The pathway of ω-3 fatty acids in hen's eggs

A ^{13}C-NMR-Study

Keywords: NMR-Spectroscopy, Fatty acids, Egg lipids

Abstract:

^{13}C-NMR-spectroscopy is shown to be an excellent tool for analyzing the fatty acid composition of different lipids [1 2 3 4]. The NMR-spectra of egg yolk lecithin and egg oil of DHA-enriched eggs as well as the special diet (single cells) are compared to obtain the differences in the relative amount and the distribution of ω-3 fatty acids in sn-1/3 and sn-2 position of the glyceride backbone. ^{13}C-NMR-spectroscopy allows the quantitation of the total content of ω-3, ω-6, ω-7, ω-9 and saturated fatty acids in a very short time and without chemical treatment of the sample.

Introduction:

Fatty acids are not statistically distributed in the triglycerides, phospholipids or glycolipids of a biological source. That applies to plant sources as well as to animal sources. Even within a single lipid class there are different fatty acid distributions. For instance triglycerides of animals sources show considerable changes in their fatty acid distribution in different organs adapted to their physiological function. It is well known that the fatty acid composition of animals can be controlled by a special diet. This investigation deals with the main lipids in egg yolk, the triglycerides and the phospholipids PC and PE. We used „DHA-eggs", which are commercially available in Germany. These eggs are enriched with ω-3 fatty acids by a special feeding of the hens with single cells. The distribution of ω-3 fatty acids in egg oil and egg yolk lecithin is analysed by ^{13}C-NMR-Spectroscopy and compared to that in the single cells. The well documented NMR-method of analyzing triglycerides can be transferred to glycolipids[5] and phospholipids[6] after a special sample preparation. ^{31}P-NMR spectroscopy[7] was used to quantify the phospholipid distribution of the egg lecithin and the different fractions.

Results:

The experiments have been done twice. We tested two different lots of DHA eggs, one in June 1996 and the other in April 1997. The results are in very good agreement with respect to the fatty acid distribution.

The lipids are separated by column chromatography to obtain purified triglyceride, PC and PE. The fractionation was controlled by ^{1}H- and ^{31}P-NMR-spectroscopy (Fig. 1).

All carbonyl signals of PC and PE are well separated in the ^{13}C-NMR-spectra even in a mixture, actually a separation of this phospholipids is not necessary. As the amount of PE is approx. only a forth in comparison to PC and the ω-3 fatty acids are not the main components, a separation of PC and PE is recommended.

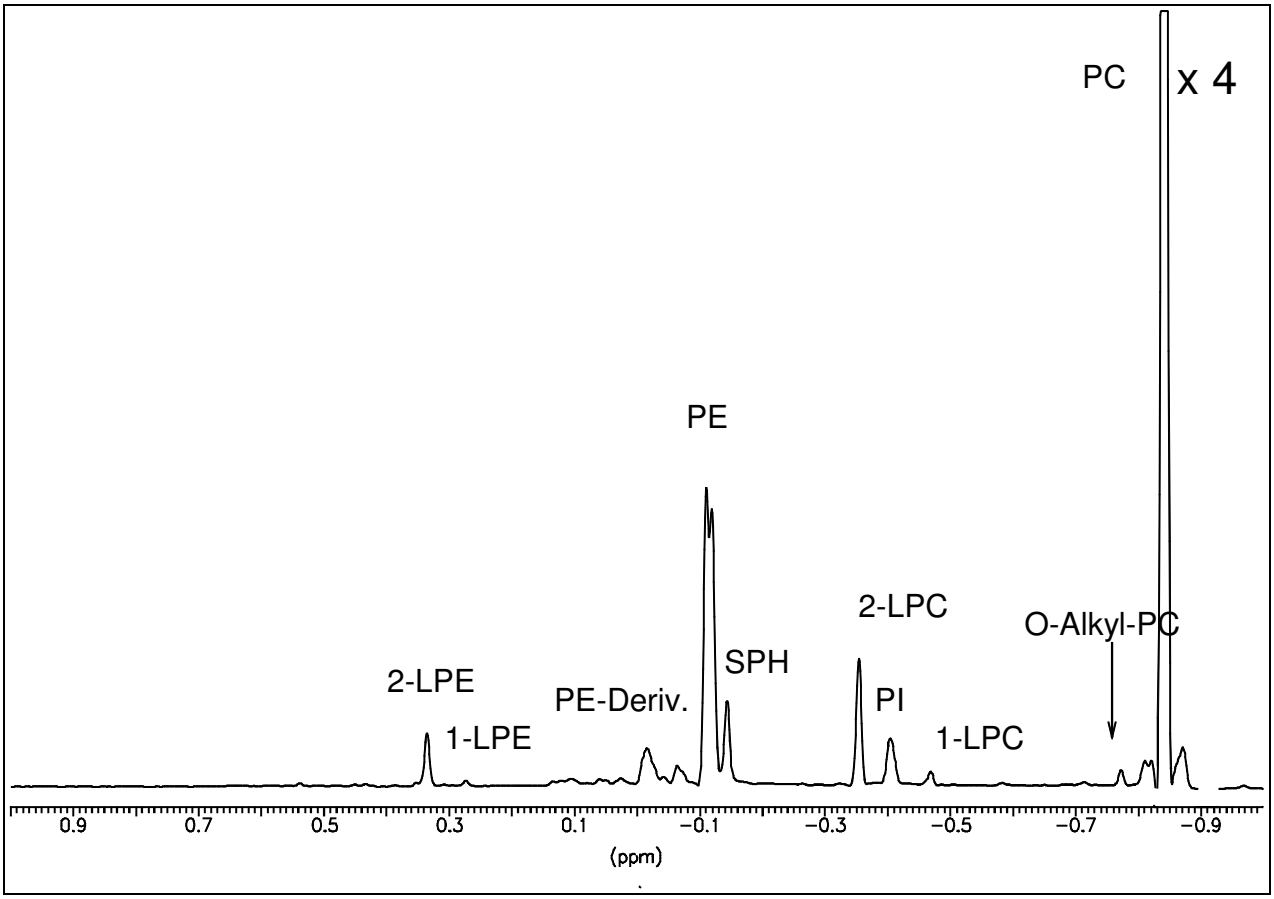

Fig. 1 ^{31}P-NMR-spectrum of DHA egg yolk lecithin

Tab. 1 Molar distribution of phospholipids in DHA eggs

DHA-Egg	PC	O-Alkyl-PC	L-PC	PI	SPH	PE+ Deriv.	L-PE
6/1996	74,2	0,3	4,0	2,2	1,7	15,8	1,8
4/1997	77,0	0,3	3,2	1,6	1,4	15,0	1,4

The ultra high resolved ^{13}C-NMR-spectra of the methyl region of the triglyceride and the phospholipid fraction differentiate between ω-3, ω-6, ω-7, ω-9 and saturated fatty acids. It is possible to quantify the total amount of these different types of fatty acids without any chemical treatment.

Tab. 2 Total ω-n fatty acids (mol%) in triglyceride fraction of common- and DHA-eggs

triglyceride	saturated	ω-9	ω-7	ω-6	ω-3
Common-egg 6/1996	29,6	47,1	5,7	16,8	0,8 (18:3)
Common-egg 8/1996	32,0	44,9	5,2	17,1	0,8 (18:3)
DHA-egg 6/1996	32,4	47,8	4,7	12,7	2,4
DHA-egg 4/1997	28,6	49,6	5,2	15,4	1,2

Tab. 3 Total ω-n fatty acids (mol%) in phospholipid fraction of common- and DHA-eggs

phospholipids	saturated	ω-9	ω-7	ω-6	ω-3
Common-egg 6/1996	47,0	25,5	2,5	22,4	2,7
Common-egg 8/1996	47,2	26,1	3,0	21,0	2,7
DHA-egg 6/1996	52,5	25,5	-	16,0	6,0
DHA-egg 4/1997	49,0	26,7	-	18,3	6,0

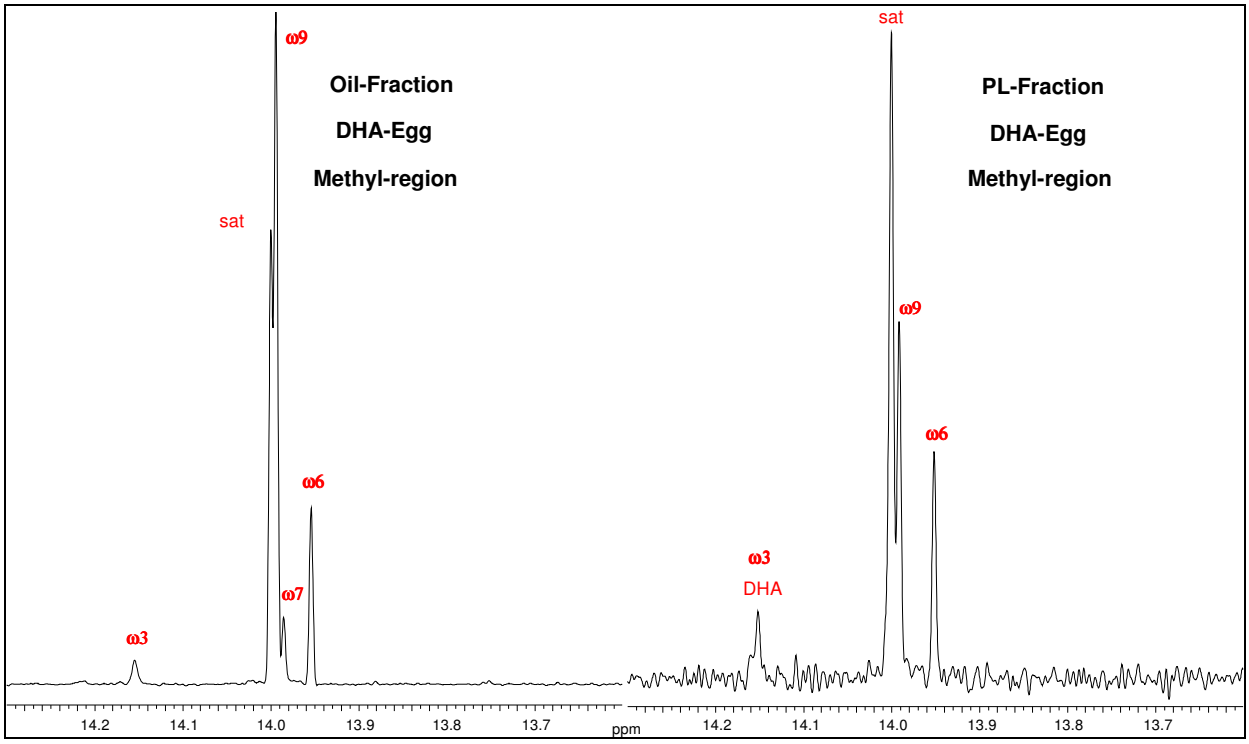

Fig. 2 Methyl region in ^{13}C-NMR-spectra of triglyceride (left) and phospholipid fraction (right)

Using the carbonyl signals of the ^{13}C-NMR-spectra even the position at the glycerol backbone can be determined. The amount of ω-3 fatty acids and especially of DHA is much higher in the DHA-eggs than in those of common feeding. The distribution (mol%) is reported in Tab. 2 and Tab. 3.

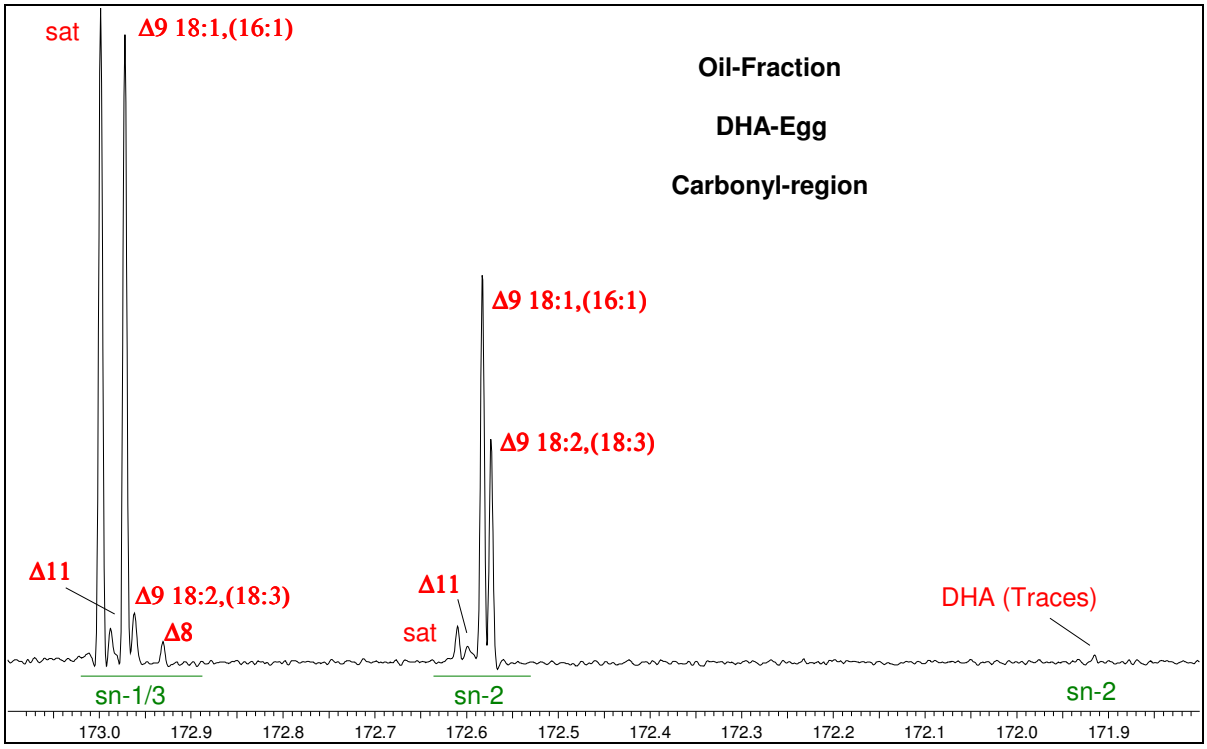

Fig. 3 Carbonyl region in ^{13}C-NMR-spectra of triglyceride fraction of DHA-egg

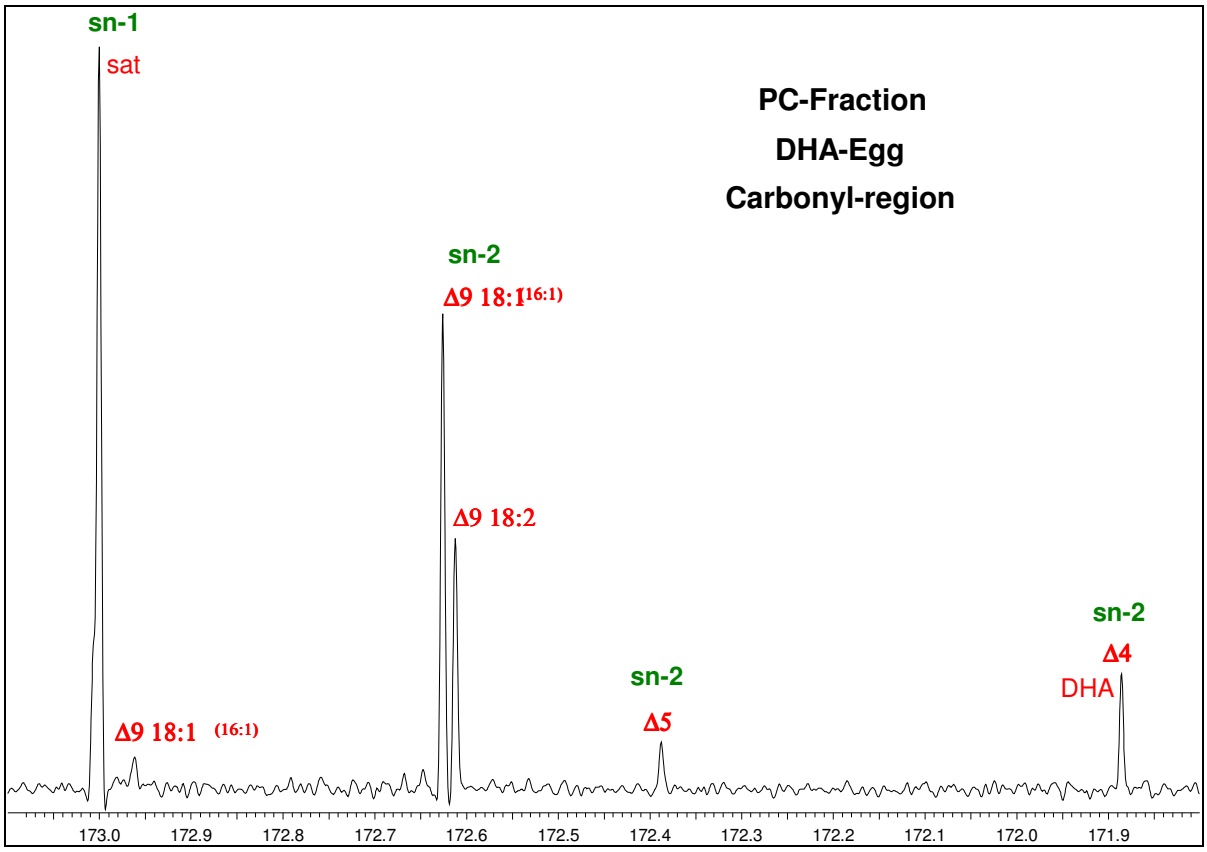

Fig. 4 Carbonyl region in ^{13}C-NMR-spectra of PC fraction of DHA-egg

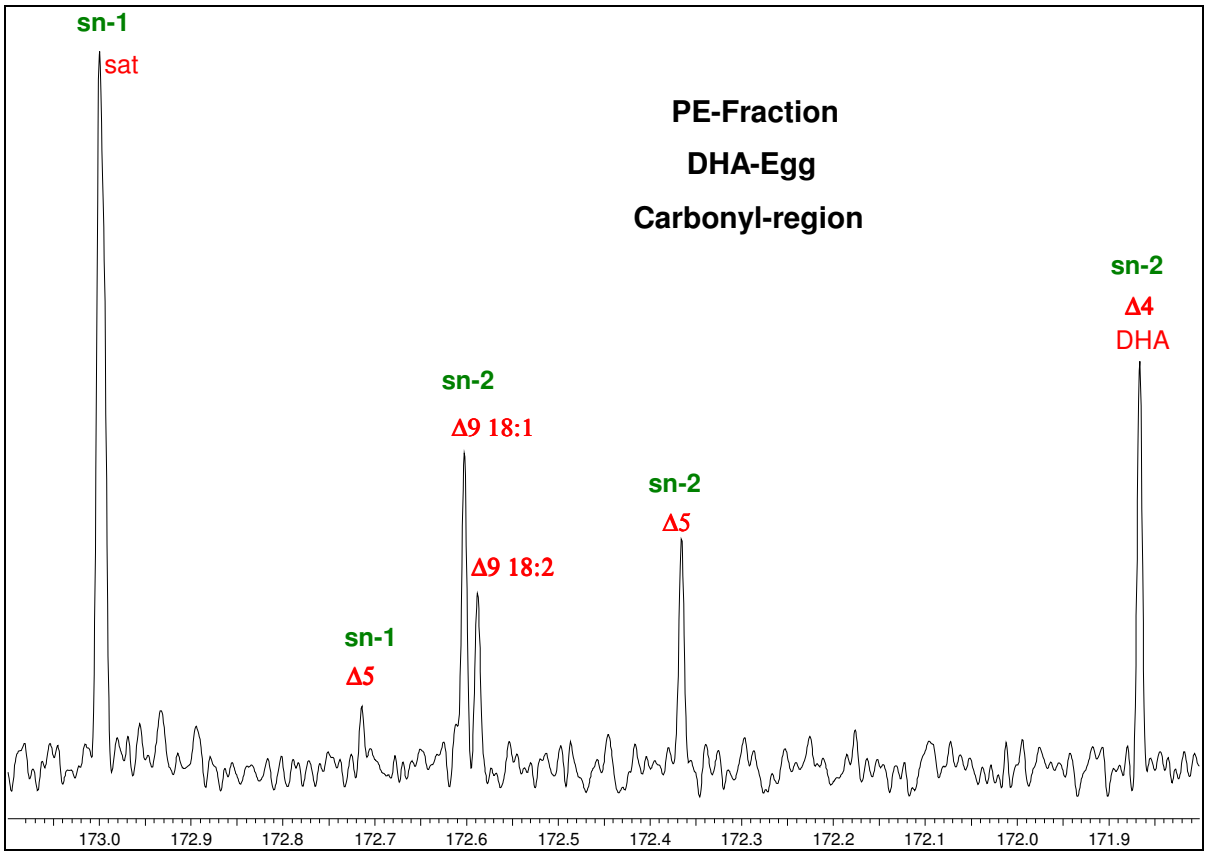

Fig. 5 Carbonyl region in ^{13}C-NMR-spectra of PE fraction of DHA-egg

Tab. 4 Position of fatty acids at the glycerol backbone (6/1996)

6/1996	sat	Δ11	Δ9+Δ8	Δ4	sat	Δ9	Δ5	Δ4
position	sn-1	sn-1	sn-1	sn-1	sn-2	sn-2	sn-2	sn-2
Oil	30,7	1,8	34,2	-	2,8	29,9	-	<0,5
PC	47,6	-	2,4	-	-	41,2	2,8	6,0
PE	50,0	-	-	-	-	23,0	10,0	17,0

Tab. 5 Position of fatty acids at the glycerol backbone (4/1997)

4/1997	sat	Δ11	Δ9+Δ8	Δ4	sat	Δ9	Δ5	Δ4
position	sn-1	sn-1	sn-1	sn-1	sn-2	sn-2	sn-2	sn-2
Oil	30,7	2,0	34,0	-	1,9	31,0	-	<0,5
PC	48,0	-	2,0	-	-	42,2	2,2	5,6
PE	49,5	-	-	-	-	24,8	8,9	16,3

In ^{13}C-NMR-spectra the carbonyl and methyl groups have the longest spin lattice relaxation times of all carbon atoms in a fatty acid or the glycerol. This leads to very sharp signals so that even small differences in the chemical shift of these atoms can be resolved. At 75 MHz the influence of different structures within the chain can be measured over 13 bonds, a differentiation of saturated and Δ13 to Δ4 fatty acids is possible using the carbonyl signals (Fig. 3 to Fig. 5), a differentiation of saturated and **ω**-9 to **ω**-3 fatty acids is successful using the methyl groups (Fig. 2). The evaluation is precise and reliable as there is no response factor within a group of carbonyl signals or a group of methyl signals.

Unfortunately the resolution at a magnetic flux density of 7.050 Tesla (corresponding to 300 MHz proton frequency) allows not to separate different Δ4 fatty acids. The carbonyl signals of DHA (docosahexaenoic acid Δ4,**ω**-3) and DPA (docosapentaenoic acid Δ4,**ω**-6) are in coincidence. The methyl group signals of **ω**-3 or **ω**-6 fatty acids can not be differentiated as well. The methyl signal of ARA (Arachidonic acid Δ5,**ω**-6) coincides with that of DPA. To overcome these problems we have to analyse the double bond signals in addition to the carbonyl atoms. These atoms „feel“ both endings of the chain. Within the region between 135 and 126 ppm all unsaturated and especially the polyunsaturated fatty acids have at least one signal which is not interfering with signals of other fatty acids. From signals with a distance up to 11 atoms from the carbonyl end of the fatty acid a splitting due to sn-1/3 or sn-2 position is detectable and a differentiation of the position even of DHA / DPA mixed oils and phospholipids is possible.

Fig. 6 shows the double bond region of the phospholipids compared with that of oil Fig. 7. The molar ratio of DHA and DPA in the phospholipids is approx. 10:1. In triglyceride fraction only traces of Δ4 fatty acids are found.

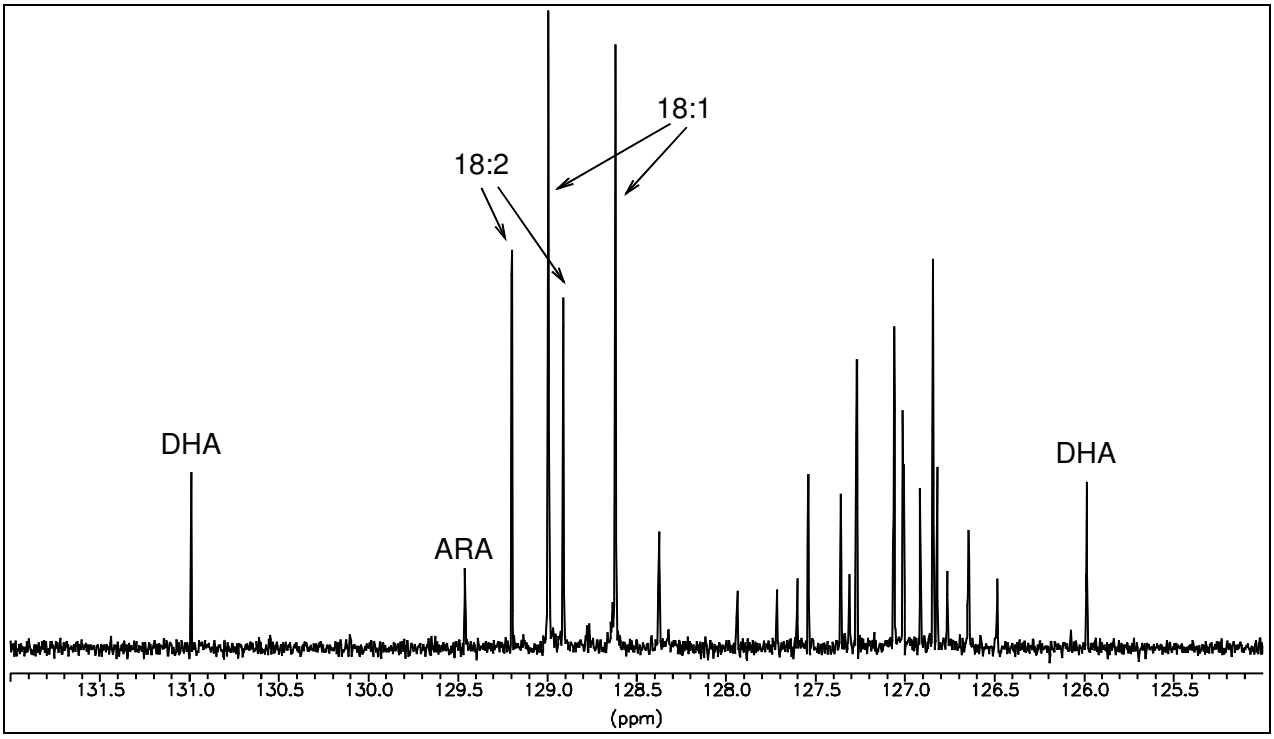

Fig. 6 Double bond region of egg PC

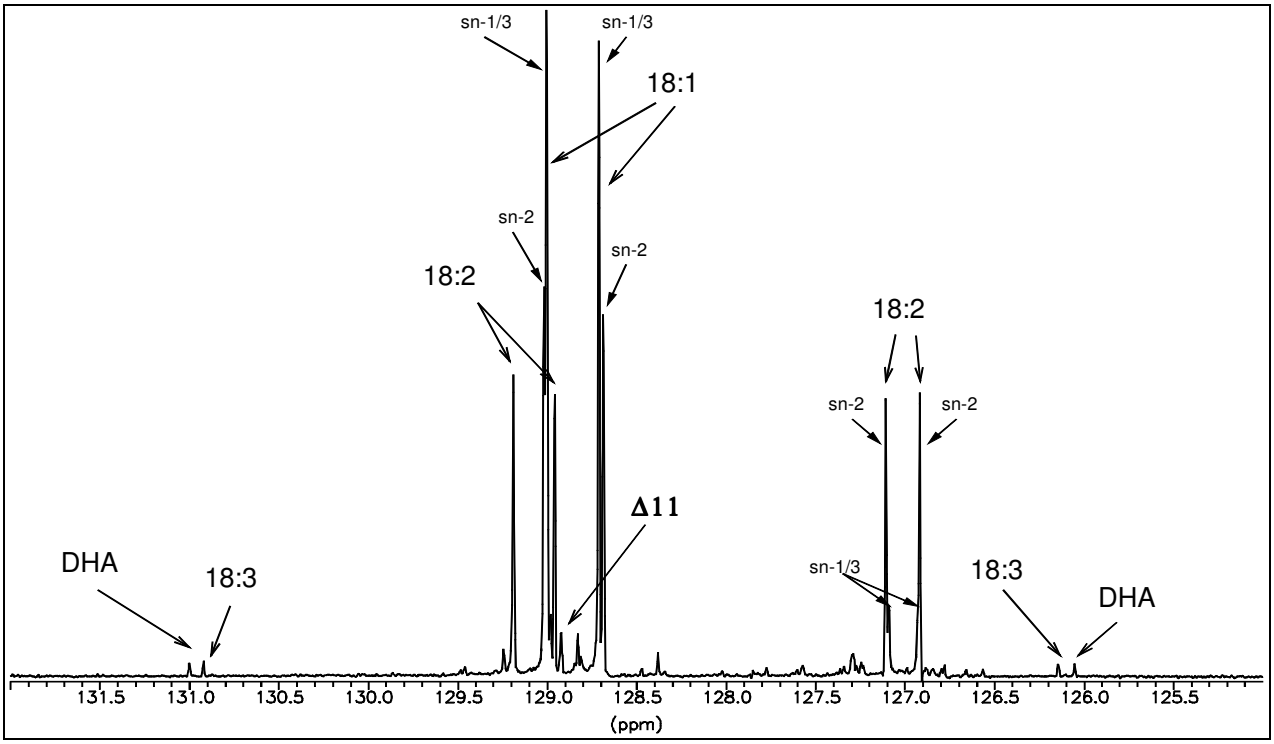

Fig. 7 Double bond region of egg triglyceride

The ^{13}C-NMR-spectra of egg lipids demonstrate the asymmetric distribution of fatty acids. Nearly all high unsaturated fatty acids are found in the phospholipids, the highest amounts in phosphatidylethanolamine (PE). The determination of fatty acids (> 0,5%) using NMR are very precise due to the lack of response factors between different fatty acids within a chosen signal group. This even allows the calibration of FAME standards for GC analysis. An analysis of the SCO-feed is necessary for more detailed information about the pathway of ω–3 fatty acids in egg triglycerides and phospholipids.

The chemical shift and with that the difference between signals is a linear function of the magnetic flux density. Modern NMR-spectrometers have up to 18 Tesla (corresponding to 800 MHz proton frequency), the chemical shift differences increase up to a factor of 2.7 accompanied by a 10 times better sensitivity . In the field of food design by biotechnology an gentechnology the NMR analysis will be an important instrument in the lipid analysis.

Experimental:

Triglycerides and phospholipids were separated using the „acetone soluble/insoluble method“. For purification of PE and PC column chromatograpy on silicagel was used. The $CHCl_3$/MeOH eluent was changed stepwise from pure $CHCl_3$ in several steps to pure methanol. The fractions were controlled by ^{31}P-NMR-spectroscopy.

Sample preparation for NMR measurements:

Triglycerides are dissolved up to 200 mg in 0.5 ml $CDCl_3$ and measured under high and ultra high resolution conditions.

The separated phospholipid samples are dissolved in 0.6 ml Methanol/$CDCl_3$ (1:2 v/v) and 0.4 ml Cs-EDTA-solution are added. The mixture is shaken vigorously. Then 0.2 ml water are added and the sample is shaken again. Two phases are formed. The sample is centrifuged and poured over completely into the NMR tube . If the organic phase (lower) has cleared the measurement of either ^{13}C-NMR-spectra or ^{31}P-NMR-spectra can be started.
Cs-EDTA-solution: 0.2m EDTA in D_2O is titrated with $CsCO_3$ to pH 8,5. This solution is diluted 1:4 v/v with methanol.

All spectra were recorded on AC-P 300 (BRUKER, Karlsruhe, Germany) equipped with automated sample changer and QNP-head for nuclei ^{1}H, ^{13}C, ^{19}F and ^{31}P.

High resolved ^{31}P-NMR-spectra were recorded with CPD proton decoupling and a pulse angle (PW) of 30°. The sweep width (SW) is set to 10 ppm, time domain (TD) is 16k, the acquisition time (AQ) is approx. 5 sec. After resolution enhancement with gaussian multiplication and zero filling the signal of O-Alkyl-PC is baseline separated from the PC signal. For calibration the PC signal is set to -0,84 ppm. In some cases a splitting of phospholipid signals due to the fatty acid composition occurs.

High resolved ^{13}C-NMR-spectra were recorded with minimal 5 sec acquisition time and a TD of 128k. The pulse program use CPD proton decoupling and a PW of 30°. For ultra high resolved spectra of different regions, e.g. carbonyl, methyl or double bonds, a small window of approx. 2 to 10 ppm is used. Long acquisition times are necessary to minimize artifacts of Fourier transformation after resolution enhancement with gaussian multiplication and zero filling. Depending on a good sample preparation and the NMR-parameters the peak to peak resolution of two signals can be in the range of 5 ppb. To compare different lipid spectra the signal of the saturated fatty acid in sn-1/3 position is adjusted to 173 ppm. This is the ester signal in the carbonyl region at lowest field. The molar amounts of fatty acids can be calculated from area ratios of signals.

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