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The use of developed chromatographic techniques to determine vitamin E and conjugated linoleic acid isomers in milk of cows

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ABSTRACT

The effect of vitamin E in feed enriched in rape seed oil or sunflower oil on the concentration of vitamin E in the cow milk was studied by chromatographic assay. The experiment was conducted on forty Holstein-Friesian cows. Addition of sunflower oil to the feed resulted in lowering the concentration in the milk compared with the concentration of vitamin E in the milk of cows fed the ration with rape seed oil. Described GLC procedure provided the new accurate analytical tool for quantification of CLA isomers and other fatty acids in milk and blood plasma of cows.

KEY WORDS: vegetable oils, vitamin E, conjugated linoleic acid, milk, blood plasma, cows

INTRODUCTION

Vitamins and some fatty acids (FA) are life sustaining organic compounds and very important for many physiological processes occurring in animals and humans. Among them is vitamin E and the last decade's much focused conjugated linoleic acid (CLA) isomers (Jensen et al., 1999; Wahle et al., 2004; Schneider, 2005). Evidence suggests that deficiency of vitamin E and CLA isomers plays an important role in numerous livestock and humans' diseases. Vitamin E is important for the oxidative stability of animal products (meat and milk), especially derived from animals fed diets enriched in unsaturated fatty acids (Giannenas et al., 2005).

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The aim of the present chromatographic studies was to examine the effect of vitamin E and vegetable oils supplementation on the concentration of vitamin E in cow milk. Attention has also been paid to improve quantification of CLA isomers in milk and blood plasma. Thus, long capillary gas-liquid chromatography method was also developed to analyse positional and geometric CLA isomers in the presence of other FAs in the milk as well as in blood plasma.

MATERIAL AND METHODS

Animals and experimental design

The experiment was carried out on Holstein-Friesian cows. Forty cows were enrolled in a 2×2 factorial experiment. The first factor was supplementing 300 mg *all-rac*-α-tocopheryl acetate plus 1450 mg RRR-α-tocopherol (αE) per cow per day (Table 1). The second factor was rape seed oil (RO) or sunflower oil (SO) from rape seed or sunflower cake. The remaining feed consisted of maize silage (50% of DM intake) and barley. Feed was provided as total mixed ration (TMR). The experiment lasted 5 weeks and milk and blood samples were taken at the beginning of the experiment, after 1 week and again after 5 weeks. Milk samples were frozen and stored in sealed tubes at -20°C until analysed.

Table 1. Composition of experimental rations for cows

Group	Treatment with rapeseed oil				Treatment with sunflower oil			
	RO (without E)		RO+E (with E)		SO (without E)		SO+E (with E)	
Feed components	kg[1]	%	kg	%	kg	%	kg	%
Rapeseed cake, 13% fat	6.81	32.6	6.81	32.6	-		-	
Barley	2.98	14.3	2.98	14.3	2.98	14.2	2.98	14.2
Sunflower cake					6.81	32.5	6.81	32.5
Maize silage	10.43	49.9	10.43	49.9	10.43	49.9	10.43	49.9
Barley straw	0.43	2.1	0.43	2.1	0.43	2.1	0.43	2.1
Mineral Type 0	0.15	0.7	0.15	0.7	0.15	0.7	0.15	0.7
Calcium carbonate	0.08	0.4	0.08	0.4	0.13	0.6	0.13	0.6
RRR-α-tocopherol, mg			1430				1430	

[1] dry matter (DM)

Reagents and chromatographic equipment

HPLC grade n-heptane (99.5%) and 2-propanol were purchased from Lab-Scan (Ireland), other reagents were analytical grade. The CLA isomer standard and vitamin E were provided by Sigma (USA).

A Perkin Elmer HPLC system (USA), equipped with a 4 × 125 mm HS-5-Silica column (Perkin Elmer) with fluorescence detector. The fluorescence detection of vitamin E was taken at the optimum excitation and emission wavelengths: at $\lambda_{ex}/\lambda_{em}$=290/327 nm. All direct assays of vitamin E were carried out at a column temperature of 22-24°C. The isocratic elution system was operated with a flow-rate of 3 mL/min; the eluent was prepared from heptane modified with 0.5% 2-propanol; duration of an analysis 3 min.

Saponification and vitamin E extraction

Vitamin E analysis in milk samples was performed as previously described by Jensen et al. (1999). Briefly, one mL milk sample was carefully vortexed at 40°C and then placed into a centrifuging tube. Next, 2 mL of ethanol (96%), 0.5 mL of methanol (100%), 1 mL of water containing ascorbic acid (20% w/w) and 0.7 mL of water were added. Saponification was performed at 80°C for 30 min. It is recommended to protect the resulting solution from light. After cooling vitamin E was extracted 2 times using 5 mL heptane followed by centrifugation at 3000 *g* for 10 min. The 100 µL of the combined heptane phase was injected onto the HS-5-Silica column.

Preparation of fatty acids methyl esters (FA-MEs)

In a vial 0.4-1 mL of milk or blood plasma was treated with 2 mL of methanol and 1 mL of chloroform. The mixture was then vigorously mixed for 1 min and next 1 mL of water was added. The vial was vigorously vortexed for 1 min followed by 2 mL of chloroform. Finally, the mixture was again vortexed for 1 min followed by centrifugation at 3000 *g* for 10 min. The bottom layer was transferred to a tube and evaporated on the warm plate set at 40°C under a stream of nitrogen.

To the residue, 0.8 mL of 0.5M NaOH in methanol was added, then flushed with nitrogen. The resulting solution in a tightly closed vial was then reacted for 15 min at 100°C. After cooling the reaction mixture, 1 mL of 20% BF_3 in methanol was added, flushed with nitrogen, and again heated for 45 min at 100°C. To a cooled resulting mixture, 4 mL of saturated NaCl solution was added and then FA-MEs were extracted with 2 mL of heptane. The obtained mixture was vortexed for 1 min followed by centrifugation at 2000 *g* for 10 min. The clear supernatant was transferred to a vial. Separation of FA-MEe was carried out using long capillary gas-liquid chromatography (GLC). The CLA isomers in the saponified milk samples (Czauderna et al., 2005) were identified using silver-ion and the reversed-phase high-performance chromatography with photodiode array detection (Czauderna et al., 2002, 2003).

Analytical conditions

The analyses were performed on an Agilent 6890N GC equipped with CP7489 fused silica capillary column (100 m × 0.25 mm i.d. × 0.2 µm film thickness; Varian, USA) and FID (the front detector temperature: 255°C), while split injections (from 1:1 to 1:5) were performed using an Agilent 7683 autosampler (the front inlet temperature: 250°C). Injection volumes were 2 µL.

Statistical analysis

Statistical analyses of the effects of addition of vitamin E to the feed enriched in RO or SO were conducted using the non-parametric Mann-Whitney U (The Statistica, version 6) (Statistica, 2002) test for comparing independent experimental groups.

RESULTS AND DISCUSSION

Vitamin E concentration in the milk of cow fed experimental feeds is presented in Table 2. Results indicated that addition of RO to the feed resulted in smaller decrease of concentration of vitamin E in the milk compared with supplementation with SO (after 35 days of feeding; $P<0.01$). The concentration of vitamin E in milk from cows fed extra vitamin E in the feed enriched in SO was lower ($P<0.05$) compared with the concentration of vitamin E in the milk from cows fed RO and vitamin E. Based on above studies we suggest that dietary SO evoked higher oxidative stress in examined cows than RO added to the feed. The reason for this difference may be related to higher concentration of polyunsaturated fatty acids in SO than in RO.

Table 2. The concentration of vitamin E in milk of cows fed feed supplemented with α-tocopherol (E), rape seed oil (RO) or sunflower oil (SO)

Day of measurement	Treatment with rape seed oil[1]		Treatment with sunflower oil[1]	
	RO (without E)	RO+E (with E)	SO (without E)	SO+E (with E)
Initial	0.47 ± 0.11^{A}	0.45 ± 0.14^{Aa}	0.41 ± 0.08^{AC}	0.49 ± 0.10^{a}
7	0.40 ± 0.07	0.61 ± 0.16^{ab}	0.27 ± 0.06^{AB}	0.60 ± 0.12^{a}
35[2]	0.35 ± 0.11^{AY}	0.82 ± 0.20^{Abx}	0.15 ± 0.05^{CBY}	0.59 ± 0.19^{x}

[1] mean with standard deviation (n=10) in columns with the same letter are significantly different at ^{A,B} $P<0.01$ or at ^{a,b} $P<0.05$ level

[2] the row with the letters Y and x are significantly different at $P<0.01$ and at $P<0.05$ level, respectively

CLA isomers in cows' milk have a wide range of beneficial effects, such as anti-carcinogenic, anti-diabetic, anti-obesity and anti-atherogenic. Therefore,

considering the above properties of CLA isomers it is extremely necessary to develop the versatile method to quantification of CLA isomers in the presence of other fatty acids in cows' milk as well as in tissues originated from ruminants (particularly in blood plasma and meat) using high-resolution capillary GLC. Our previous study documented that some additives in a diet affected the accumulation of CLA isomers in the body of laboratory animals (Czauderna et al., 2004).

In our previous chromatographic studies of positional and geometric profiles of CLA isomers revealed that the use of the column temperature program permitted satisfactory determination of all chemical form of CLA isomers, as well as separation of all isomers from other fatty acids present in milk and plasma samples. As can be seen from Figure 1, the elution order on a used capillary column is: all the *cis*,*trans* isomers followed by the *cis,cis* isomers and finally, all *trans,trans* CLA positional isomers.

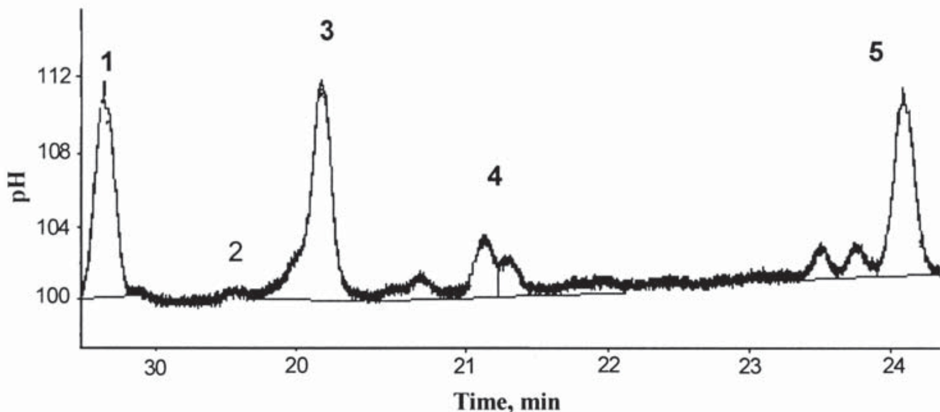

Figure 1. The part of typical GLC chromatogram for CLA isomers in cow milk (from 87 to 95 min). The methylated CLA isomers are: 1 - *cis9,trans11*CLA; 2 - *cis11,trans13*CLA; 3 - *trans10, cis12*CLA; 4 - the group of *cis,cis*CLA isomers (i.e. *8,10; 9,11; 10,12* and *11,13*); 5 - the group of *trans,trans*CLA isomers (i.e. *11,13; 8,10; 9,11* and *10,12*). The capillary GLC column was operated at 70°C for 5 min, then temperature programmed at 12°C/min to 150°C, held for 6 min, programmed at 8°C/min to 168°C, held for 27 min, programmed at 0.75°C/min to 190°C, held for 10 min, programmed at 1.8°C/min to 210°C, held for 15 min, programmed at 6°C/min to 234°C, held for 7 min, programmed at 6°C/min to 236°C, held for 20 min; the post run at 250°C for 7 min. Nominal initial He flow: 1 mL/min; the mode separation: constant pressure (i.e. 211.2 kPa)

Moreover, GLC analyses of processed plasma samples or the standard of a mixture of CLA isomer methyl esters (Czauderna et al., 2003) also confirmed that proposed GLC method permitted satisfactory fractionation of geometric and positional CLA isomers in milk and plasma samples. Obviously, all CLA isomer peaks were absent from blank, when current developed GLC method was

used. Accuracy and reliability of the assay of composition of the CLA isomers in the milk were also assessed by using high-resolution silver-ion (Ag^+-HPLC) (Czauderna et al., 2003, 2005) or the reversed-phase (RP) high-performance liquid chromatography (RP-HPLC) with the photodiode array detection (Czauderna et al., 2002). Replicate injections of underivatized or methylated CLA isomers of the milk or the standard of the CLA isomer mixture onto silver-ion or RP columns documented that all geometric and positional CLA isomer peaks are ”pure” (Czauderna et al., 2003). So, it is clear from UV spectra analysis of CLA isomers in effluents that all CLA isomer peaks are free from the presence of endogenous substances in the UV range applied (from 190 to 310 nm). Moreover, comparison of all results revealed that the profiles of methylated CLA isomers in the milk, blood plasma and the standard of CLA isomer mixture are similar using GLC, Ag^+-HPLC and RP-HPLC analyses.

Given together the results presented here, the developed new capillary GLC-FID method provided the accurate and precise analytical tool for simultaneous quantification of CLA isomers, as well as other fatty acids (Czauderna et al., 2005) in milk and blood plasma of cows. As can be seen from chromatographic analyses of milk samples, the concentration of *cis9,trans11*CLA being approximately ten times greater than the concentration of *trans10cis12*CLA; these isomers constituted 55-65% of the total amount of CLA isomers in cows’ milk, while the concentrations of other *cis,trans*CLA isomers are considerably lower (3-5%). Moreover, the percentage contribution of *cis,cis*CLA isomers is only 2-3% of the total CLA isomers, whereas *trans,trans*CLA is 20-30% of the pool of CLA isomers. The results for quantification of CLA isomers in the milk samples demonstrated that feeding the diet enriched in rape seed or sunflower, regardless of the presence of vitamin E, resulted in not-consistent changes in the concentration of CLA isomers in milk. The obtained concentrations of CLA isomers in assayed milk samples were in the range from 300 to 900 µg/mL milk.

CONCLUSIONS

The current chromatographic studies showed that the concentration of vitamin E in the milk was significantly affected by diets enriched in vitamin E. Addition of rapeseed cake to the feed resulted in higher concentration of vitamin E in milk compared with the concentration of vitamin E in milk of cows fed the diet enriched in sunflower cake. Furthermore, the proposed new capillary GLC method enabled simultaneous quantification of CLA isomers and other fatty acids. So, our current GLC method is a valuable analytical tool of the effect of dietary manipulations on the concentrations of fatty acid, particularly CLA, in products derived from domestic animals.

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