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Identification of Fatty Acid Methyl Esters as Minor Components in Fish Oil by Multidimensional GC-MSD: New Furan Fatty Acids

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ABSTRACT

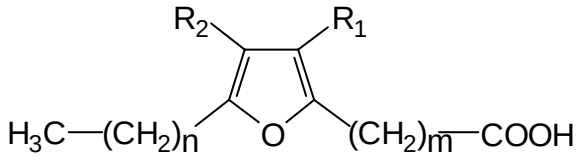
The separation and analysis of very low concentrated furan fatty acids and other minor component fatty acids in complex sample matrices, such as fish oil or lipids derived from liver and testes, require several pre-analytical separation steps in order to obtain sufficient resolution in single column gas chromatography: after extraction and transesterification hydrogenation, urea complex precipitation and Ag^+-TLC were applied prior to GC-analysis of furan fatty acids.

By using a multidimensional GC-MSD-System with cooled injection and flow controlled column switching with cold trapping in between, it is now possible to identify directly the methyl esters of furan fatty acids without any further pre-analytical separations.

The most common of the furan fatty acids can be subdivided into two groups, bearing either a propyl or a pentyl side chain in the 5-position of the furan ring. In addition to the known eight furan fatty acids in fish oil, six new ones were identified, four with a propyl and two with a pentyl side chain. Four of them were reported earlier to be found in the hepatopancreas of crayfish and in fish tissue, whereas the propyl group 16,19-epoxy-17,18-dimethyldocosa-16,18-dienoic acid and the pentyl group furan fatty acid 6,9-epoxy-7-methyltetradeca-6,8-dienoic acid are hitherto unknown ones.

INTRODUCTION

Furan fatty acids (F-acids, **Figure 1**) were found for the first time in *Exocarpus* seed oil in 1966 [1] and subsequently synthesized [2,3]. A series of propyl- and pentyl-side chain F-acids (F1-F8, F-acid 11, **Table I**) were later demonstrated to be present in different species of fish [4-9], in soft corals [10], in different plants [11,12], vegetable oils [13,14] and in mammals [15], including man [16]. Studies on the hepatopancreatic lipids of the crayfish *Procambarus clarkii* revealed a total of 30 F-acids, including tri- and tetrasubstituted ones [17,18]. The only 2,5-disubstituted F-acid so far reported, is the one found in *exocarpus* seed oil [1].



R_1, R_2:	H,CH_3
m:	2,4,6-12,14
n:	2-6

Figure 1. *Furan Fatty acids.*

The dominant F-acid in all material examined always was F6 followed by F4 and F2 in fish oil [9], by F5 and F2 in fish liver and testes [6], and by F3 and F4 in man [16] and crayfish [18].

Studies on the distribution of F-acids in the different lipid classes revealed a distinct pattern: in fish, F-acids account for 26% of total lipids in liver as the dominant cholesteryl ester and for 43% of total lipids in testes, where they are confined almost exclusively to the triglycerides [6]. In the hepatopancreas of crayfish the F-acids were found mainly in the fraction of cholesteryl ester and only traces in the triglycerides and phospholipids [18]. On the other hand the main class of lipids in human and bovine plasma were the phospholipids [16]. The transportation of F-acids in fish from the liver to the testes at spawning time [6,18] pointed out a possible, as yet not proven physiological role.

The presence of dibasic furanpropionic acids in human urine [19] and blood [20] caused further metabolic studies in which the F-acids were regarded as possible precursors [21-24]. 3-Carboxy-4-methyl-5-propyl-2-furanpropionic acid as one of the two major furanpropionic acids proved to be a metabolite with several adverse biochemical and physiological effects [25,26], especially in connection with chronic renal failure, where it is found at highly elevated levels in plasma [27-30]. The analysis of F-acids is hampered by two facts: in most cases as in fish oil they amount to only 1% or less of total fatty acids [7,9] and in spite of their structural difference their methyl esters show similar chromatographic behaviour as several highly concentrated straight chain monoene or polyene fatty acids.

In order to obtain sufficient resolution in single column gas chromatography several pre-analytical steps were applied in the past: after extraction and transesterification selective hydrogenation with PtO_2 under 56 kPa hydrogen pressure in chloroform [4,5] or in methanol [15] for 20 min affected only the unsaturated fatty acids and left the furan ring of virtually all F-acids unsaturated. Other catalysts used were 5% Pd-charcoal in methanol or Lindlar catalyst in hexane under atmospheric hydrogen pressure for 3 hours [17,18].

Following hydrogenation urea fractionation was used to separate F-acids from saturated straight chain fatty acids, as the F-acids methyl ester do not form urea inclusion complexes [4,5,7-9,15,17,18]. An alternative way of separating the F-acids from unsaturated fatty acids is offered by Ag^+-TLC, where the F-acids methyl esters migrate closely behind the saturated and mono-ene ones [1,5-9,12]. The mixture of F-acids, saturated, and mono-ene fatty acid methyl esters can again be separated by urea fractionation. Yet another method applied is the hydrogenation of the furan ring with 5% Rh-aluminumoxide in hexane under 150 kPa hydrogen pressure for 2 hours, yielding tetrahydrofuran-derivatives. The now different chromatographic behaviour allows identification and quantitation by single column chromatography [11,16].

This report describes a method for the analysis of minor components in complex sample matrices such as fish oil. After transesterification by alkaline methanolysis furan fatty acid methyl esters can directly be identified by the use of a multidimensional GC-MSD system with cooled injection and flow controlled column switching.

EXPERIMENTAL

Sample preparation. All steps were carried out under a nitrogen atmosphere to avoid oxidation of the polyunsaturated fatty acids. 500 mg of fish oil (EICOSAPEN®, Hormon-Chemie München GmbH, Germany) were transesterified by alkaline methanolysis [31], extracted with toluene and concentrated to a final volume of about 10 ml.

GC-MS system. The fatty acid methyl esters were first analyzed by gas chromatography - mass spectrometry. Electron-impact ionization was performed with a Hewlett-Packard (Waldbronn, FRG) HP 5890/5971 GC-MS system equipped with a HP 7673 automatic sampler and a 25 m x 0.2 mm i.d. HP-1 (dimethylpolysiloxane) column. The column head pressure was set to 60 kPa and the injection volume was 1 µl with a split ratio of 1:10. The injector and the transfer line temperatures were 280 and 290°C, respectively; after injection the column temperature was programmed from 130 to 260°C at 2°C/min and then at 40°C/min to 300°C, held for 20 min.

Multidimensional GC-GC-MSD. **Figure 2** represents a scheme of the various components used to configure the system employed for this work. The apparatus consists of a temperature programmable cold injection system (CIS-3, Gerstel GmbH, Mülheim an der Ruhr, Germany), two HP 5890 GC ovens (Hewlett-Packard, Avondale, USA), connected by a cryotrap interface (CTS-1, Gerstel GmbH, Mülheim an der Ruhr, Germany). The second oven is equipped with a mass selective detector (HP 5971 A, Hewlett-Packard, Avondale, USA).

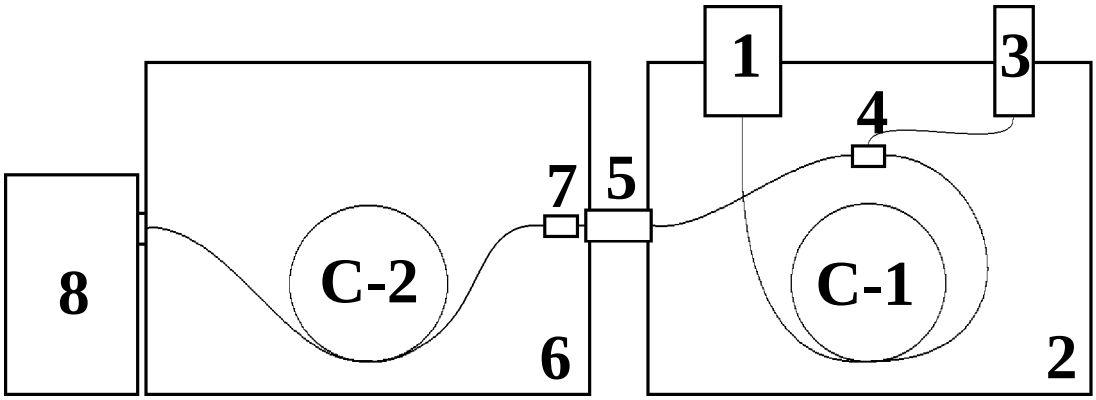


Figure 2. *Schematic diagram of the applied system which consists of a temperature programmable cold injection system with a septumless sampling head (1), a GC (2) configured with a monitor FID (3), column switching device (4) and pneumatics, connected via a heated transfer line incorporating a cryotrap (5) to a second GC (6) which has a second switching device (7) installed after the the transfer line with the main column to the MSD (8).*

Analysis conditions.

Columns:

Pre-column in GC 1	25 m HP-1 (Hewlett-Packard)		
	d_i = 0,32 mm	d_f = 1,05 µm	
Main column in GC 2	30 m Stabilwax (Restek Corp.)		
	d_i = 0,25 mm	d_f = 0,25 µm	

Pneumatics:

Carriergas	He	p_i = 130 kPa	split x:20
Control flow		p_c = 40 kPa	10 ml/min
		p_{c1} = 35 kPa	
FID	H_2, 30 ml/min	Air, 300 ml/min	N_2, 30 ml/min

Temperatures:

CIS	80°C;	↗ 300°C;	12°C/s
Oven 1	200°C;	↗ 300°C;	5°C/min
Oven 2	180°C;	↗ 240°C;	5°C/min
CTS	280°C;	↘ -150°C;	12°C/s
		↗ 280°C;	12°C/s
FID	320°C		
MSD	280°C		

Detectors:

Monitor detector in GC 1	FID	
Main detector in GC 2	MSD	(Mass-selective)
		Scan 50-450 amu

For column switching and transfer of cuts the cryotrap is cooled with LN_2 from its normal temperature of 200°C to -150°C two minutes before the cut, followed by heating at 12°C/second to 280°C for "re-injection" of the focused cut to the second column and to the MSD.

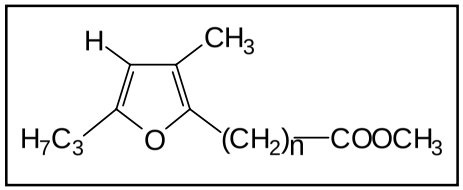
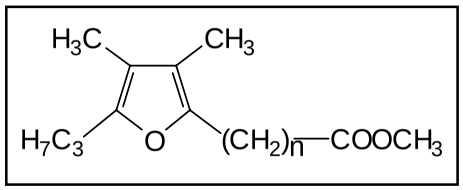
Results and Discussion

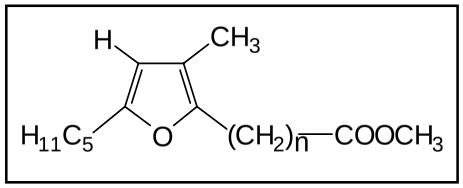
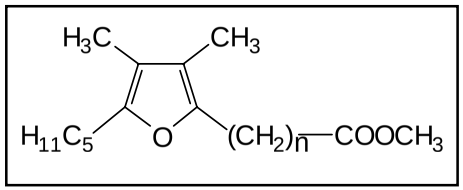
Fish oil composition. In this study the multidimensional GC-MSD system was used for the identification of furan fatty acids and only relative amounts were reported for the fatty acids composition of the fish oil investigated:

(a) more than 30 fatty acids can be identified, ranging from C 12 to C 26, including odd numbers of carbon atoms;
(b) saturated acids (25%) are mainly palmitic (18%), followed by myristic (5%) and stearic (3%);
(c) monoene acids (24%) include isomeric C 18:1 (13%), C 16:1 (9%) and C 20:1 (2%) next to a few minor ones;
(d) in the group of polyene families the two major fatty acids are 5,8,11,14,17-eicosapentaenoic (ω-3 C 20:5, EPA, 20%) and 4,7,10,13,16,19-docosahexaenoic acid (ω-3 C-22:6, DHA, 13%);
(e) F-acid 10 was the most abundant furan fatty acid (0.2% of total fatty acids), followed by F-acid 4, 2 and 5 (**Table I**).

Single column GC-MSD. Considering the small amounts of F-acids expected in the fish oil sample the column had to be overloaded a bit to detect all the F-acids. This chromatogram (**Figure 3**) was used to determine the conditions for the first column acquisition parameters and the settings of the heart-cuts for transfer onto the second column in the multidimensional GC-MSD system. The retention times of the F-acids, evaluated in single column GC-MSD with selected ion monitoring, were used for this purpose (**Table I**). Only the F-acid 10 yielded a separated peak in this chromatogram and showed a fairly good MS-spectrum (**Figure 4**). All other peaks of the F-acids had to be background subtracted and selected ion chromatograms were used to locate them at all in order to set the heart-cut times.

Mass spectra of F-acid methyl esters. The characteristic ions in the mass spectra of F-acid methyl esters are listed in **Table I** and for F-acid 10 shown in **Figure 4** and **5**. Allylic cleavage of the alkylcarboxyl chain at the furan ring produces the base peak m/z 179 (F-acids 7, 10 and 13) and m/z 165 (F-acids 1, 2, 5, 9 and 12) for the pentyl group, m/z 151 (F-acids 4, 8, 11 and 14) and m/z 137 (F-acids 3 and 6) for the propyl group F-acids. Allylic cleavage of the alkyl chain in 5-position yields ions M^+-C_2H_5 and M^+-C_4H_9, respectively. The furan ring itself gives rise to ions m/z 109 for the trisubstituted and m/z 123 for the tetrasubstituted F-acids, produced by cleavage of both allylic positions with hydrogen rearrangement.

Propyl-Furan Fatty Acids	n	Mol. peak (m/z)		$M^+ - C_2H_5$ (m/z)		Base peak (m/z)	Furan ring (m/z)		F-acids	RT (min)	Lit.
	4	238		209		137	109		u	-	-
	6	266		237		137	109		u	-	-
	8	294	(13%)	265	(4%)	137	109	(7%)	3	34.3	[18]
	10	322	(16%)	293	(5%)	137	109	(6%)	6	43.4	[18]
	12	350		321		137	109		u	-	-
	14	378		349		137	109		u	-	-
	4	252		223		151	123		u	-	-
	6	280		251		151	123		n.d.	-	[18]
	8	308	(19%)	279	(25%)	151	123	(10%)	4	39.6	F1
	10	336	(20%)	307	(24%)	151	123	(9%)	8	47.8	F4
	12	364	(25%)	335	(25%)	151	123	(9%)	11	55.5	[8]
	14	392	(34%)	363	(24%)	151	123	(13%)	14	62.5	-

Pentyl-Furan Fatty Acids	n	Mol. peak (m/z)		$M^+ - C_4H_9$ (m/z)		Base peak (m/z)	Furan ring (m/z)		F-acids	RT (min)	Lit.
	4	266	(16%)	209	(8%)	165	109	(8%)	1	24.0	-
	6	294	(16%)	237	(7%)	165	109	(16%)	2	33.1	[18]
	8	322	(15%)	265	(6%)	165	109	(11%)	5	42.2	F2
	10	350	(18%)	293	(6%)	165	109	(12%)	9	50.6	F5
	12	378	(16%)	321	n.d.	165	109	(11%)	12	58.3	F7
	14	406		349		165	109		n.d.	-	[18]
	4	280		223		179	123		n.d.	-	[18]
	6	308		251		179	123		n.d.	-	[18]
	8	336	(28%)	279	(42%)	179	123	(16%)	7	46.8	F3
	10	364	(29%)	307	(34%)	179	123	(17%)	10	54.4	F6
	12	392	(30%)	335	(25%)	179	123	(19%)	13	61.1	F8
	14	420		363		179	123		u	-	-

F-acids : 1-14 detected in fish oil by multidimensional GC-MSD; u: unknown; n.d.: not detected.
Lit. : F1-F8 listed in references [5, 8, 18]; [8] and [18] first reported in crayfish [18] and fish [8].
RT : Retention times for single column GC-MSD.
shaded : new F-acids or found in fish oil for the first time.

Table I. *F-acids methyl ester*

Multidimensional GC-GC-MSD. Here three examples (cut 1, 2 and 3) were choosen to demonstrate the power of the multidimensional technique to detect minor components in a very complex matrix. F-acid 10 (cut 2, **Figure 3**) now is well separated (**Figure 5**) and the mass spectrum is of high quality with no need of background subtraction in contrast to the single column chromatogram (**Figure 4**). F-acid 2, not detectable in single column GC-MSD with selected ion monitoring, could be identified for the first time in fish oil (**Figure 6**). The cut times used for this chromatogram is shown in **Figure 3** (cut 1). In addition to the pentyl side chain F-acid 2 three more propyl side chain furan fatty acids were identified for the first time in fish oil (F-acids 3, 6 and 11, **Table I**).

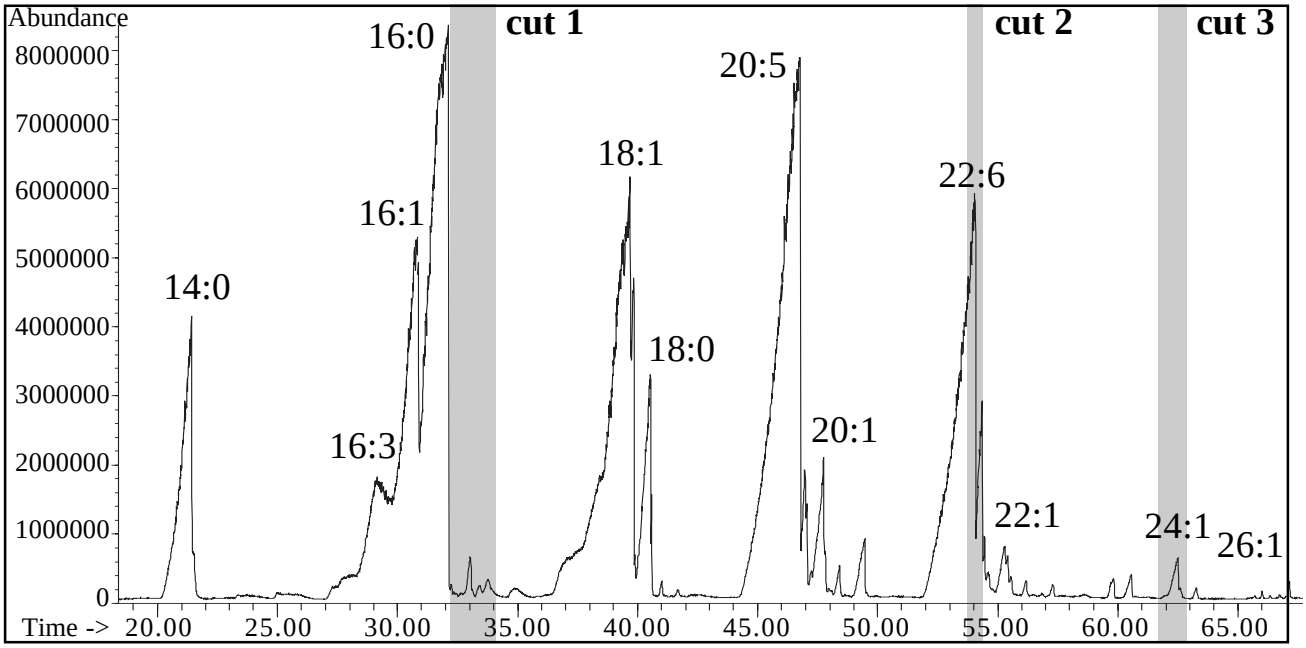


Figure 3. *Total ion chromatogram of single column GC-MSD.*

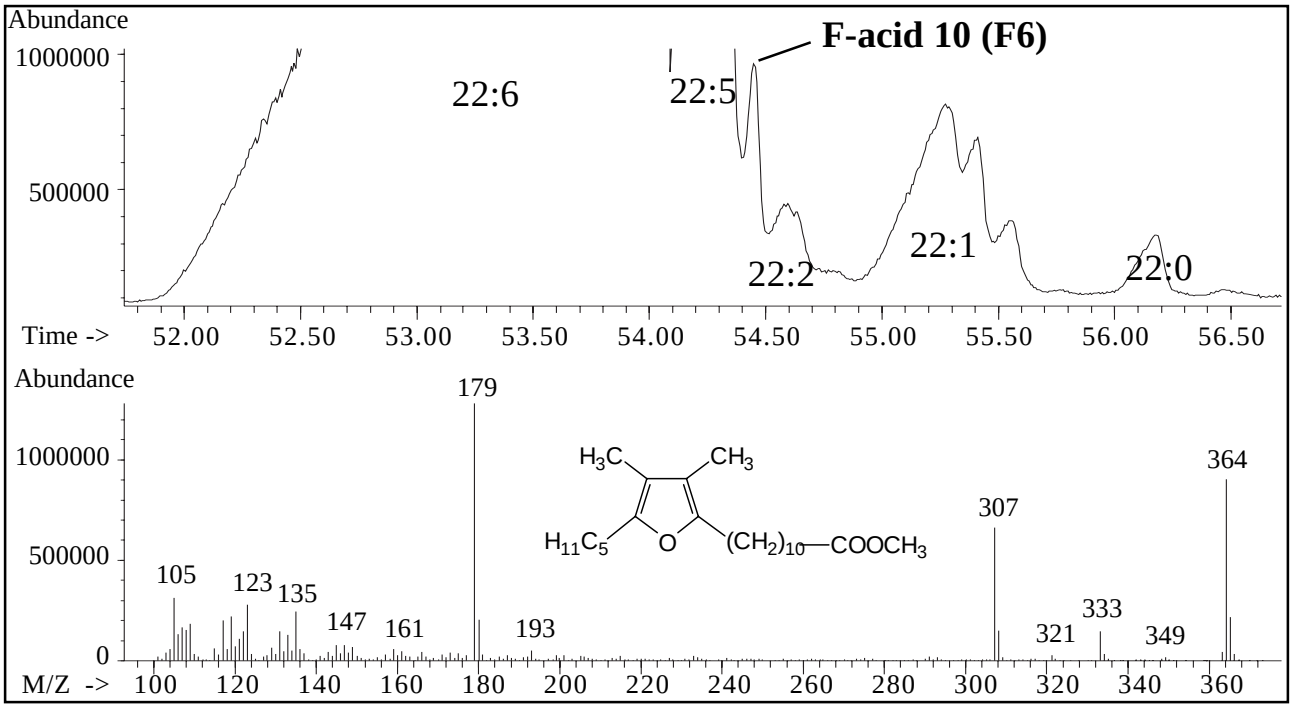


Figure 4. *Identification of F-acid 10 (F6) by single column GC-MSD.*

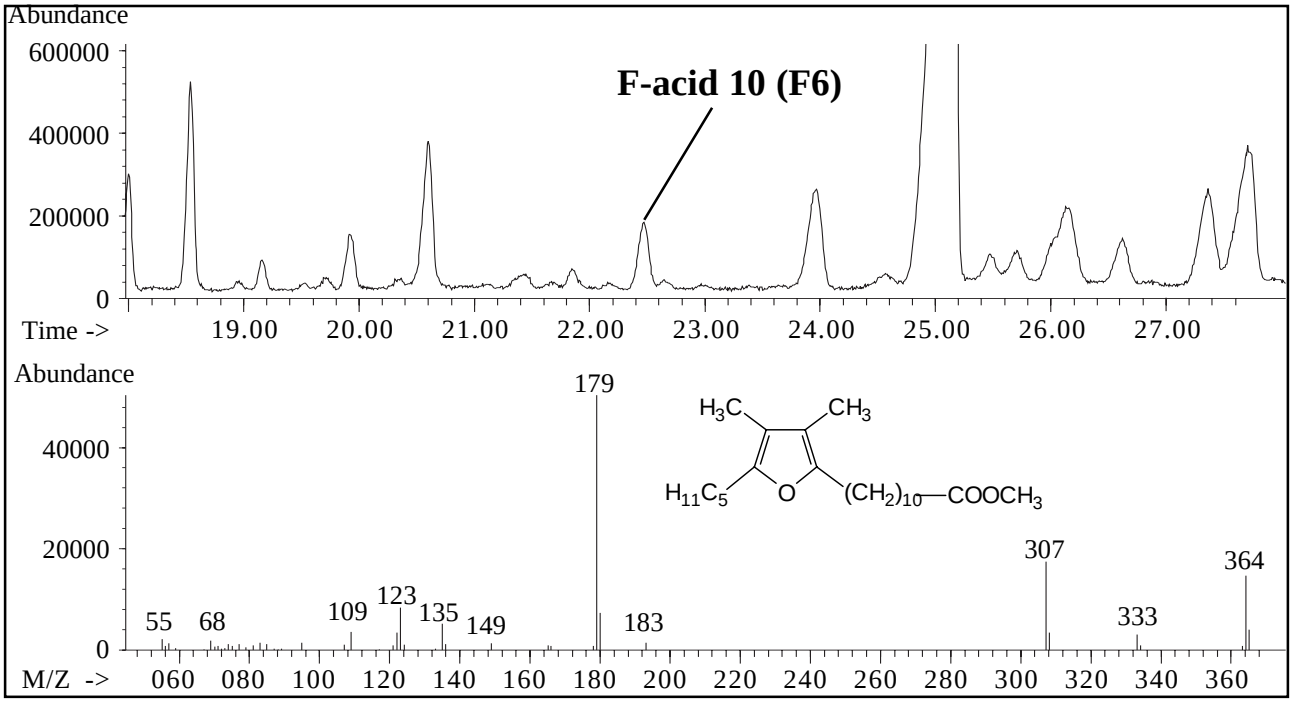


Figure 5. *Identification of F-acid 10 (F6) by multi column GC-GC-MSD (cut 2).*

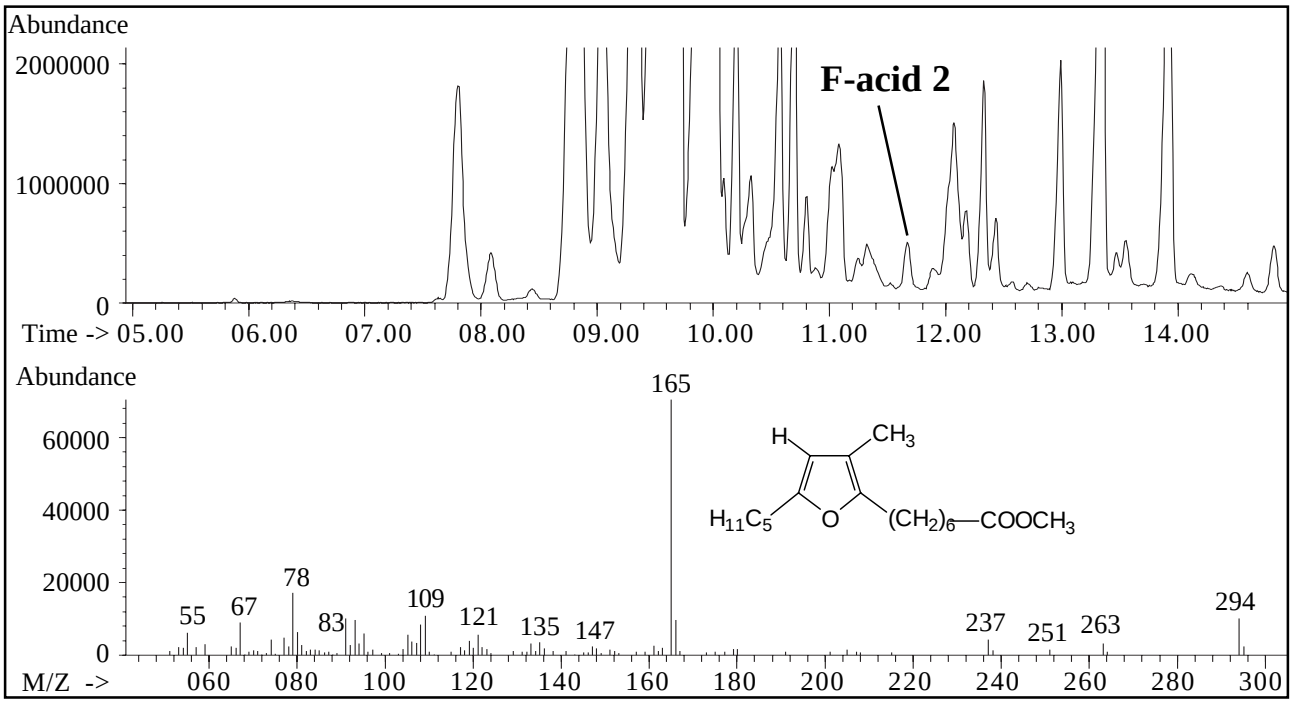


Figure 6. *Identification of F-acid 2 by multi column GC-GC-MSD (cut 1).*

Considering structure, mass fragmentation patterns and retention times of known furan fatty acids we were able to detect two new ones: the pentyl group furan fatty acid 6,9-epoxy-7-methyltetradeca-6,8-dienoic acid (F-acid 1, **Table I**) and 16,19-epoxy-17,18-dimethyldocosa-16,18-dienoic acid, belonging to the propyl group (F-acid 14, **Table I** and **Figure 7**).

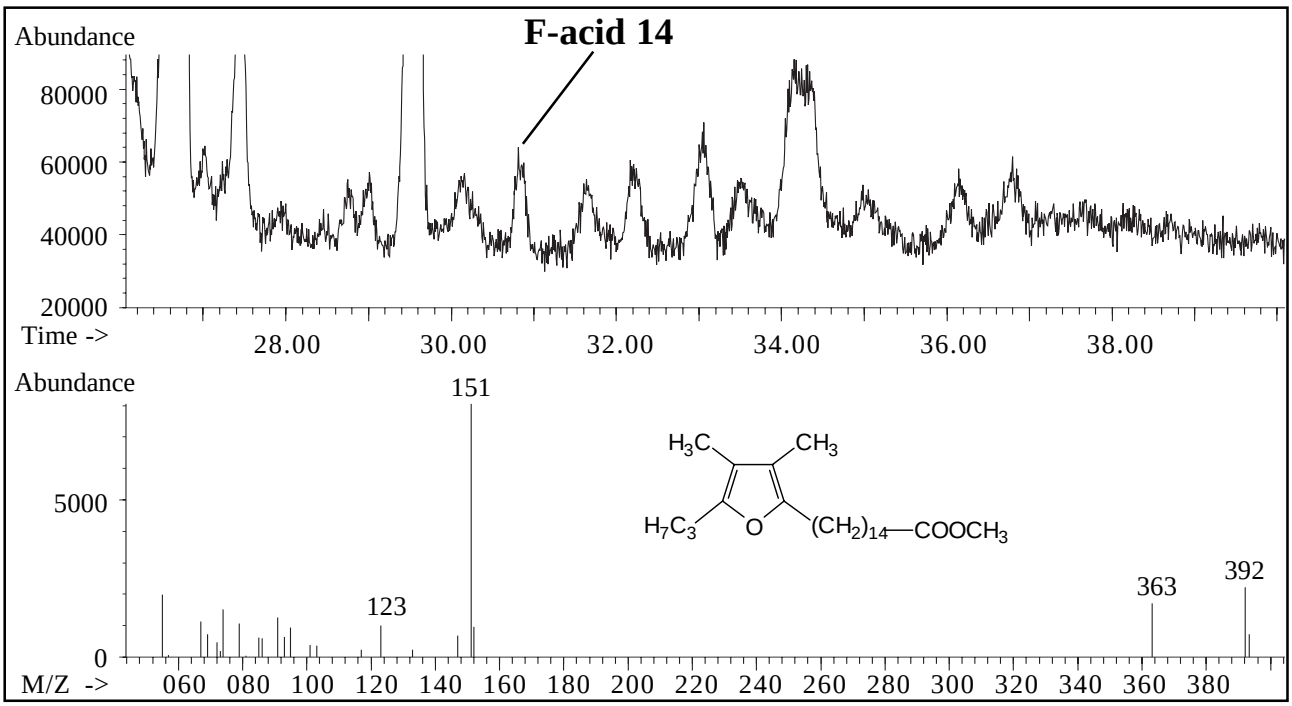


Figure 7. *Identification of F-acid 14 by multi column GC-GC-MSD (cut 3).*

Conclusion

The multidimensional GC-MSD-System described has proven to be a powerful tool for the identification of furan fatty acids in fish oil and can be applied to the analysis of any other low concentrated minor component fatty acids in complex sample matrices such as vegetable oils. It allows to separate small peaks adjacent to or covered by major component peaks just by choosing the right heart-cut time and the use of a focusing cryo-trap in between the two columns. On the other hand chromatographic separation can be improved by combination of different columns in separated GC-ovens with individual temperature programming.

Using this system we were able to detect 14 F-acids in commercially available fish oil, six of them reported for the first time in fish oil and two hitherto unknown ones. The only pre-analytical step was the transesterification, which was done by alkaline methanolysis. The multidimensional GC-MSD system, in this study used only for identification of furan fatty acids, should also be applicable for

quantitation.

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