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Quantification of Cholesterol and Cholesteryl Ester by Direct Flow Injection High Resolution FTMS Utilizing Species-Specific Response Factors

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ABSTRACT: The quantification of free cholesterol (FC) and cholesteryl-ester (CE) in mammalian serum samples of great interest for basic science and clinical lipidomics. Here, we evaluated the feasibility of direct flow injection analysis (FIA) coupled to electrospray ionization-high resolution mass spectrometry (ESI-HRMS) to quantify FC and CE in lipid extracts from human serum, cultured cells and mouse liver. Despite poor ionization efficiency of FC, the limit of quantitation was sufficient for precise and accurate quantification of FC by multiplexed HRMS (MSX) analysis without using a derivatization step. However, it was demonstrated that upon full scan Fourier transform MS (FTMS) quantification, CE species show substantial differences in their analytical responses depending on number of double bonds, length of the acyl chain, infused lipid concentration and other lipid components. A major determinant for these response differences is their susceptibility to in-source fragmentation. In particular, introduction of double bonds lowers the degree of in-source fragmentation. Therefore, CE species-specific response factors need to be applied for CE quantification by FTMS to achieve accurate concentrations. Method validation demonstrated that FIA-ESI-HRMS (MSX and FTMS) is applicable for quantification of FC and CE in samples used in basic science as well as clinical studies such as cultured cells, tissue homogenates and serum.

Cholesterol is an essential lipid component for mammalian cells and occurs mainly as non-esterified, free cholesterol in membranes (FC) and as cholesteryl esters (CE) stored in lipid droplets and transported in lipoprotein particles. FC has unique biophysical properties which play an important role in regulation of membrane fluidity and cellular lipid homeostasis. Particularly, this feature of FC requires its accurate quantification also together with other lipid classes because small changes in its content may influence membrane properties. Moreover, blood and lipoprotein cholesterol concentrations are applied in patient diagnostics and treatment for decades. Therefore, quantification of both FC and CE in serum or lipoprotein fractions needs to be reproducible and accurate to translate lipidomic tools finally to clinical applications.

While electrospray ionization tandem mass spectrometry (ESI-MS/MS) is routinely applied to quantify CE species as ammoniated adduct ions, quantification of FC frequently includes derivatization either to cholesteryl acetate or cholesterol sulfate. Direct quantification of FC by ESI is hampered by its poor ionization efficiency and in-source fragmentation of [FC+NH4]+. Recently, Gallego et al. evaluated the performance of direct quantification of FC in lipid extracts from human serum by direct infusion chip-based nano-ESI (~200 nL/min) coupled to high resolution Fourier transform mass spectrometry (FTMS). Comparison of quantification by full scan FTMS (FTMS), parallel reaction monitoring (PRM) and multiplexed PRM (MSX) demonstrated superior performance for MSX in FC quantification. In MSX experiments, multiple precursor ions (frequently analyte and internal standard) are collected and simultaneously fragmented and analyzed.

Here, we report the evaluation and validation of quantification of FC and CE by direct flow injection analysis (FIA; 10 µL/min) ESI-MSX and FTMS for human serum and samples from cultured cells and mouse liver. Reproducible and accurate quantification of FC was demonstrated for all samples. For CE, we show that accurate quantification requires species-specific response factors.

EXPERIMENTAL SECTION

Reagents and Lipid Standards. Chloroform and 2-propanol were purchased from Roth (Karlsruhe, Germany) and methanol from Merck (Darmstadt, Germany). All solvents were HPLC grade. Ammonium formate and all unlabeled cholesterol and cholesteryl ester standards were purchased from Sigma-Aldrich (Taufkirchen, Germany), all with purities higher than 95%. [25,26,26,27,27- D7]-cholesterol was obtained from Cambridge Isotope Laboratories (Andover, MA, USA) with isotope purity higher than 98%. Acetyl chloride of the highest analytical grade available was purchased from Fluka (Buchs, Switzerland). All CE standards were dissolved in hexane/2-propanol (1:1 v/v) and D7-cholesterol was dissolved in chloroform/methanol (9:1 v/v) with 1 g/L butylated.
hydroxytoluene. The concentrations of the dissolved CE standards were determined by GC-MS using a certified FAME Mix (Supelco 37 Component FAME Mix; Sigma-Aldrich, Taufkirchen, Germany) as described previously.  

**Samples.** Human serum samples were collected from residual patient material after clinical routine diagnostics. Total cholesterol and triacylglycerols were measured by enzymatic assays on Siemens Dimension Vista (Siemens Health Care GmbH, Germany). Murine liver samples were pooled residuals of previous studies. Primary human skin fibroblasts were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with L-glutamine, nonessential amino acids, and 10% FCS at 5% CO₂ in a humidified incubator at 37°C as described previously.  

**Sample Preparation.** Samples were spiked with internal standards (IS; D₃₇-cholesterol 6.36 nmol for serum and 9.53 nmol for cells/tissue, CE 17:0 3.17 nmol for serum and 0.78 nmol for cells/tissue; CE 22:0 2.98 nmol for serum and 0.71 nmol for cells/tissue) prior to lipid extraction (solvent of standards was removed by vacuum centrifugation). Serum 10 µL, cell homogenate containing 100 µg of protein or tissue homogenate containing a wet weight of 2 mg were subjected to extraction. The samples were extracted according to the procedure described by Bligh and Dyer with a total chloroform volume of 2 mL. 500 µL of the separated chloroform phase was transferred into a sample vial by a pipetting robot (Tecan Genesis RSP 150) and vacuum dried. Samples subjected to derivatization were incubated with 200 µL of acetyl chloride/chloroform (1:5 v/v) for 60 min at room temperature before vacuum drying. The residues were dissolved in 1.4 mL of 7.5 mM ammonium formate in chloroform/methanol/2-propanol (1:2:4 v/v/v).  

**Direct Flow Injection High Resolution MS.** Lipid quantification was performed by direct flow injection on a hybrid quadrupole-Orbitrap mass spectrometer QExactive (Thermo Fisher Scientific, Bremen, Germany) equipped with a heated electrospray ionization source. The ion source was operated using the following settings: spray voltage of 3.5 kV, S-lens RF level 50, capillary temperature of 250°C, aux gas heater temperature of 100°C and settings of 15 for sheath gas and 5 for aux gas. All data were acquired in profile mode. 50 µL of the reconstituted sample extracts were injected by a PAL autosampler (CTC Analytics, Zwingen, Switzerland) equipped with an UltiMate 3000 isocratic pump (Thermo Fisher Scientific, Waltham, MA, USA). Chloroform/methanol/2-propanol (1:2:4 v/v/v) was delivered at an initial flow rate of 100 µL/min until 0.25 min followed by 10 µL/min for 2.5 min and a wash out with 300 µL/min for 0.5 min. Positive ion mode FTMS data were recorded in m/z range 500 – 1000 for 1 min with a maximum injection time (IT) of 200 ms, an automated gain control (AGC) of 1·10⁶, three microscans and a target resolution of 140,000 (at 200 m/z), MS/MS was applied to obtain a homologous series varying in the number of double bonds (CE 10:0, CE 14:0, CE 16:0, CE 18:0 and CE 22:0), 3 monounsaturated species (CE 16:1, CE 18:1 and CE 20:1) and 2 polyunsaturated species (CE 18:2 and CE 18:3) to obtain a homologous series varying in the number of double bonds (CE 18:0 - CE 18:3). CE 17:0 was used as internal standard (0.46 pmol/µL; for concentrations see Figure S1). The response of CE only includes peak assignment and intensity picking. The extracted spectra were processed using the ALEX software which is based on theoretical isotope distributions; not performed for data generated with Orbitrap Fusion. Peak assignment applied a mass accuracy of less than 3 ppm. Quantification was achieved by multiplication of the spiked-in IS amount with the analyte-to-IS intensity ratio after isotope correction. Annotation of FC and CE is based on the fact that cholesterol represents by far the most abundant sterol with 27 carbons and one double bond (ST 27:1) in the investigated mammalian samples. In rare diseases of the cholesterol biosynthesis pathway like lathosterolosis the cholesterol isomers lathosterol and zymostenol may be present at higher level (their identification needs chromatographic separation).  

**Determination of Instrument Response.** For determination of instrument response we prepared a mixture of CE species composed of a homologous series of 5 saturated species varying in chain length (CE 10:0, CE 14:0, CE 16:0, CE 18:0 and CE 22:0), 3 monounsaturated species (CE 16:1, CE 18:1 and CE 24:1) and 2 polyunsaturated species (CE 18:2 and CE 18:3) to obtain a homologous series varying in the number of double bonds (CE 18:0 - CE 18:3). CE 17:0 was used as internal standard (0.46 pmol/µL; for concentrations see Figure S1). The instrument response was defined as molar ratio (after deisotoping):

\[
\text{response} = \frac{n(\text{detected})}{n(\text{expected})}
\]

The responses determined in Figure 3 were used to establish a mathematical model for response calculation. This model is based on the following considerations: First, there is a linear relationship of response to number of C-atoms (mean of the regression line slopes for saturated and monounsaturated species was used). Second, there is a linear relationship of response to number of double bonds (see insert of Figure 3). Third, response is affected also by other lipid components – this factor was derived from changes in response upon addition of triacylglycerols(TG) and phosphatidylcholines (PC) (described in Figure 6). This matrix factor A was defined as:

\[
A = \frac{\text{response CE with TG & PC}}{\text{response CE only}}
\]

Species-specific response for correction of CE quantification in plasma/serum was calculated using the following equation (C is the number of carbon atoms of the acyl chain, DB the number of double bonds):

\[
\text{response(calc)} = (0.049 \cdot C + 0.57 \cdot DB) \cdot A
\]
Measured CE concentrations were multiplied by the calculated species-specific response factors (Table S1).

**RESULTS AND DISCUSSION**

**Quantification of FC.** Direct quantification of FC was recently described using a chip-based direct infusion nano-ESI-MSX-FTMS/MS-based approach for human and murine plasma samples. Here, we evaluated whether direct quantification of FC is also feasible using FIA-ESI-MSX-FTMS/MS with a quadrupole-Orbitrap instrument in human serum, cultured cells and murine liver samples.

As described by Gallego et al. ammoniated FC and CE undergo in-source fragmentation to yield a protonated cholestadiene fragment at m/z 369.3516. Therefore, the extent of in-source fragmentation was assessed in our setting by infusing neat analytical FC and CE 17:0 standards. The percentage of intact analyte was calculated out of the added intensities of the ammoniated species and the protonated cholestadiene fragment. The experiment revealed significant differences in their susceptibility to in-source fragmentation: 27% of FC and 74% of CE 17:0 are unimpaired.

To achieve accurate FC quantification, we took advantage from multiplexing (MSX) of analyte and internal standard pairs in PRM (parallel reaction monitoring). In an initial experiment, FC concentrations of human serum samples were determined by MSX at mass resolution 17,500 (FWHM at 200 m/z) and then compared to our previously established method using FIA-MS/MS and an acetyl chloride derivatization on a triple quadrupole mass spectrometer. FC concentrations determined by these methods did not sufficiently correlate, with markedly higher values observed by the MSX method. Mass resolution of the MSX-method was increased, to evaluate whether an isobaric interference is present. Product ion spectra at increased mass resolution revealed an isobaric fragment ion at m/z 369.337 (Figure 1) which most likely derives from monoacylglycerol MG 20:0 (FTMS showed matching ions at 404.3734 m/z [M+NH₄]+ in positive and 431.3378 m/z [M+HCOO]+ in negative ion mode). In order to ensure an unambiguous differentiation of cholestadiene and this isobaric interference (mass difference of 0.015 Da), we chose a mass resolution setting of 140,000 (at 200 m/z) providing baseline separation of the peaks.

**Quantification of CE.** Cholesterol is mainly stored or transported as CE which is one of the most abundant lipid classes in human plasma. In laboratory testing, cholesterol is determined as total cholesterol (TC = esterified and non-esterified) with enzymatic tests certified for clinical diagnostics. In an initial experiment, we compared TC concentrations of 18 human serum samples determined by MSX for FC and FTMS for CE (MSX/FTMS) with those determined enzymatically. MS- and enzymatically-derived TC concentrations show a good correlation but MS-values are ~50% higher (Figure 2, grey circles). Since previous studies indicated that lipid species response may be influenced by length and unsaturation degree of the acyl chains, we investigated the analytical response of CE species in more detail.

**Figure 1.** FTMS/MS spectra of FC [M+NH₄]+ in human serum recorded at different resolutions. A series of spectra recorded in positive ion mode at mass resolution settings (at 200 m/z) 17,500, 35,000, 70,000 and 140,000 reveals isobaric ions of protonated cholestadiene and MG 20:0 [M+H-H₂O]+ (Δm/z = 0.015).

Because the signal intensity of FC [M+NH₄]+ is relatively low we wanted to evaluated the accuracy of the high resolution MSX-method. Therefore, we compared our acetyl chloride derivatization with direct MSX analysis in human serum, cultured cells and murine liver samples. The derivatization increased the intensity of FC and D-FD ~400-fold. Nevertheless, all analyzed samples were in good agreement with in average 3 to 6% higher concentrations for the derivatized samples (Table S2), indicating that the direct analysis performs as well as the acetyl chloride derivatization method.
double bonds. CE 17:0 was used as internal standard for the response of C18-CE species plotted against the number of species. Due to a limited number of polyunsaturated CE species, with a slope similar to saturated and monounsaturated species, increases systematically with double bond count (Figure 3, insert). Based on these data, we expect a linear dependency for monounsaturated (dark red) and polyunsaturated species (green) increases linearly with the acyl chain length (Figure 3). Notably, however, the acyl chain unsaturation has a significantly higher influence on the response, which also decreases the susceptibility of [CE+NH₄]⁺ ions to undergo in-source fragmentation, signifying a pronounced charge stabilizing effect. Moreover, an increased chain length decreases in-source fragmentation, although to a lower extent compared to double bonds.

To study these effects, a mixture containing five saturated and five unsaturated CE standards was compiled, analyzed by FTMS and the response determined as specified in the Experimental section. Two things are apparent from these studies - the instrument response of saturated and monounsaturated species increases linearly with the acyl chain length (Figure 3). Notably, however, the acyl chain unsaturation has a significantly higher influence on the response, which also increases systematically with double bond count (Figure 3, insert). Based on these data, we expect a linear dependency for increasing acyl chain lengths for each series of polyenoic CE species, with a slope similar to saturated and monounsaturated species. Due to a limited number of polyunsaturated CE species commercially available we could not test their response in detail.

Another question was, whether such response differences are limited to conventional ESI? Therefore, we analyzed CE mixtures by chip-based nano-ESI coupled to an Orbitrap Fusion (Figure S2). The linear increase of response with double bond count (Figure S2, insert) matches very well the results obtained by conventional ESI (Figure 3, insert). The trend for the acyl chain length appears similar but seems not to follow a linear trend at higher carbon numbers. It has been shown that CE species undergo in-source fragmentation to cholestadiene-based fragment ions in nano-ESI. Therefore, we asked whether their species-specific responses may be related to in-source fragmentation. Individual CE species were analyzed and the percentage of intact ions was calculated out of the added intensities of the unfragmented species [M+NH₄]⁺ and the protonated cholestadiene fragment (Figure 4). The trends observed are similar to those noticed for the response (Figure 3), suggesting that the in-source fragmentation behavior is a main cause for response differences of CE species. The presence of double bonds in the CE acyl chain substantially decreases the susceptibility of [CE+NH₄]⁺ ions to undergo in-source fragmentation, signifying a pronounced charge stabilizing effect. Moreover, an increased chain length decreased in-source fragmentation, although to a lower extent compared to double bonds.
range of ~0.05 to 9.5 pmol/µL; data not shown). However, concentration had only negligible effects on the susceptibility to undergo in-source fragmentation. Therefore, a potential explanation could be that aggregation of highly hydrophobic CE at higher concentration results in less efficient ionization. Here, it has to be considered that the response was calculated using CE 17:0 as saturated internal standard which explains that relative response of saturated species is not influenced by concentration since they aggregate in a similar way. Unexpectedly, monounsaturated CEs seem to behave like saturated species in this respect. Polyunsaturated CE species however, appear to be less susceptible to aggregation resulting in an increased relative response due to enhanced aggregation of the saturated internal standard. To accurately compensate for this, spike-in of unsaturated CE internal standards could be advantageous. Of note, the concentrations applied here fit very well to previously described recommendations for lipid concentrations of infusates in shotgun-mass spectrometry.\(^{27,30}\)

**Figure 5.** Effect of total CE concentration on CE species response. Responses of CE 18:0, CE 18:1, CE 18:2 and CE 18:3 are plotted against total CE concentration of the injected standard mix. CE 17:0 was used as internal standard. Displayed are mean ± SD of three independent experiments (for more details Figure S1).

Typically, biological samples comprise a complex mixture of different lipid classes and species. Therefore, we asked how responses of CE lipids are influenced by addition of triacylglycerols (TG) and phosphatidylcholines (PC), representing the other main components of human serum. To a constant concentration of the CE mixture, we spiked in either PC, TG, or both, in concentrations resembling the "physiological" range for these lipids (see legend of Figure 6 for details), and then analyzed those mixtures by FIA-FTMS. Inclusion of PC and TG reduced the response of CE species to a similar extent and this effect was more pronounced for polyunsaturated CE species (Figure 6). Simultaneous addition of both TG and PC showed an additional response reduction especially for polyunsaturated species. We checked the in-source fragmentation in presence of TG and/or PC but could not detect any changes (data not shown). Potentially addition of other lipid classes influences aggregation of CE species.

**Figure 6.** Effect of addition of TG and/or PC on the CE species response. A CE mixture (9.41 pmol/µL total CE concentration, see Experimental section) was analyzed in presence of PC (5.8 pmol/µL total PC concentration), TG (5.4 pmol/µL total TG concentration) and both lipid classes together. The PC mix is composed of PC 28:0 (1.13; all concentrations in pmol/µL), PC 34:0 (1.18), PC 34:2 (1.18), PC 36:4 (1.14) and PC 44:0 (0.98). The TG mixture consists of TG 42:0 (1.23), TG 48:0 (1.11), TG 51:0 (1.05), TG 54:3 (1.02) and TG 57:0 (0.96).

To further understand the influence of sample concentration on CE response, we selected three patient serum samples, #1 with high TC and low TG, #2 with high TC and high TG and #3 with low TC and low TG levels. These samples were diluted sequentially and analyzed at 8 different concentrations by FIA-FTMS/MSX. Total CE concentration decreased to 86% related to the non-diluted sample for both high TC samples and to 90% for the low TC sample (Table 1) and, as expected, dilution effects were most pronounced for PUFA CE species confirming our results obtained with synthetic standard mixtures. Dilution effects for both high-TC/low-TG and high-TC/high-TG samples are almost identical which argues for a minor influence of TG compared to TC concentration on CE species response (total lipid concentration versus lipid class concentration). Moreover, dilutions of more than 8-fold did not result in a further decrease of total-CE and PUFA-CE species concentrations. Notably, also for most diluted samples total cholesterol concentrations determined by FTMS were about 30% higher than those analyzed enzymatically. Hence, an accurate quantification of CE by FTMS requires application of species-specific response factors even in diluted samples. Remarkably, when these samples were subjected to analysis of lipid classes other than FC or CE, we observed that a dilution of more than 4-fold reduced signals of low abundant lipid species like minor lipid species (e.g. LPC 20:4, ceramides) below the LoD due to a relative increase of ions of the chemical background (data not shown). Since such lipid species have been shown to be relevant in previous studies,\(^{31-35}\) we chose to omit further sample dilution and to consider the concentration effect within the detected species-specific response.

The gathered information about CE response behavior (including in-source fragmentation, concentration and addition of TG & PC) have been used to compile a model to calculate species-specific response factors of individual CE species for serum/plasma samples (see Experimental section - Determination of Instrument responses). These factors (Table S1) have been applied to correct CE concentration of human serum samples in our initially performed experiment (Figure 2). FTMS/MSX derived TC concentrations were in good agreement with total cholesterol concentrations determined by an enzymatic method.\(^{27,30}\)

**Figure 6.** Effect of addition of TG and/or PC on the CE species response. A CE mixture (9.41 pmol/µL total CE concentration, see Experimental section) was analyzed in presence of PC (5.8 pmol/µL total PC concentration), TG (5.4 pmol/µL total TG concentration) and both lipid classes together. The PC mix is composed of PC 28:0 (1.13; all concentrations in pmol/µL), PC 34:0 (1.18), PC 34:2 (1.18), PC 36:4 (1.14) and PC 44:0 (0.98). The TG mixture consists of TG 42:0 (1.23), TG 48:0 (1.11), TG 51:0 (1.05), TG 54:3 (1.02) and TG 57:0 (0.96).
agreement after correction of the individual CE response arguing that accurate quantification of CE by FTMS needs implementation of CE species-specific response factors.

**Method Validation.** Finally, FIA-FTMS/MSX was validated for human serum and samples from cultured cells. Intraday CVs for FC and CE species in serum were 5% and below (Table 2). Also for day-to-day, CVs for FC and abundant CE species, as detected in serum, were not above 11%. CVs up to 15% were observed for CE species in the analyzed cells because their concentrations were close to LoQ for some species (Table S3).

The limits of quantification (LoQ) were determined functionally as previously described for LC-MS/HRMS methods. Sample matrix has a substantial influence on LoQ and was therefore assessed for both, human serum and cultured cell samples. To this end, CE 22:0 and D-FC were spiked in the samples at various concentrations close to the LoQ, the samples were extracted and analyzed by FIA-FTMS/MSX. LoQ was defined as the concentration where each CV reaches 20% or accuracy leaves the range of 80 – 120% (Figures S3, S4). For serum, LoQs were in the range of 200 µM, for FC and 2 µM for CE (Table S3). For cells, FC had a LoQ of 14 nmol/mg protein and CE 0.05 nmol/mg protein. The high LoQs for FC are related to its poor ionization efficiency and high rate of in-source fragmentation. However, we would like to emphasize that FC is a main component in most mammalian samples with concentrations markedly higher than LoQ. Thus, despite the high LoQs MSX can provide an accurate FC quantification in typical samples (Table S2).

**CONCLUSION**

Here we present, to our knowledge, the first lipidomics application using FIA with a conventional LC system coupled to a high-resolution FTMS instrument. As a first step, the method was evaluated here for analysis of FC and CE and will be extended to other lipid classes, as described for chip-based infusion nano-ESI-FTMS. Method validation demonstrated that the precision and sensitivity of FIA-HRMS is sufficient for applications involving both large clinical sample sets measuring serum, and for basic research measuring, e.g., cultured cells. Method comparison to a certified enzymatic test indicates that accurate quantification of CE species by FTMS requires utilization of individual response factors. The marked, up to three-fold, response differences between CE species are related to structural features, like the length and double bond number of constituent acyl chains that affect CE in-source fragmentation. Comparison of ESI and nano-ESI demonstrated that the huge effect of double bond number is not limited to conventional ESI. Remarkably, the response trends observed for both ion sources were similar despite disparate flow rates of 10 µL/min and 200 nL/min. This indicates that mathematical models for calculation of CE species-specific response factors may be applicable in different instrument setups. However, such models should be adjusted and validated for the individual setup. Utilization of response factors is highly relevant not only for direct infusion methods but also for LC-MS based CE quantification. CE in-source fragmentation as main cause for different responses is probably influenced by the build of the mass spectrometer, gas flow and temperature, which need to be assessed with great care when developing lipidomic methods.

**ASSOCIATED CONTENT**

**Supporting Information**

Figures S1-S4 and Tables S1-S3 are included as PDF. Additional information as noted in text. The Supporting Information is available free of charge on the ACS Publications website.

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**Author Contributions**

The manuscript was written through contributions of all authors. / All authors have given approval to the final version of the manuscript.

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**REFERENCES**


Table 1. Dilution effect on CE species concentrations in human serum samples. Samples had the following concentrations determined by enzymatic testing: Sample 1 - high TC (8.74 mM) and low TG (1.48 mM); sample 2 - high TC (8.84 mM) and high TG (3.66 mM); sample 3 - low TC (4.58 mM) and low TG (2.09 mM). Dilution effect was calculated from the non-diluted related to mean concentration of 16-fold and higher dilutions. Each value represents the average of n=3 technical replicates.

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<th>4 mM</th>
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<th>16 mM</th>
<th>32 mM</th>
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Fre cholesterol is not influenced by sample dilution, however, in dilutions of more than 8-fold FC signals were below LoD. Therefore, the FC concentration measured at 8-fold dilution was used to calculate TC for further dilution steps.
Table 2. Coefficient of variation (CV) of intra- and interday precision of FC and CE species determined in human serum and fibroblast cell homogenates by FIA-FTMS/MSX. Number of independent analyses n=5 for both intra- and interday.

<table>
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