A new internal standard for HPLC assay of conjugated linoleic acid in animal tissues and milk

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ABSTRACT: A new method for the quantification of underivatized conjugated linoleic acid (CLA) isomers and CLA-metabolites by silver ion liquid chromatography (Ag^+-HPLC) with photodiode array detection (DAD) is described. Conjugated fatty acids (CFA) and sorbic acid as the internal standard (IS) were separated on two 5 µm Chrompac ChromSpher Lipids columns (250 × 4.6 mm). Biological samples were hydrolyzed with 1M KOH in methanol and 2M KOH in water at room temperature for 12 h. Hydrolyzates were acidified and the free fatty acids were extracted with dichloromethane. The organic solvent was removed and then the residue was re-dissolved in hexane and centrifuged. The supernatant was injected onto the columns. The mobile phase of 1.6% acetic acid and 0.0125% acetonitrile in hexane was chosen as the optimum mobile phase for fractionation of IS, CLA isomers and CLA-metabolites in all assayed biological samples. The use of two silver ion-exchange columns with direct UV detection (Ag^+-HPLC-DAD) offers satisfactory precision of the IS quantification and low limits of detection of IS and CLA isomers (0.60 and 0.21–0.35 ng, respectively). The presented simple Ag^+-HPLC-DAD method with sorbic acid as the IS can be used for direct determination of underivatized CLA isomers in specimens of animal origin.

Keywords: sorbic acid; internal standard; CLA isomers; HPLC; photodiode array detection; biological samples

Isomers of conjugated linoleic acid (CLA) and their metabolites (i.e. fatty acids containing conjugated double bonds, CFA) exert a beneficial influence on physiological processes occurring in humans and animals, reducing the risk of such chronic diseases as obesity, arteriosclerosis or carcinogenesis (Hur et al., 2007; Park, 2009). Molecules of CLA isomers contain two double bonds separated by one single bond (Banni et al., 1996) which are located at carbon 6 and 8, 7 and 8 or 10, 11 and 13 or 12 and 14 (Delmonte et al., 2005). These isomers can appear in each geometric configuration *cis-trans* (*ct*), *trans-cis* (*tc*), *cis-cis* (*cc*) or *trans-trans* (*tt*) (Czauderna et al., 2003). Two of the above-mentioned isomers, CLA – *c*9*t*11 and *t*10*c*12, which are considered to be particularly biologically active, are produced by bacteria in the rumen in biohydrogenation processes, whereas *c*9*t*11CLA may also be formed endogenously *via* Δ9-desaturation of *t*11C18:1 in tissues of ruminant and monogastric animals. CLA isomers exert positive effects on cancer, cardiovascular disease, diabetes, body

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composition, immune system, and bone health in humans and animals. Therefore many studies were aimed at the influence of CLA isomers in food and living organisms on different physiological effects in humans and animals, as well as alterations in performance, meat quality and tissue fatty acid profiles in farm animals.

Chromatographic quantification of fatty acids (FA) in biological samples was usually achieved using an internal standard, e.g. odd chain fatty acids (like C9:0, C17:0, C19:0 or C23:0), *c15*C24:1, [$^2H_{31}$]-palmitic acid or fatty acid anilides (Perry et al., 1996; Zaitseva et al., 1999; Czauderna and Kowalczyk, 2002; Roach et al., 2002; Aldai et al., 2005; Lin and McKeon, 2005; Pichini et al., 2008). Unfortunately, these existing chromatographic methods are still complicated and not satisfactory for direct and accurate determination of individual CLA isomers and/or other CFA using silver ion liquid chromatography with photodiode array detection (Ag^+-HPLC-DAD) (Fritsche et al., 2000; Winkler and Steinhart, 2001; Czauderna and Kowalczyk, 2002; Roach et at., 2002; Aldai et al., 2005). We hypothesized that the use of sorbic acid as the internal standard (IS) would ensure the direct and satisfactory monitoring of the extraction yield of CLA isomers and CFA from hydrolyzed biological samples. Therefore the aim of our study was an attempt to elaborate the original method to improve the accuracy and precision of direct determination of CLA and CFA isomers in tissues and products of animal origin using sorbic acid (*c2c4*C6:2) as IS and Ag^+-HPLC-DAD.

MATERIAL AND METHODS

Chemicals and materials

Acetonitrile (99.9%) and *n*-hexane (95%) purchased from Lab-Scan (Eire) were HPLC grade. Acetic acid, dichloromethane and other reagents were of analytical grade and were from POCh (Gliwice, Poland). The mixture of free CLA isomers (95–97%), other fatty acid standards, and sorbic acid (*c*2*c*4C6:2) were supplied by Sigma (St. Louis, MO, USA).

Femoral muscles and livers from sheep (Niedźwiedzka et al., 2008) or rats fed a diet containing 2% of a CLA isomer mixture (Czauderna et al., 2009) were frozen and then stored at –25°C until CLA isomers and CFA were analyzed (Czauderna et al., 2003).

Preparation of biological samples for Ag^+-HPLC-DAD analysis

To 50–100 mg of animal tissue, 0.2–0.5 ml of milk or blood plasma, or ca 1 mg standard CLA isomer mixture were added 1 ml 1M KOH in methanol, 1 ml 2M KOH in water and 25 µl IS (6 mg *c2c4*C6:2 in 15 ml chloroform). The obtained mixture was flushed for ca 1 min with a stream of argon and left overnight at room temperature for hydrolysis. Next, 1.5 ml of water were added and the solution was acidified with 6M HCl to pH ≈ 2. The extraction of free fatty acids was carried out with dichloromethane (4 times 1.5 ml each). Pooled extracts were dried with 0.1 g anhydrous Na_2SO_4 while dichloromethane was removed (at < 35°C) under a stream of argon. The residue was re-dissolved in 0.5 ml of hexane and the obtained solution was vortexed. Finally, the resulting solution was centrifuged at 2 500–3 000 g for 10 min. Afterwards the clear supernatant was transferred into an HPLC vial and 5–95 µl of the solution were injected onto the Ag^+-HPLC columns.

Analysis of CLA isomers and CFA using Ag^+-HPLC-DAD

The HPLC system comprised a 515 Waters pump, 712 WISP autosampler and 996 Waters photodiode array detector (DAD) (Czauderna et al., 2003). The DAD was operated in a UV range from 195 to 400 nm with a spectral resolution of 1.2 nm and measurement frequency of one spectrum per second. Development of the HPLC method, data collection and analyses were performed using Millennium 2001 software (version 2.15) and a Pentium III computer (Compaq). Two analytical ion-exchange columns loaded with silver (Ag^+) ions (250 × 4.6 mm Chrompac ChromSpher 5 µm Lipids columns; The Netherlands) were used in conjunction with a guard column of 10 × 3 mm containing the same stationary phase. The ambient temperature was 22–24°C, while a column heater maintained the temperature at 26°C. Minimum tubing diameter and distance were used between the autosampler injector and detectors. The Ag^+-HPLC-DAD system pressure was 4.78 ± 0.03 MPa.

The samples injected onto the columns were subjected to isocratic elution (1 ml/min) using a mobile phase composed of *n*-hexane, acetic acid and acetonitrile (98.4 ml, 1.6 ml and 12.5 µl, respectively). The columns were equilibrated with the

freshly prepared mobile phase at least 35 min before sample injection. The mobile phase was carefully stirred before HPLC analysis as the reproducibility of the fractionation was sensitive to small fluctuations in the concentration of acetic acid and more to the concentration of acetonitrile. CLA isomers and other fatty acids containing conjugated double bonds (i.e. CLA isomer metabolites) were detected at 234 nm, whereas the internal standard (IS) was monitored at 259 nm. The limits of detection (L_D) were defined as a signal-to-noise ratio of three (Lin and McKeon, 2005), whereas the noise level under the analytical peak was calculated from the baseline on the left and right side of the analytical peak. Purity analyses of IS and *tt*, *ct*, *tc*, and *cc* isomers of CLA peaks were based on relationships between the monitoring wavelength and peak areas (S_n^{sample}) of added IS and CLA isomers in assayed biological samples and peak areas ($S_n^{standard}$) of IS and *tt*, *ct*, *tc*, *cc* isomers of CLA standards dissolved in hexane (Czauderna and Kowalczyk, 2002). The IS peak purity analyses were carried out in the UV range from 235 to 280 nm, while the purity analyses of *tt*, *ct*, *tc*, *cc* CLA isomer peaks were performed in the UV range from 222 to 245 nm.

The analyses of IS and CLA isomers as methyl esters (FAME) in standard solutions or in biological samples were performed on a SHIMADZU GC-MS-QP2010 Plus EI (GC-MS) equipped with a BPX70 fused silica capillary column (120 m × 0.25 mm i.d. × 0.25 μm film thickness; SHIM-POL), quadrupole mass selective (MS) detector (Model 5973N) and injection port. Helium as the carrier gas operated at a constant pressure (223.4 kPa) and the initial flow rate was 1 ml/min. Injector and MS detector temperatures were maintained at 200 and 240°C, respectively. The methyl esters of IS and CLA isomers in standard solutions or in biological samples were prepared as described by Czauderna et al. (2007), while the methyl ester profiles of IS and CLA isomers in 1 μl samples at a split ratio of 10:1 were determined using the column temperature gradient programme (Czauderna et al., 2009).

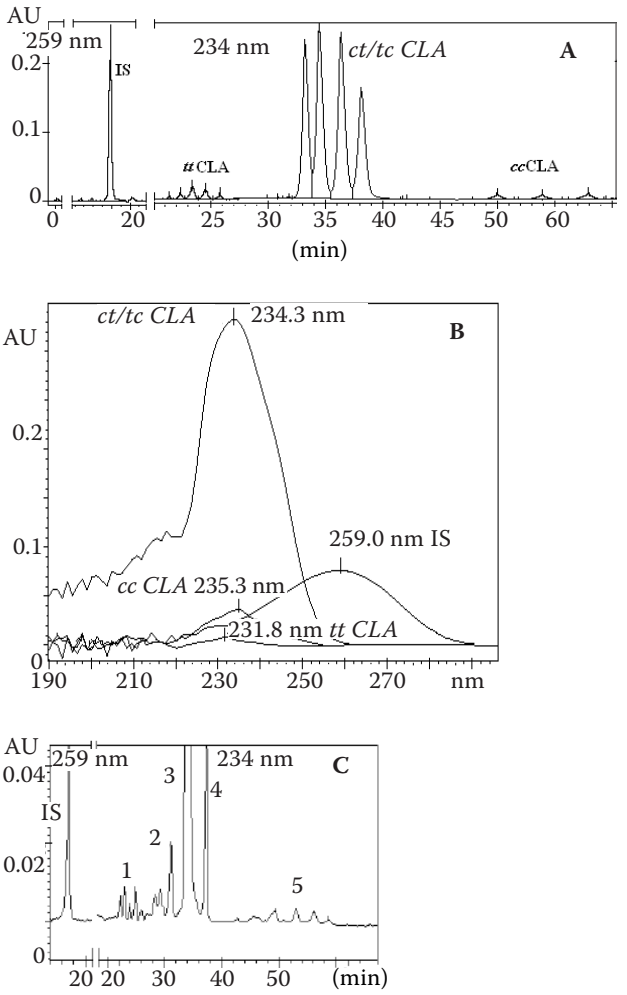

Figure 1. **A** – A typical chromatogram for sorbic acid (IS) as the internal standard (UV detection 259 nm) and mixture of *tt*, *ct/tc* and *cc* isomers of CLA standard (UV detection 234 nm); AU = the absorbance unit. **B** – UV spectra of IS and *tt*, *ct*/*tc* and cc isomers of CLA; the *tt*, *ct/tc* and *cc* isomers of CLA and IS showed average absorbance maximums at 231.8, 234.3, 235.3 and 259 nm, respectively. **C** – A typical chromatogram for the internal standard (IS) and CLA isomers for goat milk (UV detection for IS at 259 nm and for CLA isomers at 234 nm); peaks: 1 = *tt* CLA; 2 = metabolites of CLA isomers; 3 = *c*9*t*11 CLA; 4 = *c*8*t*10 CLA; 5 = *cc* CLA

RESULTS AND DISCUSSION

Chromatography of a standard mixture of geometrical and positional isomers of CLA succeeded in clearly separating all of the isomers into three well-defined groups of peaks (Figure 1). The first group of small peaks: CLA isomers in the *tt* configuration (from 21 to 26 min), the second group of the highest peaks: CLA isomers in *ct* and *tc* configurations (from 32 to 38 min), and the third group of small peaks: isomers in the *cc* configuration (from 49 to 64 min). Really, the silver ion liquid chromatography method (Ag^+-HPLC) allows better fractionation of positional CLA isomers within each geometrical group (Figure 1A) compared with gas chromatography techniques (GC) and reversed-phase C_{18}-HPLC methods (Banni et al., 1996; Cross

et al., 2000; Winkler and Steinhart, 2001; Czauderna and Kowalczyk, 2002; Roach et al., 2002; Czauderna et al., 2003; Aldai et al., 2005). Fortunately, our proposed Ag^+-HPLC-DAD method showed excellent separation of sorbic acid, the internal standard (IS), from all CLA isomers as well as from background fluctuation in processed standard solutions of IS and CLA isomers. The underivatized IS was substantially retained on the Ag^+-columns (retention time 18.0 ± 0.1 min) and was satisfactorily distinct from mobile phase impurities and baseline noise (Figure 1A). No changes in the concentrations of IS in chloroform solutions were observed when these IS solutions were protected from the light and stored for 30–40 days at –25°C. Moreover, our proposed isocratic elution system and the UV detection in the spectral range of 225–360 nm were found to provide excellent baseline stability for IS and all CLA isomers in the assayed biological samples (i.e. the muscles and liver of rats and sheep, as well as blood plasma or milk of goats, sheep and cows). What is especially puzzling, however, is that only *n*-hexane obtained from LabScan ensured very small baseline fluctuation and substantial retention of all assayed CLA isomers on silver ions-columns. On the other hand, 95% n-hexane (HPLC grade) from Sigma (USA) also provided satisfactory baseline stability, however, all CLA isomers were eluted faster. Unexpectedly, HPLC grade 95% *n*-hexane (a batch of 55 litres) purchased from J.T. Baker (The Netherlands) in 2003 could not be used as the main component of the mobile phase because of high baseline noise (i.e. ca ± 0.05 of the absorbance unit for UV detection at 234 nm).

Detailed analysis of chromatograms revealed that practically identical resolution and a similar profile of *tt*, *ct*, *tc* and *cc* isomers were obtained using CLA isomer mixtures purchased from Sigma (USA) or Larodan Fine Chemicals AB (Malmö, Sweden) (Czauderna et al., 2003). As can be seen from the UV spectra, the absorption spectra of *tt*, *ct*, *tc* and *cc* isomers of CLA bear a close resemblance (Figure 1B). Importantly, the absorbance maximum is slightly dependent upon the geometric form of the assayed isomer (i.e. at 231.8 ± 0.1 nm for *tt* CLA, 234.3 ± 0.2 nm for *ct* or *tc* isomers of CLA and 235.3 ± 0.2 nm for *cc* isomers). The high molar absorptivity of underivatized IS and CLA isomers in particular, the close proximity of their absorbance maxima to 259 nm (Figure 1B) and ~ 234 nm make IS and CLA isomers possessing their own chromophores almost ideally suited for direct analyses with photodiode array detectors. As can be seen from detailed chromatographic analyses, detection at 259 nm permitted the excellent precision of the IS quantification in the presence of other CLA isomers in blood plasma or goat milk, i.e. the

Table 1. Assessment of the quantification reliability of sorbic acid as the internal standard in the presence of *tt*, *ct*, *tc* and *cc* isomers of the CLA standard (Sigma, St. Louis, MO, USA)

Item	Sorbic acid	
	UV detection at 259 nm	UV detection at 234 nm
Equation[a]	y (μg) = 6.374 × 10^{-8} S_n + 0.014	y (μg) = 2.070 × 10^{-7} S_n + 0.021
Correlation coefficient (r)	0.9996	0.9995
Standard error in slope	1.65 × 10^{-9}	0.56 × 10^{-8}
L_D (ng)	0.60	0.89
RSD (%)[b]	0.68	0.93
$S_H/\tilde{O}_H$ [c]	5046	3406
Recovery (%)[d]	101.37	101.29
$^{IS}S_n/^{CLA}S_n$ [e]	3.016	0.929

[a]S_n and y (μg) are the IS peak area and the IS amount (μg) in a sample, respectively

[b]n = 5 (preparation and injection)

[c]the ratio of the IS peak high to the average noise high on both sides of the IS peak

[d]recovery of 30 μg IS added to 0.5 ml samples containing 10 μg IS

[e]ratio of peak areas (S_n) of 1 μg IS detected at 259 or 234 nm to 1 μg *tt, ct/tc* and *cc* isomer mixture of CLA (Sigma, St. Louis, MO, USA) detected at 234 nm

relative standard deviation (RSD) ≈ 0.78–1.20%. Indeed, CLA isomers and especially IS were monitored at unique wavelengths (i.e. 234 and 259 nm, respectively), therefore, other species present in the biological samples did not interfere with the assayed analytes. The precision of the proposed procedure was assessed by examining the relative standard deviation (RSD, %) of a known amount of sorbic acid in the presence of the CLA isomer standard (Table 1). Chromatographic analyses revealed that UV monitoring at 259 nm assured an excellent relation between the IS peak high (S_H) to the average noise high ($\tilde{O}_H$) on both of its sides ($S_H/\tilde{O}_H$), satisfactory precision of IS determination (RSD = 0.68%), accuracy (i.e. a recovery of ≈ 100%), and sensitivity (L_D = 0.6 ng), whereas UV monitoring at 234 nm permitted satisfactory sensitivity and precision of the detection of all geometrical and positional, *tt, ct, tc,* and *cc*, isomers of CLA standards (i.e. $L_D \leq 0.35$ ng; RSD ≈ 1%, respectively; Table 2). Detection at 259 nm permitted the satisfactory purity of the IS peak (i.e. 98 ± 1%) in blank samples containing 10 µg IS. As expected, the peaks of IS as well as all CLA isomers were absent from the blank signal when the proposed isocratic chromatographic elution and photodiode array detection (at 259 and 234 nm, respectively) were used.

Considering the above results, we argue that the use of photodiode array detection can be recommended for UV monitoring of *c*2*c*4C6:2 as the internal standard (IS) for quantifying the extraction yield of free fatty acids from hydrolyzates as well as for quantifying the concentrations of CLA isomers and identifying the geometric and positional configuration of the CLA isomers in assayed biological samples.

The proposed procedure of hydrolysis, chromatographic separation and UV detection of IS and fatty acids containing double bonds was applied to determine CLA isomers in biological samples from goat, rats and nutrias. Based on the chromatographic analyses (Figure 1B), it can be concluded that the proposed method of alkaline hydrolysis, preparation of samples for Ag^+-HPLC-DAD analysis, and composition of the mobile phase offer satisfactory separation of the endogenous compounds present in all assayed biological samples from IS detected at 259 nm and from all positional and geometrical forms of CLA isomers and their metabolites monitored at 234 nm. Fortunately, IS monitoring at 259 nm considerably improved sensitivity, as the endogenous substances appearing before 20 min of elution revealed extremely low molar absorption coefficients at 259 nm in comparison with absorption at 234 nm (Table 1).

Table 2. Limits of detection (L_D) derived from determination of underivatized *tt*, *c9t11*, *t10c12* and *cc* isomers of the CLA standard (UV detection at 234 nm)

CLA isomers	L_D (ng)
*tt*CLA[a]	0.35
*t*10*c*12CLA	0.33
*c*9*t*11CLA	0.33
*cc*CLA[b]	0.21

[a]group of *t*11*t*13, *t*10*t*12, *t*9*t*11 and *t*8*t*10 isomers of CLA
[b]group of *c*11*c*13, *c*10*c*12 and *c*9*c*11 isomers of CLA

The satisfactory precision (RSD) and purity of the IS peak (RSD < 0.96%; purity 97 ± 2%) and *tt*, *ct*, *tc* or/and *cc* isomers of CLA peaks (RSD < 1.3%; purity 96 ± 5%) in chromatograms obtained for samples of blood plasma of goats and liver, muscles, pancreas and perigonadal fatty tissue of rats and nutria demonstrate that the described procedure with sorbic acid as the internal standard can be applied for direct quantification of CLA isomers and their metabolites in assayed biological materials.

Comparison of IS and CLA isomers monitoring efficiency of Ag^+-HPLC-DAD with capillary gas chromatography and mass spectrometry (GC-MS).

In order to validate the proposed Ag^+-HPLC-DAD method with sorbic acid as IS, we analyzed the profile of CLA isomers and sorbic acid in standard solutions and biological samples using a diametrically different chromatographic technique. Therefore, we also fractionated IS and CLA isomers as FAME by capillary gas chromatography (GC) with selective mass spectrometry (MS) (Czauderna et al., 2007, 2009). As expected, high-resolution capillary gas chromatography with selective MS resulted in complete recovery of IS and all CLA isomers from standard solutions and tissues of monogastric animal origin (e.g. liver, muscle, and fat tissue), gave a well-separated IS peak, and a true picture of the CLA isomer composition (Czauderna et al., 2007). Moreover, the chemical composition of IS, CLA isomers and other important fatty acid standards in a GC-MS chromatogram (Figure 2) agreed closely with the profile of these standards in Ag^+-HPLC-DAD chromatograms (Figure 1A). Unfortunately, in our column temperature gradi-

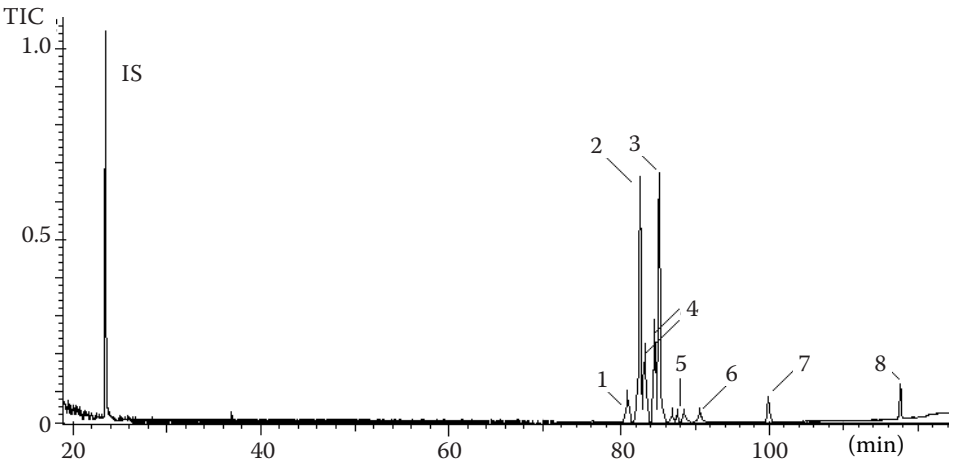

Figure 2. Part of a typical GC-MS chromatogram for standards of sorbic acid (IS), α-linolenic acid (*c*9*c*12*c*15C18:3), *tt*, *ct*/*tc* and *cc* isomers of CLA, arachidonic acid (*c*5*c*8*c*11*c*14C20:4) and C24:0; peaks: 1 = *c*9*c*12*c*15C18:3; 2 = *c*9*t*11CLA; 3 = *t*10*c*12CLA; 4 = other *ct*/*tc* isomers of CLA; 5 = three *cc* isomers of CLA; 6 = the sum of tt isomers of CLA; 7 = *c*5*c*8*c*11*c*14C20:4; 8 = C24:0. TIC = total ion current: × 1 000 000 pA

ent programme (Czauderna et al., 2009), sorbic acid added to tissues of ruminant origin co-eluted with an insignificant peak of C15:0. Therefore, the addition of sorbic acid as the internal standard to tissues of ruminant origin resulted in inaccurate C15:0 determinations using the column temperature gradient programme described by Czauderna et al. (2007).

CONCLUSION

Taken together, IS and CLA isomer profile monitoring with GC-MS is in good agreement with the profile of these acids obtained by Ag^+-HPLC-DAD. Therefore, sorbic acid as the internal standard was found to be a useful marker for direct quantification of underivatized CLA isomers and other CFA in biological materials using silver ion-exchange columns and photodiode array detection.

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