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Novel characterisation of minor α -linolenic acid isomers in linseed oil by gas chromatography and covalent adduct chemical ionisation tandem mass spectrometry

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Abstract

Discrimination between polyunsaturated fatty acid isomers with three double bonds is a great challenge, due to structural similarities and similar polarities. In this study, we report the identification of four minor geometrical isomers of α -linolenic acid (ALA) present in linseed oil samples: (9*E*,12*Z*,15*E*)-, (9*Z*,12*Z*,15*E*)-, (9*Z*,12*E*,15*Z*)- and (9*E*,12*Z*,15*Z*)-octadeca-9,12,15-trienoic acids, chromatographically resolved by gas chromatography (GC) using a new and highly polar ionic phase column (SLB-IL111). Gas chromatography-electron ionisation mass spectrometry (GC-EIMS) determined that the four unknown compounds were C18:3 *n*-3 isomers. The positional 9-12-15 C18:3 configuration was achieved by covalent adduct chemical ionisation tandem mass spectrometry (CACI-MS/MS) while geometrical configuration was established with analytical standards based on relative retention. We hypothesised that these isomers are formed during linseed oil deodorisation and postulate preferred and unfavoured isomerisation pathways of ALA.

Keywords

omega-3 fatty acids; α -linolenic acid; geometrical isomer; isomerization; SLB-IL111; CACI-MS/MS

1. Introduction

Linseed oil is derived as an oil seed crop with unique properties due to its fatty acid (FA) composition. It represents a rich source of omega-3 fatty acids (*n*-3 FA) which play a fundamental role in growth, coronary heart disease prevention and reduction in the risk of high blood pressure and atherosclerosis (Ueshima et al., 2007; Pan et al., 2012). α -Linolenic acid ((9*Z*,12*Z*,15*Z*)-octadeca-9,12,15-trienoic acid, ALA) is commonly the only *n*-3 FA

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reported in vegetable oils and it accounts for around 50% of total FA in linseed oil (Kostik, Memeti & Bauer, 2013). Nonetheless, other minor (15*E*)-FA could also be formed through ALA isomerisation (Wolf, Nour & Bayard, 1996). The characterisation and quantification of these (*E*)-FA would be of vital importance as they could be related to an increase of total- to HDL-cholesterol ratio and a higher risk for cardiovascular disease in humans (Chardigny, Bretillon, & Sébédio, 2001).

Gas chromatography (GC) is by far the most commonly used analytical procedure for FA analysis. FAs are converted into their corresponding methyl esters (FAME) to increase volatility and reduce hydrogen bonding that degrades chromatographic resolution. After derivatisation, FAME are commonly analysed with a high polarity capillary column by GC; typically cyanopropyl columns are the most widely used. The separation of positional and geometric isomers of mono and diunsaturated FA is routinely possible even in complex FA mixtures. However, discrimination between geometrical isomers with three or more double bonds with very similar polarities, such as FA in linseed oil, remains a challenge for analytical chemists (Kramer, Blackadar & Zhou, 2002). Some pairs of (*E*)-octadecatrienoic isomers, such as (9*Z*,12*E*,15*E*)- and (9*E*,12*Z*,15*E*)- or (9*Z*,12*Z*,15*E*)- and (9*E*,12*E*,15*Z*)- octadeca-9,12,15-trienoic acids are not well separated with 100 m CPSil88, the most popular cyanopropyl column. Recent availability of new extremely polar stationary phase columns could be a useful tool. Their high selectivity towards FAME can provide unique elution patterns, together with column bleed reduction (Delmonte, Fardin Kia, Sidisky, Kramer, Mossoba & Rader, 2011; Delmonte et al., 2012; De la Fuente, Rodríguez-Pino & Juárez, 2015).

The aim of this work was to profile C18:3 minor isomers in linseed oil. For that purpose, the separation of the FA was carried out using an extremely polar (100 m SLB-IL111) column. We then combined the information obtained from gas chromatography-electron ionisation mass spectrometry (GC-EIMS) with information obtained from covalent adduct chemical ionisation tandem mass spectrometry (CACI-MS/MS) to characterise four minor (*E*)-ALA isomers in linseed oil.

2. Materials and methods

2.1. Oil Samples and Analytical Standards

Two linseed oil samples from Manuel Riesgo S.A. (Madrid, Spain) and Gustav Heess S.L. (Barcelona, Spain) were acquired in a local supermarket and kept under refrigeration until FAME analysis. To study the thermal stability of the oils, 1 mL of linseed oil was aliquoted into 2-mL amber vials with N₂ in the headspace and stored at two different temperatures (+4 °C and ambient temperature). The stability of the oils was evaluated during a month (after 0, 7, 14, 21 and 28 days of storage) by comparing the FA compositions.

FAMEs were prepared by base-catalysed methanolysis of the glycerides (KOH in methanol) according to ISO-IDF procedure (ISO-IDF, 2002). FAMEs were prepared in hexane and BHT was added to prevent oxidation. C18:3 *n*-6, C18:3 *n*-3 standards and α -linolenic acid methylester isomer mixture (4-7792; Supelco) containing all eight geometrical isomers of ALA were purchased from Sigma-Aldrich (Madrid, Spain).

2.2. Analytical Methods

The FAME profile was determined on a GC fitted with a flame ionization detector (FID). Each oil sample was analysed 6 times using the following conditions. Injector and detector temperatures were 250 °C and helium was the carrier gas. FAMES were separated on an SLB-IL111 column (100 m × 0.25 mm × 0.20 µm film thickness; Varian, Middelburg, The Netherlands). Sample (1 µL) was injected by split injection (1:100) using a gradient temperature program. Initial oven temperature was isothermal 163 °C, and after 32 min it was raised at 10 °C min⁻¹ to 205 °C and held for 38 min.

FAMES were also analysed on a GC equipped with a mass spectrometer detector (Agilent 6890 N Network System, MS 5973N; Palo Alto, CA). The filament trap current was 400 µA at 70 eV. Helium was the carrier gas and the injection volume was 1 µL. FAME were injected by split injection (1:100) using the same gradient temperature program described above.

Samples were also analysed by CACI-MS/MS to provide more detailed structural information on ALA isomers. Analyses were performed on a GC (Varian Star 3400CX) coupled to a Varian Saturn 2000 3D ion trap (Varian Inc., Walnut Creek, CA). Acetonitrile was used as the reagent gas, and ion trap parameters were as follows: trap temperature, 150 °C; manifold temperature, 45 °C; transfer line temperature, 200 °C; precursor isolation window, 3 *m/z*; emission current, 5 µA; excitation amplitude, 0.41–0.47 V. The chemical ionisation storage level, corresponding to the lowest mass stored in the ion trap during ionisation of reagent gas, was set to *m/z* 22. Other parameters were ejection amplitude, 8.0 V; maximum ionisation time, 2000 µs; maximum reaction time, 120 µs; and pre-scan ionisation time, 200 µs. The excitation storage level, representing the radio frequency storage level in the ion trap when the dissociation waveform is applied, ranged from *m/z* 81.9–82.3.

2.3. Statistical Analysis

Statistical analysis was conducted with JMP Version 9 (SAS Institute, Cary, NC). Paired comparisons, using Student's *t*-test, were used to compare linseed oil FA profiles. Significant differences were declared at *p* < 0.05.

3. Results and discussion

A partial GC chromatogram of the C18:2 *n*–6 to C18:3 *n*–3 region in linseed oil is illustrated in Figure 1. Under our chromatographic conditions, linseed oil samples showed four unidentified peaks in different quantities depending on the sample. Two commercial linseed oil samples were analysed by the GC-FID method described in section 2.2. Four unidentified peaks (**A**, **B**, **C**, **D**) accounted for (**A**) 0.4%, (**B**) 3.7%, (**C**) 0.6% and (**D**) 3.1% of total FAMES in the first linseed oil sample analysed. Although those peaks, later identified as geometrical isomers of α-linolenic acid, were also detected in the second linseed oil, their amounts were significantly lower (Table 1). A GC-FID sample chromatogram was superimposed with all-(*Z*) C18:3 *n*–6 and C18:3 *n*–3 standards, but unidentified compounds differed from the standards (Figure 1).

Figure 2 shows the mass spectra of ALA and one of the unknown peaks obtained by conventional GC-EIMS of FAMES. The spectra of the four target compounds were similar to each other (data not shown) and presented a similar pattern of fragmentation to α -linolenic acid. All spectra exhibited a molecular ion of m/z 292 which supports a C18:3 structure and ruling out a C18:2 moiety (Gómez-Cortés, Tyburczy, Brenna, Juárez & De la Fuente, 2009). Moreover, they showed the m/z 108 ion which is characteristic for FAMES of polyunsaturated FAs with an $n-3$ terminal group (Brauner, Budzikiewicz & Boland, 1982). These spectra suggest that the four unidentified compounds were C18:3 $n-3$ isomers, but, unfortunately, EIMS is not capable of discriminating among positional or geometrical isomers of unsaturated FAMES.

Tandem mass spectrometry has proven to be an effective technique to locate double bonds in polyunsaturated FAs (Jensen, Tomer & Gross, 1985; Adams & Gross, 1987). In particular, CACI-MS/MS using acetonitrile chemical ionisation reagent enables the location of unsaturated bonds through the direct analysis of FAMES (Van Pelt & Brenna, 1999; Van Pelt, Carpenter & Brenna, 1999; Alves & Bessa, 2007). This technique has advantages for quantitative analysis compared with conventional methods, is suitable for high-throughput analysis and can be implemented on a relatively inexpensive table-top GC-MS/MS based on ion trap technology (Brenna, 2005). According to theory, acetonitrile forms a dimer (m/z 81) and decomposes to form (1-methyleneimino)-1-ethenylum ($\text{CH}_2=\text{C}=\text{N}^+=\text{CH}_2$, m/z 54), generated by CACI-MS conditions, which reacts with the analyte double bond to yield molecular ions with 54 mass units above the parent analyte. Isolation and collisional activation of $[\text{M} + 54]^+$ ions yields fragments indicative of double bond position while prompt dissociation in CACI-MS yields ions indicative of general structure. The fragmentation patterns of α - and γ -linolenic acids and the unidentified **B** and **D** peaks are presented in Figure 3. As shown in Figure 3, MS/MS data obtained upon collisional activation of the $[\text{M} + 54]^+$ ion yields diagnostic ions at m/z 276 and 148 for all $n-3$ peaks, characteristic of 9-12-15 double bond locations (Van Pelt & Brenna, 1999). These ions originate from cleavages on either side of the centre double bond and yield fragments containing the ester group (α diagnostic ion, m/z 276) or the methyl end of the molecule (ω diagnostic ion, m/z 148). The diagnostic ions for all FAME with 3 double bonds behave in this way (Van Pelt & Brenna, 1999), indicating a favourable decomposition channel for trienes. The other two unidentified isomers (**A** and **C**) presented less clear mass spectra due to their limited presence in linseed oil (Table 1). However, both isomers showed similar but less intense spectra to those shown in Figure 3 (i.e. **B** and **D**), suggesting that the four unknowns are 9-12-15 C18:3 isomers.

Elucidation of the geometrical configurations of the unknown C18:3 isomers was deduced taking into account the order of elution and the retention times of a standard mixture that contains the eight geometrical isomers of 9-12-15 C18:3. The elution order of the 9-12-15 C18:3 geometrical isomers on the highly polar SLB-IL111 capillary column is *E/E/E*, *Z/E/E*, *E/Z/E*, *E/E/Z*, *Z/Z/E*, *Z/E/Z*, *E/Z/Z* and lastly *Z/Z/Z* (Delmonte et al., 2012; De la Fuente et al., 2015). This standard was run under the same GC conditions as the linseed oil samples (Figure 4) confirming the molecular structure of the four target peaks (**A**) (9*E*,12*Z*,15*E*)-,

(**B**) (9Z,12Z,15E)-, (**C**) (9Z,12E,15Z)- and (**D**) (9E,12Z,15Z)-octadeca-9,12,15-trienoic acids previously quantified in linseed oil (Table 1).

The presence of these minor C18:3 isomers in linseed oil could be related to the edible oil deodorisation process or subsequent storage of the oil. The stability of linseed oil was evaluated during a month at refrigeration and room temperatures, but no significant differences were observed in its FA composition (data not shown). This would suggest that the (*E*)-octadecatrienoic isomers were formed during the deodorisation of the oil, an operation generally conducted at 230–250 °C for several hours in the presence of steam and under vacuum (Wolf, 2015). Previous research on ALA isomerisation shows that the central (12Z)-ethylenic bond is rather protected from thermal degradation (Wolf et al., 1996). A preferred isomerisation pathway would explain why the two external mono-(*E*)-isomers (i.e. (9Z,12Z,15E)- and (9E,12Z,15Z)-octadeca-9,12,15-trienoic acids) were detected in larger amounts than the other two C18:3 isomers characterised (Figure 5). In the ALA isomerisation process, an unstable conjugated (Z-Z)-radical is formed which would give rise, after rotation of the former (Z)-ethylenic bond, to a more stable (Z-E)-methylene-interrupted structure (Wolf et al., 1996). As (9Z,12E,15Z)-octadeca-9,12,15-trienoic acid cannot produce the (Z-Z)-radical, it would be the end-product of a less favoured isomerisation of ALA (Figure 5). In contrast, (9E,12Z,15E)-octadeca-9,12,15-trienoic acid could be formed after a secondary isomerisation of either (9Z,12Z,15E)- or (9E,12Z,15Z)-octadeca-9,12,15-trienoic acids, the former being somewhat more prone to a second geometrical isomerisation than the latter (Wolf et al., 1996).

In summary, the present work identifies the occurrence of (9E,12Z,15E)-, (9Z,12Z,15E)-, (9Z,12E,15Z)- and (9E,12Z,15Z)-octadeca-9,12,15-trienoic acids in linseed oil. These four minor ALA isomers were well resolved using an ionic liquid coated SLB-IL111 column. Their 9-12-15-octadecatrienoic structure was deduced by combination of GC-EIMS and CACI-MS/MS data while the geometrical configurations were determined with analytical standards. Further research is needed to determine the presence of these FA in other foodstuffs and their putative biological effects.

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Highlights

We identified 4 minor isomers of α -linolenic acid (ALA) in linseed oil.

Isomers were resolved by GC using a new and highly polar phase column (SLB-IL111).

The positional 9-12-15 C18:3 configuration was achieved by CACI-MS/MS.

Geometrical configuration was established with standards based on relative retention.

We postulated preferred and unfavoured isomerization pathways of ALA.

Figure 1.

Partial gas chromatogram of the C18:2 *n*-6 to C18:3 *n*-3 region of linseed oil using a 100 m SLB-IL111 capillary column including C18:3 *n*-3 and C18:3 *n*-6 standards (dotted line).

Final peak identification: **(A)** (*9E,12Z,15E*)-, **(B)** (*9Z,12Z,15E*)-, **(C)** (*9Z,12E,15Z*)- and **(D)** (*9E,12Z,15Z*)-octadeca-9,12,15-trienoic acids.

Figure 2.

Electron impact ionisation mass spectra of (9Z,12Z,15Z)-, (9Z,12Z,15E), and (6Z,9Z,12Z)-octadecatrienoic acids.

(*) m/z = 108 and m/z = 150 are characteristic of $n-3$ and $n-6$ polyunsaturated fatty acids, respectively.

Figure 3. Acetonitrile CACI-MS/MS spectra of (9Z,12Z,15Z)-, (6Z,9Z,12Z)-, (9Z,12Z,15E)- and (9E, 12Z, 15Z)-octadecatrienoic acids.

Figure 4. Partial chromatograms of the C18:3 region using a 100 m SLB-IL111 capillary column of linseed oil (solid line) and a C18:3 mixture containing all eight 9-12-15 geometrical isomers of α -linolenic acid (dotted line). Initial oven temperature was 163 °C. After 32 min, oven temperature was raised at 10 °C min⁻¹ to 205 °C and held for 38 min.

Figure 5.

Isomerisation pathways of α -linolenic acid involved in the formation of the geometrical 9-12-15-octadecatrienoic isomers characterised in the present study.

Table 1

Fatty acid profile of the two commercial linseed oils analyzed in the current study. Data expressed as g/100 g of total fatty acid methyl esters (mean $\pm$ SD).

	Linseed Oil	
	1	2
C14:0	0.05 $\pm$ 0.00	0.05 $\pm$ 0.00
C15:0	0.03 $\pm$ 0.00	0.03 $\pm$ 0.01
C16:0	5.49 $\pm$ 0.01	5.53 $\pm$ 0.02
C17:0	0.07 $\pm$ 0.00	0.06 $\pm$ 0.00
(9Z)-C16:1	0.08 $\pm$ 0.00	0.07 $\pm$ 0.00
C18:0	4.10 $\pm$ 0.01 ^a	3.79 $\pm$ 0.01 ^b
(9Z)-C18:1	17.49 $\pm$ 0.02 ^a	16.55 $\pm$ 0.03 ^b
(11Z)-C18:1	0.71 $\pm$ 0.03	0.68 $\pm$ 0.04
(11E,15Z)-C18:2	0.09 $\pm$ 0.00 ^b	0.15 $\pm$ 0.01 ^a
(9Z,12Z)-C18:2	15.62 $\pm$ 0.01 ^b	15.76 $\pm$ 0.01 ^a
(9E,12Z,15E)-C18:3 (A ^I)	0.42 $\pm$ 0.01 ^a	0.02 $\pm$ 0.00 ^b
(6Z,9Z,12Z)-C18:3	-	0.02 $\pm$ 0.00
(9Z,12Z,15E)-C18:3 (B ^I)	3.66 $\pm$ 0.01 ^a	0.28 $\pm$ 0.01 ^b
(9Z,12E,15Z)-C18:3 (C ^I)	0.59 $\pm$ 0.01 ^a	0.04 $\pm$ 0.00 ^b
(9E,12Z,15Z)-C18:3 (D ^I)	3.08 $\pm$ 0.01 ^a	0.07 $\pm$ 0.00 ^b
(9Z,12Z,15Z)-C18:3	46.59 $\pm$ 0.02 ^b	56.02 $\pm$ 0.03 ^a
C22:0	0.16 $\pm$ 0.00 ^a	0.12 $\pm$ 0.00 ^b

^I A, B, C and D stand for the fatty acids characterized in the present work.

^{a-b} Means within a row with different superscripts differ significantly.