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Short communication

Identification of conjugated linoleic acid elongation and β-oxidation products by coupled silver-ion HPLC APPI-MS

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Abstract

Atmospheric pressure photoionisation (APPI) was used in combination with silver-ion (Ag^+)-HPLC for detection of (conjugated) fatty acid methyl esters (FAME) by tandem-mass spectrometry. APPI-MS of methyl esters of conjugated linoleic acid showed an increase in signal-to-noise ratio by a factor of 40 compared to atmospheric pressure chemical ionization in the positive mode. It was possible to identify double bond position, configuration and chain length of FAME based on chromatographic separation and mass detection. The developed LC–MS method is useful for the analysis of CLA elongation and β-oxidation products, especially with *trans*,*trans*-configuration, which are difficult to analyze by conventional GC–MS techniques.

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1. Introduction

Conjugated linoleic acids (CLA) are a naturally occurring minor group of positional and geometric isomers of linoleic acid (c9c12-C18:2), which have been shown to have potentially positive nutritional effects such as fat mass reduction in humans [1], anti-atherosclerotic effects in mice [2] and anti-cancerogenic effects in mice [3]. These conjugated fatty acids are formed during the biohydrogenation of linoleic and linolenic acid by microorganism *Butyrivibrio fibrisolvens* in ruminants [4]. Main CLA isomer is the c9t11-C18:2 (rumenic acid) [5] which amounts for nearly 90% of total CLA content in beef and milk and some 19 CLA isomers have been identified in milk fat with double bond position between Δ12Δ14 and Δ7Δ9 in *cis*- and *trans*-configuration [6,7]. The biological mechanisms of action are still subject to intensive research and there is evidence for significant differences in biologic activity of single isomers [8]. Highly sensitive methods for the analysis of fatty acids and their metabolites (such as prostaglandins and isoprostanes) from food and biological matrices have been established in the past such as negative chemical ionization (NCI) GC–MS of their pentafluorobenzyl (PFB) derivatives [9] or isobutane positive chemical ionization (PCI) GC–MS/MS of fatty acid methyl esters (FAME) [10]. Characterization of double bond position and configuration of FAME was established by acetonitrile PCI GC–MS/MS [11]. However, CLA isomers cannot be completely resolved by GC [12] and mass spectral data for structural determination of the fatty acids are often compromised due to co-eluting compounds. Alternatively, silver ion HPLC (Ag^+-HPLC) has become the most favourable method for CLA analysis as it is capable of resolving most isomers [13,14]. Advantage of Ag^+-HPLC is that compounds are separated into a *trans*,*trans*-, a *cis*,*trans*/*trans*,*cis*- and a *cis*,*cis*-group depending on the configuration of double bonds and on the double bond position within each group. The drawback, the carbon chain length has no influence on the separation within such a

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group. This results in co-elution of elongation (C20:2) and β-oxidation (C16:2) metabolites of CLA with other conjugated fatty acids (e.g. c11t13-C18:2 co-elutes with c11t13-C20:2) as shown recently in human coronary artery smooth muscle cells (HCASMC) after incubating with different CLA isomers [15]. Differences in metabolisation of *trans* fatty acids depending on the configuration and position of double bond may explain positive or negative nutritional effects [15–17]. Therefore, Ag^+-HPLC is a very powerful technique to study the fatty acid elongation and desaturation metabolism of conjugated fatty acids but so far additional GC–MS measurements for confirmation of the absence of co-eluting compounds were necessary. Aim of this study was to hyphenate Ag^+-HPLC for separation of CLA and their elongation and β-oxidation metabolites with selective mass-spectrometric detection using lipids from HCASMC as model system. Atmospheric pressure photoionization (APPI) was tested for efficient ionisation of the non-polar methyl ester derivatives and compared with atmospheric pressure chemical ionization (APCI).

2. Experimental

2.1. Samples

c7t9-C16:2 and c11t13-C20:2 β-oxidation and elongation products of CLA have been identified in a previous study by Ag^+-HPLC-DAD, GC–FID and GC–MS as main metabolites in human coronary artery smooth muscle cells (HCASMC) incubated with 50 μM c9t11-CLA [15]. Unfortunately, these metabolites are not commercially available as pure standard compounds at the moment. Therefore, lipid extracts from HCASMC were used as model lipids for HPLC-MS method development. The metabolites were absent in control cells. Lipids were extracted from the cells three times with chloroform/methanol (2/1, v/v) after addition of 100 μL internal standard solution in *n*-hexane (100 μg heptadecenoic acid methyl ester, c10-C17:1). Lipids were *trans*-methylated with 5% potassium methylate solution in methanol (30 min at 60 °C) and subsequent acidic esterification of free fatty acids by 0.5 M sulphuric acid in methanol (15 min at 60 °C) [18]. After addition of saturated sodium chloride solution FAMEs were extracted from the aqueous phase with hexane. Internal standard c10-C17:1 and CLA methyl ester mix (98%) were purchased from Sigma, Seelze, Germany. All solvents, sulphuric acid (96%) and sodium chloride were of analytical grade and purchased from Merck, Darmstadt, Germany. Potassium methylate was obtained as 30–35% solution in methanol from the same supplier.

2.2. Liquid chromatography

HPLC system consisted of an Agilent 1100 LC binary pump, an Agilent 1100 column oven (20 °C), a CTC PAL autosampler and an Agilent 1100 diode-array detector (234 nm). Separation was performed using three Chromspher 5 Lipids columns in series (250 mm × 4.6 mm, 5 μm) with a pre-column (50 mm × 4.6 mm, 5 μm) of the same column material (Varian, Darmstadt, Germany). 0.2% propionitrile in *n*-hexane (both Merck, Darmstadt, Germany) was used as eluent [19] at a flow rate of 0.6 mL min^{-1}. Analysis was performed after cleaning the columns with 8% propionitrile in *n*-hexane for 1 h and equilibrating the columns with the eluent for 1 h.

2.3. Mass-spectrometry

The LC-system was equipped with an API 4000 QTrap mass spectrometer detector (Applied Biosystems, Darmstadt, Germany/MDX Sciex, Toronto, Canada). APPI and APCI interfaces were tested. Experiments were performed either by direct flow injection or by Ag^+-chromatography with an injection volume of 10 μL. For APPI experiments toluene was used as dopant for the ionization. The toluene was co-injected into the APPI ion source via the auxiliary gas (gas2) at a flow rate of 30 μL min^{-1}. The source temperature was 300 °C for APPI and APCI experiments. Needle current for APCI was set to 3 μA. Detector/interface-parameters: full scan parameters: enhanced MS (EMS, Linear Ion Trap (LIT) capability of the tandem MS was used), polarity: positive, scan rate: 4000 amu s^{-1}, LIT fill time: dynamic, scan range: 200–400 amu, curtain gas 10 (arbitrary units), ion transfer voltage IS: 730 V, nebulizer gas (gas 1): 45 psi, auxiliary gas (gas 2): 30 psi, interface heater: on, system pressure 4.8 10e−5 Torr, declustering potential: 50 V. Multiple reaction monitoring (MRM) parameters: polarity: positive, curtain gas: 10 (arbitrary units), ion transfer voltage IS: 730 V, nebulizer gas (gas 1): 45 psi, auxiliary gas (gas 2): 30 psi, interface heaeter: on, collision gas (nitrogen): medium (system pressure 3.8 10e−5 Torr). Precursor/product ions and MRM parameters, which were optimized automatically by injection of CLA methyl ester standard mix using Analyst 1.4.1 software, are listed in Table 1.

Table 1
Multiple reaction monitoring parameters for different FAME

Fatty acid	Precursor ion $[M]^+$ (amu)	Product ion $[M - CH_3OH]^+$ (amu)	Dwell time (ms)	DP	CE	CXP
C18:2	294.26	262.1	70	51	13	16
C16:2	266.30	234.3	70	50	15	13
C18:3	292.30	260.3	70	50	15	13
C20:2	322.33	290.2	70	46	13	16
C20:3	320.30	288.3	70	50	15	13
C20:4	318.30	286.3	70	50	15	13

DP, declustering potential; CE, collision energy; CXP, collision cell exit potential.

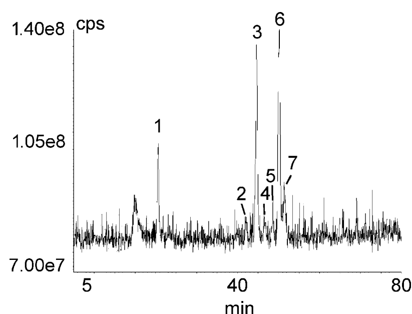

Fig. 1. Total ion chromatogram of methylated lipid fatty acids of HCASMC incubated with 50 μM c9t11-CLA for 24 h. Peak assignment: (1) saturated fatty acid methyl esters (C16:0, C18:0), (2) c11t13-C20:2, (3) c9t11-CLA, (4) c7t9-C16:2, (5) c11-C18:1, (6) c10-C17:1 (internal standard) and (7) c9-C18:1.

3. Results

3.1. Comparison of sensitivity

The signal-to-noise ratio for APPI analysis of a standard CLA methyl ester solution with toluene as dopant was enhanced by a factor of approximately 40 compared to the same concentration analyzed with APCI. Therefore, APPI interface was considered as the most sensitive ionization technique and used for further experiments. Test calibration for CLA methyl ester was linear between 1 $\mu g\,L^{-1}$ and 1 $mg\,L^{-1}$ for direct flow injection analysis in MRM mode (transition *m/z* 294/262) ($r = 0.9993$) reaching detection limits of 1 pg on column.

3.2. Fragmentation of FAME

Photoionization with post-column addition of toluene as dopant showed similar fragmentation pattern for FAME compared to GC–MS with chemical ionization. Ionization of CLA methyl esters lead to the formation of high abundant $[M]^+$ and $[M+H]^+$ ions (*m/z* 294/295, 100%) and main fragments of *m/z* 262 (35%), 110 (10%). A full scan (200–400 *m/z*) total ion chromatogram of methylated lipid fatty acids of human coronary artery smooth muscle cells (HCASMC) incubated with 50 μM c9t11-CLA for 24 h is presented in Fig. 1 and three exemplary mass spectra in Fig. 2, respectively. CLA show typical $[M]^+$ and $[M+H]^+$ ions of *m/z* 294/295 and $[M-32]^+$ fragments of *m/z* 262 (methanol loss) as can be seen in Fig. 2A. Other FAMEs like heptadecenoic acid methyl ester (c10-C17:1, *m/z* 283/250, Fig. 2B) or oleic acid methyl ester (c9-C18:1 *m/z* 296/264, not shown) also form $[M+H]^+$, $[M]^+$ and $[M-32]^+$ ions. Monoenic FAMEs were also separated depending on the position and configuration of double bond (Fig. 1, peaks 5–7). Major saturated fatty acid methyl esters were not separated by the Ag^+-columns and eluted as one peak (Fig. 1, peak 1). Stearic acid methyl ester (C18:0) and palmitic acid methyl ester (C16:0) form only the molecular ions $[M+H]^+$ (Fig. 2C, *m/z* 299 and 271, respectively).

3.3. Analysis of CLA elongation and β-oxidation metabolites in HCASMC

Fig. 3 shows four different MRM traces of HCASMC lipid fatty acids mentioned above. Using these ion traces we screened for fatty acid elongation, β-oxidation and desaturation products (transitions: *m/z* 294/262 C18:2, 266/234 C16:2, 322/290 C20:2, and 292/260 C18:3). Only the incorporated c9t11-CLA (Fig. 3A), its β-oxidation (c7t9-C16:2, Fig. 3B) and its elongation product (c11t13-C20:2, Fig. 3C) were detectable but no desaturation to conjugated linolenic acid (Fig. 3D). The FAs t9t11-CLA (Fig. 3A) and t7t9-C16:2 (Fig. 3B), respectively, were also detectable but not t11t13-C20:2 (Fig. 3C).

4. Discussion

Analysis of *cis-/trans*-isomers of fatty acids is of increasing importance as recent studies showed positive and negative nutritional effects depending on the configuration as well as on the position of double bonds [8] and the differences in metabolisation of *trans,trans*-CLA isomers [15–17]. *Trans* fatty acids and conjugated fatty acids occur naturally in food of animal origin such as beef, milk, cheese but are also formed during processing and storage of fats [20]. These fatty acids show very similar chromatographic behavior in GC and HPLC [12–14] and are difficult to analyze. This is also the case in respect to other minor lipid oxidation products such as hydroxy- and keto-fatty acids. Ag^+-HPLC with two or three columns in series permits an improved resolution of CLA (especially for the *trans-/trans*-isomers) in comparison to GC on polar columns. Fig. 4 shows a CLA methyl ester mixture in a partial Ag^+-HPLC chromatogram and in a partial GC–FID chromatogram from a 100 m CP Sil 88 column.

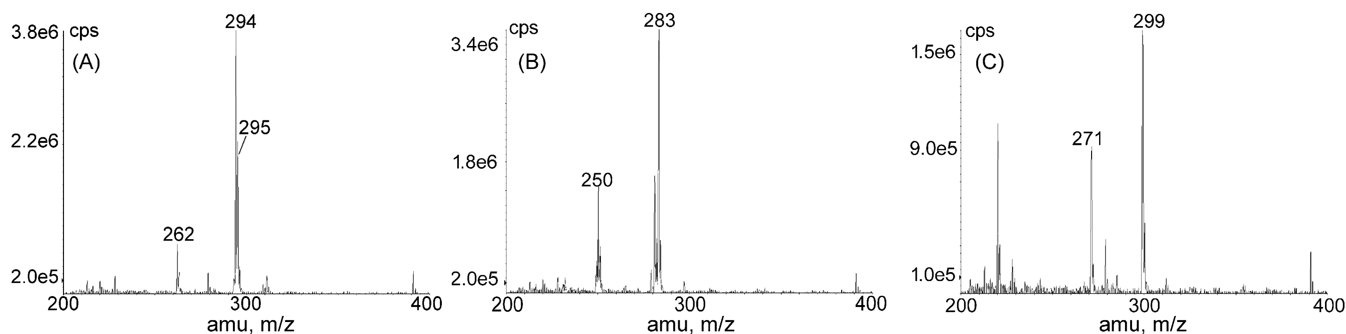

Fig. 2. Full scan mass spectra of FAMEs: (A) c9t11-CLA (peak 3 in Fig. 1); (B) c10-C17:1 (internal standard; peak 6 in Fig. 1); (C) C16:0 and C18:0 (peak 1 in Fig. 1).

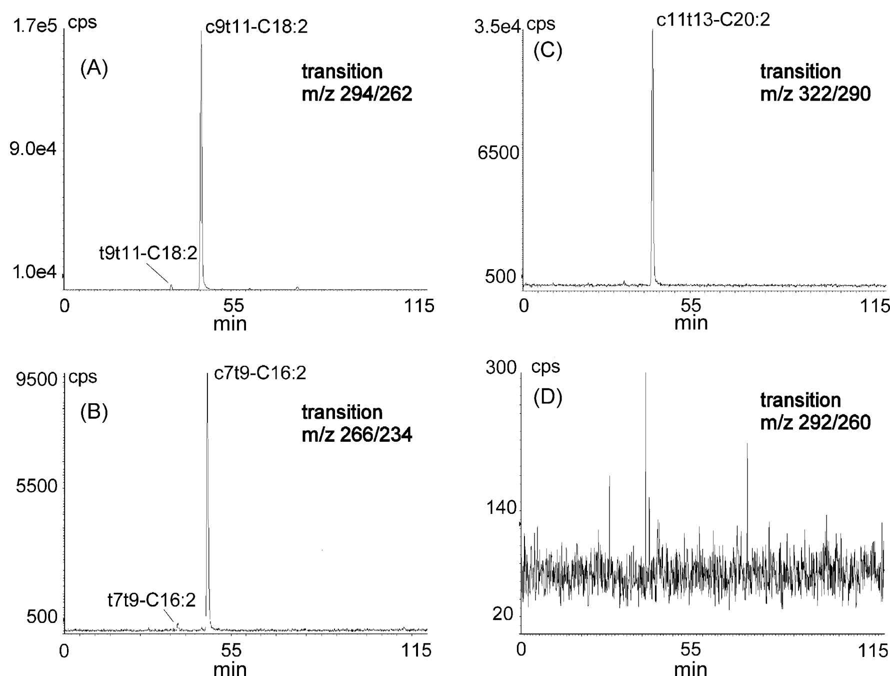

Fig. 3. Extracted ion chromatogram traces of FAMEs in HCASMC incubated with 50 μM c9t11-CLA for 24 h, analyzed by multiple-reaction-monitoring (MRM). (A) C18:2 trace (*m/z* 294/262), (B) C16:2 trace (*m/z* 266/234), (C) C20:2 trace (*m/z* 322/290) and (D) C18:3 trace (*m/z* 292/260).

Ag^+-HPLC offers an improved resolution, especially for the six *trans*,*trans*-CLA-isomers (peaks 1–6) which eluted as two peaks in the GC analysis. In complex (physiological) samples for metabolic studies of *trans*,*trans*-CLA metabolism GC–MS techniques are not sufficient, not because of a lack in sensitivity but of their inability to separate all *trans*-/*trans*-isomers necessary for reliable qualification and quantification. Ag^+-HPLC is superior for this specific analytical problem as Ag^+ ion can form charge-transfer-complexes of different strength and the double bond configuration and position can be exactly determined by their retention times in comparison to known standards [14]. Unfortunately, HPLC of lipids normally requires apolar solvent mixtures which are unfavorable for commonly used atmospheric pressure electrospray ionization (AP-ESI) interfaces in LC–MS. As an alternative, APCI and APPI interfaces are available for ionization of unpolar analytes. APPI was firstly introduced by Robb et al. for LC–MS [21] as an adaptation of the already known photoionization detector (PID) for GC and was successfully used for analysis of e.g. respiratory quinones [22] or phytosterols [23]. After evaporation of the HPLC eluent, compounds are ionized by irradiation of a strong UV-lamp with energy of approximately 10 eV which is not sufficient to ionize the solvents resulting in low background [21]. As APCI has been successfully used in combination with Ag^+-HPLC for triglyceride analysis [24] both interfaces were tested to elucidate differences in the sensitivity of analysis of a CLA mixture. Flow injection analysis of a CLA mixture showed that CLA are very susceptible for APPI analysis resulting in an increase in signal-to-noise ratio by a factor of 40 in comparison to APCI. This sensitivity is sufficient for common analysis of CLA isomers, their metabolites and isomerization products even at detection limits. Stability of retention times is a problem in Ag^+-HPLC for correct peak assignment. We showed recently that propionitrile as modifier greatly enhances the stability of retention times in comparison to the commonly used acetonitrile for Ag^+-HPLC [19]. The stability of the eluent system is sufficient for identification of compounds on the basis of retention times in comparison to an analysis of a standard mixture. APPI was regarded as optimal choice for CLA analysis as the eluent of 0.2% propionitrile in *n*-hexane did not influence sensitivity (data not shown). Background from HPLC grade solvents was detectable in full scan mass spectra (Fig. 1, *m/z* 380, *m/z* 200–230) but did not interfere in MRM mode. Ionization mechanisms and fragmentation patterns for analysis of FAME

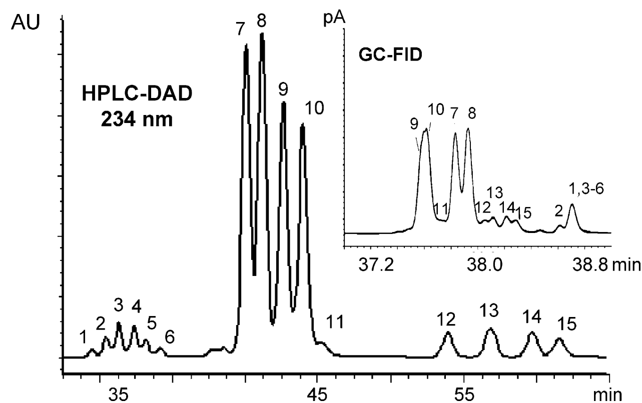

Fig. 4. Partial Ag^+-HPLC chromatogram (three ChromSpher five lipids columns, 1 mL min^{-1} 0.2% propionitrile in hexane as eluent, UV-detection at 234 nm) of a CLA methylester mixture. The insert shows the partial GC-chromatogram of the respective sample separated on a 100 m CPSil 88 GC column. Peak assignment: (1) t12t14-CLA, (2) t11t13-CLA, (3) t10t12-CLA, (4) t9t11-CLA, (5) t8t10-CLA, (6) t7t9-CLA, (7) c11t13-CLA, (8) t10c12-CLA, (9) c9t11-CLA, (10) t8c10-CLA, (11) t7c9-CLA, (12) c11c13-CLA, (13) c10c12-CLA, (14) c9c11-CLA and (15) c8c10-CLA.

are noteworthy. Saturated FAME preferably form the $[M+H]^+$ ions and did not show any further fragmentation. For these stable molecules, ionization occurs by means of a proton transfer via toluene/solvent as dopant. Monounsaturated FAME such as c10-C17:1 preferably form the $[M+H]^+$ ion, fragmenting by a loss of the proton and methanol to the $[M-32]^+$ ion. CLA were shown to form the $[M]^+$ as most abundant ion, followed by methanol loss (*m/z* 262). These differences can be explained by increasing ionization sensitivity of fatty acids with increasing number of double bonds. Mild ionization conditions causing high abundant molecular ion and small fragmentation are volitional for CLA analysis as number, position and configuration of double bonds can be deduced from chromatography [14,15,17]. Therefore, the different techniques must be discussed not only for their sensitivities but also for the structural information obtained. Negative CI-GC–MS of PFB derivatives can be used preferably for sensitive detection of fatty acid and especially their eicosanoid metabolites [9] reaching detection limits of 10 fg but hardly gives any structural information for double bond configuration of closely related compounds as the different CLA isomers. Positive APPI-MS reaches detection limits for CLA in the lower pg range which is an order of magnitude lower than PCI-GC–MS of PFB derivatives [9] or isobutane PCI-GC–MS of FAME [10,25]. Hyphenation of Ag^+-HPLC with APPI-MS detection is convenient way for identification of CLA metabolites. For complete structure elucidation of CLA other fatty acids derivatives have to be synthesized (e.g. 4,4-dimethyloxazoline derivatives) and configuration of the double bonds have to be analysed by (GC)-Fourier Transformation Infrared Spectroscopy (FTIR) [12,25]. These methods require high sample amounts and may be subject to artifact formation as fatty acids have to be derivatized at high temperatures (180 °C for 4–16 h) [26]. Identification of double bond configuration by acetonitrile CI-GC–MS/MS in positive mode is based on the formation of an $[M+54]^+$ ion via a six-membered ring intermediate after reaction with acetonitrile in the gas phase. Collisional dissociation fragments (α and ϖ ions) are characteristic for the double bond position. The α/ϖ ratio can be used for deducing double bond configuration [11]. The six *trans*,*trans*-CLA-isomers ($\Delta7\Delta9$-$\Delta13\Delta15$) elute as two peaks on a CPSil 88 GC column [12,27] the quality of the spectra of these isomers by GC–MS may be comprised at low concentrations. Ag^+-HPLC clearly separates the six isomers. Co-elution does occur but only for compounds with different chain lengths and identical double bond configuration. The resulting mass difference of at least *m/z* 28 is easily detectable by MS compared to a mass difference of 2 *m/z* for co-eluting positional CLA isomers in GC–MS. Ag^+-HPLC-APPI-MS/MS is a suitable tool for detailed analysis of double bond position and configuration for conjugated FAMEs with two double bonds and might become a complementary technique to NCI-GC–MS for specific analytical problems. It was possible to show that no isomerization of c9t11-CLA and its elongation and β-oxidation product to other *cis-/trans*-isomers occurred but small isomerization to the C18:2 and C16:2 *trans*,*trans*-isomers, respectively. No *trans*,*trans* isomerization occurred for the c11t13-C20:2 metabolite suggesting that isomerization is not caused by sample preparation. The described HPLC-MS technique is advantageous for analysis of labile compounds because mild chromatographic and ionisation conditions can be applied minimising artifact formation. The FAs c7t9-C16:2 and c11t13-C20:2 were clearly confirmed as main rumenic acid metabolites in HCASMC, which otherwise can hardly be distinguished from the respective C18:2 isomers by diode array detection [6]. Without the further need of RP-HPLC pre-fractionation of interfering compounds [28], the method will help analyzing CLA, *trans*-fatty acids, their metabolites and oxidation products to survey their effects in human nutrition. As requested in the Dietary Guidelines for Americans (2005) "research is needed to determine whether differences exist in the health effects of industrial versus animal sources of *trans* fat" [29], which differ substantially in the isomer distribution of conjugated fatty acid and C18:1 isomers.

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