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ORIGINAL PAPER

Identification and quantification of dicarboxylic fatty acids in head tissue of farmed Nile tilapia (*Oreochromis niloticus*)

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Abstract

Nile tilapia (*Oreochromis niloticus*) was grown in Bangladesh with four different feeding treatments as part of a project that aims to produce fish in a cost-effective way for low-income consumers in developing countries. Fillet and head tissue was analysed because both tissues were destined for human consumption. Gas chromatography with mass spectrometry (GC/MS) analyses of transesterified fatty acid methyl ester extracts indicated the presence of ~ 50 fatty acids. Major fatty acids in fillet and head tissue were palmitic acid and oleic acid. Both linoleic acid and polyunsaturated fatty acids with three or more double bonds were presented in quantities > 10% of total fatty acids in fillet, but lower in head tissue. Erucic acid levels were below the newly proposed tolerable daily intake in the European Union, based on the consumption of 200 g fillet per day. Moreover, further analysis produced evidence for the presence of the dicarboxylic fatty acid azelaic acid (nonanedioic acid, Di9:0) in head tissue. To verify this uncommon finding, countercurrent chromatography was used to isolate Di9:0 and other dicarboxylic acids from a technical standard followed by its quantification. Di9:0 contributed to 0.4–1.3% of the fatty acid profile in head tissue, but was not detected in fillet. Fish fed with increasing quantities of flaxseed indicated that linoleic acid was the likely precursor of Di9:0 in the head tissue samples.

Keywords Farmed fish · Nile tilapia · Fatty acid · Dicarboxylic acid · Azelaic acid · Countercurrent chromatography

Introduction

Fish is a rich source of easily digestible proteins, omega-3 fatty acids and essential micronutrients. Hence, various dietary recommendations suggest the regular intake of 1–2 servings of fish and seafood per week [1]. On average, about 20 kg of fish per capita is annually consumed worldwide [2]. To satisfy this high marked demand, aquaculture has significantly increased since the 1970s [2]. In 2018, the production volume of animals from aquaculture reached 82 million tonnes worldwide, with 62.5% from inland aquaculture (51 million tonnes). After carp (*Cyprinidae*), the production of Nile tilapia (*Oreochromis niloticus*) (8.3% of total finfish production in 2018) established as second largest mass of farmed finfish in the last decade [2]. Tilapia is mostly raised in Southeast Asia (with China being the biggest producer) and mainly farmed extensively to semi intensively [2].

Nile tilapia was selected for the development of cost-effective methods to improve the production and nutritional quality of tilapia for low-income consumers in developing countries [3, 4]. Suitable quality markers for nutritional value are the content of polyunsaturated fatty acids (PUFAs) which include the essential fatty acids eicosapentaenoic acid (20:5*n*-3, EPA) and docosahexaenoic acid (22:6*n*-3, DHA) [1]. Moreover, in several cultures not only fillet, but also head tissue is consumed. We thoroughly analysed the fatty acid pattern of fillet and head issue of tilapia raised with different feed. Initial screening resulted in the detection of an uncommon fatty acid which indicated the presence of the dicarboxylic acid azelaic acid (HOOC–$(CH_2)_7$–COOH, IUPAC name: nonanedioic acid).

The goal of this study was to verify the presence of azelaic acid and to determine its concentrations in the fish samples. To gain insights into its possible origins, fillet and head tissue from fish raised by four different feed compositions were analysed for fatty acids. The data were used to trace back the origins of this fatty acid. For this reason,

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azelaic acid and further dienoic fatty acids (DiFAs) were isolated from a technical standard and purified by countercurrent chromatography (CCC). CCC is a (semi)preparative all-liquid based sample preparation method preferably used in natural product isolation [5]. Isolated compounds were used for structure determination and the preparation of a standard mixture for GC/MS analysis.

Materials and methods

Chemicals and standards

Methanol, *n*-hexane (both HPLC grade), cyclohexane (p.a.) and acetonitrile (99.5%, p.a.) were from Th. Geyer (Renningen, Germany). Ethyl acetate (p.a.) was from Sigma Aldrich (Steinheim, Germany), sulphuric acid (96%, p.a.) and sodium chloride (> 99.5%, p.a.) from Carl Roth (Karlsruhe, Germany), azelaic acid (nonane-1,9-dioic acid, Di9:0) (purity 80%) and brassylic acid (tridecane-1,13-dioic acid, Di13:0) (purity > 96.0%) were from TCI (Antwerpen/Belgium). Additional fatty acids as well as a 37 component FAME mix were purchased from Supelco (Taufkirchen, Germany). The internal standards 10,11-dichloroundecanoic acid (DC-11:0) and myristic acid ethyl ester (14:0-EE) were synthesised in our research group [6]. Nitrogen (quality 4.8) and helium (quality 5.0) were from Westfalen Company (Münster, Germany).

Sample description

Samples were obtained during 2018 within the BMZ/GIZ supported AquaLINC (*Aquaculture for Low Income Consumers*) project [7] hosted by WorldFish. In this project, genetically improved farmed Nile tilapia (*Oreochromis niloticus*), hereafter tilapia, was grown in Bangladesh with four different feed compositions (FC1-FC4) based on increasing amounts of flax seed powder and orange sweet potato (Table 1). Fish were raised in cages for five months to an average weight of 300–350 g. Fillet and head tissue were taken from five individuals per treatment. Fillet ($n = 20$) and head tissue ($n = 20$) samples were freeze-dried and shipped to the partner laboratory in Germany for fatty acid analysis. Samples were kept frozen until analysis within a few weeks upon arrival.

Fatty acid analysis

Freeze-dried samples were homogenised with a laboratory mill (IKA basic, Staufen, Germany). Between 0.3 and 0.5 g fillet or head tissue was used for lipid extraction by means of accelerated solvent extraction (Dionex ASE 350, Thermo Fisher Scientific, Waltham/MA, USA) using the azeotropic mixture of cyclohexane and ethyl acetate (46:54, *w*/*w*) [8]. The lipid extract was concentrated in a rotary evaporator and adjusted to a defined volume. An aliquot (1 mL) was used for the gravimetric determination of the fat content in dry matter [6]. For fatty acid determinations, ~ 0.2 mg lipids and the internal standard DC-11:0 (ISTD I) were placed in a 6 mL test tube. Transesterification was performed by adding 2 mL methanol complemented with 1% sulphuric acid. The resulting sample solution was heated to 80 °C for two 2 h and afterwards cooled on an ice bath. Then, 2 mL *n*-hexane, 0.5 mL saturated sodium chloride solution and 0.5 mL demin. water were added and the sample was thoroughly shaken. After phase separation, the upper organic phase was separated [9]. Another internal standard (14:0-EE, ISTD II) was added before analysis by gas chromatography with mass spectrometry (GC/MS) [6]. Measurements were carried out with a 5890 series II/5972A GC/MS system equipped with a 7673 autoinjector (Hewlett-Packard/Agilent, Waldbronn, Germany). Helium (purity 99.9990%) was used as the carrier gas at a constant flow rate of 1 mL/min. An Rtx-2330 fused silica column (60 m length, 0.25 mm i.d., 0.1 µm film

Table 1 Feed compositions (FC1–FC4) [%][a] of the four treatments of farmed Nile tilapia (*Oreochromis niloticus*)

	FC1		FC2		FC3		FC4	
	Start	Grow	Start	Grow	Start	Grow	Start	Grow
Flax seed powder	–	–	5	5	7.5	7.5	10	10
Sweet potato	–	–	5	5	7.5	7.5	10	10
Wheat flour	19	18	14	12	14	12	14	12
Maize	6	10	4.5	10	4.5	10	4.5	10
Rice polish-A	5	6	5	6.5	5	4		
De-oiled rice bran (DORB)	14.4	14.6	11	10	6	7.5	6	6.5
Di-calcium phosphate	0.7	0.6	0.7	0.6	0.7	0.6	0.8	0.6
Limestone powder	0.51	0.51	0.38	0.56	0.34	0.52	0.18	0.47
Lysine HCL	0.1	0.12	0.12	0.15	0.15	0.18	0.19	0.22
DL-methionine	0.27	0.15	0.28	0.17	0.29	0.18	0.31	0.19

[a]Ingredients without changes in the four treatments are not listed

thickness; Restek, Bellefonte, PA, USA) was installed in the GC oven. The GC oven was programmed as follows: after 1 min at 60 °C, the temperature was increased at 6 °C/min to 150 °C, then at 4 °C/min to 190 °C and finally at 7 °C/min to 250 °C (hold time 7 min). In selected ion monitoring (SIM) mode, *m/z* 74, *m/z* 79, *m/z* 81, *m/z* 87, *m/z* 88, *m/z* 101 [10, 11] were recorded from 8 min (end of solvent delay) to the end of the run (41.65 min). Ion abundance ratios and retention times were used for peak assignment. Final peak verification was performed by GC/MS operated in full scan mode (*m/z* 50–500). Injector and detector temperatures were set at 250 and 280 °C, respectively.

Dicarboxylic fatty acid dimethyl esters (DiFA-diMEs) in fish samples were analysed with a 5890 GCDPlus/5971 GC/MS system equipped with a 7673 autoinjector (Hewlett-Packard/Agilent, Waldbronn, Germany). A DB5-MS UI column (30 m length, 0.25 mm i.d., 0.25 µm film thickness; Agilent, Waldbronn, Germany) was used in combination with the carrier gas helium (see above) and the following temperature program: After 1 min at 55 °C, T was raised at 10 °C/min to 300 °C (hold time 4 min). In full scan mode, *m/z* 50–500 was recorded throughout the run after a solvent delay of 7 min. GC/MS-SIM measurements were based on ten *m/z* values, i.e. *m/z* 74 and *m/z* 87 for saturated FAME in general, *m/z* 88 and *m/z* 101 for the internal standard FAEE [6] along with the $[M–OMe]^+$ ($[M-31]^+$) ions of DiFA-diMEs, namely *m/z* 171 (Di8:0-diME), *m/z* 185 (Di9:0-diME), *m/z* 199 (Di10:0-diME), *m/z* 213 (Di11:0-diME), *m/z* 227 (Di12:0-diME) and *m/z* 241 (Di13:0-diME). DiFA-diMEs were quantified by means of *m/z* 74.

Countercurrent chromatography (CCC) fractions obtained from the injection of Di9:0-diME (see next section) were analysed on a 5890 GC/FID system (Hewlett-Packard/Agilent, Waldbronn, Germany) equipped with a DB5-MS UI column (30 m length × 0.25 mm i.d. × 0.25 µm film thickness; Agilent, Waldbronn, Germany). Peak areas, normalised by the internal standard, were used for creating the elution profiles. A DB5-MS UI column with the same parameters and GC oven program as shown above was used for the measurements.

Countercurrent chromatographic (CCC) fractionation of technical azelaic acid standard (purity 80%)

Three solvent systems, i.e. acetonitrile/water 1:1 (*v*/*v*), *n*-hexane/benzotrifluoride (THF)/acetonitrile 20:7:13 (*v*/*v*/*v*) [12] and *n*-hexane/methanol/water 700:350:4 (*v*/*v*/*v*) [13] were initially selected for shake flask experiments according to Ito [14]. For this purpose, the technical azelaic acid standard (80% purity) was twofold methylated to give Di9:0-diME. An aliquot of technical Di9:0-diME (0.15 mg) was added to 1 mL of each separated phase of the solvent system and thoroughly shaken. After phase separation, aliquots of both fractions were taken and analysed by GC/FID and the partition constant ($K_{U/L}$ value) was calculated from the peak ratio. The technical Di9:0-diME standard featured four additional DiFA-diMEs those $K_{U/L}$ values were determined as well.

CCC separations were performed with an AECS Quikprep MK8 CCC instrument (AECS, Downend, UK) equipped with two coils each in two bobbins of ~ 120 mL each [15]. Coils 2 and 3 were selected (total volume 236 mL) [16] for separations in head-to-tail mode (lower phase served as mobile phase) using the instrument´s maximum rotational speed of 870 rpm. The solvent system *n*-hexane/methanol/water 700:350:4 (*v*/*v*/*v*) according to Kapp and Vetter [13] was selected for CCC separations. After equilibration of the system (17 mL displacement of stationary phase), 120 mg technical Di9:0-diME, dissolved in a mixture of 2 mL of both phases, was injected and the flow rate was set to 2 mL/min. Fractionation of 41 subsequent CCC fractions of 6 mL each was started after 50 mL. The individual fractions were concentrated to ~ 2 mL using a heating block maintained at 40 °C and a gentle stream of nitrogen and filled up to a volume of 5 mL in a volumetric flask. To remove traces of water used in the solvent system an aliquot of 1 mL was filtrated through sodium sulphate. These fractions were analysed by GC/MS for peak identification followed by GC/FID analysis using with the same conditions for generation of the elution profiles purity controls and quantitation.

Results and discussions

Fatty acid profile in fillet and head tissue

Lipid content in fillet (mean value of all treatments) was ~ 7% in dry matter. Fifty-two fatty acids were detected and mostly verified in the samples (Table S1). Discussion will be based on the most relevant fatty acids while low abundant FA (< 1% contribution to the fatty acid pattern) was summarised as minor FAs (Table 2). The fatty acid pattern of fillet from all treatments was dominated by ~ 25–29% oleic acid (18:1*n*-9) and ~ 22.5–25.5% palmitic acid (16:0) (Table 2). These and further saturated and monoenoic fatty acids resulted in a shared contribution of on average 73% to the total fatty acids (Fig. 1a). The remaining share of 26% originated from dienoic fatty acids (~ 13%, Fig. 1a) with main contributions of linoleic acid (18:2*n*-6; ~ 12%) and PUFAs with more than two double bonds (~ 14%), including 3.5% docosahexaenoic acid (DHA, 22:6*n*-3) (Fig. 1a; Table 2). Phytanic acid contributed with 0.1% to the total fat [11] (Table 2). Considering the food compositions of the individual treatments (Table 1), the most striking differences in fatty acid profiles were an increase in the contributions of dienoic FAs (12.9–13.6% vs. 12.5% without flaxseed powder) and PUFAs with more

Table 2 Fatty acid profile [%] of the most abundant fatty acids in fillet (left columns) and head tissue (right columns) of Nile tilapia (*Oreochromis niloticus*) raised with four feed compositions (FC1–FC4, see Table 1)

	Fillet				Head			
	FC1	FC2	FC3	FC4	FC1	FC2	FC3	FC4
Lipid in DM [%]	7.1	15.1	7.9	5.0	39.0	39.7	41.9	45.5
14:0	2.5	2.6	2.4	2.0	3.5	3.6	3.5	3.8
Di8:0	n.d	n.d	n.d	n.d	0.06	0.10	0.14	0.19
16:0	25.5	23.3	22.8	22.6	32.5	27.6	31.0	30.8
Phytanic acid	0.1	0.1	0.1	0.1	n.d	n.d	n.d	n.d
Di9:0	n.d	n.d	n.d	n.d	0.4	0.7	1.0	1.4
18:0	6.5	6.8	6.5	6.8	6.6	6.4	7.7	7.1
16:1	0.7	0.6	0.6	0.6	0.9	0.9	0.9	0.9
16:1*n*-7	6.3	4.7	5.1	4.2	7.1	6.3	6.2	6.2
18:1*n*-9	28.7	28.6	28.8	25.3	33.4	35.9	37.1	37.2
18:1	2.8	2.6	2.6	2.3	3.2	3.3	3.3	3.2
20:1*n*-9	1.4	1.4	1.3	1.2	1.7	1.8	1.7	1.7
22:1*n*-9	0.4	0.4	0.5	0.5	0.5	0.4	0.5	0.6
18:2*n*-6	11.6	12.8	12.8	12.6	5.4	7.2	4.0	3.6
18:3*n*-6	0.5	0.4	0.4	0.4	0.10	0.20	0.05	n.d
18:3*n*-3	2.3	4.3	6.3	8.0	1.0	1.8	0.7	0.6
20:2	0.4	0.5	0.4	0.5	0.7	0.4	0.1	0.8
20:3*n*-6	0.7	0.7	0.5	0.6	0.09	0.15	n.d	n.d
20:4*n*-6	1.4	1.8	1.6	2.3	0.1	0.4	0.1	0.1
20:5*n*-3	0.2	0.3	0.2	0.3	0.03	0.04	n.d	n.d
22:5*n*-3	1.2	1.7	1.6	1.3	0.1	0.3	0.05	n.d
22:6*n*-3	3.4	3.7	2.9	4.9	0.2	0.4	0.1	n.d
Minor FAs[a]	5.5	5.0	4.6	5.7	5.5	6.3	4.9	6.4
Saturated FAs	36.1	34.0	32.9	33.5	44.2	38.9	43.7	43.4
Monoenoic FAs	40.7	38.5	39.2	34.3	47.0	48.8	49.9	50.1
Dienoic FAs	12.5	13.6	13.5	13.3	6.9	8.6	5.3	5.6
Polyenoic FAs	10.7	13.9	14.5	18.9	1.8	3.5	0.9	0.7

[a]Individual contributions < 1% to total fatty acids, i.e. saturated FAs (12:0, 13:0, 19:0, 20:0, 22:0 and 24:0), methyl branched FAs (*i*15:0, *a*15:0, *i*16:0, *i*17:0, *a*17:0, *i*18:0), monoenoic and dienoic FAs (14:1, 16:1, 17:1, 22:1, 16:2, two 20:2 isomers and six unknown PUFAs

than two double bonds (12.5–18.9 vs. 10.7% without flaxseed powder, Table 2) with increasing shares of flax seed powder in the feed (Fig. S1). Specifically, both the share of α-linolenic acid (18:3*n*-3; ALA), i.e. the major constituent in flaxseed oil (40–60% of total fat) as well as docosahexaenoic acid (22:6*n*-3; DHA) increased from 2.3% (FC1, 0% flaxseed powder) to 8.0% (FC4, 10% flaxseed powder) and 2.8% (FC1) to 4.9% (FC4), respectively. This trend was in agreement with the findings of Visentainer et al. [17]. In contrast, the share of saturated fatty acids decreased slightly when more flax seed powder was applied with the feed (Table 2). Notably, the fillet samples also featured between 0.4 and 0.5% erucic acid (22:1*n*-9, Table 2). Erucic acid is considered toxic to humans and a maximum tolerable daily intake of 7 mg/kg body weight was recently proposed by the European Union [18]. Since fish is a known dietary source of erucic acid [19, 20], the daily intake was calculated for body weights of 40 kg (children, ~ 10 years old) and 75 kg (adults), respectively, i.e. 280 and 525 mg erucic acid per day [20]. Based on a portion size of 200 g fillet, erucic acid concentrations were transferred on a wet weight basis (Table S2). Based on the values, the mean intake via consumption of tilapia was 10–54 mg erucic acid. Accordingly, even the highest amount in an individual fish sample was 230 mg erucic acid which is below the proposed TDI of erucic acid [18, 20].

When compared with fillet, head tissue (mean value of all treatments) showed a much higher lipid content (42% fat of the dry matter) and also a different fatty acid profile. Oleic acid (35%), palmitic acid (32%) and the share of saturated and monoenoic fatty acids (> 83%) was more prominent than in fillet (Table 2). Also, erucic acid (22:1n-9) was higher concentrated in head tissue, with mean concentrations in the four treatments of ~ 100–210 mg/200 g fresh weight (Table S2) which is close to the proposed TDI of erucic acid for young people and ~ 50% of the proposed TDI of adults

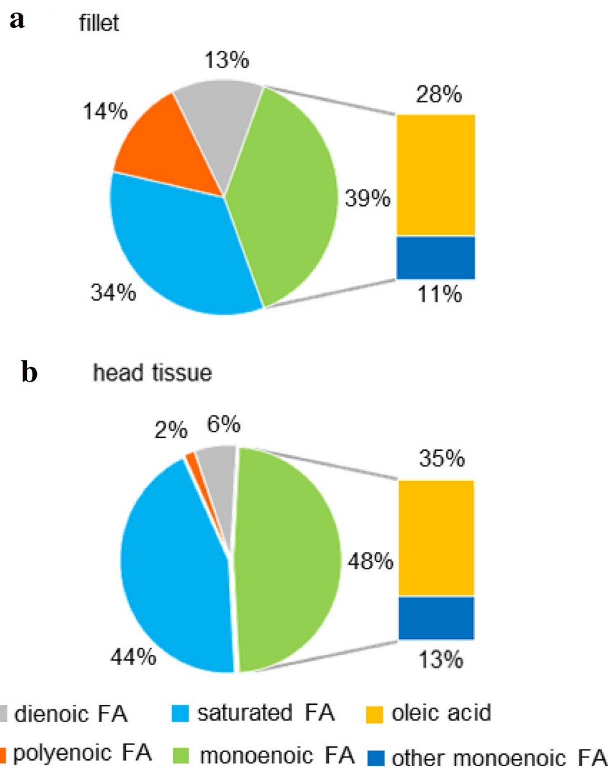

Fig. 1 Mean profile of fatty acid groups [%] in **a** fillet and **b** head tissue of Nile tilapia (*Oreochromis niloticus*) raised with four food compositions

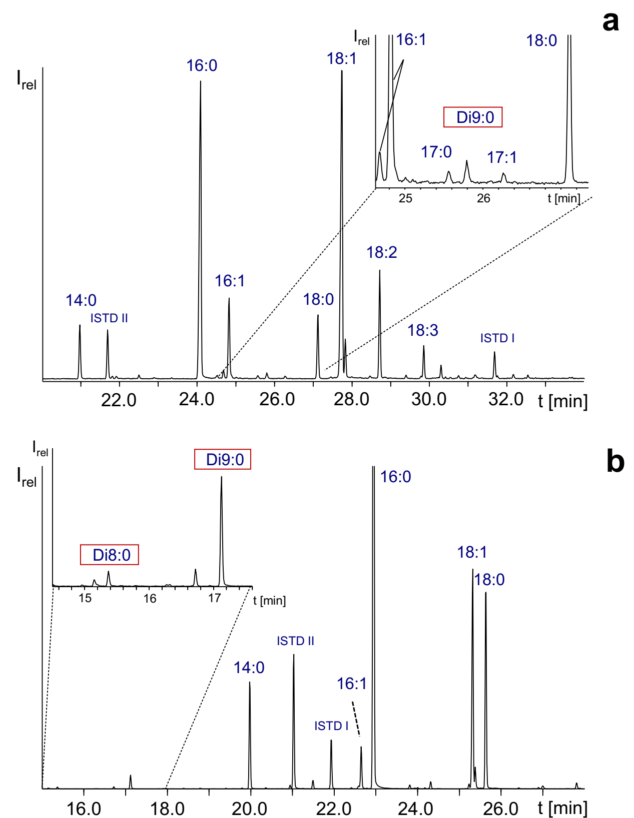

Fig. 2 GC/MS full scan chromatograms of the fatty acid methyl esters prepared from the lipids of Nile tilapia head tissue extract measured on **a** a polar GC column (Rtx-2330) and **b** a nonpolar GC column (DB-5)

[18, 20]. In addition, 5% linoleic acid and only < 2% PUFAs with more than two double bonds (DHA > 1%) were present while phytanic acid was not detected at all (Fig. 1b; Table 2). Most remarkably, the samples contained additional peaks that were not detected in fillet samples (see next section).

Identification of dicarboxylic acids in head tissue of tilapia

GC/MS chromatograms of the FAME solution obtained from head tissue extracts featured one additional peak between the retention time of 16:0-ME and 18:1*n*-9-ME (Fig. 2a). The full scan GC/MS spectrum indicated the presence of the (di-) methyl ester of azelaic acid (Di9:0-diME) due to its good match with the compound in the instrument´s NIST library of mass spectra. Characteristic GC/MS fragment ions of Di9:0-diME were *m/z* 185 ([M-31]$^+$), *m/z* 152 ([M-64]$^+$, [M–2CH$_3$OH]$^+$), *m/z* 111 ([M-105]$^+$), along with diagnostic fragment ions of saturated fatty acids, i.e. *m/z* 74 (base peak) and *m/z* 87, as well as *m/z* 83 (Fig. 3) [10]. Presence of Di9:0 (as dimethyl ester) in the head tissue samples could be verified by means of a commercial technical reference standard of Di9:0 (80% purity). Specifically, Di9:0-diME eluted between 17:0-ME and 17:1-ME from the polar Rtx-2330 GC column (Fig. 2a). Actually, it showed the same retention time as a standard of phytanic acid methyl ester (which was not present in the head tissue sample, see above). The long retention time was surprising because M$^+$ of Di9:0-diME (*m/z* 216) is much lower than M$^+$ of 17:0-ME (*m/z* 284) and phytanic acid-ME (*m/z* 326). Yet, switching to a nonpolar

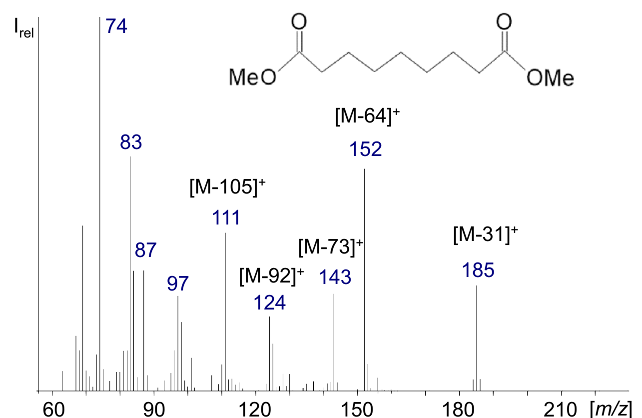

Fig. 3 GC/MS spectrum (full scan mode) of azelaic acid dimethyl ester (Di9:0-diME) in a Nile tilapia head tissue sample

DB-5 phase enabled a full GC separation of Di9:0-diME from all conventional FAMEs in the fish samples, which is in agreement with its lower molecular weight (Fig. 2b). Using this protocol, Di9:0-ME eluted much earlier, i.e. in the t_R range of 12:0-ME (M^+, *m/z* 214). Moreover, the early and noninterfered elution of DiFA-DiMEs on the nonpolar column allowed the additional detection of Di8:0-diME at lower abundance (Fig. 2b, insert). Subsequently, a GC/MS-SIM run was programmed based on the characteristic ions of DiFA-DiMEs (by shifting diagnostic fragment ions of Di9:0-ME by Δ14 u in both directions, Table 3). This targeted GC/MS-SIM method enabled the detection of the DiFAs Di7:0-diME, Di8:0-diME, Di9:0-diME, Di10:0-diME, Di11:0-diME, Di12:0-diME and Di13:0-diME in the samples. Owing to the insufficient purity of the technical Di9:0 standard (~80%, see "Materials and methods") which contained lower shares of Di7:0-diME, Di8:0-diME and Di10:0-diME to Di12:0-diME), an exact quantification was not possible at this point. Accordingly, the commercial Di9:0 standard was purified by means of countercurrent chromatography (CCC).

Purification of dicarboxylic fatty acids (DiFAs) by countercurrent chromatography

An aliquot of the technical Di9:0 standard (purity ~80%, along with homologues Di7:0, Di8:0, Di10:0, Di11:0, Di12:0) was methylated to give DiFA-diMEs which were subjected to shake flask experiments [14]. Three biphasic solvent systems were tested (see Materials and Methods). While $K_{U/L}$ values in acetonitrile/*n*-hexane and the BTF system were low and unsuited, the solvent system *n*-hexane/methanol/water 700:350:4 (*v*/*v*/*v*) [13] provided $K_{U/L}$ values of Di9:0-diME and three of the four homologues in the so-called sweet spot range of $K_{U/L} = 0.4 - 2.5$ [21] (Table 4). Moreover, the predicted elution profile (freeware: ProMISE—probabilistic model for immiscible phase separations and extractions [22]) indicated a sufficient separation of Di9:0-diME in head-to-tail mode using a flow rate of 2 mL/min (lower phase used as mobile phase).

Accordingly, 120 mg of the impure technical Di9:0-diME standard was injected into the calibrated CCC system and fractionated as shown in the section Materials and Methods.

Table 3 GC/MS data of the saturated dicarboxylic fatty acid dimethyl esters Di7:0-diME–Di13:0-diME with the molecular- ion (usually undetected) along with the diagnostic fragment ions

Name	$[M]^+$	$[M-31]^+$ $[M–CH_3O]^+$ (identifica-tion ion)	$[M-64]^+$ $[M–2CH_3OH]^+$	$[M-73]^+$ $[M–CH_3OCO–CH_2]^+$	$[M-92]^+$/$[M-91]^+$ $[M–2CH_3OH–CO]^+$/ $[M–CH_3OH–CH_3OCO]^+$	$[M-105]^+$
Di7:0-diME	188[a]	157	124[c]	115	96	83
Di8:0-diME	202[a]	171	138	129	110	97
Di9:0-diME	216[a]	185	152	143	124	111
Di10:0-diME	230[a]	199	166	157	138	125
Di11:0-diME	244[a]	213	180	171	152	139
Di12:0-diME	258[a]	227	194[b]	185	166	153
Di13:0-diME	272[a]	241				

[a]Not detected

[b]Very low abundant

Table 4 $K_{U/L}$ values of the dimethylated dicarboxylic acids (DiFA-diMEs) in the technical standard of azelaic acid dimethyl ester (purity 80%) in three solvent systems (columns 2–4) and the corresponding elution profile and isolation characteristics after separation with the solvent system *n*-hexane/methanol/water 700:350:4 (*v*/*v*/*v*)

	Shake flask experiments			CCC fractionation characteristics with *n*-hexane/methanol/water 700:350:4 (*v*/*v*/*v*)			
	Acetonitrile/water 1:1	*n*-hexane/BTF/acetonitrile 100:35:65	*n*-hexane/methanol/water 700:350:4	Elution time [min]	Elution volume [mL]	Quantity [mg]	Purity [%]
Di7:0-diME	n.d.	n.d.	n.d.	43–46	86–92	0.15	100
Di8:0-diME	0.0	0.05	0.0	49–55	98–110	1.4	97
Di9:0-diME	0.2	0.08	0.4	58–64	116–128	30.3	100
Di10:0-diME	0.2	0.14	0.5	79–82	158–164	0.24	96
Di11:0-diME	0.2	0.24	0.8	97–106	194–212	1.9	97
Di12:0-diME	1.0	0.35	0.8	121–130	242–260	0.18	98

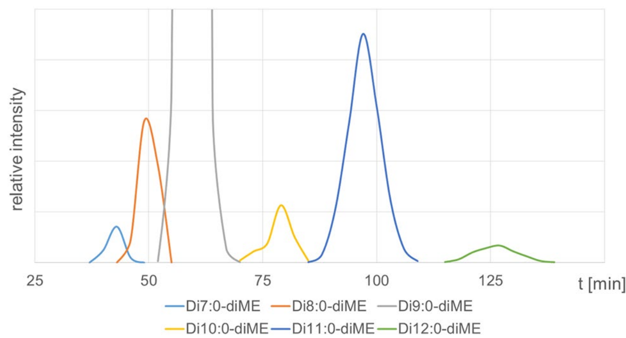

Fig. 4 CCC elution profile of six dicarboxylic acids in the technical dimethylated standard of azelaic acid (Di9:0-diME) of 80% purity. The elution profile was generated from normalised peak areas as determined by GC/FID. The flow rate was 2 mL/min and fractions were taken every sixth minute after an offset of 25 min

Elution profiles were generated by means of normalised peak areas in the resulting GC/FID chromatograms of the individual CCC fractions (Fig. 4). Next to the five DiFA-diMEs initially detected (Table 4), several CCC fractions also featured Di7:0-diME (see also above). Moreover, all six DiFA-FAMEs were present in selected CCC fractions with purities of 96–100%, respectively (Table 4). Hence, CCC fractionation not only allowed to detect Di7:0-diME, but also to isolate it in sufficient amounts and with high purity (Table 4). Aliquots of the isolated individual DiFA-diMEs were mixed to give a quantitative mixture which was checked by GC/FID for equal response. The resulting solution was used as external standard for quantitative analysis of DiFA-diMEs by GC/MS-SIM.

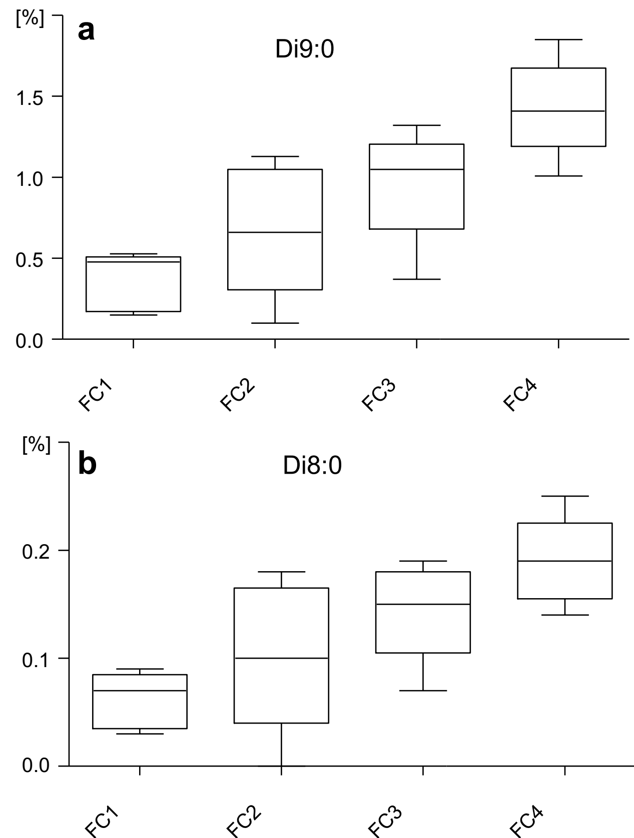

Fig. 5 Concentrations of **a** Di9:0 and **b** Di8:0 in Nile tilapia head tissue (in % of total fatty acids) in dependence of the different treatments (feed compositions) with different shares of flaxseed powder and sweet potato (FC1: 0%, FC2: 5%, FC3: 7.5% and FC4: 10%, respectively)

Concentrations of dicarboxylated fatty acids (DiFA-diMEs) in tilapia head tissue

Quantitative GC/MS-SIM analysis on the nonpolar GC column resulted in mean contributions of 0.4–1.4% Di9:0 to the total fatty acids in head tissue (Fig. 5a). The second most abundant DiFA in the head tissue samples, Di8:0, contributed on average with > 0.1–0.3% to the total fatty acids (Fig. 5b). Interestingly, the share of the most relevant DiFAs (Di7:0-Di11:0) steadily increased from FC1 to FC4 (Table S3, Fig. S2).

DiFAs are known degradation products of unsaturated fatty acids formed by oxidative cleavage. Assumedly, the formation of DiFAs starts with peroxidation (Fig. 6) [23]. Subsequent α-cleavage leads to an *n*-oxo-FA which then is oxidised to give a DiFA (Fig. 6). Three peroxidation processes could be involved, i.e. (1) autoxidation [23], (2) photooxidation with singlet oxygen and (3) enzymatic oxidation.

In the case of monoenoic fatty acids, Di9:0 was found to be formed from 18:1*n*-9 (oleic acid) and 16:1*n*-7 (palmitoleic acid) [23–25]. However, the amounts of 18:1*n*-9 would barely be changed by losses corresponding to the amount of Di9:0 in the head samples. Autoxidation of monoenoic acids should lead to similar amounts of Di8:0 and Di9:0, photooxidation would generate similar amounts of Di9:0 and Di10:0, while enzymatic oxidation requires at least two double bonds in a FA (which is not the case in 18:1*n*-9). Similarly, 16:1*n*-7 should also generate similar amounts as much lower concentrated than 18:1*n*-9 and very similar abundant in treatment FC2–FC4, Fig. S2). Hence, no direct dependency between the monoenoic fatty acids and the DiFAs could be established in the present samples.

However, PUFAs showing the first double bond on C-9 position like linoleic acid (18:2*n*-6) and α-linolenic acid (18:3*n*-3) can also be precursors of azelaic acid [26, 27]. All three peroxidation processes will favor the formation of the saturated DiFA Di9:0 from both PUFAs. In agreement with that the highest amounts of Di9:0 were determined in the treatments with the highest shares of flax seed powder in the feed (FC3 and FC4, Fig. 5a). Moreover, the amount of flaxseed powder (and thereby the PUFAs) correlated with the amount of Di9:0 determined in head tissue (Fig.

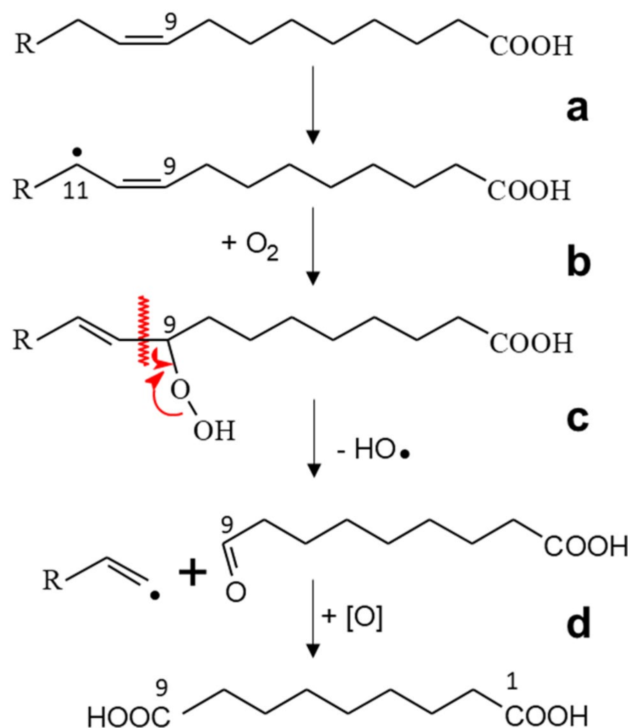

Fig. 6 Proposed mechanism of the formation of Di9:0 initiated by lipid oxidation of a fatty acid showing the first double bond in C-9 position. Several other products are formed, but only the decisive way is shown. **a** Radical formation on C-11 is followed by **b** attack of oxygen on C-9 leading to the hydroperoxide. Finally, **c** α-cleavage on the left hand side (leads to the aldehyde on C-9 which is **d** oxidised) to give Di9:0

S1, S2). Unfortunately, feed samples were not available for analysis. However, Di9:0 was not detected in tilapia fillet, which pointed rather to the formation of Di9:0 in head tissue although a selective transfer and enrichment in this tissue could not be fully ruled out.

Previous reports on azelaic acid (Di9:0) and other DiFAs in fish are scarce. This could be due to the fact that head tissue is not commonly analysed and that DiFAs were not detected in fillet. Yet, DiFAs were previously detected in skin mucus of fish [28]. It was noted that it is produced naturally by *Pityrosporum ovale*, a yeast that lives on skin [29, 30]. Also, *Candida tropicalis*, a ubiquitous yeast, is a known producer of azelaic acid [31]. This pointed towards the involvement of microorganisms in the formation of DiFAs, which are likely more present in head tissue than in fillet. In addition, Di9:0 was shown to occur naturally in whole grain cereals such as barley [32] and sorghum [33, 34] as well as in red and green seaweed [35]. Also in these examples in the literature, involvement of microorganisms in the formation of DiFAs cannot be ruled out. Di9:0 was shown to be involved in the immune-signal pathway, called systemic acquired resistance (SAR) triggering the AZI1 enzyme to prime plants for salicylic acid accumulation and immune-related genes [36] and described in *Arabidopsis* [37]. Furthermore, DiFAs are used as an agent for the therapy of acne in cosmetics [38].

Conclusions

In contrast to the corresponding fillet samples, DiFAs were present in all heads tissue samples of tilapia. Although their formation could not be fully clarified, lipid oxidation of PUFAs were found to be the most relevant precursors, with possible impact of microorganisms. Although oral administration indicated that acelaic acid is a nontoxic substance, which is excreted via urine [36], more data on the occurrence of dicarboxylic acids (DiFAs) in head or rather brain tissue in fish would be important. The methods shown in this article, including the isolation of reference standards by CCC, application of a targeted GC/MS-SIM method and the last but not least, the use of a nonpolar GC stationary phase will be beneficial to reach this goal.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Compliance with ethics requirements This article does not contain any studies with human subjects. Fish samples were obtained from a fish farm producing for human consumption.

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