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Short communication

2-Propanol in the mobile phase reduces the time of analysis of CLA isomers by silver ion-HPLC

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ABSTRACT

Individual isomers of octadecadienoic acid (C18:2) with conjugated double bonds (conjugated linoleic acids; CLA) exert different biological activities. Their distribution in food and tissues differs. Therefore, the separation of the various positional and geometric isomers is important. The time of analysis using silver ion-high performance liquid chromatography can extend up to 90 min. The aim of this study was to reduce this time. The time of analysis reduced from ca. 90 min onto 45 to 35 min, respectively, by the addition of 0.05% or 0.1% (v/v) 2-propanol to the mobile phase [acetonitrile (0.1%; v/v) and diethyl ether (0.5%; v/v) in *n*-hexane]. There was no effect on resolution of the 17 individual CLA isomers of the CLA mixture. Regarding the lowest coefficient of variation and an adequate baseline separation the use of 0.05% 2-propanol in the mobile phase is recommended, without any disadvantages and adverse effects on the service life of columns. In conclusion, adding 0.05% or 0.1% 2-propanol to the mobile phase shortens the time of analysis of CLA isomers, saves solvents and reduces costs.

1. Introduction

In recent years, it became evident that the analysis of the individual isomers of octadecadienoic acid (C18:2) with conjugated double bonds, called conjugated linoleic acids (CLA), is important. Especially the amounts and distribution of minor CLA isomers [e.g., *trans*(*t*)11,*cis*(*c*)13] are insufficiently investigated and only preliminary results from *in vitro* studies are available [1].

Individual CLA isomers have different biological activities (e.g., *t*10,*c*12-CLA *vs.* *c*9,*t*11-CLA) and their distribution in food differs depending on their origin (industry or ruminant) (reviewed by Wahle et al. [2]). CLA distribution in ruminant lipids is determined by the species, ruminal flora and feeding [3]. In human tissues, it depends on food intake and Δ9-desaturation of CLA precursors [4].

Silver ion-HPLC (Ag^+-HPLC) is an important tool in the lipid analysis [5]. Using silver-impregnated analytical columns is an efficient technique for the separation of geometric and positional isomers of CLA as fatty acid methyl esters (FAME) [6,7]. Using three silver-impregnated coupled columns, the time of analysis at a flow rate of 1.0 mL/min can extend up to 90 min, depending on column age and sample matrix. This time limits a high sample throughput and the analysis expends high volume of solvents.

In the present communication, a composition of a mobile phase which enables to reduce the time of analysis of 17 CLA isomers by more than half and which does not impair the resolution and the service life of the columns is reported.

2. Method

2.1. Silver ion-high performance liquid chromatography (Ag^+-HPLC)

The CLA isomers were analysed as FAME by Ag^+-HPLC (LC10A, Shimadzu, Kyoto, Japan) equipped with an autoinjector (SIL-10A, Shimadzu), a pump (LC-10AD, Shimadzu) and an UV spectrophotometric detector (SPD-10A, Shimadzu) operating at 233 nm. Three silver-impregnated analytical columns (Chrompack ChromSpher 5 Lipids; each: 250 mm × 4.6 mm i.d., 5 μm; VARIAN Inc., Middelburg, Netherlands) were used in series. A guard column was connected upstream of the three analytical columns (ChromGuard HPLC column, replacement cation exchanger, VARIAN Inc.). A degasser (DGU-4A, Shimadzu) intermittently degassed the mobile phase. The mobile phase and the columns were maintained at 21 °C. The columns were equilibrated with the mobile phase 1-h prior to analysis. The isocratic mobile phase contained acetonitrile (0.1%; v/v) in *n*-hexane, with diethyl ether (0.5%; v/v) in order to minimize retention volume drift. The pre-

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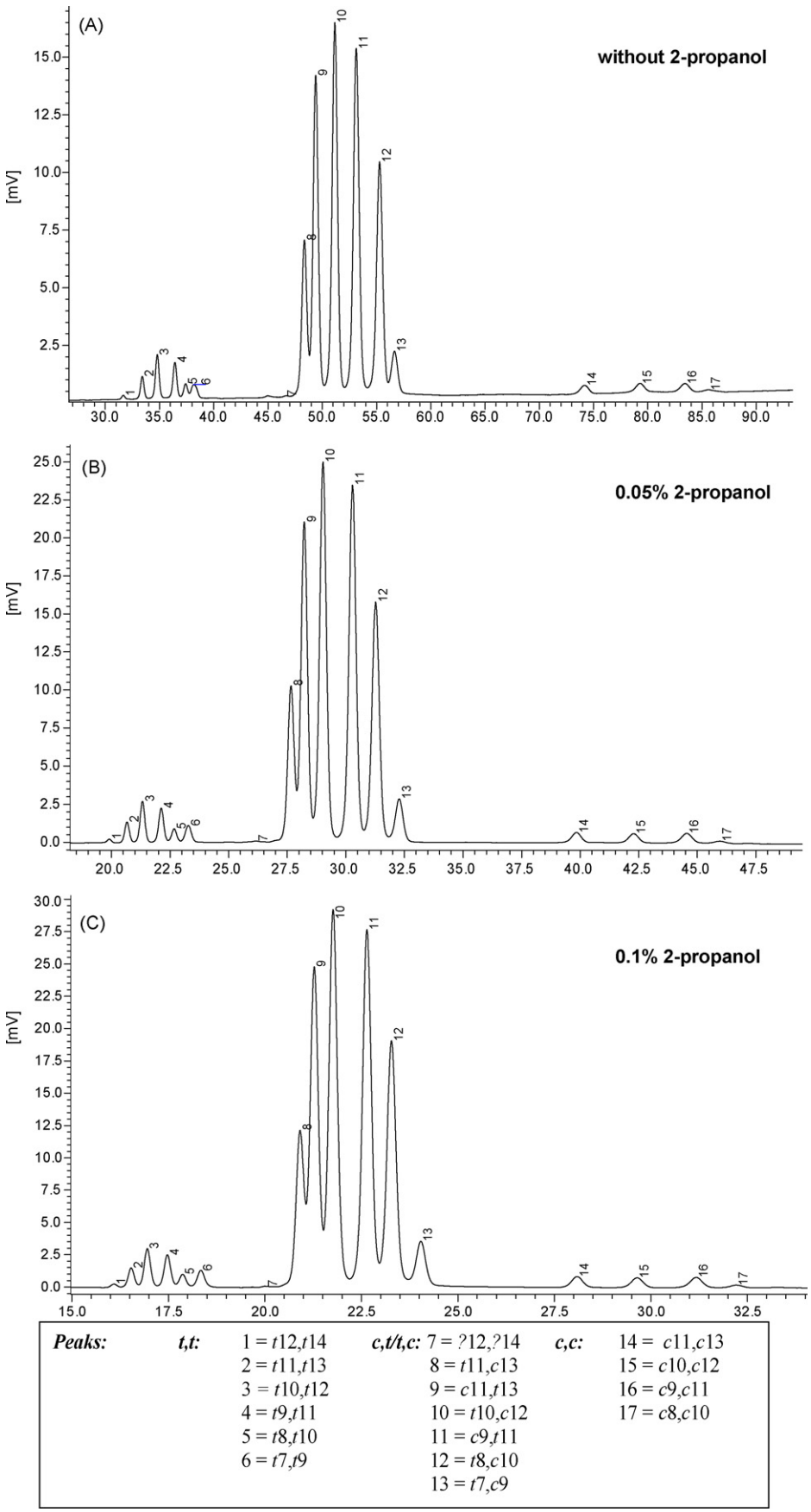

Fig. 1. Partial three-column Ag^+-HPLC chromatograms of 5 μL injections [sample size: 1.5 μg] of a CLA mixture (Sigma–Aldrich® added with single isomers *t*7,*c*9- and *t*11,*c*13-CLA) using mobile phase 0.1% acetonitrile, 0.5% diethyl ether in *n*-hexane including [A] no 2-propanol, [B] 0.05% 2-propanol or [C] 0.1% 2-propanol. Flow rate: 1.0 mL/min, 21 °C. UV detection at 233 nm.

Table 1
CLA distribution of 5 μL injections [sample size: 1.5 μg] of a CLA mixture (Sigma–Aldrich® added with single isomers *t*7,*c*9- and *t*11,*c*13-CLA) analysed with mobile phases containing different proportions of 2-propanol (area% of total CLA).

Mobile phase	Without 2-propanol $n=15$			0.05% 2-propanol $n=15$			0.1% 2-propanol $n=15$		
CLA isomers	Retention time [min]* max† ± 4.5	Area% of total CLA (Mean ± SD)		Retention time [min]* max† ± 2.5	Area% of total CLA (Mean ± SD)		Retention time [min]* max† ± 1.0	Area% of total CLA (mean ± SD)	
∑*trans*,*trans*		*5.89*[a]	0.55		*5.65*[a]	0.29		*5.88*[a]	0.56
*t*12,*t*14	34.6	**0.13**[a]	0.07	19.9	**0.13**[a]	0.02	16.1	**0.13**[a]	0.05
*t*11,*t*13	37.7	**0.92**[a]	0.39	20.7	**0.86**[a]	0.12	16.5	**0.92**[a]	0.34
*t*10,*t*12	39.3	**1.72**[a]	0.25	21.3	**1.75**[a]	0.12	17.0	**1.76**[a]	0.29
*t*9,*t*11	41.2	**1.59**[a]	0.30	22.1	**1.52**[a]	0.09	17.5	**1.54**[a]	0.22
*t*8,*t*10	42.1	**0.78**[a]	0.22	22.7	**0.63**[b]	0.05	17.9	**0.68**[b]	0.09
*t*7,*t*9	43.3	**0.74**[a]	0.18	23.3	**0.78**[a]	0.16	18.3	**0.84**[a]	0.23
∑*cis*,*trans*/*trans*,*cis*		*91.39*[a]	0.66		*91.49*[a]	0.36		*91.32*[a]	0.55
?12,?14	52.0	**0.06**[a]	0.03	26.2	**0.07**[a]	0.03	20.0	**0.04**[b]	0.02
*t*11,*c*13	53.0	**9.25**[a]	0.92	27.7	**9.30**[a]	0.58	20.9	**8.86**[a]	0.65
*c*11,*t*13	54.1	**18.46**[a]	1.17	28.2	**18.47**[a]	0.80	21.3	**18.73**[a]	1.19
*t*10,*c*12	56.2	**22.24**[a]	0.83	29.0	**22.43**[a]	0.68	21.8	**22.34**[a]	0.83
*c*9,*t*11	58.4	**22.09**[a]	1.12	30.3	**22.00**[a]	0.86	22.6	**22.22**[a]	0.70
*t*8,*c*10	60.9	**15.88**[a]	1.15	31.3	**15.89**[a]	0.57	23.3	**15.68**[a]	0.78
*t*7,*c*9	62.4	**3.40**[a]	0.62	32.3	**3.33**[a]	0.28	24.0	**3.45**[a]	0.55
∑*cis*,*cis*		*2.70*[a]	0.23		*2.85*[a]	0.16		*2.80*[a]	0.45
*c*11,*c*13	81.1	**0.83**[a]	0.10	39.8	**0.86**[a]	0.04	28.1	**0.81**[a]	0.13
*c*10,*c*12	84.9	**0.81**[a]	0.13	42.3	**0.82**[a]	0.05	29.6	**0.83**[a]	0.17
*c*9,*c*11	89.2	**0.85**[a]	0.06	44.5	**0.92**[a]	0.05	31.2	**0.90**[a]	0.16
*c*8,*c*10	90.8	**0.21**[a]	0.07	46.0	**0.25**[a]	0.06	32.2	**0.26**[a]	0.08
CV		0.20			0.07			0.18	

[a,b] Indicate significant differences (Scheffé, $P \leq 0.05$). CV, coefficient of variation.
* Represents the exact retention time of the data shown in Fig. 1.
† The retention time varied maximally from ±1.0 to ±4.5 min depending on the used mobile phase.

viously used mobile phase (without 2-propanol) was compared with mobile phases containing 0.05% or 0.1% (v/v) 2-propanol. All three mobile phases (with HPLC grade solvents, Merck, Germany) were freshly prepared prior to the analyses and stirred continuously. The same flow rate of 1.0 mL/min was used with all three mobile phases. Method's reproducibility was intermittently determined of one operator, same environmental conditions, e.g., temperature, in the same laboratory and with same instruments. In addition, the same CLA mixtures were analysed at different concentrations and different sample matrices as well (not shown).

The presented CLA standard mixture (including 15 CLA isomers) was analysed as FAME (Sigma–Aldrich®, Inc.). Two further CLA isomers were added; *t*7,*c*9-CLA and *t*11,*c*13-CLA (synthesised by the former work group of H. Steinhart; University Hamburg, Germany; purity ≥88% of total CLA, both substances contain the corresponding *t*,*t*- and *c*,*c*-isomers: sum ~10%). The three mobile phases were compared with various injection volumes from 5 to 25 μL of the CLA mixture, representing approximately 1.5–7.5 μg FAME (data not shown). The shown data originate from 5 μL injections of the same CLA mixture representing 1.5 μg. The peak areas were integrated using Labsolution software (LC Solution Vers. 1.21 SP1, Shimadzu).

2.2. Statistical analysis

All statistical analyses were performed using SPSS statistics, version 17.0 (©2009 SPSS Inc., Illinois, USA) with $P \leq 0.05$ to indicate significant differences. The results are stated as means with their standard deviation (SD). The Kolmogorov–Smirnov test was used to test the distribution of the data. All data were normally distributed (asymptotic significance $P \leq 0.05$). The multivariate analysis of variance was employed to compare the portion of each CLA isomer (area% of total CLA) between the different methods. The post hoc Scheffé test was used. The repeated analyses (replicates with injection volumes of 5 μL, sample size 1.5 μg FAME; $n=15$) with one mobile phase were randomized over weeks to include day to day variations. The coefficient of variation (CV) was calculated as SD/mean.

3. Results and discussion

Using 0.1% acetonitrile and 0.5% diethyl ether in *n*-hexane as mobile phase the time of analysis requires approximately 70–90 min depending on age of the columns ([3] and Fig. 1; first chromatogram). The portion of acetonitrile was already optimised [8]. Previous analyses showed that the connection of three columns in series allows an optimal resolution of the individual CLA isomers in different matrices (e.g., milk [3], serum, adipose tissue, faeces). Nuernberg et al. [9] achieved no further resolution with four columns.

To reduce the time of analysis various portions of 2-propanol in the mobile phase were tested. By adding 0.05% or 0.1% (v/v) 2-propanol to the mobile phase, the time of analysis could be reduced from ca. 90 min onto 45 to 35 min, respectively, without impact on the separation of the 17 CLA isomers (Fig. 1, Table 1). The sensitivity increased and retention drift could be reduced when 2-propanol was added (Fig. 1, Table 1). Only differences for the portions of the minor CLA isomers *t*8,*t*10 and *t*,*c* or *c*,*t*12,14 (<1% of total CLA) were found between the tested mobile phases ($P=0.044$, 0.048; Table 1). The tested CLA concentrations were generally low (~1.5 μg). There were no differences between different mobile phases of the separation of larger sample sizes of the CLA mixture (data not shown).

Other authors used for the separation of a CLA mixture (8 isomers) a dual-column Ag^+-HPLC with isocratic conditions (acetonitrile in hexane; 23 °C) and higher flow rates (e.g., 2.0 mL/min) and could achieve a faster separation ≤30 min [10]. However, in case of the increasing number of CLA isomers, the increasing portion of *trans*,*trans* CLA and/or complex biological matrices only a suboptimal resolution could be achieved. In addition, the increased

pressure (from 6.5 to 13.7 MPa) could lower long-term pump capacity.

After a more than 1-year successful experience with the 0.05% 2-propanol-containing mobile phase (v/v) in the analyses of various matrices (lipids of food, plasma and tissues) any disadvantages and adverse effects on the service life of columns can be excluded and no loss of resolution could be observed. The CLA mixture was measured with the same columns over the entire time. Regarding the lowest CV, the adequate baseline separation and our experience the use of 0.05% 2-propanol is recommended.

In conclusion, adding 0.05% or 0.1% 2-propanol to the mobile phase shortens the time of analysis, saves solvents and reduces costs.

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