PL-ISSN 0015-5497 (print), ISSN 1734-9168 (online)
Folia Biologica (Kraków), vol. 61 (2013), No 1-2

doi: 10.3409/fb61_1-2.107

The Content of Conjugated Linoleic Acid (CLA) Isomer Groups in Milk of two Polish Sheep Breeds Determined by Silver Ion Liquid Chromatography (Ag+-HPLC)

Agnieszka ROZBICKA-WIECZOREK, Aurelia RADZIK-RANT, Witold RANT, and Marian CZAUDERNA

Accepted November 22, 2012

ROZBICKA-WIECZOREK A., RADZIK-RANT A., RANT W., CZAUDERNA M. 2013. The content of conjugated linoleic acid (CLA) isomer groups in milk of two Polish sheep breeds determined by silver ion liquid chromatography (Ag+-HPLC). Folia Biologica (Kraków) **61**: 107-111.

Sheep milk is rich in CLA isomers which are biologically active components influencing human health. There are four geometric CLA isomer pairs: *cis,trans; trans,cis; trans,trans* and *cis,cis*. The aim of the present study was the analysis of CLA isomer groups content by Ag+-HPLC in milk fat of Żelazneńska (ZS) and Wrzosówka (WS) sheep breeds. The ewes of both breeds were kept under the same environmental and nutritional conditions. Milk samples were collected from 60 suckling ewes (30 from each breed), at the age of 3-4 years and in their 4th week of lactation. A higher total amount of all CLA isomer groups was obtained in milk of ZS ewes, however, this result was statistically insignificant. The percentage of the main *c*9,*t*11 isomer in total CLA was higher in Wrzosówka milk (68% vs. 74%). The content of the *trans,trans* isomer group in milk fat of the studied breeds was similar. The percentage of this group in total CLA in milk of WS and ZS constitutes 7.2% and 7.7%, respectively. The amount of *cis,cis* isomers in milk fat of ZS was higher thanWS ($P \leq 0.01$).

Key words: Sheep, milk, Ag+-HPLC, CLA isomers.

Agnieszka ROZBICKA-WIECZOREK, Marian CZAUDERNA, Polish Academy of Sciences, The Kielanowski Institute of Animal Physiology and Nutrition, Instytucka 3, 05-110 Jabłonna, Poland.
Aurelia RADZIK-RANT, Witold RANT, Warsow University of Life Sciences-SGGW, Department of Animal Breeding, Ciszewskiego 8, 02-786 Warsaw, Poland.
E-mail: aurelia_radzik_rant@sggw.pl

Among products of animal origin, sheep milk is rich in biologically active components positively affecting human health. Conjugated linoleic acid isomers undoubtedly belong to these components. The most recognizable include the isomer from *cis,trans* group *c*9,*t*11 called rumenic acid (RA) and the *t*10,*c*12 isomer from the *trans,cis* group. The *c*9,*t*11 isomer comprises 75-90% of total CLA for which potential anticarcinogenic and antiatherogenic effects have been highlighted, whereas *t*10,*c*12 CLA is a minor isomer and is probably responsible for effects on body composition, i.e. lowering of body weight and fat mass (COLLOMB *et al*. 2006; PARODI 2003). The other minor CLA isomers, of little or trace content, appear as geometric pairs *cis,cis* and *trans,trans* (PARK *et al*. 2007).

Most studies on ruminant milk fat quantified CLA isomers by gas chromatography (GC) and have shown that the GC peak can include more than one component and minor CLA isomers masked by the RA peak (LUNA *et al*. 2005). A more precise method, allowing quantification of isomers other than RA CLA or at least determining their group, is silver-ion high performance liquid chromatography (Ag+ HPLC). This method can provide separation of CLA not attainable by other means. *Trans,trans* isomers elute first, followed by *cis,trans/trans,cis* and then *cis,cis* (DELMONTE *et al*. 2005).

It is not easy to obtain repeatable results using Ag+HPLC. Potential sources of error include variation in silver loadings of the lipid columns, differences in instrument configuration, changes in elution volumes, solvent composition, and lack of internal standards as well as the number of analyzed samples (ADLOF 2003).

Attempts at determining CLA isomer content in milk of Polish sheep have been undertaken earlier, but in animals kept in different environmental and nutritional conditions, and using different analytical methods (BODKOWSKI *et al*. 2008; GABRYSZUK *et al*. 2007; RADZIK-RANT *et al*. 2010; SZUMACHER--STRABEL *et al*. 2008; SZUMACHER-STRABEL *et al*. 2011).

The aim of the present study was to analyze CLA isomer group content including *c*9,*t*11 by Ag+HPLC in milk fat of the Żelazneńska strain of Polish Lowland Sheep and Wrzosówka breed, kept under the same environmental and nutritional conditions.

Material and Methods

The study was carried out according to the guidelines of the Third Local Ethics Committee in Warszawa (No. 54/2010 from the 14th July 2010).

Animals and treatment

Milk samples were collected from 60 suckling ewes of two breeds (30 ewes from each breed): Żelazneńska strain of Polish Lowland Sheep (ZS) and Wrzosówka sheep (WS), which were maintained in one flock belonging to the Experimental Farm of the Warsaw University of Life Sciences, SGGW.

The diet of ewes of both breeds was based on local feeds: cereal meal, hay, straw and a mineral mixture. The chemical composition and nutritional value of fodder are presented in Table 1.

Table 1

The chemical composition and nutritional value of fodder given to ewes

Specification	Cereal meal	Hay	Straw
Dry matter (%)	89.24	90.82	91.1
Crude protein (%)	11.8	8.8	3.06
Ether extract (%)	1.72	1.36	1.24
Crude fiber (%)	4.89	34.55	41.24
Ash (%)	2.00	6.81	6.23
EN (MJ/1kg s.m.)	7.54	4.27	–
UFL/1kg s.m.	1.06	0.6	–
UFV/1 kg s.m.	1.02	0.5	–
PDI/1kg s.m.	69	68	–

EN – net energy; UFL – unit energy for milk production; UFV – unit energy for meat production; PDI – protein digested in the small intestine.

All sampled sheep were at the age of 3-4 years and in the 4th week of lactation. The length of lactation as well as litter size for both breeds was similar.

The lambs were separated from their dams two hours before milk collection, then ewes were hand milked and 100 ml samples for chemical analysis and CLA isomer determination were taken.

Chemical analysis

Chemical composition of fodder was analyzed according to procedure AOAC (1990). Each milk sample was analyzed for fat, protein, lactose, total solids (TS) and solids non-fat (SNF) with IR spectrometry using Milkoscan FT-120 (Poland).

Milk fat was extracted according to the Röse-Gottlieb method (AOAC 1990). CLA isomers were determined using a liquid chromatograph (Waters 625LC), a photodiode detector (DAD: model 996, Waters) and professional software Millennium 32 (ver.4.00). The isomers were separated by two silver-ion columns (Chrom. Sphere, 5μm Lipids: 250x4.6 mm; Chrompack), secured by the protective column (10x3 mm). The mobile phase consisted of hexane (98.39% v/v); acetic acid (1.60% v/v) and acetonitrile (0.013% v/v), at a flow rate of 1ml/min; the diode array detector was adjusted to 234 nm. Calibration of the HPLC system was conducted using individual standards of CLA isomers *c*9,*t*11 and *t*10,*c*12 and a mixture of CLA isomers (Sigma, USA; Larodon Fine Chemicals AB, Sweden) (CZAUDERNA *et al*. 2003).

Statistical analysis

Statistical treatment of the data of chemical composition of milk and CLA isomer groups of fat milk was performed using the SPSS 14.0 software (2003) based on one-way ANOVA, for $P \leq 0.05$.

Results and Discussion

The milk of Wrzosówka ewes was characterized by higher fat, lactose and TS content, but lower protein and SNF content than milk of Żelazneńska sheep, although differences were statistically insignificant (Table 2). Similar protein (4.68%) and lactose (5.12%) content, but lower fat portion (5.84%) in milk of the Wrzosówka breed was observed in earlier studies by NOWAK and NIŻNIKOWSKI (1996). RADZIK-RANT (2005) also reported lower fat content in milk of the Żelazneńska strain at the peak of lactation compared to the present study (6.22% vs. 8.21%), whereas the protein and lactose portions were similar. A much higher fat content reaching 12.6% was noted by WOHLT *et al*. (1981) for the

Table 2

The chemical composition of milk of the studied ewes

Analyzed traits	Wrzosówka n=30	Żelazneńska n=30	SE	P value
	LSM	LSM		
Fat (%)	8.91	8.21	0.25	0.17
Protein (%)	4.97	5.13	0.05	0.15
Lactose (%)	5.21	5.15	0.05	0.51
TS (%)	20.04	19.51	0.24	0.28
SNF (%)	11.13	11.3	0.11	0.52

TS – total solids; SNF – solids not fat; P>0.05 – not significant effect.

Table 3

Groups of CLA isomers in milk fat of the investigated ewes (g/100g fat)

CLA isomers	Wrzosówka n=30	Żelazneńska n=30	SE	P value
	LSM	LSM		
Total CLA	0.69	0.78	0.05	0.25
Trans,trans CLA	0.05	0.06	0.00	0.54
Cis,cis CLA	0.08	0.14	0.01	0.01
Cis,trans/trans,cis CLA	0.56	0.58	0.05	0.77
in this: *c*9,*t*11CLA	0.51	0.53	0.05	0.71

P≤0.05 – significant effect; P>0.05 – insignificant effect.

Dorset breed. The studies mentioned above confirm that this component is highly variable.

A higher total amount of all CLA isomer groups was obtained in the milk of ZS ewes (Table 3), although this result was statistically insignificant. The main *c*9,*t*11 isomer content was also insignificantly higher in milk of Żelazneńska sheep. Compared to the present study, a lower content of this isomer (0.4% of total fatty acids) in crossbred sheep of Polish Merino and Frisian breed was obtained by SZUMACHER-STRABEL *et al*. (2008), while a higher amount of the *c*9,*t*11 isomer (0.62g/100g total fatty acids) was recorded in milk fat of Polish dairy sheep line 05 (SZUMACHER--STRABEL *et al*. 2011). Although the *c*9,*t*11 isomer content in the present study was slightly higher for Żelazneńska sheep, its percentage in total CLA was higher in Wrzosówka milk (68% vs. 74%). In preliminary research, using the Ag+HPLC method for analysis of CLA isomers in milk fat of Polish breeds, a twofold greater amount of *c*9,*t*11 isomer for Polish Merino and Polish Mountain Sheep was reported (RADZIK-RANT *et al*. 2010). The percentage of this isomer was also higher in milk of Polish Merino (79.4%) in comparison to the present study. A similar percentage of this main isomer of total CLA was obtained for Portuguese sheep breeds (67-70%) by PARTIDARIO *et al*. (2008) and for Polish Mountain Sheep (74%) by RADZIK-RANT *et al*. (2010). A higher percentage of *c*9,*t*11 isomer for Friesian sheep (85%) using the GC method for designation of CLA isomers was reported by BODKOWSKI *et al*. (2008). PARK *et al*. (2007) described that the proportion of rumenic acid in sheep milk could vary from 76% to 82%.

The amount of other isomers belonging to the same group that *c*9,*t*11 CLA was similar in milk of both studied breeds, although their percentage in total CLA was higher for WS than ZS (7.2% vs.6.4%) (Table 3). A higher and lower proportions of these isomers in milk fat of Polish Merino and Polish Mountain Sheep, respectively, was observed by RADZIK-RANT *et al*. (2010).

There are *t*7,*c*9 and *t*10,*c*12 isomers within *cis,trans/trans,cis* groups of CLA. The *t*7,*c*9 is quantitatively the second most important isomer present in ruminant fat and constitues 8.5% to 16% of the total CLA in sheep milk (PARTIDARIO *et al*. 2008). In milk fat, this isomer originates almost exclusively *via* endogenous synthesis by Δ9 desaturase. PIPEROVA *et al*. (2002) confirmed that the *t*7,*c*9 isomer was absent in ruminal fluid and was present in only small quantities in the duodenal flow. KHANAL & DHIMAN (2004) described *t*10,*c*12 as the third most predominant isomer in ruminant milk. In this study the amount of *t*10,*c*12 CLA was not detected. The content of *t*10,*c*12 ranged from 0.03 to 0.1% of total fatty acids in other studies of CLA isomers in milk of Polish sheep (BODKOWSKI *et al*. 2008; SZUMACHER-STRABEL *et al*. 2008).

The differences in total content of *trans,trans* isomers in milk fat of the studied breeds were not statistically confirmed (Table 3). The proportion of this isomer group in milk of WS and ZS constitutes 7.2% and 7.7%, respectively. Similar results were reported by RADZIK-RANT *et al*. (2010) in milk of Polish Merino and Pomorska sheep and by PARTIDARIO *et al*. (2008) in milk of Portuguese breeds. However, in sheep cheese, the sum of the *trans,trans* isomers contributed 5-9% of total CLA (SEHAT *et al*. 1998).

The sum of *cis,cis* isomers in milk fat of ZS was higher (P≤0.01) than in milk of WS (Table 3).

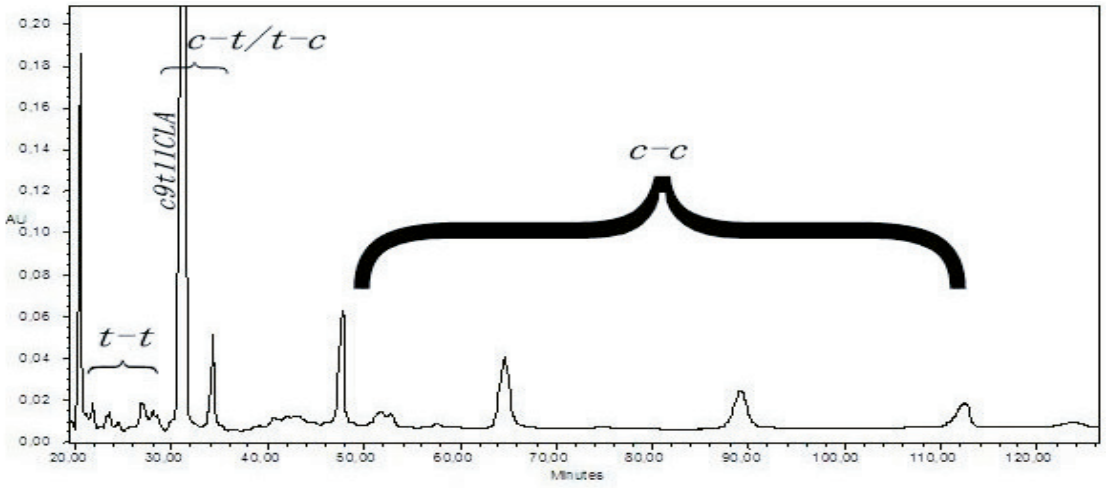

Fig. 1. The example of silver-ion HPLC separation of CLA isomer groups (Żelazneńska sheep – sample No 14).

The proportions reached 18% of total CLA for Żelazneńska sheep, but attained 11.6% for the Wrzosówka breed. The area of the *cis,cis* isomer elution is presented on an exemplary chromatogram (Fig. 1). The presence of this isomer group in milk fat of other Polish sheep breeds was also recorded in earlier research by RADZIK-RANT *et al.* (2010). *Cis,cis* isomers were undetected in milk and cheese derived from Portuguese sheep, even if the same Ag+HPLC method was applied (PARTIDARIO *et al*. 2008). In the present study, the high content of this group could be the result of the presence of other acids, masking isomers from *trans,cis/cis,trans* groups, which should be revealed earlier on this section of chromatogram (Fig. 1).

In all groups of CLA isomers, including *c*9,*t*11 C18:2 were negatively, but not significantly, correlated with milk fat content (Table 4). These results are consistent with those reported by TSIPLAKOU *et al*. (2006) for the main *c*9,*t*11 isomer in milk of grazed sheep and goats. Also NAŁĘCZ-TARWACKA *et al*. (2008) obtained a negative relationship between RA and fat content in cow milk. A completely contrary relation between fat amount and CLA isomers was noted by RADZIK-RANT *et al*. (2010) in an earlier study.

Table 4

The correlation coefficients between milk fat content and groups of CLA isomers

CLA isomers	Milk fat	P value
Total CLA	-0.32	0.22
Trans,trans CLA	-0.41	0.12
*cis,cis*CLA	-0.03	0.90
Cis,trans/trans,cis CLA in this:	-0.30	0.25
*c*9,*t*11CLA	-0.29	0.27

P>0.05 – insignificant effect.

In conclusion, it is possible to ascertain that the study of groups of CLA isomer content by Ag+HPLC in milk of sheep breeds kept under the same environmental conditions has confirmed preliminary research for sheep kept in different conditions. For greater repeatability of results, further research on the determination of CLA isomers by the Ag+HPLC method are required.

References

ADLOF R. O. 2003. Application of silver-ion chromatography to the separation of conjugated linoleic acid isomers. (In: J.-L. Sébédio, W.W. Christie and R. ADLOF ed. Advances in conjugated linoleic acid research) vol. 2**,** AOCS Press, Champaign, IL, USA: 37–55.

AOAC 1990. Association of Official Analytical Chemists. Food Composition Additives Natural Contaminants 2.4 Oils and Fats: 963.

BODKOWSKI R., PATKOWSKA-SOKOŁA B., WALISIEWICZ--NIEDBALSKA W. 2008. Effect of supplementing isomerised poppy seed oil with high concentration of linoleic isomer t10, c12 and c9, t11 on fat level in sheep milk and its fatty acids profile. Züchtungskunde **80**: 420-427.

COLLOMB M., SCHMID A., SIEBER R., WECHSLER D., RYHÄNEN E. L. 2006. Conjugated linoleic acids in milk fat. Variation and physiological effects. Int. Dairy.J. **16**: 1347-1361.

CZAUDERNA M., KOWALCZYK J., WĄSOWSKA I., NIEDŹWIEDZKA K. M. 2003. Determination of conjugated linoleic acid isomers by liquid chromatography and photodiode array detection. J. Anim. Feed Sci. **12**: 369-382.

DELMONTE P., KATAOKA A., CORL B. A., BAUMAN D. E., YURAWECZ M. P. 2005. 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. Lipids **40**: 509-514.

GABRYSZUK M., STRZAŁKOWSKA N., KRZYŻEWSKI J. 2007. Effect of pre- and post-partum injections of Se, Zn and vitamin E on the concentration of cholesterol, CLA isomers and fatty acids in ovine milk. Anim. Sci. Papers and Reports **25**: 87-95.

KHANAL R. C., DHIMAN T. R. 2004. Biosynthesis of conjugated linoleic acid (CLA) a review. Pakistan Journal of Nutrition **3**: 72-81.

LUNA P., FONTECHA J., JUÁREZ M., DE LA FUENTE M. A. 2005. Identification of conjugated isomers of linoleic acid in ewes milk fat. J. Dairy Res. **72**: 415-424.

NAŁĘCZ-TARWACKA T., GRODZKI H., KUCZYŃSKA B., PRZYSUCHA T. 2008. The influence of non-nutritional factors on health-promoting components of cow milk. Roczniki Nauk. PTZ **4**: 115-124. (In Polish with English summary).

NOWAK W., NIŻNIKOWSKI R. 1996. The influence of chosen factors on milk traits of Wrzosówka ewes nursing lambs. Zeszyty Naukowe PTZ **23**: 145-160. (In Polish with English summary).

PARK Y. W., JUÁREZ M., RAMOS M., HAENLEIN G. F. W. 2007. Physico-chemical characteristics of goat and sheep milk. Smmall Rum. Res. **68**: 88-113.

PARODI P. W. 2003. Anti-cancer agents in milk fat. Australian Journal of Dairy Technology **58**: 114-118.

PARTIDARIO A. M., RIBERIO J. C. M., PRATES J. A. M. 2008. Fatty acid composition and nutritional value of fat in three PDO ewes milk Portuguese cheeses. Dairy Sci. of Tech. **88**: 683-694.

PIPEROVA L. S., SAMPUGNA J., TETER B. B., KALSCHEUR M. P., YURAWECZ M.P ., KU Y., MOREHOUSE K. M., ERMAN R. A. 2002. Duodenal and milk trans octadecanoic acid and conjugated linoleic acid (CLA) isomers indicate that postabserptive synthesis is the predominant source of cis-9-containing CLA in lactating dairy cow. J. of Nutr.**132**: 1235-1241.

RADZIK-RANT A. 2005. The modification of fatty acids content in muscle tissue of lambs by diet enriched of different source oils. Postdoctoral Habilitation Thesis, ed. SGGW Warszawa. (In Polish with English summary).

RADZIK RANT A., ROZBICKA-WIECZOREK A., CZAUDERNA M., RANT W. 2010. The preliminary determination of conjugated linoleic acid isomers in milk of Polish sheep breeds by silver-ion liquid chromatography (Ag+-HPLC). Roczniki Naukowe PTZ **6**: 353-361. (In Polish with English summary).

SEHAT N. J, KRAMER K. G, MOSSOBA M. M, YURAWECZ J. A, ROACH G., EULITZ K., MOREHOUSE K. M, KU. 1998. Identification of conjugated linoleic acid isomers in cheese by gas chromatography, silver ion high performance liquid chromatography and mass spectral reconstructed ion profiles. Comparison of chromatography elution sequences. Lipids **33**: 963-971.

SPSS 2003. User's Guide 14.0. SPSS Inc.

SZUMACHER-STRABEL M., CIEŚLAK A., NOWAKOWSKA A., POTKAŃSKI. 2008. The effect of rapeseed oil and a combination of linseed and fish oils in the diets for sheep on milk fatty acid profile. Züchtungskunde **80**: 412-419.

SZUMACHER-STRABEL M., CIEŚLAK A., ZMORA P., PERS-KAMCZYC E., BIELIŃSKA S., STANISZ M., WÓJTOWSKI J. 2011. *Camelina sativa* cake improved unsaturated fatty acids in ewe's milk. J. Sci. Food Agric. **91**: 2031-2037.

TSIPLAKOU E., MOUNTZOURIS K. C., ZERVES G. 2006. Concentration of conjugated linoleic acid in grazing sheep and goat milk. Livestock Science **105**: 74-84.

WOHLT J. E., KLEYN D. H., VANDERNOOT G. W., SELFRIDGE D. J., NOVOTNEY C. A. 1981. Effect of stage of lactation, age of ewe, sibling status and sex of lamb on gross and minor constituents of Dorset ewe milk. J. Dairy Sci. **64**: 2175-2184.