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DETERMINATION OF CONJUGATED FATTY ACID IN OVINE MILK, MEAT, FAT AND INTESTINAL DIGESTA

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ABSTRACT

Positional and geometric isomers of geometric isomers of linoleic acid (CLA) were separated from interfering species on commercially available two reversed-phase C_{18}-columns (Nova Pak, Waters) in gradient systems composed of acetonitrile and water, utilizing photodiode array detection. The biological samples were hydrolyzed with 2 M NaOH for 35 min at 85°C. After cooling, the hydrolysates were acidified with 4 M HCl and the free fatty acids were extracted with dichloromethane. The CLA isomers were determined directly using UV detection at 234.5 nm or after pre-column derivatization with 2,4'-dibromoacetophenone in the presence of triethylamine and UV detection at 256 and 235 nm. HPLC system with pre-column derivatization enables more efficient fractionation of the CLA isomers than the direct HPLC system. On the other hand, elimination of derivatization procedure provides a less expensive, more specific and simpler analytical tool for determination of CLA than HPLC method with pre-column derivatization. The presented HPLC methods provide analytical tools for simple quantification of CLA in ovine meat, milk, fat and intestinal digesta samples.

Keywords: conjugated fatty acid, milk, meat, fat, intestinal digesta, sheep, HPLC

INTRODUCTION

Conjugated linoleic acid (CLA) is a mixture of seventeen positional and geometric isomers of linoleic acid with conjugated double bonds

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located at positions 11,13-, 10,12-, 9,11-, 8,10- or 7,9- on the carbon chain [1]. The CLA isomers are produced by bioconversion of linoleic acid by the bacterium *Butyrivibrio fibroselvens* in the rumen [2] and are an intermediary product in the successive biohydrogenation of linoleic acid to *trans-11*-octadecenoic acid and then to stearic acid. The *cis, trans/trans, cis-8,10-; -9,11-; -10,12-* and *-11,13-* isomers accounts for the major ones, however, the most abundant CLA isomer is *cis-9, trans-11*-octadecadienoic acid. Although CLA are a relative minor component of the total fatty acid composition of feed, CLA exhibit significant health benefits for humans and has been shown to elevate lean mass in beef cattle and significantly decreased excess body fat [2]. Moreover, CLA appears to have antioxidative and anticarcinogenic properties [1-3]. In addition, CLA has been shown to stimulate immune response and protect against arteriosclerosis [2-6]. Food products from ruminants, red meat and particularly dairy products are the principal dietary source of CLA for humans. Considering the above facts, it is essential to provide an accurate, sensitive and specific HPLC methods for simple determination of CLA in ovine milk, meat, subcutaneous fat and intestinal digesta offering satisfactory estimation of the nutritive value of meat, dairy products and to study metabolic pathway of CLA. We have also demonstrated that our earlier HPLC methods [7,8] may be successfully adapted to analyze derivatized CLA isomers using a newest HPLC apparatus.

MATERIALS AND METHODS

All reagents were of analytical grade; water and all organic solvents were of HPLC grade. Acetonitrile, dichloromethane, acetic acid, benzoic acid and acetone were purchased from POCh (Poland). Triethylamine and 2,4'-dibromoacetophenone were from Merck (Germany). All saturated and unsaturated fatty acids were purchased from Sigma (USA). The mobile phases were filtered through a 0.45 μm filter membrane (Millipore, Canada) and then degassed for 2-3 min in vacuum with ultrasonication prior to use.

HPLC configurations

An alliance separation module (model 2690, Waters) with a Waters 996 photodiode array detector (DAD) was used for gradient elution system I. A Waters 625LC system that included a controller for gradient elution system II, two Waters 501 pumps and a Waters 515 pump, was applied. The apparatus coupled to a Waters™717plus WISP autosampler, a Waters 996 photodiode array detector (DAD) and computer data handling system. Separations were performed on two Nova Pak C_{18} columns (4 μm, 250×4.6 mm I.D., Waters) in conjunction with a Waters guard column of 10x6 mm I.D. (Nova Pak). Solutions of internal standards were prepared by dissolved ~135 mg of nonanoic acid (for system I) and ~135 mg of benzoic acid (for system II) in 25 ml of acetonitrile.

Mobile phases and binary gradient elution systems

Mobile phases A and B were acetonitrile and water, respectively. For analysis of the derivatized CLA isomers, the gradient elution system I was used, while for quantification of underivatized CLA isomers the gradient system II (Table 1).

Preparation and hydrolysis of samples

Milk, meat, subcutaneous fat and duodenal digesta samples were collected from sheep. All samples were frozen, lyophilised and then meat (~70 mg), fat (~10 mg), milk (~47 mg) or intestinal digesta (~100 mg) were hydrolysed with 3.5-4.0 ml of 2M NaOH at ~85°C for 35 min in sealed tubes. Next, to hydrolysates were added the internal standard (20 - 30 µl of nonanoic acid for system I; 60 - 80 µl of benzoic acid for for system II). The hydrolysates were acidified with 4 M HCl to pH ~2; free fatty acids were extracted for times with 3.5 ml of dichloromethane. The organic layer was dried with Na_2SO_4 and then dichloromethane was removed under a stream of argon. The residue was dissolved in 1 ml of acetonitrile (system II) or was used for derivatization as described below (system I). The solutions of underivatized fatty acids were directly injected onto HPLC columns.

Derivatization procedure (system I)

To a residue in a reacti-vial, 0.5 ml of 2,4'-dibromoacetophenone (12 g/L in acetone) and 1.5 ml of triethylamine (10 g/L in acetone) were added. The content was mixed and reacted for 2 h at 50°C. The derivatization reaction was stopped by adding 50 µl of acetic acid (2 g/L in acetone). The derivatizing procedure for standard was the same as for biological samples.

RESULTS AND DISCUSSION

Our initial monitoring CLA levels in milk and intestinal digesta samples was based upon duplication of analysis published previously by Czauderna et al. [7]. We had found that trace amounts of CLA isomers can be detected in rumen fluid, duodenal digesta, while slightly greater amounts in milk samples. Unfortunately, in the early gradient elution system [7], the CLA isomers of these biological samples tend to co-elute with isomers of di-unsaturated fatty acids (linoleic and linolelaidic acids). Therefore, the presented study was undertaken to develop a gradient elution system for determination of the CLA isomers in the presence of other unsaturated and saturated fatty acids. By adjustment of percentage of water in acetonitrile (Table 1), the seven positional and geometrical derivatized CLA isomers eluted as six peaks (three large and three small peaks).

Tab. 1. Gradient elution system I used for analysis of pre-column derivatized CLA and other fatty acids (columns temperature: 36°C). Gradient elution system II used for direct analysis of underivatized CLA (columns temperature 31°C).

System I					System II				
Time Min	Flow rate	Composition, %			Time Min	Flow rate	Composition, %		
		Acetonitrile	Water	Curve[b]			Acetonitrile	Water	Curve[b]
0	2.4	68.0	32.0	-	0	2.0	54.0	46.0	-
5.0	2.4	68.0	32.0	6	10.0	2.0	96.0	4.0	6
23.0	2.4	76.5	23.5	6	15.0	2.0	100.0	0	6
34.5	2.7	85.0	15.0	6	15.5	2.2	100.0	0	6
80.9	2.7	85.0	15.0	6	20.0	2.4	100.0	0	6
81.0	3.0	100.0	0	6	21.0	2.2	54.0	46.0	6
95.9	3.0	100.0	0	11	23.0	2.0	54.0	46.0	6
96.0[c]	2.6	68.0	32	6	30.0	2.0	54.0	46.0	11

[a] - from 80.1 to 95.0 min the columns were cleaned in 100 % solvent A. However, the columns should be cleaned for 25 min when injected samples contain behenic acid;
[b] - Millennium software user's guide. 1994 Waters Corporation, Milford, MA 01757 USA;
[c] - from 96 min the columns were equilibrated for 14 min.

Moreover, the presented HPLC method was able to resolve all derivatized CLA isomers from critical geometrical isomers of di-unsaturated fatty acids (linoleic and linolelaidic acid) and two close eluted saturated fatty acids (palmitic and myristic acids). Thus, the new binary gradient elution system is more successful in separating the CLA isomers from other critical fatty acids peaks in comparison with separation obtained in our previous binary gradient system [7]. Unfortunately, in milk, meat, subcutaneous fat and internal digesta samples, the CLA isomers tent to co-elute with unidentified unsaturated fatty acids (Fig. 1A), but by using the ratio of detector response set at 235 and 256 nm for the CLA peaks, the CLA content in all assayed biological samples can be simply calculated [7]. The total yield of extraction process and derivatization reaction was calculated using area of nonanoic acid peak as the internal standard. We found that response of photodiode detector to the concentration of CLA isomers is linear function. Therefore, this HPLC method with *pre*-column derivatization are suitable for simultaneous quantification of CLA, saturated and unsaturated non-conjugated fatty acids.

In recent years, intensive studies have been conducted on RP-HPLC separations of underivatized CLA and direct UV detection at ~235 nm [7-11]. Indeed, conjugated dienes showed the very high band in the spectral range from 215 to 250 nm due to the presence of conjugated double bonds. Fortunately, saturated and unsaturated non-conjugated fatty acids possess a high absorbance at only short UV wavelengths (i.e. λ<205 nm). Thus, in this present study HPLC method with UV detection at 234.5 nm was used to quantify of underivatized CLA isomers. The extraction yield of CLA isomers was calculated applying benzoic acid peak as the internal standard.

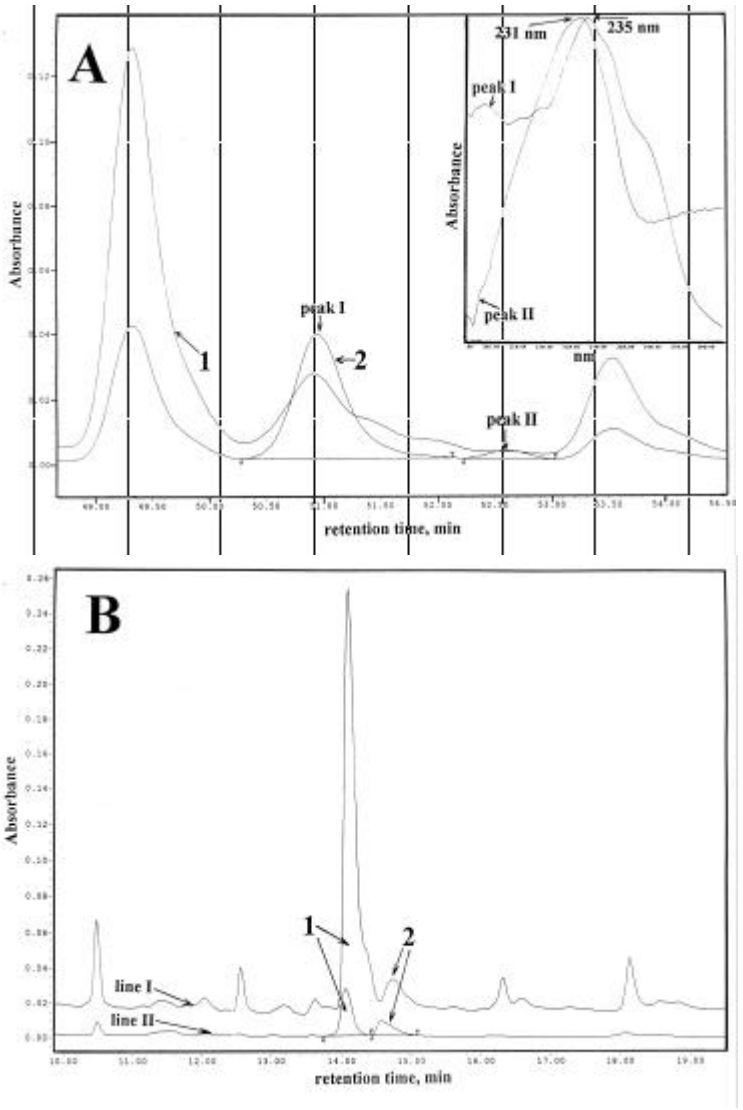

Fig. 1. The part of typical HPLC chromatograms for derivatized (system I) and underivatized (system II) CLA. Chromatogram A: derivatized CLA isomers (peaks I and II) in a milk sample (line 1 – detection at 256 nm; line 2 – detection at 235 nm). In right corner – typical the stop-flow UV spectra of CLA in milk samples. Chromatogram B: direct HPLC separation of the CLA isomers in milk (line I) and a duodenal digesta sample (line II). Peaks 1 and 2 – the CLA isomers.

As is shown in Fig. 1B in the gradient elution system II (Table 1) developed in this study all CLA isomers were substantially retained on the C_{18} columns and as pair of large peaks (from 14 to 15 min of HPLC run) clearly distinct

from background interferences and unidentified species. Moreover, trace amounts of other unidentified conjugated dienes in milk and duodenal digesta samples were detected after 18 min of elution. It is possible that these dienes contained more than eighteen carbon atoms since the retention times of these species significantly increased with decreasing polarity, i.e. increasing alkyl chain length. As expected the response of the photodiode detector to content of CLA was linear function. Obviously, all derivatized and underivatized CLA peaks were absent from the blank when gradient elution systems I and II were used. The purity of CLA peaks in milk, meat, duodenal digesta and subcutaneous fat was examined by analysis of the UV spectra (from 190 to 310 nm) of CLA in the effluents. As expected, the absorption spectra of CLA peaks in standard and all other assayed biological samples bear the very close resemblance. The separation efficiency of CLA from endogenous species was also assessed by investigating relationships between the monitoring wavelength (λ_{nm}, nm) and the ratios (R^{nm}) of CLA peaks in assayed biological samples (R^{nm}_{sample}) and calibration standards ($R^{nm}_{standard}$) (i.e. $R^{nm} = R^{nm}_{sample}/R^{nm}_{standard}$; for details see in Ref. 7). As expected, the ratio (R^{nm}) of CLA for milk and duodenal digesta were nearly 1 in the UV range of 230 - 240 nm (i.e. average ratios were: 1.13±0.14 and 1.01±0.09, respectively). So, it is clear from presented results that both CLA peaks are "pure" and free from the close presence of endogenous substances in the UV range applied.

One of the main advantage of HPLC system II is that a very simple method for preparation of samples was used. Really, this preparation procedure consist of hydrolysis and extraction only. Elimination of *pre*-column derivatization yields a more rapid and very specific method for analysis of CLA in comparison with the gradient elution system I. Moreover, the lower detection (0.30 vs. 0.57 pg) and quantification (0.98 vs. 1.89 pg) limits for CLA points to better sensitivity of direct HPLC system II. However, the HPLC system I with *pre*-column derivatization enables more efficiently fractionation of the CLA isomers than the direct HPLC system II.

GENERAL ASSESSMENT

The develop new gradient conditions using newest HPLC apparatus, like our earlier HPLC methods [7,8] provide the accurate, universal and reproducible new analytical tools for sensitive quantification of derivatized CLA isomers in meat, milk, fat and intestinal digesta samples. The underivatized CLA isomers and the internal standard (measurer of extraction yield) were successfully separated from interfering species using proposed here gradient elution system II.

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