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Characterization of conjugated diene fatty acids in milk, dairy products, and lamb tissues

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Conjugated diene isomers of linoleic acid (CLA), possess anticarcinogenic and antiatherogenic properties, but little is known about their metabolism. We have recently obtained evidence that CLA present in partially hydrogenated oil can be metabolized to conjugated linolenic and eicosatrienoic acids in rat liver. In the present study, we have investigated whether CLA are metabolized in the liver of lambs, which normally consume high levels of CLA produced in the rumen and present in their diet, consisting exclusively of milk. Conjugated linolenic, eicosatrienoic, and arachidonic acids were detected in lamb liver phospholipids showing that elongation and desaturation of CLA occur also in lamb tissues, and that all metabolites maintain the conjugated diene structure. (J. Nutr. Biochem. 7:150–155, 1996.)

Keywords: conjugated diene fatty acids; *trans* fatty acids; HPLC; second derivative UV spectrophotometry; milk; lambs

Introduction

At the present time there is a great interest in linoleic acid isomers with conjugated dienes (CLA), because they have been shown to have anticarcinogenic[1–3] and antiatherogenic[4] properties. CLA are normal constituents of certain foods such as milk,[5,6] including human milk,[7] dairy products,[7–9] meat from ruminants,[9–13] and partially hydrogenated oils (PHO).[14] CLA are formed along with *trans* fatty acids, during partial hydrogenation processes, performed either industrially,[14] or by anaerobic bacteria present in ruminant intestinal flora.[15] We and others have shown that CLA are absorbed and assimilated in rat tissues, but little is known about the metabolism of CLA.[2,3,16] Recently, we detected conjugated linolenic and conjugated eicosatrienoic acids in the liver of rats fed for 1 week a diet containing about 0.04% CLA.[17] This finding suggested that elongation and desaturation of CLA may occur in rat liver, as in the case of the parent compound, linoleic acid. However, we detected no conjugated diene isomers of arachidonic acid, the expected end products of such processes in the liver. Evidence in literature[3,17] indicates that the organ and lipid distribution, and the metabolism of assimilated CLA, including their incorporation in phospholipids, depend on at least two variables: the amount present in the diet, and length of intake. In these respects, lambs appear to offer some unique opportunities to study the metabolism of CLA, because they normally have a high intake of these isomers, either produced in their rumen, or derived from their diet, which consists exclusively of milk. For this reason, we decided to characterize the conjugated diene fatty acids and *trans* fatty acids present in milk, dairy products, liver, and adipose tissue of lambs. We then compared the results to previous findings in rats, to assess whether the metabolism of CLA depends on animal species.

Methods and materials

All solvent used were HPLC grade (Carlo Erba, Milano, Italy). The following fatty acids and their methyl esters were purchased from Sigma Chemicals Co., St. Louis, MO, USA: arachidonic, linolenic, linoleic, oleic, eicosatrienoic, vaccenic, and elaidic. Hydroperoxy-octadecadienoic acid (HPODE) was purchased from Cascade Biochem. LTD, London, UK. Desferal (deferoxamine methanesulfonate), an iron chelator, was purchased from CIBA-Geigy, Basel, Switzerland. A mixture of standard CLA was ob-

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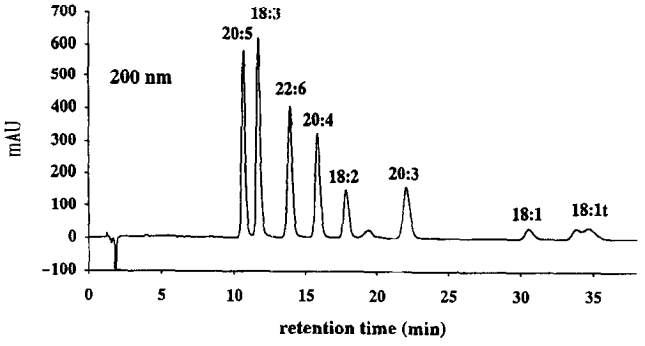


Figure 1 HPLC chromatogram of reference unsaturated non-conjugated fatty acids recorded at 200 nm.

tained from NU Chek Prep, Inc., Elysian, MN, USA. All other reagents and chemicals were of the highest available purity. Samples (six each) of sheep milk, and of liver and adipose tissue of 1-month-old lambs, were obtained from a private breeder located in Sarroch, Sardinia, Italy. The tissues were promptly removed after the animals were killed, and immediately processed as indicated. Samples (six each) of romano, parmesan, ricotta, and swiss cheese, and of cow milk and yogurt, were purchased in local stores.

Lipid extraction and preparation of fatty-acids

Total lipids were extracted from sheep and cow milk (1 g), cheese (0.25 g), yogurt (0.5 g), liver (0.5 g), and adipose tissue (0.1 g) by the Folch et al. procedure.[18] Separation of total lipids into neutral and phospholipids were performed as described by Lombardi et al.[19] Preparation of free fatty acids were obtained by a mild saponification as described by Banni et al.[14] Briefly, aliquots (3 mg) of lipid extracts were dissolved in 5 ml of ethanol, and 100 µl of desferal (25 mg/ml H_2O), 1 ml of a 25% water solution of ascorbic acid, and 0.5 ml of 10N KOH, were added. The solutions were left in the dark at room temperature for 14 hr. After addition of 10 ml of n-hexane and 7 ml of H_2O, samples were acidified with 0.35 ml of 37% HCl, to a pH 3–4 and were then centrifuged for 1 hr at 900 × g. The hexane phase containing free fatty acids was collected, the solvent evaporated, and the residue was dissolved in 0.5 ml of CH_3CN/0.14% of CH_3CO_2H (vol/vol). Aliquots (8µl) of the latter were injected into the HPLC system. All solvent evaporations were performed under vacuum, and lipids were quantified using the method of Chiang et al.[20]

Preparation of conjugated diene fatty acids by alkalinization

Alkalinization of commercial eicosatrienoic, linolenic, arachidonic, and linoleic acid methyl esters was carried out as

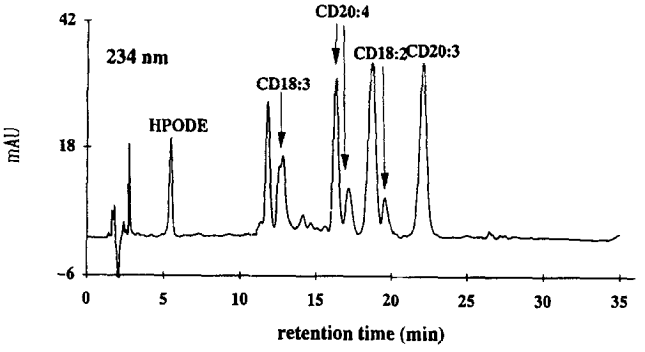
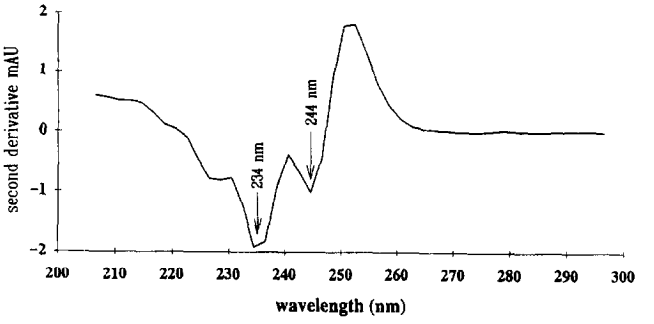


Figure 2 HPLC chromatogram of reference conjugated fatty acids recorded at 234 nm. HPODE, Hydroperoxy-octadecadienoic acid.



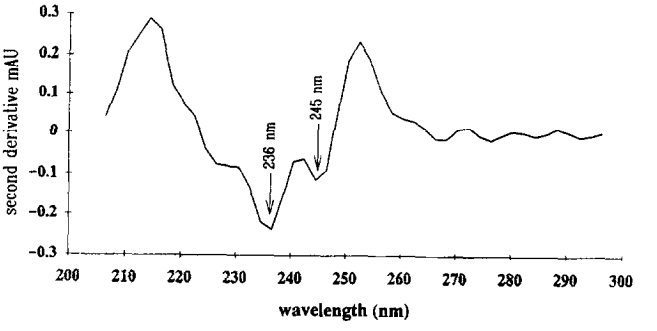
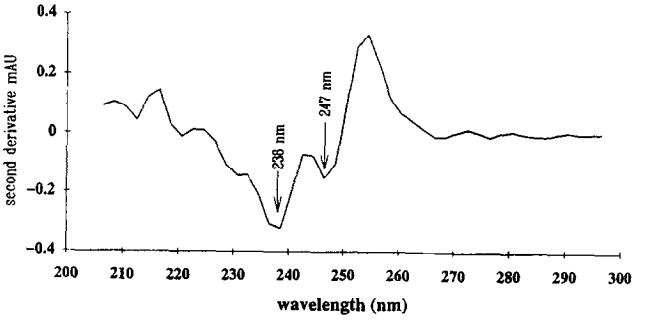


Figure 3 Second derivative UV spectra of conjugated linoleic acid (upper graph) and of conjugated linolenic acid (lower graph).



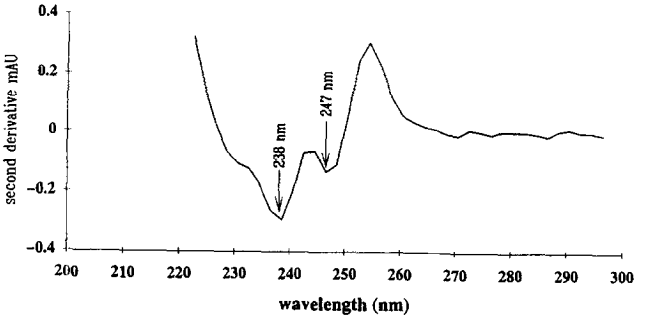


Figure 4 Second derivative UV spectra of conjugated eicosatrienoic acid (upper graph) and of conjugated arachidonic acid (lower graph).

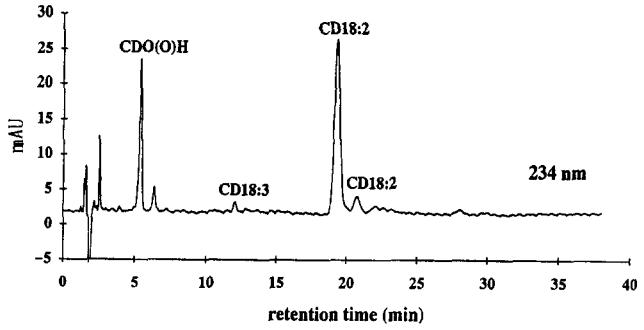


Figure 5 HPLC chromatogram of sheep milk conjugated fatty acids recorded at 234 nm.

described by Nichols et al.[21] Briefly, 5 mg of fatty acid methyl esters were dissolved in 5 ml ethylene glycol and added to 1 g of activated (200°C for 15 min) KOH. The samples were incubated for 4 h at 60°C. Free fatty acids were extracted as described in the previous paragraph.

Second derivative spectrophotometry analyses

Total lipid samples were redissolved in cyclohexane, and their conventional and second derivative U.V. absorption spectra were taken between 220 and 300 nm using a Perkin Elmer model Lambda 15 spectrophotometer. The height of the two signals with a minimum at around 233 and 242 nm were measured and added together. The concentration of total conjugated dienes in the samples was determined by using a standard reference curve, built as described in [22] for each set of experiments.

HPLC diode array detector analyses

Separation of unsaturated fatty acids was carried out with a Hewlett-Packard 1050 liquid chromatograph equipped with a diode array detector 1040M (Hewlett Packard, Palo Alto, CA). A C-18 Alltech Adsorbosphere column, 5μm particle size, 250 × 4.6 mm, was used with a mobile phase of $CH_3CN/H_2O/CH_3COOH$ (70/30/0.12, V/V/V) at a flow rate of 1.5 ml/min. Unsaturated non-conjugated fatty acids were detected at 200 nm (*Figure 1*), and conjugated diene fatty acids at 234 nm (*Figure 2*). Spectra (195–315 nm) of the eluate were obtained every 1.28 sec and were electronically stored. Second-derivative UV spectra were generated using the Phoenix 3D HP Chemstation software.

These spectra were taken to confirm the identification of the peaks, as shown in Figures *3* and *4*.

Results

Identification of sample fatty acids was carried out by comparing their retention time and second derivative UV spectra to those of reference conjugated fatty acid standards depicted in *Figures 2, 3, 4.*

Figure 5 shows a typical chromatogram of sheep milk conjugated fatty acids recorded at 234 nm. Four peaks having typical conventional and second-derivative spectra of conjugated dienes were attributed to HPODE (retention time 5.1 min), conjugated linolenic acid (retention time 11.7 min), and CLA (retention time 18 and 21 min).

Quantitative analyses of the conjugated diene fatty acids showed high levels in sheep milk and ricotta cheese, whereas much lower levels were present in cow milk and yogurt (*Table 1*). Interestingly, milk samples collected during the summer season had a much lower content of CLA, conjugated linolenic acid and *trans* fatty acids and a higher concentration of linoleic acid than samples collected during winter.

A good correlation was found between isolated *trans* 18:1 and CLA (r^2 = 0.9, $P < 0.0001$), whereas no correlation was found between CLA and the parent compound linoleic acid (data not shown).

HPLC analyses of lamb liver total lipid fatty acids recorded at 234 nm (*Figure 6*) showed at least eight peaks containing a conjugated diene chromophore attributed to conjugated linolenic acids (retention times 12 and 13 min), conjugated arachidonic acids (retention times 16 and 17 min), CLA (retention times 19, 20, and 21 min), and conjugated eicosatrienoic acid (retention time 22 min). Phospholipid analyses yielded a similar pattern of results (*Figure 7*). Neutral lipids were found to contain conjugated linolenic, CLA and conjugated eicosatrienoic acid, but not conjugated arachidonic acid (*Figure 8*). HPLC analyses of adipose tissue total lipids showed the same profile of conjugated fatty acids as in liver neutral lipids (*Figure 9*). Quantitative analyses of lamb liver conjugated fatty acids (*Table 2*) showed that CLA was present in the highest concentration, followed by conjugated linolenic, conjugated eicosatrienoic, and conjugated arachidonic acid.

Table 1 Conjugated fatty acids, *trans*-octadecenoic, and linoleic acids in Italian milk and dairy products

Samples	18:1t nmoles/mg lipids mean ± S.D.	18:2 nmoles/mg lipids mean ± S.D.	CD18:2 nmoles/mg lipids mean ± S.D.	CD18:3 nmoles/mg lipids mean ± S.D.	CD18:2/18:2 mean ± S.D.	18:1t/CD18:2 mean ± S.D.
Pecorino	178.00 ± 102.95	63.92 ± 36.99	46.48 ± 20.95	3.60 ± 1.74	0.71 ± 0.47	3.83 ± 1.76
Ricotta	322.33 ± 11.73	62.39 ± 4.92	86.24 ± 9.93	7.29 ± 1.73	1.50 ± 0.07	2.88 ± 0.25
Parmesan	114.03 ± 65.92	63.00 ± 36.39	30.83 ± 2.25	1.89 ± 0.25	0.52 ± 0.30	2.85 ± 1.64
Swiss cheese	131.08 ± 26.18	45.14 ± 3.35	50.73 ± 1.28	3.79 ± 0.95	1.21 ± 0.04	4.20 ± 0.67
Sheep milk	303.57 ± 39.00	34.89 ± 1.67	105.85 ± 8.40	6.55 ± 0.27	3.22 ± 0.09	3.69 ± 0.19
Sheep milk*	46.51 ± 5.01	87.01 ± 9.22	41.80 ± 4.61	2.70 ± 0.37	0.51 ± 0.01	9.50 ± 0.02
Cow milk	68.03 ± 8.47	56.32 ± 0.81	25.32 ± 0.38	1.86 ± 0.11	0.48 ± 0.01	3.99 ± 0.43
Yogurt	153.57 ± 21.03	89.59 ± 13.86	28.44 ± 4.32	0.58 ± 0.18	0.32 ± 0.01	1.87 ± 0.03

*= samples collected in the summer.
S.D. = standard deviation.

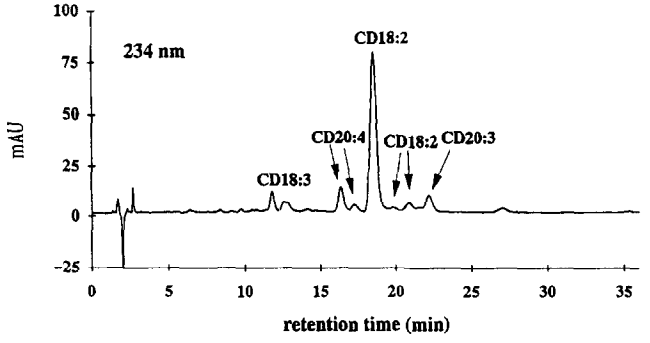


Figure 6 HPLC chromatogram of conjugated fatty acids in lamb liver total lipids recorded at 234 nm.

Discussion

CLA have been detected in tissues of patients with different pathological states,[26–31] but their origin in humans is still debated. In these regards three hypotheses have been proposed: a free radical attack on PUFA;[26–29,31] that has been strongly questioned,[37,38] an endogenous synthesis by anaerobic bacteria;[32,33] and a dietary origin.[9,30,34–36] However dietary CLA seems to be by far the predominant source in humans. Indeed, it has been shown that, consumption of food containing CLA can significantly increase their concentration in both human[9,34,35] and experimental animal[2,3,11,17] tissues. In a previous study[36] we also found a strong correlation between CLA and 18:1 *trans* present in human adipose tissue, as was found in the present study in milk and dairy products. These results support the hypothesis that in humans, CLA are mostly of dietary origin.

To our knowledge, this is the first report of the presence of conjugated diene fatty acids, other than CLA, in milk, dairy products, and lamb tissues. Though the conjugated diene fatty acids present in the milk and dairy products we analyzed were mostly CLA, as previously reported,[5–9,24,25] small but quantifiable amounts of conjugated linolenic acid were also detected. The latter may derive from biohydrogenation of linolenic acid.[23] Furthermore, we detected seasonal variations in the CLA content of milk, with higher values in samples collected during the cold season (*Table 1*). In fact, as reviewed by Riel,[25] highest values are reached when pastures are lush and rich in polyunsaturated fatty acids.

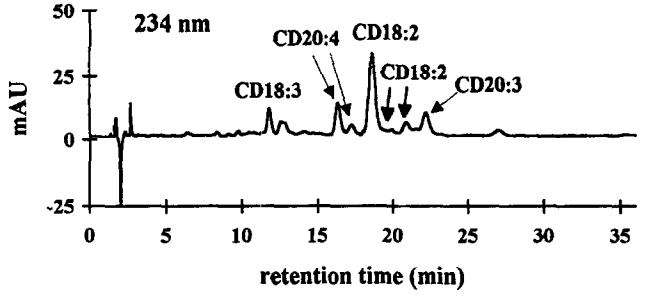


Figure 7 HPLC chromatogram of conjugated fatty acids in lamb liver phospholipids recorded at 234 nm.

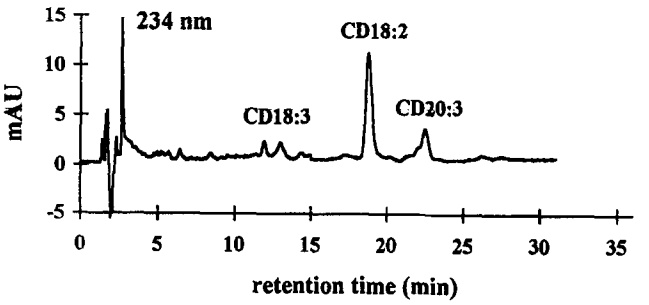


Figure 8 HPLC chromatogram of conjugated fatty acids of lamb liver neutral lipids recorded at 234 nm.

Analyses of lamb liver lipids showed that, as in rat liver,[17] elongation and desaturation of CLA do not affect the conjugated diene structure, and that these processes are carried out in the same fashion as in the case of linoleic acid. More significantly, perhaps, in lambs the conjugated diene intermediates were found to be incorporated also into liver phospholipids, and the end product, conjugated arachidonic acid, only in phospholipids. In adipose tissue, that is constituted mostly of neutral lipids, no conjugated arachidonic acid was detected. The conversion of CLA into conjugated diene arachidonic acid, and the incorporation of the latter into tissue phospholipids, appear to be the most significant findings of the present study. They raise indeed the questions of whether conjugated arachidonic acid may compete with the parent compound in the biosynthesis of eicosanoids, and exert anti-inflammatory actions on the one hand, and participate in the antiatherogenic and anticarcinogenic effects of CLA, on the other.

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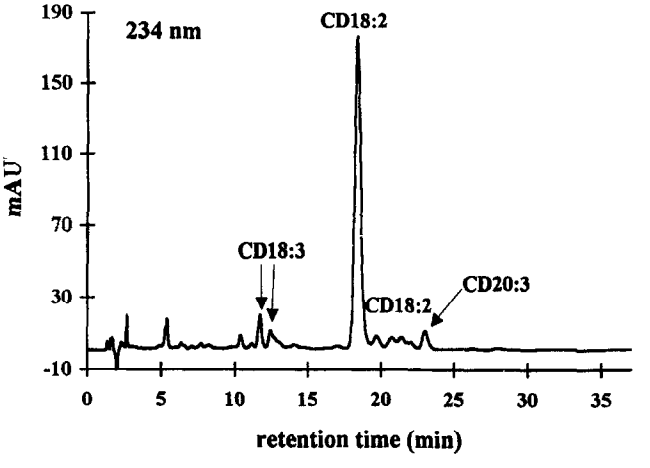

Figure 9 HPLC chromatogram of conjugated fatty acids of lamb adipose tissue total lipids recorded at 234 nm.

Table 2 Conjugated fatty acids of liver and adipose tissues of 1-month-old lambs

Samples	CD18:2 nmoles/mg lipids mean ± S.D.	CD18:3 nmoles/mg lipids mean ± S.D.	CD 20:3 nmoles/mg lipids mean ± S.D.	CD 20:4 nmoles/mg lipids mean ± S.D.	t18:1 nmoles/mg lipids mean ± S.D.
Liver	44.81 ± 3.56	6.52 ± 1.10	3.68 ± 0.74	1.63 ± 0.45	106.38 ± 49.34
Adipose tissue	60.28 ± 10.55	6.23 ± 1.50	2.00 ± 1.09	n.d.*	282.68 ± 87.22

* = not detected.
S.D. = standard deviation.

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