Relative Retention Order of All Isomers of *cis/trans* Conjugated Linoleic Acid FAME from the 6,8- to 13,15-Positions Using Silver Ion HPLC with Two Elution Systems

Pierluigi Delmonte[a], Ai Kataoka[a], B.A. Corl[b], D.E. Bauman[b], and Martin P. Yurawecz[a,*]

[a]U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition, College Park, Maryland 20740, and [b]Department of Animal Science, Cornell University, Ithaca, New York

ABSTRACT: CLA, defined as one or more octadecadienoic acids (18:2) with conjugated double bonds, has been reported to be active in a number of biological systems. GC and silver ion HPLC (Ag^+-HPLC) have been the primary techniques for identifying specific CLA isomers in both foods and biological extracts. Recently, GC relative retention times were reported for all *c,c, c/t* (*c,t* and *t,c*), and *t,t* CLA FAME from the 6,8- to the 13,15-positions in octadecadienoic acid (18:2). Presented here is the relative retention order of the same CLA FAME using Ag^+-HPLC with two different elution systems. The first elution system, consisting of 0.1% acetonitrile/0.5% diethyl ether (DE) /hexane, has been used previously to monitor CLA composition in foods. Also presented here is the retention order of CLA FAME using 2% acetic acid/hexane elution solvent, which has advantages of more stable retention volumes and a complementary elution order of CLA FAME isomers. The data are reported using retention volumes (RV) adjusted for toluene, an estimator for dead volume, and relative to *c*9,*t*11-18:2. Measurement of relative RV in the analysis of 88 samples of cow plasma, milk, and rumen fluids using Ag^+-HPLC is also presented here. The % CV ranged from 1.04 to 1.62 for *t,t* isomers and from 0 to 0.48 for *c/t* isomers.

Paper no. L9281 in *Lipids 40*, 509–514 (May 2005).

Conjugated linoleic acid and CLA are terms used to describe octadecadienoic (18:2) fatty acid(s) or ester(s) with two conjugated double bonds. Different double bond positions and geometric configurations can create a large set of compounds with similar structure but not necessarily similar chemical or biological properties. Twenty CLA isomers already have been reported in food (1). GC separation, using the best capillary columns that are currently available, typically elutes the CLA-18:2 FAME in a small interval, ~3.5 min, of a 70-min run. Insufficient resolution is obtained by GC to identify or quantify individual isomers. A typical Ag^+-HPLC run of ~60 min will spread the area of elution for CLA isomers to >30 min. The two techniques, GC and Ag^+-HPLC, are complementary and can be used successfully for separation of virtually all the isomers present in a test portion of a sample.

*To whom correspondence should be addressed.
E-mail: mpy@cfsan.fda.gov

Abbreviations: DE, diethyl ether; MeCN, acetonitrile; RRV, relative retention volume; RV, retention volume.

A major problem with Ag^+-HPLC is the retention volume (RV) drift that occurs over time (2). This Ag^+-HPLC technique, which provides a wide separation of CLA isomers, is gaining more acceptance but it is not yet in common use because the nonreproducibility of RV makes the identification of peaks difficult. To simplify the interpretation of Ag^+-HPLC chromatograms, we present here the application of relative retention volumes (RRV) to the identification of CLA isomers. The mean (μ), standard deviation (σ), and % CV ($100 \times \sigma/\mu$) are presented for the RRV of 88 sample extracts from cow plasma, milk, and rumen fluid. Lack of knowledge concerning the chromatography of CLA FAME has been a point of concern in the accurate identification of specific isomers. In many reports, responses occurring at the retention time of *c*9,*t*11-CLA have been reported only as *c*9,*t*11-CLA even though, depending on the relative quantities present, the isomers *c*6,*t*8-, *t*7,*c*9-, and both *c/t (c,t* and *t,c)* 8,10-CLA give responses at this retention time (3). The use of relative retention times (GC) or RRV (HPLC), combined with the availability of reference materials for all CLA isomers from 6,8 to 13,15, allows the development of a basis for a more conservative approach to identification.

EXPERIMENTAL PROCEDURES

A mixture of CLA FFA, CLA FAME, γ-linolenic acid (*cis*-6, *cis*-9, *cis*-12-octadecatrienoic acid) and α-linolenic (*cis*-9, *cis*-12, *cis*-15-octadecatrienoic acid) FAME were purchased from Nu-Chek-Prep, Inc. (Elysian, MN). CLA isomers of known purity (*c*9,*t*11-, *t*10,*c*12-, *c*9,*c*11-, *c*11,*t*13-, and *t*9,*t*11-18:2) were obtained as FFA from Matreya, Inc. (Pleasant Gap, PA). The CLA isomers that were not commercially available were synthesized as previously reported (4). FAME were prepared from fat extracted from milk, plasma, and rumen fluid of dairy cows as previously described (5). Acetonitrile (MeCN) and hexane were UV grade. Diethyl ether (DE) was anhydrous. BF_3 in methanol was obtained from Supelco (Bellefonte, PA). Test portions of FAME were dissolved in 0.5% toluene/hexane (or isooctane) prior to chromatographic analysis. Normalization of the chromatograms shown in this work was accomplished using default functions of Microsoft® Excel (9.0 3821 SR-1) (Microsoft Corp., Redmond, WA). Chromatograms were ex-

ported from the acquisition software as the raw signals vs. time. HPLC data were exported from Millenium (Waters Associates, Milford, MA) as ASCII (ARW) files to Microsoft Excel. The new chromatograms, in the relative scale, were obtained as X-Y scatter plots of the signal vs. RRV. Column values were recalculated in the RRV scale using the following formula:

$$RRV_a = (RV_a - RV_{tol})/(RV_{c9,t11} - RV_{tol}) \quad [1]$$

where RRV_a is the relative retention volume of a CLA isomer, RV_a is the retention volume of a CLA isomer, RV_{tol} is the retention volume of toluene, and $RV_{c9,t11}$ is the retention volume of the *c*9,*t*11-CLA.

Analytical Ag$^+$*-HPLC.* Ag$^+$-HPLC separation of the CLA FAME was carried out using a Waters 2960 chromatographic system (Waters Associates), equipped with a photodiode array detector (Waters 996) operating between 200 and 300 nm, and a Millenium 3.20 chromatography manager. Three ChromSpher 5 Lipids analytical silver-impregnated columns (each 4.6 mm i.d. × 250 mm stainless steel; 5 µm particle size; Chrompack, Bridgewater, NJ) were used in series. The temperature was maintained at 30°C. The first mobile phase, 0.1% MeCN/0.5% DE/hexane, was prepared fresh daily and introduced isocratically at a flow rate of 1.0 mL/min. The columns were conditioned with 1% MeCN/hexane then equilibrated with the elution solvent for 60 min each day prior to starting test portion analysis. Typical injections, 1–10 µL, for milk, plasma, or reference materials resulted in <100 µg FAME being loaded onto the columns. For test portions of rumen FAME, column loads of >>100 µg were used. Single chromatograms of CLA isomers were extracted at 233 nm. To obtain the RRV values of all CLA isomers, iodine-isomerized mixtures of positional CLA isomers (4) were co-injected with a small amount of *c*9,*t*11-18:2 FAME and toluene. The second mobile phase used was 2% acetic acid/hexane eluted at 1 mL/min. HPLC chromatograms were obtained using the same three ChromSpher 5 Lipids analytical silver-impregnated columns described above. Responses were measured using UV detection at 233 nm.

RESULTS AND DISCUSSION

We recently completed an exhaustive analysis of CLA isomers in 88 samples of plasma, milk, and rumen fluid from dairy cows (5). Ag$^+$-HPLC chromatograms from that study, from plasma, two samples of cow's milk and CLA reference material (Nu-Chek-Prep) are shown in Figure 1A–D, left side. As can be seen in the figure, the retention volumes (shown as retention times in the figures) shift for the elution of each isomer. The *c*9,*t*11 isomer elutes over a range of several minutes. In most of the cases, the rule that the *c*9,*t*11-isomer is the most abundant may be applied to CLA analysis by Ag$^+$-HPLC, but in several other cases, as shown in Figure 1, this rule does not apply. Chromatogram A was obtained by injection of a plasma sample from a cow that had been fed a diet supplemented with *t*10,*c*12-CLA. Our original analysis of these samples involved many co-injections of standard with samples to verify the identities. We discovered that by using toluene as a reference to approximate the dead volume

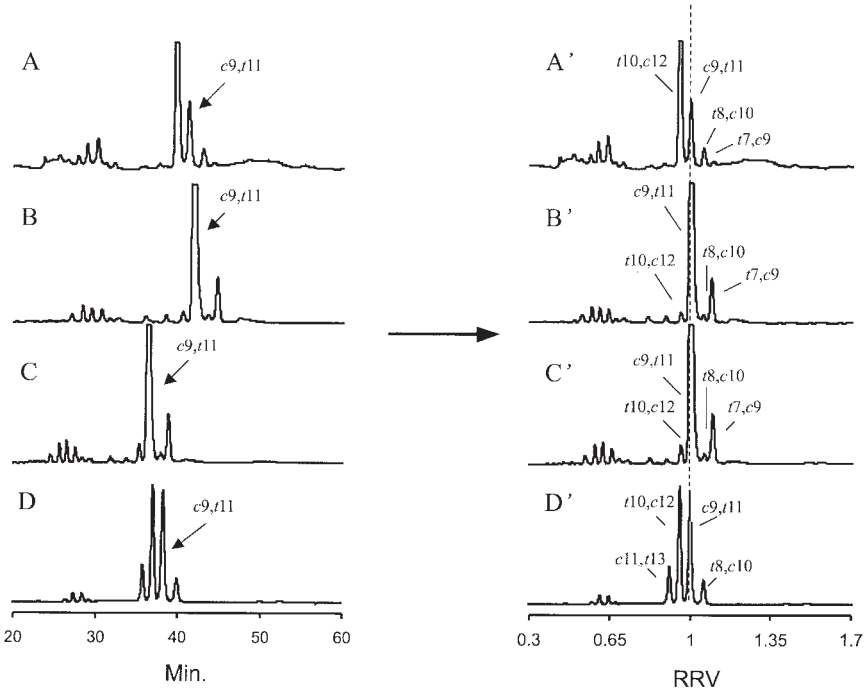

FIG. 1. Ag$^+$-HPLC chromatograms. Time vs. response (233 nm) of FAME obtained from plasma (A), milk fat (B, C), and Nu-Chek-Prep CLA (D). The corresponding graphs A′–D′ of the same sample test portions plotting response vs. relative retention volumes (RRV) are shown on the right.

TABLE 1
Relative Retention Volumes for CLA FAME from Cow Milk, Plasma, and Rumen Fluids

	Milk			Plasma			Rumen			Total			
	n	μ	σ	n	μ	σ	n	μ	σ	n	μ	σ	% CV
*t*12,*t*14	48	0.533	0.008	24	0.538	0.005	16	0.544	0.010	88	0.536	0.009	1.62
*t*11,*t*13	48	0.574	0.007	24	0.579	0.005	16	0.586	0.010	88	0.578	0.008	1.46
*t*10,*t*12	48	0.609	0.007	24	0.612	0.005	16	0.620	0.010	88	0.611	0.008	1.34
*t*9,*t*11	48	0.649	0.007	24	0.655	0.007	16	0.658	0.010	88	0.653	0.008	1.27
*t*8,*t*10	48	0.680	0.007	21	0.686	0.006	9	0.681	0.007	78	0.682	0.007	1.04
*t*7,*t*9	48	0.716	0.009	22	0.719	0.006	0	NA[a]	NA	70	0.717	0.008	1.09
*c*11,*t*13	48	0.914	0.003	24	0.911	0.002	2	0.901	0.018	73	0.913	0.004	0.48
*t*11,*c*13	48	0.893	0.005	24	0.892	0.002	1	0.894	NA	73	0.893	0.004	0.44
*t*10,*c*12	48	0.957	0.003	24	0.956	0.001	16	0.958	0.003	88	0.957	0.003	0.27
*c*9,*t*11	48	1.000	0.000	24	1.000	0.000	16	1.000	0.000	88	1.000	0.000	0.00
*t*8,*c*10	48	1.059	0.002	24	1.060	0.003	0	NA	NA	72	1.059	0.002	0.22
*t*7,*c*9	48	1.095	0.003	24	1.094	0.002	7	1.094	0.017	79	1.094	0.005	0.47

[a]Not applicable.

in the HPLC system, and *c*9,*t*11 as a retention reference, the chromatographic data could be recalculated into a reproducible format using a formula derived from standard chromatography theory. The RRV was calculated as shown in the Experimental Procedures section. By using this format, the chromatograms were regenerated using Excel as shown in Figure 1A′–D′, but these chromatograms are plotted as response vs. RRV. This application of the RRV theory is based on the assumption that, for the small variation in MeCN in hexane concentration occurring during the analysis of a sample set, the change in composition of the mobile phase will affect the elution of all of the CLA isomers in the same way.

The reproducibility of RRV in sample data using a specific chromatography system was established as shown in Table 1. The % CV measured for the 88 samples was 1.04–1.62 for the *t,t* isomers, and 0–0.47 for the *c/t* isomers. The precision was better for the plasma and milk samples, as is seen from the lower SD for these sample types in Table 1. A factor that leads to higher RRV SD of CLA isomers in rumen samples is related to critical (sample overload) chromatography conditions. CLA FAME isomers were present at trace levels in all the methylated rumen samples we analyzed, and test portions loaded onto the columns were increased to reach the detection limits of as many CLA isomers as possible. Co-eluting *cis* monoenoic FAME interfered with the CLA analysis of rumen test portions under these extreme conditions, requiring UV (232 nm) confirmation for every identification.

Ag^+-HPLC chromatograms, transformed using Equation 1, are presented in Figure 2. A small amount of *c*9,*t*11-CLA FAME was added to every sample shown in Figure 2 to aid in translating chromatograms to the RRV scale. A graphical representation in Figure 3 further elucidates the RRV pattern. The

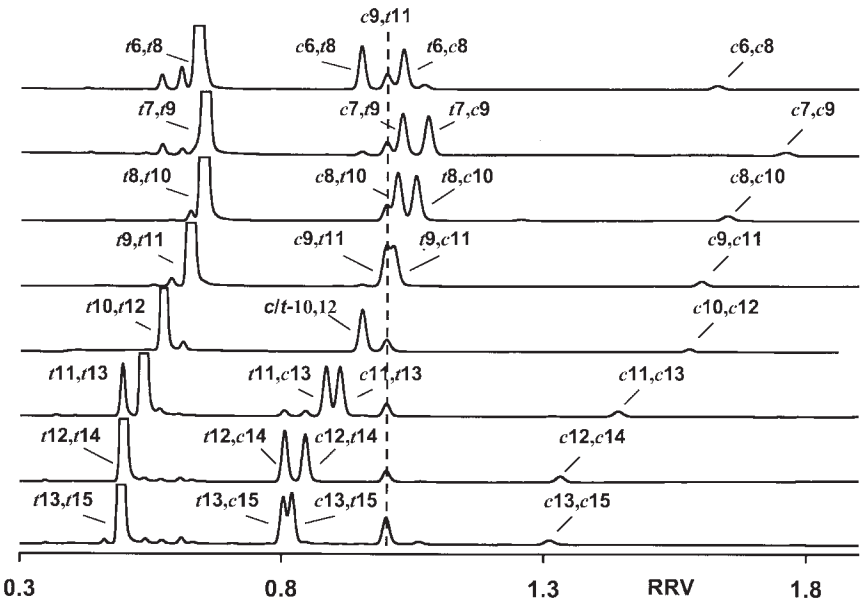

FIG. 2. Ag^+-HPLC chromatograms using 0.1% acetonitrile (MeCN)/0.5% diethyl ether (DE)/hexane elution, RRV vs. response (233 nm) of all geometric CLA isomers from the 6,8 to the 13,15 carbon–carbon double bond positions. A small quantity of *c*9,*t*11 was added to each positional mixture for reference.

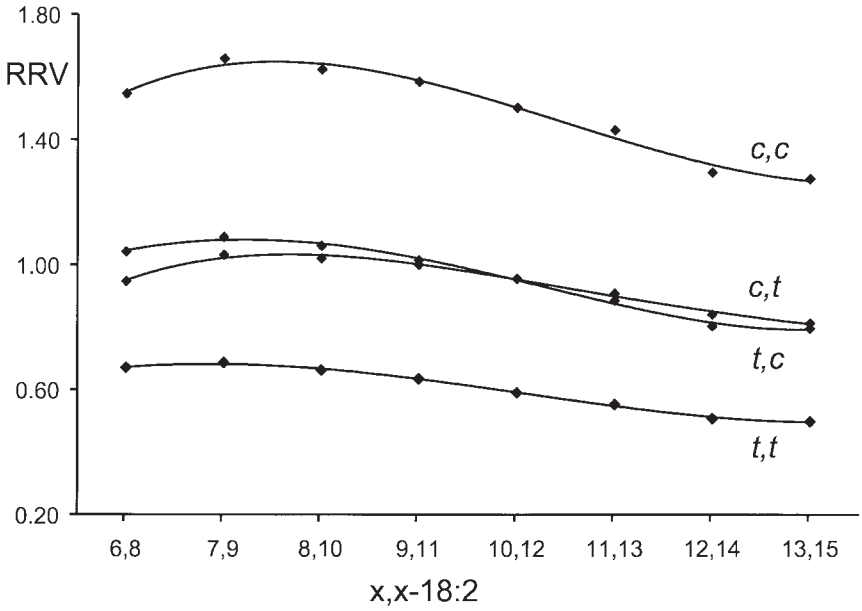

FIG. 3. Plot of RRV vs carbon-carbon double bond position for all geometric CLA isomers from the 6,8 to the 13,15 carbon–carbon double bond positions (using 0.1% MeCN/0.5% DE/hexane elution). For abbreviations see Figures 1 and 2.

RRV values of the *t,t*, *c,t*, and *t,c* isomers fit third-degree curves with R^2 greater than 0.99. The RRV values of the *c,c* isomers fit a third-degree equation with R^2 greater than 0.985. All four curves show a similar pattern, with a maximum corresponding to the 7,9 positional isomer, and an inflection point close to the 10,12 position. Isomers *c*10,*t*12 and *t*10,*c*12 have the same RRV, and the *c,t* and *t,c* curves intersect at the 10,12 position. For a given position, from 6,8 to 9,11 (all of these isomers elute after the 10,12 *c/t* isomer), the RRV of the *t,c* isomer will be higher than that of the corresponding positional *c,t* isomer. For *c/t* isomers eluting before the *c/t* isomers of 10,12 (from 11,13 to 13,15), the *c,t* isomer will elute after the *t,c* isomer.

Chromatograms obtained using the second solvent system presented here are shown in Figure 4. The set of the iodine-isomerized solutions (4) containing all the positional isomers from 6,8- to 13,15-18:2 are presented. Under these operating

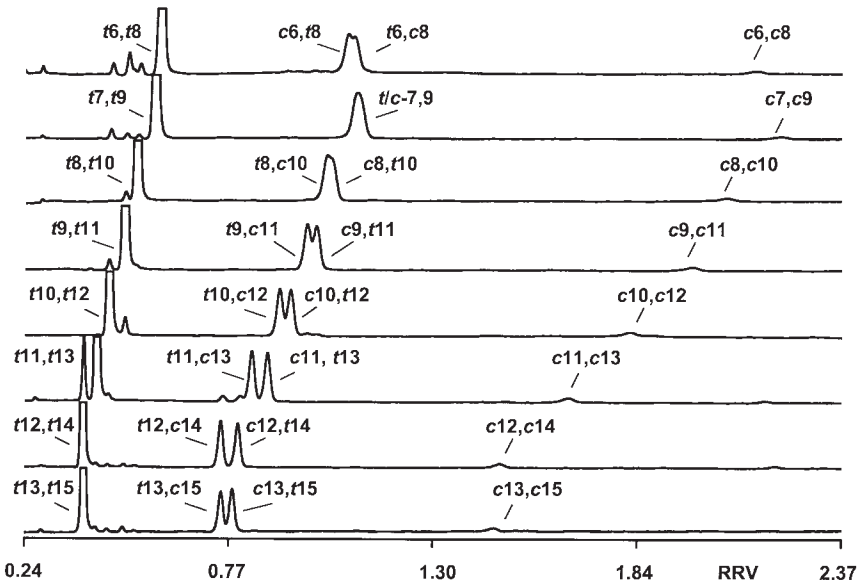

FIG. 4. Partial Ag^+-HPLC chromatograms (RRV) of the iodine-isomerized solutions from each CLA positional isomer from 6,8- to 13,15-18:2 as FAME. Chromatographic conditions: three ChromSpher 5 Lipids columns in series maintained at 25°C, 2% HOAc in hexane mobile phase at 1.0 mL/min, UV detection at 233 nm.

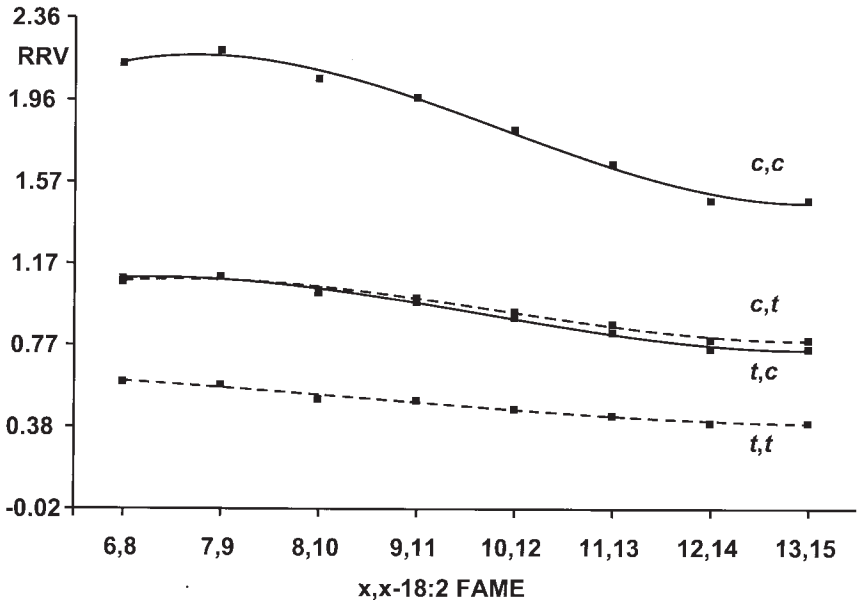

FIG. 5. Plot of RRV vs. double bond positions of CLA isomers. Three ChromSpher 5 Lipids columns maintained at 25°C were used in series, with 2% HOAc in the hexane mobile phase at 1.0 mL/min.

conditions there is no appreciable drift in RV, but the data are presented as RRV for a direct comparison of the retention order of CLA isomers eluted using either elution system. In Figure 5, the RRV of all the CLA isomers shown in Figure 4 are plotted as a function of double bond position. The plot in Figure 5 is similar to the plot in Figure 3 in that the *c,t* isomers of positional compounds near the carbonyl moiety elute before the corresponding *t,c* isomers. The plots differ in that for the 2% acetic acid/hexane mobile phase (Fig. 5) the position at which *c,t* and *t,c* isomers have the same RV is near the 7,9 position compared with the 10,12 position for the mobile phase containing MeCN (Fig. 3). The importance of this difference is emphasized by the separation of *t*10,*c*12- from *c*10,*t*12-18:2 and the partial separation of the *c*6,*t*8 from the *c/t* 7,9 isomers.

RRT are commonly used in GC analysis, but the use of RRV in HPLC analysis is less common. We believe that it is advantageous to use both in the instance of CLA analysis. The use of the *c*9,*t*11-CLA as a chromatographic reference in chromatographic analysis makes the identification of other peaks considerably easier, i.e., the analyst will know all the isomers that elute at a given RRV. The precision obtained, shown in Table 1, suggests that the drift in RV is only due to a loss of MeCN in the elution reservoir, and this is very consistent with the observation that adding more MeCN to the reservoir will re-establish the original RV. Column effects, particularly column temperature, age, and the amount of silver loaded, may, however, affect consistencies of RRV between different columns. Use of RRT (4) and RRV (presented herein) data also allows a substantially improved understanding of the GC and Ag^+-HPLC chromatographic patterns of elution. The reproducibility of RRV on a given column is excellent despite drifts in absolute RV.

The 2% acetic acid/hexane mobile phase produces a slightly different but complementary pattern of elution for CLA FAME. Its advantages include resolution of the two *c/t* 10,12 isomers and partial resolution for the *c*6,*t*8 isomer from the *t*7,*c*9 isomer. The disadvantages of this procedure are that it takes longer for a chromatographic run, 100 vs 60 min, and that in using this elution system it is not possible to use lower wavelengths, e.g., 205 nm, to monitor the chromatogram for interfering monoenoic (18:1) FAME that can interfere with CLA responses when present at a greatly elevated level.

The two Ag^+-HPLC chromatographic techniques presented here, along with GC data (4), complement each other quite well, and these data demonstrate that use of relative retention parameters allows for a more rapid initial identification.

One observation that is clear from the Ag^+-HPLC and GC (4) chromatographic patterns is that the identification of the *c*6,*t*8-18:2 CLA isomer, which is known (6) to be produced by the desaturation of *t*8-18:1 by Δ^6-desaturase, has been missed by every laboratory that depended on only GC (equipped with an FID) and Ag^+-HPLC using an MeCN/hexane system for their analysis.

REFERENCES

1. Sehat, N., Kramer, J.K.G., Mossoba, M.M., Roach, J.A.G., Yurawecz, M.P., Eulitz, K., and Ku, Y. (1998) Identification of Conjugated Linoleic Acid (CLA) Isomers in Cheese by Gas Chromatography, Silver Ion High Performance Liquid Chromatography, and Mass Spectral Reconstructed Ion Profiles: Comparison of Chromatographic Elution Sequences, *Lipids 33*, 963–971.

2. Yurawecz, M.P., and Morehouse, K.M. (2001) Silver-Ion HPLC of Conjugated Linoleic Acid Isomers, *Eur. J. Lipid Sci. Technol. 103,* 609–613.
3. Yurawecz, M.P., Roach, J.A.G., Sehat, N., Mossoba, M.M., Kramer, J.K.G., Fritsche, J., and Steinhart, H. (1998) A New Conjugated Linoleic Acid (CLA) Isomer, 7-*trans,9-cis*-Octadecadienoic Acid, in Cow Milk, Cheese, Beef, and Human Milk and Adipose Tissue, *Lipids 33,* 803–809.
4. Delmonte, P., Roach, J.A.G., Mossoba, M.M., Losi, G., and Yurawecz, M.P. (2004) Synthesis, Isolation, and GC Analysis of All the 6,8- to 13,15-*cis/trans* Conjugated Linoleic Acid Isomers, *Lipids 39*, 185–191.
5. Corl, B., Baumgard, L.H., Griinari, J.M., Delmonte, P., Morehouse, K.M., Yurawecz, M.P., and Bauman, D.E. (2002) *Trans*-7,*cis*-9 CLA Is Synthesized Endogenously by Δ^9-Desaturase in Dairy Cows, *Lipids 37*, 681–688.
6. Pollard, M.R., Gunstone, F.D., James, A.T., and Morris, L.J. (1980) Desaturation of Positional and Geometric Isomers of Monoenoic Fatty Acids by Microsomal Preparations from Rat Liver, *Lipids 15*, 306–314.

[Received March 10, 2003; accepted April 21, 2005]