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Separation of some mono-, di- and tri-unsaturated fatty acids containing 18 carbon atoms by high-performance liquid chromatography and photodiode array detection

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Abstract

Positional and geometric isomers of mono-, di- and tri-unsaturated fatty acids containing 18 carbon atoms were separated on commercially available reversed-phase columns in gradient systems composed of acetonitrile and water, utilizing photodiode array detection. The biological samples were hydrolyzed with 2 *M* NaOH for 35–40 min at 85–90°C. After cooling, the hydrolysates were acidified with 4 *M* HCl and the free fatty acids were extracted with dichloromethane. The organic solvent was removed in a gentle stream of argon. The fatty acids were determined after pre-column derivatization with dibromacetophenone in the presence of triethylamine. The reaction components were mixed and reacted for 2 h at 50°C. Separations of derivatized fatty acids were performed on two C_{18} columns (Nova Pak C_{18}, 4 μm, 250×4.6 mm, Waters) by binary or ternate gradient programs and UV detection at 254 and 235 nm. The geometric and positional isomers of some unsaturated fatty acids were substantially retained on the C_{18} columns and were distinct from some saturated fatty acids, endogenous substances in biological samples or background interference. Only slight separation of critical pairs of *cis*-9 $C_{18:1}$/*cis*-11 $C_{18:1}$ and *cis*-6 $C_{18:1}$/*trans*-11 $C_{18:1}$ was obtained. A ternate gradient program can be used for complete fractionation of a mixture of conjugated linoleic acid isomers (CLA) from *cis*-9, *cis*-12 and *trans*-9, *trans*-12 isomers of $C_{18:2}$. The CLA isomers in the effluent were monitored at 235 nm. The CLA isomers were differentiated from saturated and unsaturated fatty acids using a photodiode array detector. The utility of the method was demonstrated by evaluating the fatty acid composition of duodenal digesta, rapeseed and maize oils.

Keywords: Geometric isomers; Unsaturated fatty acids

1. Introduction

Many chromatographic methods for separating and quantifying geometric and positional isomers, especially in modified vegetable oils and fats, are of importance in human nutrition and metabolic studies [1–10]. Furthermore, analysis of fatty acids is important in the control of technical products, in medical diagnostic research, cancer research, and in testing the purity, origin, and freshness of food [3,11–13]. Because of the complexity of the samples, selective and sensitive techniques are necessary. Thus, capillary electrophoresis [11], gas chromatography–mass spectrometry [14] and various liquid chromatography methods [1,15–20] have been used for saturated and unsaturated fatty acids. No single

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chromatographic method is presently able to resolve all ~400 fatty acids [1]. Separations of fatty acids by degree of saturation using argented HPLC columns show several regions with overlapping peaks, particularly in the di- and mono-unsaturated fatty acid region [21–23]. The application of long polar capillary columns (50–100 m) for gas–liquid chromatography has improved the resolution of many positional and geometric fatty acid isomers [1]. Unfortunately, there may be difficulties in quantitatively preparing base-catalyzed esters from the great variety of fatty acids and lipid classes in intestinal digesta or milk [1]. Acid-catalyzed methylation converts all known fatty acids and lipid classes, however, under these conditions conjugated dienes are isomerized [1]. The major advantages of HPLC (reversed-phase, silver, adsorption and chiral) over GC are lower temperatures during the analysis, which reduce the risk of isomerization of double bonds, and the possibility of collecting fractions for further investigations. As fatty acids have a very low molar absorption at wavelengths above 220 nm, they are converted to a derivative containing a chromophore before being subjected to HPLC analysis with UV detection.

The aim of this study was to develop new gradient conditions for simultaneous analysis of some geometric and positional isomers of 18-carbon fatty acids. Attempts were made to simultaneously determine these unsaturated fatty acids (with emphasis on conjugated dienes) and some saturated fatty acids that are present in the rumen and duodenum of sheep. In fact, interest in ruminal and duodenal fatty acid composition is considerable as ruminant diets affect ruminal and duodenal fatty acid contents. In turn, ruminal and duodenal fatty acid compositions significantly influence the essential fatty acid profile of milk, meat, etc. Moreover, vegetable oils have been added to ruminant diets with the intention of changing the fatty acid profiles of milk or meat to better suit human dietary concerns [1]. Thus, in the present study, new HPLC methods were evaluated for the analyses of rumen fluids, duodenal digesta samples, rapeseed and maize oils.

There is growing interest in conjugated linoleic acid isomers (CLA) [1,4,24–27], considered beneficial in cancer prevention. Theoretically, eight possible geometric isomers of 9,11- and 10,12-linoleic acid exist, however the *cis*-9, *trans*-11 linoleic acid is the main product of ruminal biohydrogenation of polyunsaturated fatty acids [4,25]. Thus in this type of study, a combination of the separation of fatty acid derivatives and determination using a high resolution HPLC system equipped with a photodiode array detector should be used. In order to improve selectivity, reversed-phase C_{18} columns containing dimethyloctadecylsilyl-bonded amorphous silica were used.

Banni et al. [28] proposed the use of underivatized CLA, CLA metabolites and unsaturated non-conjugated fatty acids for HPLC analysis. This method gives satisfactory separation of these acids only in milk, dairy products and lamb tissues. However, our HPLC method for simultaneous analysis of some saturated fatty acids, and geometric or positional isomers of unsaturated fatty acids was found to be more selective than previously published methods [11,14,17,19,20,29,30]. One of the major advantage of this HPLC procedure is that a very simple method for preparation of free fatty acids extracts was used.

2. Experimental

2.1. Reagents

All reagents were of analytical grade; organic solvents were of HPLC grade. Acetonitrile, dichloromethane, glacial acetic acid, acetone, and methanol were purchased from POCH (Gliwice, Poland). Triethylamine and 2,4-dibromoacetophenone were from Merck (Darmstadt, Germany). Palmitic, stearic acids, conjugated linoleic acid (CLA) and all other *cis* and/or *trans* unsaturated fatty acids were purchased from Sigma (St Louis, MO, USA). Undecanoic (an internal standard), myristic and behenic acids were from Fluka. All other chemicals were obtained from POCH (Gliwice, Poland). Water used for the preparation of mobile phases and chemical reagents was prepared using an Elix™ water purification system (Millipore, Toronto, Canada). The mobile phases were filtered through a 0.45-μm membrane filter (Millipore) and then degassed for 2–3 min in vacuum with ultrasonication prior to use.

2.2. HPLC configuration

For the separation of derivatized fatty acids with direct UV detection, a Waters 625LC system consist-

Table 1
Binary gradient program

Time (min)	Composition (%)		Curve
	Solvent A	Solvent B	
0	0	100	
49.98	0	100	
50.50[a]	100	0	6
58.75	100	0	6
58.79	0	100	6
70.00	0	100	

Flow-rate: 3 ml/min; column temperature: 38°C.
[a] From 50.50 to 58.75 min, the columns were cleaned in 100% solvent A. However, the columns should be cleaned for 18 min when injected samples contain behenic acid.

ing of a controller for gradient elution, two Waters 501 pumps, and a Waters 515 pump, was used. The apparatus is coupled to a Waters™ 717plus WISP autosampler and a Waters 996 photodiode array detector. Data acquisition was performed on an Optimus Pentium 5P60 computer with Millennium 2001 software. The ambient temperature was 20–23°C. 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 10×6 mm I.D. containing reversed-phase C_{18} (30–40 μm) pellicular packing material.

2.3. Analytical solvents and gradient composition

Three HPLC grade solvents were used in this study. Solvent A was acetonitrile, while solvents B and C were acetonitrile–water (85:15, v/v) and water, respectively. For analysis of derivatized fatty acids in standards and biological samples, elution was carried out in simple gradient mode with a flow-rate of 3 ml/min; all separations were performed at a column temperature of 38°C (Table 1). For analysis of the CLA isomers, a ternate gradient program can also be used; the gradient composition is shown in Table 2. The maximum system pressure was 40.1 MPa. Injection volumes were 2–40 μl. Fatty acid derivative peaks were identified by the retention time of processed standards injected separately and by adding standard solutions to biological samples.

The concentrations of fatty acids in biological samples were calculated using fatty acid standards and an internal standard (undecanoic acid) as a measure of extraction yield.

The limit of detection (LOD) was calculated as a signal-to-noise ratio of three, while the limit of quantification (LOQ) was defined as 10 times the noise level [31,32]. The background under the peak was calculated from the baseline from the left and right sides of the peak.

2.4. Preparation and hydrolysis of samples

Rumen fluid, milk, and duodenal digesta samples were collected from sheep. Milk and duodenal digesta samples were frozen, lyophilized and the residues obtained were stored in sealed tubes under nitrogen at −20°C until analyzed. Rapeseed or maize oils and rumen fluid samples were stored at −20°C when not in use.

Rapeseed, maize oils (~50 mg), rumen fluids (~2 ml), lyophilized milk (~50 mg) and duodenal digesta

Table 2
Ternate gradient program used for analysis of conjugated linoleic acid (CLA)

Time (min)	Flow rate (ml/min)	Composition (%)			Curve
		Solvent A	Solvent B	Solvent C	
0	2.4	0	80	20	
1.0	2.4	0	80	20	6
20.0	2.4	0	90	10	6
30.0	2.6	0	100	0	6
50.0	2.6	0	100	0	6
53.9	2.7	5	95	0	6
54.5[a]	3.0	100	0	0	11

Column temperature: 40°C.
[a] After 54.5 min, the columns were cleaned, depending on type of sample, for 7–12 min in 100% solvent A. Next the columns were re-equilibrated for 15 min in 80% solvent B and 20% solvent C at a flow-rate of 2.4 ml/min.

(~150 mg) samples were hydrolyzed with 3–4 ml of 2 *M* NaOH at 85–95°C for 35–40 min in sealed tubes. All mixtures were protected from the light. After cooling, the hydrolysates were acidified with 4 *M* HCl to pH~2 and then free fatty acids were extracted four times with 3.5 ml of dichloromethane. The lower organic layer was dried with Na_2SO_4 and then the organic solvent was removed under a gentle stream of argon [11]. The residue was used for derivatization [11] as below.

2.5. Derivatization procedure

To a residue in a reacti-vial, 0.5 ml of dibromacetophenone (12 g/l in acetone) and 0.5 ml of triethylamine (10 g/l in acetone) were added. The contents were mixed and reacted for 2 h at 50°C. All mixtures were protected from the light. The derivatization reaction was stopped by adding 50 μl of acetic acid (2 g/l in acetone). The derivatizing procedure for standards was the same as for biological samples. The resulting solutions were injected on to the columns. It is recommended to protect all derivatized samples from the light and to store them at about −25°C until analyzed.

3. Results and discussion

Binary and ternate gradient systems (Tables 1 and 2) composed of H_2O in acetonitrile were found to provide a wide range of solvent strengths and excellent baseline stability. Geometric and positional isomers of fatty acids can be satisfactorily separated using long columns packed with a strongly hydrophobic silica-based bonded phase. Thus, binary or ternate gradient systems utilizing C_{18} columns, acetonitrile and H_2O were suitable for reproducible fatty acid derivative separations. Unfortunately, rather poor separation of fatty acid derivatives, especially of geometrical and/or positional isomers, was observed when a single column was used. Therefore, in order to improve selectivity, especially for *cis* and *trans* isomers of long-chain fatty acids, two long C_{18} columns (Nova Pak) together with photodiode array detection were applied. Fortunately, excellent peak shapes, close to symmetrical, were observed even with analyte elution times from 55 to 65 min. To decrease retention times and system pressure, all separations were performed on columns at a temperature of 38°C (the binary gradient program) or 40°C (the ternate gradient program).

The binary gradient system (Table 1) was used to determine reliability and reproducibility of the proposed HPLC method by analyzing geometrical and positional isomers of derivatized fatty acid standards. As expected, the use of two widely available long C_{18} columns in combination with a photodiode array detector set at 254 nm resulted in satisfactory resolution of fatty acid isomers. Underivatized fatty acids and their methyl esters separated by HPLC had high molar absorption at low UV wavelengths in which many suitable mobile phase components are not transparent [17]. Therefore, for analytical applications it is better to derivatize the fatty acids with reagents possessing high molar absorptivity at longer UV wavelengths. Thus, all investigated fatty acids were derivatized with dibromacetophenone in the presence of triethylamine [11]. The high molar absorptivity and the close proximity of the absorbance maximum to 254 nm make these derivatives almost ideally suited for analysis with fixed wavelength detectors. The derivatized fatty acids were substantially retained on the C_{18} columns and were distinct from other endogenous substances in digesta samples or from background interference. Indeed, all derivatives were monitored at a unique wavelength (254 nm), therefore, other species present in the samples did not interfere with fatty acid derivatives. As shown in Fig. 1 (chromatogram A), in the system developed in this study, fatty acid derivatives appeared in the chromatogram as single peaks.

Unfortunately, rather poor separation of critical pairs of *cis*-9 $C_{18:1}$/*cis*-11 $C_{18:1}$ and *cis*-6 $C_{18:1}$/*trans*-11 $C_{18:1}$ was obtained (Fig. 1, chromatogram B). Our method using the binary gradient program (Table 1) fractionates a mixture of conjugated linoleic acid (CLA) containing seven positional and geometrical *cis* and/or *trans* isomers. As shown in Fig. 1C, these CLA isomers appear as six peaks (i.e. 1–6). However, in the binary elution system, an insignificant peak of a conjugated isomer (peak 4) of linoleic acid tended to co-elute with linolelaidic acid (peak 8) (see the overlying chromatograms in Fig. 1C).

We also confirmed that no degradation of any

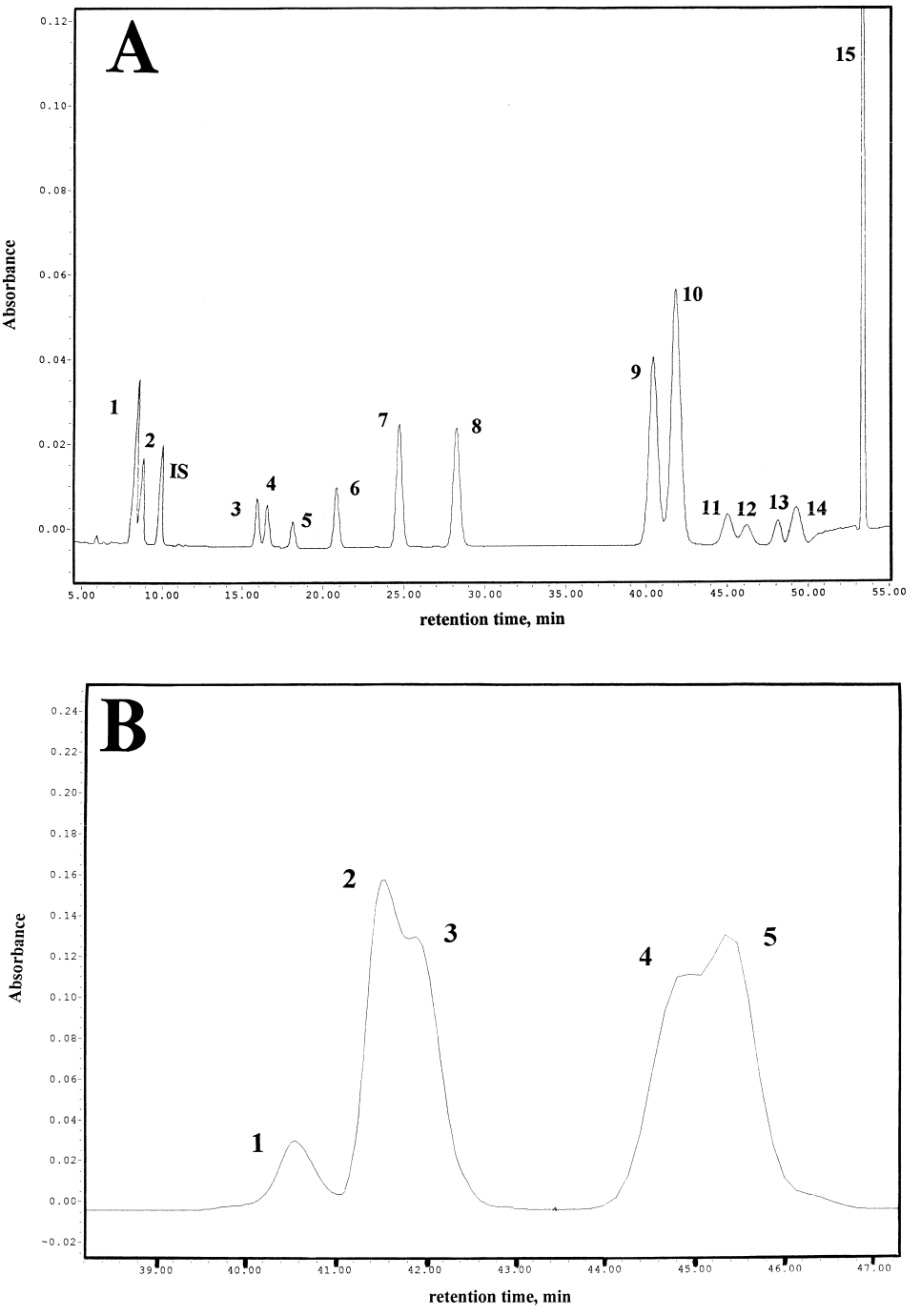

Fig. 1. HPLC chromatograms for derivatives of fatty acid standards using the binary gradient system (the detector was set at 254 nm). Chromatogram A — peaks: 1, ricinoleic acid; 2, ricinelaidic acid; IS, internal standard; 3, linolenic acid; 4, γ-linolenic acid; 5, linolenelaidic acid; 6, myristic acid; 7, linoleic acid; 8, linolelaidic acid; 9, palmitic acid; 10, oleic acid; 11, petroselinic acid; 12, elaidic acid; 13, *trans*-7-octadecenoic acid; 14, petroselaidic acid; 15, stearic acid. Chromatogram B — part of a typical chromatogram of separation of critical pair of *cis*-9 $C_{18:1}$/*cis*-11 $C_{18:1}$ (peaks 2 and 3) and *cis*-6 $C_{18:1}$/*trans*-11 $C_{18:1}$ (peaks 4 and 5) (palmitic acid — peak 1). Chromatogram C — part of a chromatogram of separation of the conjugated isomers (peaks 1–6) of linoleic acid and part of a chromatogram A (peak 7, linoleic acid; peak 8, linolelaidic acid; bold line).

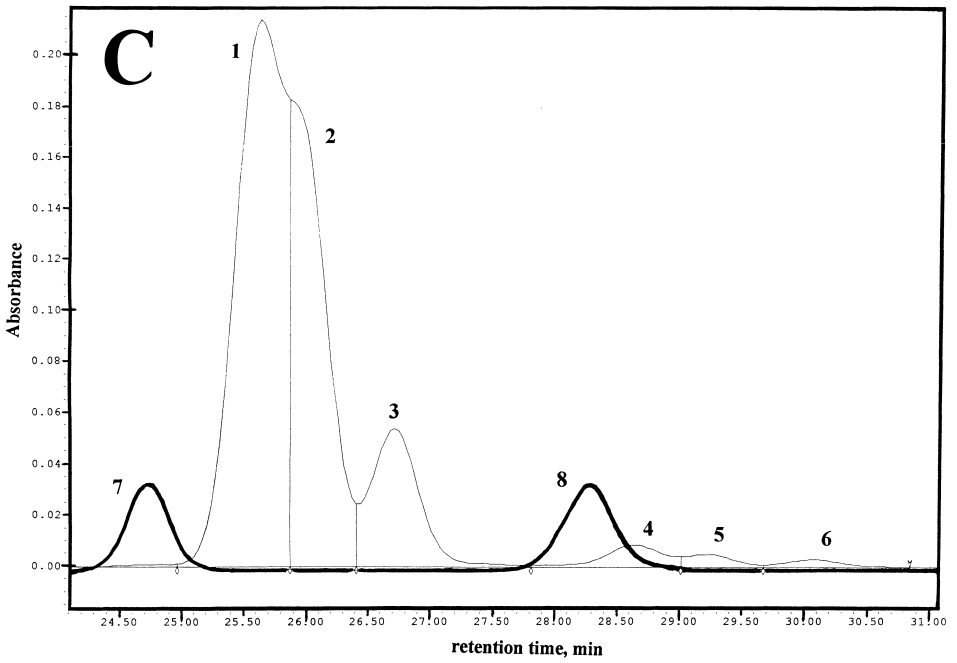

Fig. 1. (*continued*)

assayed fatty acid was observed when samples were carried through the described hydrolysis and derivatization. In fact, the same quantitative results were obtained when the same hydrolysis and derivatization procedures were performed in de-aerated fatty acid solutions (using a stream of argon). Obviously, de-aeration of reaction mixtures can eliminate even insignificant oxidation of unsaturated fatty acids. Considering the above results, it is reasonable to suggest that observable relative changes in concentrations of unsaturated fatty acid can be expected in air saturated solutions containing very low levels of these acids (i.e. the nmol or pmol ranges [29]) and/or during the hydrolysis and derivatization procedures at higher temperatures.

As can be seen in Fig. 1, the HPLC procedure resulted in separation of all derivatives in about 55 min, while the total run time of the analysis was 70 min. The retention times of the analyzed species increased with decreasing polarity, increasing alkyl chain length, and smaller number of double bonds. *Cis* isomers eluted faster than *trans* isomers (Fig. 1). Fatty acid peaks were identified by the retention time of standards injected separately and by the recovery estimation carried out by addition of fatty acid standards to duodenal digesta samples. The results of the recovery studies were satisfactory (nearly 100%) and showed that fatty acid peaks were correctly identified. Furthermore, saturated fatty acids were differentiated from unsaturated ones by the use of a photodiode array detector (Fig. 2). All fatty acid peaks were absent from the blank when the binary and ternate gradient programs (Tables 1 and 2) were used.

The accuracy of the described procedure was also assessed by examining the recoveries of determined fatty acids using different concentrations of TAG mixtures. As expected, the recoveries of assayed fatty acids calculated by the regression approach were satisfactory, i.e. the correlation coefficients (r) for determined fatty acids throughout the procedure (hydrolysis and derivatization) were close to one.

As expected, the responses of the photodiode array detector to the quantification of fatty acids are a linear function (Table 3). The correlation coefficients (r) and standard errors in slopes (SES) evidenced that the proposed UV detection provided good linearity of fatty acid derivative responses. The low

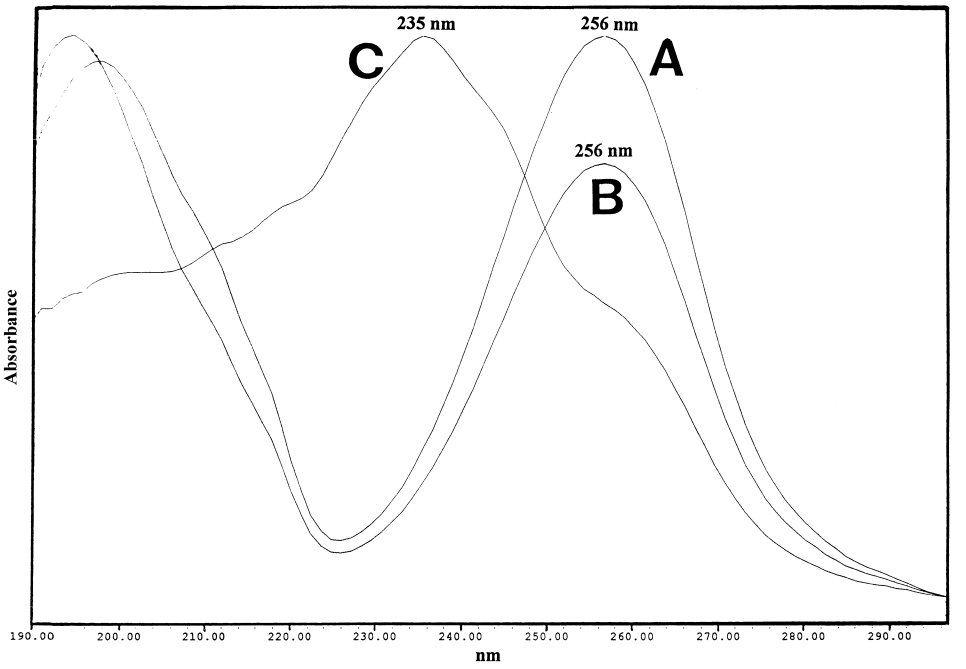

Fig. 2. Stop-flow UV spectra of the derivatized standards of saturated (A), unsaturated (B) and a mixture of the CLA isomers (C).

values of the LOD and LOQ for fatty acids of standards point to satisfactory sensitivity of the proposed method. Indeed, these values are relatively similar or smaller compared with other HPLC methods [17,19] based on UV or fluorescence detection. As the presented method was applied to biological materials (e.g. duodenal digesta samples), sensitivity was reduced to pmol levels. However, it seems clear from the LOD and LOQ values that the proposed method offers satisfactory sensitivity permitting detection and quantification of a relatively low level of fatty acids when compared with the original fatty acid contents in ruminal, duodenal digesta or milk samples. No changes in the contents of fatty acids in standards, vegetable oils, milk and duodenal digesta samples were observed when the derivatized solutions were protected from the light and stored for 30–40 days at −25°C.

3.1. Reliability of the HPLC method

The main analytical problem in the present work was to obtain suitable separation of fatty acids from interfering organic or inorganic impurities. Therefore, different monitoring wavelengths were applied to check the separation efficiency of fatty acid peaks from interfering species. Duodenal digesta, rapeseed and maize oils were evaluated by HPLC analysis. The accuracy of the method was assessed by determining relationships between the monitoring wavelength (λ_{nm}) and the ratios (R^{nm}) of individual fatty acid peaks in biological samples (R^{nm}_{sample}) and calibration standards ($R^{nm}_{standard}$) (i.e. $R^{nm}=R^{nm}_{sample}/R^{nm}_{standard}$) (for abbreviations see Table 4). Summaries of the results obtained from monitoring at various wavelengths are given in Tables 4–6. The ratios (R^{nm}) of other fatty acids for duodenal digesta samples were nearly 1 in the examined UV range (i.e. the average±SD ratios: ricinoleic acid, 0.945±0.082; ricinelaidic acid, 0.987±0.081; the internal standard, 0.991±0.040; γ-linolenic acid, 1.037±0.095; palmitic acid, 1.002±0.013; oleic acid, 1.012±0.026; petroselinic acid, 1.004±0.048; stearic acid, 1.018±0.022). Therefore, short ($\lambda<205$ nm) or long ($\lambda>225$ nm) wavelengths may be used for quantification of these fatty acids in duodenal digesta. Similarly, for maize oil samples, the ratios (R^{nm}) of palmitic, oleic and stearic acid were practi-

Table 3
Linear regression curves[a] of fatty acid standards, correlation coefficients (r), standard error in slope (SES), limits of detection (LOD), and limits of quantification (LOQ) derived from determination of fatty acid derivatives in standards

Fatty acid	Maximal amount of standard (nmol)[b]	Equation[a]	Correlation coefficient[c]				SES	LOD (ng l^{-1})	LOQ (ng l^{-1})
			$r_{standard}$	r_{rumen}	r_{rape}	r_{maize}			
Ricinoleic acid *cis*-9,OH-12 $C_{18:1}$	88.9	$y=3.14\cdot10^{-6}S_n+0.91$	0.9993	–	–	–	$0.112\cdot10^{-6}$	92	307
Ricinelaidic acid *trans*-9,OH-12 $C_{18:1}$	34.4	$y=2.68\cdot10^{-6}S_n+0.04$	0.9999	–	–	–	$0.014\cdot10^{-6}$	76	254
Linolenic acid *cis*-9,12,15 $C_{18:3}$	97.5	$y=3.36\cdot10^{-6}S_n+0.01$	1.0000	0.9830	1.0000	–	$0.003\cdot10^{-6}$	9.0	30.1
γ-Linolenic acid *cis*-6,9,12 $C_{18:3}$	65.0	$y=2.72\cdot10^{-6}S_n+0.31$	0.9999	0.9910	1.0000	–	$0.044\cdot10^{-6}$	8.4	27.8
Linolenelaidic acid *trans*-9,12,15 $C_{18:3}$	25.0	$y=2.29\cdot10^{-6}S_n+0.08$	0.9999	0.9699	–	–	$0.025\cdot10^{-6}$	13.2	43.9
Myristic acid (C14:0)	21.0	$y=0.82\cdot10^{-6}S_n+0.03$	1.0000	0.9939	0.9996	0.9954	$0.001\cdot10^{-6}$	23.9	79.7
Linoleic acid *cis*-9,12 $C_{18:2}$	111.1	$y=5.05\cdot10^{-6}S_n+0.22$	1.0000	0.9922	1.0000	0.9995	$0.002\cdot10^{-6}$	136	454
Linolelaidic acid *trans*-9,12 $C_{18:2}$	75.5	$y=3.19\cdot10^{-6}S_n+0.09$	1.0000	0.9925	–	–	$0.014\cdot10^{-6}$	80	269
Palmitic acid (C16:0)	25.5	$y=8.82\cdot10^{-6}S_n+0.11$	0.9999	0.9993	0.9996	0.9989	$0.015\cdot10^{-6}$	40	133
cis-Vaccenic acid *cis*-11 $C_{18:1}$	29.4	$y=1.59\cdot10^{-6}S_n+0.11$	1.0000	–	–	–	$0.010\cdot10^{-6}$	48	159
trans-Vaccenic acid *trans*-11 $C_{18:1}$	52.6	$y=1.49\cdot10^{-6}S_n+0.01$	1.0000	–	–	–	$0.007\cdot10^{-6}$	28	92
Oleic acid *cis*-9 $C_{18:1}$	71.1	$y=4.08\cdot10^{-6}S_n+0.01$	1.0000	0.9998	1.0000	0.9869	$0.003\cdot10^{-6}$	45	149
Petroselinic acid *cis*-6 $C_{18:1}$	22.2	$y=2.03\cdot10^{-6}S_n$	1.0000	0.9933	–	0.9967	$0.004\cdot10^{-7}$	64	213
Elaidic acid *trans*-9 $C_{18:1}$	43.2	$y=2.24\cdot10^{-6}S_n+0.20$	1.0000	0.9864	–	–	$0.029\cdot10^{-6}$	151	503
trans-7-octadecenoic acid	54.7	$y=1.49\cdot10^{-6}S_n+0.01$	1.0000	–	–	–	$0.001\cdot10^{-6}$	37	125
Petroselaidic acid *trans*-6 $C_{18:1}$	52.6	$y=2.59\cdot10^{-6}S_n+0.14$	1.0000	0.9923	–	–	$0.024\cdot10^{-6}$	74	248
Stearic acid ($C_{18:0}$)	19.0	$y=0.98\cdot10^{-6}S_n+0.09$	0.9997	0.9985	0.9991	0.9994	$0.016\cdot10^{-6}$	68	227

[a] S_n and y are the peak areas and fatty acid concentrations (μM) in a sample, respectively. Multilevel forced through zero option for generation of linear calibration curve fit (Millennium software user's guide, Waters Corporation, Milford, MA 01757 USA, vol. II (1994) pp. 12–24). Number of points used in the calibration curves: 3.

[b] Maximal amount (nmol) of fatty acid standard injected on to the columns.

[c] Correlation coefficients of fatty acids found for standards ($r_{standard}$), rumen fluid samples (r_{rumen}), rapeseed (r_{rape}) and a maize (r_{maize}) oil.

cally one (i.e. the average±SD ratios were 1.001±0.030, 1.004±0.042 and 1.050±0.058, respectively), thus these fatty acids can be determined using long or short wavelengths. Moreover, both UV monitoring ranges appear to be suitable for the analysis of linoleic, palmitic, oleic and stearic acids in rapeseed samples since the obtained ratios were also close to one (i.e. the average±SD ratios were 1.034±0.047, 0.986±0.029, 0993±0.024 and 0.992±0.082, respectively). Comparison of the obtained results indicates that, generally, in the applied UV range, integrated peaks corresponding to fatty acids in duodenal digesta and vegetable oils are pure and free from the close presence of unidentified species. Therefore, all analytical fatty acid peaks can be integrated using the total peak area method as devoid of substantial co-eluting impurities with peaks absorbing in the applied UV detection range. Obviously, the current HPLC method based on the longer wavelength UV detection ($\lambda>250$ nm) is more suitable for routine analysis of all fatty acids since unidentified endogenous species, in general, possess a relatively high absorbance in the short UV range ($\lambda<225$ nm). Therefore, the ratio (R^{nm}) in the

Table 4
Relationships (R^{nm})[a] between wavelength (λ_{nm}, nm) of fatty acid monitoring and ratios of fatty acid peaks in standards ($R^{nm}_{standard}$)[b] to fatty acid peaks in duodenal digesta samples (R^{nm}_{sample})[c]

λ_{nm}	R^{nm} for analyzed fatty acids[d]				
	cis-9,12,15 $C_{18:3}$	*trans*-9,12,15 $C_{18:3}$	*cis*-9,12 $C_{18:2}$	*trans*-9,12 $C_{18:2}$	*trans*-6 $C_{18:1}$
190	1.541	0.727	1.240	NI[e]	NI
192	1.480	0.715	1.235	NI	0.557
194	1.399	0.744	1.137	NI	0.495
196	1.345	0.753	1.094	NI	0.532
198	1.302	0.780	1.087	NI	0.586
200	1.244	0.780	1.078	NI	0.599
205	1.126	0.782	0.977	NI	0.657
226	1.086	2.047	0.980	0.365	0.680
228	1.148	1.703	0.960	0.412	0.742
230	1.033	1.547	0.956	0.344	0.732
232	0.990	1.444	0.951	0.663	0.779
234	1.069	1.219	0.991	0.570	0.733
236	1.013	1.143	0.989	0.520	0.737
238	0.989	1.081	0.970	0.611	0.753
240	0.972	1.029	0.984	0.567	0.784
242	0.955	1.007	0.978	0.816	0.816
244	0.953	0.988	0.966	0.956	0.866
246	0.958	0.986	0.980	0.985	0.902
248	0.966	0.970	0.985	1.013	0.942
250	0.975	0.970	0.981	0.998	0.958
252	0.984	0.983	0.984	1.013	0.963
254	1.000	1.000	1.000	1.000	1.000
256	1.014	1.050	0.986	0.984	1.034
258	1.027	1.030	0.985	0.962	1.003
260	1.046	1.070	0.981	0.845	1.045
264	1.096	1.105	0.997	0.986	1.049
268	1.144	1.219	0.972	0.999	1.086
272	1.168	1.300	0.979	1.148	1.019
276	1.148	1.329	0.983	1.158	1.081
280	1.079	1.329	0.986	1.390	1.037

[a] Values (R^{nm}) of ratio R^{nm}_{sample} and $R^{nm}_{standard}$: $R^{nm} = R^{nm}_{sample} / R^{nm}_{standard}$.
[b] Values ($R^{nm}_{standard}$) of ratio of the individual fatty acid peak in a standard monitored at 254 nm ($S^{254\ nm}_{standard}$) and another wavelength ($S^{nm}_{standard}$): $R^{nm}_{standard} = S^{nm}_{standard} / S^{254\ nm}_{standard}$.
[c] Values (R^{nm}_{sample}) of ratio of individual fatty acid peak in duodenal digesta samples monitored at 254 nm ($S^{254\ nm}_{sample}$) and another wavelength (S^{nm}_{sample}): $R^{nm}_{sample} = S^{nm}_{sample} / S^{254\ nm}_{sample}$.
[d] Abbreviations for fatty acids, see Table 3.
[e] Non-integrating peak.

short UV range can sometimes be greater than one (Tables 4–6) due to overlapping of some fatty acid peaks and those of unidentified impurities. On the other hand, in the short or longer UV range the background fluctuation or the close presence of some species peaks can interfere in the accurate integration of some fatty acid peak areas, so the ratio (R^{nm}) can be less than one (Tables 4 and 5). As expected, the most satisfactory accuracy and precision can be achieved by monitoring in the proximity of the maximum of absorbance of fatty acid derivatives (Fig. 2).

3.2. Determination of conjugated linoleic acid isomers (CLA)

Trace amounts of CLA isomers can be detected in duodenal digesta. By manipulation of the percentage of H_2O in acetonitrile, new HPLC procedures can be used to fractionate mixtures of principal CLA iso-

Table 5
Relationships (R^{nm}) between wavelength (λ_{nm}, nm) of fatty acid monitoring and ratios of fatty acid peaks in standards ($R^{nm}_{standard}$) to fatty acid peaks in maize oil samples (R^{nm}_{sample})

λ_{nm}	R^{nm} for analyzed fatty acids[a]			
	$C_{14:0}$	*cis*-9,12,15 $C_{18:3}$	*cis*-6,9,12 $C_{18:3}$	*cis*-9,12 $C_{18:2}$
190	0.663	2.099	0.354	1.332
192	0.550	2.020	0.561	1.312
194	0.569	1.884	0.446	1.223
196	0.725	2.284	0.571	1.190
198	0.761	1.965	0.490	1.130
200	0.725	1.701	0.440	1.057
205	0.798	1.627	0.223	1.060
226	0.364	3.627	2.242	1.354
228	0.406	3.045	1.841	1.335
230	0.457	2.544	1.309	1.284
232	0.551	2.077	0.931	1.226
234	0.653	1.627	0.767	1.177
236	0.764	1.324	0.674	1.124
238	0.836	1.181	0.564	1.093
240	0.835	1.022	0.466	1.058
242	0.866	0.954	0.425	1.023
244	0.879	0.898	0.385	1.021
246	0.991	0.867	0.412	1.008
248	0.886	0.835	0.637	1.003
250	1.002	0.876	0.704	1.000
252	1.012	1.020	0.929	1.000
254	1.000	1.000	1.000	1.000
256	1.064	1.044	1.030	1.004
258	1.009	1.061	1.097	1.081
260	1.000	1.107	1.062	1.017
264	0.856	1.214	1.039	1.029
268	0.895	1.394	1.077	1.062
272	0.600	1.803	1.225	1.064
276	0.784	1.733	1.345	1.081
280	0.909	1.165	1.525	1.090

[a] Symbols and abbreviations for fatty acids as in Tables 3 and 4.

Table 6
Relationships (R^{nm}) between wavelength (λ_{nm}, nm) of fatty acid monitoring and ratios of fatty acid peaks in standards ($R^{nm}_{standard}$) to fatty acid peaks in rapeseed samples (R^{nm}_{sample})

λ_{nm}	R^{nm} for analyzed fatty acids[a]		
	$C_{14:0}$	*cis*-9,12,15 $C_{18:3}$	*cis*-6,9,12 $C_{18:3}$
190	3.152	1.384	1.370
192	2.640	1.377	1.247
194	1.932	1.333	1.265
196	1.730	1.334	1.219
198	1.623	1.276	1.187
200	1.579	1.254	1.058
205	2.169	1.113	1.147
226	3.484	1.130	1.324
228	3.092	1.121	1.316
230	2.446	1.092	1.207
232	1.875	1.026	1.070
234	1.361	0.983	1.007
236	1.321	0.953	0.997
238	1.235	0.938	0.928
240	1.211	0.948	0.973
242	1.180	0.935	0.969
244	1.182	0.950	0.959
246	1.178	0.956	1.035
248	1.184	0.967	0.984
250	1.023	0.972	0.925
252	1.035	0.983	0.932
254	1.000	1.000	1.000
256	0.818	1.013	0.994
258	0.820	1.029	0.934
260	0.899	1.046	0.935
264	1.256	1.092	0.974
268	1.646	1.037	0.898
272	2.044	1.177	0.959
276	2.037	1.165	0.995
280	2.213	1.141	1.049

[a] Symbols and abbreviations for fatty acids as in Tables 3–5.

mers. Our HPLC method using the ternate gradient system (Table 2) fractionates mixtures of seven *cis* and/or *trans* isomers of CLA (Fig. 3A,B); these isomers appear as six peaks. As expected, in the ternate gradient system, all derivatized standards of critical saturated fatty acids (myristic and palmitic acid) and isomers of di-unsaturated fatty acids (linoleic and linolelaidic acids) completely resolved from the CLA isomers. In particular, resolution of a critical pair, peak 4 of the mixture of the CLA isomers and linolelaidic acid, was improved using the ternate gradient system (compare Figs. 1C and 3B). Unfortunately, in the ternate (Fig. 3C,D) and binary gradient systems the CLA isomers of intestinal digesta and milk samples tend to co-elute with unidentified unsaturated fatty acids. As can be seen from stop-flow UV spectra (Fig. 2) and chromatograms (Fig. 1A,C), the absorption spectra of the standards of saturated and unsaturated fatty acid isomers bear a close resemblance. Indeed, the response of the detector at a longer UV range (>225 nm) depends on the derivative agent (i.e. dibromacetophenone). Therefore, saturated or unsaturated fatty acid derivative peak response ratios of the detector set at 235 and 256 nm ($S^{235\ nm}_{FA}/S^{256\ nm}_{FA}$) are in good agreement (0.2334–0.2350) with the average value of ratio (R_{FA}) of 0.2342. On the other hand, the stop-flow UV spectra of the CLA isomers (Fig. 2)

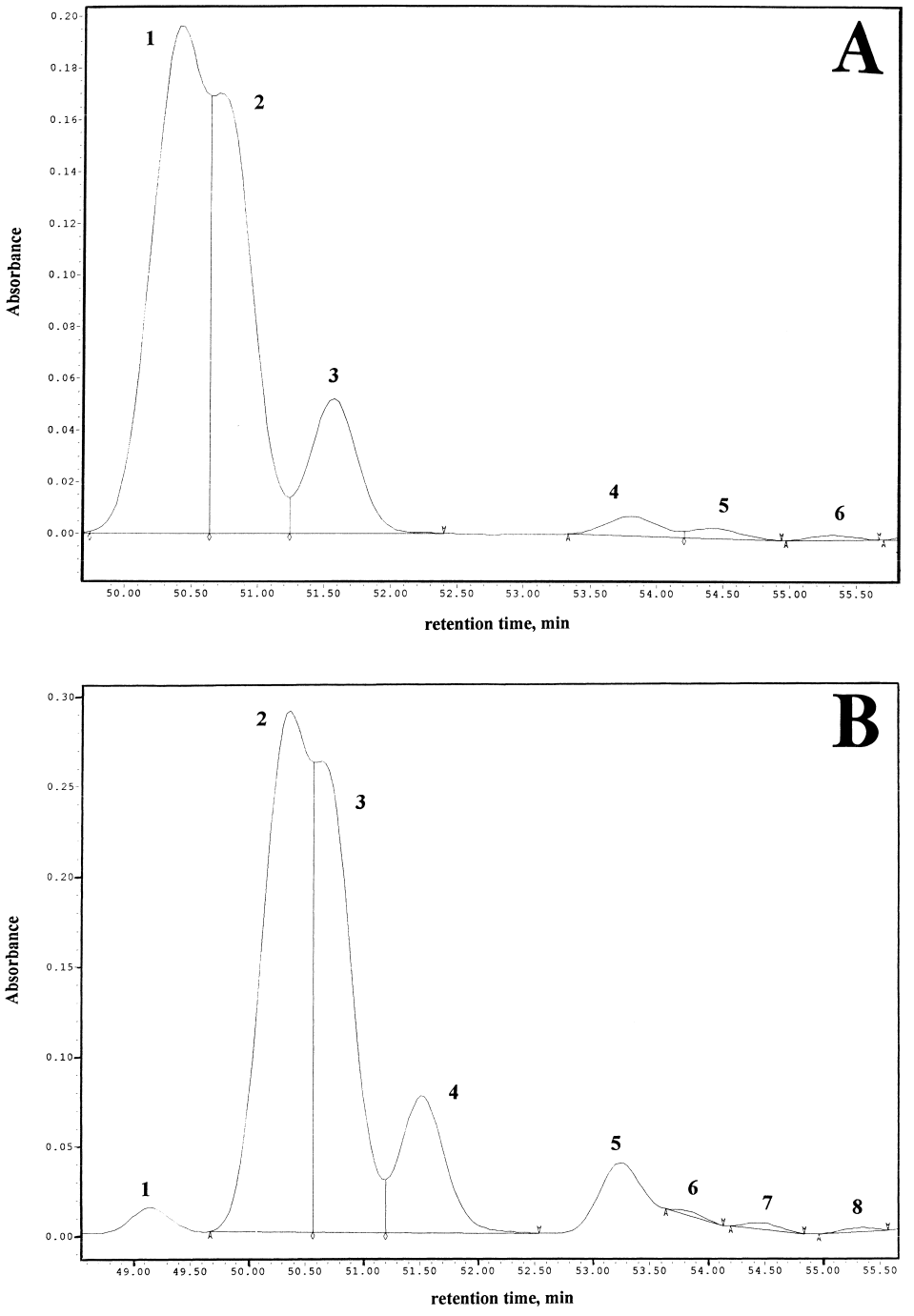

Fig. 3. Chromatograms for processed standards using the ternate gradient system (UV detection at 235 nm; columns and ambient temperatures were 40 and 20°C, respectively): (A) standard of the CLA isomers (peaks 1–6); (B) standard mixture of linoleic acid (peak 1), the CLA isomers (peaks 2–4 and 6–8) and linolelaidic acid (peak 5). Chromatograms for a biological material using the ternate gradient system (UV detection at 254 nm; columns and ambient temperatures were 40 and 25°C, respectively); (C) duodenal digesta sample (CLA-peak 1); (D) expanded area from chromatograms of a duodenal digesta sample (upper line) spiked with CLA (peak 1) and not spiked (lower line). All samples were analyzed after the derivatization procedure. Injection volumes were 5–20 μl.

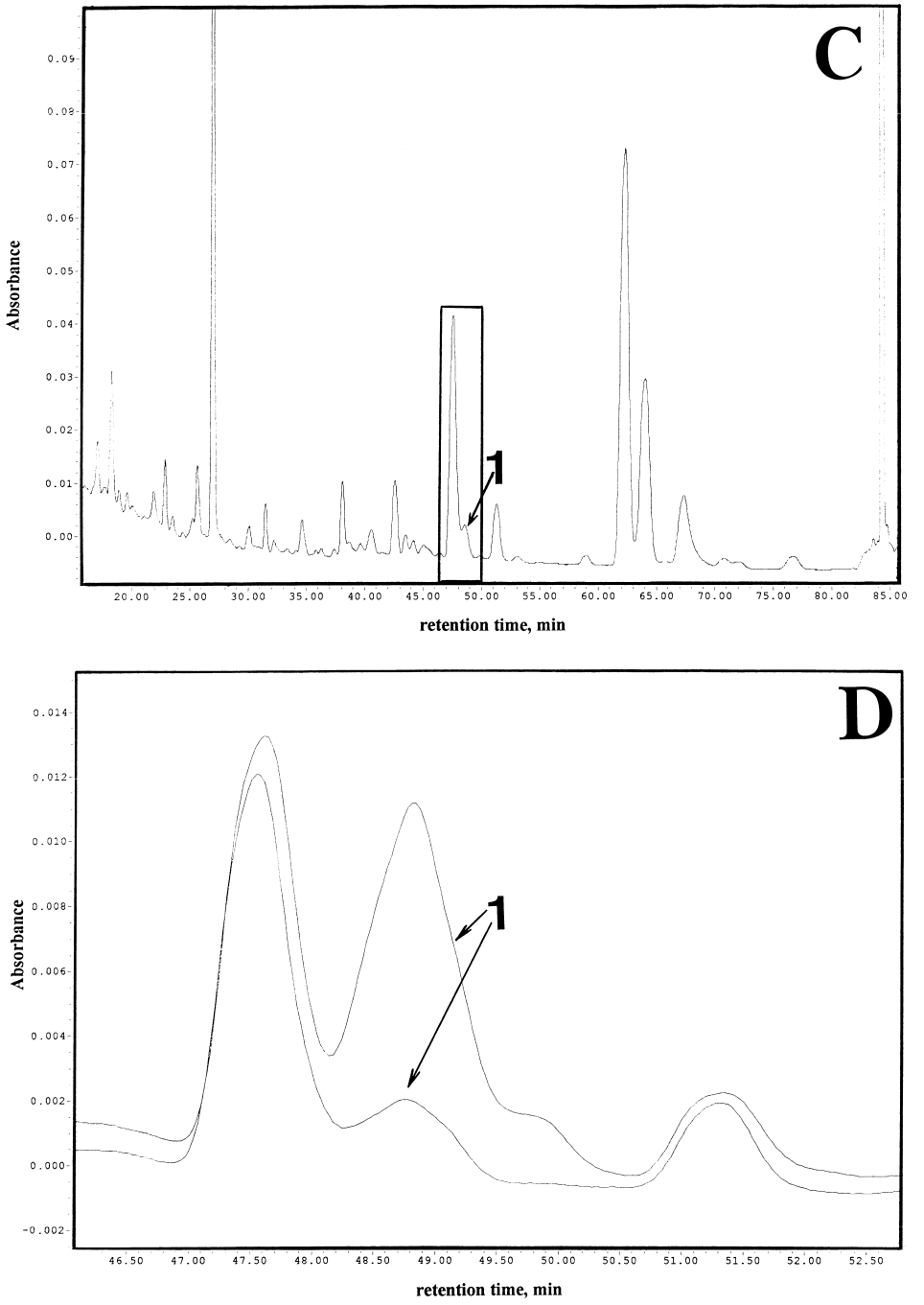

Fig. 3. (*continued*)

showed a high and broad band in the spectral range from 220 to 250 nm (a maximum at 234–236 nm) and a weaker but still noticeable band to 265 nm. Similarly, the ratio value (R_{CLA}) of detector response set at 235 and 256 nm for the CLA isomers standard was constant; the average value of $S_{CLA}^{235\ nm}/S_{CLA}^{256\ nm}$ was 1.5304. Therefore, by using these relationships and the binary or ternate gradient system, the peak area of CLA ($S_{CLA}^{256\ nm}$ or $S_{CLA}^{235\ nm}$) in biological samples can be calculated as:

$$S_{CLA}^{256\ nm} = (S_{\Sigma}^{235\ nm} - R_{FA} \times S_{\Sigma}^{256\ nm})/(R_{CLA} - R_{FA})$$

$$S_{CLA}^{235\ nm} = R_{CLA} \times S_{CLA}^{256\ nm}$$

where $S_{\Sigma}^{256\ nm}$ and $S_{\Sigma}^{235\ nm}$ represent the total peak areas of the sum of co-eluting CLA isomers and unidentified unsaturated fatty acids detected at 256 and 235 nm, respectively. $S_{CLA}^{256\ nm}$ and $S_{CLA}^{235\ nm}$ are the peak areas of the CLA isomers in the total peak (at 256 and 235 nm) corresponding to co-eluted CLA and unidentified unsaturated fatty acids. The binary gradient system with monitoring at 235 and 256 nm allows simultaneous determination of CLA and several saturated and unsaturated fatty acids in intestinal digesta and milk. On the other hand, the more selective ternate gradient system should only be used for quantitative determination of the CLA isomers in these samples. As expected, the responses of the photodiode array detector to the quantification of three main CLA isomers are linear functions (Table 7). The correlation coefficients (r) and standard errors in slopes (SES) evidenced that the UV detection at 235 nm provides good linearity of the CLA isomers. Moreover, the low values of LOD and LOQ demonstrate the satisfactory sensitivity of the method when compared with the original CLA concentration in milk (~0.8–14 μg/g DM) or duodenal digesta samples (~1.2–10.0 μg/g DM).

4. Conclusion

The HPLC method presented allows selective, accurate, and precise simultaneous determination of several saturated fatty acids and some positional or geometric isomers of unsaturated 18-carbon fatty acids in rumen fluids and duodenal digesta samples. Since fatty acids are rather poorly retained on C_{18} columns and have low molar absorptivity at wavelengths above 220 nm, we converted fatty acids into derivatives containing a chromophore. A mixture of $C_{12:0}$–$C_{18:0}$ saturated fatty acids and positional or geometrical isomers of unsaturated fatty acids containing 18 carbon atoms can be separated within 55 min using two dimethyloctadecylsilyl type columns and a binary gradient program composed of acetonitrile and water as mobile phases. Optimization of the binary gradient program and photodiode array detection can be carried out, since higher resolution

Table 7
Linear regression curves[a] for the standard of three main CLA isomers and sum of all CLA isomers[b], correlation coefficients (r), standard error in slope (SES), limits of detection (LOD) and limits of quantification (LOQ) derived from determination of CLA derivatives in the standard (the ternate gradient system)

CLA isomers[c]	Equations[d]	Correlation coefficient (r)	SES	LOD[e] (ng l^{-1})	LOQ[e] (ng l^{-1})
Peak 1	$y\ (\mu g) = 4.896 \cdot 10^{-7}\ S_n + 0.03$	0.9998	$1.07 \cdot 10^{-8}$	5.46	17.9
Peak 2	$y\ (\mu g) = 4.647 \cdot 10^{-7}\ S_n + 0.04$	0.9985	$2.52 \cdot 10^{-8}$	3.61	12.0
Peak 3	$y\ (\mu g) = 4.910 \cdot 10^{-7}\ S_n + 0.01$	0.9995	$156 \cdot 10^{-8}$	4.79	16.1
All CLA isomers[b]	$y\ (\mu g) = 5.030 \cdot 10^{-7}\ S_n + 0.08$	0.9994	$1.74 \cdot 10^{-8}$	26.6	88

[a] Abbreviations as in Table 3.
[b] Maximal amount (y, μg) of all CLA isomers in injected standards, 9.545 μg.
[c] See Fig. 3A.
[d] Number of points used in the curves: 3.
[e] The detector was set at 235 nm.

efficiency is necessary for natural samples. The nature of the separation depends on the total number of carbon atoms, double bonds and the type of geometric isomers of unsaturated fatty acids. Therefore, the presented method complements silver ion chromatography, where separation is based solely on degree of saturation. By manipulation of the percentage of water in the acetonitrile, our ternate gradient program can fractionate the geometric isomers of linoleic acid designated as CLA. This gradient system with monitoring at 235 and 256 nm should only be used for quantitative determination of conjugated linoleic acid isomers in rumen fluids, milk and duodenal digesta samples.

The presented method based on widely available C_{18} columns, simple and rapid preparation of free fatty acids extracts provides the HPLC procedure suitable for routine determination of some saturated and unsaturated fatty acids.

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