statins or ezetimibe) [2,7].

Abbreviations: ANOVA, Analysis of variance; AUC, area under the curve; BBP, benzylbutylphthalate; CS, calibration standard; CV, coefficient of variation; DBP, di-n-butyl phthalate; DEHP, di(2-ethylhexyl) phthalate; DMSO, dimethyl sulphoxide; Dv, derivatization yield; EDTA, Ethylenediaminetetraacetic acid; Ex, extraction yield; FDA, Food and Drug Administration; FID, flame ionization detector; GC, Gas chromatography; HMDS, 1,1,3,3,3-Hexamethyldisilazane; HPLC, high-performance liquid chromatography; IS, internal standard; KOH, Potassium hydroxide; LLE, liquid-liquid extraction; LOD, limit of detection; LOQ, limit of quantitation; MS, mass spectrometry; NSC, Non-cholesterol sterol; PTFE, polytetrafluoroethylene; RI, retention index; Rs, resolution factor; S/N, signal-to-noise ratio; THF, tetrahydrofurane; TMCS, Trimethylchlorosilane

☆ NCSs method validation and preanalytical problems

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Despite of its acknowledged clinical and diagnostic potential, sterol bioanalysis still lacks measurement harmonization and methodology standardization. So far, a considerable number of studies examined the clinical significance of sterols as cholesterol synthesis and absorption markers [1–7]. Nevertheless, a limited number of studies dealt with method validation and even fewer studies examined preanalytical factors which may influence the NCSs quantitation. Over the past decade, a positive trend towards reduction of laboratory errors is observed, but preanalytical phase still represents the most critical area to target [8].

Gas chromatography-flame ionization detection (GC-FID) and gas chromatography-mass spectrometry (GC-MS) methods for separation and quantification of NCSs represent an analytical challenge because NCSs concentrations in plasma are 200–1000 times lower compared to cholesterol levels [8,9]. Various authors have pointed out the importance of proper sample type selection and sample storage conditions [10]. However, hardly there is a study examining freeze-thaw cycle and analyte stability which can also represent potential sources of errors leading to unsatisfactory validation results. Sample preparation prior the NCSs quantitation requires multiple steps. Along with saponification and extraction, many authors state the necessity for the derivatization, while others try to avoid this procedure because of its complexity [8,11]. If one chooses to encompass the derivatization into the sample preparation procedure, an additional optimization of the whole process is needed. According to our best knowledge, there are no papers on this subject.

A recent study by Mackay et al. showed that humidity content and temperature which are present during the sample preparation greatly influence NCSs quantitation [8]. However, a small number of studies comprehensively examined the influence of labware on NCSs quantitation. Nevertheless, it often represents the overlooked preanalytical factor. Plastic labware and consumables are widely used in the laboratory setting, although plastics may spontaneously release its contents and interfere with the results [12,13]. Even if there is a growing concern regarding this issue, it isn't backed up with considerable literature data, especially regarding compound leakage due to the usage of organic solvents with plastic consumables during extraction and derivatization of steroid compounds.

Steroid structures are susceptible to oxidative changes. Therefore long-term preparation may cause the structural changes and represent a great preanalytical issue. It is acknowledged that this process can be accelerated by the presence of metal ions originating from the various labware components, such as tube seals [14,15]. During the employment of contemporary analytical techniques such as chromatographic methods with high-sensitive detection, every structural change of the analyte may influence the quantitation.

The aim of this study was to examine the most common variability causes, in particular those deriving from pre-analytical sample preparation, as well as gas chromatographic conditions, through comprehensive optimization process in order to ensure a reliable, validated method for further employment into the clinical studies based on NCSs quantitation.

2. Materials and methods

2.1. Reagents, samples and instrumentation

Peaks of desmosterol, lathosterol, campesterol, stigmasterol and β-sitosterol were identified by comparison with authentic standards (Supelco, Bellefonte, PA, USA). Internal standard (IS) 5α-cholestane (GC grade), 0.5 M methanolic potassium hydroxide (KOH) and tetrahydrofuran (HPLC grade) from Sigma-Aldrich (St. Louis, MO, USA) were used. KOH was purchased from POCH (Center Valley, PA, USA), and ethanol, chloroform, petroleum ether, n-hexane and acetonitrile (HPLC grade) from Fisher (Pittsburgh, PA, USA). Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) from Acros Organics (Geel, Belgium) was used as an antioxidant and Supelco's Sylon™ HTP (1,1,1,3,3,3–hexamethyldisilane + trimethylchlorosilane + pyridine, 3:1:9) was used as derivatization reagent. The C8-C40 Alkanes Mixture was used as a calibration standard for calculation of retention indices (Supelco, Bellefonte, PA, USA). Helium (5.0. purity), synthetic air free of hydrocarbons (5.0), hydrogen (5.0), and nitrogen (3.5, and 5.0.) were obtained from Messer Group (Bad Soden, Germany).

Human serum and EDTA plasma samples were obtained from 60 healthy volunteers following the guidelines laid down in the Declaration of Helsinki. Samples were used for method development pool preparation (n = 10) and clinical verification (n = 50). None of the participants was vegetarian/vegan or treated with hypocholesterolemics. All participants signed an informed consent form before enrolment.

GC analyses were performed on HP-5, DB-17MS (Agilent Technologies, USA) and ZB-5MS (Phenomenex, USA) columns, while HPLC analyses were done using EclipseXDB C-18 column (Agilent Technologies, USA).

NCSs analyses were performed on Agilent 7890A GC instrument (Agilent Technologies, USA) equipped with FID. Additional analyses were done using Agilent GC/MSD system 6890N/5975C (Agilent Technologies, USA) and Agilent 1200 HPLC/DAD system (Agilent Technologies, USA).

2.2. Preparation of glassware

Borosilicate glassware (tubes, beakers, pipettes, volumetric flasks etc.) and labware made from high-density plastics (pipette tips, Pasteur pipettes etc.) were used for sample handling during the entire preanalytical and analytical process.

In order to avoid cross-contamination, rigorous glassware washing procedure employed tap water/distilled water wash followed by an overnight immersion in chromic acid. After regular wash, drying in the oven at 100 °C, glassware was rinsed twice with acetone, followed by n-hexane. We omitted the process of pre-analysis glass silylation described by some authors [11], since our preliminary method studies showed that this time- and the reagent-consuming step wasn't necessary for obtaining satisfactory derivatization yield.

2.3. Sample preparation and GC/FID method optimization

2.3.1. Experiment 1 - sample preparation without derivatization

Initially, we tested the sample preparation method without a derivatization step. Following the protocol of Matthan and co-workers [16], 150 μL of IS (5-α-cholestane) dissolved in chloroform (1 mg/mL) was added to 1 mL of sample and saponified with 5 mL of 0.5 M methanolic KOH solution for 1 h at 100 °C. After the addition of 2.5 mL of distilled water, the NCSs were extracted in 3 mL of petroleum ether and the tubes were vortexed and centrifuged at 1500 g for 15 min. The upper layer containing the nonsaponifiable materials was then transferred into clean glass tubes. Overall, the extraction procedure was repeated three times. Combined extracts were dried under nitrogen and re-suspended in 1 mL of chloroform prior to the GC/FID analysis [16].

GC/FID parameters were initially optimized on Agilent's HP-5 column ((5%-Phenyl)-methylpolysiloxane, 30 m × 0.32 mm × 0.25 μm). Initially, the isothermal run was performed with the following GC conditions: 5 μL injection volume, split injection mode (9:1), 300 °C inlet temperature, 280 °C column temperature, 310 °C detector temperature, 9.26 psi constant pressure and helium gas flow 0.78 mL/min. Total run time was 35 min. Finally, we optimized the following instrumental method: injection volume of 5 μL, split injection mode (9:1) and 280 °C inlet temperature were applied. The column temperature was set by multiple ramp as follows: initial temperature of 150 °C was held for 3 min, linear temperature ramp (rate 30 °C/min) was applied until reaching 280 °C held for 28 min and followed by another linear ramp (rate 10 °C/min) until reaching 295 °C which was held for 10 min. Detector temperature was 300 °C; constant pressure of 9.26 psi and helium gas flow of 1.23 mL/min. The total run time was

47 min. In addition, two GC columns of different film thickness and polarity were also tested to assess whether minor adjustment of the chromatographic conditions would result in the satisfactory separation: Agilent's DB-17MS ((50%-Phenyl)-methylpolysiloxane; 30 m × 0.25 mm × 0.25 μm) and Phenomenex' ZB-5MS (5%-Phenyl-Arylene, 95%-Dimethylpolysiloxane; 30 m × 0.25 mm × 0.25 μm).

In order to further enhance the chromatographic performance both nitrogen and helium were tested as carrier gases.

2.3.2. Experiment 2 - sample preparation with derivatization

In this experiment, we tested if derivatization combined with smaller sample volume would result in better chromatographic characteristics. Sample preparation was performed according to the Wu et al. procedure with certain modifications [17]. Fresh and thawed serum and plasma pools were used for method optimization and validation. Sample (100 μL) was transferred into conical 15 mL-glass tube with polytetrafluoroethylene (PTFE) lined screw cap containing 100 μL IS (1 mg/mL 5-α-cholestane dissolved in hexane) previously added and dried under nitrogen. Then, 1 mL of ethanol was added and the mixture was briefly vortexed. Afterwards, 960 μL of freshly prepared KOH solution (8.9 mol/L) was added and the mixture was vortexed for 15 s. Subsequent 1-hour saponification at 67 °C was done. After cooling until room temperature, 1 mL of deionized water and 2 mL of n-hexane were added. The mixture was briefly vortexed and then centrifuged at 1500 rcf for 5 min. The upper organic phase was carefully transferred into clean glass tube by a glass pipette. The extraction procedure was repeated three times. In the next step, 4 mL of deionized water was added to the collected extract and then vortexed and centrifuged for 5 min at 1500 rcf. This desalting procedure was repeated twice. The upper layer was transferred into another clean glass tube and dried under nitrogen at room temperature prior to adding 220 μL of derivatization reagent. Derivatization with Sylon™ HTP was performed by 1-hour incubation at 67 °C. After cooling at room temperature and briefly vortexing, the mixture was dried under nitrogen. Solid debris was reconstituted in 300 μL of n-hexane prior to GC analysis.

Separation of the derivatized analytes was achieved by using Agilent HP-5 (5%phenyl)-methylsiloxane non-polar capillary column of the following characteristics: 30 m × 0.32 mm × 0.25 μm. The splitless liner was used for introducing 1 μL of derivatized extract into the column. The following temperature multiple ramp was established: initial temperature of 150 °C was held for 3 min, linear temperature ramp (rate 30 °C/min) was applied until reaching 250 °C, followed by another linear ramp (rate 5 °C/min) until reaching 270 °C which was held for 30 min. The constant pressure of 15 psi and flow of 2.28 mL/min were applied during the entire run-time. Helium was used as a carrier-gas. Inlet and detector temperatures were set to be 290 °C. Total run-time was 40.33 min.

2.3.3. Experiment 3 – assessment of derivatization yield, plastics influence and sample stability

In this experiment, we tested the influence of different preanalytical and analytical factors as well as sample storage time and conditions. For this experiment pooled serum and plasma were used and prepared in the manner described in experiment 2.

2.3.3.1. Derivatization yield assessment. Firstly, the cut-off value for the derivatization yield was established. To achieve this, plasma and serum pools were prepared with different derivatization reagent volumes (100, 150, 200, 220, 250, 260 μL) and run in pentaplicate. Subsequently, 30, 60 and 80 min incubation periods were tested with the optimal reagent volume.

2.3.3.2. Interference of plastic leachates. Interference of plastic leachates was examined by filling one of the Pasteur pipettes with 1 mL of n-hexane and leaving it for 30 min at room temperature (average extraction time and condition). The resulting extracts were then analysed by GC/MS and components were identified using NIST/NBS and Wiley libraries, and by comparison of their retention indices (RIs) with literature data [18]. RIs were calculated using a linear interpolation, in relation to a homologous series of n-alkanes (C8-C40 Alkanes Calibration Standard) analysed under the same operating conditions [19].

2.3.3.3. Phthalate analysis. The presence of phthalates was analysed by validated HPLC/UV method, routinely used in Institute of Public Health of Serbia “Dr Milan Jovanovic Batut”. Phthalates were extracted using THF/n-hexane, dried and reconstituted with acetonitrile and run with linear gradient mobile phase acetonitrile:water from 90:10 to 100:0.

2.3.3.4. Sample stability studies. The effect of storage on native plasma and serum samples was evaluated. The basal levels of NCSs were determined from the fresh pooled samples on day 1, whereas the remaining aliquots were frozen, stored at − 80 °C for further testing and run in pentaplicate on day 2, 7, 15, 30 and 60.

Also, the influence of storage on derivatized plasma and serum samples were assessed. After determining the basal levels of NCSs from the fresh pooled samples on day 1, the remaining aliquots were prepared, derivatized, frozen and stored at − 20 °C for further testing in pentaplicate on day 2, 7, 15 and 20.

The effect of freeze-thaw cycles on plasma and serum samples was assessed by determining the basal levels of NCSs from the fresh pools on day 1. Afterwards, the remaining pool was divided into two aliquots, blank and antioxidant-enriched pools and stored frozen at − 80 °C for further testing. Antioxidant-enriched aliquots of both plasma and serum were prepared in the following manner: 1.5 mg of the antioxidant (Trolox®) were measured and dissolved in 6 mL of pooled plasma/serum in order to obtain the final concentration of 1 mmol/L [20]. Each time both aliquots were thawed for 30 min at room temperature, prior the analysis and frozen again for the next cycle. The freeze-thaw cycles were repeated 5 times. Samples were prepared and run each time in pentaplicate.

2.3.4. Method validation

Calibration curves were generated to confirm the relationship between the peak area ratios and the concentration of each sterol. Fresh calibration standards (CS) of 6 different concentration levels were prepared, derivatized and assayed using the optimized method. Limit of detection (LOD) and limit of quantitation (LOQ) were defined experimentally as signal-to-noise ratio (S/N) equals 3 and 10, respectively [21].

Intra-run precision was determined from serum and plasma pools freshly prepared in 10-fold and run in the same day.

Inter-run precision was determined from serum and plasma pools prepared in pentaplicate and run on day 1, 2, 3, 4, 5, 8, 9, 10, 11, 12.

Recovery studies were performed from serum and plasma pools spiked with 5 different concentration-level solutions. The blank serum/plasma pool sample was regarded as the level-zero.

2.3.5. Clinical verification

For the clinical verification of method, we tested 50 plasma samples from healthy individuals. Average sterol concentrations were determined. LOD and LOQ values were confirmed by diluting the abovementioned samples to the appropriate S/N ratio (approx. 3 and 10, respectively). Intra-run precision was determined in pentaplicate for LOD and LOQ levels for each sterol and run in the same day. Additionally, inter-run precision was assessed by measuring sterol concentrations at LOD and LOQ levels for 10 days.

3. Statistical analysis

Regression analysis was used for standard curve generation and the Recovery test. Continuous variables were compared by Analysis of

Table 1
Retention times, relative retention times, and mass spectrum for internal standard and derivatized sterols.

Component	Retention time, min	Retention time, CV% (N = 10)	Relative retention time	Relative retention time, CV% (N = 10)	Characteristic ions, m/z
5-α cholestane	14.133	0.031	1	/	372.4/357.4/217.2
TMCS-desmosterol	21.048	0.016	1.489	0.038	456.5/343.3/129.1
TMCS-lathosterol	21.527	0.021	1.523	0.043	458.5/353.3/255.2
TMCS-campesterol	23.018	0.017	1.629	0.031	472.6/382.4/343.3
TMCS-stigmasterol	24.339	0.088	1.723	0.096	484.5/394.4/255.2
TMCS-β-sitosterol	26.161	0.013	1.851	0.033	486.4/396.4/357.4

CV-coefficient of variation, TMCS–trimethylchlorosilane.

variance (ANOVA). Between-group differences were tested by Tukey test. A p-value of < 0.05 was considered statistically significant. All data were analysed using IBM® SPSS® Statistics version 22 software.

4. Results

Sample preparation and instrumental conditions described in experiment 2 showed optimal separations of all five sterols. Each peak of interest was identified according to the retention time of the corresponding analytical standard after derivatization, as well as by the GC/MSD spectral analysis and concordance with the Wiley on-line mass spectral database. Retention time, relative retention time and their coefficient of variation (CV%), as well as mass spectrum for each compound of interest were shown in Table 1.

Final derivatization procedure, optimized in experiment 3, implies 220 μL of Sylon™ HTP with 60 min-incubation period which represent the optimal conditions for consistent derivatization yield of 97.4–99.7%.

4.1. Plasticizer influence

Interfering plasticizers were extracted and identified because of their influence on the derivatization yield. Their retention time, relative retention time and mass spectrum are shown in Table 2. Fig. 1 shows the GC-FID chromatogram of plasticizer peaks extracted from plastic labware with hexane.

The phthalates analysis showed that < 0.01% of benzylbutylphthalate (BBP), di(2ethylhexyl) phthalate (DEHP) and di-n-butyl phthalate (DBP) were present in the extract.

4.2. Sample stability

The results for the native samples stability showed no significant differences in NCSs' concentrations of the fresh samples comparing to the concentrations measured afterwards, during the 2-month time period storage at − 80 °C (data not shown).

Our results for the derivatized samples stability showed significantly lower concentrations of all of the NCSs', except the stigmasterol, on the 15th day in plasma ($p = 0.001$ for desmosterol, $p = 0.008$ for lathosterol, $p = 0.001$ for campesterol, $p = 0.003$ for β-sitosterol). In serum, only desmosterol concentration differed on the 15th day ($p = 0.002$).

A significant decrease in plasma and serum concentrations of desmosterol and β-sitosterol was evident after the first freeze-thaw cycle in native samples ($p = 0.037$, $p = 0.012$, respectively). In antioxidant-enriched samples, desmosterol and stigmasterol concentrations were constant during four freeze-thaw cycles while in the fifth cycle the statistically significant change was observed ($p = 0.025$, $p = 0.036$, respectively). The rest of the NCSs were stable over the all five cycles.

4.3. Method validation and clinical verification

Standard curves equations were as follows: y = 6.9985 × − 4.5139 ($r = 0.997$) for desmosterol, y = 6.0608 × − 2.3414 ($r = 0.999$) for lathosterol, y = 5.2835 × + 1.3494 (r = 0.999) for campesterol, y = 7.4974 × − 0.9926 (r = 0.999) for stigmasterol and y = 7.5013 × + 0.7386 (r = 0.999) for β-sitosterol. Results for LOD and LOQ obtained from standard solutions, as well as the final linearity concentration ranges are given in Table 3.

According to Food and Drug Administration (FDA) guidelines, the acceptable intra-run and inter-run variations for real samples (plasma and serum) were considered to be CV < 15% [22]. Both variabilities were satisfactory for each of the sterols and both sample types. These results are summarized in Table 4.

Results for the LOD and LOQ values determined in diluted plasma samples as well as corresponding average S/N values for each sterol are represented in Table 3. Intra-and inter-run precisions at LOD and LOQ concentration levels were under 20% for each sterol [22].

Satisfactory results were obtained for the difference between the expected concentrations and found concentrations over the five-level concentration range. For the established method, recovery was between 93.4 and 105.7% for plasma, and 87.5–106.9% for serum, while the acceptable values are in the ± 25% range according to FDA guidelines [22]. There was also a good correlation between spike concentrations and found concentrations for each sterol in both sample types. Results of recovery analysis are shown in Table 5.

5. Discussion

The increasing interest in cholesterol homeostasis assessment by NCSs determination is accompanied by the necessity for precise and accurate quantitative method.

The optimal sample preparation method for NCSs analysis includes the following steps: saponification, liquid-liquid extraction (LLE) and silylation as a derivatization technique.

Saponification is usually performed with methanolic or ethanolic NaOH or KOH solutions, followed by incubation for 30–120 min at 60–100 °C, while LLE of sterol compounds from plasma/serum is usually performed with non-polar solvents, such as petroleum ether, n-

Table 2
Retention times, relative retention times, calculated retention index and mass spectrum for identification fatty acid amides.

Component	Retention time, min	Retention time, CV% (N = 10)	Relative retention time	Relative retention time, CV% (N = 10)	RIexp	Characteristic ions, m/z
Palmitamide	8.118	0.035	0.575	0.030	2179.1	255.3/72.1/59.1
Oleamide	9.187	0.073	0.658	0.062	2363.0	281.3/72.1/59.1
Stearamide	9.333	0.060	0.665	0.051	2386.0	283.3/72.1/59.1

CV-coefficient of variation, RI_{exp} - calculated retention index against n-alkanes on an ZB-5MS column.

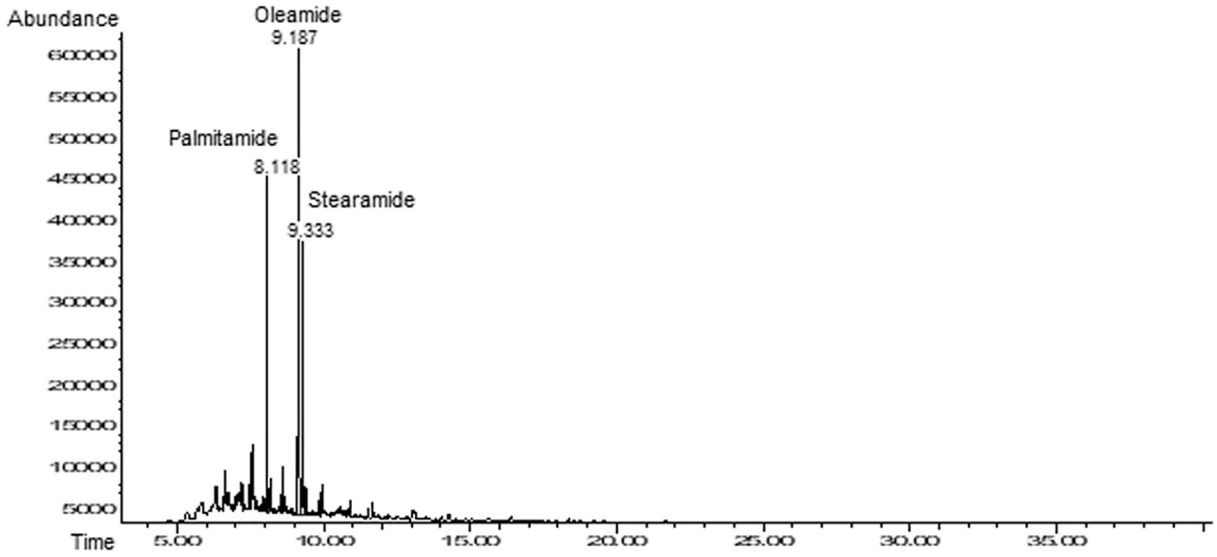

Fig. 1. Chromatogram of plasticizers peaks obtained using FID detector. For extract preparation, see experiment 3 and for chromatographic conditions, see experiment 2.

hexane, n-heptane, and cyclohexane [8]. We opted for saponification procedure with aqueous KOH solution (8.9 mol/L), pure ethanol and 1-hour incubation at 67 °C (experiment 2) and LLE procedure with n-hexane [17]. Some authors employ post-extraction washing step in order to achieve desalting of the organic layer [10,17]. We concluded that post-extraction washing step was needed to be repeated twice with 4 mL of deionized water in order to achieve neutral pH of the aqueous phase and thus optimal desalting. The efficiency of the extraction procedure was assessed by adding structurally similar and endogenously absent 5-α-cholestane as the IS prior to sample preparation procedure [17]. Afterwards, comparison of the IS peak area with medium peak area calculated after running the internal standard 10 times in a row was performed and extraction efficiency factor was calculated. Extraction efficiency was in the range of 80–95% and it was successfully corrected by implementation of the appropriate IS.

Sample preparation without derivatization resulted in the unsatisfactory separation of both desmosterol and lathosterol peaks from the dominant cholesterol peak with resolution factor (Rs) < 1.2 (experiment 1). With this in mind, we opted to include derivatization in our final sample preparation procedure and thus improve the chromatographic separation. Silylation is the most widely used derivatization technique which provides replacement of hydroxyl group in C3 position with -SiMe3 group [8,9,23]. Silylation derivatives are generally less polar, more volatile and thermally more stable. The choice of a silylating reagent is based on its reactivity and selectivity towards the compound, the intended application, the stability of the derivative, and the abundance/nature of reaction byproducts [8,23]. We opted for Sylon™ HTP (HMDS + TMCS + Pyridine, 3:1:9). Ideally, derivatization step should be rapid, reproducible and simple, resulting in efficient NCSs silylation. In practice, however, derivatization faces many difficulties. Along with the usual problems and interferences during the derivatization process, such as the optimal reagent volume, duration, reaction temperature and humidity, it is very important to report and examine additional issues that may arise [8]. According to our best knowledge the literature data regarding interferences related to plastic labware consumption, derivatization efficiency assessment and cut off for the derivatization yield are insufficient or non-existent.

It is important to point out that variations in derivatization efficiency could not be corrected by using the internal standard, because 5-α-cholestane wasn't prone to silylation due to the structural absence of the hydroxyl group. We proposed a simple way of derivatization efficiency assessment which was determined by derivatized versus non-derivatized cholesterol peak area ratio. In experiment 3, we optimized the volume of Sylon™ HTP reagent, as well as the incubation duration, in order to achieve the satisfactory derivatization yield. After experimenting with different derivatization reagent volumes we concluded that yield < 95% gave a statistically significant difference in the concentrations of campesterol ($p = 0.013$) and sitosterol ($p = 0.005$). Therefore, derivatization yields > 95% were regarded as acceptable for reliable quantitation. We used derivatization yield coefficient for correcting the peak area before calculating the final concentration by aforementioned correlation curve method: AUCsterol(corrected) = AUCsterol(measured)··· Ex ··· Dv, where AUC being the area under the curve; Ex being extraction yield coefficient (Ex = 100 + (100 − (AUCis·100 / AUCis) / 100); Dv being

Table 3
Concentration ranges of calibration curves, sterols concentration in healthy subjects, limits of detection (LOD) and quantitation (LOQ) for NCSs.

Analyte	Concentration range [μmol/L]	Average concentration in healthy subjects (N = 50) [μmol/L]*	LOD [μmol/L]			LOQ [μmol/L]		
			Determined from standard solutions	Determined from samples#	Average S/N values determined from samples#	Determined from standard solutions	Determined from samples#	Average S/N values determined from samples#
Desmosterol	1.394–22.305	10.13 ± 2.820	0.235	0.465	3.2	1.209	1.481	10.2
Lathosterol	1.587–19.049	8.89 ± 3.444	0.299	0.401	2.9	1.165	1.385	10
Campesterol	1.846–44.309	19.48 ± 6.396	0.251	0.591	3	1.259	1.952	9.9
Stigmasterol	0.981–15.694	5.91 ± 2.283	0.185	0.415	3	0.744	1.356	9.8
β-sitosterol	1.674–26.790	8.55 ± 2.184	0.370	0.406	3.2	0.914	1.243	9.8

S/N – signal to noise ratio.
* Data are expressed as mean ± standard deviation.
# Values obtained for each sterol by intra-day running of pentaplicates for LOD and LOQ levels.

Table 4
Intra- and inter run precision for plasma and serum NCSs level.

Sterol	Precision							
	Plasma				Serum			
	Intra-run		Inter-run		Intra-run		Inter-run	
	Medium concentration (μmol/L)	CV (%)	Medium concentration (μmol/L)	CV (%)	Medium concentration (μmol/L)	CV (%)	Medium concentration (μmol/L)	CV (%)
Desmosterol	13.84 ± 0.557	4.02	13.38 ± 1.031	7.70	12.69 ± 0.534	4.30	14.39 ± 1.131	7.86
Lathosterol	13.37 ± 0.457	3.41	14.13 ± 0.972	6.88	10.85 ± 0.493	4.54	13.47 ± 1.478	10.97
Campesterol	21.37 ± 0.659	3.09	21.06 ± 1.633	7.75	21.72 ± 0.925	4.26	23.60 ± 0.720	3.05
Stigmasterol	4.41 ± 0.421	9.55	4.66 ± 0.270	5.80	3.80 ± 0.217	5.72	4.64 ± 0.328	7.07
β-sitosterol	9.60 ± 0.264	2.75	9.19 ± 0.550	5.98	10.13 ± 0.314	3.10	10.37 ± 0.534	5.15

CV – coefficient of variation.

Table 5
Results of the Recovery test for all five sterols.

Sterol	Recovery assays			
	Plasma		Serum	
	Correlation coefficient (r)	Slope (b), %	Correlation coefficient (r)	Slope (b), %
Desmosterol	0.998	93.4	0.997	97.6
Lathosterol	0.994	105.7	0.997	106.9
Campesterol	0.999	100.9	0.999	104.4
Stigmasterol	0.997	102.9	0.998	87.5
β-sitosterol	0.994	100.5	0.999	105.5

derivatization yield coefficient (Dv = 100 + (100 − %Dv) / 100). Additionally, the derivatization yield coefficient cut-off value of 95% was determined and used in further analyses. Further on, we proved that minimizing the moisture content in and around the reaction tube yielded better derivatization results. As far as we know, this is the first study dealing with derivatization yield cut off estimations, as well as using derivatization yield for correcting the NCSs' values.

During method development, we encountered a problem when three unidentified peaks emerged and the derivatization yield dropped to 60%, far below the cut off value for reliable quantification. To identify the source of the problem we examined the overall preanalytical and analytical process and tracked the interference down to disposable plastic Pasteur pipettes used during the extraction protocol (Experiment 3). Accidentally the pipettes, made by different producer and from low-density plastics, were obtained during the purchase. It has been shown that when using organic solvents, low-density plastic laboratory dishes release certain plastic components [13]. With this in mind, we extracted and identified the specific components from plastics which do interfere with the derivatization process (Table 2). Palmitamide, oleamide and stearamide, fatty acid amides routinely used as non-blocking slip agents in the manufacturing process were identified as interfering leachates (Fig. 1). These compounds were not present in the n-hexane blank, neither in the samples processed with glass pipettes. Similar findings were obtained by Watson et al. [13]. These authors found that even a change of consumables lot number can cause faulty results, so special care should be taken when it comes to plastics implementation into the analytical process. They examined the behaviour of the specific disposable plastic sample-handling tips upon DMSO exposure during overnight extraction at room temperature, and identified the erucamide as principal leachate with considerable effects on specific biological assays [13]. According to Olivieri et al. [24] the leading experimental conditions which cause processing additives to leach from plastic consumables are extended periods of time and (or) at elevated temperatures. Also, Alvaro Garrido-Lopez et al. [25] have shown that the extraction of erucamide and oleamide in polyethylene films was temperature and time-dependent. Maximum extraction of erucamide and oleamide was obtained during 16 min-exposure to a temperature of 105 °C. Conditions applied in the present study (30 min extraction with n-hexane, at room temperature) resulted in the detection of palmitamide, oleamide, stearamide, but not erucamide. The current study represents, to the best of our knowledge, the first study focused on plastics' leachate interferences during NCSs analyses in plasma and serum samples.

It is known that phthalates can be present in samples due to leaching from the plastic tubing or other plastic devices and thus interfere with the GC analysis [13,26]. With this in mind, we wanted to test whether plastic labware leaches phthalates along with interfering slip agents. The extract contained < 0.01% of phthalates [13]. Since Fankhauser-*Noti* and Grob [12] have demonstrated phthalates were present everywhere and "coat" the laboratory as well as the whole environment, their presence was also noted in the hexane HPLC purity, upon opening, used for their analysis. Thus, the concentrations obtained in this experiment are not considered significant [12,13].

These results confirm the necessity of using exclusively glass labware and minimizing the usage of plastics during NCSs quantification with derivatization.

Preanalytical phase is a critical part of the entire analytical process. In practice, there is often a necessity to preserve the samples for additional analyses or repeats. With this in mind, we conducted the sample stability studies (Experiment 3).

There was no significant change in NCSs' concentrations during storage of native samples for 2-months. On the other hand, it is known that during heating, cholesterol and phytosterols undergo oxidative changes [14,27]. Since our method employs relatively long sample preparation with heating (1-hour incubation, twice repeated), we were concerned about the possibility of the oxidative modification of sterols, which can further progress during storage of derivatized samples at − 20 °C. We observed that derivatized samples are stable for one week if stored at − 20 °C. This information is useful particularly if samples cannot be prepared and run on the same day.

Also, it is documented that oxidation increases in the presence of metal ions [14]. In order to minimize the influence of oxidation during sample preparation, commonly used metal screw caps for reaction tubes were substituted with PTFE-lined screw caps.

The literature data emphasizes that in particular C5-C6 double bond of the phytosterol structure is prone to free radical chain reaction. The reaction starts with hydroperoxide formation, followed by several reaction pathways resulting in hydroxy-, keto- and epoxy- compounds formation. Beside these oxidational products, there is also the possibility of C25 oxy-products formation [27,28]. Hence, the influence of freeze and thaw was investigated in samples with and without the antioxidant. We chose hydrosoluble Trolox® as an antioxidant, assuming that it will not transfer into the hexane extract layer during the sample preparation. Also, for freeze-thaw study, we chose plasma because of its

greater antioxidative capacity compared to serum [29]. As shown by our results, the addition of the antioxidant prior freezing extends the freeze-thaw stability from one cycle without antioxidants to four cycles with antioxidant. These findings led to the conclusion that the freeze-thaw stability is prolonged by the addition of the antioxidant prior sample freezing.

The importance of examining freeze-thaw cycle stability is considerable since repeated freezing and thawing may occur in laboratory settings during procedures such as sample transport, electric-power outage and frost-free freezer usage. Many studies have addressed the influence of freezing and thawing on cholesterol level. Cuhadar et al. [30] have shown that the cholesterol concentrations measured by an enzymatic method, does not change after the ten cycles. However, sample preparation can modify the steroid structure. These changes are often undetected by the routine spectrophotometric methods, but more sensitive techniques such as gas chromatography can differentiate these compounds because the chromatographic properties of the compounds undergo a change as well.

Regarding NCSs, despite being the emerging biomarkers in various pathologies, as far as we know, there was no literature data on the freeze-thaw cycle stability previous to the current study.

All of the experiments were done in both serum and plasma, since the literature debates which sample type is generally more appropriate for NCSs quantitation [9]. Plasma is regarded as advantageous over serum due to shorter preparation time since there is no need for clotting. In the current study, this method of sample preparation and analyte detection turned out to be applicable to serum and plasma which can be validated through values of inter-run, intra-run and recovery variability coefficients (Table 4 and Table 5). Afterwards, the method was clinically verified in real plasma samples, average NCS concentrations were determined, as well as LOD and LOQ values in diluted plasma samples. Higher LOD and LOQ values in real samples were observed, compared to LOD and LOQ values determined in the standard solution. Additionally, we used the method validated in this study, and demonstrated clinical significance of NCSs in coronary artery disease patients [2].

6. Conclusion

A sensitive and precise method was optimized and validated for quantification of NCSs. Both serum and plasma were tested and it was found that both of them represent appropriate sample types for the validated GC-FID method. Derivatization, as well as derivatization yield assessment, was shown to be necessary in order to accomplish the reliable quantitation of the cholesterol precursors. Also, when applying derivatization, special care must be taken during the selection of appropriate labware and laboratory consumables.

All of the in-house procedures described in this paper proved to be useful for minimizing the preanalytical and analytical variations, as shown in the validation results and clinical verification. These results promise the future transferability of the aforementioned method between different laboratories as well as the reproducibility of the results. This is an especially valuable piece of information for studies dealing with the hypercholesterolemia-related development of atherosclerosis and accompanying ischemic disorders such as cardiovascular and cerebrovascular insult. Since clinical studies need a large number of samples which are usually being collected and stored for long time periods, the considerably long stability of the analytes is crucial. Also, after antioxidant addition, satisfactory freeze-thaw stability leaves the possibility of the additional testing for NCSs from the leftover samples.

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Conflict of interest

None declared.

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