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METHODS

Analysis of Intact Cholesteryl Esters of Furan Fatty Acids in Cod Liver

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Abstract Furan fatty acids (F-acids) are a class of natural antioxidants with a furan moiety in the acyl chain. These minor fatty acids have been reported to occur with high proportions in the cholesteryl ester fraction of fish livers. Here we present a method for the direct analysis of intact cholesteryl esters with F-acids and other fatty acids in cod liver lipids. For this purpose, the cholesteryl ester fraction was isolated by solid phase extraction (SPE) and subsequently analyzed by gas chromatography with mass spectrometry (GC/MS) using a cool-on-column inlet. Pentadecanoic acid esterified with cholesterol was used as an internal standard. GC/MS spectra of F-acid cholesteryl esters featured the molecular ion along with characteristic fragment ions for both the cholesterol and the F-acid moiety. All investigated cod liver samples ($n = 8$) showed cholesteryl esters of F-acids and, to a lower degree, of conventional fatty acids. By means of GC/MS-SIM up to ten F-acid cholesteryl esters could be determined in the samples. The concentrations of cholesteryl esters with conventional fatty acids amounted to 78–140 mg/100 g lipids (mean 97 mg/100 g lipids), while F-acid cholesteryl esters were present at 47–270 mg/100 g lipids (mean 130 mg/100 g lipids).

Keywords Furan fatty acids · Steryl esters · Cholesterol · GC/MS · Cod liver · Solid phase extraction

Abbreviations

7D5	7-(3,4-Dimethyl-5-pentylfuran-2-yl)-heptanoic acid
9D3	9-(3,4-Dimethyl-5-propylfuran-2-yl)-nonanoic acid
9D5	9-(3,4-Dimethyl-5-pentylfuran-2-yl)-nonanoic acid
9M5	9-(3-Methyl-5-pentylfuran-2-yl)-nonanoic acid
11D3	11-(3,4-Dimethyl-5-propylfuran-2-yl)-undecanoic acid
11D5	11-(3,4-Dimethyl-5-pentylfuran-2-yl)-undecanoic acid
11M3	11-(3-Methyl-5-propylfuran-2-yl)-undecanoic acid
11M5	11-(3-Methyl-5-pentylfuran-2-yl)-undecanoic acid
13D3	13-(3,4-Dimethyl-5-propylfuran-2-yl)-tridecanoic acid
13D5	13-(3,4-Dimethyl-5-pentylfuran-2-yl)-tridecanoic acid
CE	Cholesteryl ester
DHA	Docosahexaenoic acid (22:6n-3)
EPA	Eicosapentaenoic acid (20:5n-3)
F-acid	Furan fatty acid
F-CE	Furan fatty acid cholesteryl ester
FAME	Fatty acid methyl ester
GC/MS	Gas chromatography with mass spectrometry
HPLC	High performance liquid chromatography
NMR	Nuclear magnetic resonance
PUFA	Polyunsaturated fatty acid
SIM	Selected ion monitoring
SPE	Solid phase extraction
TIC	Total ion current

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Introduction

Furan fatty acids (F-acids) are a class of naturally occurring fatty acids characterized by a furan moiety within the acyl chain (Fig. 1). Typically, the 2-position of the furan moiety is substituted with a carboxyalkyl chain with an odd number of carbon atoms (typically 7–13) while the 5-position is substituted with a propyl or pentyl chain [1]. In addition, the hydrogen atoms in 3-position or 3- and 4-positions are usually substituted with methyl groups (Fig. 1) [1, 2]. F-acids are potent antioxidants and radical scavengers and thus have been classified as valuable minor compounds in foodstuff [1, 3, 4].

F-acids were first identified in the liver lipids of northern pike in 1974 [5]. Since then, they have been repeatedly detected in lipids of other fish, plants and milk fat [2, 6–10]. However, fish remains the type of food with the highest amounts of F-acids per gram fat [4]. Despite the comparably low concentrations of F-acids in fish filet (~3–30 mg/100 g [9]), F-acids represented the majority of the fatty acids in the cholesteryl ester fraction of liver lipids of different fish species [5, 11]. These results were obtained after separation of the cholesteryl ester fraction from bulk lipid classes followed by hydrolysis and determination of the fatty acids as methyl esters [5, 11]. Schödel and Spiteller reported that transesterification procedures may generate artifacts in the form of unsaturated F-acids with a double bond in the carbon chains [12]. For this reason it seemed important to study the presence of esters of F-acids and other fatty acids in the intact cholesteryl ester fraction.

Cholesteryl esters (CEs) can be analyzed by either high-performance liquid chromatography (HPLC) or gas chromatography (GC). Gas chromatography was reported to offer better resolution, but due to the low volatility of intact CEs, high temperatures are necessary [13, 14]. Analysis of F-acids (usually as methyl esters), on the other hand, is exclusively performed by gas chromatography with flame ionization detection (GC/FID) or coupled to mass spectrometry (GC/MS) [2, 7–10]. Accordingly, data about the elution order and GC/MS spectra are available, which is not the case for HPLC.

The goal of our study was to develop a GC/MS method suited for the identification and quantification of fatty acid cholesteryl esters in fish lipids. Due to the low contribution of the CEs to the total fish lipids, an enrichment step had to be carried out prior to the GC/MS measurement. Of the different methods suggested for this purpose (e.g., thin layer chromatography, column chromatography or high-performance liquid chromatography [13, 15–17]), we chose column chromatography. We developed a convenient solid-phase extraction (SPE) method on silica gel and fractionated the fish lipids into (1) hydrocarbons and (2) CEs, thus separating the CEs from the far more abundant triacylglycerides, which eluted later. The CE fraction was analyzed by means of GC/MS to study both gas chromatographic behavior and the GC/MS spectra of intact F-acid cholesteryl esters (F-CEs). With information about retention time and fragmentation of the F-CE, GC/MS in the single ion monitoring (SIM) mode was used for a thorough analysis of the samples and quantification of individual F-CE.

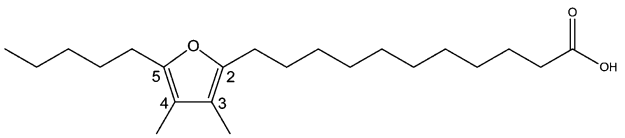

Fig. 1 Chemical structure of 11-(3,4-dimethyl-5-pentylfuran-2-yl)-undecanoic acid (11D5)

Materials and Methods

Nomenclature

Since the full chemical names of F-acids are long and complicated, several abbreviation terms have been suggested [2, 18, 19]. In this article, we use the abbreviated "number-letter-number" codes suggested by Vetter et al. [2]. The first number represents the length of the carboxyalkyl chain, the letter in the middle stands for the methyl substituents in the 3 and 4 positions (i.e., "M" for methyl, "D" for dimethyl- or "F" for non-substituted F-acids), and the length of the alkyl chain is denoted by the last number. Accordingly, the F-acid 11-(3,4-dimethyl-5-pentylfuran-2-yl)-undecanoic acid was abbreviated as 11D5 (Fig. 1).

Chemicals and Samples

n-Hexane and methanol (both HPLC grade) were from Th. Geyer (Renningen, Germany); ethyl acetate (puriss., >99.5 %), pentadecanoic acid (99 %), α-linolenic acid (80 %, GC), oleic acid (>99 %) and silica gel (silica gel 60, for column chromatography) were from Sigma-Aldrich (Steinheim, Germany). Palmitic acid (>98 %) was from Riedel-de Haën (Seelze, Germany). Helium (purity 99.9990 %) was from Westfalen (Münster, Germany). Thionyl chloride (p.s.) and cholesterol (puriss.) were from Merck (Darmstadt, Germany), toluene (p.s., >99.5 %) was from Carl Roth (Karlsruhe, Germany), and α-cholestane (98 %) was from Acros Organics (Geel, Belgium). Concentrated sulfuric acid was from BASF (Ludwigshafen, Germany). Individual cod livers were taken from cans bought in retail stores in Stuttgart, Germany, in

April 2014. According to the nutrition fact label, the cods (*Gadus Morhua*) were caught in the Northeast Atlantic Ocean, i.e., Major Fishing Area 27 (Food and Agriculture Organization of the United Nations, FAO 27; http://www.fao.org/fishery/area/Area27/en). The fish oil sample was from fish oil capsules (750 mg) bought in a store in Stuttgart, Germany.

Lipid Extraction of the Cod Liver Samples

From each can two cod liver samples (about 20 g) were placed in a 400-ml beaker glass, and 200 ml of *n*-hexane was added. The samples were crushed using an Ultraturax T25 (IKA, Staufen, Germany) and subjected to ultrasonication for 10 min. The hexane-liver suspension was filtered and the residue washed twice with 50 ml of *n*-hexane. The combined organic phases were condensed by means of a rotary evaporator. The residue was transferred into an amber glass vial and stored at 5 °C in a refrigerator until further analysis.

Synthesis of Cholesteryl Esters

The recovery standard pentadecanoic acid cholesteryl ester (hereafter referred to as 15:0-CE) was prepared according to Lusby et al. [20] with slight modifications. Pentadecanoic acid (121 mg, 0.5 mmol) was placed in a 50-ml flask and dissolved in 10 ml of toluene. About 120 µl thionyl chloride was added, and the solution was refluxed for 4 h to obtain the acid chloride. Afterwards, the remaining thionyl chloride and parts of the toluene were removed under reduced pressure. To the residue (about 2 ml), 153 mg cholesterol (0.4 mmol) dissolved in 10 ml toluene was added, and the solution was stirred overnight at room temperature. The solvent was removed on a rotary evaporator, and the residue was re-dissolved in 4 ml *n*-hexane. This solution was purified by SPE. For this purpose, 15 g of silica gel deactivated with 20 % water (w/w) was given into a glass column (i.d. 2.5 cm) and pre-conditioned with *n*-hexane. After 50 ml of *n*-hexane (fraction 1), three 50-ml fractions (fractions 2–4) were collected with *n*-hexane/ethyl acetate (99:1, v/v) as eluent. Fractions 3 and 4 yielded 154.3 and 64.8 mg of 15:0-CE. The purity in both fractions was >99 % according to GC/MS and NMR spectroscopy analysis.

The syntheses of cholesteryl palmitate, oleate and α-linolenate (18:3n-3-CE) were performed analogously. However, in the case of 18:3n-3-CE, the fatty acid chloride was prepared by the reaction with oxalyl chloride as described by Bauer (1946), with the exception that toluene was used as solvent instead of benzene [21]. All subsequent steps were carried out as described in the synthesis of 15:0-CE.

Solid-Phase Extraction (SPE) of Cod Liver Lipid Extracts

The CE fraction was isolated by SPE on silica gel. For this purpose, 5 g of silica deactivated with 20 % water (w/w) was loaded onto a 1-cm i.d. glass column, and the chromatographic system was conditioned with *n*-hexane. Sample solutions (80–120 mg of the crude *n*-hexane extract in 0.5 ml of *n*-hexane) were spiked with 39 µg of the internal standard 15:0-CE and placed on the pre-conditioned column. Elution started with 30 ml *n*-hexane (fraction 1) followed by 40 ml of *n*-hexane/ethyl acetate 99:1, v/v (fraction 2). All fractions were collected in amber pear-shaped glass flasks. CEs were eluted into fraction 2. The solvent was removed by rotary evaporation; the residue was re-dissolved in 1 ml *n*-hexane and transferred into a 1.5-ml screwcap vial. This solution was diluted to a final concentration of about 50–100 µg/ml CE and subjected to GC/MS analysis after addition of the second internal standard α-cholestane (3 µg/ml), which was used to even out instrumental variations from injection to injection.

Conversion of Fatty Acid Cholesteryl Esters (CEs) into Fatty Acid Methyl Esters (FAMEs)

For the determination of the fatty acid composition of the CE fraction, the fatty acids esterified to cholesterol of one sample were transferred into methyl esters (FAMEs). An aliquot of fraction 2 ("Solid-Phase Extraction (SPE) of Cod Liver Lipid Extracts") corresponding to about 0.2 mg CE was placed in a 6-ml test tube, and the solvent was evaporated with a gentle stream of nitrogen. Then, 2 ml of methanol with 1 % sulfuric acid (prepared from 0.5 ml conc. sulfuric acid in 49.5 ml methanol) was added, and the test tube was tightly sealed and heated to 80 °C for 1 h. After cooling to room temperature, 1 ml of deionized water and 2 ml of *n*-hexane were added, and the tube was shaken vigorously. After phase separation, the organic phase was transferred into a 1.5-ml vial and subjected to GC/MS analysis.

Gas Chromatography with Mass Spectrometry (GC/MS)

CEs were analyzed on a HP 6890 GC/5973 MSD system equipped with a cool-on-column inlet (Hewlett-Packard/Agilent, Waldbronn, Germany) and a pre-column (2 m, 0.53 mm i.d., deactivated with 1,3-diphenyl-1,1,3,3-tetramethyldisilazane, BGB Analytics, Boeckten, Switzerland). The pre-column was connected by a press fit to a 15-m, 0.25-mm i.d. capillary column coated with 0.1-µm film thickness dimethyl polysiloxane (ZB-1, Phenomenex, Aschaffenburg, Germany). Injections (1 µl) were made with a 7683 autosampler system (Hewlett-Packard/Agilent,

Table 1 Chemical names and abbreviations according to Vetter et al. [2] of F-acids, which could be identified as cholesteryl esters by GC/MS in the full scan mode

Chemical name of the F-acid	Abbreviation	Methyl esters		Cholesteryl esters[a]				
		M^+	Base peak	M^+	(*a*)[b]	(*b*)[b]	(*c*)[b]	(*d*)[b]
9-(3,4-Dimethyl-5-propylfuran-2-yl)-nonanoic acid	9D3	308	151	662 (9)	293 (100)	151 (76)	265[c]	277[c]
9-(3,4-Dimethyl-5-pentylfuran-2-yl)-nonanoic acid	9D5	336	179	690 (16)	321 (100)	179 (51)	265[c]	305[c]
11-(3-Methyl-5-pentylfuran-2-yl)-undecanoic acid	11M5	350	165	704 (4)	335 (100)	165 (70)	279[c]	319[c]
11-(3,4-Dimethyl-5-propylfuran-2-yl)-undecanoic acid	11D3	336	151	690 (9)	321 (100)	151 (93)	293 (21)	305 (7)
11-(3,4-Dimethyl-5-pentylfuran-2-yl)-undecanoic acid	11D5	364	179	718 (10)	349 (100)	179 (84)	293 (24)	333 (7)

Relative intensities of the fragments and molecular ions of the cholesteryl esters are listed in parentheses

[a] *m/z* values were rounded to unity mass resolution for better readability

[b] Fragmentation pathways according to Fig. 2b

[c] The relative intensity was too low to be properly determined

Waldbronn, Germany). Helium was used as carrier gas with a flow rate of 1.2 ml/min. The GC oven was programmed as follows: After 1 min at 55 °C, the temperature was raised at 30 °C/min to 250 °C, then at 7 °C/min to 320 °C and then at 3 °C/min to 350 °C. This final temperature was held for 5 min leading to a run duration of 32.5 min. The temperatures of the transfer line, ion source and quadrupole were set at 350, 230 and 150 °C, respectively. The temperature of the injector port was set to track the oven temperature (i.e., 55 °C at the beginning of the run). In the GC/MS full-scan mode, data were collected after a solvent delay of 6 min from *m/z* 50–800. In the selected ion monitoring (SIM) mode, three time windows were used. The ions representing *m/z* 137.1, 151.1, 165.1, 179.1 and 368.3 were recorded throughout the run. In addition, *m/z* 217.1, 357.3, 372.3, 279.2 and 293.2 were recorded from 6 to 10 min (time window 1), *m/z* 293.2, 307.2, 321.2, 335.2 and 349.2 from 10 to 21 min (time window 2), and *m/z* 321.2, 335.2, 349.2, 363.3 and 377.3 from 21 to 32 min (time window 3). The dwell time was set to 45 ms.

FAMEs were analyzed on the same instrument except for using a split/splitless injector and a HP-5MS column (5 %-phenyl, 95 %-methyl polysiloxane, 30-m, 0.25-mm i.d., 0.25-µm film thickness, Agilent, Waldbronn, Germany). Helium was used as carrier gas at 1.0 ml/min. The GC oven was programmed as follows: After 1 min at 60 °C, the temperature was raised at 13 °C/min to 180 °C, then at 3 °C/min to 250 °C and then at 20 °C/min to 300 °C. This final temperature was held for 5 min. The temperatures of the injector, transfer line, ion source and quadrupole were set to 250, 280, 230 and 150 °C, respectively. After a solvent delay of 7 min, data were recorded from *m/z* 50–650.

Quantification of Cholesteryl Esters

Quantification of individual CEs was performed by GC/MS-SIM. Conventional CEs were quantified using *m/z* 368.3, and F-CEs were quantified using the base peak, i.e., *m/z* 321.2 for 11D3-CE and *m/z* 349.2 for 11D5-CE [see fragmentation (a), Table 1]. The contribution of *m/z* 368.3 to the full-scan mass spectrum of four conventional CEs was on average 11.1 ± 1.0 % (Supplementary Material, Table S1). The contribution of *m/z* 321.2 to 11D3-CE and *m/z* 349.2 to 11D5-CE amounted to 7.5 ± 0.5 and 8.9 ± 0.6 % (average 8.2 ± 0.9 %) (Supplementary Material, Table S2). The lower contribution of the SIM ion of F-acids was taken into account by correcting the peak area with the conversion factor of 11.1/8.2 = 1.354. Since no authentic F-CEs were available as recovery standards, the recovery of F-CEs in the individual samples could not be determined accurately. Instead, it was estimated indirectly by re-analysis of the CE fraction by a second SPE procedure ("Quality Control"). This procedure verified that the F-CEs could be analyzed with the present method. However, the recovery rate of F-CE determined by the second SPE was not taken into account for the calculation of the contribution of F-CEs. Similarly, all peaks of conventional CEs were only corrected with the recovery of 15:0-CE.

Quality Control

Recovery Tests of the SPE Procedure

In a first step, we determined the recovery rate of 15:0-CE and 18:3n-3-CE standards ($n = 3$) by application of a solution containing about 40 µg of each compound to SPE. The recovery of the major F-CEs was determined by subjecting an aliquot of the CE fraction 2 (in which they had been determined) to a second SPE ($n = 4$, Supplementary Materials, Table S3). The recovery rate of cholesteryl esters of monounsaturated and polyunsaturated fatty acids was determined by the analysis of a fish oil capsule in the same way (re-analysis of CE fraction 2 by SPE). Here, the recovery of the individual fatty acids was determined after

transesterification as FAME relative to 15:0 methyl ester (Supplementary Materials Table S4).

Reproducibility of the Lipid Extraction and SPE Procedure

The reproducibility of the extraction of the raw lipids from the cod liver was checked by means of one cod liver sample that was divided into two parts. Both sample aliquots were extracted separately ("Lipid Extraction of the Cod Liver Samples"), and the CE fractions were isolated by SPE and analyzed by GC/MS. Furthermore, five aliquots from the same lipid extract were subjected to SPE ("Solid Phase Extraction (SPE) of Cod Liver Lipid Extracts"), and the composition of CE fraction 2 was determined by GC/MS (Supplementary Materials Table S5 + S6).

Storage Stability of F-CEs

Solutions of two isolated CE fractions were reanalyzed by GC/MS after being stored for almost 4 months at 4 °C in the refrigerator, and the compositions of the CE fraction and levels of F-CEs were compared to the original analyses.

Results and Discussion

Isolation of the Fatty Acid Cholesteryl Ester (CE) Fraction from Fish Lipids

By means of SPE, CEs were eluted into fraction 2 and could be separated from the far more abundant triacylglycerides and free cholesterol, which eluted later. Fraction 1 contained hydrocarbons such as squalene. Fraction 2 could be analyzed for CEs without additional purification steps.

The performance of the SPE method was first checked by the fractionation of 15:0-CE and 18:3n-3-CE because both cholesteryl esters were available as reference standards ("Quality Control"). The recovery rates ($n = 3$) of 15:0-CE and 18:3n-3-CE in fraction 2 were 97 ± 5 and 64 ± 6 %, respectively. In the sample solutions, the recovery of 15:0-CE was 92 ± 6 % ($n = 8$). Due to a lack of authentic standards, the recovery rates of F-CEs were estimated indirectly by repeating the SPE with CE fraction 2 ("Solid Phase Extraction (SPE) of Cod Liver Lipid Extracts"). Afterwards, the abundances of the F-CE relative to the internal standard 15:0-CE of the first SPE were compared with the second SPE. We observed a recovery of 85 ± 6 % for 11D3-CE and 84 ± 5 % for 11D5-CE relative to 15:0-CE ($n = 4$). Corrected for the recovery of 15:0-CE (92 ± 6 %), the overall recovery for F-CEs was ~78 % (Supplementary Material, Table S2).

Since the contribution of PUFAs in the CE fraction of cod liver lipids was reported to be rather low [22], we used the cholesteryl ester fraction from a fish oil capsule for the determination of recovery of PUFA-CE. As described for F-CEs, the recovery rates for CEs with polyunsaturated fatty acids other than 18:3n-3 as well as important monounsaturated fatty acids were determined by repeated SPE of CE fraction 2. However, cholesteryl esters with fatty acids of the same chain length but different numbers of double bounds could not be separated on the nonpolar ZB-1 phase. For this reason, the cholesteryl ester fraction was transesterified to FAME ("Solid Phase Extraction (SPE) of Cod Liver Lipid Extracts", "Conversion of Fatty Acid Cholesteryl Esters (CEs) into Fatty Acid Methyl Esters (FAMEs)"). By this measure, the recovery rates of CEs with 20:5n-3 (EPA) and 22:6n-3 (DHA) relative to the internal standard (15:0) could be determined to be 67 ± 3 and 69 ± 4 %, respectively (Supplementary Material, Table S4). Accordingly, the recovery rates of EPA-CE and DHA-CE were comparable with those of 18:3n-3-CE (~70 %), while those of CEs with 16:1 and 18:1 isomers were the same as for the internal standard 15:0 because no changes in the relative abundances were observed.

To test the reproducibility of the lipid extraction, a cod liver sample was cut in half, and both parts were extracted and analyzed separately. The compositions of the CE fraction were almost identical (Supplementary material, Table S1). Also, when five aliquots from the same cod liver were subjected to SPE and analyzed, the compositions of the CE fractions were very well in accordance (Supplementary material, Table S2). Potential changes in the CE concentrations or pattern were investigated by re-analysis of a sample that had been stored for about 4 months at 4 °C in a refrigerator ("Quality Control"). The differences in the concentrations of F-CE were less than 5 %. Thus, the sample preparation method was suitable and used for the isolation of the CE fraction of cod liver samples.

Gas Chromatographic Separation of Fatty Acid Cholesteryl Esters (CEs) in Fish Lipids

In all samples, fraction 2 only contained CEs (Fig. 2a) and no esters with other sterols or long alcohols (wax esters). As expected from the literature, the nonpolar GC column (100 % dimethyl polysiloxane) used in this work separated CEs very well by the total carbon number of the fatty acid (retention time of C_{14} ester < C_{15} ester < C_{16} ester) [14]. However, CEs that differed in the degree of unsaturation (e.g., esters of stearic and oleic acid) could not be separated. Compared to this rather limited separation power, it was surprising that cholesteryl esters with isomeric F-acids (positional isomers) could be resolved under these conditions. In agreement with F-acid methyl esters, F-CEs eluted the earlier the longer the

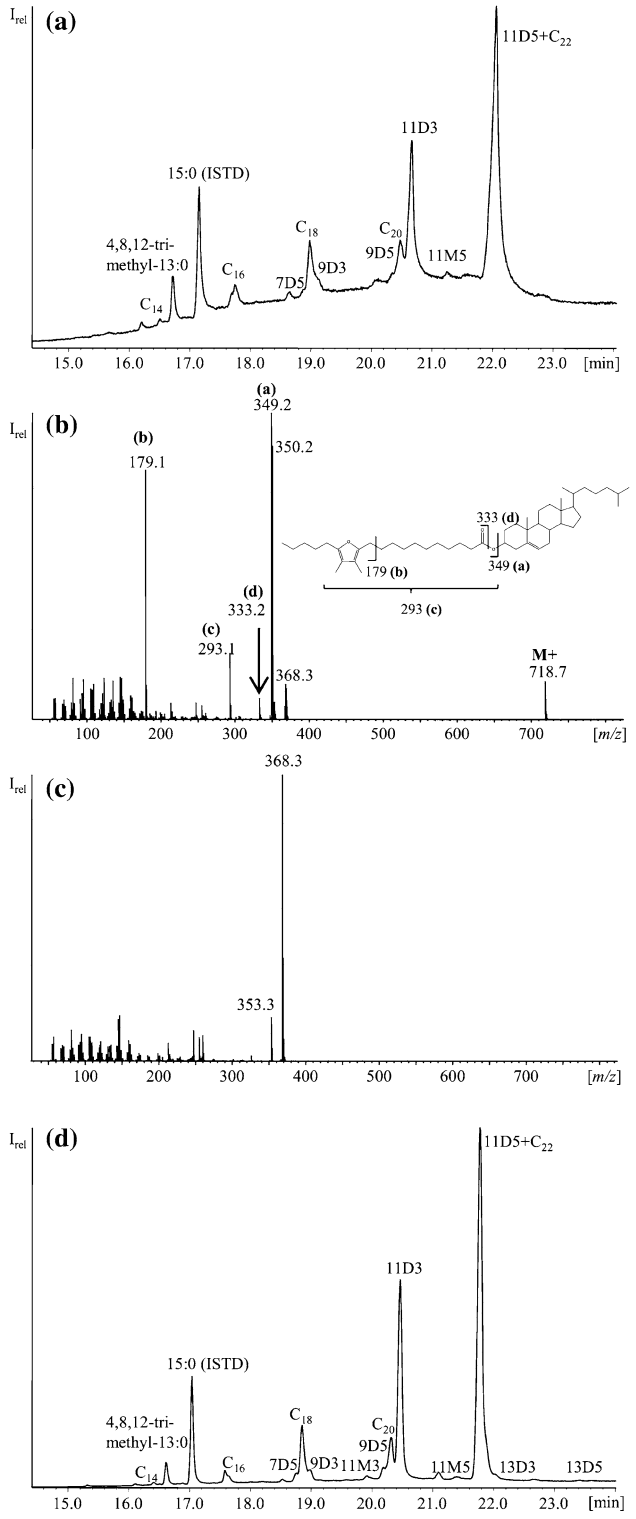

Fig. 2 **a** GC/MS full-scan chromatogram of the cholesteryl ester fraction of cod liver oil sample 4.1 and GC/MS spectra of **b** 11D5-CE and **c** 15:0-CE as well as **d** GC/MS-SIM chromatogram of the cholesteryl ester fraction of sample 4.1. All peaks in **a** and **d** are cholesteryl esters. The peak labeling refers to the fatty acid moiety

terminal alkyl chain was [2]. For this reason, 7D5-CE (9D5-CE) eluted before 9D3-CE (11D3-CE) and both pairs of isomers could be baseline separated from each other (Fig. 2a). F-CEs were eluted in the range of conventional CEs with the same carbon number despite the slightly higher molecular weight of F-acids. As a consequence, the retention time increased in the order 7D5-CE < C_{18}-CE < 9D3-CE, and those peaks were almost baseline separated. In the group of fatty acids with 20 carbons, 9D5-CE and C_{20}-CE could not be fully resolved, and both were eluted prior to 11D3-CE (Fig. 2a). Hence, this GC setup provided a good separation by the total carbon number and of positional isomers of the F-acid moiety, but coelutions of F-CEs with conventional CEs could not be avoided.

GC/MS Spectra of Furan Fatty Acid Cholesteryl Esters (F-CEs)

As noted in the literature, GC/MS spectra of conventional CEs provide only little structural information [20, 23]. The mass spectra were characterized by the base peak formally arising from the elimination of the fatty acid moiety ($[M\text{-}ROOH]^+$, *m/z* 368.3, Fig. 2c). The molecular ion and further fragment ions in the higher mass range were frequently not detected [20, 23].

On the contrary, GC/MS spectra of F-CEs provided substantial structural information. The molecular ion was generally visible with a relative abundance of 4–16 % of the base peak (Table 1). For instance, the molecular ion of 11D5-CE at *m/z* 718.7 was detected at a relative intensity of about 10 % of the base peak (Fig. 2b; Table 1). It is worth noting that the mass of the molecular ion (*m/z* 718.7) was almost one atom mass unit (u) higher than the nominal mass because of the mass defect caused by the 82 hydrogen atoms in the molecule (Fig. 2b). The base peak (*m/z* 349.2 in the case of 11D5-CE), which corresponded to $[ROO]^+$ (fragmentation (a) in Fig. 2b; Table 1), may be formed as an alternative to the α-cleavage of the ester [*m/z* 333.2, fragmentation (d) in Fig. 2b]. In addition, the McLafferty ion corresponding to $[ROOH]^+$ (*m/z* 350.2 in the case of 11D5-CE) was almost as prominent as the base peak (Fig. 2b). A further prominent fragment ion in the mass spectrum of 11D5-CE was detected at *m/z* 179.1. This fragment ion is well known from GC/MS spectra of F-acid methyl esters (Fig. 4b) [2, 18]. It arises from an allyl cleavage in the carboxyalkyl chain [fragmentation (b) in Fig. 2b; Table 1] [2]. Together with the molecular ion, this diagnostic fragment ion provided valuable information about the length of the alkyl chain and the number of methyl group(s) on the furan moiety (Table 1).

At lower relative intensity (about 15 % of the base peak), the corresponding ion formed by allyl cleavage in the alkyl chain was detected at *m/z* 293.1 [fragmentation (c) in Fig. 2b]. Interestingly, the fragment ion formally representing $[M\text{-}ROOH]^+$, which is the base peak in the GC/MS spectra of steryl esters of conventional fatty acids (i.e., *m/z* 368.3 for CEs), was of rather low abundance with about 10 % relative intensity (Fig. 2b, c). The fragment ion at *m/z* 333.2 in the mass spectrum of 11D5-CE and at *m/z* 305.2 in the mass spectrum of 11D3-CE (Table 1) (Supplementary material, Figure S1) was most likely formed by α-cleavage on the ester side of the carbonyl group [fragmentation (d) in Fig. 2b].

Aside from the molecular ion in the high mass range, GC/MS spectra of F-CEs and the corresponding F-acid methyl esters looked very similar (Figs. 2b, 4b; Table 1). The major difference originated from fragment ions, which included the intact ester moiety of F-acid methyl esters such as *m/z* 364 (M^+) and *m/z* 307 (allyl cleavage in the alkyl chain) because the corresponding ions in the mass spectra of F-CEs were found shifted at 14 u to lower mass (i.e., *m/z* 350 and *m/z* 293) (Table 1). Most importantly, conventional CEs and F-CEs could be distinguished by means of their respective GC/MS spectra.

GC/MS Determination of Cholesteryl Esters of Furan Fatty Acids in the Full Scan and Selected Ion Monitoring Mode

Five F-CEs could be identified, i.e., the cholesteryl esters of 9D3, 9D5, 11D3, 11M5 and 11D5, by GC/MS in the full scan mode (Table 1; Fig. 2a, supplementary material, Figure S1-S4). In order to achieve a higher sensitivity and selectivity, the CE fraction was additionally analyzed by GC/MS-SIM. For this purpose, the fragment ions representing fragmentations (a) and (b) (Fig. 2b) were selected along with *m/z* 368.3, which was present in all cholesteryl esters. Since the alkyl chain in F-acids is almost exclusively a propyl or pentyl chain [1], the number of methyl groups on the furan moiety and the alkyl chain of F-CE can be deduced with just four distinct ions formed by fragmentation (b) (arising from allyl cleavage in the carboxyalkyl chain, Fig. 2b): *m/z* 137 for "M3", *m/z* 151 for "D3", *m/z* 165 for "M5" and *m/z* 179 for "D5." As fragment ion (a) includes the entire F-acid structure, and fragment ion (b) contains the furan moiety including the alkyl chain, these two ions can be used for deducing the structure of the F-acid moiety.

The analysis of the CE fraction by GC/MS in the more sensitive SIM mode allowed us to detect another five F-CEs, i.e., 7D5-CE, 9M5-CE, 11M3-CE, 13D3-CE and 13D5-CE in addition to those already found in the full scan mode (Tables 1, 2; Fig. 2d). Alongside the sensitivity, the selectivity of the method was greatly increased by the SIM analysis and allowed the detection of F-CE even when these were coeluting with other, more abundant CEs.

This GC/MS-SIM method was also used to estimate the relative contributions of individual cholesteryl esters in the samples. CEs with conventional fatty acids were determined by means of the $[M\text{-}ROOH]^+$ fragment ion at *m/z* 368.3, while the base peak of F-CE (fragmentation (a), Fig. 2b) was selected for this purpose. In the next step, the contribution of these ions to the total ion chromatograms was calculated ("Quantification of Cholesteryl Esters"). In conventional CEs, *m/z* 368.3 contributed ~11.1 % to the total ion current (TIC), while the base peak of F-CE contributed ~8.2 % to the TIC for 11D5-CE and 11D3-C3 ($n = 3$). With these values, the peak areas from the SIM determination could be normalized. The contributions of all F-CEs were determined using the GC/MS-SIM response of 11D5-CE and 11D3-CE.

It has to be noted that the responses of other F-CEs might differ substantially from those studied by us, but since no authentic standard substances were available at this point and individual response factors for low-abundant F-CEs could not be determined accurately, the approach of a single response factor for all F-CEs was chosen.

As a further measure, the results of the GC/MS analyses of the CE composition of sample 1.1 in full-scan and SIM mode were compared to the fatty acid composition after transesterification ("Conversion of Fatty Acid Cholesteryl Esters (CEs) into Fatty Acid Methyl Esters (FAMEs)"). We noted a good agreement between the contributions obtained by the SIM method and the transesterified sample for the major compounds such as 11D3 and 11D5 or C_{18} fatty acids (Table 3). Only for CEs present at low abundance, higher deviations between the analysis of the intact esters and the transesterified sample were observed. Thus, the GC/MS-SIM method was used to analyze the composition of the CE fraction.

Composition of the Fatty Acid Cholesteryl Ester Fraction from Eight Cod Livers

The quantitative contributions of individual CEs to the total CE content were determined relative to 15:0-CE ("Quantification of Cholesteryl Esters"). The total CE concentrations in the lipid extracts ranged from 140–380 mg (mean 230 mg) per 100 g lipids. CEs with conventional fatty acids contributed 78–140 mg (mean 97 mg) per 100 g lipids, which represented 28–75 % (mean 47 %) of the total CEs. Likewise, F-CEs represented 25–72 % (mean 53 %) of total CEs [concentration range 47–270 mg (mean 130 mg) per 100 g lipids (Fig. 3)].

In all samples, 11D5-CE was the most abundant F-CE followed by 11D3-CE (Fig. 3). Both F-CEs contributed

Table 2 Fatty acid cholesteryl esters (CE) identified in the cod liver samples by GC/MS-SIM

Fatty acid moiety of the cholesteryl ester	Abbreviated	t_R[a]	Identified by m/z[b]
F-acids			
7-(3,4-Dimethyl-5-pentylfuran-2-yl)-heptanoic acid	7D5	18.82	179, 293, 368
9-(3,4-Dimethyl-5-propylfuran-2-yl)-nonanoic acid	9D3	19.06	151, 293, 368
9-(3-Methyl-5-pentylfuran-2-yl)-nonanoic acid	9M5	19.67	165, 307, 368
11-(3-Methyl-5-propylfuran-2-yl)-undecanoic acid	11M3	20.01	137, 307, 368
9-(3,4-Dimethyl-5-pentylfuran-2-yl)-nonanoic acid	9D5	20.26	179, 321, 368
11-(3,4-Dimethyl-5-propylfuran-2-yl)-undecanoic acid	11D3	20.55	151, 321, 368
11-(3-Methyl-5-pentylfuran-2-yl)-undecanoic acid	11M5	21.20	165, 335, 368
11-(3,4-Dimethyl-5-pentylfuran-2-yl)-undecanoic acid	11D5	21.88	179, 349, 368
13-(3,4-Dimethyl-5-propylfuran-2-yl)-tridecanoic acid	13D3	22.15	151, 349, 368
13-(3,4-Dimethyl-5-pentylfuran-2-yl)-tridecanoic acid	13D5	23.53	179, 377, 368
Conventional fatty acids			
C_{14}	–	16.48	t_R
4,8,12-Trimethyl-13:0	–	16.68	t_R, after transesterification[c]
15:0 (internal standard)	–	17.10	Standard substance
C_{16}	–	17.65	Standard substance, t_R
C_{18}	–	18.92	Standard substance, t_R
C_{20}	–	20.41	t_R
C_{22}	–	21.98	t_R

[a] GC retention time

[b] Rounded to full mass units

[c] Identified with an authentic spectrum from the AOCS lipid library [30]

Table 3 Relative contributions of cholesteryl esters by GC/MS in SIM and full scan mode compared to the fatty acid composition after transesterification (FAME)

Fatty acid/ester	FAME (%)	SIM (%)	Full scan (%)
C_{14}	0.8	0.3	0.2
4,8,12-Trimethyl-13:0	2.4	2.7	2.1
C_{16}	7.0	2.3	2.1
C_{18}	11.8	9.2	8.1
C_{20}	7.5	8.7	5.6
C_{22}	3.7	4.6	Coelution with 11D5
9D3	0.8	0.8	1.5
9D5	0.8	1.5	1.9
11D3	18.1	21.3	23.2
11M5	0.3	1.0	0.8
11D5	46.9	47.6	54.3[a]

[a] 11D5 + C_{22}

between 25 and 70 % to the total CEs, while residual F-CEs represented less than 5 % to the total CEs in the samples (Fig. 3). The remaining share originated from conventional CEs. Gunstone et al. [22] reported that F-acids contributed 10–83 % to the CEs of different fish with the predominance of 11D5 and 11D3 and minor contributions by 9D3, 9M5, 9D5 and 11M5. All these F-acids could also be detected in our samples as F-CEs. Moreover, we were able to identify low contributions of 7D5, 11M3, 13D3 and 13D5 in the CE fraction in our samples. Except for the minor compounds, the F-acid pattern obtained from the direct analysis of the CE fraction compared well to the literature data, which were obtained after conversion of the isolated CE fraction into FAME [11, 22]. Interestingly, individual cod livers taken from the same can partly showed distinct differences in the share of F-acids in the CE fraction. For instance, F-CEs contributed 25 vs. 55 % to the total CEs in samples 3.1 and 3.2, which represented individual livers taken from the same can. Likewise, the concentrations of F-CEs in the liver extracts varied widely from 47 to 270 mg/100 g lipids, although all cans contained livers originating from the same fishing area (FAO 27). The reasons for these individual differences remained unclear. The data indicate that larger sample numbers or larger sample pools are required for determining a typical concentration range of F-CEs in a given region.

Analysis of Fatty Acids after Transesterification

Conversion of an aliquot of fraction 2 that contained the CEs ("Conversion of Fatty Acid Cholesteryl Esters (CEs) into Fatty Acid Methyl Esters (FAMEs)") into FAMEs verified the presence of the F-acids in the investigated cod liver samples (Fig. 4a). In addition, the conventional fatty acids could be determined as well. Aside from F-acids,

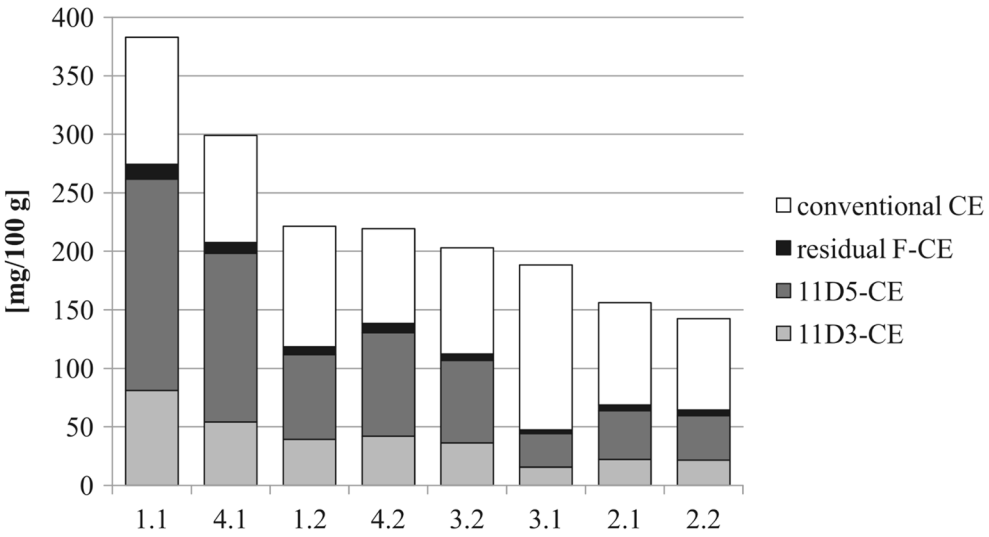

Fig. 3 Levels of cholesteryl esters of 11D5, 11D3, other F-acids and conventional fatty acids (mg/100 g lipids) in the eight cod liver oil samples analyzed

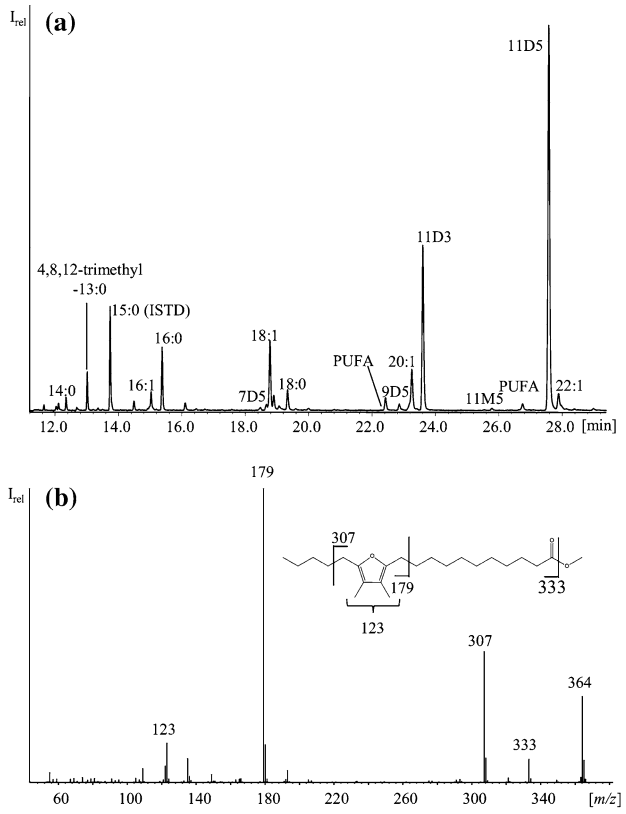

Fig. 4 **a** GC/MS full-scan chromatogram of the fatty acids in the cholesteryl ester fraction of a cod liver oil sample after conversion into methyl esters and **b** GC/MS spectrum of 11D5 methyl ester

the majority of the conventional fatty acids originated from monoenoic followed by saturated fatty acids. In contrast, PUFAs contributed <5 % to the fatty acids in the CE fraction (Fig. 4a), but this may be attributable to a loss of CEs with PUFAs during SPE ("Isolation of the Fatty Acid Cholesteryl Ester (CE) Fraction from Fish Lipids"). We also noted a minor contribution of 4,8,12-trimethyltridecanoic acid in the CE fraction (Fig. 4a). Gunstone et al. [22] reported similar low PUFA and high F-acid contributions in the CE fraction of liver lipids from starved fish. These findings are remarkable, since studies have suggested that CEs with PUFAs are susceptible to oxidation and could promote the formation of cholesterol oxidation products, which are thought to contribute to the formation of atherosclerotic plaque [24–26]. Accordingly, the high abundance of radical scavenging F-acids [1] in the cholesteryl ester fraction could be a protective measure to prevent cholesterol oxidation.

Conclusions

The cholesteryl ester fraction of fish lipids could be isolated from a lipid extract, and the intact cholesteryl esters were analyzed by means of GC/MS-SIM. Although a good GC separation of F-CEs could be obtained, some coelutions of F-CEs with conventional CEs (e.g., 11D5-CE with 22:1-CE) could not be avoided. Since no authentic reference standards were available, identification was based on GC/MS spectra only. Similarly, quantification was performed using the internal standard 15:0-CE. Future improvements could be the search for alternative GC stationary phases, which provide a better resolution of conventional CEs. A further attempt to improve the separation of intact CEs by GC/MS may be achieved by switching from the carrier gas helium to hydrogen [27, 28]. However, the use of hydrogen

requires much attention regarding leak tightness and potential reactions of analytes with the carrier gas [29], which limits the feasibility for routine application. Given the demonstrated relevance of F-CEs in fish oil, our methods along with the alternatives just mentioned may be used for the determination of F-acids in the steryl ester fraction of other sample matrices such as plant oils (olive oil, soybean oil) that contain different phytosteryl esters.

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