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Conjugated linoleic acid (CLA). *Cis* 9, *trans*11 and *trans* 10, *cis*12 isomer detection in crude and refined corn oils by capillary GC

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RESUMEN

Acido linoleico conjugado (CLA). Detección de isómeros *cis* 9, *trans 11* and *trans* 10, *cis* 12 en aceites de maiz crudos y refinado mediante cromatografía de gases.

El ácido linoleico conjugado (CLA) parece exhibir efecto protector frente a enfermedades cardiovasculares y varios tipos de cáncer. En este trabajo, se establece un mátodo analítico mediante cromatografía de gases con columna capilar para la determinación cualitativa y cuantitativa de los isómeros *cis* 9,*trans* 11 y *trans* 10, *cis* 12 en aceites de maiz crudo y refinado. El isómero *cis* 9, *trans*11 C18:2 fue el mayoritario encontrándose en concentraciones de 0.62% en el aceite cru,do y de 1.24 % en el aceite refinado. La cantidad total de CLA encontrada en el aceite refinado (n = 9) ($p < 0.01$) fue superior al doble de la encontrada en el aceite crudo. Se realiza la validación del método analítico, aportándose datos de calibración (R^2 = 0.9999) y de recuperación [y = 2.782x+0.046 (R^2 = 0.9999)]. El método cromatográfico propuesto podría ser usado para el control de calidad de los aceites vegetales.

PALABRAS-CLAVE: *Aceite de maiz – Acido linoleico conjugado – CLA – GC – Isómeros – Refinación.*

SUMMARY

Conjugated linoleic acid (CLA). *Cis* 9, *trans 11* and *trans* 10, *cis* 12 isomer detection in crude and refined corn oil by capillary GC.

Conjugated linoleic acids (CLAs) exhibit protective effects against various types of cancer and heart diseases. With the newly developed capillary gas chromatographic method (GC), *cis*9, *trans*11 and *trans*10, *cis*12 octadecadienoic acid isomers of CLA (C18:2) were determined in crude and refined corn oils as qualitative and quantitative measurements . *Cis* 9, *trans*11 C18:2 (*c*9, *t*11 CLA) was the major CLA isomer in both oils. It was found that *c*9, *t*11 CLA was 0.62% of the total lipid in crude oil and 1.24% of the total lipid in refined oil. Using the refining process, the total CLA was 1.38% whereas that of crude corn oil was 0.62%. An approximate 2.2 fold increase in the total CLA was found in refined oil (n = 9) ($p < 0.01$). The alteration in CLA could be used as a quality indicator for the refining process due to the contrubution of CLA to polyunsaturated fatty acids. Analytical method validation, calibration (R^2 = 0.9999) and recovery data [y = 2.782x + 0.046 (R^2 = 0.9999)] were performed ($p < 0.01$). The proposed chromatographic procedure could be used for vegetable oil quality control.

KEY-WORDS: *CLA – Conjugated linoleic acid – Corn oil – GC – Isomer – Refining.*

INTRODUCTION

Conjugated linoleic acid (CLA) is a term now used for a group of positional and geometrical isomers of linoleic acid and characterized by the presence of conjugated double bonds. It has been found that CLA exhibit a plethora of biological activities including its protective effects against various types of cancer and heart diseases (Pariza, 1999). It has also been shown that various CLA isomers inhibit platelet aggregation (Torres-Duerte and Vanderhoek, 2003). Conjugated linoleic acids (CLAs) are isomers of octadecadienoic acid containing conjugated double bonds at carbons 10 and 12 or 9 and 11, in all possible *cis* and *trans* combinations.

Pariza *et al.* (2001) have recently reviewed the numerous positive biological effects of CLAs including anticarcinogenic, antidiabetic and antilipogenic. According to latest research, it is reported that CLA has anticancer effects on MCF-7 breast cancer cells (Guo *et al*., 2007), has positive effects on bone formation and rheumatoid arthritis problems (Hur and Park, 2007), has an important role in weight control, reducing body fat, obesity and psychiatric treatment (Katzman *et al*., 2007; Watras *et al*., 2007). It is described that CLA increases skeletal muscle ceramide content and decreases insulin sensitivity in overweight, non-diabetic humans (Thrush *et al*., 2007).

It was demonstrated that *cis*9, *trans*11 C18:2 prevented the growth of human mammary cancer cells more effectively than *trans*10 ,*cis*12 C18:2. Ruminant meat and milk are the primary sources of CLA in the food chain. The quantitative amount of CLA ranges from 3.4 to 8.0 mg/g lipid in milk and dairy products and ranges from 2.7 to 5.6 mg/g lipid

in ruminant meat products depending on the animal species, tissue and diet (Lin *et al*., 1995; Chin *et al.,*1992) while butter contains approximately 720 mg/100g food (wet weight) (Britton *et al*.,1992). It has been found that ruminant meats and milk contain ~80% of *cis*9, *trans*11 CLA and ~10% of *trans*10, *cis*12 CLA (Fogerty *et al*.,1988).

It has been reported that CLA content increases with manufacturing processes and hydrogen donor effects (Lin *et al*., 1995). The occurrence of CLA isomers in probiotic yogurt enhanced with fructooligosaccharide (FOS) and concentrated cream "kaymak" manufactured from cow's milk were identified (Akalin and Tokuşoğlu *et al*., 2005; 2007).

The content of CLA in animal products is much higher than in plant oils. (Chin *et al.,* 1992; Ackman *et al.,* 1981; Fogerty *et al.,*1988). It has been shown that CLA ranges from 0.2-0.7 mg/g lipid in vegetable oils (Evans *et al.,* 2002). Vegetable oils including safflower oil, sunflower oil, corn oil and peanut oil contain large amounts of linoleic (C18:2), oleic acid (C18:1); especially soybean oil contains high amounts of linolenic acid (C18:3). Not only their oils but also their meals have been used as animal feed for ruminant and nonruminant animals.

High oleic (*cis*9-C18:1) or high linoleic (C18:2*n*-6) feeding has affected the CLA formation in ruminant tissues. *Cis*9-C18:1 (oleic acid) and *trans*9-C18:1 (elaidic acid) are competitive inhibitors of linoleate isomerase activity (Kepler *et al.,* 1970).If present in large amounts, *trans* 9-C18:1 (derived from 18:2*n*-6 or *cis*9, *trans*11-C18:2 hydrogenation) and c*is*9-C18:1 could alter the profiles of intermediates produced in the rumen, thereby affecting the levels available for absorption in the small intestine. In this context, vegetable oils are important sources for CLA formation

Due to the animal feed source and directly consumable food, vegetable oils are important for nutrition and it is necessary to know the quantitative CLA alterations in oil processing technology. The aim of this research is to develop a rapid GC method for CLA identification in corn oil and to monitor the conjugated linoleic acid (CLA) isomers in crude and refined corn oil by using this effective gas chromatographic method.

MATERIAL AND METHODS

Research Material and Standards

Corn oil was obtained from "*Yonca Ege Oil Company*, Manisa, Turkey" in April, 2003. Three bottle of raw corn oil (n = 9) and three bottle of refined corn oil (n = 9) were used for the analyses. Each was analyzed in duplicate .

Conjugated linoleic acid (CLA) of pure standard, a fatty acid (FA) standard mixture and boron trifluoride (BF_3)-methanol (14%) complex were purchased from Sigma Chem. Co., St Louis U.S.A. Methanol (99.8%) (HPLC grade) from J.T Baker (Deventer, The Netherlands) and chloroform and *n*-hexane (analytical grade) were obtained from Merck (Darmstadt,Germany).

Refining Process of Crude Oils

The conventional refining operation consisted of 4 steps . The first step was degumming to remove phospholipids. Secondly, FFAs were neutralized with sodium hydroxide (NaOH) to produce a soap which was then removed with remaining phospholipids by centrifugation. The pigments were then adsorbed by acid-activated bleaching clays. Finally, the oil was steam distilled under high vacuum to strip out trace amounts of FFAs, aldehydes, ketones and other volatile compounds (Tokuşoğlu and Ünal, 2003).

Proximate Composition of Crude and Refined Corn Oils

The moisture content of a 5.0g sample was determined at 110°C for 24 h in an oven by the procedure described by AOAC (1999). The total lipids in 5.0g of corn oil was determined by the procedure described by AOAC (1999). All proximate detections were done in triplicate.

Fatty Acid Methyl Esters of Corn Oil Samples

The fatty acid methyl esters (FAs) of corn oil samples were prepared from extracted lipids from each samples by esterification reaction with 14% boron trifluoride (BF_3)-methanol complex according to the modified method described by Tokuşoğlu and Ünal (2003).

Extracted lipids from corn oil was refluxed with a NaOH solution containing 0.05 N methanol for 5 min and was esterified with 14% boron trifluoride (BF_3)-methanol complex for 15 min and then equilibrated to room temperature (25 °C). All solutions were fractionated with hexane and saturated NaCl and then the methyl ester phase was separated. Anhydrous sodium sulfate (Na_2SO_4) was then added to the methyl ester phase. Prior to GC injection, extract was sonicated for 2 min to remove oxygen and 1 μL of extract was injected into the GC.

Capillary Gas Chromatography (GC)

Subsequent FA profiles and *cis*-9, *trans*-11 and *trans*-10,*cis*-12 octadecadienoic acid isomers of CLA was obtained through Gas-liquid chromatography (GC) by the newly developed method using a 60 m-capillary column (with 0.25 μm film tickness), 0.25-mm-inside-diameter WCOT fused-silica SGE (BP70X GC) capillary column installed on a Perkin Elmer (Auto System) Gas-liquid chromatograph with a Flame Ionization Detector (FID).

The gas chromatograph was temperature-programmed to start at 70 °C for 1 min isoterm

(initial temp.) and to increase at 10 °C/min to 150 °C and was held for 2 min isoterm (Ramp1) and then to increase at 2 °C/min to 265 °C and was held for 20 min isoterm (Ramp2). Injector and detector temperatures were set at 250 °C. Carrier gas was helium at a flow rate of 1.5 mL/min and split ratio was 50:1. The injection amount was 1 μL. FAs and CLA determinations were performed from 3 separate lipid extractions and esterifications. Each was injected in triplicate (n = 3).

Analytical Validation and Analytical Quality Control

Retention times (RT) were compared with known standards of FAMEs and CLA. Both FA standard mixture and CLA standard had linear calibration curves through the origin (R^2 = 0.9999) ($p < 0.01$). Analytical GC method was validated for FA determination of corn oil within the 95% confidence limits. Mean analytical recoveries determined from individual fatty acids in corn oil samples changed from 99.92% to 100%.

In 95% statistical confidence limits, an analytical standard addition of 5-20 μg mL-1 CLA standard for refined "*Yonca*" corn oil was performed and recovery data were obtained [y = 2.782x+0.046 (R^2 = 0.9999)]. Mean recoveries were %100 and %99.97 for *cis*-9, *trans*-11 and *trans*-10,*cis*-12 octadecadienoic acid isomers, respectively. All other analytical parameters are shown in Table 1.

Statistical Analysis

Data were analyzed with Statistica (1998) by one-way analysis of variance (Kruskal-Wallis ANOVA) with corn oil individual fatty acids and conjugated linoleic acid isomers as the source of variance.

RESULTS AND DISCUSSION

Figure 1 shows standard GC chromatogram of conjugated linoleic acid *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers and Figure 2 shows good separation of the above-mentioned compounds with other fatty acids simultaneously in refined corn oil at the same chromatographic conditions (Figure 1 and Figure 2). Conjugated linoleic acid *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers were perfectly separated with the proposed chromatographic conditions. This GC procedure provided the good baseline separation of *t*10, *c*12 CLA (CLA isomer 1) ($t_{\text{retention time}}$ = 20.37 min) and *c*9, *t*11 CLA (CLA isomer 2) ($t_{\text{retention time}}$ = 20.58 min) as shown in standard chromatogram (Figure 1). CLA isomer 1 ($t_{\text{retention time}}$ = 20.39 min) and CLA isomer 2 peaks ($t_{\text{retention time}}$ = 20.58 min) were confirmed in the GC chromatogram of refined corn oil (Figure 2) .

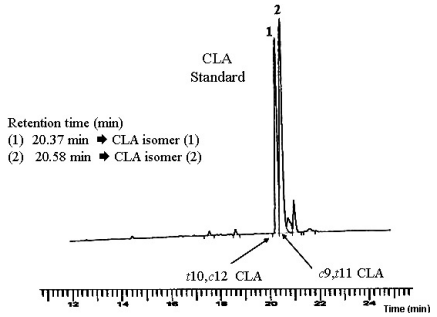

Figure 1
Standard GC chromatogram of CLA isomers

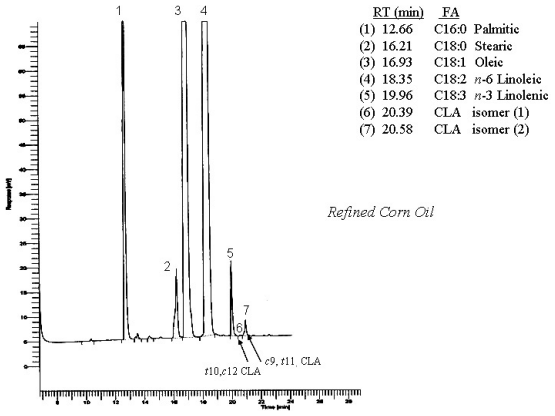

Figure 2
GC chromatogram of refined corn oil

The analytical parameters of crude and refined corn oils are shown in Table 1. Interday precision, relative standard deviation (RSD), recovery, and detection limit data of *cis*-9, *trans*-11 CLA and

Table 1
The analytical parameters of corn oil CLA determination

Analytical Parameters		*cis*-9, *trans*-11 CLA	*trans*-10, *cis*-12 CLA
Interday precision, n = 9	μg 100 mL-1 corn oil	1.25 ± 0.06	0.12± 0.03
	RSD (%)	1.48	1.22
	Recovery (%) n=9	100.00	99.97
Detection limit	μg 100 mL-1 corn oil	0.48	0.09
	μg 100 mL-1 assay	0.01	0.05

trans-10, *cis*-12 CLA were supported to good resolution (Table 1).

With the proposed chromatographic method, it was found that *c*9, *t*11 CLA was 0.62% of the total lipid content (as percent of total lipid) in crude oil whereas 1.24% of total lipid in refined oil ($n = 9$) ($p < 0.01$) (Table 2). With the refining process of oil, it was found that this CLA isomer increased 1.99 fold. *t*-10, *c*-12 CLA isomer was 0.14 % in refined corn oils whereas this isomer was not detected in crude corn oils. It is shown that *t*-10, *c*-12 CLA increased with the refining process (Table 2).

Some quality parameters of crude and refined corn oils including total lipid, volatile matter data and fatty acid contents were also determined. In particular, the total lipid level is most important in order to evaluate CLA content in oils (Table 3). It has been shown that crude oil contains 99.35 ± 0.03g oil /100g sample whereas refined oil contains 99.62 ± 0.02g oil /100g sample (Table 3). According to some proximate data table (Table 3), volatile matter was 0.38% in refined corn oils and was 0.65% in crude corn oils ($n = 9$) ($p < 0.01$). With the refining process, volatile matter has been decreased (Table 3).

Table 3
Moisture and total lipid composition of refined and crude corn oil

CORN OIL	Moisture (%) (Volatile matter)	Total Lipid (%)
Crude oil	0.65 ± 0.01	99.35 ± 0.03
Refined oil	0.38 ± 0.01	99.62 ± 0.02

Due to the refining process, the total CLA content of refined corn oil was 1.38% whereas that of crude corn oil was 0.62% (Table 2). An approximate 2.2 fold alteration for lipid constituent CLA was found in refined oil. In this context, the alteration in the CLA amount could be used an indicator for refining process quality due to CLA contribution to polyunsaturated fatty acids (PUFAs) ($n = 9$) ($p < 0.01$) (Table 2).

As shown in Table 2, fatty acids (FAs) in crude and refined oils were determined in the same chromatographic conditions. With the refining process used, total polyunsaturated acid (Σ PUFA) amounts were 57.02%. It is shown that Σ PUFA content had increased ~1.0 fold with refining ($n = 9$) ($p < 0.01$) (Table 2). Total saturated FA levels (Σ SAT) were 13.24% and had reduced ~1.05 fold ($n = 9$) ($p < 0.01$). Linoleic acid (omega-6) (C18:2*n*-6) and linolenic acid (omega-3) (C18:3*n*-3) increased in trace amounts with refining whereas palmitic acid (C16:0) decreased 1.07 fold (Table 2).

Current studies and clinical investigations have made evident that polyunsaturated fatty acids (PUFAs) exert beneficial effects on human health and play an important role in the prevention and treatment of coronary artery disease, hypertension, inflammatory, autoimmune disorders and cancer (Ricardo Uauy and Valenzula, 2000).

Crude vegetable oils are obtained by oilseed crushing, followed by solvent extraction. Crude vegetable oils contains 95% triacylglycerols and remaining compounds such as phospholipids, free fatty acids (FFAs), pigments, sterols, carbohydrates, proteins and degradation products (Tokuşoğlu, 2003).

Preexpelled and solvent extracted crude oil is not appropriate for dietary use. Crude vegetable oils undergo a complex refining process to achieve the desired quality and to produce a more stable product because the above-mentioned substances may impart undesirable flavor and color and shorten the shelf life of the oil shelf-life (Tokuşoğlu, 2003).

Refining is the final process for high quality vegetable oil. In our study we determined that CLA levels increased after the refining process. This may occur due to the manufacturing process of oil.

According to Ha *et al.* (1987) in the presence of oxygen and catalysts such as metals, the autooxidation of linoleic acid produces hydroperoxy acids that have the specific characteristics of a *cis*- or *trans*-conjugated dienic system (Ha *et al*., 1987).

Jung *et al*. (2002) reported that the CLA formation in oils during the hydrogenation process as affected by catalyst types, catalyst contents, hydrogen pressure, and oil species.

Table 2
Fatty acid (FA) composition of corn oil samples as percent (%) of total lipid content

FATTY ACIDS	C16:0	C18:0	C18:1*n*-9	C18:2*n*-6	C18:3*n*-3	c-9, t-11 CLA	t-10, c-12 CLA	Σ CLA[a]	Σ SAT[b]	Σ MUFA[c]	Σ PUFA[d]
Crude Corn oil	12.07	1.86	30.31	54.34	1.28	0.62	ND	0.62	13.93	30.31	56.24
Refined Corn Oil	11.24	2.00	30.24	54.35	1.29	1.24	0.14	1.38	13.24	30.24	57.02

[†]Mean ± SD (n=9) (p<0.01);
C16:0 = Palmitic acid , C18:0 = Stearic acid , C18:1n-9 = Oleic acid, C18:2n-6 = Linoleic acid C18:3n-3 = Linolenic acid ;
c-9, t-11 CLA= cis-9,trans-11 Conjugated Linoleic Acid ; t-10, c-12 CLA= trans-10,cis-12 Conjugated Linoleic Acid
[a]Σ CLA = Total Conjugated Linoleic Acid
[b]Σ SAT = Sum total area percentage of C16:0 and C18:0
[c]Σ MUFA = Total area percentage of C18:1n-9
[d]S PUFA = Sum total area percentage of C18:2n-6 , C18:3n-3 and CLA isomers.

It has been reported that the autoxidation of linoleic acid produces four isomeric hydroperoxy acids: 13-hydroperoxy-*cis*-9, *trans*-11-octadecadienoic acid; 13-hydroperoxy-*trans*-9, *trans*-11-octadecadienoic acid; 9-hydroperoxy-*cis*-10, *trans*-12-octadecadienoic acid; and 9- hydroperoxy-*trans*-10,*trans*-12-octadecadienoic acid (Chan and Lewett, 1977ab).

The lipids in food undergo a variety of chemical changes as a result of heat treatment through manufacturing processes, cooking, roasting, frying, pasteurization, etc. or the extrusion process. Pakdeechanuan *et al*. (2007) indicated that the effect of extrusion parameters on the CLA of corn extrudates. It is reported that CLA content increased from 1.2 mg/g of oil in feed to 7.8 mg/g of oil in corn extrudates at 150 °C.

In this research, with the developed GC method, we effectively separated CLA isomers in both crude and refined corn oil. This above-mentioned procedure provided a reproducible and sensitive quantitative fatty acid and CLA detection and can be used in the quality control area of the oil manufacturing industry. The study is in progress under different refining conditions and CLA separation. As it is known, recently, the commercial membrane separation processes and the use of a combination of membrane and supercritical fluid (SCFE) technologies in oilseed processing and edible oil refining are possible in the areas of minor ingredient purification such as tocopherols, phosphatidyl-choline and lecithin except for protein purification and waste treatment applications. CLA purification from vegetable oils in the refining process may be possible using these technologies.

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