Three-, Six- and Nine-Carbon Ozonolysis Products from Cottonseed Oil and Crude *Chlorella* Lipids

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ABSTRACT

Two naturally occurring lipids were subjected to ozonolysis. Isolated cottonseed oil and crude, mixed lipid extracts of the alga *Chlorella* yielded three-, six-, and nine-carbon compounds. Both mono- and difunctional acids and aldehydes were quantifiable. Monofunctional C9 compounds occur in higher than expected yields, apparently at the expense of difunctional C3 and C9 compounds. Yields of the compounds of interest varied from ca. 35 to 150%, depending on exact conditions. The possibility of utilizing ozonolysis of natural lipids for the production of reactive organic chemicals helps justify further research in algal lipid production.

INTRODUCTION

Algal biomass has been considered a possible source of chemicals and feeds (1) for a number of years. Likewise, the concept of waste stream treatment with algal biomass and subsequent digestion of the algal substance to produce methane fuels was proposed long ago (2). Various analytical and applied research results were collated and edited in 1953 by John Burlew (3). The data reported pertain to detailed chemical analyses of biomass, general physiology, and productivity in algal cultures. An increasing world problem with respect to energy sources has led to a revitalized interest in the use of biomass as a source of fuels (4), or source of energy-rich chemicals (5,6).

Reports of triglyceride accumulation, over 50% dry weight, in algae (7), focus thoughts on the concept of large-scale production of green, photosynthetic biomass high in this energy-rich chemical. Furthermore, production costs may be offset by environmental gains if the algal culturing process can reclaim waste streams (8). Research has justifiably begun in this area (9).

One problem area of current research involves the induction of lipid accumulation in algae. In general, the controls over triglyceride levels in eukaryotic cells including algae are unknown (10). To justify further study of algal accumulation of triglycerides, or other lipids, data as to potential uses for algal lipids are needed.

In attempting to establish any possible use for algal neutral lipids, previously developed technology should be considered. Chemical oxidation, including the process of ozonolysis, has historically been used in processing unsaturated lipid substances (11). Ozonolysis specifically has been employed in the successful production of commercial organic acids (12). Results of a laboratory study of the ozonolytic products from crude total lipid extracts of *Chlorella* sp. (University of Texas #2168) and cottonseed oil are reported here.

MATERIALS AND METHODS

Culture and Harvest of Algae

Chlorella sp. (Utex 2168) was obtained from the Culture Collection of Algae at the University of Texas at Austin, Texas. Three culture flasks, each containing 50 mL of Spoehr and Milner C2 medium (7), were sterilized and inoculated with Utex 2168. These three inocula grew to a bright green, dense culture within 3 days under continuous fluorescent illumination at 26-28 C, with shaking. They were each used in their entirety to initiate three respective 5-8L cultures in large spinner flasks containing the same C2 medium. The large flasks were stirred, gassed with 1-5 % CO_2 in air at a rate of 100-200 mL/min, and held at 26-28 C, again under continuous fluorescent lighting. After two weeks of growth, the dark green biomass was harvested by centrifugation and washed once with deionized water. The cell pellet was lyophilized overnight to give thoroughly dry samples.

Extraction of Crude Algal Lipids

Freeze-dried *Chlorella* samples of various sizes were extracted with 2:1 chloroform/methanol (13). Each sample was suspended in three separate volumes of solvent corresponding to 20-50 times the sample weight (weight to volume). The cell debris was removed by centrifugation. The resulting extracts were washed with 2N KCl, and reduced in volume by evaporation.

Fatty Acid Analysis

The fatty acids in crude lipid extracts and cottonseed oil were analyzed as methyl esters. Lipid samples were transmethylated in 2.5 % HCl in anhydrous methanol (14) and quantitatively analyzed by gas chromatography on a 6-ft SP 2330 column. Octanol was added as an internal standard, and the column oven temperature was programmed from 130 to 225 C. Standard calculations were performed using the automatic processing available on the Hewlett Packard Model 5840A gas chromatograph.

Ozonolyses

Utilizing hydrogen and Lindlar catalyst. Ozone was passed through a solution of natural oil at a rate of 2.25 g/hr generated from air with an Ozone Research and Equipment Corporation, Phoenix, AZ, ozonator model 03V5-0. Ethyl acetate and methanol, 160 and 40 mL, respectively, were used as a solvent mixture in ozonating cottonseed oil, and 25 % chloroform in methanol was used for *Chlorella* lipids. Each reaction was carried out at 0 C, and taken to completion as indicated by a KI trap for excess O_3 in the effluent gas. Starch solution was used as a visual indicator. The treatment was continued for 30 min beyond completion to ensure oxidation of unsaturated bonds in the lipids. Total ozonation times were between 90 and 120 min.

Lindlar catalyst was added to the reaction vessel, and hydrogen reduction, filtration, and methylation of each sample was performed in a manner similar to that of Fitton, et al. (15).

Utilizing methanolic boron trifluoride. Small samples of lipids or cottonseed oil were dissolved in 1.5 mL methanol and exposed to a stream of ozone in conical test tubes. After 8-10 min of exposure, during which ozone was generated at a rate of ca. 0.8 g/hr, some solvent had evaporated, so the volume was adjusted to 1 mL with methanol. One quarter of a milliliter of 14 % BF_3 in methanol was added and methylation was performed according to Sebidio and

TABLE I

Relative Fatty Acid Composition for Cottonseed Oil and Crude Lipid Extracts of *Chlorella* sp. (average of three analyses)

Fatty acid	Mole percent	
	Cottonseed oil	*Chlorella* lipids
14:0	0.6	–
16:0	15.9	39.8
18:0	1.6	2.2
18:1	15.3	23.7
18:2	65.6	12.2
18:3	1.1	22.1

Ackman (16). The tubes were tightly capped and held in a temperature block at 100 ± 5 C for 90 min. One mL chloroform and 2 mL of water were added to each tube, and the tubes were shaken. The chloroform extract was removed to another tube and the water/methanol phase was extracted again with one mL of chloroform. The pooled chloroform extracts (2 mL) containing methylated ozonolysis products were washed once with water and transferred to a third dry tube.

Analysis of Methylated Ozonolysis Products

Quantitative gas chromatography. The ozonolytic processes employed yielded methylated products which were analyzed by gas chromatography. An OV 101 column was used in a Hewlett Packard Model 5840A gas chromatograph temperature-programmed to run from 60 to 200 C. An internal standard of methyl tridecanoate was added to each sample.

Mass spectral confirmation of analyses. The ozonolysis products quantitatively detected by gas chromatography were confirmed using gas chromatography-mass spectral analysis (GC-MS). A Hewlett-Packard 5985 system was employed. Masses in the range 25-400 were scanned; the system was capable of reconstructing a GC trace from total ion counts. A column differing from the OV 101 used in quantitative analysis, either a 15-m fused quartz SP 2100 capillary or 6-ft SP 2330 packed column, was installed in the chromatograph oven. Temperature ramps went from 60 to 240 C at 6 C/min, and 60 to 200 C at 4 C/min, respectively.

Chemicals and Standards

Dimethyl acetal of methyl azelaaldehydate was prepared as one of the products from methyl oleate ozonolysis (17-19). Its mass was determined gravimetrically, and the gas chromatographic response factor was then calculated. All other chemicals and standards were purchased from the Aldrich Chemical Company, Milwaukee, WI, the Sigma Chemical Company, Saint Louis, MO, or Supelco Inc., Bellefonte, PA. Solvents and reagents were obtained from the J.T. Baker Company of Phillipsburg, NJ.

RESULTS

Cottonseed Oil

The products expected in the ozonolytic reaction of cottonseed oil are determined by the unsaturated compounds found in the oil. Gas chromatographic analysis of the cottonseed oil utilized showed linoleic (C18:2) acid to be greatly dominant over all other fatty acids present (Table I). One saturated fatty acid, palmitic acid (C16:0), was found in a noticeable percentage. Overall, the ratio of unsaturated to saturated fatty acid residues was ca. 6:1. Therefore, a strong ozone reaction occurred with cottonseed oil, yielding a pale yellow product mixture. The cleavage products of the acyl acid chains were mainly C3, C6 and C9 compounds.

A complex pattern of products, including both aldehydes and acids, emerged when catalytic hydrogenation was used (Table II). Quantifiable amounts of both compound types were found for C6 and C9 species, but only the tetramethyl acetal of malonaldehyde was recovered. The theoretical yields were near 35 % for difunctional C9, difunctional C3, and monofunctional C6 compounds. The yield for monofunctional C9 compounds was consistently greater than 100 %. A little dimethyl acetal of methyl malonaldehydate was detectable in the mass spectral scans of hydrogen-reduced samples, but absolute amounts were not measurable. No dimethyl malonate appeared. A quantitative recovery of methyl palmitate showed that transmethylation of the triglyceride linkages was complete (see Fig. 1).

The presence of boron trifluoride in the ozonolysis mixture caused the formation of only organic acid methyl esters as expected (20). It should be noted that preliminary experiments in which the warming period was shortened to 60 min yielded a product mixture similar to that observed for hydrogenation. Table III lists the amounts recovered for

TABLE II

Methylated, Hydrogen-Reduced Ozonolysis Products of Cottonseed Oil (average of three trials)

Compound type	Compound	Amount		% Yield
		Detected (m mol)	Expected (m mol)	
C6 monofunctional	DMC	2.8	–	–
	MC	1.3	–	–
	Total	4.1	12.0	34.2
C3 difunctional	TMM	4.2	12.6	33.3
C9 monofunctional	DMN	2.5	–	–
	MN	1.5	–	–
	Total	4.0	2.7	148.1
C9 difunctional	DMMA	2.3	–	
	DMA	3.1	–	
	Total	5.4	14.9	36.2

DMC = Dimethyl acetal of caproaldehyde; MC = methyl caproate; TMM = tetramethyl acetal of malonaldehyde; DMN = dimethyl acetal of nonaldehyde; MN = methyl nonanoate; DMMA = dimethyl acetal of methyl azelaaldehydate; DMA = dimethyl azelate.

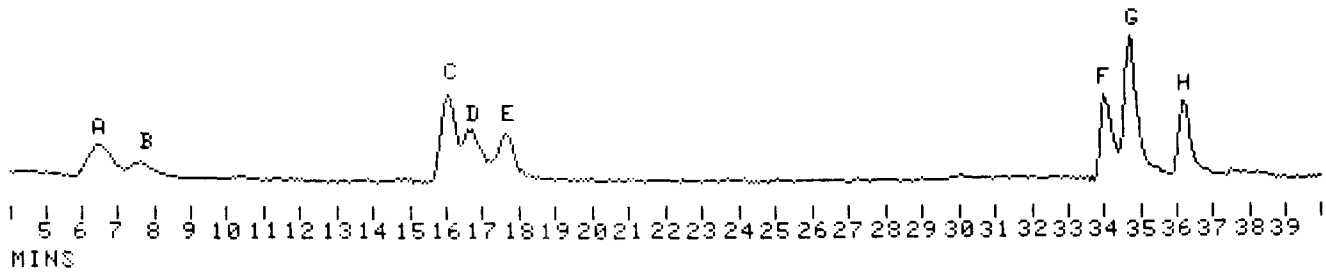


FIG. 1. GC of methylated, hydrogen-reduced ozonolysis products of cottonseed oil, reconstructed from GC-MS data (6-ft SP 2330 column). (A) Dimethyl acetal of caproaldehyde; (B) methyl caproate; (C) tetramethyl acetal of malonaldehyde; (D) dimethyl acetal of nonaldehyde; (E) methyl nonanoate; (F) methyl palmitate; (G) dimethyl acetal of methyl azelaaldehydate; (H) dimethyl azelate.

TABLE III

Products of Cottonseed Oil Ozonolysis in the Presence of BF_3 (average of three trials)

Compound type	Compound	Amount Detected (m mol)	Amount Expected (m mol)	% Yield
C6 monofunctional	MC	1.96×10^{-2}	2.95×10^{-2}	66.2
C9 monofunctional	MN	7.91×10^{-3}	6.99×10^{-3}	113.2
C9 difunctional	DMA	3.60×10^{-2}	3.70×10^{-2}	97.2

MC = Methyl caproate; MN = methyl nonanoate; DMA = dimethyl azelate.

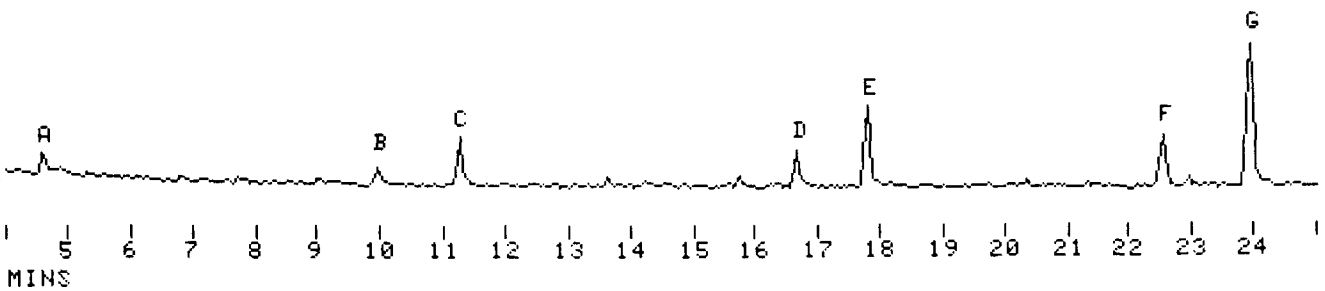


FIG. 2. GC of methylated, hydrogen-reduced ozonolysis products of crude *Chlorella* lipids, reconstructed from GC-MS data (15-M SP 2100 capillary column). (A) Tetramethyl acetal of malonaldehyde; (B) methyl nonanoate; (C) dimethyl acetal of nonaldehyde; (D) dimethyl azelate; (F) dimethyl acetal of methyl azelaaldehydate; (F) unknown; (G) methyl palmitate.

TABLE IV

Methylated, Hydrogen-Reduced Ozonolysis Products of Crude *Chlorella* Lipids (average of three trials)

Compound type	Compound	Amount Detected (m mol)	Amount Expected (m mol)	% Yield
C9 monofunctional	DMN	0.097		–
	MN	0.033		–
	Total	0.130	0.119	109.2
C9 difunctional	DMMA	0.068		–
	DMA	0.090		–
	Total	0.158	0.302	52.3

DMN = Dimethyl acetal of nonaldehyde; MN = methyl nonanoate; DMMA = dimethyl acetal of methyl azelaaldehydate; DMA = dimethyl azelate.

methyl caproate, methyl nonanoate, and dimethyl azelate. The percent yields were significantly improved in using BF_3; methyl caproate was recovered at ca. 66 % of the theoretical yield and C9 compounds were found at ca. 100 % of the theoretical yield. The methyl nonanoate yield is once again greater than 100%, but the problem is not as marked as in the case of H_2 reduction. C3 compounds are not detectable, probably due to their solubility in water/methanol solutions (16). Also the mass spectral peak and quantitative recovery of palmitate shows successful cleavage of the triglycerides. No anomalous C8, C5, or C6 compounds were produced in detectable amounts.

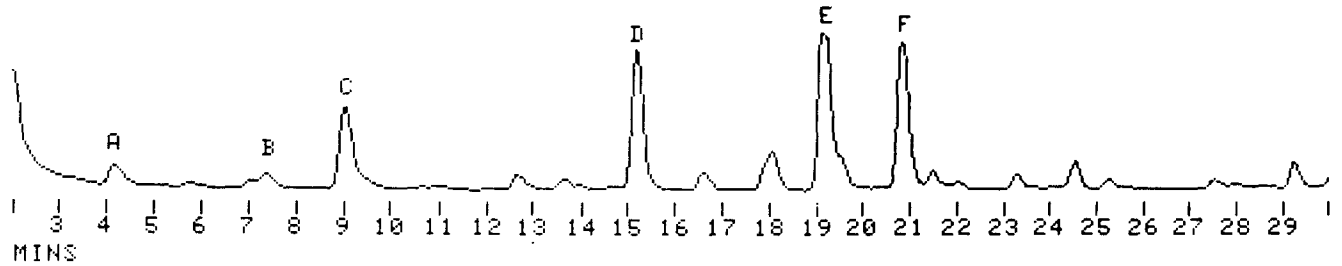


FIG. 3. GC of ozonolysis products of crude *Chlorella* lipids in the presence of BF_3, reconstructed from GC-MS data (6-ft SP 2330 column). (A) Methyl caproate; (B) methyl octanoate; (C) methyl nonanoate; (D) methyl tridecanoate (added standard); (E) methyl palmitate; (F) dimethyl azelate; other peaks unknown.

TABLE V

Products of Crude *Chlorella* Lipid Ozonolysis in the Presence of BF_3 (average of 3 trials)

Compound type	Compound	Amount Detected (m mol)	Amount Expected (m mol)	% Yield
C6 monofunctional	MC	2.40×10^{-3}	2.32×10^{-3}	103.5
C9 monofunctional	DMN	0.11×10^{-3}	–	–
	MN	4.74×10^{-3}	–	–
	Total	4.85×10^{-3}	4.52×10^{-3}	107.3
C9 difunctional	DMMA	0.07×10^{-3}	–	–
	DMA	10.43×10^{-3}	–	–
	Total	10.50×10^{-3}	11.45×10^{-3}	91.7

MC = methyl caproate; DMN = dimethyl acetal of methyl nonaldehyde; MN = methyl nonanoate; DMMA = dimethyl acetal of methyl azelaaldehydate; DMA = dimethyl azelate.

Crude Algal Lipids

The high percentages of C18:1, C18:2, and C18:3 fatty acids (Table I) in the total lipids of *Chlorella* sp. comprise more than half of the total present. Palmitic acid is also present in large percentages. Thus the predicted ozonolytic reaction products are C9 difunctional, C9 monofunctional, C6 monofunctional and C3 difunctional compounds. The azelate and malonate analogs should predominate.

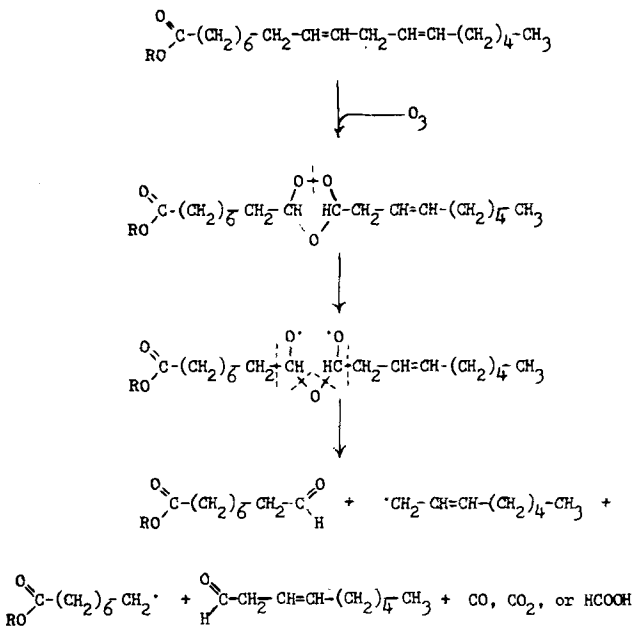
The pattern for results with crude algal lipids as reactants was similar to that where cottonseed oil was the starting material. Hydrogenation procedures yielded a mixture of esters and acetals (Fig. 2 and Table IV). Ozone attack in methanolic BF_3 produced mainly the methyl esters predicted (Fig. 3 and Table V). Some tetramethylacetal of malonaldehyde is identifiable, but is not measurable, in hydrogenated samples; none is seen when boron trifluoride is employed. In contrast, only the BF_3 samples show any C6 compounds, but also show a minor amount of methyl octanoate. In both treatment procedures, minor products formed which were not identified. The mass spectral data associated with Figures 2 and 3 indicate these to have been mostly complex carbonyl or carboxyl compounds.

The theoretical yields were near 100 % for all recoverable reaction products except for dimethyl azelate in catalytically reduced samples (Table V). Also, the yields for methyl nonanoate were still consistently above unity, and those for dimethyl azelate consistently below.

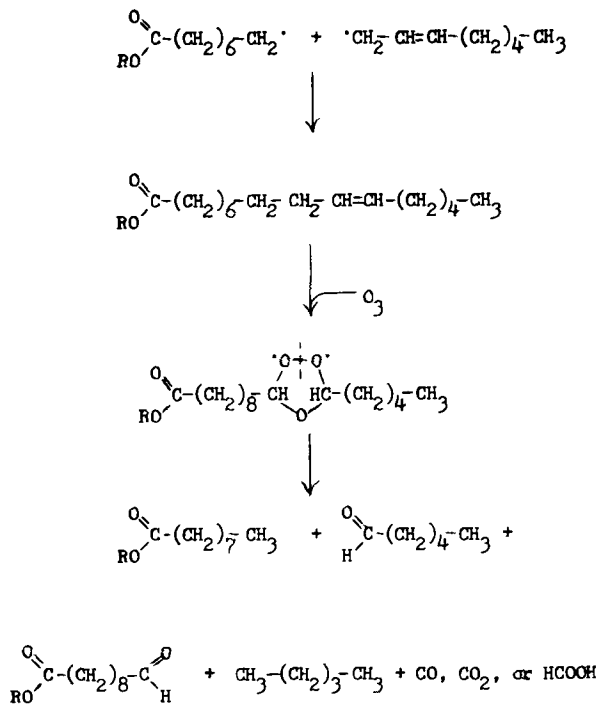
DISCUSSION

The reaction products of the most interest in the ozonolysis of vegetable oils are three-, six-, and nine-carbon compounds. Difunctional azelaic and monofunctional pelargonic acids have long been of industrial interest in lubrication and polymer chemistry (11), and may find new applications in the same areas (21). Malonaldehydes and azelaaldehydates are of some potential interest in thin-film technology (22,23). In this respect, the yields reported for C9 compounds are reasonable when either catalytic reduction with hydrogen or methylation in the presence of BF_3 is used. The results are closer to theoretical (Tables III and V) for esters produced in BF_3 samples. However, the possibility of recovering at least some acetals of malonate analogs exists only in the process utilizing hydrogen (Table II). The yield of 33.9% for C3 compounds observed occurred almost exclusively as tetramethyl acetal of malonaldehyde. In all of the samples analyzed the low yield for both six-carbon and three-carbon compounds is explicable by their increased solubility in highly polar solvents including water, as well as their increased volatility (16).

The only stark anomaly in the data collected is the consistently elevated yield for C9 methyl esters. These vary from 107 % to 148 % of the theoretical yield. Since each sample was analyzed using at least two gas chromatographic columns, and mass spectral data are not contradictory, the results in excess of 100 % appear to be real. The result cannot be explained adequately without assuming radical production is occurring. Noticeable yields of unexpected compounds from ozonolyses have been explained or examined via radical mechanisms by numerous workers (24). Privett and Nickel (25) proposed a scheme for hydrocarbon production during ozonlyses which involved radical production in thermal ozonide decomposition. Scheme 1 is in accordance with their observations, and illustrates two possible radicals which could arise from a linoleate ester. The possibility of localized ozone shortages during initial stages of chemical reaction have been indicated (25), and the formation of the stable vinyl radical is therefore not impossible. The intermolecular combination of the vinyl and octanoate radicals is plausible (26) and would produce an ester of a hexadecenoic acid which could yield at least some nonanoic ester as shown in Scheme 2. Thus it is possible to explain



SCHEME I



SCHEME II

the excessive yields of nonanoate ester using radical mechanisms known to occur during ozone treatments, and especially in protic solvents, under peroxidative conditions (27), like the methanol used in this study. The probability of the above proposed schemes is supported in the data by two facts. The lowered C3 yields, even though solubilities must be remembered, could result from the one-carbon losses implicit in the radical mechanisms (23). Likewise, one of the small peaks in Figure 3 seems to be methyl octanoate, as expected from Schemes 1 and 2.

Other than the elevated nine-carbon ester yield, the yield data agree well with that formerly published for ozonolyses in methanolic boron trifluoride (16, 28), participating solvents (29), and ozonolyses using Lindlar's catalyst and hydrogen for reductive cleavage (15).

The several unidentified peaks present in reconstructed gas chromatographs for ozonlysis products of crude algal lipid are not surprising. The starting material was a complex green mixture. Mass spectra corresponding to most of these peaks contain either the 75 m/e ion or 74 m/e ion which are McLafferty rearrangement products (30) indicative, respectively, of acetals and esters. Unsaturations in the carbon skeletons of the several pigments known to be present in algae can easily account for numerous carbonyl and carboxylic products not predictable from fatty acid cleavages. Although the overall complexity, and undoubtedly yields in general, are affected by such extra products, the hoped-for C9 products were obtained through the ozonolysis of crude algal lipid extracts.

The cost of ozonation of either algal culture in situ or extracts thereof to produce chemicals derived from lipids has not been calculated, but the 1981 costs for ozonlytic production of C6 and C9 compounds from olive, palm, and soybean fatty acids have been estimated (23). The production of free acids, esters, or acetals through ozonolyses in participating solvents or ozonlyses with catalytic hydrogenation, appeared potentially competitive. If one considers the economic returns possible when linking chemical production to waste stream reclamation, continued study in the areas of algal aquaculture and lipid composition does seem justifiable.

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Determination of Ultratrace Metals in Hydrogenated Vegetable Oils and Fats

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ABSTRACT

Ultratrace levels of nickel, chromium, copper and iron occurring in hydrogenated vegetable oil products were estimated by dispersion of the samples in 4 methyl-2-pentanone and atomic absorption analysis by the graphite furnace technique. The principal goals in establishing the analytical methods were improved sensitivity to metals at low levels and applicability to limited amounts of products. Using reproducibility and linearity of response as criteria, optimum oil concentration in solvent and instrument parameters were established. For a series of commercial products, the method of standard additions was adopted to correct for matrix differences between the products and salad oil-based standards. The range for the metals was determined in five cooking oils: Ni, 29–207 ppb; Cr, 1–5 ppb; Cu, 13–37 ppb; and Fe, 138–301 ppb; in recovered oils from five margarines: Ni, 34–70 ppb; Cr, 2–12 ppb; Cu, 26–58 ppb; and Fe, 239–540 ppb; and in five solid shortenings: Ni, 592–2772 ppb; Cr, 8–35 ppb; Cu, 26–108 ppb.

INTRODUCTION

The transition metal content of edible oils has a significant impact on stability and shelf life of products (1). Salad-grade soybean oil has very low background levels of a variety of trace metals, but the potential exists for increased levels due to oil treatments, i.e., nickel from hydrogenation and copper, chromium and iron from processing equipment.

There have been few published reports on the transition metal content of edible vegetable oil products. Several studies (2–6) have reviewed the methods of measuring low levels of such metals and reported on developments in use of the graphite furnace atomic absorption spectrometer directly on the oil (2,6).

The present research has been directed further to assess graphite furnace atomic absorption spectrometry, to develop methods to obtain maximum instrument response, and to accurately determine several metals. A method requiring a minimum of sample quantity, handling and surface exposure was also desired. The developed method was used to determine the trace metal content of several commercial vegetable oil products.

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PROCEDURE AND METHODS

Apparatus

A Perkin-Elmer Corp. Model 372 Atomic Absorption instrument was equipped with a model 2200 graphite furnace and a deuterium lamp background corrector.

Product Selection and Sample Preparation

Two containers with the same lot number for each of five cooking oils, margarines and solid shortenings were obtained at local retail outlets (designated subsamples). Margarine oils were recovered by melting samples in acid-washed beakers on a hot plate, allowing phase separation at 95–98 C in an oven overnight, drying the decanted oil layer on a rotary evaporator at 90 C for 1 hr and, finally, decanting the clear oil from the top of the granular material at 70 C. Cooking oils and margarine oils, recovered as above, were dispersed in redistilled 4-methyl-2-pentanone (methyl isobutyl ketone, MIBK) at 50% w/v in acid-washed glassware. Shortenings were sampled in a semisolid state using a core sampler fabricated from a plastic disposable tuberculin syringe. The final dispersion was likewise 50% w/v in MIBK. These dispersions required warming at the time of analysis to achieve a homogenous solution.

Standard Solutions

Organometallics in the form of *tris*(1-phenyl-1,3-butandieno) chromium III, *bis*(1-phenyl-1,3-butandieno) copper II and *tris*(1-phenyl-1,3-butandieno) iron III were dissolved in small quantities of 2-ethyl hexanoic acid and xylene, and diluted to make stock solutions in MIBK. Nickel cyclohexanebutyrate was dissolved as above, but was dispersed directly in a salad oil stock solution. Using two or more portions of each stock, singular working standards, which were 50% w/v salad oil in MIBK, were prepared at metal concentrations slightly above and slightly below the expected range for each respective sample.

Evaluations

Three design criteria were considered for measuring metals in each commercial fat: (a) reproducibility between containers, (b) repeatibility among assays, and (c) uniformity of response at two levels of metal content. The standard