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Quality Control of Krill Oil by Nuclear Magnetic Resonance (NMR) Spectroscopy: Composition and Detection of Foreign Species

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TABLE OF CONTENTS LISTING

The table of contents for the journal will list your paper exactly as it appears below:

Quality Control of Krill Oil by Nuclear Magnetic Resonance (NMR) Spectroscopy: Composition and Detection of Foreign Species
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SPECTROSCOPY

Quality Control of Krill Oil by Nuclear Magnetic Resonance (NMR) Spectroscopy: Composition and Detection of Foreign Species

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ABSTRACT

Krill oil is currently among the most highly promoted products in the dietary supplement market, which, due to its high price, can be potentially adulterated with fish species and artificial oil. For a holistic control of krill oil quality, ^{1}H, ^{13}C, and ^{31}P nuclear magnetic resonance (NMR) spectroscopies were used. The fatty acid and phospholipid composition as well as secondary ingredients, such as homarine, amino acids, and chitin, were examined. The following phospholipid species were detected: phosphatidylcholine (75–85 mol %) and phosphatidylethanolamine (4–7 mol%) and their lyso derivatives 1-lysophosphatidylcholine (1–2 mol%)–2-lysophosphatidylcholine (10–16 mol%) and lysophosphatidylethanolamine (1 mol%). In the -2 position of phospholipids, the content of eicosapentaenoic acid (mean 68.23%; relative standard deviation 2.23%) was twice as high as the content of docosahexaenoic acid (mean 31.77%; relative standard deviation 4.79%). ^{13}C NMR spectroscopy was used to distinguish between krill and fish oil-based dietary supplements. The adulteration of krill oil can be detected by fatty acid distribution in the *sn*-2 triacylglycerol position. The sensitivity of the method is about 10% (w/w) of fish content in blends, which is enough to detect deliberate adulteration. The same methodology can be used to recognize synthetically modified krill oil. The method was successfully applied to 30 commercially available krill and fish oil supplements.

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Introduction

Quality control is an essential component of the food industry, whose aim is to protect customers and to ensure food safety. Furthermore, food quality should comply with food laws and other official regulations, which include appropriate ingredient specifications. Certain food products, such as dietary supplements, are defined by regulations called *standards of identity*, which are responsible for assuring the identity of a product and its ingredients (Vasconcellos 2003).

Due to the high phospholipid content and other beneficial healthy ingredients, in the last few years krill oil and krill oil-based dietary supplements received an increased attention by producers, consumers and researchers (Marik and Varon 2009; Ramprasath et al. 2013; Berge

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et al. 2014; Kwantes and Grundmann 2015). It has been reported that krill oil offers a potential to reduce risk of cardiovascular diseases by lowering serum triglycerides and treatment of conditions, such as hyperlipidemia, inflammation, and arthritis (Marik and Varon 2009; Ramprasath et al. 2013; Berge et al. 2014; Kwantes and Grundmann 2015). Moreover, some promising results were obtained about better bioavailability of eicosapentaenoic and docosahexaenoic acids in krill oil in comparison with fish oils because in krill oil eicosapentaenoic and docosahexaenoic acids were bound primarily to phospholipid species (Ramprasath et al. 2013; Kwantes and Grundmann 2015; Köhler 2016). Among other ingredients, phospholipids, amino acids, and secondary ingredients such as 1-methyl-2-pyridiniumcarboxylate (homarine) and chitin are present in the krill oil matrix and should be routinely determined as well.

Krill oil is currently viewed as a viable alternative to fish oil (Berge et al. 2014). It should be mentioned, however, that krill oil is generally more expensive to produce. Therefore, it has a potential to be adulterated with fish oil (Kwantes and Grundmann 2015). This situation requires the development of assays to determine the animal source of oil and, broadly speaking, to control the ingredient composition of krill-oil dietary supplements. This demand has been confirmed by some studies on commercial fish, krill, and algae oil supplements, where the eicosapentaenoic and docosahexaenoic acids contents were systematically found under the labeled amount (Ritter, Budge, and Jovica 2013; Kleiner, Cladis, and Santerre 2015).

Sophisticated methods such as liquid chromatography electrospray tandem ion trap mass spectrometry (LC–MS/MS), liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS), gas chromatography-mass spectrometry (GC-MS), and HPLC–LC-ESI-MS/MS have been utilized to control the triglycerides, free fatty acid and phospholipid composition in krill oil (Grandois et al. 2009; Zhou et al. 2012a, 2012b; Araujo et al. 2014; Nash, Schlabach, and Nichols 2014; Kleiner, Cladis, and Santerre 2015). In most of these studies, krill oil was analyzed among several other food matrices, therefore, only the concentrations of limited number of samples are available (Zhou et al. 2012a, 2012b; Araujo et al. 2014; Nash, Schlabach, and Nichols 2014). Moreover, there are no studies devoted to distinguishing commercial krill and fish oil capsules.

To fill the gap regarding screening analytical methods, we recently proposed a fast and elegant way to simultaneously determine phospholipids and fatty acids in krill oil by nuclear magnetic resonance (NMR) spectroscopy (Burri et al. 2016). The results were shown only on one representative sample of krill oil. No (semi)quantitative procedure was proposed to detect krill oil adulteration by fish species.

In this article, we proceeded one step further and analyzed a representative set of fish ($n = 15$) and krill ($n = 15$) oil capsules (dietary supplements) available on the German market by NMR including new quality parameters such as eicosapentaenoic and docosahexaenoic acids, as well as other ingredients chitin, homarine, and amino acids. Moreover, regiospecific characterization of lipids obtained during ^{13}C NMR experiments was used to develop a methodology to distinguish samples according to its origin.

Materials and methods

Samples and sample preparation

Off-the-shelf krill ($n = 15$) and fish ($n = 15$) oil capsules were purchased from different retailers in Germany. The sample set for analysis can be seen as a representative for the current dietary supplement market in Germany.

Chloroform-d_1 ($CDCl_3$, degree of deuteration 99.8% with 0.03% (v/v) tetramethylsilane (TMS)), dimethylsulfoxide-d_6 (DMSO, degree of deuteration 99.8% with 0.03% (v/v) TMS), deuteriumoxide (D_2O, degree of deuteration 99.9%) and deuterium chloride (DCl, degree of deuteration 99.9%) were purchased from Euriso-top (Saarbrücken, Germany), ethylenediaminetetraacetic acid (EDTA) was from AppliChem GmbH (Darmstadt, Germany). Triphenylphosphate was purchased from Tokyo Chemical Industry (Tokyo, Japan). Cesium carbonate was obtained from Rockwood Lithium GmbH (Langelsheim, Germany).

The stock Cs-EDTA solution was prepared by weighting of approximately 2.9 g of EDTA and 6 g of Cs_2CO_3 and dissolving in D_2O. The pH was adjusted to 9.0. Volume ratios in mixed solvents were used for sample preparation.

Water-soluble compounds

For the detection of homarine and its degradation product methylpyridinium chloride, 200 mg of krill oil was dissolved in 1 ml $CDCl_3$/DMSO (4:1) and was directly measured.

For the determination of chitin and amino acid profiling, 100 mg of krill oil were hydrolyzed in 1 ml DCl for three hours at 100°C. The solvent was evaporated and the residue was dissolved in 1 ml D_2O.

Nonpolar compounds

For the determination of the phospholipids, 600 mg krill oil and 25 mg triphenyl phosphate were dissolved in $CDCl_3$, MeOD, and Cs-EDTA/D_2O in a ratio of 3:5:5. For the analysis of the fatty acid profile 100 mg of the krill oil were dissolved in 1 ml $CDCl_3$/MeOD in a 1:1 ratio.

To determine the animal origin of krill oil, 300 mg of a sample were dissolved in 1 ml $CDCl_3$ and were directly measured. A series of blends with a total weight of 300 mg and 5, 10, 25, 50, and 75% (w/w) of fish species was prepared from representative krill and fish oil supplements.

NMR measurements

Nuclear magnetic resonance measurements were performed using 500 MHz Avance III and 600 MHz Avance III spectrometers (Bruker, Karlsruhe, Germany) equipped with BBO Prodigy cryo and QNP cryo probes, respectively. The data were recorded automatically under the control of ICON-NMR (Bruker Biospin, Rheinstetten, Germany).

The following parameters were used for acquisition of water-soluble compounds by ^{1}H NMR spectrometry: 32 scans, 128k time domain, 12,020 Hz spectral width, relaxation time of 1 s, and acquisition time of 5.45 s.

The following parameters were used for acquisition of ^{13}C NMR spectra for krill oil authentication regarding animal origin: 256 scans, 256k time domain, 39,060 Hz spectral width, relaxation time of 2 s, and acquisition time of 3.36 s. ^{31}P NMR parameters were described in Burri et al. (2016). Totally, 2048 scans were necessary to authenticate animal origin of krill oil.

The acquisition of ^{31}P NMR spectra for accurate quantification of phospholipids was previously described in Burri et al. (2016). 2D NMR (P,H correlation spectroscopy) experiments as well as comparison with the 1D NMR spectra of standard solutions were used for signal assignments.

Results and discussion

Qualitative and quantitative analysis of krill oil ingredients

Holistic control involves the determination of different components such as phospholipids, fatty acids, amino acids and secondary ingredients. Some of these quality parameters were examined with ^{1}H, ^{13}C, and ^{31}P NMR spectroscopy and have been published by Burri et al. (2016).

One of the most important information is the distribution of fatty acids and phospholipids. Recently a comprehensive review on NMR techniques used for structural and analytical characterization of unsaturated lipids was published (Alexandri et al. 2017). By comparing the signals of –COOH (δ 179.0–180.5 ppm) and –COOR (δ 172.0–174.5 ppm) in the ^{13}C NMR spectrum, the ratio of free and esterified fatty acids such as mono-, di- and triglyceride as well as fatty acids bound to phospholipids was determined. In the examined krill oils on average 9.38% (RSD 74.34%) of the fatty acids were present in their free form and 90.6 ± 7.7% in their esterified form, Figure 1. On an average, 29.2 ± 12.7% of the free fatty acids was eicosapentaenoic acid, 19.8 ± 13.4% docosahexaenoic acid and 50.9 ± 11.3% other fatty acids such as saturated and ω9-fatty acids. N-3 polyunsaturated fatty acids were dominated by eicosapentaenoic and docosahexaenoic acids. Eicosapentaenoic and docosahexaenoic acids were asymmetrically distributed in the nonpolar triglyceride and the polar phospholipid parts, highly concentrated in the polar lipid part of krill oil, with an accumulation in the *sn*-2 position in the triglyceride and phospholipids. In the *sn*-2 position of phospholipids the eicosapentaenoic acid content (mean 68.2 ± 2.2%) was twice as high as the docosahexaenoic acids content (mean 31.8 ± 4.8%).

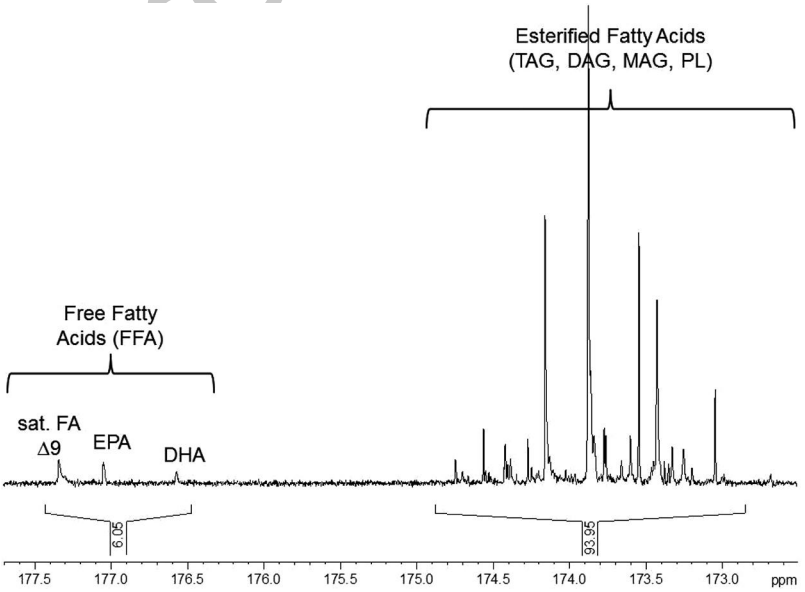

Figure 1. Details of the ^{13}C NMR spectrum of krill oil acquired in $CDCl_3$:MeOD (1:1) mixture; free (–COOH) and esterified (–COOR) fatty acids. *Note*: DAG, diglyceride; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acids; MAG, monoglyceride; PL, phospholipids; TAG, triglyceride.

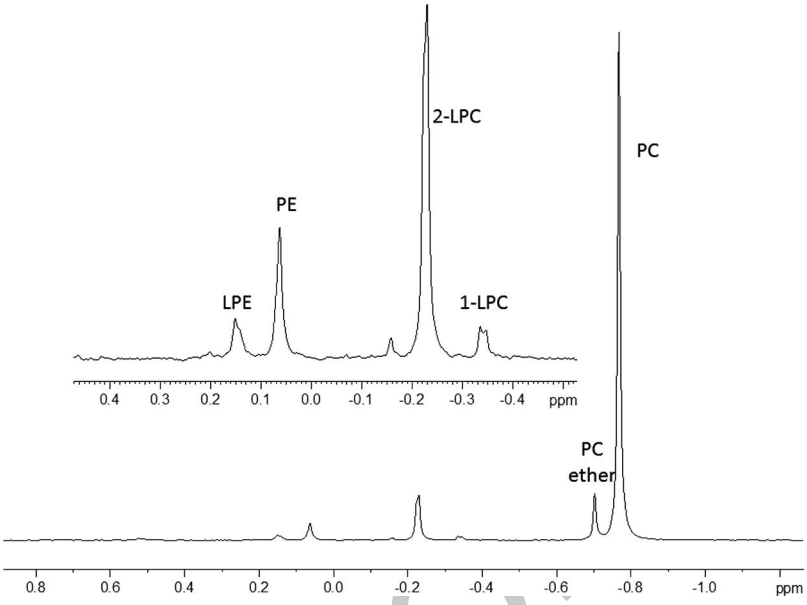

Figure 2. ^{31}P NMR spectrum of krill oil with the assignments of several phospholipid species and their ethers. *Note*: LPE, lysophosphatidylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; 1-LPC, 1-lysophosphatidylcholine; 2-LPC, 2-lysophosphatidylcholine. The organic phase obtained from the liquid–liquid extraction (ternary solvent mixture $CDCl_3$:MeOD:Cs-EDTA:D2O = 3:5:5) was used.

An example of ^{31}P NMR spectrum of a krill oil sample with the signal assignment is presented in Figure 2. A representative set of krill oil samples ($n = 15$) was analyzed according to the previously described validated procedure (Burri et al. 2016). The results were summarized for each individual phospholipid species as mean values together with the standard deviation (Table 1). According to our quantitative analysis, krill oil is a mixture of several phospholipid species being dominated by phosphatidylcholine (75–85 mol%) and phosphatidylethanolamine (4–7 mol%) and their lyso derivatives 1-lysophosphatidylcholine (1–2 mol%)–2-lysophosphatidylcholine (10–16 mol%) and lysophosphatidylethanolamine (1 mol%) (Table 1). The variation in phospholipid distribution depended on the krill and on the oil extraction process. Traces of other phospholipids such

Table 1. Mean values (%, w/w), standard deviation (%, w/w) and relative standard deviation (%), maximum (%, w/w) and minimum values (%, w/w) for the individual phospholipid species and the total phosphorus amount in krill oil capsules ($n = 15$).

	Maximum (%, w/w)	Minimum (%, w/w)	Mean (%, w/w)	Standard deviation (%, w/w)	Relative standard deviation (%)
Phosphatidylcholine	26.6	12.0	18.2	4.0	22.0
1-Lysohosphatidylcholine	0.7	0.1	0.4	0.2	43.3
2-Lysohosphatidylcholine	5.1	0.6	2.9	1.2	42.8
Phosphatidylethanolamine	2.3	0.4	1.5	0.5	32.2
Lysophosphatidylethanolamine	0.4	0.1	0.2	0.1	40.3
Acylphosphatidylethanolamine	2.0	0.2	1.3	0.4	32.5
Other phospholipids	1.6	0.03	0.5	0.4	71.8
Sum	37.1	16.8	25.0	4.7	18.7
Phosphorus	1.6	0.7	1.0	0.2	19.1

as phosphatidylserine, phosphatidylglycerol, and diphosphatidylglycerol/cardiolipin were also present in the investigated samples. The detection of a signal of phosphatidic acid was an indication for degradation, which was not present in the examined krill oil samples.

Using ^{1}H NMR spectroscopy, other ingredients were detected in krill oil such as homarine and its degradation product methylpyridinium chloride, as well as chitin, peptide-bound amino acids, and ethanol as a by-product from processing. By comparing the ^{1}H NMR spectra from an extract of fresh and processed krill oil, it was determined that nonprocessed krill oil contained homarine, whereas process krill oil such as krill oil capsules contained in addition the degradation product methylpyridinium chloride (Figure 3). According to our observations, the ratio of homarine and methylpyridinium chloride was dependent on the process sequence.

Another crustacean-specific ingredient is chitin, which is a primary component of the exoskeletons of crustaceans. This is an interesting quality parameter because the interaction of chitin has a great importance in the research of immune system response (Gómez-Casado and Díaz-Perales 2016; Rovenich, Zuccaro, and Thomma 2016). The very slight water solubility of chitin complicated the analysis. To overcome this challenge, krill oil samples were hydrolyzed for several hours. The hydrolyzed chitin was more soluble and thus its qualitative determination in krill oil was possible (Figure 4). The exact contents of homarine and chitin in krill oil were not quantified because they vary significantly among capsules.

The results indicated that peptides and not free amino acids were the dominant f krill proteins (Figure 5). To determine the total amino acid composition of peptides and

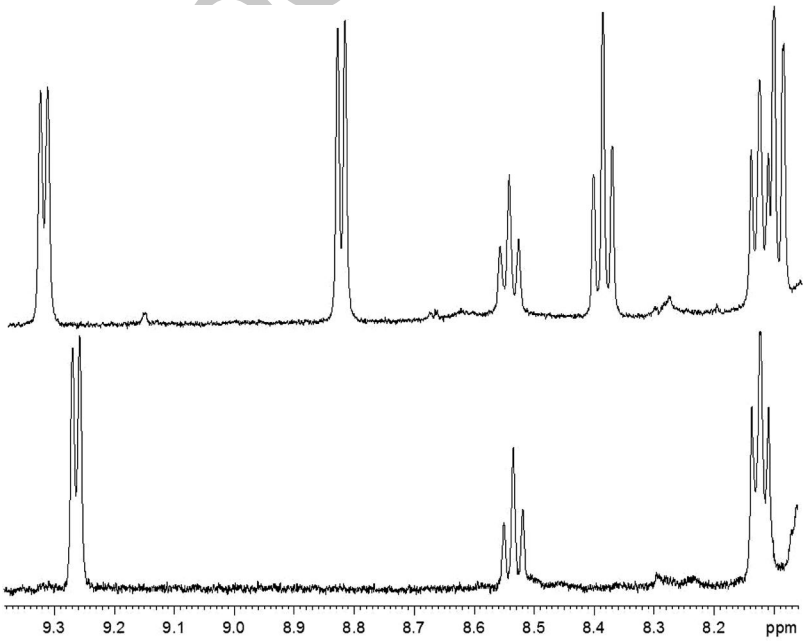

Figure 3. Details of the ^{1}H NMR spectra of krill oil samples with homarine (bottom) in fresh krill oil, and with its degradation product methylpyridinium chloride (top) in processed krill oil. Both samples were dissolved in the binary solvent $CDCl_3$/DMSO (4:1).

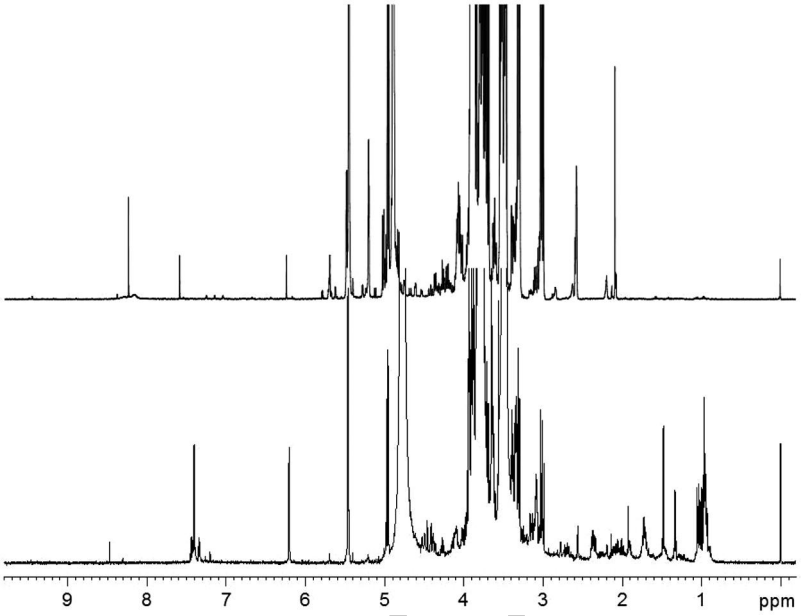

Figure 4. ^{1}H NMR spectra of hydrolyzed krill oil sample (bottom) and hydrolyzed chitin (top). The hydrolysis conditions are described in "Materials and Methods" section.

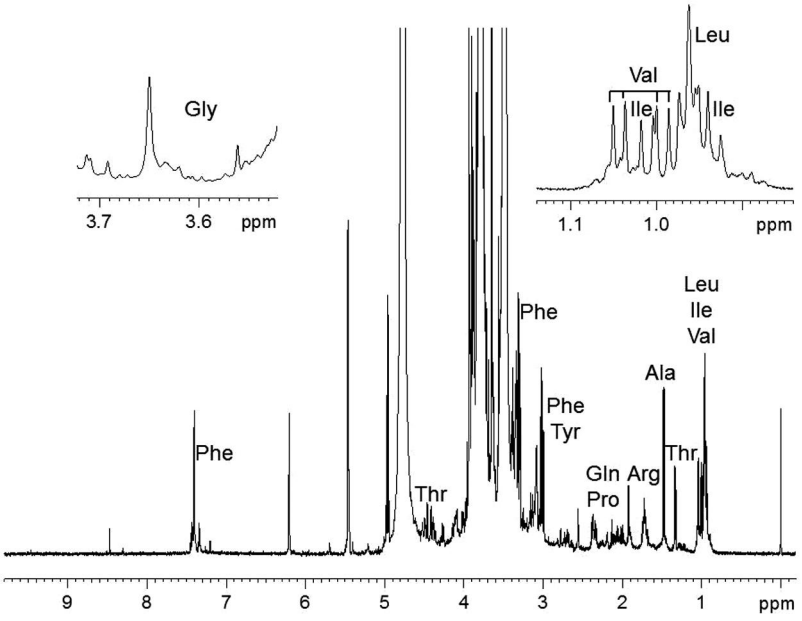

Figure 5. ^{1}H NMR spectra of hydrolyzed krill oil sample with signal assignment of amino acids. *Note*: Ala, alanine; Arg, arginine; Gln, glutamine (Gln); Gly, glycine; Ile, isoleucine; Leu, leucine; Phe, phenylalanine; Pro, proline; Thr, threonine; Tyr, tyrosine; Val, valine. The hydrolysis conditions are described in "Materials and Methods" section.

proteins, krill oil was hydrolyzed. By the hydrolysis of krill oil proteins and peptides were cleaved, the amino acids were analyzed in their free form. In Figure 5, the signals were assigned to the respective amino acids (alanine, isoleucine, leucine, valine, glycine, threonine, arginine, proline, phenylalanine (Phe), tyrosine, and glutamine). The amino acid distribution in different investigated samples demonstrated the natural fluctuations depending on environmental and biochemical impacts. Similarly to homarine and chitin, the quantitative determination of amino acids was outside the scope of the current study.

Authenticity of animal origin

It was observed that the identity of krill oil can be confirmed by ^{1}H and ^{31}P NMR spectroscopy based on the presence of the signals of phytanic acid derivatives (markers of chlorophyll-containing algae feeding), astaxanthin (red-color antioxidant), homarine, inosine (characteristic for crustaceans) as well as several phospholipid species, into which the majority of krill oil omega-3 fatty acids were incorporated (Burri et al. 2016). These compounds are highly specific for krill oil and are not detected in fish oil.

However, for the authenticity control, it would be advantageous to find a suitable fish oil marker, based on which adulteration of krill oil could be reliably justified. Among NMR techniques, ^{13}C NMR measurements were found the most suitable to authenticate animal origin of oil due to the possibility to provide regioselective analysis of the fatty acid distribution, especially in the *sn*-2 position (Burri et al. 2016). It was also an excellent tool to analyze the fatty acid composition without chemical treatment of the sample, like it is required in the traditional analysis by gas chromatography (Brenna 2013).

The fatty acid profile at the *sn*-2 position of triglyceride in commercial krill oil supplements is presented in Figure 6. The natural variability of the samples composition was limited by 1- and 99% quantiles calculated for 15 commercial krill oil supplements (Figure 6). The ^{13}C NMR spectrum shows the presence of low-intensive signals of docosahexaenoic (δ 69.53 ppm) and eicosapentaenoic (δ 69.45 ppm) acids (Figure 6). The more intensive peak of the saturated fatty acids (δ 69.36 ppm) had a −0.2 ppm shift related to the docosahexaenoic acid signal. This observation confirmed that krill oil was characterized by a lower content of polyunsaturated fatty acids in the *sn*-2 position of triglyceride than those of phospholipids.

In comparison, the individual asymmetric distribution of polyunsaturated fatty acids in the *sn*-2 position of triglyceride for commercial fish oil supplements is shown in Figure 7. The accumulation of eicosapentaenoic and docosahexaenoic acids in the *sn*-2 position in triglyceride structures was obvious. Moreover, the differentiation among mono-, di- and, potentially, three- substituted triglyceride, which contained docosahexaenoic (δ 69.1–69.2 ppm) or eicosapentaenoic (δ 69.0–69.1 ppm) acid in the *sn*-2 position was possible. The eicosapentaenoic/docosahexaenoic acids signals in fish oil were characterized by more than fivefold increase in intensity compared with the krill oil profile (Figures 6 and 7). Clearly, polyunsaturated fatty acids profiles at the *sn*-2 position of krill and fish oil supplements were distinguishable, which could be used for authentication purposes (Figures 6 and 7).

The results discussed above could be only used to differentiate between individual krill and fish oil supplements. It is clear, however, that the quantity of the foreign material (e.g., fish oil) was also of interest for consumers and control authorities. ^{13}C NMR spectra of a representative krill oil sample spiked with 5, 10, 25, 50, and 75% (w/w) fish oil content are

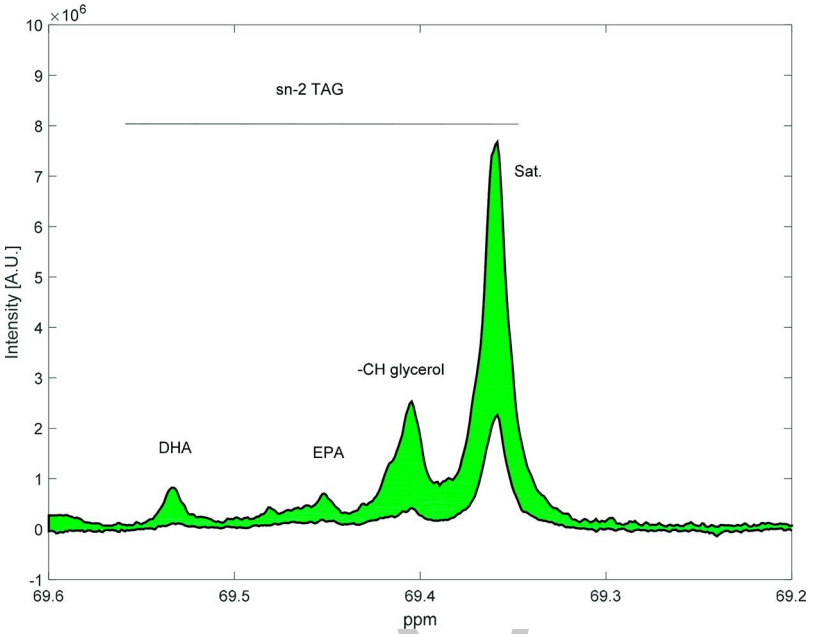

Figure 6. Details of the ^{13}C NMR spectra of krill oil samples dissolved in $CDCl_3$/MeOD (1:1) in the region of *sn*-2 glycerol carbon atoms. The borders depict 1%- and 99% quantiles calculated for 15 commercial krill oil supplements. *Note*: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; Sat., saturated fatty acids; TAG, triglyceride.

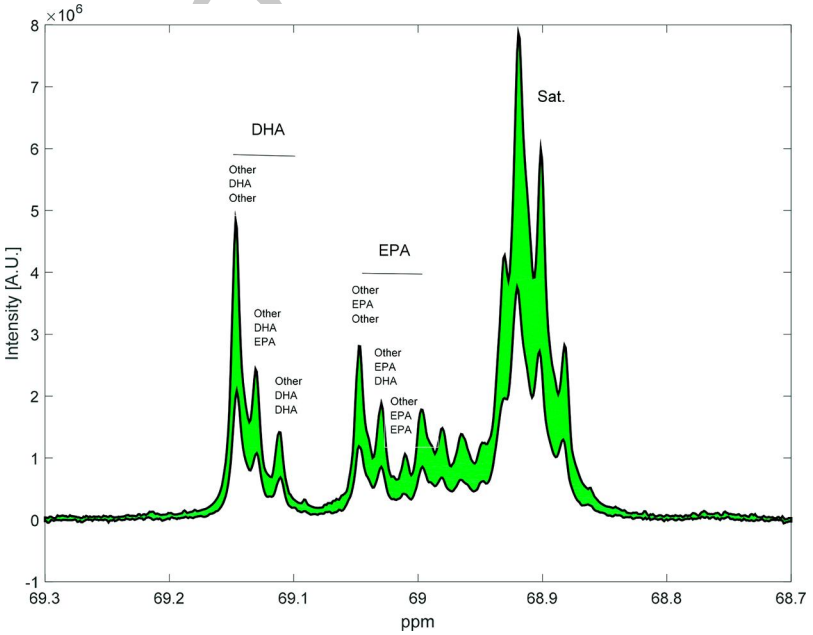

Figure 7. Details of the ^{13}C NMR spectra of fish oil samples dissolved in $CDCl_3$/MeOD (1:1) in the region of *sn*-2 glycerol carbon atoms. The borders depict 1%- and 99% quantiles calculated for 15 commercial fish oil supplements. *Note*: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; Sat., saturated fatty acids.

shown in Figure 8. The samples for spiking experiments were selected as the closest to the 50% quantile for krill and fish oil *sn*-2 profile, respectively (Figures 6 and 7). The addition of fish species to krill capsules was unambiguously observed by the increase of all eicosapentaenoic and docosahexaenoic acids signals, whereas the signals of saturated fatty acids and glycerol remained unchanged. The mentioned differences allowed to postulate krill oil adulteration qualitatively starting from approximately 10–15% (w/w) of fish oil in blends (NMR spectrum at the 10% (w/w) adulteration level had approximately the same intensity as 99% quantile of the krill oil profile). The same finding was obtained using authentic fish oil material.

The NMR profiles of the investigated commercial krill oil capsules were compared with the authentic krill oil. The identity of the ^{13}C NMR fingerprints showed the correctness of our conclusions. Apart from the animal origin, the source of fatty acids in dietary supplements is of great importance for health effects. The manufacturing of synthetically produced fish and krill oils or addition of high doses of synthetics to oil supplements is constantly growing due to lower production costs (Reicks et al. 1990). Since the estimation of the differences in health benefits between synthetic and natural oil is the subject of ongoing research, there are no analytical methods to differentiate both types of supplements.

An *sn*-2 triglyceride profile of a synthetically modified krill oil supplement is shown in Figure 9. Similarly to natural krill oil, this ^{13}C NMR profile was characterized by high amounts of eicosapentaenoic and docosahexaenoic acids in *sn*-2 position of triglyceride. However, the signals of mono-, di-, and tri-substituted TAG in case of both eicosapentaenoic and docosahexaenoic acids had approximately the same intensity (Figure 9). Therefore, the distribution of eicosapentaenoic and docosahexaenoic acids in *sn*-2 position

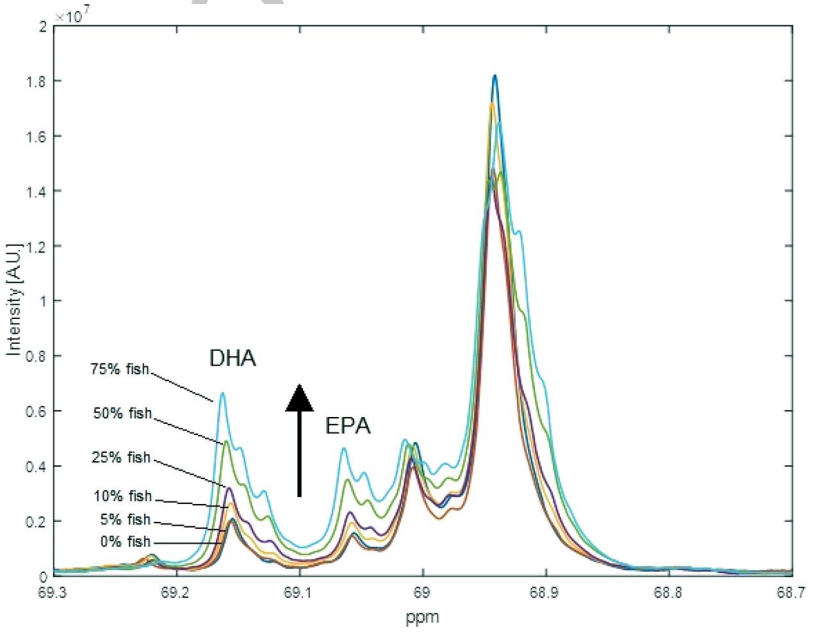

Figure 8. Details of the ^{13}C NMR spectra of fish-krill oil mixtures dissolved in $CDCl_3$/MeOD (1:1) in the region of *sn*-2 glycerol carbon atoms with 0–75% (w/w) fish oil content. The percentage of fish species material (%, w/w) is shown on the plot. *Note*: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.

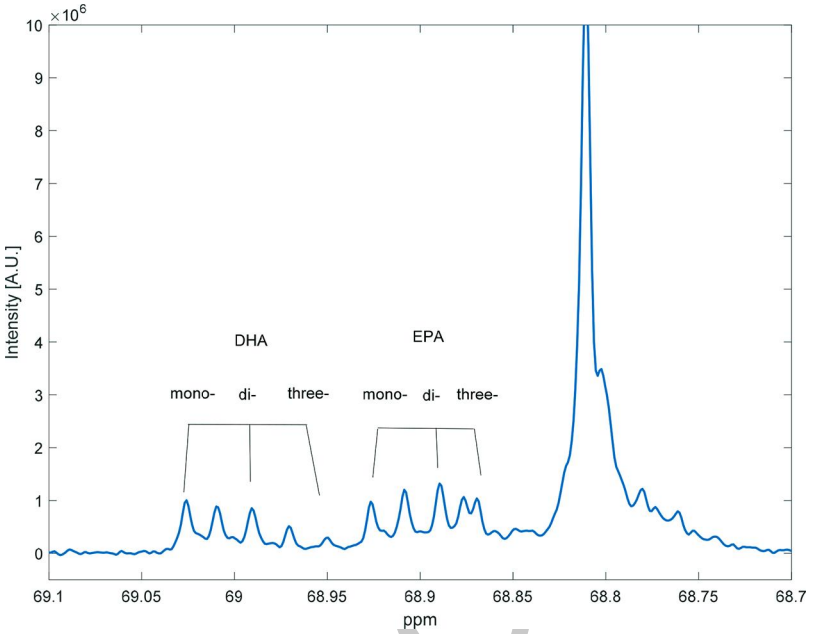

Figure 9. Details of the ^{13}C NMR spectra of the synthetically modified krill oil sample dissolved in $CDCl_3$/MeOD (1:1) in the region of *sn*-2 glycerol carbon atoms. *Note*: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.

was statistical, which was useful to postulate synthetic and semi- synthetic nature of the sample. In this regard, ^{13}C NMR spectrometry is a unique technique, because most of alternative analytical methods cannot assess regiospecific fatty acid composition.

Conclusion

The current situation on the growing market of krill oil-based dietary supplements demands the development of reliable analytical methodologies to ensure its regulatory compliance (Ritter, Budge, and Jovica 2013; Kleiner, Cladis, and Santerre 2015). The combination of ^{1}H, ^{31}P, and ^{13}C NMR spectroscopic methods is a unique methodology to rapidly and efficiently control the quality and to simultaneously characterize the animal origin of a given krill oil sample.

Qualitative and quantitative analysis of krill oil ingredients including eicosapentaenoic and docosahexaenoic acids, phospholipids distribution, chitin, aminoacids, homarine was achieved in an effective way. Moreover, an adulterated product with added fish oil can be detected by an increase in the omega-3 polyunsaturated fatty acids content in the *sn*-2 triglyceride fraction. The sensitivity of the method is about 10% (w/w) of fish content in blends, which is sufficient for primary screening. Moreover, a clear distinction from synthetically modified krill oil has been demonstrated.

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