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Quantitative gas chromatographic method for the analysis of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers of the conjugated linoleic acid in liver

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Abstract

A quantitative GC method for conjugated linoleic acid (CLA) isomers of physiological significance (*cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA) as non-esterified fatty acids (NEFA) or triacylglycerols (TAG) was developed. Furthermore, the effect of the internal standard addition point (sample or fat extract) was studied. Response linearity, recovery and precision assays, detection and quantification limits were determined. Linearity was demonstrated over a range from 0.1 to 10 μg/mL. When CLA isomers were present as NEFA, the recovery significantly decreased ($P \leq 0.05$) from 76% to 27.1% (*cis*-9, *trans*-11 CLA) and 28.5% (*trans*-10, *cis*-12 CLA) when the standards were added to the fat extract or to the initial tissue, respectively. As an application, liver samples from hamsters fed a diet supplemented with both CLA isomers were analyzed. The CLA isomers in liver samples were detected with reasonable reproducibility.

Keywords: Quantitative method; GC; CLA analysis; Liver

1. Introduction

Conjugated linoleic acid (CLA) refers to a group of positional and geometric isomers of octadecadienoic acid first described by Dr Pariza's group in the 1980s [1]. These isomers possess conjugated double bonds (from 2, 4 – 18:2 to 15, 17 – 18:2) instead of the typical methylene interrupted configuration. The position of double bonds and the isomeric configuration (*cis* or *trans*) make numerous CLA isomers possible [2]. However, only a few are naturally found in foods such as ruminant meats, dairy products and processed cheeses, the *cis*-9, *trans*-11 CLA being the predominant isomer [3], and even fewer are commercially available.

In order to establish the complete isomeric distribution it is essential to use a combination of analytical methods (Ag^+-HPLC, GC/MS) [4–6]. However, in physiological and mechanistic studies only *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA isomers are important at present, since beneficial effects including anticarcinogenic, antiatherogenic, antidiabetogenic, antiobesity and immunomodulatory activities have been attributed to them [7,8]. Therefore, Kramer et al. [9] suggested that a good GC analysis might be sufficient for their quantitative analysis when the study is applied to monogastric animals or humans.

Thus, considerable efforts have been made to supply data related to CLA isomer content in biological samples, most of them, however, being related as percentage of total fat [10–12] usually making it difficult to reach metabolic conclusions. This is because these isomers are always in limited amounts, which are linked to changes in the profile of the major fatty acids when they are expressed as normalized area. Nevertheless, when quantitative data are provided another doubt is raised, since a review of the literature shows that there is a lack of consistency concerning the addition point of the internal standard, it being applied either to the fat extract [13,14] or to the initial tissue [15,16].

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As a consequence, due to the potential health benefits of the *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA isomers reported in the literature, accurate quantification of these fatty acids in biological samples is crucial in order to establish the biochemical mechanism(s) of the physiologically relevant CLA isomers, including their incorporation to the fat depots, and to definitively verify CLA-related response [17–19].

Therefore, the aim of this work was to describe a method that would provide accurate data of the CLA isomers of physiological significance (*cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA) in liver as non-esterified fatty acids (NEFA), or in the main storage form, esterified as triacylglycerols (TAG), focusing on the incidence of the internal standard addition point.

2. Material and methods

2.1. Animals, diets and experimental design

Sixteen male Syrian Golden hamsters (105 + 1 g) purchased from Harlan Ibérica (Barcelona, Spain) were individually housed in polycarbonate metabolic cages (Techniplast Gazzada, Guguggiate, Italy) following procedures in accordance with the institution's guide for the care and use of laboratory animals. Animals were randomly assigned to two groups ($n = 8$) and fed semi-purified atherogenic diets supplemented with 0.5 g/100 g diet of *cis*-9, *trans*-11 (Diet A) or *trans*-10, *cis*-12 CLA (Diet B) in free fatty acid form, respectively. At the end of the experimental period, animals were killed under anaesthesia (diethyl ether) with cardiac puncture. Liver was dissected, weighed and immediately dropped in liquid nitrogen. Samples were stored at −80 °C until analysis.

2.2. Chemicals and gases

All reagents were of analytical grade (Merck, Sigma–Aldrich, Panreac). 99% purity standards of triacylglycerol and free fatty acid were purchased from Sigma–Aldrich except for CLA isomers which were acquired from Matreya (Matreya Inc., Pleasant GAP, PA, USA). Gases used were of 99.999% purity (Alphagaz, Air-Liquide, Spain).

2.3. Sampling and lipid extraction

Samples of 0.5–1 g of liver were accurately weighed in screw-capped glass tubes and, at this point, 1 mg of *n*-nonadecanoic acid (19:0) and 1 mg of trinonadecanoylglycerol were added as internal standards in dry form. After flushing the tubes with nitrogen, lipid extraction was carried out with trichloromethane:methanol (2:1, v/v) and the sealed tubes were incubated for 30 min at room temperature (20 °C) following the method described by Folch et al. [20]. Samples were flushed with nitrogen until dry and were dissolved in 1 mL of trichloromethane.

2.4. Separation

TAG and NEFA were separated by TLC on pre-coated silica plates (DC-Platten Kiesegel 60 F254, Merck, Frankfurt, Germany) with *n*-hexane:diethyl ether:glacial acetic acid 70:30:1 (v/v/v) for NEFA. The solvent mixture used for TAG was trichloromethane:*n*-hexane:glacial acetic acid 65:35:1 (v/v/v) to avoid migration problems. The lipids were visualised with Rhodamine 0.05% in absolute ethanol and bands were identified by using standards for TAG, trioleylglycerol, and NEFA, capric (10:0), palmitic (16:0) and arachidonic (20:4) acids to determine the R_F, scraped off, dissolved in trichloromethane and filtered, first through a glass wool and then through a syringe filter (Millipore, PVDF 0.22 μm 13 mm diameter). The sample obtained was dried under nitrogen and redissolved in 1 mL trichloromethane.

2.5. Methylation

Methylation of TAG: 500 μL of sodium methoxide in methanol (0.5 M) (Sigma–Aldrich, Steinheim, Germany) was added to lipid extracts in a screw-capped test tube to prepare the fatty acid methyl esters (FAMEs). The mixture was heated at 50 °C for 10 min and the reaction was stopped with 100 μL of glacial acetic acid. Water (5 mL) was added, vortexed and the required esters were extracted twice with *n*-hexane (5 mL). The *n*-hexane layer was dried over anhydrous sodium sulphate (Merck, Frankfurt, Germany) and filtered with a Whatman filter paper (#1). The solvent was removed under reduced pressure on a rotary evaporator (20 °C) and dissolved in 500 μL of *n*-heptane containing 50 ppm of butylated hydroxytoluene (BHT) as stabilizer [21]. Completion of methylation was checked by TLC with *n*-hexane:diethyl ether:glacial acetic acid 70:30:1 (v/v/v). Finally, samples were transferred into vials and stored at −80 °C until analysis.

Methylation of NEFA: 400 μL of (Trimethylsilyl)diazomethane (TMS-DM) in *n*-hexane (2 M) (Sigma–Aldrich) was added to lipid extracts. After 30 min at room temperature, the reaction was stopped with glacial acetic acid added dropwise (5–10 drops) until the yellow colour had gone. The solvent was evaporated under a gentle nitrogen stream, and samples reconstituted in 500 μL of *n*-heptane containing 50 ppm of BHT, and stored in capped vials at −80 °C [22]. Despite TMS-DM is a safer alternative source of diazomethane, the whole process was carried out in a fume-hood. Completion of methylation was also checked by TLC at the aforementioned conditions.

2.6. Analytical conditions

Samples were analyzed by GC using a Hewlett Packard HP6890 gas chromatograph (Hewlett Packard, Palo Alto, CA, USA) equipped with a flame ionization detector (FID), split/splitless injection port, HP7673 autosampler (Hewlett Packard). The analytical column was a fused silica capillary SP-2380 column (100 m, 0.32 mm i.d., 0.20 μm film thickness) from Supelco (Bellafonte, PA, USA). The oven temperature was initially programmed at 150 °C (hold 4 min) and raised to 220 °C at a 4 °C/min rate (hold 25 min). Injection (1 μL) was run in split (40:1) mode. Helium was the carrier gas at constant flow (1 mL/min) and make-up gas for the FID. The injector was maintained at 200 °C and detector at 250 °C. Chromatographic