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Conjugated Linoleic Acid Oils

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1. INTRODUCTION

The discovery of conjugated linoleic acid (CLA) dates back to 1933, when it was found that treatment of polyunsaturated fatty acids with alkali increased the UV absorbency (1–3). It was later found that the treatment produced a one to one mixture of cis-9-, trans-11- (9,11-ct) and trans-10-, cis-12 (10,12-tc) CLA. In 1935, it was noted that UV absorbency at 230 nm of milkfats was higher in milk from cows fed polyunsaturated fatty acids than cows fed saturated fats (4). This phenomenon was shown to be a result of conjugation of the double bonds of the polyunsaturated fatty acids (5). The predominant isomer in dairy products (milk, cheese, butter) or meat is 9,11-ct CLA (6).

Since the time of their discovery, conjugated fatty acids have been the subject of intense investigation. Spectroscopic analysis using ultraviolet light was the major analytical instrument available to researchers in the 1930s. As a result of high absorbance of UV light at 230 nm or higher, conjugated fat became a useful research tool for the study of fat metabolism. The first animal study used naturally conjugated fats of tung oil, but it was poorly tolerated (7). During this period, essential fatty
acids were discovered. Aaes-Jorgensen (8) studied the possibility of using CLA to treat/prevent the symptoms of essential fatty acid-deficiency. They observed that in essential fatty acid-deficient animals, CLA could not prevent deficiency but showed toxicity. Synthetic conjugated fatty acids produced from linoleic acid (LA), usually mixtures of 9,11-ct and 10,12-tc CLA, replaced the natural substances as a preferred biological marker.

Over the last two decades, the conjugated fats and, particularly, conjugated linoleic acid (CLA) have been intensively studied for their biological activity. As a result of the ease of synthesis, blends of two CLA isomers, namely 9,11-ct and 10,12-tc-CLA, have been the focus of most research into biological activity. Recent research, however, has been expanding to include pure or enriched isomer preparations.

2. METABOLISM

Early studies using a CLA mixture revealed that animals absorb and incorporate some of the CLA in their tissues in phospholipid, glycolipid, and acylglycerol fractions. In 1950, Reiser (9) observed faster and better absorption and incorporation of CLA when administered as triacylglycerol (TAG) versus free fatty acid. Following administration of CLA as TAG, the maximal levels appear in blood, liver, and organs at 16 hours compared with 24 hours for the free fatty acid form. Incorporation was higher in mesentery fat followed by perirenal and subcutaneous fat (9). Barnes et al. (10), in 1941, reported the kinetics of CLA absorption from the mixture. The absorption rate was highest in the first hour following administration and then gradually declined. Following a single oral dose, over 50% of conjugated CLA was absorbed in neutral mucosal lipids in the first hour followed by a decline, whereas in mucosal phospholipids, the incorporation was much slower, reaching maximum at about 8 hours followed by decline. In 1951, it was found that poultry incorporate CLA into egg lipids (5). Recent studies confirm the earlier finding of preferential incorporation in neutral lipids followed by phospholipids in liver (11) and mammary tissues (11, 12). Incorporation of CLA in tissues is associated with a reduction in the amounts of arachidonic acid and linoleic acid in neutral lipids (11) and in the liver TAG levels with an increase in the levels of 18:0 (13). In pig heart lipids, 11,13-ct isomer was the major CLA isomer followed by 9,11-ct isomer following an oral administration of CLA mixture (14). CLA isomers compete with desaturases and elongases to produce desaturated and elongated products maintaining the geometry of double bonds (15–17). CLA has been shown to inhibit delta-9-desaturase enzyme in vitro (18) and in vivo (19). CLA feeding also resulted in a decrease in arachidonic acid, and also reduced the desaturation of linoleic acid without affecting the desaturation of alpha-linolenic acid to any significant level. In rat, 9,11-ct isomer was preferentially metabolized to conjugated C20:3, whereas 10,12-tc isomer was metabolized to conjugated C16:2 and 18:3 compounds (19). The CLA mixture and its individual isomers (9,11-ct and 10,12-tc) inhibited basal and calcium ionophore stimulated production of prostaglandin from human sapheneous vein endothelial cells in
a dose-dependent manner. The mixture of CLA isomers was reported to inhibit the production of eicosanoids at all doses, whereas 10,12-tc isomer was shown to inhibit production at lower dose but stimulate at higher dose (20).

3. PHYSIOLOGICAL ACTIONS OF CLA

3.1. Effect on Body Composition

While working with essential fatty acid deficient rats in 1951, Holman observed that CLA-fed rats had significantly less total fat than control rats, and that they lost weight (21). Subsequent studies demonstrated that CLA inhibited fat accumulation and promoted lean muscle mass in growing animals, including pigs and mice (22–24). The results on the effect of CLA on body fat composition in animals are unequivocal, whereas studies in humans are providing mixed results. In a double blind, randomized clinical trial on obese and overweight humans, Blankson et al. (25) observed a clinically significant reduction in body fat mass in groups administered various doses of CLA ranging from 1.7 g per day to 6.8 g per day. Reduction in body fat mass was significant for groups administered 3.4 g CLA per day and 6.8 g CLA per day. Interestingly, this study found that 3.4 g of CLA per day provided maximum reduction in body fat mass; increasing the dose above this level provided no additional effect (25). Lean body mass and body mass index were similar in all the groups, although there was a slight increase in lean body mass in the CLA group. The increase in lean body mass did not achieve significance compared with a placebo group administered olive oil. Additionally, the CLA group presented a significant reduction in total-, LDL-, and HDL-cholesterol (25). In another study, Riserus et al. (26) observed a reduction in sagittal abdominal diameter in abdominally obese humans without affecting total body weight. A reduction in total fat mass in healthy, nonobese, exercising males was observed when CLA was given at a total daily dose of 1.8 g (divided in 3 doses) for 12 weeks. Body weight was not affected in this double blind clinical trial (27). A recent study in type II diabetic patients who were not on any medication found an inverse relationship between plasma CLA levels and weight loss and serum leptin levels (28). The inverse relationship was significant for 10,12-tc isomer of CLA and not for 9,11-ct isomer. A study in nonobese individuals using a mixture of CLA isomers containing about 20% each of 9,11-ct and 10,12-tc-CLA isomers, along with 20% to 25% other isomers, did not observe a reduction in body fat mass (29). Another study investigated the effect of CLA on weight regain after weight loss in overweight subjects (30). This study observed no effect of CLA on weight gain after weight loss; however, the weight gain in the CLA group was a result of an increase in fat free mass and was independent of dose (30). Comparison between studies is difficult as these studies differed in the degree of obesity of the subjects, duration of treatment, dose, and the isomer composition of the CLA preparation. Earlier commercial products of CLA contained equal amounts of 9,11-ct and 10,12 –tc isomers with other isomers in small amounts. The other isomers include all trans-isomers as well as other
cis-, trans-isomers, including 11,13-cis, 11,13-trans, 8,10 cis, 8,10-trans, etc. This illustrates the need for research on specific isomers and standardized protocols.

These reported actions of CLA could be mediated through a number of physiological mechanisms including increased fat oxidation (31) or inhibition of lipid accumulation in fat cells. Recent studies have started to investigate the physiological actions of individual isomers. It appears that the 10,12-cis isomer of CLA is mainly responsible for the effect of CLA on adiposity (32–34).

It was demonstrated that 10,12-cis-CLA reduces leptin, a hormone involved in regulation of fat deposition, in cultured fat cells (35), and in mice (36). In the latter study, feeding 10,12-cis-CLA to mice caused a comparatively small gain in weight with no gain in adipose fat. The 10,12-cis isomer of CLA was also shown to inhibit differentiation of preadipocytes in murine (3T3-L1) (37) and human preadipocytes (38). This was associated with decreased accumulation of TAGs in differentiating preadipocytes; inhibition of peroxisome proliferator-activated receptor gamma (PPAR-\(\gamma\)) gene (38) and its downstream gene products including lipoprotein lipase (LPL), GLUT-4 (glucose transporter gene 4), and inhibited expression of fatty acid synthase (FAS) gene. CLA isomer 9,11-cis, on the other hand, increased accumulation of TAGs in adiposities and also stimulated GLUT-4 and LPL. This suggests that isomer 10,12-cis may be responsible for inhibition of glucose uptake and oxidation in the adipocytes, leading to decreased TAG accumulation. These actions also underlie the effect of 10,12-cis isomer in inducing insulin resistance leading to lipoatrophic diabetes observed in animal (39, 40) and human studies (41).

3.2. Anticancer Properties

In 1985, Pariza and Hargrave discovered an antimutagenic fraction in cooked and raw beef during their studies on identification of carcinogenic compounds present in cooked beef (42). This fraction was identified to be a mixture of 4 isomers of CLA (9,11-cis, 9,11-trans, 10,12-cis, and 10,12-trans) (43). Studies in animal models and cell lines demonstrated the antimutagenic activity of a CLA mixture against known chemical carcinogens (7,12-dimethylbenz[a]anthracene, DMBA, and benzo (a) pyrene) (43–47). CLA has been shown to have anticancer effects against breast, colon, and prostate cancer cell lines (48–50). In rat models of breast cancer, CLA was reported to affect the breast structure when given during development stages. In this study, dietary CLA reduced the proliferation of terminal end bud and lobuolaveolar bud structures, whereby breast tissue became resistant to neoplastic transformations associated with cancer at later stages in life (48–50).

The exact mechanism of anticancer effects of CLA is not clear, and several possible mechanisms could underlie the anticancer properties of CLA. These actions may include its ability to interfere with the proliferation of cancer cells, increased apoptotic cell death, inhibition of angiogenesis, or increased oxidative stress. In a study comparing the effects of CLA on estrogen receptor positive human breast cancer cells (MCF-7) and estrogen receptor negative (MDA-MB 231) cells, CLA was shown to selectively inhibit proliferation of estrogen receptor positive cells.
CLA-treated MCF-7 cells selectively remained in G0/G1 phase and the expression of c-myc was inhibited. CLA had no effect on the growth of MDA-MB 231 cells. This study suggests that CLA acts by interfering with the estrogen-mediated second messenger system (51). CLA is also known to interfere with eicosanoids pathway and inhibits production of prostaglandin E2 (PGE2) (50, 52). Reduced production of PGE2 may play a role in anticancer actions of CLA. CLA is also shown to stimulate apoptotic death of cancer cells (50, 53, 54). CLA isomers increased apoptosis by stimulating the expression of caspase 3 and 9 activities and by reducing the expression of Bcl-2, an apoptosis repressor gene. The 10,12-tc isomer of CLA was found to be more potent in mediating these actions than either the 9,11-ct isomer or a mixture of the two (54). The other possible mechanism for anticancer properties of CLA is its ability to inhibit angiogenesis. In a mouse model of breast cancer, both isomers inhibited angiogenesis in mammary fat pad and reduced the concentration of vascular endothelium-derived growth factor (55). The CLA isomer 10, 12-tc also inhibited secretion of leptin and induced apoptosis in white and brown adipocytes, whereas 9,11-ct isomer was without effect on these parameters. CLA was also shown to reduce cell proliferation by reducing the expression of proteins involved in cell cycle regulation (p16 and p27) and DNA synthesis (56).

The above discussion focused on the role of CLA as a chemoprotective agent. Information regarding its effect on cancer treatment is limited. Feeding CLA for four or eight weeks after carcinogen administration was reported to be ineffective in preventing tumor formation, whereas continuous administration protected against tumor development (12, 57). A recent case control study in Finnish women suggested that dietary CLA may be protective against breast cancer (58). The role of CLA in prevention or treatment of cancer in humans is not clear and requires more research.

3.3. Insulin Resistance and Diabetes

CLA has been shown to normalize impaired glucose tolerance and improve hyperinsulinemia in prediabetic ZDF rats (59). These actions appeared to be mediated through PPAR-γ pathway as CLA treatment induced expression of mRNA for aP2. Recently, it has been observed in Zucker diabetic rats that either a 50:50 mixture of 9,11-ct-CLA and 10,12-tc-CLA isomers or a 90% 9,11-ct-CLA isomer stimulated insulin action in fat and muscle cells (60). These observations suggest that CLA can prevent or delay the onset of diabetes and that the 9,11-ct isomer may contribute much of this activity. Recently, 10,12-tc isomer of CLA was shown to induce insulin resistance and hyperinsulinemia in mice, whereas 9,11-ct isomer had no effect (61). Both these isomers were shown to be equally efficient in stimulating PPAR-α and γ receptors, indicating that the hyperinsulinemia may not be mediated through nuclear receptor pathway. In another study using either high metabolic rate mice or low metabolic rate mice, Hargrave et al. (62) demonstrated that CLA increased the insulin resistance in high metabolic rate mice only, whereas in Zucker diabetic rat, CLA was shown to prevent a rise in insulin and glucose levels that might have been mediated through an increased production of adiponectin, a hormone released by adipose tissues (63). In pigs, dietary CLA had no effect
on plasma glucose or insulin levels or on the ability of insulin to mobilize plasma glucose (64). These studies indicate that the effect of CLA in diabetes is not clear and the reported differences may be species specific. In human subjects, the effects of CLA on insulin resistance and glucose homeostasis are not well studied. In one clinical trial, the 10,12-tc isomer, but not a mixture of 9,11-ct and 10,12-tc isomers, was shown to increase the insulin resistance and blood glucose levels in abdominally obese people (41), whereas another study on normolipidemic subjects failed to observe any effect of either a 50:50 or 80:20 mixture of 9,11-ct and 10,12-tc isomers on blood glucose or insulin levels (65). Belury et al. (28) observed a reduction in fasting plasma glucose levels in type II diabetics when they were treated with 6.0 g of a mixture of CLA for eight weeks. This necessitates the need for controlled studies in human to delineate the effect of CLA and its isomers on blood glucose homeostasis and insulin resistance.

3.4. Cardiovascular Actions

An isomeric mixture of CLA was reported to inhibit the development of atherosclerosis in rabbits (66, 67) or hamsters (68) fed a high cholesterol diet. The CLA mixture caused a regression of atherosclerosis in rabbits (67) and Apo E-/- mouse (69). In hamsters, CLA was shown to lower the levels of total- and LDL-cholesterol and TAG levels (33, 70). The reduction in plasma levels of cholesterol could be mediated via an increase in LDL receptor expression in the liver leading to increased clearance from the circulation (71). CLA has also been reported to reduce secretion of apolipoprotein B in animals (72). The effect of CLA could be mediated through induction of the PPAR-γ pathway. CLA has been shown to inhibit cyclooxygenase enzyme in vitro (69). Its anti-inflammatory actions might be playing a role in prevention of atherosclerosis but does not appear to play a role in regression of atherosclerosis in animals. The 9,11-ct, and 10,12-tc isomers of CLA and the metabolite of 9,11-ct isomer (13-hydroxy-9c,11t-octadecadienoic acid) have been reported to inhibit arachidonic acid and collagen-induced platelet aggregation (73), which could have been mediated through inhibition of thromboxane A2 formation from arachidonic acid. CLA was also shown to prevent the development of hypertension in Zucker diabetic rats (63) that was associated with increased expression of mRNA for adiponectin, a hormone released by adipose tissues. Similar results on prevention of hypertension were shown in Otsuka Long-Evans Tokushima fatty (OLETF) rats (74). In this rat model, the 10,12-tc isomer of CLA was shown to inhibit angiotensinogen production from adipose tissues. The effect of CLA on plasma cholesterol levels and atherogenic potential in human is not clear. In healthy, normcholesterolemic human subjects, Benito et al. (75) reported no effect of CLA on cholesterol levels, platelet aggregation, or bleeding time, whereas Noone et al. (65) reported a TAG lowering effect of 50:50 mixture of CLA isomers. These differences could be due to differences in the composition of CLA, as Noone et al. (65) used 50:50 or 80:20 mixtures of 9,11-ct and 10,12-tc isomers and Benito et al.’s (75) preparation contained 11.4% 9,11-ct, 10.8% 8,10-tc, 15.3% 11,13-ct, and 14.7% 10,12-tc, with 6.7% c,c and 5.9% tt isomers of CLA. To establish the effects
of CLA on cardiovascular risk factors in humans, more research is needed using pure isomers or standardized mixture of CLA isomers.

3.5. CLA and Immune Function

In animal studies, CLA was reported to enhance immune response and attenuate allergic reactions (76, 77). CLA has also been shown to prevent age associated reductions in immune function (78). In Guinea pigs, feeding CLA was shown to reduce the release of histamine and PGE$_2$ from isolated trachea challenged with antigens (79), suggesting a strong antiallergic action. In mice, feeding CLA dose dependently increased splenic lymphocyte proliferation in response to phytohemagglutinin but not to lipopolysaccharide or concalin A, suggesting that CLA has selective actions on immune function. CLA stimulated IL-2 production (80) and also increased the basal and mitogen stimulated natural killer cell activity of splenic lymphocytes in mice that was associated with increased number of NK-cells but no change in the ratio of NK-cells to total splenic lymphocytes (81). CLA has also been shown to reduce the release of proinflammatory mediators, including PGE$_2$, IL-1, and IL-6, from macrophages stimulated by interferon gamma (IFN-$\gamma$) (82). These actions appeared to be mediated through stimulation of PPAR-$\gamma$ pathway. When fed to pigs, CLA enhanced cellular immunity by modulating white blood cell types that control adaptive and innate immunity (83, 84). Feeding CLA to pregnant and lactating pigs caused an increase in IgG levels of colostrums without affecting the serum IgG levels (85). In the same study, feeding CLA to suckling piglets resulted in increased serum levels of IgG and lysosomes. These results indicate enhancement of immune function in piglets. Following the feeding of CLA to young healthy women, no effects were observed on several measures of immune function including delayed type hypersensitivity response and numbers of circulating white cells and antibodies to vaccine, although an increase in CLA content of mononuclear cells was observed (86, 87). Differences in immune responses may be due to the selection of a young healthy population with optimal immune function, species differences, or dietary CLA isomer composition.

CLA has recently been studied for its actions on peroxisome proliferator-activated receptors (PPARs), most notably of the PPAR$\gamma$. As PPAR$\gamma$ plays a role in macrophage activity, Yu et al. (82) observed a stimulation of PPAR$\gamma$ in RAW264.7 mouse macrophage (RAW) cells by various CLA isomers. CLA also decreased the production of PGE$_2$, TNF-$\alpha$, nitric oxide (NO), IL-1$\beta$, and IL-6 in RAW cells treated with interferon-$\gamma$ (IFN-$\gamma$) (82). The inhibition of production of these inflammatory mediators was associated with a reduced expression of mRNA for cyclo-oxygenase 2 (COX2), inducible NOS (iNOS), and tumor necrosis factor alpha (TNF-$\alpha$). Cheng et al. (88) observed similar inhibition of COX-2 and NOS mRNA in lipopolysaccharide (LPS) stimulated macrophage cell, which was associated with an inhibition of LPS-induced protein expression of the cytoplasmic phosphorylated inhibitor kappaBalpha (IxB$\alpha$) and nuclear p65 as well as NF-kappaB nuclear protein-DNA binding affinity. This observation indicate a role of NF-kappaB in regulation of anti-inflamaotry actions of CLA.
4. STRATEGIES TO INCREASE DIETARY INTAKE OF CLA

As the interest in beneficial effects of CLA is increasing, so are the efforts to increase its dietary intake. Various strategies are being employed, which include increasing the content of CLA in eggs, milk, and meat. In 1951, hens were shown to incorporate CLA into egg lipids following dietary administration (5), but the egg production was significantly reduced. Recently, strategies to reduce the population of problem birds, based on feeding CLA to the females to reduce the egg hatchability, have been patented (89). Feeding 0.5% CLA in the diet of hens was shown to increase the CLA content of egg lipids, which was associated with a significant increase in saturated fatty acids and a reduction in monounsaturated fatty acids in the egg lipids. These changes in the egg composition also changed the properties of the egg yolk in that it became hard when stored at cool temperatures (refrigerator). This observation suggests that feeding CLA to poultry may not be an attractive strategy to increase the CLA content of eggs as poultry breeders cannot bear the economic burden of significantly reduced fertility. Strategies are needed to increase the content of CLA without affecting hatchability of eggs. Recently, methods to increase the content of CLA in eggs have been patented (90, 91). These strategies include incorporating CLA along with monounsaturated (91) or mono- and polyunsaturated fatty acids (90) in the diet. Similarly, feeding CLA in a diet to cattle increased CLA content in milk and meat and simultaneously reduced total milkfat content (92) and increased milkfat saturated fatty acids. Other strategies include feeding 11-trans-octadecaenoic (C18:1) acid (93) and other sources of polyunsaturated fatty acids to cattle. Feeding either fresh forage or supplementing the cattle diet with high linoleic acid meals/oils is another effective way of increasing the content of CLA in milkfat (94–97) and muscle. Most common sources of polyunsaturated fatty acids include sunflower oil, linseed (flaxseed oil), safflower oil, fish oil, or marine algae (98–101). When CLA is fed to cattle, it is better to protect it from rumen hydrogenation by converting to calcium salts.

5. COMMERCIAL PRODUCTION OF CLA

Industrial conjugated linoleic acid (CLA) is a poorly defined blend of compounds (102). Early commercial syntheses focused on maximizing total CLA content. Many early products were rich in CLA but contained a number of positional isomers. Market demand has now shifted for a product that contains two predominant isomers, specifically 9,11-c,t-octadecadienoic acid and 10,12-tc-octadecadienoic acid. It is not surprising that alkali isomerization produced some undesirable positional isomers of CLA. In 1970, Mounts and Dutton (103) had shown unequivocally that when potassium t-butoxide was used, at least four positional isomers of CLA were produced. It was not until 1997, after the use of CLA as a dietary supplement
began, that Christie et al. (102) elegantly demonstrated that commercial CLA was a blend of positional isomers. In response to this discovery, new commercial CLA products have been introduced that have comparatively high levels of the preferred isomers. In spite of the improvements, all current available commercial CLA products contain some level of the less desirable isomers and other components, which may or may not be desirable.

Commercial processes for the synthesis of any compound of economic value is normally proprietary information and the commercial methods of CLA production are no exception. The process by which each brand of commercial CLA is synthesized is not known by the authors of this review. Therefore, this review is directed at the patent literature on CLA synthesis, major problems encountered in CLA synthesis, and analysis of CLA from commercial suppliers.

5.1. CLA Production Raw Materials

The raw material for CLA production must be a material that is rich in linoleic acid. This product could be in the triacylglycerol form, fatty acids or fatty acid esters. The concentration of CLA in the final product is directly dependent on the level of linoleic acid in the starting material. The highest level of linoleic acid available from botanical sources is not available in commercial products. Extraction and refining equipment would be required to obtain oils with the highest linoleic acid levels. Table 1 lists the commercial and noncommercial sources of oil and fatty acids that are known to be rich in linoleic acid and their availability as TAGs and fatty acids.

If commercial CLA is to be synthesized from a fatty acid, it must be recognized that commercial fatty acids are generally not intended for use in the production of CLA. Commercial fatty acids are usually produced by the reaction of water (steam) and TAG oil at high temperatures in a continuous reaction (Reaction 1).
Reaction 1: Alkali or acid hydrolysis of acylglycerols.

\[
\begin{align*}
\text{RO} & \xrightarrow{H^+ \text{ or } H_2O} \text{RO}^- + 3 \text{HO} \\
\text{OH} & \xrightarrow{\text{OH}^-} \text{OH} + 3 \text{RCHO}
\end{align*}
\]

This reaction is accelerated through the use of a solid phase acid catalyst, which is readily separated from the fatty acid and glycerol products after hydrolysis (105). The disadvantage of this hydrolysis process is that the reaction is reversible and products generated by this process contain appreciable amounts of mono- and diacylglycerols, which may have undesirable side reactions in CLA synthesis. Fatty acids may also be produced by the hydrolysis of TAGs in a pressurized reactor at 200 °C without the addition of a catalyst (105). This reaction may be catalyzed at lower temperatures using zinc oxide in a batch reactor (105). The product of these batch reactions also contains substantial amounts of mono- and diacylglycerols.

Hydrolysis of TAGs is possible using water and strong base to produce soaps (Reaction 1). This reaction proceeds to completion and can be conducted at the modest temperatures required to maintain the reaction mixture as a fluid. More than three moles of potassium hydroxide or sodium hydroxide are required to hydrolyze one mole of TAG oil. As the caustic alkali cannot catalyze the reverse reaction, this process can produce soaps that are virtually free of acylglycerols in a single step (105). The soaps from alkali hydrolysis of TAGs are readily converted to fatty acids by acidification with the addition of citric acid or strong mineral acids, which include HCl, H2SO4, or H3PO4. Regardless of the method chosen for production of fatty acids, the acids should be dried under vacuum after washing with brine or by a combination of other acceptable methods (105).

There are commercially available fatty acids suitable for use in CLA production. For example, Henkel Corporation (106) sells a series of fat products including those shown in Table 2. However, none of the products listed in Table 2 would be preferred as starting materials for CLA production for reasons that will be discussed.

5.2. Enrichment of Linoleic Acid

A commercial interest may wish to produce CLA at concentrations greater than can be obtained by modifying high linoleic acid plant oils. Several methods exist that will improve the starting material by increasing the concentration of linoleic acid,
but only a few methods are used in industrial settings. Industrial separation of fatty acids has been reviewed by others (104, 107). A limited discussion of these methods will be presented.

Crystallization is used to separate saturated fats and oleic acid from linoleic acid. If a highly concentrated product is required, the linoleic acid may be crystallized once or repeatedly as the last step in purification. Crystallization is a mild procedure but usually requires the use of a solvent (108) such as acetone or methanol. The use of low boiling point and flammable solvents raises concerns over plant safety, government regulations on manufacturing, and market acceptance of the product. Furthermore, the removal of oleic acid by crystallization in solvent is only possible by lowering the temperature of the liquor to below $-40^\circ C$ (108). To crystallize linoleic acid, the temperature must be reduced to $-75^\circ C$.

Dry or solvent free crystallization is also possible; but these methods often require the addition of crystal modifiers that become incorporated into the product (108). Losses during crystallization can be very high as the crystals entrain large amounts of fatty acid. However, these losses may be reduced by physically pressing the crystals to remove the entrained solution (109). Linoleic acid-rich products of dry crystallization would be preferred starting materials for CLA production over those of solvent crystallized products; but the losses incurred in dry crystallization may prohibit this method of manufacture. Crystal modifiers may be selected so that they do not adversely affect the quality or acceptance of the final product.

Specific fatty acids may be concentrated by sequentially removing contaminating fatty acids as urea adducts and forming the urea adduct of the desired fatty acid. This process requires dissolving the fatty acids or esters in urea and hot methanol (or other alcohol) and cooling to effect adduct formation. The adduct is filtered from the liquor and, if conditions are carefully controlled, the adducts can be used to sequentially crystallize saturates, monounsaturates, diunsaturates, and triunsaturates. A urea adduct rich in linoleic acid could be produced by first removing adducts of saturates and monounsaturates from a suitable oil and then forming the desired adduct. Once formed, the adduct may then be decomposed by the addition of water to the solid phase. Enriched linoleic acid could be recovered by solvent extraction of the urea:water solution with a nonpolar solvent such as hexane.

All problems associated with crystallization in solvent mentioned previously also occur in formation of urea adducts, with the exception of the requirement for

### TABLE 2. Henkel Products (106).

<table>
<thead>
<tr>
<th>Product</th>
<th>14:0 (%)</th>
<th>16:0 (%)</th>
<th>18:0 (%)</th>
<th>16:1 (%)</th>
<th>18:1 (%)</th>
<th>18:2 (%)</th>
<th>18:3 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soya fatty acids (Emersol 610)</td>
<td>0.5</td>
<td>16</td>
<td>4</td>
<td>1</td>
<td>25.5</td>
<td>48</td>
<td>5</td>
</tr>
<tr>
<td>Linoleic acid (Emersol 315)</td>
<td>0.5</td>
<td>3.5</td>
<td>0.5</td>
<td>Trace</td>
<td>19.5</td>
<td>65.5</td>
<td>10.5</td>
</tr>
<tr>
<td>Methyl Linoleate (Emery 2221)</td>
<td>0.5</td>
<td>3.5</td>
<td>0.5</td>
<td>Trace</td>
<td>19.5</td>
<td>65.5</td>
<td>10.5</td>
</tr>
</tbody>
</table>
very low temperatures. Typically, urea adducts form between room temperature and 0°C (108).

Fatty acids may also be enriched by the use of various absorption media. Molecular sieves can separate saturated fatty acids from unsaturated fatty acids dissolved in acetone (110). Oleic acid and linoleic acid dissolved in blends of solvents, including acetonitrile, tetrahydrofuran, water, and formamide, may be separated using cross-linked polystyrene polymers such as Amberlite™ XAD-2 or XAD-4.

Selective extraction methods using two-phase solvent systems may also be used to enrich fatty acids. Solvent systems such as dimethyl formamide, hexane, and ethylene glycol can form a two-phase system that effectively partitions sunflower oil TAGs rich in linoleic acid from those depleted in linoleic acid (111). TAGs partitioned in this way may contain up to 84.7% linoleic acid. This method would not likely be used in industry because the magnitude of the losses are usually unacceptable.

5.3. Approaches to CLA Production

CLA has been produced by the reaction of soaps with strong alkali bases in alcohol, ethylene glycol, and glycerol (112–114) (Reaction 2).

Reaction 2: Isomerization of cis,cis-9,12-octadecadienoyl soaps.

The CLA product is generated by acidification of the soap solution with a strong acid (sulfuric or hydrochloric acid) and repeatedly washing the product with brine or an aqueous CaCl₂ solution.
CLA has been synthesized from fatty acid and soap blends using SO$_2$ in the presence of a substoichiometric amount of soap forming base (115). This reaction produced predominantly the all trans-configuration of CLA.

Of these methods, alkali isomerization of soaps is the least expensive process for bulk preparation of CLA isomers; however, the use of either monohydric or polyhydric alcohols in alkali isomerization of CLA can be problematic. Lower alcohols are readily removed from the CLA product, but they require that the production facility be constructed to support the use of flammable solvents. Higher molecular weight alcohols and polyhydric alcohols are considerably more difficult to remove from the product and residual levels of these alcohols (e.g., ethylene glycol) may not be acceptable in the CLA product.

Water may be substituted for the alcohols in the production of CLA by alkali isomerization of soaps (116, 117). When water is used in this reaction, it is necessary to perform the reaction in a pressure vessel, whether in a batch (116) or continuous mode of operation (117). The process for synthesis of CLA from soaps dissolved in water still requires a complex series of reaction steps. Bradley and Richardson (118) were able to produce CLA directly from TAGs by mixing sodium hydroxide, water, and oil in a pressure vessel. Their method eliminated the need to synthesize fatty acids followed by soap formation prior to the isomerization reaction. However, the authors reported that they were able to produce an oil with only 40% CLA. Quantitative conversion of the linoleic acid in soybean oil to CLA would have produced a fatty acid mixture with approximately 51% CLA.

5.4. Reaction Kinetics and Production of Positional Isomers

The kinetics of conversion of linoleyl soaps to conjugated linoleyl soaps has been well described by first-order reaction kinetics (119). Total conjugation is readily measured by simple methods such as increases of UV absorbance at 231.5 nm. In industry, it is necessary to allow the reaction to proceed until most of the linolyl soaps are conjugated but desirable to stop the reaction as soon as possible after that point. The reaction is between 97% and 98.5% complete after 5 half-lives and 6 half-lives respectively. There is little advantage in continuing the reaction longer than this time and, as will be discussed below, undesired reactions may occur with longer reaction times. The reaction constant is readily determined early in the reaction when changes in the level of conjugation are large. The task of determining the rate constant in a large reactor is complicated by the mass of the reactor and its contents. A large batch reactor often requires several hours to reach the optimum heat of reaction, at which time the reaction may be almost complete. Similarly, cooling the contents of a large reactor as a means of stopping a reaction is usually impractical. A reaction that nears 99% completion in 2 hours in a laboratory has a half life of less than 20 minutes. Control of a batch reaction in a commercial operation may use analytical data from periodic sampling, but the analytical method must be very rapid to be an effective tool for decision making.

The authors have found that the half-life of the reaction may vary by approximately 10% per 1°C. It follows that precise reactor temperature control is essential.
to standardize quality control. A reaction planned to continue for 6 half-lives could vary from 5 half-lives to 7 half-lives if the reactor temperature control is ±2 °C.

The isomerization reaction that leads to the production of positional isomers (Reaction 3) has similar first-order kinetics. Using this assumption, we have modeled the sequential conversion of linoleyl soaps to a mixture of 9,11-cis-, trans-octadecadienoyl and 10,12-trans-, cis-octadecadienoyl soaps (Reaction 2) and finally, the conversion of these two soaps to 8,10-octadecadienoyl and 11,13-octadecadienoyl soaps. Reaction 3 shows the mechanism of one of these two isomerization processes.

Reaction 3: Isomerization of cis-,trans-9,11-octadecadienoyl soap to trans-,cis-8,10-octadecadienoyl soap via sigmatropic rearrangement.

\[
\begin{align*}
R &= (\text{CH}_2)_6\text{COOH} \\
R_1 &= (\text{CH}_2)_5\text{CH}_3
\end{align*}
\]

In an earlier review of commercial CLA production, Reaney et al. (120) developed a model of the base catalyzed sequential conversion of unconjugated linoleic acid to a mixture of two isomers, 9,11-c,t- and 10,12-t,c-CLA, by first-order kinetics. After the formation of the two primary isomers, two additional isomers, 8,10-t,c-CLA and 11,13-c,t-CLA, were produced by a sequential reaction. It was later reported by Saebo (121) that the second reaction producing the additional isomers was actually a thermal sigmatropic rearrangement as shown in reaction 3. The intramolecular rearrangement is independent of catalyst concentration and, thus, the accumulation of additional isomers is a function of the reaction temperature and the duration of reaction. Saebo (121) reports that prolonged heating results in the accumulation of isomers but that reaction solvent that allows for low-temperature reactions may be used to prevent sigmatropic rearrangement and the consequent formation of isomers.

5.5. Solvents Used in Production of CLA by Alkali Catalysts

Research reports describe the use of at least eight solvent systems for the production of CLA using alkali catalysts (112–116) (Table 3). The choice of solvent greatly affects the reaction conditions of CLA production. The choice of solvent by the manufacturer is determined by a number of considerations. Many markets will not accept low levels of ethylene glycol, ethylene glycol monomethylether, t-butanol, dimethyl sulfoxide (DMSO), or dimethyl formamide (DMF) in the final product. This limitation could restrict the choice of solvents to only molten alkali, glycerol, propylene glycol, water, and ethanol. The reaction in water and ethanol only proceeds above the boiling temperature of these solvents and, therefore, a pressure reactor would be required to operate using these solvents. Glycerol is
expensive, but it could be recovered from a commercial operation that produces its own fatty acids. The quality of glycerol necessary to produce high-quality CLA has not been investigated, but refining this glycerol stream to remove the salt might prove difficult to a small operation. Recovery would have to be very efficient as fatty acid production only generates 10% of the weight of the oil as glycerol.

5.6. Catalyst Selection

Numerous catalysts have been used in the production of CLA. We have found that hydroxides of lithium, sodium, and potassium are all capable of generating CLA in various solvents. As fatty acids neutralize the catalyst, it is necessary to add at least one mole of catalyst for every mole of fatty acid in the reaction to ensure soap is generated. We have found that, on a molar basis, potassium hydroxide has proven to be a more effective catalyst than sodium hydroxide, with lithium hydroxide the least effective and not suited for industrial CLA production. On a weight basis, sodium and potassium hydroxide have similar efficiency of conversion. Although sodium hydroxide is much less expensive than potassium hydroxide, the disposal costs for the waste neutralized alkali should also be considered. Potassium salts are easily used as fertilizer and can be applied to fields, whereas sodium salts cannot be disposed of in a similar fashion.

The effective form of the catalyst is not necessarily determined by the added catalyst itself but rather by the solvent used. When water is used as the solvent and sodium ethoxide is the catalyst, the effective form of the catalyst is likely the hydroxide ion. If t-butanol is used as the solvent and sodium methoxide is added

<table>
<thead>
<tr>
<th>TABLE 3. Summary of Solvents Used in CLA Production.</th>
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<tbody>
<tr>
<td>Solvent (reference)</td>
</tr>
<tr>
<td>----------------------</td>
</tr>
<tr>
<td>Ethylene glycol (148)</td>
</tr>
<tr>
<td>Glycerol (148)</td>
</tr>
<tr>
<td>Propylene glycol (149)</td>
</tr>
<tr>
<td>t-butyl alcohol (103)</td>
</tr>
<tr>
<td>Water (118)</td>
</tr>
<tr>
<td>DMSO (149)</td>
</tr>
<tr>
<td>DMF (149)</td>
</tr>
</tbody>
</table>

^1Phase Separation = Yes if a two-phase system is formed after acidification of the soap.
^2Color = As described in references.
fp = flash point.
^*Superscript numbers refer to Vacuum in mm of Hg.
DMF, Dimethylformamide; DMSO, Dimethylsulfoxide.
to catalyze the reaction at 90°C, the reaction mixture will quickly release methanol vapours and t-butoxide ion will become the effective catalyst.

Water consumes alkoxide catalysts and, in industrial production, maintaining alkoxide catalysts in a water-free environment is difficult. Water is produced by the neutralization of fatty acids with alkali hydroxide. As many alkoxide catalysts contain some alkali hydroxide, it is not uncommon for the catalyst to be consumed by this reaction.

5.7. Reaction Vessels

Alkali isomerization of linoleyl soaps requires a containment vessel that is both tolerant of heat and caustic. When a low boiling point solvent such as ethanol or water is used, the vessel must also be capable of maintaining the reaction under pressure. There are a limited number of materials that will meet these criteria. Polytetrafluoroethylene and other fluoropolymers are capable of withstanding both the heat of the reaction and the caustic environment, but they cannot withstand pressure and are poor heat conductors (122). Fluoropolymer coated parts and nickel and nickel alloys such as Monel may be used in the construction of reaction vessels for production of CLA. The high cost of these materials rules out their use in construction of large batch reactors. Furthermore, none of these materials has sufficient strength for use in pressure reactors if a reaction in water or alcohol is planned. The preferred choice for reactor construction is nickel-plated steel, which has the desired strength, heat transfer, and chemical properties for conducting reactions in strong caustic solutions. A coated vessel of this design requires regular inspection, as a flaw in the coating could lead to vessel failure.

5.8. Microbial Production of CLA

Pariza and Yang (123) have recently described the microbial production of 9,11-cis,t-CLA from linoleic acid using cultures of *Lactobacillus sp*. In their patented method, early stationary phase *Lactobacillus* cultures were incubated with linoleic acid dissolved in propylene glycol. A total CLA level of 7 mg/g cells was produced, which was over 96% 9,11-cis,t-CLA. This type of conversion may lead to improved CLA products in the future.

5.9. Synthesis of CLA by Dehydration of Ricinoleic Acid (12-Hydroxy-cis-9-Octadecadienoic Acid)

The most attractive method for production of pure 9,11-cis,t-CLA is through the dehydration of ricinoleic acid. Synthesis from this relatively inexpensive starting material has proven elusive as it is difficult to control the formation of dehydration products (124). Synthesis of 9,11-cis,t-CLA from ricinoleic acid has been reported (125), which, although an efficient reaction, uses expensive elimination reagents such as 1,8-diazobicyclo-(5,4,0)-undecene. For most applications, the high cost of the elimination reagent increases the production cost beyond the level at which commercial production of CLA is economically viable.
5.10. The Quality of Commercial CLA Products

The fate of other fatty acids and minor components during processing has not been investigated. The conditions used to conjugate linoleic acid have little or no effect on either monounsaturated or saturated fatty acids, however, any polyunsaturated fatty acids may be conjugated. The products of the reaction of alkali catalysts on these fatty acids are more complex than that discussed for linoleic acid (Reaction 4) and will not be discussed except to note that these reactions may produce undesirable products.

Reaction 4: Isomerization of \( \text{cis}, \text{cis}, \text{cis}-9,12,15\)-octadecatrienoyl soap to isomers.

From our observations, glycerol does not form undesirable compounds under the conditions of alkali isomerization of linoleic acid. However, we have found that a number of commercial fatty acids and CLA preparations contain appreciable levels
of monoacylglycerols (MAGs). MAGs themselves are not toxic, but it is possible
that toxic compounds (such as ethylene glycol) may be incorporated into the final
product through alcoholysis of the MAG by the low volatility alcohols used as a
reaction medium (Reaction 5).

Reaction 5: Alcoholysis of MAG with ethylene glycol.

\[
\begin{align*}
\text{O} & \quad \text{O} \\
\text{O} & \quad \text{O} \\
\text{OH} & \quad \text{OH} \\
\text{OH} & \quad \text{OH} \\
\text{HO} & \quad \text{O} \\
\text{O} & \quad \text{O} \\
\end{align*}
\]

NMR and liquid chromatographic analysis of CLA samples from all sources
indicated that, although CLA was the predominant compound, CLA esters and other
unknown compounds may also have been formed.

The minor components of vegetable oil, such as tocopherol, sterol, or squalene,
are stable to heat a strong alkali. However, tocopherol and other components pre-
sent in vegetable oil readily react with oxygen in the presence of metals. Tocopherol
stabilizes vegetable oils against oxidation and tocopherol loss through processing or
high metal content may lead to a decreased shelf-life of the CLA product. Three
commercial CLA products were analyzed for metal content using ICP. The metal
contents are given in Table 4.

The CLA samples tested were free of potentially toxic metals at the levels tested,
with the exception of a trace level of barium in product C. Products A and B had
very low total metal contents, whereas product C had appreciable levels of calcium.
The soaps probably derived from a CaCl₂ wash in a late stage of processing.
Product C also contained iron and chromium suggesting the use of a stainless-steel reactor for processing. Even these small levels of metals may contribute to rapid oxidation of product C, and it probably has a shortened shelf-life when compared with the other products.

Three commercial samples of CLA were subjected to a series of tests, which are reported in Table 5. Viscosity was measured using ASTM test D445. It was presumed that increased viscosity might be related to increased oxidation if it had occurred. Large differences in viscosity were not found. Color of the three samples is reported, but it is only a subjective statement regarding the apparent quality of the three materials. Included in the color analysis is the absorbance maximum observed for CLA dissolved in hexane (1:1000 CLA:hexane) using a Cary UV/Vis spectrophotometer. The UV spectra indicated that conjugated dienes were the major source of absorption of the oil samples.

Proton and carbon 13 NMR spectra were obtained from the three commercial samples. A series of unknown spectral components were observed in product A at 3.68 ppm, 3.98 ppm, 4.08 ppm, 4.15 ppm, 5.05 ppm, and 5.17 ppm. Comparison of the unknown peaks with the spectra of MAGs indicated that most of the observed peaks correlated with those present in the spectra of MAGs. An exact assignment of the spectra was not attempted as many forms of MAGs are possible. Observation of the spectral region of the olefinic protons revealed that the three samples were

![Table 4. Metal Contents of Three Commercial CLA Products (ppm).](image)

![Table 5. Summary of Analyses of Three Commercial CLA Products.](image)
predominantly cis-, trans- or trans-, cis-fatty acids (126). We have found that a convenient measure of the CLA content of an oil can be obtained by comparing the integrated values of the protons at 6.0 ppm and 6.3 ppm with the integrated value of alpha methylene group (adjacent to the carbonyl) at 2.3 ppm. The olefinic protons were chosen as they do not overlap with oleic acid olefinic protons or olefinic protons from conjugated linoleic acid with trans-, trans-configurations. The ratio of cis-, trans-olefin to alpha methylene protons is a useful measure of CLA purity.

Carbon-13 olefinic carbons were observed at 400 MHz. The carbon-13 spectrum clearly demonstrates the formation of positional CLA isomers. As pure standards were not available, it was not possible to unequivocally assign the spectra of 8,10- or 11,13- CLA, but it is clear that these isomers are predominantly cis-, trans- or trans-, cis-fatty compounds.

Size exclusion chromatography was performed with a Waters GPC-Styragel™ HR 0.5 column (7.8 × 300 mm) at 35 °C using tetrahydrofuran as a solvent flowing at 1.0 mL/min and detecting compounds with both UV absorbance and evaporative light scattering detection (ELSD, 40 °C, 4.2 L/min gas flow). The goal of this analysis was to observe polymerization of the CLA products or the occurrence of acylglycerols. Under these conditions, 18-carbon fatty acids had a retention time of 6.6 minutes and MAGs had a retention time of 5.9 minutes. All three samples had two peaks observed by ELSD, one at the position expected for 18-carbon fatty acids and the other as expected for their respective MAGs. Product A had the largest peak at the position expected for MAGs. Observations made using UV absorbance at 233 nm reflected the observations made with the ELSD.

Reversed phase liquid chromatography was performed on a 150 mm × 3.0 mm Waters Symmetry™ C-8 column at 30 °C and a flow rate of 1.0 mL/min. The solvent phase was acetonitrile:tetrahydrofuran:0.1% aqueous phosphoric acid (50.4:21.6:28v/v/v). Under these operating conditions, most of the UV absorbance occurred as a peak at 3.83 minutes for all samples. Chromatograms of all samples had some small peaks (presumably the more polar compounds) eluting prior to the major peak. Product C presented a small but significant UV absorbing peak that eluted after the peak at 3.83 minutes.

### 5.11. Rapid Analytical Methods

Industrial CLA syntheses must be controlled to both maximize the content of preferred isomers such as 9,11-cis-, trans-octadecadienoic acid and minimize the formation of undesirable isomers. For the analysis to be useful, the results of the analysis should be available online or as quickly as possible. The authors are not aware of any existing online tests for the quality of CLA preparations, but several methods may have promise for use offline.

The potential offline analytical methods include UV, FTIR, proton NMR, carbon-13 NMR, gas chromatography, and capillary electrophoresis. With the exception of capillary electrophoresis and UV absorbance, none of these methods can
effectively analyze soap solutions and, therefore, for most analytical methods, neutralization of soaps would be necessary.

A reaction medium that contains 40% soaps by weight can usually be dissolved in ethanol. We have found that 100 mg of reaction mixture will totally dissolve in 10 mL of 95% ethanol when glycerol, water, or ethylene glycol are the reaction solvent and alkali hydroxides are used as the catalyst. It is then possible to dilute the reaction mixture solution 1000-fold to determine the UV absorbance at 231.5 nm. When there is no other interfering UV absorbance, this method is an excellent indication of the total conjugated double bonds. This method is also sensitive to the presence of conjugated linolenic acids derived from linolenic acid, which is indicated by a UV absorbance at 268 nm. None of the samples observed show three conjugated double bonds.

To obtain more detailed information regarding the composition of fatty acids requires additional analytical methods, some of which require extensive and time-consuming sample preparation. For these methods, we have found that it is possible to rapidly prepare a free fatty acid fraction. Alkali soaps from most reaction mixtures are readily dissolved in a mixture of hexane and ethanol (1 : 1v/v) or ethanol alone. When the soaps are neutralized by the addition of hydrochloric acid and water, a two-phase system is evolved. The fatty acids remain in the nonpolar phase while the polar solvent used in the reaction medium dissolves in the water. The solution of dissolved fatty acids can be directly injected onto a GC column specifically designed for separation of free fatty acids, such as the DB-FFAP column, or a suitable nonpolar GC column, such as the HP-5 column. Analysis by chromatography without derivitization affords the potential for rapid analytical feedback. We have found that a 30 m, DB-FFAP column (0.32 mm id, 0.25 μm film thickness) gave baseline resolution of most fatty acids without derivitization (program 50 °C for 1 min, 50–200 °C @ 25°C/min, 200–220 °C @ 2°C/min, hold 20 min, He carrier 3 ml/min). The nonpolar HP-5 column (0.32 ID, 0.25 film) gave poorer resolution of underivatized fatty acids with some tailing (program 50 °C for 1 min, 50–150 °C @ 25°C/min, 150–290 °C @ 10°C/min, hold 6 min, He carrier 2 ml/min). Both columns also partially separated isomers of CLA. It was possible to observe the formation of the all trans-isomers, but detailed analysis of positional isomers was not possible without additional effort.

6. ANALYSIS OF CONJUGATED LINOLEIC ACIDS

An analyst needs to recognize three major variables before the selection of a suitable method of analysis of CLA. CLA preparations can differ in degree of conjugation, position of double bonds, and configuration of double bonds. The most basic variable is the degree of conjugation of the double bonds. Not all of the isolated double bonds may become conjugated either in biological or chemical conversions. The second issue to be addressed is the position of the double bonds in conjugated linoleic acids. Initially, in linoleic acid, the double bonds begin at the ninth and twelfth carbon atoms. After alkali isomerization, the predominant positional isomers
are 9,11 and 10,12 dienes. However, minor amounts of other positional isomers are also reported (127). In biological samples, the number of positional isomers is more varied with 16 isomers being separated by silver-ion chromatography (128). The final consideration is the geometric isomers present in CLA. Whereas the double bonds in linoleic acid are $\text{cis}$, $\text{cis}$, the isomerized product contains a predominance of $\text{cis}$, trans- and trans-, cis-isomers plus lesser amounts of $\text{cis}$, cis- and trans-, trans-isomers. Thus, the task for the analyst is to decide what information is required and then select a method or methods that will provide that information.

### 6.1. Ultraviolet Spectroscopy

The early observation of an increase in the ultraviolet absorption (UV) near 233 nm was recognition that the composition of milkfat was not consistent throughout the year (129). This increase in absorption was not initially attributed to the increase in conjugated dienes present in milk fatty acids; however, it is now accepted that this is the case. There are subtle differences in the absorption maxima for the geometric isomers. Czauderna (130) reported a maximum of 231.9 nm for the trans-, trans-isomer, 234.3 nm for the cis-, trans- (or trans-, cis-) isomer and 235.4 nm for the cis-, cis-isomer. These small differences would not likely be discernable in isomer mixtures typically found in either biologically or chemically produced CLA. The UV measurement provides no information on the position of the double bonds. The advantage of using UV is the comparatively low cost of the spectrophotometer and the fact that it can be performed on either intact acylglycerols, fatty acids, or esters. In Figure 1, the UV spectra of linoleic acid and a commercial CLA product are shown.

![UV-Vis Spectra of Linoleic Acid and CLA](image)

Figure 1. The ultraviolet spectra of linoleic acid and conjugated linoleic acid (CLA).
6.2. Infrared Spectroscopy

Infrared spectroscopy (IR) initially used double beam optics, photocells, and alkali halide sample cells. These features at times tended to reduce the sensitivity of the instrument and to decrease the signal to noise ratio. Newer instruments have improved the signal to noise ratio by using Fourier Transform (FT) methodology and modern electronics. The application of infrared spectroscopy to the problem of identification of geometric isomers was first reported about 50 years ago. It was recognized that cis-, trans- (or trans-, cis-) dienes had a characteristic doublet at 948 cm\(^{-1}\) and 982 cm\(^{-1}\) (131). The corresponding trans-, trans-diene had a strong absorption band at 988 cm\(^{-1}\). Using FTIR and a direct deposit interface, Mossaba (132) reported the cis-, trans-doublet to occur at 949 cm\(^{-1}\) and 988 cm\(^{-1}\) and the trans-, trans-singlet at 993 cm\(^{-1}\) for 4,4-dimethyloxazoline (DMOX) derivatives of CLA. An example of the spectra of linoleic acid and two CLA positional isomers obtained with a FTIR using a synthetic sample disc are shown in Figure 2A. In addition to these absorptions, there are other characteristic frequencies attributed to carbon-hydrogen stretching. In one report of 4,4-dimethyloxazoline derivatives (DMOX) of CLA isomers, it was possible to attribute unique differences among geometric isomers (133). The cis-, trans-isomers had characteristic bands at 3020 cm\(^{-1}\) and 3002 cm\(^{-1}\) (see Figure 2B); the cis-, cis-isomers had bands at 3007 cm\(^{-1}\) and 3005 cm\(^{-1}\); and the trans-, trans-isomer had a single band at 3017 cm\(^{-1}\). The use of these bands to determine geometric composition may be complicated by the much more intense absorption bands from other carbon-hydrogen bands present in the same region of the spectrum. Although it is theoretically possible to obtain a quantitative measurement of the amount of a substance present by IR, it is not often done. In the case of CLA, the bands that needed to be measured are minor bands compared with other bands, making the determination imprecise and difficult.

![Figure 2. Infrared spectra of linoleic acid and 2 cis-, trans-, isomers of conjugated linoleic acid (A) 1000 to 900 nm of the =C–H vibration band and, (B) 3050 to 2950 nm of the C–H stretching bands.](image-url)
6.3. Thin Layer Chromatography

Thin layer chromatography (TLC) has proven very useful in the separation of a vast array of chemicals both synthetic and naturally occurring. When it comes to separation of closely related diene isomers, it is a difficult task with unmodified adsorbents such as silica gel. Silver nitrate-modified thin layer chromatography (Ag⁺-TLC) has been used to separate CLA isomers. Some cis-, trans-isomers of CLA have been separated as their methyl esters using hexane/diethyl ether, benzene, or toluene (102). The initial solvent mixture caused the cis-, trans-isomers to migrate faster than the cis-monoenes. Either of the later solvents resulted in the cis-, trans-isomer migrating between the cis- and trans-monoenes. Ackman (134) reported that the cis-, trans-isomer had a relative retention (R_f) of 0.61, whereas the trans-, trans-isomer had a R_f of 0.65. Although the difference in the R_f values is not great, it is possible to separate the isomers to either collect larger quantities or investigate possible metabolites.

6.4. Gas Chromatography

Gas chromatography (GC), initially using packed columns and later using capillary columns, provides another method for the analysis of fatty acids and esters or other derivatives. As binding of the fatty acids to the column was problematic, fatty acid methyl esters (FAME) were traditionally used. The method of forming the methyl esters can be divided into three procedures: acid-catalyzed, base-catalyzed, and diazomethane alkylation (135). No one method is suitable for all situations and all suffer some deficiency.

Acid-catalyzed procedures typically are either boron trifluoride/methanol (BF₃/CH₃OH) or hydrochloric acid/methanol (HCl/CH₃OH). Many analysts use sulfuric acid instead of hydrochloric acid. Acid-catalyzed procedures are used for free fatty acids, phospholipids, or triacylglycerols, often at elevated temperatures. Although the procedures are relatively efficient at production of methyl esters, there will be some isomerization of some cis-, trans-isomers to the trans-/trans-isomers (136). This isomerization can be reduced by using lower temperatures, for instance 60°C, for HCl/CH₃OH or room temperature for BF₃/CH₃OH (137). However, under these milder conditions, some phospholipids may not be esterified (137). In addition, methoxy adducts may be formed and hydroxy fatty acids may produce artifacts.

A base, such as sodium methoxide (NaOCH₃), is useful in esterifying lipids as found in acylglycerols, sterol esters, and phospholipids (138). Free fatty acid and N-acyl lipids in sphingolipids will not be methylated. The NaOCH₃ method does not apparently change the cis-, trans-isomer composition or form methoxy artifacts. However, some artifacts that could interfere with shorter chain fatty acids were observed.

Diazomethane is effective in esterifying free acids to their corresponding methyl esters under mild conditions and is fast. It will not produce methyl esters from acylglycerols, cholesterol esters, or phospholipids. Many researchers are concerned about the potential hazard of the diazomethane, its precursors, and the actual
preparation of the reagent. Trimethylsilyl diazomethane is commercially available and can be used as a source of diazomethane. However, some artifacts (trimethylsilyl CLA esters) and other trimethylsilyl impurities may interfere with analysis (139). As there are limitations to each method of making methyl esters, the analyst will need to select reagents and conditions that are most appropriate for the substrate. For intact acylglycerols, sterol esters, and phospholipids, the NaOCH3 method would be appropriate. If only free fatty acids are present, the diazomethane method would be appropriate, particularly if double bond isomerization is of concern.

The earliest of GC analyses were performed on columns packed with a solid support coated with a nonvolatile liquid phase. Packed columns are not frequently used today as they have been replaced by capillary columns where the liquid phase is immobilized on the internal surface of the capillary. As there are numerous liquid phases available, it is now possible to obtain commercial columns that will separate not only the methyl esters but also the underivatized fatty acids. This advancement obviates the need for derivatization and the associated problems. A typical chromatogram of free fatty acids is displayed in Figure 3. Individual isomers of CLA are now available to aid in the identification of isomers in the chromatogram. Gas chromatography can provide quantitative information on the degree of conjugation, positional, and geometric isomer distribution when suitable standards are available.

6.5. High-Performance Liquid Chromatography

High-performance liquid chromatography (or less common, high-pressure liquid chromatography, HPLC) is a preferred method of analysis for many compounds because it does not require the high temperatures used in gas chromatography. Separations in HPLC can be based on either a size exclusion or on an adsorption principle. The size exclusion mode is useful for separating fatty acids from
acylglycerols and has been applied to CLA analysis. Reaney et al. (120) have used a Waters GPC-StyragelHR™ column for the purpose of observing possible polymerization of CLA or the presence of acylglycerols. In three commercial samples of CLA, the 18-carbon fatty acids could be separated from the MAGs. Both UV detection at 233 nm and evaporative light scattering detection (ELSD) were used. There was no reported separation of individual fatty acids or CLA isomers. Use of a reverse phase column (Waters C-8 Symmetry™ column) for separation of commercial CLA resulted in a major peak being observed that would correspond to the free fatty acids (120). As with the size exclusion column, there was no separation of either isomeric conjugated fatty acids or acids of different chain length. A preferred technique is silver ion-modified high-performance liquid chromatography (Ag⁺-HPLC). Using this modification, several groups have reported successful separation of both positional and geometric isomers of CLA (14, 140).

6.6. Mass Spectrometry

Mass spectrometry (MS) is a method to determine the mass of either an intact ionized molecule or an ionized fragment. When MS is combined with the separation power of gas or liquid chromatography, much valuable information can be obtained for structural determination. Although GC-MS or HPLC-MS may seem to be an ideal tool for CLA analysis, it suffers a major problem as originally practiced. In order to obtain a mass spectrum, it is necessary to produce an ionized species. With CLA, as well as other molecules, some ionization conditions cause the double bonds to migrate to new positions. If the double bonds migrate, it is only possible to obtain information on chain length and number of double bonds. To allow for less harsh ionization conditions, it is possible to make derivatives at either the carboxylic acid (remote site) or at the site of the conjugated double bonds (on site). The derivatives have been selected to allow for easier ionization. In the case of the on-site derivatives, the position of the diene is fixed at one location. At the carboxylic acid site pyrrolidide, picolinyl ester and 4,4-dimethyloxazoline (DMOX) derivatives are used. The fragmentation of the remote site derivative basically produces ions that are 14 mass units lighter than the previous ion for each methylene (CH₂) group. When a double bond is encountered, the decrease observed is 12 mass units. A review on this subject has been compiled (141).

Two different approaches have been reported for on-site or double bond site derivatization of fatty acids or other conjugated dienes. One method involves the complete hydroxylation of the double bonds with osmium tetroxide (health caution) followed by trimethylsilylation (142). The second method is based on the well known Diels-Alder cyclo addition. MTAD (4-methyl-1,2,4-triazoline-3-5-dione) has been shown to be a useful reagent for adduct formulation with FAME conjugated dienes. Fragmentation patterns of adducts are usually dominated by cleavage fragments that include the ring formed during the cyclo addition plus either of the residual carbon chains. In Figure 4 of the MTAD derivative of methyl cis-, trans-9,11-octadecadienoate, these fragments occur at 322 m/z and 250 m/z, indicating
that the starting FAME was a 9,11 diene (143). Methyl cis-, trans-9,11-octadecadienoate and methyl trans-, trans-9-11-octacadienoate readily formed adducts with similar mass spectra but with different retention times when analyzed by GC-MS (143). Methyl cis-, cis-9,11-octadecadienoate reacted more slowly to produce two products with similar fragmentation patterns but with different retention times. Unfortunately, it was demonstrated that the cis-, cis- and cis-, trans-isomers produced the same adduct, limiting usefulness when studying geometric isomers of CLA products.

6.7. Nuclear Magnetic Resonance

Nuclear magnetic resonance (NMR) is a powerful technique for CLA analysis (144, 145). NMR spectroscopy can provide information on the environments of both the proton (1H) and the carbon-13 (13C) atoms in CLA and also provide correlation between the two atoms. In one of the most basic analyses, it is possible to observe the disappearance of the signals as a result of the isolated olefinic protons (5.35 ppm, Figure 5A) and the isolated methylene group (2.75 ppm) linoleic acid as well as the appearance of new signals associated with the conjugated diene (5.2–6.5 ppm, Figure 5B). Reaney et al. (120) reported that a convenient measurement of CLA content can be obtaining by comparing the integrated areas of the olefinic protons at 6.0 ppm and 6.3 ppm with the area of the methylene protons adjacent to the carbonyl (2.3 ppm). The NMR 13C spectra for both linoleic and cis-, trans-9,11-octadecadienoic acids are presented in Figure 5C and 5D, respectively. The complete assignment for this and other isomers of CLA has been reported (146, 147). A notable feature for CLA is the appearance of four well separated signals between 129–135 ppm (Figure 5D) compared with the two pairs of
narrowly separated doublets observed in linoleic acid spectrum (Figure 5C). Detailed $^1$H and $^{13}$C and NMR spectroscopy have the potential to provide information on both positional and geometric isomers of CLA and can provide semiquantitative information on CLA concentration.

**Figure 5.** (A) The $^1$H spectrum of linoleic acid; (B) the $^1$H spectrum of cis-, trans-9,11-octadecadienoic acid; (C) the $^{13}$C spectrum of linoleic acid; and, (D) the $^{13}$C spectrum of cis, trans-9,11-octadecadienoic acid.
7. CONCLUSIONS

The research on CLA in growing animals is consistently showing effect on modulation of body mass and fat, however, the effect in humans is not consistent. More research is needed to delineate the effect of CLA and isomers on body composition in humans. Major research emphasis, at present, is focused on the effects of CLA and its isomers on body composition and carcinogenesis. Other areas that are attracting attention include the effects of CLA and isomers on cardiovascular, metabolic, and immune functions and the strategies to increase the content of CLA isomers in meat and dairy products.

At the same time, research is still needed to improve the commercial production of CLA. Although the content of desirable isomers in commercial CLA products has improved, there is still a demand for highly enriched or pure 9,11-cis-, trans-octadecadienoic acid products. The kinetic control of CLA synthesis will allow the development of CLA products that are virtually free of isomers other than 9,11-ct and 10,12-tc. Kinetic control of reactions requires exceedingly rapid analytical techniques that can be applied inexpensively and online or virtually online.

REFERENCES

REFERENCES
