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Quantification of Branched Chain Fatty Acids in Polar and Neutral Lipids of Cheese and Fish Samples

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Branched chain fatty acids (*iso*- and *anteiso*-fatty acids) are common minor compounds of the lipids found in dairy products and fish (<1−3%) and major fatty acids of Gram-positive bacteria. Their presence in food has been associated with bacterial sources. Bacterial lipids usually consist of polar lipids and virtually no triacylglycerides while food lipids are predominantly composed of triacylglycerides. In this study, we examined the difference in the *iso*- or *anteiso*-fatty acid content and composition of neutral and polar lipids in fish and cheese samples. Neutral lipids (triacylglycerides) were separated from the polar lipids (phospholipids) by means of solid-phase extraction (SPE). Deuterium-labeled internal standards were used to verify the successful performance of the accelerated solvent extraction and particularly the SPE. The separated lipid fractions were transformed into their corresponding fatty acid methyl esters, and the concentrations of seven *iso*- and *anteiso*-fatty acids were determined by means of gas chromatography coupled to electron ionization mass spectrometry operated in the selected ion monitoring mode (GC/EI-MS-SIM). No coelutions of branched chain fatty acids with other fatty acids were obtained on the medium polar column used for quantification. The branched chain fatty acid content of 17 cheese and 7 fish samples ranged between 0.2% and 1.9% in polar lipids and between 0.1% and 1.7% in neutral lipids. The concentration of total branched chain fatty acids in fish was 2−10 times lower than that found in cheeses, and the relative distribution of *iso*-17:0 and *iso*-15:0 increased compared to their *anteiso* homologues. While branched chain fatty acids in polar lipids of cheese constituted only ∼1% of the content in total lipids, their contribution in fish was significantly higher (6% to >30%).

KEYWORDS: GC/MS; selected ion monitoring; methyl-branched fatty acids; *anteiso*-fatty acids; *iso*-fatty acids; fish; dairy products; phospholipids

INTRODUCTION

iso- and *anteiso*-fatty acids bear a methyl substituent on the penultimate ($n-1$) (*i*FAs) or the antepenultimate ($n-2$) (*a*FAs) carbon of the otherwise straight chain alkyl chain. These branched chain fatty acids (BCFAs) are characterized as anticancer agents against human tumor cells, similar to conjugated linolenic acid (*1*, *2*). In addition, they are able to regulate the membrane fluidity of bacterial membranes when exposed to low temperatures (*3*). BCFAs are commonly distributed in the environment and in foodstuffs (particularly in dairy products and fish oils (*4*−*9*)), albeit their concentrations in food are generally low (1−3% of the total lipid content). However, they dominate in the lipid bilayer of various Gram-positive bacteria (*10*). In the latter, FAs are mainly found in phospholipids (i.e., phosphatidylcholine, -serine, -ethanolamine, and -inositol (*11*)), while FAs in food are mainly stored in the nonpolar triacylglycerols.

Major BCFAs in food include 12-methyltridecanoic acid (*i*14:0), 13-methyltetradecanoic acid (*i*15:0), 12-methyltetradecanoic acid (*a*15:0), 14-methylpentadecanoic acid, (*i*16:0), 15-methylhexadecanoic acid (*i*17:0), 14-methylhexadecanoic acid (*a*17:0), and 16-methylheptadecanoic acid (*i*18:0). These seven BCFAs represent between 65% and 90% of the fatty acids in *Staphylococcus aureus* (*12*) and *Listeria monocytogenes* (*13*), respectively; this occurrence has been determined to indicate that BCFAs in foodstuffs are linked to bacterial sources (*14*, *15*). To date, data on the distribution of BCFAs in the neutral lipids (NLs) and polar lipids (PLs) of food are scarcely available. The determination of low amounts of BCFAs in the presence of 50 or more fatty acids poses a formidable challenge, as no suitable method for routine analysis was available.

The aim of this study was to develop a method for the routine analysis of BCFAs in both the NLs and PLs of food lipids. For this purpose, we separated the NLs and PLs using solid-phase extraction (SPE) (*16*) and added suitable internal standards to control the separation of both lipid classes. After transesterification, the resulting fatty acid methyl esters (FAMEs) in the two lipid fractions were separately quantified using gas chromatography with electron ionization mass spectrometry operated in the selected ion monitoring mode (GC/EI-MS-SIM). A medium polar GC column was used in the final method and applied to a range of cheese and fish samples.

MATERIALS AND METHODS

Chemicals and Standards. Ethyl acetate (purest; Acros Organics, Geel, Belgium) and cyclohexane (purest; VWR, Darmstadt, Germany)

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were combined (1:1 v/v) and distilled to obtain the azeotropic mixture (54:46 v/v). Ethanol, *n*-hexane, methanol (all HPLC gradient grade), and silica gel 60 (particle size 0.063−0.2 mm, 70−230 mesh) as well as ethanolic BF_3 (∼10%) were ordered from Fluka (Taufkirchen, Germany), while methanolic BF_3 (∼13%) was purchased from Riedel-de-Haën (Taufkirchen, Germany). The deuterated lipid standard 1,2-di(stearoyl-d_{35})-*sn*-glycero-3-phosphatidylcholine (DSPC-d_{70}), the 37-component FAME mix, and the individual standards of *anteiso*- and *iso*-fatty acids were purchased from Larodan (Malmö, Sweden). Tri(palmitoyl-d_{31})-glycerol (PPP-d_{93}) was purchased from Sigma-Aldrich (Taufkirchen, Germany).

FAMEs were prepared using the boron trifluoride method as recently reported (*17*). Accordingly, stearic acid ethyl ester (18:0 EE), used as an internal standard (added to sample and standard solutions in order to adjust for variations in the GC/MS peak areas from injection to injection), was prepared from ethanolic solutions of 0.5 M KOH and ethanolic BF_3 (*17*). Methyl esters of *i*14:0, *a*14:0, *i*15:0, *a*15:0, *i*16:0, *a*16:0, *i*17:0, *a*17:0, *i*18:0, and *a*18:0 were separately prepared, and aliquots of each BCFA were combined and filled up with *n*-hexane to give 80−100 ng/μL for each FAME (BCFA-ME standard mix).

Food Samples Analyzed. The following cheese samples were analyzed (product information is given in parentheses): gouda (pasteurized milk cheese, 48% lipids), emmental (raw milk cheese, 45% lipids), butter cheese (pasteurized milk cheese, 45% lipids), organic camembert (raw milk cheese, 45% lipids), organic brie (raw milk cheese, 60% lipids), organic alpine cheese (raw milk cheese, 62% lipids), organic curd cheese (pasteurized milk cheese, 40% lipids), cow mozzarella (pasteurized milk cheese, 45% lipids), buffalo mozzarella (pasteurized buffalo milk cheese, 50% lipids), cow feta cheese (pasteurized milk cheese, 45% lipids), ewe feta cheese (pasteurized ewe milk cheese, 45% lipids), goat cheese (pasteurized goat milk cheese, 50% lipids), goat cream cheese (pasteurized goat milk cheese, 45% lipids), Bavaria blue (pasteurized milk, 68% lipids), limburger (pasteurized milk cheese, 40% lipids), romadur cheese (pasteurized milk cheese, 40% lipids), and roquefort cheese (raw ewe milk, 52% lipids).

The following fish samples were also analyzed: tuna (fillet without skin, 6% lipids in dry matter), salmon (fillet without skin, 33% lipids in dry matter), pollack (fillet without skin, 4.2% lipids in dry matter), rainbow trout (fillet with skin, 19% lipids in dry matter), gilthead seabream (fillet with skin, 44% lipids in dry matter), sea bass (fillet with skin, 36% lipids in dry matter), and brown trout (fillet with skin, 16% fat in dry matter).

Gas Chromatography in Combination with Electron Ionization Mass Spectrometry (GC/EI-MS). An HP GCD Plus system (Hewlett-Packard, Waldbronn, Germany) was used to perform GC/EI-MS analyses. One microliter of standard/sample solution was injected in splitless mode (split opened after 2 min) via an HP 6890 autosampler (Hewlett-Packard). The injector and transfer line temperatures were set at 250 and 280 °C, respectively. Helium (purity 99.9990%; Sauerstoffwerke, Friedrichshafen, Germany) was used as the carrier gas at a constant flow rate of 0.9 mL/min. The ionization energy was 70 eV, and the temperature of the ion source was set at 165 °C. Mass spectrometric data were collected after a run time of 8 min. For GC/EI-MS in the selected ion monitoring (SIM) mode, eight fragment ions (*m*/*z* 74, *m*/*z* 87, *m*/*z* 81, and *m*/*z* 79 for FAMEs, *m*/*z* 101 and *m*/*z* 88 for fatty acids ethyl esters (FAEEs) (*17*, *18*), and *m*/*z* 77 and *m*/*z* 91 for d_{31}-16:0 and d_{35}-18:0 methyl esters) were recorded throughout the run. Two capillary columns (50 m, 0.25 mm internal diameter, 0.20 μm film thickness), both from Varian/Chrompack, Middelburg, The Netherlands, were investigated with the following optimized GC oven temperature programs. For the CP-Sil 19 (14% cyanopropyl, 86% dimethyl polysiloxane): started at 60 °C (hold time 2 min), ramped at 10 °C/min to 190 °C, followed by 3 °C/min to 223 °C (hold time 3 min), and finally ramped at 3 °C/min to 280 °C (hold 12 min). For the CP-Sil 88 (100% bis(cyanopropyl) polysiloxane): started at 60 °C (hold time 1 min) and ramped at 3 °C/min to 230 °C (hold time 7 min) (*19*).

Extraction of Food Samples. Lipids of cheese and fish samples (see section Food Samples Analyzed) were extracted by means of accelerated solvent extraction (ASE 200; Dionex, Idstein, Germany) as recently described (*16*, *20*). In brief, ∼1 g of freeze-dried sample was loaded into an 11 mL extraction cell. Headspace was filled with approximately 2 g of diatomaceous earth (isolute-HM-N; Separtis, Grenzlach-Wyhlen, Germany). Each sample was then extracted with three portions of 40 mL of ethyl acetate/cyclohexane (54:46 v/v) as well as with two portions of 40 mL of methanol/ethyl acetate (1:1 v/v). The extracts were then combined, concentrated in a rotary evaporator (180 mbar, 30 °C water bath temperature), and adjusted to a defined volume (10−25 mL). Aliquots were evaporated to dryness and gravimetrically analyzed to verify the lipid content provided by each sample's respective label. Quantitative polar lipid recovery rates for ASE were determined by spiking several cheese and fish samples with DSPC-d_{70} (addition of 110−150 μg of DSPC-d_{70} to 0.5−1.0 g of freeze-dried food sample; see also below).

Solid-Phase Extraction (SPE) of Food Samples. The dry lipid extracts were redissolved in a defined volume of cyclohexane/ethyl acetate/MeOH (9:9:2 v/v/v) (*16*) to obtain a final concentration of 35−50 mg/mL. One hundred microliters of each solution was spiked with the internal standards (IS) used to monitor the recovery (15 μg of DSPC-d_{70} and 0.2 mg of PPP-d_{93}). Each solution was applied to the SPE column containing ∼350 mg of deactivated silica gel preconditioned with cyclohexane/ethyl acetate (1:1 v/v) (*16*). NLs were eluted with 23 mL of cyclohexane/ethyl acetate (1:1 v/v), and thereafter, PLs were eluted with (i) 10 mL of ethyl acetate/methanol (1:1 v/v), (ii) 10 mL of methanol, and finally (iii) 10 mL methanol/H_2O (98:2 v/v) into one flask (*16*). The separated NLs and the PLs were concentrated by rotary evaporation (30 °C, 180 mbar) to about 1 mL, transferred into derivatization tubes, and finally evaporated to dryness under a gentle stream of nitrogen (35 °C). The dry lipid fractions were finally converted into their FAMEs (see above), and the volume of the FAME containing PLs and NLs was adjusted with *n*-hexane to 1 and 2 mL, respectively.

Quantification Procedure. Ten microliters of the BCFA-ME standard mix (see above and ref *17*) and 10 μL of 18:0 EE (c = 100 ng/μL) were diluted to 100 μL (the final concentrations were 8−10 ng/μL for MEs and 10 ng/μL for 18:0 EE). Accordingly, 90 μL of a 1:10 dilution of a 37 component FAME mix was spiked with 10 μL of 18:0 EE. The concentrations of the individual 37 FAMEs (C_4−C_{24}) were 18, 36, or 54 ng/μL. For the quantification of BCFAs in food samples, 10 μL of 18:0 EE was added to 90 μL of the FAME-containing PL fraction, while 25 μL of 18:0 EE was added to 10 μL of the FAME- containing NL fraction. The volume of each fraction was increased to 100 μL with *n*-hexane. The PL content was estimated by division of the total amount of fatty acids from this fraction with the so-called "dipalmitoylphosphatidylcholine equivalent" ($DPPC_{eq}$ = 0.696) as recently described (*16*) (Tables S1 and S2, Supporting Information). Random samples were selected for repeated fractionation of lipid classes and BCFA determination, in order to assess the reproducibility of the applied method. Standard deviations of BCFAs were 2−10% in the NLs and 3−21% in the PLs.

RESULTS AND DISCUSSION

Selection of a GC Column for the Noninterfered Determination of *iso*- and *anteiso*-Fatty Acids. Highly polar columns coated with up to 100% *bis*(cyanopropyl) polysiloxanes (CP-Sil 88) are optimal for FAME analyses because they provide excellent separations of positional and geometrical isomers of unsaturated FAMEs (*21*). However, coelutions of monoenoic and branched chain FAMEs (e.g., *i*/*a*17:0 with 16:1 isomers and *a*15:0 with 14:1 isomers) were unavoidable even on 100 m columns (*22*−*24*). For instance, the variety of 16:1 isomers (ranging from the first eluting *trans* isomer 16:1(4*tr*) to the last eluting *cis* isomer 16(14)) covered a wide elution range (*25*), and thus the BCFAs could not be fully resolved regardless of the GC oven temperature program used (*19*). Such overlaps hamper the correct quantification of BCFAs in food samples. Recently, it was brought to mind again that weakly polar GC columns are also suitable for the determination of saturated and unsaturated straight chain fatty acids (*26*).

Being primarily interested in BCFAs, we then tested whether BCFAs could be eluted free of interferences from the semipolar CP-Sil 19 column. This column was selected because unsaturated fatty acids are not retarded to the same degree as on polar columns. In fact, BCFAs were fully resolved from monounsaturated fatty acids (MUFAs) on the semipolar CP-Sil 19 (**Figure 1**),

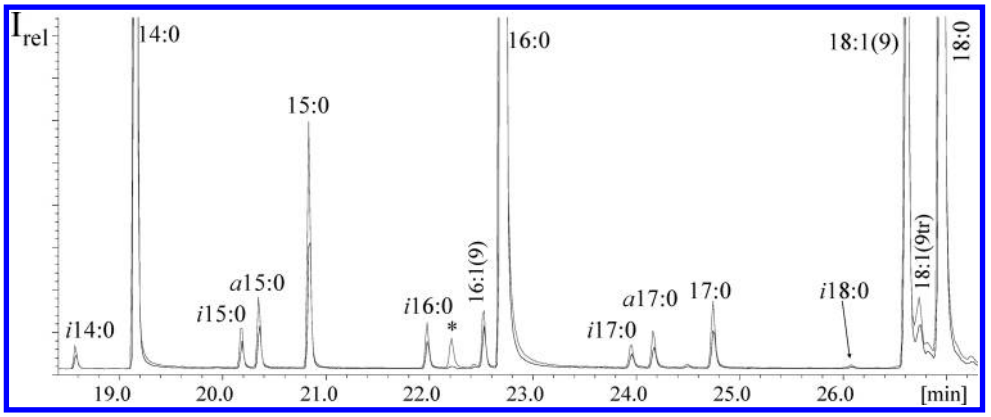

Figure 1. GC/MS ion chromatogram of a cheese sample obtained on CP-Sil 19 (*m/z* 74 (black line) and *m/z* 87 (gray line)). *, internal standard.

and the resolution of C_{18} and C_{20} isomers was also adequate (data not shown). Moreover, coelutions of phytanic acid and pristanic acid, which are both found in fish and dairy products (*6*), were not observed. Phytanic acid eluted shortly before *i*18:0 and pristanic acid before *i*16:0 from the CP-Sil 19. However, MUFAs shorter than C_{16} were not separated from their saturated analogues. This disadvantage was deemed acceptable given the unadulterated elution of BCFAs. In addition, the CP-Sil 19 phase was less sensitive to oxygen and more tolerant against high oven temperatures (*27*) compared to the polar CP-Sil 88 phase, which in turn provided better S/N ratio and lower detection limits. Thus, for this selected problem the medium polar phase was better suited than highly polar columns.

Analysis of Deuterium-Labeled Standards. Internal standards (IS) were used to verify quantitative recoveries of PLs and NLs during both ASE and SPE. Initially, we used dipalmitoylphosphatidylcholine (DPPC) for PLs and triolein (OOO) for NLs (*16*). Since both FAs present in this IS mix (i.e., 16:0 and 18:1(9)) are also found in food, they had to be replaced with PL and NL nonfood FA standards. For this purpose, we used the commercially available DSPC-d_{70} for the PLs and PPP-d_{93} for the NLs, in which the acyl chains of the fatty acids were perdeuterated. Transmethylation of the IS thus resulted in d_{35}-18:0 ME and d_{31}-16:0 ME. Both compounds were baseline separated from their native analogues, and they did not coelute with any other FAME in the food sample extracts.

The fragment ions used for the quantification of FAMEs (*m/z* 74 and *m/z* 87) were analyzed for the formation of perdeuterated homologues. The McLafferty ion of FAMEs (*m/z* 74) was shifted by 3 units to higher mass (*m/z* 77), whereas *m/z* 87 (formed by γ-cleavage) was shifted to *m/z* 91 in the GC/EI-MS spectra of the methyl esters of perdeuterated fatty acids (**Figure 2**). The low mass fragment ion *m/z* 55 ($[C_4H_7]^+$) was even shifted to *m/z* 62 ($[C_4D_7]^+$) due to the septuple replacement of hydrogen by deuterium (**Figure 2**). The fragment ions *m/z* 55, *m/z* 87, and *m/z* 74 were of low relative intensity (I_{rel}) in the GC/EI-MS spectra of the perdeuterated FAs ($I_{rel(m/z\,55)} < 0.2\%$, $I_{rel(m/z\,87)} < 0.14\%$, and $I_{rel(m/z\,74)} \approx 2.4\%$), and native saturated and monounsaturated FAMEs gave no response to *m/z* 91, *m/z* 62, and *m/z* 77 ($I_{rel(m/z\,91)} < 0.1\%$, $I_{rel(m/z\,62)} < 0.02\%$, and $I_{rel(m/z\,77)} < 1.2\%$), in the elution range of the MEs mentioned. Finally, the molecular ions were shifted by 35 and 31 u, respectively, toward a higher odd mass (*m/z* 301, *m/z* 333, **Figure 2**). Based on these evaluations, determination of the perdeuterated IS was possible by using *m/z* 91 for quantification and *m/z* 77 for verification.

Application of Internal Standards. Quantitative recoveries of NLs for the applied ASE method were recently described (*17*). Recovery rates of PLs for both ASE and SPE were examined by

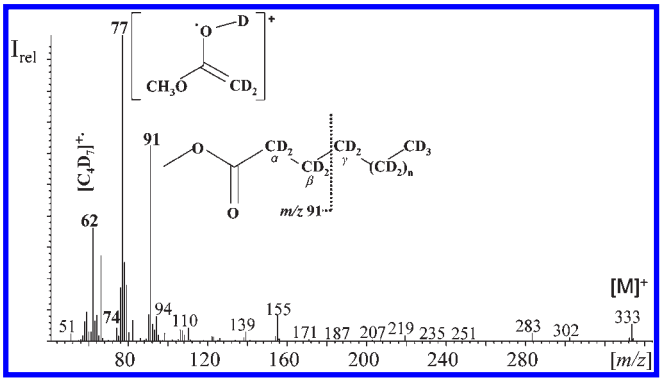

Figure 2. Mass spectrum of the d_{35}-18:0 methyl ester obtained after methylation of DSPC-d_{70}.

spiking DSPC-d_{70} (110−150 μg) into freeze-dried food samples prior to the ASE step. After extraction, solvent adjustment, SPE, and transesterification, the recovery rate of DSPC-d_{70} (determined with d_{35}-18:0) was 88−102% for three fish (trout, gilthead seabream, and sea bass) and three cheese (goat cheese, alpine cheese, and buffalo mozzarella) samples. These results also confirmed the suitability of the ASE and the SPE methods for the determination of FAs in the PLs. However, addition of the IS to the samples before ASE required rather high amounts of IS (110−150 μg of DSPC-d_{70} and ∼20 mg of PPP-d_{93}) because only aliquots were subjected to succeeding steps (e.g., SPE). Therefore, lower amounts (15 μg) of DSPC-d_{70} (IS for PLs) and PPP-d_{93} (IS for NLs) (0.2 mg) were added to aliquots of the ASE extracts just before SPE. The recovery rates of the IS for all 17 cheese and 7 fish samples were 93 ± 10% for PLs and 101 ± 6.7% for NLs.

Polar Lipid Content of Cheese and Fish. The amount of PLs in fish (6−19% of total lipids) was 3−60 times higher than in the cheese samples (0.3−1.9% of total lipids) (**Tables 1** and **2**). These amounts are in agreement with data obtained from other authors for fish (*28*−*31*) and cheese samples (*32*,*33*) based on lipid weight. For instance, a PL content of 0.85% in salmon fresh weight (*28*) corresponds to 6% in lipid weight, given that ∼13% of fresh weight consists of lipids.

Similar PL amounts were determined in the lipids of tuna and pollack fillet (6−8%) (**Table 2**). A higher PL content was detected in the lipids of fish processed with their skin (i.e., rainbow trout, brown trout, gilthead seabream, and sea bass). The highest amount of PLs was determined in the lipids of brown trout (18.8%). This is more than 10-fold of the highest PL content in cheese (**Tables 1** and **2**).

BCFAs in Cheese Samples. The cheeses varied with regard to the origin (animal species), the method of production (made from

Table 1. Content (g/100 g) of BCFAs in Neutral Lipids (NLs) and Polar Lipids (PLs) (in Parentheses) of Different Types of Cheese

	BCFA (g/100 g of NLs) [(g/100 g of PLs)] at t_R (min)																
	*i*14:0 at 18.53		*i*15:0 at 20.13		*a*15:0 at 20.30		*i*16:0 at 21.92		*i*17:0 at 23.89		*a*17:0 at 24.10		*i*18:0 at 26.01		∑BCFAs		
cheeses	NLs	(PLs)	NLs	(PLs)	NLs	(PLs)	NLs	(PLs)	NLs	(PLs)	NLs	(PLs)	NLs	(PLs)	NLs	(PLs)	% PL
gouda	0.05	(0.07)	0.16	(0.24)	0.35	(0.39)	0.14	(0.19)	0.22	(0.36)	0.42	(0.54)	0.03	(0.06)	1.38	(1.84)	0.76
emmental	0.07	(0.07)	0.18	(0.20)	0.35	(0.64)	0.15	(0.19)	0.22	(0.23)	0.38	(0.54)	0.04	(0.07)	1.39	(1.94)	0.34
butter cheese	0.05	(<0.04)[a]	0.14	(<0.04)	0.31	(<0.04)	0.15	(<0.04)	0.21	(<0.04)	0.41	(<0.04)	0.03	(<0.04)	1.30	(<0.04)	0.57
org camembert	0.08	(0.09)	0.18	(0.19)	0.39	(0.38)	0.14	(0.17)	0.19	(0.21)	0.33	(0.41)	0.02	(0.05)	1.34	(1.49)	0.71
org brie	0.09	(0.04)	0.21	(0.15)	0.43	(0.27)	0.19	(0.12)	0.26	(0.16)	0.45	(0.29)	0.05	(<0.04)	1.67	(1.07)	0.68
org alpine cheese	0.08	(<0.04)	0.20	(0.22)	0.38	(0.36)	0.16	(0.17)	0.23	(0.24)	0.37	(0.38)	0.04	(<0.04)	1.46	(1.38)	0.29
org curd cheese	0.06	(0.04)	0.17	(0.25)	0.40	(0.46)	0.15	(0.20)	0.26	(0.21)	0.46	(0.45)	0.04	(<0.04)	1.53	(1.62)	0.50
cow mozzarella	0.05	(<0.04)	0.12	(0.17)	0.29	(0.31)	0.09	(0.20)	0.16	(0.29)	0.30	(0.49)	0.02	(0.08)	1.03	(1.53)	0.53
mozzarella	0.09	(0.05)	0.17	(0.25)	0.34	(0.32)	0.18	(0.18)	0.10	(0.27)	0.25	(0.47)	0.01	(0.10)	1.14	(1.65)	0.53
cow feta	0.05	(0.04)	0.18	(0.15)	0.38	(0.28)	0.13	(0.09)	0.20	(0.09)	0.35	(0.27)	0.02	(<0.04)	1.31	(0.93)	0.77
feta	0.07	(0.07)	0.15	(0.25)	0.38	(0.42)	0.13	(0.16)	0.20	(0.23)	0.33	(0.43)	0.03	(<0.04)	1.28	(1.58)	1.47
goat cream	0.04	(0.06)	0.11	(0.15)	0.22	(0.27)	0.12	(0.17)	0.16	(0.19)	0.28	(0.36)	0.01	(0.06)	0.94	(1.26)	0.81
goat cheese	0.04	(0.06)	0.08	(0.24)	0.18	(0.29)	0.09	(0.15)	0.12	(0.16)	0.23	(0.29)	0.01	(0.04)	0.76	(1.23)	1.85
Bavaria blue	0.07	(0.14)	0.08	(0.24)	0.27	(0.42)	0.14	(0.22)	0.13	(0.18)	0.27	(0.38)	0.03	(<0.04)	1.01	(1.58)	1.12
limburger	0.07	(0.06)	0.18	(0.37)	0.37	(0.31)	0.16	(0.17)	0.24	(0.23)	0.40	(0.42)	0.04	(0.07)	1.47	(1.63)	0.55
romadur	0.07	(0.08)	0.13	(0.36)	0.28	(0.42)	0.11	(0.15)	0.13	(0.17)	0.22	(0.31)	0.01	(0.04)	0.94	(1.53)	1.45
roquefort	0.07	(0.10)	0.15	(0.23)	0.32	(0.38)	0.13	(0.17)	0.16	(0.19)	0.28	(0.45)	0.02	(<0.04)	1.13	(1.51)	0.65
mean	0.06	(0.06)	0.15	(0.22)	0.33	(0.35)	0.14	(0.16)	0.19	(0.20)	0.34	(0.38)	0.03	(0.04)	1.22	(1.43)	0.80
median	0.07	(0.06)	0.16	(0.23)	0.35	(0.36)	0.14	(0.17)	0.20	(0.21)	0.33	(0.41)	0.03	(0.04)	1.29	(1.53)	0.70

[a] <0.04: limit of quantification, typically 0.04% for PLs.

Table 2. Content (g/100 g) of BCFAs in Neutral Lipids (NLs) and Polar Lipids (PLs) (in Parentheses) of Different Fish Samples

	BCFA (g/100 g of NLs) [(g/100 g of PLs)] at t_R (min)																
	*i*14:0 at 18.53		*i*15:0 at 20.13		*a*15:0 at 20.30		*i*16:0 at 21.92		*i*17:0 at 23.89		*a*17:0 at 24.10		*i*18:0 at 26.01		∑BCFAs		
fish samples	NLs	(PLs)	NLs	(PLs)	NLs	(PLs)	NLs	(PLs)	NLs	(PLs)	NLs	(PLs)	NLs	(PLs)	NLs	(PLs)	% PL
tuna (fillet)	<0.004[a]	(0.03)	<0.004	(0.10)	<0.004	(0.13)	<0.004	(0.07)	<0.004	(0.13)	<0.004	(0.13)	<0.004	(0.03)	<0.004	(0.62)	6.12
salmon (fillet)	0.01	(0.01)	0.04	(0.04)	0.02	(0.05)	0.02	(0.02)	0.04	(0.05)	0.02	(0.04)	0.01	(0.01)	0.15	(0.22)	6.03
pollack (fillet)	<0.004	(<0.004)	<0.004	(0.03)	<0.004	(0.03)	<0.004	(0.01)	<0.004	(0.05)	<0.004	(0.03)	<0.004	<0.004	<0.004	(0.16)	8.30
trout rainbow	0.02	(<0.004)	0.09	(0.02)	0.04	(0.02)	0.02	(0.02)	0.06	(0.07)	0.04	(0.04)	0.02	(0.01)	0.29	(0.19)	10.30
gilthead seabream	<0.004	(0.04)	0.06	(0.34)	0.02	(0.12)	0.02	(0.10)	0.03	(0.22)	0.03	(0.17)	0.01	(0.03)	0.16	(1.01)	10.60
sea bass	0.01	(0.02)	0.05	(0.20)	0.02	(0.06)	0.02	(0.08)	0.04	(0.23)	0.02	(0.15)	0.01	(0.02)	0.17	(0.77)	12.50
brown trout	0.01	(0.02)	0.18	(0.23)	0.03	(0.07)	0.02	(0.04)	0.05	(0.15)	0.03	(0.09)	<0.004	(0.01)	0.32	(0.62)	18.80
mean	0.01	(0.02)	0.07	(0.13)	0.02	(0.07)	0.02	(0.05)	0.04	(0.13)	0.03	(0.09)	0.01	(0.02)	0.18	(0.51)	10.38
median	0.01	(0.02)	0.05	(0.10)	0.02	(0.06)	0.02	(0.04)	0.04	(0.13)	0.03	(0.09)	0.01	(0.01)	0.16	(0.62)	10.30

[a] <0.004: limit of quantification, typically 0.004% for NLs and PLs.

pasteurized or raw milk, organically or conventionally produced), and the type (hard/soft cheese, mold cheese). This set of samples was not suitable for a statistical evaluation. Nevertheless, the amounts (**Table 1**) and ratios (**Figure 3**) of individual BCFAs were comparable in all samples. Altogether, seven BCFAs (five *i*FAs and two *a*FAs) were detected in both lipid fractions (**Figure 3** and Figure S1, Supporting Information), except for butter cheese in which BCFAs were only detected in the NLs. The *a*15:0 and the *a*17:0 dominated the BCFA pattern of NLs from all cheeses (0.18−0.46%). Even-numbered *a*FAs were not detectable in any cheese, which agrees with results obtained in bovine milk (*5*, *34*). In food, *a*16:0 has only been detected after selective enrichment of BCFAs by urea complexation (*35*). In agreement with literature (*17*, *36*), the odd-numbered *i*FAs were 2−10 times higher concentrated than the even-numbered homologues. In the NLs, the sum of BCFAs ranged between 0.8% and 1.7%, spread around a mean value of 1.2%, which was only slightly lower than the median (**Table 1**), indicating a normal distribution. Since PL-BCFAs only moderately contributed to the total BCFA content of the samples (see below), the quantities of branched chain fatty acids determined in the NLs are in good agreement with the amounts reported elsewhere for total lipids (*17*, *36*, *37*).

In the PLs, the sum of BCFAs ranged from 0.9% in cow feta cheese to 1.9% in emmental cheese. The concentrations of individual BCFAs in the PLs ranged between < 0.01% and 0.64%, with *a*15:0 (0.27−0.64%) and *a*17:0 (0.27−0.54%) again being predominant and *i*14:0 and *i*18:0 contributing the least (< 0.01−0.04%).

The relative contribution of BCFAs to PLs was on average 17% higher than that of NLs (median: +19% higher), except in the cases of butter cheese (see above), organic brie, alpine cheese, and cow feta where BCFAs were higher in the NLs. No significant difference (**Table 1**) regarding the total content of BCFAs in the PLs and NLs could be established. In both lipid fractions, the relevance of mean and median values decreased in the order *a*17:0 ≈ *a*15:0 > *i*17:0 > *i*15:0 > *i*16:0 > *i*14:0 ≈ *i*18:0 (**Figure 3** and Figure S1, Supporting Information). Concentrations of individual BCFAs varied slightly more in the PLs than in the NLs (**Figure 3**, error bars). The relative amount of *i*14:0 was 23% higher in the NLs while *i*15:0 was 21% higher in the PLs. All other BCFAs were comparable in both lipid fractions (**Figure 3**, **Table 1**).

BCFAs in Fish Samples. The total amounts and the relative pattern of individual BCFAs in fish differed among the different

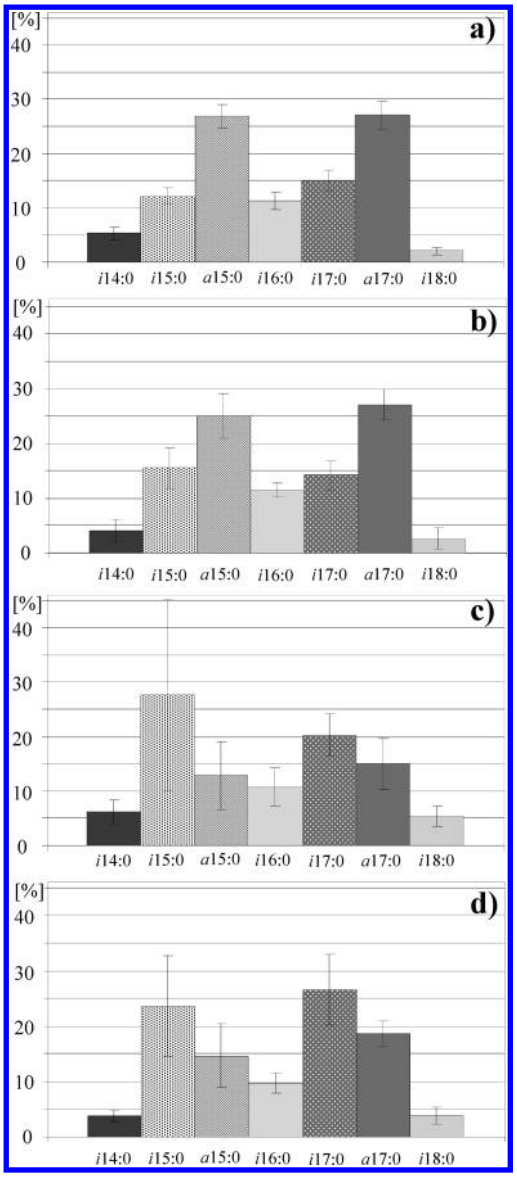

Figure 3. Relative distribution of branched chain fatty acids in (a) neutral lipids and (b) polar lipids of cheeses and (c) neutral lipids and (d) polar lipids of fish.

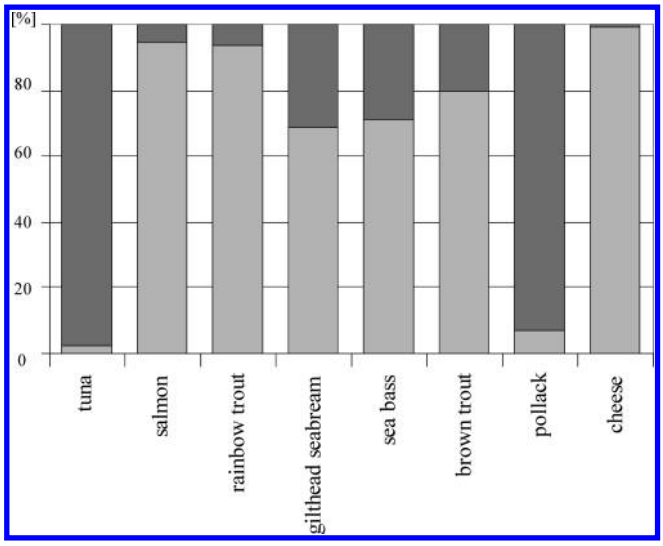

Figure 4. Relative contribution (%) of branched chain fatty acids in their neutral lipids (light gray) and polar lipids (dark gray) to the total lipid content in fish and cheese (mean value). BCFAs not detected were taken into account with one-tenth of the limit of quantification (signal-to-noise ratio = 10).

fish samples and also from those in cheese (see **Table 2** and **Figure 3c**,**d**). The largest amounts of BCFAs in the NLs were found in brown trout and rainbow trout (0.26%). BCFAs were not detected in the NLs of pollack and tuna (< 0.004 g/100 g) (**Table 2**). The percentages of individual BCFAs in the NLs (0.01−0.3%) were in the same magnitude as those recently described for total lipids (*6*, *17*, *29*). In contrast to the cheese samples, the BCFA pattern of fish was dominated by the odd-numbered *i*FAs (*i*17:0 and *i*15:0) followed by *a*15:0 and *a*17:0 (**Figure 3c**,**d**). For example, *i*15:0 accounted for 57% of the BCFA content in the NLs of brown trout lipids. A similar abundance of the *i*FAs in total lipids was observed by Thurnhofer et al. in total lipids of fish (*17*). Even-numbered *i*FAs were only found in traces (0.01 g/100 g) while even-numbered *a*FAs were not detected at all (**Table 2**).

In the PLs, the amount of total BCFAs ranged between 0.16% (pollack) and 1% (gilthead seabream) (**Figure 3d**), which was up to ten times higher than their respective amounts of NLs (**Table 1**). Consequentially, the individual amounts of *i*15:0 (up to 0.34%), *i*17:0 (up to 0.23%), and *a*17:0 (up to 0.17%), which constituted the bulk of total BCFAs, were also significantly higher in the PLs (**Table 2**). Interestingly, BCFAs were only detected in the PLs of pollack (0.16% PLs) and tuna (0.62%) but not in the NLs. An analytical error was excluded due to the high recovery rate for the IS d_{31}-16:0-ME. In general, the relative distribution of BCFAs varied between the lipid classes and also from fish to fish (see also error bars in **Figure 3c**,**d**). The total amount of BCFAs in fish samples was 2−10 times lower than that found in cheese samples.

Contribution of BCFAs in the NLs and PLs to the Total Lipid Amount in Fish and Cheese. Although the total amount of BCFAs in fish lipids was significantly lower than in cheese (discussed above), PL-BCFAs in fish lipids contributed a larger portion to the BCFA content in total lipids compared to cheeses. The PL-BCFAs in fish samples consisted of between ∼6% and 20−31% of the BCFA content in total lipids (**Figure 4**), except for pollack and tuna (> 97% and 93%, respectively; see above). In contrast, PL-BCFAs in cheese samples constituted only ∼1% of total BCFAs (**Figure 4**). Due to the low PL content of cheeses (∼0.8% mean), the content of BCFAs in total lipids was not influenced by the PLs and was virtually identical with the BCFA content in the NLs. The higher variations in the BCFA content of PLs from fish were surprising. The same effect was also found for straight chain fatty acids with odd carbon number (15:0 and 17:0, Table S3, Supporting Information). However, the samples were from different species, and a statistical evaluation was not possible due to the small sample size. It is possible that the larger occurrence of BCFAs in the PLs could also be due to bacterial infestation. The method presented here is suited for a thorough investigation of this problem in the future. Such follow-up studies should include a sufficient number of samples suitable for a thorough investigation and statistical evaluation of this hypothesis.

Supporting Information Available: Concentrations of individual schemes of individual fatty acids in cheese (Table S1) and fish (Table S2), proportions of 15:0, 17:0, and some branched chain fatty acids found in the polar lipids of fish and cheese (Table S3), and relative amounts of individual BCFAs in the samples (Figure S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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