International descriptive and interventional survey for oxycholesterol determination by gas- and liquid-chromatographic methods

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Abstract

Increasing numbers of laboratories develop new methods based on gas-liquid and high-performance liquid chromatography to determine serum concentrations of oxygenated cholesterol metabolites such as 7α-, 24(S)-, and 27-hydroxycholesterol. We initiated a first international descriptive oxycholesterol (OCS) survey in 2013 and a second interventional survey 2014 in order to compare levels of OCS reported by different laboratories and to define possible sources of analytical errors. In 2013 a set of two lyophilized serum pools (A and B) was sent to nine laboratories in different countries for OCS measurement utilizing their own standard stock solutions. In 2014 eleven laboratories were requested to determine OCS concentrations in lyophilized pooled sera (C and D) utilizing the same provided standard stock solutions of OCS. The participating laboratories submitted results obtained after capillary gas-liquid chromatography-mass selective detection with either epicoprostanol or deuterium labelled sterols as internal standards and high-performance liquid chromatography with mass selective detection and deuterated OCS as internal standard. Each participant received a clear overview of the results in form of Youden-Plots and basic statistical evaluation in its used unit. The coefficients of variation of the concentrations obtained by all laboratories using their individual methods were 58.5-73.3% (survey 1), 56.8-60.3% (survey 2); 36.2-35.8% (survey 1), 56.6-59.8, (survey 2); 61.1-197.7% (survey 1), 47.2-74.2% (survey 2) for 24(S)-, 27-, and 7α-hydroxycholesterol, respectively. We are surprised by the very great differences between the laboratories, even under conditions when the same standards were used. The values of OCS´s must be evaluated in relation to the analytical technique used, the efficiency of the ample separation and the nature of the internal standard used. Quantification of the calibration solution and inappropriate internal standards could be identified as major causes for the high variance in the reported results from the different laboratories. A harmonisation of analytical standard methods is highly needed.

Keywords: oxysterol; bile acid precursors; gas-liquid chromatography; high performance-liquid chromatography; mass spectrometry; isotope dilution

Abbreviations: OCS, oxycholesterol; CV, coefficient of variation;

1. Introduction

Ring and side-chain oxidized cholesterols or oxycholesterols (OCS) are essential transport forms for reverse cholesterol transport from the periphery [1,2], for cholesterol homeostasis of the human brain [3] and serve as precursor molecules for bile acid and steroid hormone synthesis [4]. Analysis of serum OCS concentrations as surrogate markers for cholesterol metabolism, degradation and excretion of cholesterol, allows evaluation of cholesterol homeostasis in the human physiology [5–7]. Detection of abnormal serum concentrations of OCS, combined with other indicators enables the diagnosis of certain inherited lipid storage diseases i.e. Nieman-Pick diseases [8–10], cerebrotendinous xanthomatosis [11] and a subtype of hereditary spastic paresis [12]. Therefore, reliable determination and quantification of OCS is essential for diagnosis and adequate patient care. During the last decades, fundamentally important understanding of the influence of OCS has been achieved with respect to development and progress of atherosclerosis [13], cardiovascular diseases [14] as well as neurodegenerative diseases i.e. Alzheimer disease [15]. Knowing that oxysterols may function as signalling molecules in cholesterol metabolism [16], makes them attractive candidates for a wide field of physiological actions. The ratio of the amount of plasma or CSF 24(S)-hydroxycholesterol to 27-hydroxycholesterol is suggested to assess cholesterol metabolism in the brain, integrity of blood-brain barrieror to characterize pathological conditions in neurological disorders [17,18] (2x Leoni, clin chem. Lab 2004, J Lipid Res 2002, Björkhem I J Int Med] . Recently side-chain and B ring oxysterols have been identified as tumour promoters in several cancers or as tumour suppressors according to their structure. [19-23] These circumstances increase the need for analytical laboratories and research facilities to accurately quantify oxysterols in human serum and to develop new more accurate analytical procedures and methods.

The present international OCS survey was designed with two parts taking place in the year 2013 and 2014. The first survey provides information about the variation of reported results from the different laboratories involved and was designed as “descriptive”. The second survey was designed as “interventional”.

2. Materials and Methods

The participants submitted results from three different separation and detection methods for OCS determination: capillary gas-liquid chromatography-mass selective detection using epicoprostanol (GC-MS-epi) or deuterated OCS (GC-MS-deuterium) as internal standard and high-performance-liquid-chromatography with mass selective detection (LC-MS-deuterium) using deuterated OCS as internal standard (Table 1). The explicit values and statistical data are additionally listed in Table 2. Note, 27-hydroxycholesterol is also called (25R)26-hydroxycholesterol, taking into account stereochemistry at C-25. However, 27-hydroxycholesterol is the more common name and will be used here. The lyophilized probes A-D were sent by ordinary mail with no temperature or air pressure control. This includes the possibility of autoxidation of cholesterol into oxysterols, which are not exclusively formed by enzymatic cytochrome P450 supported oxidation. One example for this could be 7α-hydroxycholesterol, which is formed from cholesterol by both, enzymatic and radical oxidation. In knowledge of this fact, we limited the analysis of oxysterols to 24(S)-, 27- and 7α-hydroxycholesterol, the first two of which are almost exclusively formed enzymatically [24]. [22]. The work-up procedures and analytical settings are strikingly different for each participant since each laboratory was requested to use its own specific routine method (Table 1). However, each laboratory used alkaline hydrolysis in order to deconjugate fatty acid esterified oxysterols and thus analyzing total oxysterol serum concentrations after

derivatisation of the free hydroxyl groups. It should be emphasized that the individual sample work-up procedures or detection specifications cannot be reported within this article because of the anonymization of the participants. Inconsistent values are defined within the survey as values obtained from sample A and B concentrations which are impaired by different systematic errors. For example, the value for point a of sample A in Figure 1 A is slightly to high reported but the value of sample B is much too low determined. The participant failed to obtain results with the same, reproducible error. Therefore, in both samples A and B two different errors occurred and these inconsistent values must be regarded as failed. The ratios of 24(S)-hydroxycholesterol to 27-hydroxycholesterol are listed in Table 3.

3. Statistics

Data are given as mean ± SD. Coefficient of variation (CV), minimum and maximum values are also given in Table 2 for the first and second survey. The calculated statistics from all participants are greatly influenced by presence of single values more than 50% different from the calculated mean value of the submitted OCS concentrations. Such individual values were regarded as outliers (see Figure 1). For 7α-hydroxycholesterol selected parameters of the above-mentioned data were additionally calculated without the outlier values and reported within the manuscript (Table 2). The statistical evaluation of the data sets was weakened by the fact that only 13 laboratories participated, and that only eight out of 13 laboratories participated in both surveys.

4. Results

4.1 First survey

Nine laboratories specialized in chromatographic lipid analysis participated in this first survey. A set of two different lyophilized serum pools (sample A and B) were sent to each participant and analysed with the individual determination method of each laboratory. After a period of three months the laboratories were requested to submit their results to the Referenzinstitut für Bioanalytik at Bonn, Germany for further data evaluation. The data were given in the units individually used by the participant (ng/mL, mg/dL, µmol/L) and was converted into ng/mL for comparison. Each participant received his results presented in the form of Youden-Plots and basic statistical data. Each laboratory was informed about the results in the individual units, which were used by the participant.

4.2 Second survey

Eleven laboratories participated in this second survey including four laboratories that were not included the first survey. One group that participated in the first survey was not able to participate in the second survey. Two different lyophilized serum pools (sample C and D) were analysed by each participant. In contrast to the first part of the OCS survey the second part was designed as interventional, focusing on the influence of the calibration solutions used. Each participant was thus requested to use allocated OCS stock solutions (containing 7α-, 24(S)-, 27-hydroxycholesterol, each 10 µg/mL) for the quantification of the lyophilized sample material. Data submission and notification of individual results were performed as described above.

4.3 24(S)-Hydroxycholesterol

In the first survey for chromatographically determined 24(S)-hydroxycholesterol nine different laboratories participated. The mean 24(S)-hydroxycholesterol concentration determined by all nine participants was calculated for sample A (61.5±36.0 ng/mL; CV 58.5%) and B (59.0±43.3 ng/mL; CV 73.3%). One participant used the GC-MS-epi and five used the GC-MS-deuterium methods. Three laboratories provided results obtained from LC-MS methods using deuterated sterols as internal standard. Two out of ten participants using GC-MS-deuterium and LC-MS-deuterium (Figure 1A) did not determine 24(S)-hydroxycholesterol concentrations within a ± 50% range of the calculated average. In the second survey eleven different laboratories participated of which eight contributed to both surveys. The mean 24(S)-hydroxycholesterol concentration determined by all eleven participants were calculated for sample C (61.0±36.7 ng/mL; CV 60.3%) and D (38.0±21.6 ng/mL; CV 56.8%). In the second survey one laboratory used GC-MS-epi, and six used GC-MS-deuterium methods. Four laboratories provided results obtained from LC-MS-deuterium methods. Of the eleven participants, one who used LC-MS-deuterium method did not determine 24(S)-hydroxycholesterol concentrations within a ± 50% range of the calculated average.

The results of 24(S)-hydroxycholesterol from the first and second survey are shown in figure 1 A (Samples a and b are identified as outliers, sample c submitted inconsistent values) and figure 1B (Sample a is identified as outlier, sample a, b and c submitted inconsistent values) and values for all subgroups are listed in detail Table 2 and 3.

4.4 27-Hydroxycholesterol

In the first survey for chromatographically determined 27-hydroxycholesterol nine different laboratories participated. The mean 27-hydroxycholesterol concentration determined by all nine participants was calculated for sample A (107.3±38.8 ng/mL; CV 36.2%) and B (121.7±43.6 ng/mL; CV 35.8%). One laboratory used GC-MS-epi and five used GC-MS-deuterium methods. Three laboratories provided results obtained by LC-MS methods using deuterated sterols as internal standard. Two of the participants using GC-MS-deuterium and LC-MS-deuterium, did not determine 27-hydroxycholesterol concentrations within a ±50% range of the calculated average.

In the second survey eleven different laboratories participated from which eight contributed to both surveys. The mean 27-hydroxycholesterol concentration determined by all eleven participants was calculated for sample C (159.5±90.4 ng/mL; CV 56.6%) and D (162.6±97.2 ng/mL; CV 59.8%). In the second survey one laboratory used GC-MS-epi, and six used GC-MS-deuterium methods. Four laboratories provided results obtained by LC-MS-deuterium methods. Three of the participants, using GC-MS-epi and LC-MS-deuterium did not determine 27-hydroxycholesterol concentrations within a ±50% range of the calculated average.

The results of 27-hydroxycholesterol from the first and second survey are shown in Figure 1 C (samples a and b are identified as outlier) and Figure 1 D (samples a to c are identified as outlier, sample a and c submitted inconsistent values) and values for all subgroups are listed in detail in Table 2 and 3.

4.5 7α-Hydroxycholesterol

In the first survey seven different laboratories participated. The mean 7α-hydroxycholesterol concentration determined by all seven participants were calculated for sample A (427.8±261.3 ng/mL; CV 61.1%) and B (1918.7±3793.2 ng/mL; CV 197.7%). Among all laboratories five used GC-MS-deuterium methods and one laboratory provided results obtained from LC-MS methods using deuterated sterols as internal standard. The seventh laboratory using GC-MS with 19-hydroxcholesterol as internal standard was omitted from the survey because of very high concentrations of 610 ng/mL and 10500 ng/mL for sample A and B, respectively. After exclusion of this participant the mean 7α-hydroxycholesterol were calculated for sample A (397.5.8±272.4 ng/mL; CV 68.5%) and B (488.4±289.2 ng/mL; CV 59.2%). In the second survey eight different laboratories participated among which five contributed to both surveys. The mean 7α-hydroxycholesterol concentrations determined by all eight participants were calculated for sample C (86.1±40.7 ng/mL; CV 47.2%) and D (83.9±62.3 ng/mL; CV 74.2%). In the second survey six laboratories used GC-MS-deuterium methods. Two laboratories provided results obtained by LC-MS-deuterium methods.

The results of 7α-hydroxycholesterol from the first and second survey are shown in Figure 1 E and Figure 1 F, respectively, and values for all subgroups are listed in detail in Table 2.

4.6 Ratio 24(S)-hydroxycholesterol to 27-hydroxycholesterol

Since the ratio of 24(S)-hydroxycholesterol to 27-hydroxycholesterol has reached importance in clinical diagnosis for the integrity of the blood-brain barrier and description of the state of neurological diseases we calculated this ratio for sample A to D for all participants. We accepted a difference of +/- 50% in each sample. Four participants in both survey did not

submit values. The average value of all participants and the identified outliers (bold) are listed in Table 3.

5. Discussion

The survey can be regarded as an attempt to explain the marked differences in concentrations of ring and side-chain oxidized cholesterols reported in literature. A multitude of different variants of gas- and liquid-chromatographic methods have been used in different reported studies. Our group initiated the first survey for chromatographic OCS (7α-, 24(S)-, 27-hydroxycholesterol) determination by different laboratories in order to compare different methods and try to identify possible sources of errors. It must be emphasized that the determination of OCS species in human serum is associated with numerous potential sources of errors which may bias reported oxysterol concentrations in research articles or even worse in connection with diagnosis and patient care.

In the first survey, 24(S)-hydroxycholesterol levels determined by laboratories using LC-MS-deuterium revealed higher coefficients of variation than levels obtained with GC-MS-deuterium methods (68.7-73.3% vs. 58.8-49.2%). Conversely, 27-hydroxycholesterol values determined by laboratories using LC-MS-deuterium methods revealed slightly lower coefficients of variation than reported with GC-MS-deuterium methods (23.2-25.4% vs. 35.1-38.2%). However, the above coefficients of variation do not reflect the wide distribution of the reported values. As shown in Figure 1 single laboratories had difficulties to get consistent results or reported substantially higher values than the other laboratories. These substantially higher levels were regarded as outliers here. In the second interventional survey the participants were requested to quantify their sample concentrations against a provided standard solution to evaluate the influence of the calibration curve on the results. For 24(S)-hydroxycholesterol CV values determined by GC-MS-deuterium methods are lower than in the first survey. The already good results for 27-hydroxcholesterol from the first survey have also been enhanced by the use of common calibration solution. The results of the participants in both ring trials did improve by using a common standard solution.

The ratio of plasma or csf 24S- to 27-hydroxycholesterol is often used as marker of blood-brain barrier integrity or a marker for the state of neurological diseases [17, 18]. We here present the absolute values of 24S- and 27-hydroxycholesterin in Table 1 A-D and the ratios of both in Table 3. Interestingly, in one participant (#11, Table 3), we do receive unconspicous ratios in comparison to the ratios from other participants. However, in Figure 1 the absolute values of this participant appear as outliers. The reason for this could be that the levels from the standard curves for both 24S- and 27-hydroxycholesterol are equally increased or decreased. Thus, it is of importance for further publication to show the basic individual oxysterol levels before calculating ratios. In case that only the data from a standard curve for 24S-hydroxycholesterol are too high and those for 27-hydroxycholesterol are normal or too low, the ratio 24S- to 27-hydroxycholesterol could give us a wrong impression of the real state of the blood-brain barrier or a neurological disease.

Since the samples were not specifically prepared to prevent cholesterol autoxidation during the lyophilisation process, storage nor shipping, the levels of 7α-hydroxycholesterol were widely distributed in the sample. Therefore, a clear statement about the 7α-hydroxycholesterol cannot be made.

However, it must be noted that also here the use of a common standard appears to reduce the variation of the reported results and brought values together. Therefore, we conclude that even when using individual sample work-up procedures and different analytical methods similar results could be obtained when common quantified stock solutions are used.

Despite this it is evident that some laboratories do have problems with measurement consistence and/or absolute quantification. For LC-MS methods it is crucial to use internal standard compounds of the same chemical constitution co-eluting with the analyte. Therefore, corresponding deuterated oxysterols are necessary for a precise absolute quantification of

OCS. In most instances, the participants in this survey did not use the isotope labelled internal standard. The results of both surveys show how extremely the reported concentrations could vary between individual laboratories.

6. Conclusions and outlook

Comparing the results obtained with different analytical methods and with use of common standard solutions we conclude that the present state for the quantification of oxysterols from serum or plasma samples is quite unsatisfactory. We acknowledge that our pre-analytical protocol was not optimal and that variation in quantification could equally well be a consequence of hydrolysis methods used, independent of LC- of GC-MS measurement.

We do need further attempts to harmonize our different analytical methods in order to compare OCS levels in individual subjects.

Acknowledgment: The basic initiatives for the performance of these surveys was started during the annual meetings of the European Network for Oxysterol Research (ENOR; https://www.oxysterols.net/). Most of the survey participants are members of the ENOR group. We gratefully acknowledge funding for materials, statistics, and travel expenses to the Foundation for Pathobiochemistry and Molecular Diagnostics, German Society of Clinical Chemistry and Laboratory Medicine, Germany. The work in University of Valencia was financed by CICYT-FEDER (AGL2008-02591-C02-01) and MINECO-FEDER (AGL2012-39503-C02-01).

References

[1] G. Brufau, A.K. Groen, F. Kuipers, Reverse cholesterol transport revisited: contribution of biliary versus intestinal cholesterol excretion, Arteriosclerosis, thrombosis, and vascular biology 31 (8) (2011) 1726–1733.

[2] van der Velde, A. E., Reverse cholesterol transport: from classical view to new insights, World journal of gastroenterology WJG 16 (47) (2010) 5908–5915.

[3] I. Björkhem, Crossing the barrier: oxysterols as cholesterol transporters and metabolic modulators in the brain, Journal of internal medicine 260 (6) (2006) 493–508.

[4] A. Crosignani, M. Zuin, M. Allocca, M. Del Puppo, Oxysterols in bile acid metabolism, Clinica chimica acta; international journal of clinical chemistry 412 (23-24) (2011) 2037–2045.

[5] L. Goedeke, C. Fernandez-Hernando, Regulation of cholesterol homeostasis, Cellular and Molecular Life Sciences 69 (6) (2012) 915–930.

[6] van der Wulp, M. Y., H.J. Verkade, A.K. Groen, Regulation of cholesterol homeostasis, Molecular and cellular endocrinology 368 (1-2) (2013) 1–16.

[7] I. Björkhem, Do oxysterols control cholesterol homeostasis?, The Journal of clinical investigation 110 (6) (2002) 725–730.

[8] S. Boenzi, F. Deodato, R. Taurisano, D. Martinelli, D. Verrigni, R. Carrozzo, E. Bertini, A. Pastore, C. Dionisi-Vici, D.W. Johnson, A new simple and rapid LC-ESI-MS/MS method for quantification of plasma oxysterols as dimethylaminobutyrate esters. Its successful use for the diagnosis of Niemann-Pick type C disease, Clinica Chimica Acta 437 (2014) 93–100.

[9] X. Jiang, R. Sidhu, F.D. Porter, N.M. Yanjanin, A.O. Speak, D.T. te Vruchte, F.M. Platt, H. Fujiwara, D.E. Scherrer, J. Zhang, D.J. Dietzen, J.E. Schaffer, D.S. Ory, A sensitive

and specific LC-MS/MS method for rapid diagnosis of Niemann-Pick C1 disease from human plasma, Journal of lipid research 52 (7) (2011) 1435–1445.

[10] G. Klinke, M. Rohrbach, R. Giugliani, P. Burda, M.R. Baumgartner, C. Tran, M. Gautschi, D. Mathis, M. Hersberger, LC-MS/MS based assay and reference intervals in children and adolescents for oxysterols elevated in Niemann-Pick diseases, Clin Biochem (2015).

[11] S. Pajares, A. Arias, J. Garcia-Villoria, J. Macias-Vidal, E. Ros, J. de las Heras, M. Giros, M.J. Coll, A. Ribes, Cholestane-3beta,5alpha,6beta-triol: high levels in Niemann-Pick type C, cerebrotendinous xanthomatosis, and lysosomal acid lipase deficiency, Journal of lipid research 56 (10) (2015) 1926–1935.

[12] R. Schüle, T. Siddique, H.-X. Deng, Y. Yang, S. Donkervoort, M. Hansson, R.E. Madrid, N. Siddique, L. Schöls, I. Björkhem, Marked accumulation of 27-hydroxycholesterol in SPG5 patients with hereditary spastic paresis, Journal of Lipid Research 51 (4) (2009) 819–823.

[13] A. Zmyslowski, A. Szterk, Current knowledge on the mechanism of atherosclerosis and pro-atherosclerotic properties of oxysterols, Lipids in health and disease 16 (1) (2017) 188.

[14] A.B. Reiss, I. Voloshyna, J. de Leon, N. Miyawaki, J. Mattana, Cholesterol Metabolism in CKD, American journal of kidney diseases the official journal of the National Kidney Foundation 66 (6) (2015) 1071–1082.

[15] M. Heverin, N. Bogdanovic, D. Lütjohann, T. Bayer, I. Pikuleva, L. Bretillon, U. Diczfalusy, B. Winblad, I. Björkhem, Changes in the levels of cerebral and extracerebral sterols in the brain of patients with Alzheimer's disease, Journal of lipid research 45 (1) (2004) 186–193.

[16] J. Wong, C.M. Quinn, I.C. Gelissen, A.J. Brown, Endogenous 24(S),25-epoxycholesterol fine-tunes acute control of cellular cholesterol homeostasis, The Journal of biological chemistry 283 (2) (2008) 700–707.

[17] V. Leoni, T. Mastermann, F.S. Mousavi, B. Wretlind, L-O. Wahlung, U. Diczfaulsy, J. Hillert, I. Björkhem, Diagnostic use of cerebral and extracerebral oxysterols, Clin chem lab med 42 (2004) 185-191.

[18] V. Leoni, T. Mastermann, P. Patel, S. Meaney, U. Diczfalusy, I. Björkhem, Side chain oxidized oxysterols in cerebrospinal fluid and the integrity of blood-brain and blood-cerebrospinal fluid barriers, J of lipid research, 44 (2003) 793-799.

[19] M. Voisin, P. de Medina, A. Mallinger, F. Dalenc, E. Huc-Claustre, J. Leignadier, N. Serhan, R. Soules, G. Segala, A. Mougel, E. Noguer, L. Mhamdi, E. Bacquie, L. Iuliano, C. Zerbinati, M. Lacroix-Triki, L. Chaltiel, T. Filleron, V. Cavailles, T. Al Saati, P. Rochaix, R. Duprez-Paumier, C. Franchet, L. Ligat, F. Lopez, M. Record, M. Poirot, S. Silvente-Poirot, Identification of a tumor-promoter cholesterol metabolite in human breast cancers acting through the glucocorticoid receptor, Proceedings of the National Academy of Sciences of the United States of America 114 (44) (2017) E9346-E9355.

[20] M. Soncini, G. Corna, M. Moresco, N. Coltella, U. Restuccia, D. Maggioni, L. Raccosta, C.-Y. Lin, F. Invernizzi, R. Crocchiolo, C. Doglioni, C. Traversari, A. Bachi, R. Bernardi, C. Bordignon, J.-A. Gustafsson, V. Russo, 24-Hydroxycholesterol participates in pancreatic neuroendocrine tumor development, Proceedings of the National Academy of Sciences of the United States of America 113 (41) (2016) E6219-E6227.

[21] P.K. Sandhu, S.M.A. Musaad, A.T. Remaley, S.S. Buehler, S. Strider, J.H. Derzon, H.W. Vesper, A. Ranne, C.S. Shaw, R.H. Christenson, Lipoprotein Biomarkers and Risk of Cardiovascular Disease: A Laboratory Medicine Best Practices (LMBP) Systematic Review, The journal of applied laboratory medicine 1 (2) (2016) 214–229.

[22] M. Poirot, S. Silvente-Poirot, The tumor-suppressor cholesterol metabolite, dendrogenin A, is a new class of LXR modulator activating lethal autophagy in cancers, Biochemical pharmacology 153 (2018) 75–81.

[23] E.R. Nelson, S.E. Wardell, J.S. Jasper, S. Park, S. Suchindran, M.K. Howe, N.J. Carver, R.V. Pillai, P.M. Sullivan, V. Sondhi, M. Umetani, J. Geradts, D.P. McDonnell, 27-Hydroxycholesterol links hypercholesterolemia and breast cancer pathophysiology, Science (New York, N.Y.) 342 (6162) (2013) 1094–1098.

[24] G.J. Schroepfer JR, Oxysterols: Modulators of cholesterol metabolism and other processes, Physiological reviews 80 (1) (2000) 361–554.

Table 1: List of methodological parameters during work-up within the different laboratories and of the analytical instruments used.

sample volume [µL]	25; 100; 200; 400; 500; 1000
internal standards [ng]	2; 5; 10; 15; 20; 35.74; 50; 100; 150; (or 2µmol)
hydrolysis time [min]	20; 60; 120; 180
hydrolysis temperature [°C]	room temperature; 25; 60; 75
hydrolysis NaOH/KOH concentration [mol/L]	0.35; 0.5; 0.71; 1.0; (or 50%)
extraction solvents	hexane; chloroforme; cyclohexane; isooctane; chloroforme: methanol (2:1)
solvent volume for extraction [mL]	2; 3; 5; 7.2; 9; 18
silylation time [min]	30
silylation temperature [°C]	60
silylation reagents (GC only)	HMDS/TMCS/pyridine (3:1:9)(Supelco) BSTFA/TMCS 99:1 + pyridine HMDS/TMCS/Pyridine 2:1:3 MSTFA: Pyridin (2:1) 1% TMCS
reconstitution volume [µL]	50; 70; 300; 1400
Injection volume [µL]	1; 2; 20; 40; 50
split mode injection (GC only)	splitless
column lenght (GC only) [m]	30; 50 (5cm and 25cm for LC systems)
column diameter	250µm; 2.1mm; 4.6 mm
film thickness	0.25µm; 5µm
column film thickness (GC only) [µm]	0.1; 0.25
columns (GC only)	HP-5MS Hypersil Gold RP DB-5-MS revers phase C18 Supercoil LC18S C18 HPLC Zorbax Eclips plus C18 guard column c HP-5MS 5% phenylmethyl siloxane
separation instruments	GC-Agilent Technologies 6890N Shimadzu GC-MSQP2010 LC/MS model LC2010A Shimadzu
detectors	LTQ orbitrap XL LTQ-orbitrap Velos MS AgilentMS 5973N LCMS-2010A mass spectrometer with APCI interph SCIEX API 3000 triple quadrupol Photospray

Table 2: Results of 7α-, 24(S)-, and 27-hydroxcholesterol submitted by GC-MS and LC-MS methods in the interventional survey.

	First survey								Second surve				
	Sample A				Sample B				Sample C				
	mean ± SD[1)]	CV[2)]	min[3)]	max[4)]	mean ± SD[1)]	CV[2)]	min[3)]	max[4)]	mean ± SD[1)]	CV[2)]	min[3)]	max[4)]	m
total 24(S)-OH-cholesterol	**61.5 ± 36.0**	**58.5%**	**24.4**	**120.0**	**59.0 ± 43.3**	**73.3%**	**15.8**	**150.0**	**61.0 ± 36.7**	**60.3%**	**17.7**	**161.9**	**38**
GC-MS (total)	58.3 ± 30.1	51.6%	24.4	108.0	47.5 ± 21.1	44.4%	15.8	72.5	50.5 ± 16.8	33.2%	17.7	68.0	33
GC-MS (epi)	76.4				64.3				68.0				
GC-MS (deuterium)	54.6 ± 32.1	58.8%	24.4	108.0	44.2 ± 21.7	49.2%	29.2	72.5	47.5 ± 16.3	34.2%	17.7	62.0	31
LC-MS (deuterium)	68.1 ± 46.8	68.7%	29.2	120.0	82.0 ± 60.1	73.3%	36.0	150.0	79.3 ± 56.9	71.7%	40.3	161.9	45
total 27-OH-cholesterol	**107.3 ± 38.8**	**36.2%**	**47.3**	**170.0**	**121.7 ± 43.6**	**35.8%**	**51.6**	**190.0**	**159.5 ± 90.4**	**56.6%**	**84.0**	**349.0**	**162**
GC-MS (total)	90.8 ± 31.8	35.1%	47.3	129.0	109.0 ± 43.6	40.0%	51.6	164.0	129.7 ± 43.4	33.4%	84.0	217.4	153
GC-MS (epi)	68.3				72.4				217.4				
GC-MS (deuterium)	95.3 ± 33.4	35.1%	47.3	129.0	116.3 ± 44.5	38.2%	51.6	164.0	115.1 ± 21.6	18.7%	84.0	142.2	113
LC-MS (deuterium)	140.2 ± 32.5	23.2%	105.6	129.0	147.2 ± 37.3	25.4%	121.5	164.0	211.6 ± 133.3	63.0%	95.6	349.0	177
total 7α-OH-cholesterol	**397.5 ± 272.4**	**68.5%**	**77.5**	**884.0**	**488.4 ± 289.3**	**59.2%**	**99.4**	**844.0**	**86.1 ± 40.7**	**47.2%**	**40.3**	**164.0**	**83**
GC-MS (deuterium)	429.7 ± 291.5	67.9%	77.5	884.0	534.3 ± 298.0	55.8%	99.6	844.0	68.4 ± 23.7	34.7%	40.3	102.1	56
LC-MS (deuterium)	236.5		236.5	236.5	259.5		259.5	259.5	139.4 ± 34.7	24.9%	114.9	164.0	167

[1)] mean value with standard deviation [ng/mL] [2)] coefficient of variation [3)] minimum value [ng/mL]

Table 3 Ratios of 24(S) to 27-hydroxycholesterol from individual laboratories as measured in the first (A, B) and second (C,D) trial.

	Ratio 24(S)- to 27-OH-cholesterol			
Participant	**A**	**B**	**C**	**D**
1	0.45	0.44	0.45	0.26
2	**1.12**	**0.89**	0.31	**0.11**
3	0.51	0.59	0.50	0.39
4	**0.90**	**0.11**		
5			0.52	0.40
6	0.28	0.30	0.44	0.28
7			**0.14**	0.18
8			0.42	0.22
9	0.71	**0.79**	0.21	0.13
10	0.55	0.54	0.51	0.26
11	0.38	0.46	0.53	0.35
12	0.39	0.39	0.41	0.25
mean	0.59	0.50	0.40	0.26
SD	0.27	0.24	0.13	0.09
CV [%]	46.5%	47.4%	32.3%	36.9%
upper cut off	0.88	0.75	0.61	0.39
lower cut off	0.29	0.25	0.20	0.13

Data given in bold are outliers below or above the 50% cut-off levels.

The empty fields indicate missing data in 2013 or 2014 from different laboratories.

Figure 1

Distribution of 24S-,27-, and 7α-hydroxcholesterol concentrations analyzed by GC-MS and LC-MS methods in the first descriptive and second interventional survey. Values a, b, and c in the figures A, B, C, and D are regarded as outliers or inconsistent sample analysis.