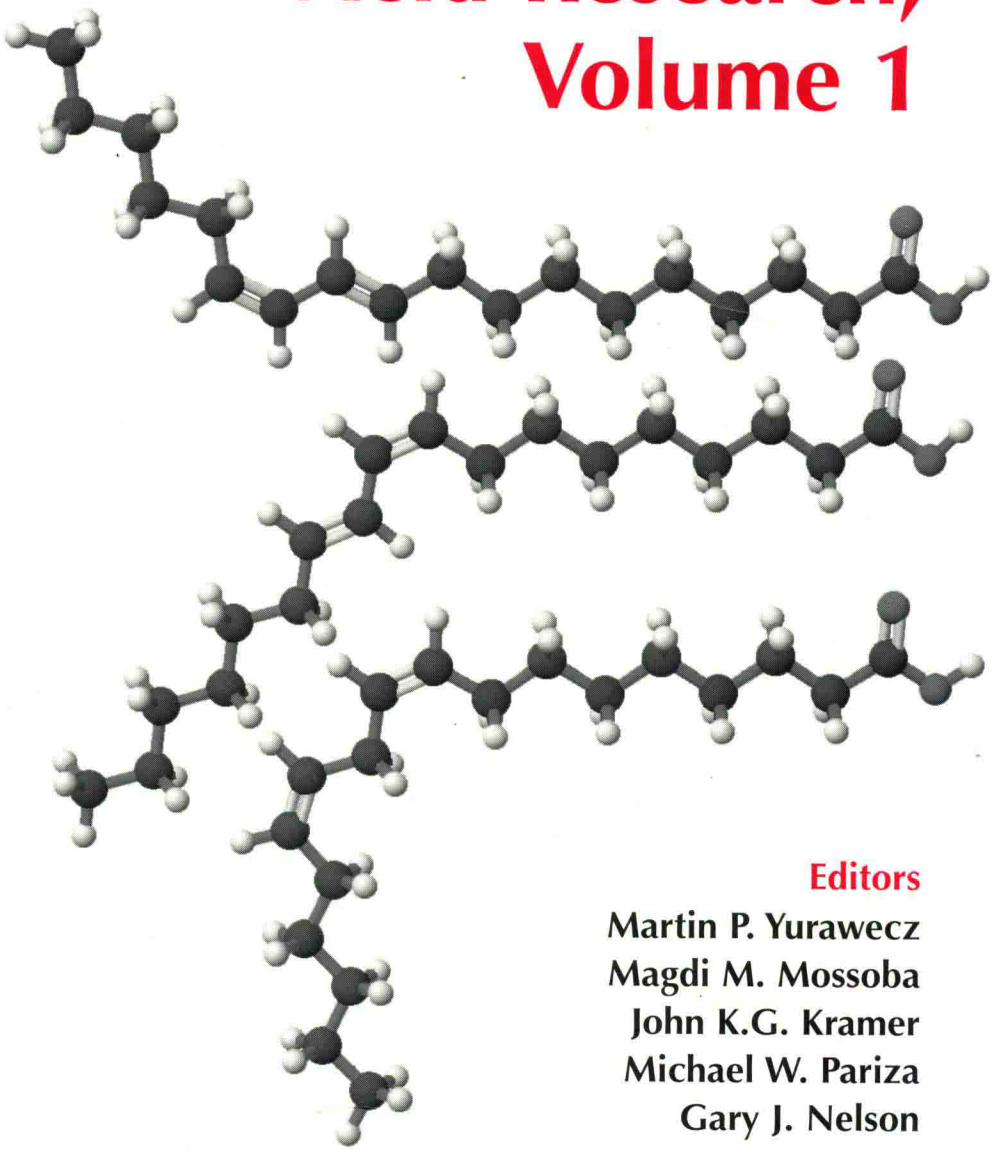
Advances in Conjugated Linoleic Acid Research, Volume 1



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Preface

This is the first book to be devoted solely to the subject of conjugated linoleic acid (CLA). It is the Editors' intent that it should document the state of knowledge about CLA as the 20th century draws to a close.

It has been known for more than 50 years that fatty acids with conjugated double bonds are present at varying levels in dairy products and other foods derived from ruminant animals. These conjugated fatty acids are common, though usually minor products of microbial lipid metabolism, which occur in the rumen of cattle, sheep, and other *Ruminantia*. Interest in conjugated fatty acids has increased substantially in recent years, following the observation that conjugated linoleic acid (CLA) is anti-carcinogenic in a number of rodent models. Since then, CLA has also been reported to induce a number of additional physiologic effects in a number of species, including the inhibition of atherosclerosis, enhancement of immunologic function, protection against the catabolic effects (cachexia) induced by immune stimulation, and nutrient repartitioning such that body fat is significantly reduced, whereas lean body mass is increased. These findings continue to drive CLA research.

As one should expect with any evolving scientific field, research to date on CLA has provided more questions than answers: How is it possible that CLA produces so many physiologic effects? What are the roles of the various CLA isomers in these effects? What are the precise biochemical mechanisms for these effects? What is the role of CLA metabolism, for example, elongation and desaturation of the various CLA isomers? Are there common mechanistic threads that tie these seemingly diverse effects together? Are the effects observed in animal models applicable to humans? How much of the various CLA isomers are in the diet? What are the best ways to analyze for CLA in foods/feeds and biological materials? These are some of the questions that are addressed (but not necessarily answered) in this book.

The nomenclature for the title compounds, "conjugated linoleic acid" and "CLA," varies from chapter to chapter. Sometimes the *cis*-9,*trans*-11 CLA isomer is referred to as "rumenic acid" or "RA." Conjugated linoleic acid is sometimes included in a larger class called "conjugated fatty acids" or "CFA." The term "octadecadienoates" is also sometimes used synonymously with CLA. The editors have not attempted to unify the nomenclature, preferring to leave this until consensus is achieved on the physiologic importance of the various CLA isomers.

Although this book is an up-to-date report of work that is still in progress, it is also clear that substantial progress has been made in some areas of CLA research. This is particularly so in the development of analytical methodologies. The reader is encouraged to utilize the newest methods as reported in this book, rather than older methods reported in the earlier literature.

At least 48 new peer-reviewed papers on CLA were published in 1998. If the reader wishes to keep track of CLA progress, an updated listing can be found on the internet at http://www.wisc.edu/fri/clarefs.htm.

Finally, the editors thank each of the contributors for their outstanding contribution, for their insight into the latest research findings, and the vision they have provided to make this book a truly valuable addition to the CLA literature.

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Contents

Chapter 1

Conjugated Linoleic Acid: The Early Years

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Introduction

During the early 1980s, Michael Pariza and his colleagues at the University of Wisconsin found that an isolate from grilled minced beef could inhibit carcinogenesis. The anticarcinogenic isolate was shown to consist of isomers of conjugated octadecadienoic acid in which the constituent double bonds are separated by a single carbon-to-carbon bond instead of a methylene group (1). The isomers were referred to collectively as conjugated linoleic acid (2) for which the acronym CLA is now used.

Since that time, an ever increasing number of studies using synthetically prepared CLA have shown that it can suppress cancer development at a number of sites in animal models and inhibit the growth of a large selection of human cancer cell lines *in vitro*. In addition, CLA possesses antiatherogenic, growth-promoting, lean body mass–enhancing properties; it can modulate food allergic reactions and normalize impaired glucose tolerance in noninsulin-dependent diabetes. These topics will be discussed in the following chapters. This chapter outlines the history of CLA detection in biological specimens and the determination of its structure.

Discovery of CLA in Milk Fat

In 1932, scientists from the National Institute for Research in Dairying at the University of Reading, collaborating with the Dunn Nutritional Laboratory, University of Cambridge, commenced a study of the seasonal variation in the vitamin content of milk. Vitamin A was estimated colorimetrically by measuring the intense blue color produced on treatment with antimony trichloride in chloroform solution—the Carr-Price reaction. However, they found that butterfat inhibited the blue color of the Carr-Price reaction. Further, it was established that inhibition was greater in summer butter, when cows were fed fresh pasture, than in the winter butter of stall-fed cows (3). In an associated study, this group noted that free fatty acids prepared from summer butter also differed from those prepared from winter butter in exhibiting much stronger spectrophotometric absorption at 230 μm as well as a much more rapid brown color formation when treated with antimony trichloride reagent (4).

Booth *et al.* (5) next reported that fatty acids obtained from butter fat by short-time saponification had definite absorption at 230 μm. On the other hand, the major unsaturated components of butter fat, oleic, and linoleic acids, treated in a similar manner, did not exhibit absorption at 230 μm. Another arm of this study showed that when the cow is turned out to pasture after winter, the fatty acids of milk fat showed

a greatly increased absorption at 230 μm. Values were more than doubled during summer months when the cows had access to pasture. Although the nature of the fatty acid responsible for absorption at 230 μm was unknown at this time, this is the first study to record the seasonal variation in CLA content of milk fat.

In the same year, this group prepared mixed fatty acids from cod-liver oil, sardine oil, rapeseed oil, and linseed oil and showed that they exhibited little absorption at 230 μm. However, when the oils were fed to cows, the secreted mixed acids of milk fat possessed much greater absorption at 230 μm (6). This is the first study to demonstrate that the CLA content of milk fat could be increased by dietary manipulation.

What Causes Absorption at 230 μm?

Studies by Gillam *et al.* (7) and Edisbury *et al.* (8) showed that, although a selection of triglycerides of animal or vegetable origin lacked absorption in the ultraviolet region, the mixed acids derived from them by prolonged saponification often showed intense and highly selective absorption. Knowledge was extended when Dann and Moore (9) and Dann *et al.* (6) found that saponification of cod-liver oil was complete within a few minutes, and the resulting acids exhibited little selective absorption. However, prolonged heating with alcoholic potash greatly enhanced absorption at a number of wavelengths. Oxidation was ruled out as a cause of increased absorption. It was postulated that a molecular rearrangement occurs, probably with acids having two or more double bonds, with the production of an isomeric form.

On the other hand, Edisbury *et al.* (10) considered the most plausible explanation for the spectroscopic phenomenon to be cyclization to form polycyclic hydroaromatic compounds. Moore (11) then noted that the prolonged saponification of linseed oil produced two acids, one with intense absorption at 230 μm and the other, a solid, with an absorption maximum at 270 μm. This latter acid appeared similar to elaeostearic acid from tung oil. At this time, the absorption at 270 μm for elaeostearic acid (*cis*-9,*trans*-11,*trans*-13-octadecatrienoic acid) was considered to be due to the presence of three conjugated double bonds. Next, Moore (12) hydrogenated a sample of tung oil. This resulted in a change in the absorption maximum of the mixed acids from 270 to 230 μm. Moore (12) then concluded that absorption at 230 μm was the result of two conjugated double bonds.

Spectrophotometric Determination of Polyunsaturated Fatty Acids

It was now known that the wavelength of the absorption maxima depends on the number of double bonds present in the acids, and the intensity is proportional to the amount of the acid present. Thus alkali isomerization of polyunsaturated fatty acids, which lack absorption bands, to conjugated forms that have specific absorption bands allowed the detection and quantitative measurement of dienoic, trienoic, tetraenoic, pentaenoic, and hexaenoic acids as classes.

During the 1940s and early 1950s, a number of spectrophotometric methods, involving alkaline isomerism and the use of empirically determined constants, were

developed for the determination of polyunsaturated fatty acids. These methods, which were performed rapidly and more accurately than precious techniques, such as ester fractionation analysis, are reviewed by Holman (13).

Polyunsaturated Fatty Acids of Milk Fat

Because of the nutritional significance of polyunsaturated fatty acids and their importance in the oxidation of milk fat, dairy scientists took advantage of the new spectrophotometric techniques to determine their composition. Riel (14) reviewed studies conducted in various countries up until 1963, which can be considered the period before gas–liquid chromatography (GLC). The review details the composition of monounsaturated, conjugated dienoic, trienoic, and tetraenoic fatty acids, together with nonconjugated polyunsaturated fatty acids with 2–6 double bonds.

Table 1.1 lists the range of conjugated dienoic acids for the major studies. The highest values occurred during the summer period. With the exception of the New Zealand study, the other studies were conducted in the Northern hemisphere where cows are stall-fed during winter. In these countries there was a two- to threefold increase in conjugated diene content when cows were turned out to fresh pasture.

Structure of the Conjugated Acid

Determination of Chain Length. Studies of milk fat fractions by Hilditch and Jasperson (23,24) and Mattsson (15) suggested that the conjugated unsaturation was associated with the C_{18} polyunsaturated fatty acids.

Infrared Absorption. Jackson *et al.* (25) examined the infrared spectra of conjugated isomers of linoleic acid. They found that *trans,trans*-conjugated linoleate was characterized by a strong absorption band at 988 cm^{-1}, whereas *cis,trans* (*trans,cis*)-conjugated linoleate was distinguished by a doublet at 948 and 982 cm^{-1}. No characteristic absorption was found for the *cis,cis*-conjugated isomer.

TABLE 1.1 Spectrophotometric Determination of Conjugated Diene Fatty Acids in Milk Fat

Country	Conjugated diene Range (wt%)	Reference
Sweden	0.6–3.7	15
New Zealand	0.7–1.4	16
Germany	0.5–2.3	17
U.S.	0.7–1.1	18
Holland	0.6–2.8	19
Canada	0.5–1.8	20
Sweden	0.4–1.6	21
France	0.4–1.9	22
Canada	0.2–2.0	14

Utilizing this infrared information, Lloyd Smith and colleagues at the University of California found that the C_{18}–C_{20} polyunsaturated fatty acid fraction of milk fat had strong infrared absorption bands at 948 and 982 cm^{-1}. This suggested *cis,trans-* or *trans,cis*-conjugated unsaturation. However, their spectra did not preclude the presence of small amounts of *trans,trans*-isomers (26). Conjugated *trans,trans*-diene was later detected in a concentrated unsaturated ester fraction of milk fat, further separated by urea-adduct stepwise crystallization with the use of ultraviolet and infrared spectroscopy (27). By using differential infrared spectroscopy Bartlet and Chapman (28) found that conjugated and isolated *trans* unsaturation were present in a constant ratio in milk fat. They used this characteristic as a basis for determining adulteration of milk fat.

Gas–Liquid Chromatography Studies

With the advent of GLC, this technique was soon utilized to determine the fatty acid composition of milk fat. Because milk fat contains >400 different fatty acids, it presents a complex GLC elution pattern. Magidman *et al.* (29) subjected methyl esters of milk fat fatty acids to distillation and silicic acid column chromatography to obtain fractions of less complexity for GLC analysis. With the aid of ultraviolet and infrared spectroscopy they were able to detect peaks representing octadecadienoic acids with *cis,trans-* (18:2 *ct*-conj.) or *trans,cis-* and *trans-trans*-conjugated unsaturation. The presence of the conjugated double bond system increased the retention time of the ester over that of a similar fatty acid with methylene interrupted double bonds. In a following report (30), the 18:2 *ct*-conj. and 18:2 *tt*-conj. acids were found to be present in a sample of milk fat at the 0.63 and 0.09% level, respectively.

Thin-Layer Chromatography Studies

The recently developed technique of silver ion absorption thin-layer chromatography (TLC) was used by Kuzdzal-Savoie (31) to separate the methyl esters of milk fat by the number and geometry of their double bonds. A band eluting between the *trans*-monounsaturated and *cis-* monounsaturated acids was thought to consist of conjugated dienes. A later study by Kuzdzal-Savoie *et al.* (32) used a combination of GLC, ultraviolet, and infrared spectroscopy, together with mass spectrometry to identify the presumed conjugated diene band as containing 18:2 *ct-* conj. or 18:2 *tc*-conj.

It is interesting to note that during silver ion absorption TLC, conjugated diene esters migrate with *cis*-monoenes when the common solvent system hexane:diethyl ether is used for development. However, when benzene or toluene is used as the solvent, they elute between the *cis*-monoenes and *trans*-monoenes (33,34). During studies with milk fat conjugated dienes, this author noted that they charred a brown color on silver nitrate–impregnated TLC plates after heating with sulfuric acid. On the other hand, other unsaturated acids produced a black color.

The Conjugated Fatty Acid Is cis-9, trans-11-Octadecadienoic Acid

Parodi (34) fractionated the methyl esters of milk fat by preparative GLC using a polyester phase. The effluent represented by the peak containing 18:2*ct* (*tc*)-conj. was collected and separated from co-eluting 18:3 and 20:1 by preparative silver ion absorption TLC. The band representing 18:2*ct* (*tc*)-conj. had a strong ultraviolet absorption maximum at 233 μm, indicating conjugated unsaturation. The infrared spectrum showed strong absorption at 949 and 982 cm^{-1}, designating conjugated, *cis,trans*- or *trans,cis*-unsaturation. GLC equivalent chainlengths (ECL) from both polar and nonpolar columns suggested that the isolated band was mainly *cis,trans*- or *trans,cis*-octadecadienoic acid.

The conjugated diene was subjected to reductive ozonolysis with the result that 90% of the cleavage products were represented by a C-7 aldehyde and a C-9 aldehyde-ester. This indicated that the original double bonds were at positions 9 and 11. Stereochemistry of the double bonds was determined by partial hydrazine reduction of the conjugated *cis,trans*-(*trans,cis*)-octadecadienoic acid. The resulting *cis*- and *trans*-monoene fractions were separated by preparative silver ion absorption TLC, then subjected to reductive ozonolysis. Cleavage products of the *cis*-monoene fraction consisted almost entirely of C-9 aldehyde and C-9 aldehyde-ester, indicating that the double bond at carbon 9 had the *cis*-configuration. The cleavage products of the *trans*-monoene fraction were mainly C-7 aldehyde and C-11 aldehyde-ester, showing that the double bond at carbon 11 had the *trans*-configuration. Thus, in milk fat, the conjugated dienoic acids are essentially *cis*-9,*trans*-11-octadecadienoic acid.

At various stages during the isolation and structural determination, small amounts of *trans,trans*-octadecadienoates were detected, and also octadecadienoates with double bonds at the 8,10- and 11,13-positions. Because of the labile nature of conjugated acids and the propensity of the *cis*-double bond to isomerize to the *trans*-configuration, it was considered that the minor components could have resulted from manipulative procedures.

cis-9, trans-11-Octadecadienoic Acid Acquires a Name

By reason of the importance now ascribed to synthetic CLA because of its many beneficial biological reactions, trivial names for the natural *cis*-9,*trans*-11-isomer have been suggested. McGuire *et al.* (35) proposed bovinic acid, but this name is considered too restrictive because the isomer is also produced in the rumen of a number of other species of commercial importance. For this reason, Parodi suggested rumenic acid, a name that is now gaining acceptance (36).

CLA in Milk Phospholipids

A study by Mattsson and Swartling (21) appears to be the first to measure conjugated fatty acids in milk phospholipids. They could not detect any absorption at 232 μm in phospholipids isolated from butter, collected during summer and winter, although the triglycerides from these butters contained 1% or more conjugated diene. The

authors reported a high background absorption in the region of 230 μm. This may have obscured any absorption due to conjugation. A year later, Smith and Jack (37) reported that mixed phospholipids extracted from buttermilk powder, a rich source of phospholipid, contained 2.3% conjugated diene. This value was nearly twice that for triglycerides from the same source. Knowledge of conjugated dienes in phospholipids was extended when Hay and Morrison (38) presented the composition of the fatty acids from the *sn*-1 and *sn*-2 positions of phosphatidyl choline and phosphatidyl ethanolamine, prepared from spray-dried buttermilk powder. They reported that phosphatidyl ethanolamine contained 0.9 and 0.7% conjugated *cis,trans*-diene and *trans,trans*-diene, respectively. Phosphatidyl choline contained 0.65 and 0.35% of these isomers. The two conjugated isomers were nearly equally distributed between the *sn*-1 and *sn*-2 position. This distribution was in contrast to the *cis,cis*-, *cis,trans*-, and *trans,trans*-nonconjugated octatadecadienoates that exhibited preference for the *sn*-2 position.

Of interest here is the study of Christie (33), which showed that bile phosphatidyl cholines from cows and sheep contained 1.1 and 4.7% of *cis*-9,*trans*-11-18:2, respectively. In both cases, the conjugated diene was esterified exclusively at the *sn*-2 position. The question arises, was the *trans,trans*-conjugated diene reported by Hay and Morrison (38) in milk phospholipids, and by other early investigators in milk fat triglycerides, factual or artifactual? Conjugated double bonds are very labile, even at room temperature, and the *cis*-double bond can isomerize readily to the *trans*-form (39). No doubt heating during pasteurization and the subsequent processing of dairy products can result in isomerism. In addition, it is realized now that the method employed to prepare methyl ester derivatives for GLC analysis is critical. Methods used during the early years of CLA investigation produced considerable amounts of the *trans,trans*-isomer (40,41).

The Origin of CLA

During the 1930s, biological chemists began to realize that the cow could convert dietary nonconjugated fatty acids to a conjugated component. However, the mechanism for this transformation and the location at which it was effected were unknown. By 1950, it was established that there was a species-to-species difference in the unsaturated fatty acid content of body fat from pasture-fed animals. Linolenic acid is the predominant pasture fatty acid. When ruminant animals such as cows and sheep consume this acid, only trace amounts appear in body tissues or milk. On the other hand, the horse, a nonruminant, transfers a considerable proportion of dietary linolenate to its depot fat. Shorland (42) designated such fats as "heterolipoid" and "homolipoid," respectively.

Reiser (43) incubated rumen contents with an emulsion of linseed oil. This process reduced the linolenic acid content of the mixture with a corresponding increase in the linoleic acid content; the other acids remained unchanged. Later, Shorland *et al.* (44) showed that the hydrogenation process was more extensive than suggested by Reiser (43). When linolenic acid was incubated with sheep rumen contents, the process produced monoene as well as diene fatty acids and introduced *trans*-unsaturation.

The presence of conjugated diene was also established. Shorland *et al.* (45) next extended their study to include oleic and linoleic acid. In all cases, stearic acid was produced as well as *trans* fatty acids. A considerable quantity of conjugated diene was produced but only when linloeic acid was used as the substrate.

Kepler *et al.* (46) later showed that the conjugated diene was *cis*-9,*trans*-11-(or *trans*- 9,*cis*-11-, or both)octadecadienoic acid, and was produced as a stable first intermediate when the common rumen bacterium *Butryivibrio fibrisolvens* was incubated with linoleic acid. In a second step, the conjugated diene was hydrogenated to a mixture of *trans*-11-18:1 and *trans*-9-18:1. The enzyme responsible for the initial isomerism of linoleic acid to *cis*-9,*trans*-11-18:2 was isolated from *B. fibrisolvens* by Kepler and Tove (47) and identified as a linoleate *cis*-12,*trans*-11- isomerase.

CLA in the Depot Fat of Ruminants and Nonruminants

Observations by Reiser (43) and Shorland *et al.* (44) that rumen contents could hydrogenate polyunsaturated fatty acids to produce acids with *trans*-unsaturation provided a biological explanation for the presence of *trans*-fatty acids in the depot fat of sheep and oxen, noted as early as 1928 by Bertram (48). Because of its spectroscopic absorption at 230 μm, conjugated linoleic acid was used by Reiser (49) as a marker to monitor absorption and transfer of dietary fatty acids to the tissue lipids of rats.

In a study with pasture-fed horses, Shorland *et al.* (50) found that their tissue triglycerides and phospholipids contained conjugated diene. Next, this New Zealand group examined the *trans*-unsaturated fatty acid content of fat from a selection of ruminant and nonruminant animals (51). As expected, the depot fats from ruminants (Sambur, fallow deer, ox, and sheep) contained conjugated diene at around the 0.5% level. An unexpected finding was the presence of considerable conjugated diene in the body fat of the nonruminant marsupials, wallaby (3.5%), and quokka (2.9%). Depot fat *trans*-monounsaturated fatty acid content from these marsupials was also exceptionally high, 19 and 21%, respectively. The high conjugated diene and trans-monoene content is explained by their possession of a ruminant-like digestion. The stomach contents contain a rich microbial population similar to, but rather simpler than that of cows and sheep (52).

Hansen and Czochanska (53) tentatively identified *cis*-9,*trans*-11-18:2 in the depot fat of pasture-fed lambs on the basis of ECL from GLC analysis.

CLA in Vegetable Oils

A wide range of fatty acids with conjugated unsaturation occur in seed oils of various plant families (39). On the other hand, conjugated unsaturation is absent in fatty acids from the native form of common dietary vegetable oils. The small amount of CLA found in refined corn and peanut oil (54) no doubt results from heating, bleaching, and deodorization during the refining process (55).

Partial hydrogenation of vegetable oils containing linoleic acid results in the formation of isomers with conjugated unsaturation. Spectrophotometric methods were

used to measure this conjugated unsaturation in various vegetable oil–based products. In the U.S., commercial shortenings and processed soybean oil contained from 0.3 to 0.6% conjugated diene (56). Different types of margarines had a range of 0.4 to 1.9% (57) and 0.6 to 0.8% (58). The conjugated diene content of Australian shortening ranged from 0.2 to 0.6%, whereas the level in various types of margarine was between 0.3 and 0.8% (59).

During this period, there appeared to be little interest in detecting the conjugated dienes by GLC or determining their structure. Currently the world-wide trend is to produce zero-*trans* margarines and shortenings. This will also result in the absence of CLA from these products.

The End of the Beginning

Imagine the biological chemists of the 1930s era being alive today. They would be astonished at the evolving literature, available analytical techniques, and the animal studies that have shown such a wide range of advantageous physiologic events, for the compound they struggled to isolate and identify. May CLA research continue to prosper.

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