Food Chemistry 200 (2016) 141–145

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

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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ARTICLE INFO

Article history:
Received 3 October 2015
Received in revised form 14 December 2015
Accepted 6 January 2016
Available online 9 January 2016

Keywords:
Omega-3 fatty acids
α-Linolenic acid
Geometrical isomer
Isomerisation
SLB-IL111
CACI-MS/MS

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.

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 (Pan et al., 2012; Ueshima et al., 2007). α-Linolenic acid ((9*Z*,12*Z*,15*Z*)-octadeca-9,12,15-trienoic acid, ALA) is commonly the only *n*–3 FA 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 (Wolff, 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 (De la Fuente, Rodríguez-Pino, & Juárez, 2015; Delmonte et al., 2011, 2012).

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

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http://dx.doi.org/10.1016/j.foodchem.2016.01.023
0308-8146/

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 N_2 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.

FAME were prepared by base-catalysed methanolysis of the glycerides (KOH in methanol) according to ISO-IDF procedure (ISO-IDF, 2002). FAME were prepared in hexane and BHT was added to prevent oxidation. C18:3 *n*–6, C18:3 *n*–3 standards and α-linolenic acid methyl ester 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 ionisation 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. FAME 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.

FAME were also analysed on a GC equipped with a mass spectrometer detector (Agilent 6890N 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 Fig. 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 FAME in the first linseed oil sample analysed. Although those peaks, later identified as geometrical isomers of α-linolenic acid, were 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 (Fig. 1).

Fig. 2 shows the mass spectra of ALA and one of the unknown peaks obtained by conventional GC–EIMS of FAME. 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 FAME of polyunsaturated FA 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 FA (Adams & Gross, 1987; Jensen, Tomer, & Gross, 1985). In particular, CACI-MS/MS using acetonitrile chemical ionisation reagent enables the location of unsaturated bonds through the direct analysis of FAME (Alves & Bessa, 2007; Van Pelt & Brenna, 1999; Van Pelt, Carpenter, & Brenna, 1999). 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-ethenylium ($CH_2{=}C{=}N^+{=}CH_2$, *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 $[M+54]^+$ 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 Fig. 3. As shown in Fig. 3, MS/MS data obtained upon collisional activation of the $[M+54]^+$ 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 Fig. 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