



A matrix assisted laser desorption ionization time-of-flight mass spectrometry investigation to assess the composition of cod liver oil based products which displayed a different in vivo allergenic power

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ABSTRACT

Cod liver oil is a well-known “nutraceutical”, which contains a wide range of substances, including triacylglycerols (TAGs), mono- and di-acylglycerols, free fatty acids, vitamins and *n*–3 polyunsaturated fatty acids. Topically applied, cod liver oil contributes to faster wound healing and improvement in skin quality. We recently reported a case of allergic contact dermatitis to cod liver oil contained in a topical ointment, in whom the patch test reaction with the ointment containing cod liver oil at a concentration of 40% was stronger than the reaction induced by a pure cod liver oil at the same concentration. We hypothesized that the different reactivity could be explained by differences in composition of the two products. In order to verify this hypothesis, we assessed the composition of those products using a matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). The results obtained showed that the spectra of the ointment and of the cod liver oil samples were very similar, even if a major number of peaks were observable in the higher mass range of the spectra relevant to the analysis of the ointment sample, that have been assigned to higher molecular weight TAGs. Our results suggest that the different reactivity to the two products could be due to differences in the amount of contained TAGs. TAGs may favor the penetration of the allergen(s) or may be the direct culprit substances, taking into account that TAGs have been reported to have sensitizing properties.

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1. Introduction

Fish is considered an important source of essential fatty acids, which are fundamental from a physiological viewpoint (Belitz and Grosch, 1999). Cod liver oil (CLO), in particular, is a well-known “nutraceutical” and is widely consumed especially in Northern European countries. This food contains a wide range of major compounds, i.e. triacylglycerols (TAGs), mono- and di-acylglycerols, free fatty acids, and minor compounds, i.e. an abundant amount of specific vitamins (A, D and E) and *n*–3 polyunsaturated fatty acids (commonly known as omega-3 fatty acids), especially eicosapentanoic acid (EPA) and docosahexaenoic acid (DHA), and, due to this, is widely used as a dietary supplement (Brustad et al., 2004). The cod family (*Gadus* sp.) is one of the largest families of fish, found in all oceans of the world. There are more than 90 species of cod, of which 40 are commercially available as oils. Even in the same waters, different species of the *Gadus* family can have a

great variation in fatty acid profile (Wetzel, 2005) and vitamin A and D content.

Topically applied, cod liver oil contributes to faster wound healing and improvement in skin quality (Terkelsen et al., 2000). However, we recently reported a case of allergic contact dermatitis to cod liver oil contained in a topical ointment (Foti et al., 2007). The main allergen was unknown. The negative patch tests with vitamins A and D led us to exclude the role of these vitamins as responsible for the allergic reactions. Moreover we observed that the reaction with the topical ointment containing cod liver oil at a concentration of 40%, white wax and petrolatum, was really stronger (vesicular-bullous) (Fig. 1A) compared with the reaction induced by a pure cod liver oil (Fig. 1B). We hypothesized that the reactions could be due to TAGs, that have been reported (Laube et al., 2002, 2003) to have sensitizing properties, and that the different reactivity to the patch test could be explained by the different compositions of the two products, in particular in the TAGs content. In order to verify this hypothesis, we assessed the composition of those products using a matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS), a technique that has emerged (Asbury

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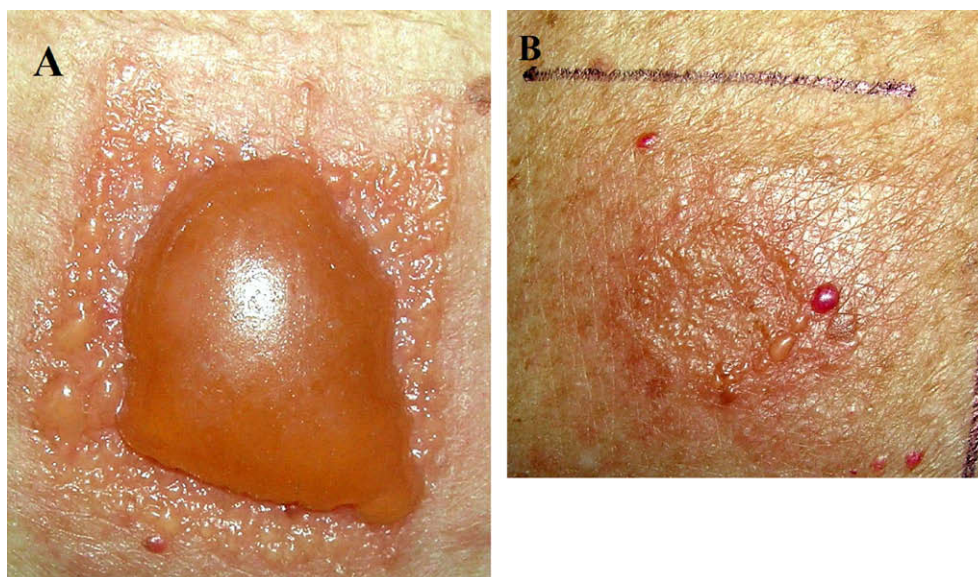


Fig. 1. (A) Vesico-bullous reaction to patch test with the cod liver oil based ointment; (B) vesicular reaction to patch test with pure cod liver oil.

et al., 1999; Ayorinde et al., 1999a,b,c; Ayorinde et al., 2000; Hlongwane et al., 2001; Jakab et al., 2002; Robins and Limbach, 2003; Al-Saad et al., 2003; Schiller et al., 2002a,b; Calvano et al., 2005, 2007) as a useful tool for different oils characterization, offering the advantages of fast and easy sample preparation without the need for analyte-derivatization.

2. Experimental

2.1. Chemicals

All solvents used (Sigma–Aldrich, St. Louis, MO, USA) were HPLC grade and were used without further purification. The matrices

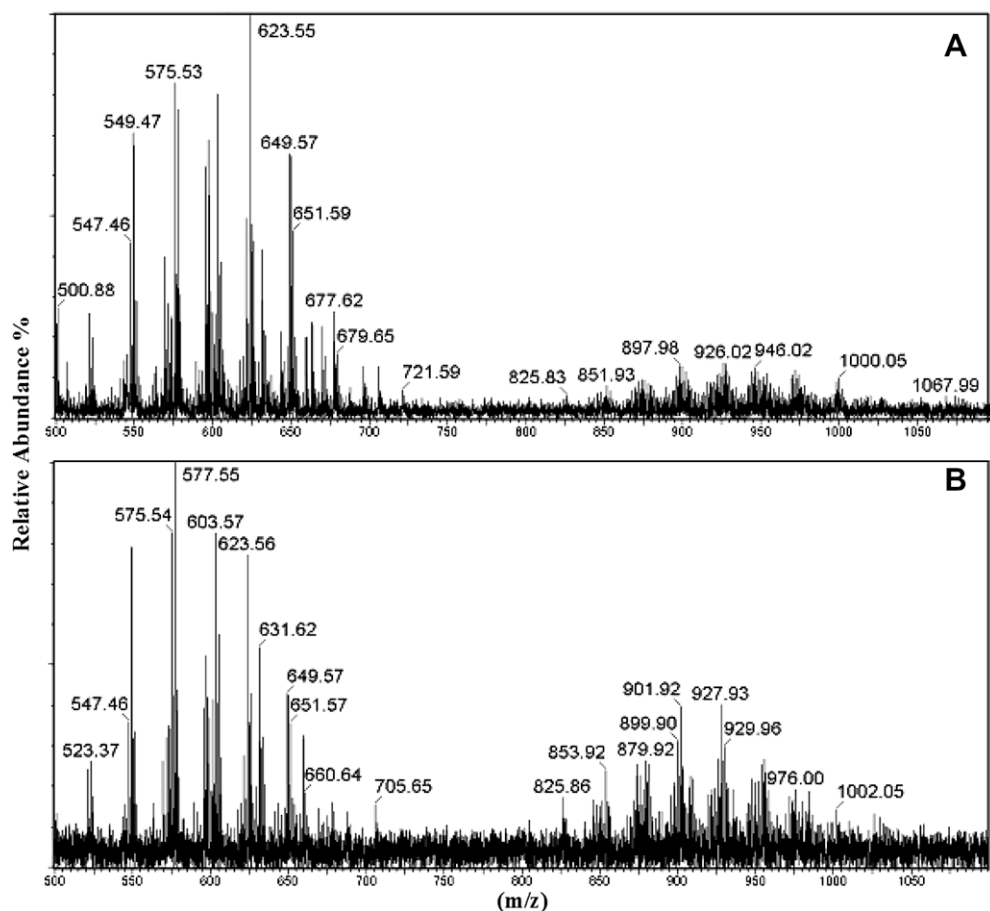


Fig. 2. MALDI-TOF-MS spectra obtained in the analysis of (A) a sample of the cod liver oil based ointment responsible for the reported allergic contact dermatitis, and (B) a sample of pure cod liver oil, both dissolved in pentane as described in the experimental section.

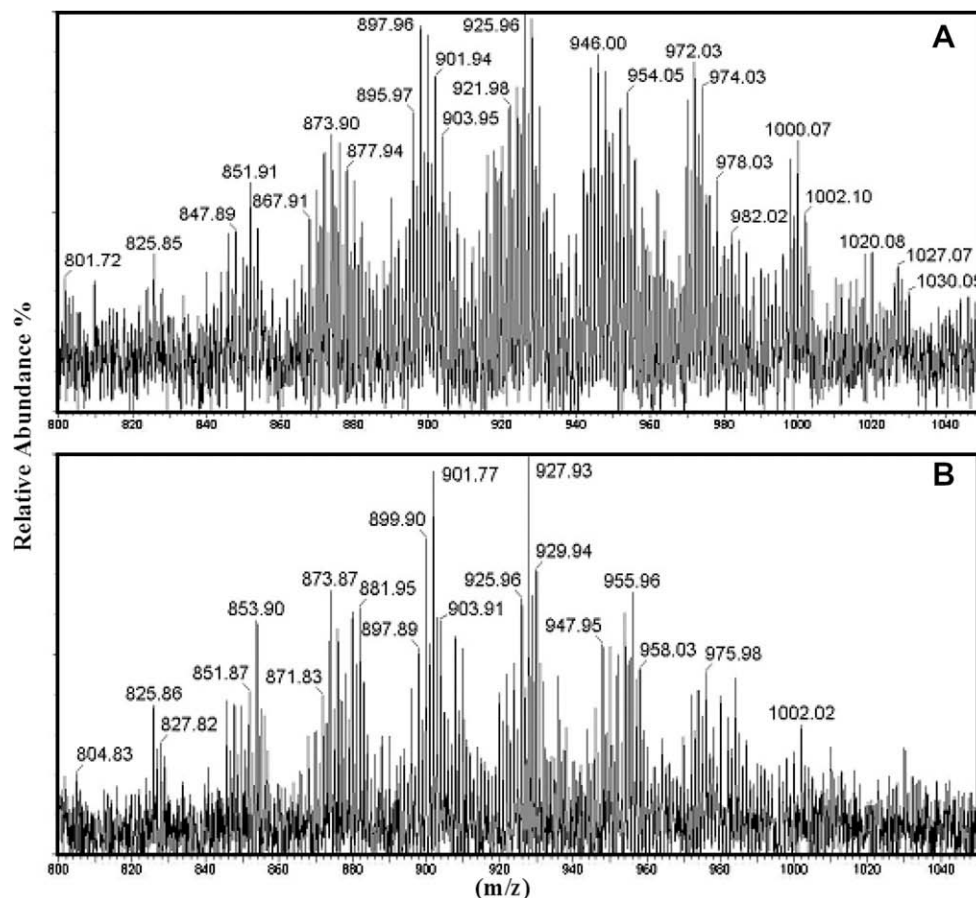


Fig. 3. Expanded views in the high-mass region of the MALDI-TOF-MS spectra of Fig. 2A and B, respectively.

(α -cyano-hydroxycinnamic acid, CHCA, and 2,5-di-hydroxybenzoic acid, DHB) used for MALDI experiments were obtained from Sigma. Dermovitamina® (the cod liver oil containing cream) was obtained from Pasquali S.R.L., Sesto Fiorentino, Florence, Italy. Cod liver oil was purchased from Marco Viti Farmaceutici SpA, Mozzate, Italy.

2.2. Apparatus

MS experiments were performed using a Micromass M@LDI™ – LR (Waters MS Technologies, Manchester, UK) time-of-flight mass spectrometer equipped with a nitrogen UV laser (337 nm wavelength), a precision flat target plate sample introduction system bearing a micro-titer target plate (96 well, 3 mm diameter, 4.5 mm pitch, rectangular 12 by A–H with additional 24 near-point calibration wells), reflectron optics with effective path length of 2.3 m, a fast dual micro-channel plate (MCP) detector, and a high magnification (70 $\times$) camera system.

2.3. Instrumental conditions

Positive ion spectra were acquired in reflectron mode in the m/z range from 500 to 1100. The following voltages were applied: pulse, 2580 V; source, 15,000 V; reflectron, 2000 V; MCP 1850 V. The laser energy employed was 40% of its maximum value, corresponding approximately to 188 μJ per laser shot. The laser power densities were in the range of 10^7 – 10^8 W cm^{-2} with a laser pulse duration of 4 ns. The laser firing rate was 5 Hz, and 60 laser shots were used for each well. The 60 resulting spectra were averaged, background subtracted and smoothed by a Savitzky–Golay algorithm. A time lag focusing (TLF) delay of 500 ns was used between the laser pulse and the application of the accelerating voltage.

2.4. Cod liver oil and cod liver ointment samples

Immediately before analysis, oil and ointment samples (50 mg) were dissolved in 1 ml of pentane and vortexed for 1 min. Then, different sample preparation procedures have been tested for MALDI analysis but the “sandwich method” showed to provide the most satisfactory results. According to this sample preparation protocol, 1 μL of DHB dissolved in a highly volatile solvent (160 mg/ml in acetone) was deposited on the target plate and a thin crystalline film was generated within few seconds during evaporation of the solvent. On the top of the DHB layer, 1 μL of the mixture (1:1) composed by an oil (or cream) pentane solution and DHB (40 mg/ml in 0.1% TFA in methanol) was deposited and allowed to dry. The target plate was then loaded in the MALDI sample chamber.

3. Results

From previous findings, it results that complex polyunsaturated oils (Laube, 2003) tend to provide signals with low intensities. Thus, great attention was paid to the optimization of the sample preparation step. Different techniques have been tested using different matrices for MALDI analysis. The use of α -cyano, as reported in the literature (Smirnov et al., 2004; Keller and Li, 2000) was found to give spectra with a large number of interfering peaks arising from the matrix itself in the explored mass region. The DHB matrix showed to provide better results, in particular by adopting the “sandwich” sample preparation approach. In fact, the formation of a homogeneous analyte/matrix crystalline phase on the target plate was observed and, consequently, more reproducible spectra and higher signal intensities were produced.

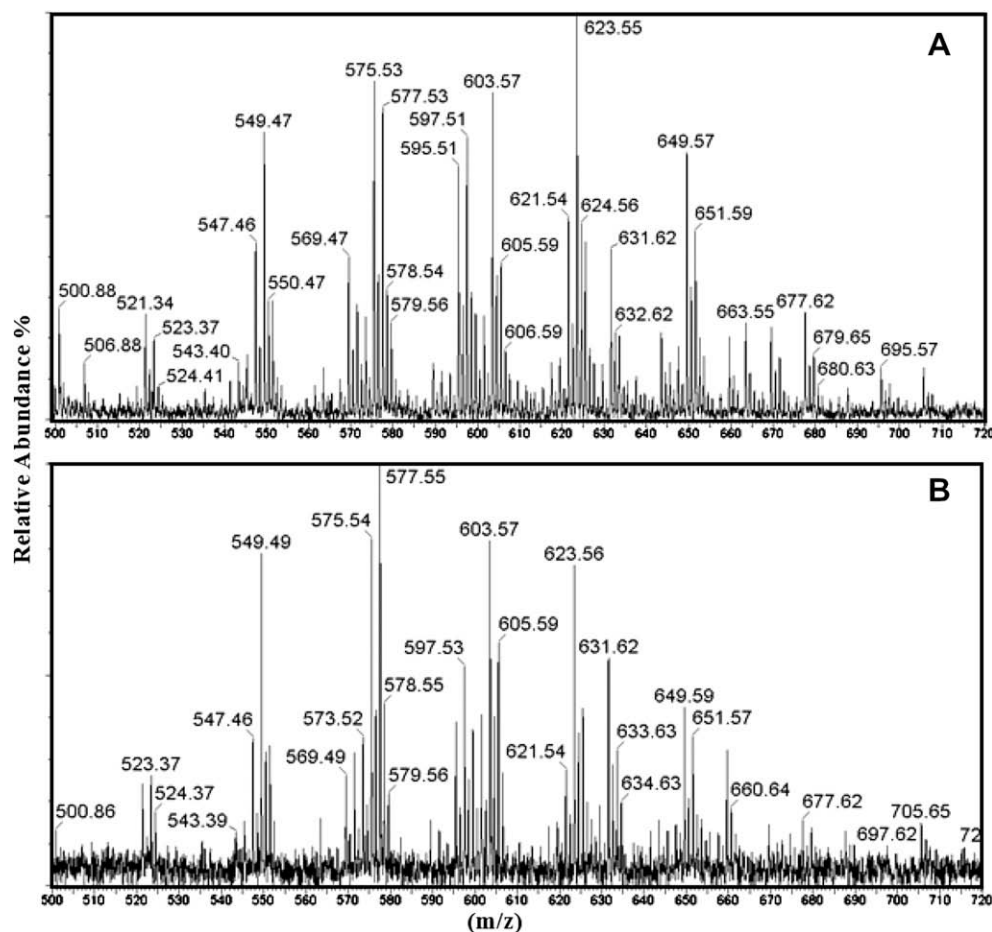


Fig. 4. Expanded views in the low-mass region of the MALDI-TOF-MS spectra of Fig. 2A and B, respectively.

Fig. 2 reports the MALDI-TOF-MS mass spectra obtained from the analysis of (A) a sample of the cod liver oil based ointment responsible for the reported allergic contact dermatitis, and (B) a sample of pure cod liver oil, both dissolved in pentane as described in the experimental section. The explored mass range was selected on the basis of the theoretical range for sodiated triacylglycerols in the MALDI-TOF-MS spectra of CLO, based on its fatty acid profile.

Figs. 3A, B and 4A, B show expanded views in the high and the low-mass regions of the spectra of Fig. 2A and B, respectively.

Table 1 reports the list of the peaks observable in the spectra obtained from both samples (with the exception of those arising from the matrix), their attributions and the presence (+) or absence (–) in each spectrum.

4. Discussion

At a first sight (see Fig. 2) the commercial cod liver oil and the cod liver oil based ointment samples appear to have similar MALDI-TOF mass spectral profiles, except for some differences in signal intensities.

According to previous reports (Asbury et al., 1999) dealing with the MALDI-TOF-MS analysis of TAGs, no protonated molecules were observed in the spectra; sodium ions adducts $[M + 23]$ were the main observable species (probably due to the presence of impurities in the solvent and/or on the target plate). In both spectra of Fig. 2, sodium adducts of the main triacylglycerols (TAGs) known to be present in cod liver oils (Ayorinde et al., 1999c) were easily detectable and were present as clusters with one or two predominant fatty acids in each cluster. Even if a “soft ionization”

technique was used, peaks attributable to the fragmentation of the most abundant TAGs, corresponding to the loss of RCOO^-Na^+ molecules, with the consequent formation of the shorter positively charged diglycerides, were easily detected in the low-mass region of both spectra.

From a careful inspection of Figs. 3 and 4, some interesting information could be retrieved. A detailed assignment of the main peaks observed in the MALDI-TOF mass spectra of both the cod liver oil and the cod liver oil based ointment samples was accomplished. Almost seventy signals (see Table 1) were assigned, confirming the known complexity of these samples, where a complex fatty acid profile is described together with no predominance in the fatty acid composition (Ayorinde et al., 1999c). It should be noted that many other isomeric assignments are possible in principle, since a MALDI analysis could not discriminate among isobaric ions.

As can be seen in Table 1, the majority of the peaks was present in both spectra; however, some differences were observed between the two samples. In particular in the high m/z region, i.e. 985–1003, the ointment sample showed the presence of some peaks not observable in the cod liver oil spectrum, that could be attributed to higher molecular weight TAGs (probably also present in the cod liver oil samples, but at a “not-detectable” concentration level). In accordance with this experimental evidence, also the diglycerides fragment ions arising from these peaks were present only in the spectrum relevant to the ointment sample. Our results suggest that the different reactivity to the two cod liver oil-based products (see Fig. 1A and B) could be due to differences in their composition and, in particular, in the amount of contained TAGs. TAGs may favor the penetration of the allergen(s) or may be the

Table 1

Attributions of the main peaks observable in the spectra of [fig. 1](#) and their presence (+) or absence (–) in each spectrum.

<i>m/z</i>	Relative abundance (%)		Attribution	Appearance	
	Oil	Cream		Oil	Cream
521	25	26	[M ¹ P] ⁺ , [MP] ⁺	+	+
523	21.5	15	[MP] ⁺	+	+
543	–	14	[M ¹ Ln] ⁺	–	+
545	9	13	[M ¹ L] ⁺	+	+
547	32.5	40	[M ¹ L] ⁺ , [P ¹ P] ⁺	+	+
549	73	62	[MO] ⁺ , [P ¹ P] ⁺	+	+
551	17	15	[PP] ⁺ , [MS] ⁺	+	+
561	–	8	Dg[M ¹ P+Na] ⁺	–	+
563	15.5	11	Dg[MP+Na] ⁺	+	+
567	–	10	[M ¹ A ⁵] ⁺	–	+
569	26	38	[MA ⁵] ⁺	+	+
571	26	20	[P ¹ Ln] ⁺	+	+
573	28.5	20	[P ¹ L] ⁺ , [PLn] ⁺	+	+
575	76	78	[PL] ⁺	+	+
577	100	60	[PO] ⁺	+	+
579	0.8	8	[PS] ⁺	+	+
589	15	14	Dg[MO+Na] ⁺	+	+
591	23.5	9	Dg[MS+Na] ⁺	+	+
593	13	9	[P ¹ A ⁵] ⁺	+	+
595	42	60	[P ¹ A ⁵] ⁺ , [LnLn] ⁺	+	+
597	45	57	[PA ⁵] ⁺ , [LnL] ⁺	+	+
599	27	12	[LL] ⁺	+	+
601	32	20	[OL] ⁺	+	+
603	74	75	[OO] ⁺ , [SL] ⁺	+	+
605	41	23	[OS] ⁺	+	+
607	–	3	[SS] ⁺	–	+
617	15	14	[LnA ⁶] ⁺	+	+
619	13	12.5	[LnA ⁵] ⁺	+	+
621	27	46	[LA ⁵] ⁺ , [P ¹ D ⁶] ⁺	+	+
623	71	100	[OA ⁵] ⁺ , [PD ⁶] ⁺	+	+
625	15	23	[SA ⁵] ⁺	+	+
627	7.5	5	[LnA ¹] ⁺	+	+
629	15	10	[LA ¹] ⁺	+	+
631	49	39	[OA ¹] ⁺	+	+
633	21.5	12	[SA ¹] ⁺	+	+
641	12	–	[A ⁵ A ⁶] ⁺	+	–
643	13	21	[A ⁵ A ⁵] ⁺	+	+
645	9	10	[D ⁶ Ln] ⁺	+	+
647	12	15	[D ⁶ L] ⁺	+	+
649	39	62	[D ⁶ O] ⁺	+	+
651	27	33	[D ⁶ S] ⁺ , [A ⁵ A ¹] ⁺	+	+
653	9	6	[D ⁵ S] ⁺	+	+
659	32	20	[D ¹ O] ⁺	+	+
663	–	24	[DS] ⁺	–	+
669	–	23	[A ⁵ D ⁶] ⁺	–	+
671	–	11	[A ⁵ D ⁵] ⁺	–	+
677	15	26	[A ¹ D ⁶] ⁺	+	+
679	10	10	[AD ⁶] ⁺	+	+
695	–	13	[D ⁶ D ⁶] ⁺	–	+
697	–	6	[D ⁵ D ⁶] ⁺	–	+
705	14	12.5	[D ¹ D ⁶] ⁺	+	+
825	17	5	[MP ¹ O+Na] ⁺	+	+
827	9	–	[PP ¹ P+Na] ⁺	+	–
843	–	5	[MM ¹ D ⁶ +Na] ⁺	–	+
845	16	5	[MMD ⁶ +Na] ⁺	+	+
847	12	5	[P ¹ P ¹ Ln+Na] ⁺	+	+
849	12	4	[P ¹ PLn+Na] ⁺	+	+
851	14	6.5	[PPLn+Na] ⁺	+	+
853	21	5	[PPL+Na] ⁺	+	+
855.78	10	–	[PPO+Na] ⁺	+	–
869	13	7.5	[MLnA ⁵ +Na] ⁺	+	+
871	14	7	[P ¹ LnLn+Na] ⁺	+	+
873	23	7.5	[PLnLn+Na] ⁺	+	+
875	18	7.5	[PLLn+Na] ⁺	+	+
877.71	14	6.5	[PLL+Na] ⁺ , [POLn+Na] ⁺	+	+
879.75	21	6	[POL+Na] ⁺	+	+
881.72	21	5	[POO+Na] ⁺	+	+
883.73	7	6	[POS+Na] ⁺	+	+
893	11	–	[P ¹ LnA ⁶ +Na] ⁺	+	–
895	15.5	10	[P ¹ LnA ⁵ +Na] ⁺	+	+
897	18.5	11	[P ¹ LA ⁵ +Na] ⁺	+	+
899	28	10	[PLA ⁵ +Na] ⁺	+	+

Table 1 (continued)

<i>m/z</i>	Relative abundance (%)		Attribution	Appearance	
	Oil	Cream		Oil	Cream
901	33	9	[LLL+Na] ⁺	+	+
903.72	16	7	[OLL+Na] ⁺	+	+
905.74	9.5	5	[OOL+Na] ⁺	+	+
907.75	16	5	[OOO+Na] ⁺	+	+
909.72	17	5	[SOO+Na] ⁺	+	+
911.73	–	4	[SSO+Na] ⁺	–	+
913	–	5	[SSS+Na] ⁺	–	+
915	–	7.5	[M ¹ D ⁶ A ⁶ +Na] ⁺	–	+
917	–	7	[M ¹ D ⁶ A ⁵ +Na] ⁺	–	+
919	17	7.5	[MD ⁶ A ⁵ +Na] ⁺	+	+
921	16	8.5	[P ¹ LnD ⁶ +Na] ⁺	+	+
923	16	9	[PLnD ⁶ +Na] ⁺	+	+
925	23	12	[PLD ⁶ +Na] ⁺	+	+
927	37	11	[POD ⁶ +Na] ⁺	+	+
929	22	8	[PSD ⁶ +Na] ⁺	+	+
931	13	5	[LLA+Na] ⁺	+	+
933	–	6	[OLA+Na] ⁺	–	+
935	19	–	[OLA ¹ +Na] ⁺	+	–
937	–	6	[SLA ¹ +Na] ⁺	–	+
939	–	5	[SOA ¹ +Na] ⁺	–	+
941	–	7	[SSA ¹ +Na] ⁺	–	+
943	13	10	[SSA+Na] ⁺	+	+
945	13	10	[LA ⁵ A ⁵ +Na] ⁺	+	+
947	19	9	[OA ⁵ A ⁵ +Na] ⁺	+	+
949	17	7	[SA ⁵ A ⁵ +Na] ⁺	+	+
951	17	8	[LnA ⁵ A ¹ +Na] ⁺	+	+
953	21.5	9	[OOD ⁶ +Na] ⁺	+	+
955	22	6	[SOD ⁶ +Na] ⁺	+	+
957	14	5	[SSD ⁶ +Na] ⁺	+	+
959	–	–	[SSD ⁵ +Na] ⁺	–	–
961	–	7.5	[OLD ¹ +Na] ⁺	–	+
963	–	5	[OOD ¹ +Na] ⁺	–	+
969	13	10	[LnA ⁵ D ⁶ +Na] ⁺	+	+
971	14	10	[LA ⁵ D ⁶ +Na] ⁺	+	+
973	14	8.5	[OA ⁵ D ⁶ +Na] ⁺	+	+
975	15	5	[SA ⁵ D ⁶ +Na] ⁺	+	+
977	10	6	[LnA ¹ D ⁶ +Na] ⁺ , [SA ⁵ D ⁵ +Na] ⁺	+	+
979	13	4	[LA ¹ D ⁶ +Na] ⁺	+	+
981	11	5	[OA ¹ D ⁶ +Na] ⁺	+	+
983	16	4.5	[SA ¹ D ⁶ +Na] ⁺	+	+
985	–	4	[SAD ⁶ +Na] ⁺	–	+
987	–	4	[LnA ¹ D ¹ +Na] ⁺	–	+
989	–	5	[LA ¹ D ¹ +Na] ⁺	–	+
997	–	9	[D ⁶ D ⁶ L+Na] ⁺	–	+
999	–	7.5	[A ⁶ A ¹ D ⁶ +Na] ⁺ /[D ⁶ D ⁶ O+Na] ⁺	–	+
1001	–	5	[A ⁵ A ¹ D ⁶ +Na] ⁺ /[D ⁵ D ⁶ O+Na] ⁺	–	+
1003	–	4	[A ⁵ A ¹ D ⁵ +Na] ⁺ /[D ⁵ D ⁵ O+Na] ⁺	–	+

M = myristic acid (C14:0); M¹ = (C14:1); P = palmitic acid (C16:0); P¹ = palmitoleic acid (C16:1); S = stearic acid (C18:0); O = oleic acid (C18:1); L = linoleic acid (C18:2); Ln = linolenic acid (C18:3); A = arachidic acid (C20:0); A¹ = (C20:1); A⁵ = eicosapentaenoic acid (EPA, C20:5); D = docosanoic acid (C22:0); D¹ = docosaenoic acid (C22:1); D⁶ = docosahexaenoic acid (DHA, C22:6).

direct culprit substances, taking into account that TAGs have been reported ([Laube et al., 2002](#); [Laube, 2003](#)) to have sensitizing properties.

5. Conclusions

In this report MALDI-TOF-MS was used to investigate a recently described case of allergic contact dermatitis to an ointment containing cod liver oil, with a strongly positive patch test result to the ointment as is. Interestingly, the patch test to the pure cod liver oil at the same concentration of that present in the ointment caused a positive reaction with a significantly less severe intensity. It was hypothesized that the different reactivity to patch test to the two products could be related to their different compositions, in particular in the TAGs content.

The experimental evidences showed that the MALDI-TOF-MS spectra of the ointment and of the cod liver oil samples were very similar, even if a major number of peaks was evident in the higher mass range of the spectrum relative to the analysis of the ointment sample, that have been assigned to higher molecular weight TAGs, thus highlighting differences in TAGs content between the two products. This finding might explain the different reactivity to patch test with the two products, although our results did not permit to establish if TAGs were the true sensitizing substances or alternatively nonspecific cofactors favoring and enhancing the skin response.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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