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ORIGINAL PAPER

An Efficient Methodology for the Preparation of Alkoxyglycerols Rich in Conjugated Linoleic Acid and Eicosapentaenoic Acid

Carlos F. Torres · Luis Vazquez · Francisco J. Señoráns · Guillermo Reglero

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Abstract Saponification of a raffinate obtained by supercritical fluid fractionation of shark liver oil was utilized for the production of an unsaponifiable fraction enriched in nonesterified alkoxyglycerols. Re-esterification of the alkoxyglycerols produced with conjugated linoleic acid (CLA) and eicosapentaenoic acid in the presence of *Candida antarctica* lipase B was then studied. High yields (>90%) of diesterified alkoxyglycerols were obtained via esterification and transesterification with CLA, their respective ethyl ester, and eicosapentaenoic ethyl ester. The effect of a continuous purge stream of nitrogen on the kinetics of the reactions was also evaluated.

Keywords Alkoxyglycerols · CLA · EPA · Lipase · Novozym 435 · Re-esterification · Supercritical fluid extraction

Introduction

Increased understanding of the nutritional values of lipids, in particular, the metabolic effects associated with consumption of lipids containing substantial levels of specific fatty acid residues, has led to the development of novel technologies for modifying fats and oils to enhance the health benefits resulting from ingestion of these substances [1].

Ether lipids or alkoxyglycerols have been the subject of much attention because of their special physiological functions in humans [2, 3]. Anti-carcinogenic and immune stimulatory properties have been attributed to dietary ingestion of these substances [4, 5]. They are located in the human body mainly in the cells of the immune system and in higher doses in human milk. In some circumstances, the endogenous synthesis is reduced and the oral administration of alkoxyglycerols is recommended.

Shark liver oil is a natural source of alkoxyglycerols among other lipid classes such as squalene, triacylglycerols, and cholesterol. The 1-*O*-alkyl-*sn*-glycerols are highly valuable compounds and can be prepared from the unsaponifiable fraction of shark liver oil. However, in order to isolate pure alkoxyglycerols, squalene needs to be removed. For this process, supercritical fluid technology has shown very interesting properties [6].

Conjugated linoleic acid (CLA) is a mixture of positional and geometric isomers of octadecadienoic acid with conjugated double bonds. Nutritional studies based on animal models have demonstrated a variety of beneficial health effects from dietary ingestion of CLA, including anticarcinogenic, antiatherogenic, antiobesity, and antidiabetic effects, as well as immune system enhancement [7, 8]. Conjugated linoleic acid (CLA) is a natural component of ruminant-derived food products. An intermediate in rumen biohydrogenation is *cis*-9, *trans*-11 CLA, the major CLA isomer in milk fat representing 80–90% of the total CLA. The total concentration of CLA in dairy products typically ranges from 3 to 7 mg/g of fat [9].

The various health effects of CLA may relate to specific CLA isomers, and this is an active area of research. However, it has been established that the *cis*-9, *trans*-11 isomer of CLA is an active anticarcinogenic agent when included in the diet as a natural component of food [10], and several biological functions in humans are under clinical study.

C. F. Torres · L. Vazquez · F. J. Señoráns · G. Reglero (✉)
Sección Departamental Ciencias de la Alimentación,
Facultad de Ciencias, Universidad Autónoma de Madrid,
28049 Cantoblanco, Madrid, Spain
e-mail: guillermo.reglero@uam.es

Factors affecting the CLA content of milk fat have been reviewed, and diet plays a major role in determining CLA levels in milk fat [11]. However, previous investigations have observed substantial variations in the CLA content of milk fat from individual cows fed the same diet [12, 13].

Research has demonstrated that long chain polyunsaturated fatty acids (PUFA) have specific physiological effects in humans [14]. Fish oil is an important natural source for PUFA, especially 5, 8, 11, 14, 17-eicosapentaenoic (EPA, C20:5) and 4, 7, 10, 13, 16, 19-docosahexaenoic (DHA, C22:6) acids. The numerous health-promoting effects of EPA and DHA in human subjects have been reviewed [15].

One logical approach to assure ingestion of appropriate levels of both PUFA and CLA is to produce fats and oils enriched in these substances via enzymatic processes. Enzyme catalyzed esterification [16], hydrolysis [17] transesterification [18], and acidolysis [19] reactions have all been employed to obtain selective enrichment of naturally occurring triacylglycerols in both PUFA and CLA.

The production of alkylglycerols enriched with EPA and DHA has been previously described [20] via transesterification under vacuum. The study reported by Haraldsson et al. shows an alternative methodology for the isolation of alkylglycerols and transesterification reaction from those reported in the present study. In addition, our research also includes the esterification and transesterification of alkylglycerols with CLA and CLAEE, respectively. Hence, the present study focuses on the lipase-catalyzed synthesis of alkoxyglycerols rich in CLA or EPA. A two-step methodology has been utilized. First, saponification of a raffinate obtained by supercritical fluid extraction of shark liver oil to obtain nonesterified alkoxyglycerols that can subsequently react with a new acyl donor. The second step consists of producing alkoxyglycerols that contains new fatty acid via esterification or transesterification reactions. The effect of a nitrogen purge stream and the type of acyl-donor on the kinetics of these reactions has been investigated.

Materials and Methods

Shark liver oil was obtained from Lysi (Reykjavik, Iceland) and Crystalfishing (La Coruña, Spain). CLA (purity of 90% w/w) and CLA ethyl ester (purity of 80% w/w) was a gift from Natural ASA (Sandvika, Norway), the eicosapentaenoic acid ethyl ester (EPAEE, Incromega E7010) was kindly donated by Croda (Yorkshire, England). The composition of this product was EPA 75% and DHA 11%. The balance of the product was comprised of other fatty acid ethyl esters (mainly C20:1 ca. 3%). The immobilized lipase B from *Candida antarctica* (Novozym 435) was a gift from Novozymes (Bagsvaerd Denmark).

All solvents used were HPLC grade from Lab-Scan (Dublin, Ireland).

Methods

Supercritical Fluid Extraction

Supercritical fluid extraction with countercurrent CO_2 in a pilot plant scale [21] was utilized to extract the squalene contained in the original shark liver oils. Different extraction conditions were explored in order to produce raffinates with negligible amounts of squalene (ca. 3% w/w). The best results were attained at the following conditions: temperature of the extraction column 65 °C, pressure of the column of 180 and 210 bar, with ratios of solvent to feed of 40 and 25, respectively.

Saponification

Five grams of the raffinate obtained as described above were mixed with 16 mL of a solution of potassium hydroxide 3.7 N in ethanol/water (50/50 v/v) with 0.15% p/v of EDTA. The mixture was heated to 60 °C during a 1 h reaction with mixing at 300 rpm. Then, the reaction was stopped by adding 4 mL of water and the unsaponifiable fraction was extracted from the aqueous phase with three 20-mL portions of diethyl ether. Then the ether phase was dried with sodium sulphate and evaporated under nitrogen to obtain the unsaponifiable residue.

HPLC Analyses

The analyses were effected on a Kromasil silica 60 column (250 mm by 4.6 mm, Análisis Vinicos, Tomelloso, Spain) coupled to a CTO 10A VP 2 oven, a LC-10AD VP pump, a gradient module FCV-10AL VP, a DGU-14A degasser, and a evaporative light scattering detector ELSD-LT from Shimadzu (IZASA, Spain). The ELSD conditions were 2.2 bar, 35 °C, and gain 3. The flow rate was 2 mL/min. A splitter valve was used after the column and only 50% of the mobile phase was directed through the detector. The column temperature was maintained at 35 °C. The utilized mobile phase has been previously reported by Torres et al. [22]. The retention times of squalene (SQ), fatty acid ethyl ester (FAEE), diesterified alkoxyglycerol (DEAG), free fatty acid (FFA), monoesterified alkoxyglycerol (MEAG), and nonesterified alkoxyglycerol (NEAG), were 1.7, 8.7, 12, 18.2, 25.5, and 44.3 min, respectively.

Transesterification Reaction

The unsaponifiable residue (500 mg) and CLAEE (818 mg), CLA (818 mg), or EPAEE (1,000 mg) were added to a

30-mL flask and mixed by swirling. Then the lipase (10% w/w) was added. The flasks were stoppered and placed in an orbital shaker (200 rpm) at 55 °C. Samples (30 μL) were withdrawn periodically.

Analysis of reaction products—methylation to prepare methyl esters of free and esterified fatty acids. Samples of 250 μL were methylated by addition of 1 mL of 0.2 M methanolic HCl. This mixture was allowed to stand for 4 h at 60 °C. After addition of 200 μL water, the mixture was subjected to the extraction, drying, and centrifugation procedures noted in the previous paragraph.

Analysis of the Reaction Products by Gas Chromatography

Sample of 1 μl was injected into a Perkin-Elmer autosystem XL (Wellesley, MA, USA) gas chromatograph fitted with a 30-m BTR-Carbowax column (0.25-mm i.d.). Injector and detector temperatures were set at 220 and 230 °C, respectively. The temperature program was as follows: starting at 100 °C and then heating to 180 °C at 20 °C/min; followed by heating from 180 to 220 °C at 15 °C/min. The final temperature (220 °C) was held for 30 min. Identification of the methyl esters of the various fatty acids was based on a menhaden oil fish standard (#4-7085) obtained from Supelco (Bellefonte, PA, USA). Identification of the methyl ester of CLA and the associated retention time was accomplished by direct injection of this ester as obtained by reaction in methanol containing 0.2 M HCl.

Results and Discussion

To produce alkoxyglycerols from commercial shark liver oil containing high levels of omega-3 or CLA fatty acid residues, the following procedure was employed: Supercritical fluid extraction of shark liver oil was followed by a saponification of the raffinate obtained and a subsequent reaction of the unsaponifiable residue with CLAEE, CLA, or EPAEE. Composition of the original shark liver oil, raffinatte, and unsaponifiable residue are shown in Table 1.

Inspection of Table 1 shows that the squalene content of shark liver oil is very high (ca. 60%). High concentration of squalene in the liver indicates that sharks belong to deep-sea species [23].

Hence, to obtain an unsaponifiable residue rich in alkoxyglycerols, squalene needs to be removed from the original shark liver oil. Table 1 also indicates that supercritical fluid extraction eliminated more than 95% of the original content of squalene in the shark liver oil to produce a raffinate with a very low content in squalene (ca. 3% w/w).

However, this raffinate is unsuitable for the production of alkoxyglycerols because of the presence of triacylglycerols which can undergo undesired transesterification reactions in the presence of lipases. Hence, saponification was effected to efficiently remove all triacylglycerols and to obtain an unsaponifiable residue highly enriched in nonesterified alkoxyglycerols.

Table 1 Composition of the original shark liver oil, raffinate obtained by extraction with supercritical CO_2, and unsaponified residue

Percentage (w/w)	Shark liver oil	Raffinate	Unsaponifiable
SQ	60	3	8
CHE	ND	<1	ND
DEAG	26	63	ND
TAG	13	33	ND
CH	ND	ND	6
NEAG	ND	ND	86

ND not detected, *CHE* cholesterol ester, *CH* cholesterol

Transesterification Reaction

A schematic representation of the lipase catalyzed esterification of alkoxyglycerols is shown in Fig. 1. These reactions take place in two consecutive steps. During the first acylation mono-esterified alkoxyglycerol (MEAG) is formed followed by a second acylation to obtain diesterified alkoxyglycerol (DEAG).

The transesterification reaction between CLAEE and the nonesterified alkoxyglycerol (NEAG) from the unsaponifiable residue was also carried out under conditions such that nitrogen was continuously bubbled through the reaction mixture in order to volatilize the ethanol produced by reaction. Elimination of ethanol from the reaction mixture shifts the equilibrium towards the production of DEAG.

The results (Fig. 2) indicate a rapid loss of NEAG followed by a slower reaction where MEAG is esterified to produce DEAG.

The difference in the rates of reaction could be explained by the higher reactivity of the primary hydroxyl group present in NEAG which leads to the production of MEAG, compared to that of the secondary hydroxyl group, responsible for the formation of DEAG.

Detailed inspection of Fig. 2 shows that in the absence of a nitrogen purge stream, the consumption of NEAG is ca. 30% (Fig. 2b) and in the presence of a continuous purge of nitrogen the disappearance of NEAG is ca. 75% during the first 30 min of reaction (Fig. 2a). At longer reaction times the conversion of NEAG never exceeded 50% without bubbling nitrogen into the reaction mixture. In contrast, ca. 95% of DEAG was obtained when the nitrogen was continuously bubbled through the reaction mixture.

The transesterification reaction between CLAEE and the nonesterified alkoxyglycerol (NEAG) from the

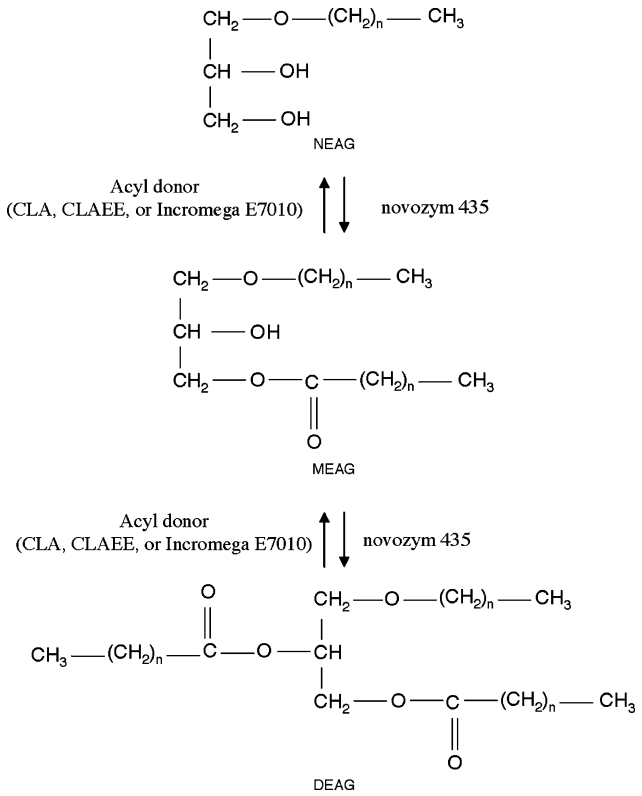

Fig. 1 Schematic representation of the reaction. *NEAG* nonesterified alkoxyglycerol, *MEAG* monoesterified alkoxyglycerol, *DEAG* diesterified alkoxyglycerol

unsaponifiable residue was also scaled-up to 12 g of reaction mixture. Similar results to those at analytical scale (Fig. 2a and c, hole symbols) were obtained. The differences observed between the experiments on the analytical scale and on a bigger scale could be based on the slight variations of the composition of the unsaponifiable residue obtained in both experiments. In our opinion, the process should be readily scaled up without drastically affecting the reaction rates and final yields.

Examination of Fig. 2c indicates that more than 95% of the original CLAEE was consumed which indicates the high efficiency of the reaction.

Esterification of NEAG with CLA

In order to compare the transesterification reaction with the direct esterification of NEAG, the synthesis of DEAG was also carried out with CLA. This reaction was also effected with a continuous purge of nitrogen. Similar to the observations in the transesterification reaction, it is possible to distinguish two consecutive stages in the course of the reaction (Fig. 3). The first stage, during the first 30 min, was comprised of a conversion of NEAG higher than 90%, and the second stage reached a yield of DEAG ca. 80%.

If one compares the transesterification (Fig. 2a) and the direct esterification (Fig. 3a), it can be observed that the

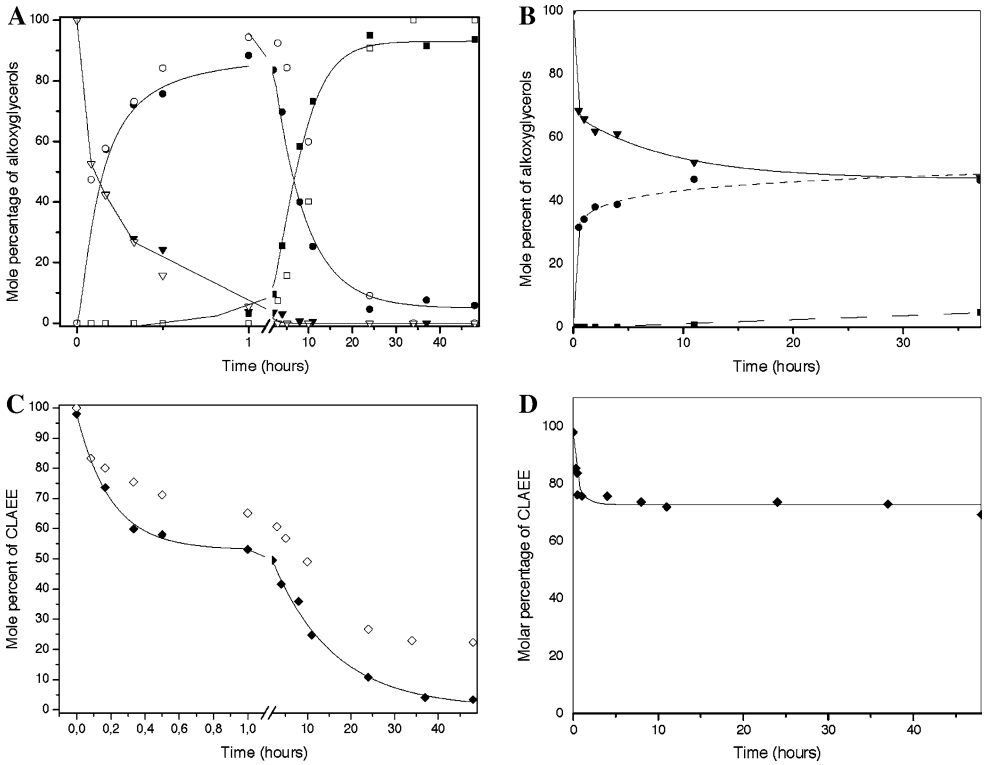

Fig. 2 Time course of the lipase-mediated transesterification of CLAEE with the NEAG from the unsaponifiable residue. **a** and **c** with nitrogen purge stream and scaled-up experiment (*hole symbols*); **b** and **d** without nitrogen purge stream. **a** and **b** *filled squares* DEAG; *circles* MEAG; *inverted triangles* NEAG. **c** and **d** *diamonds* CLAEE

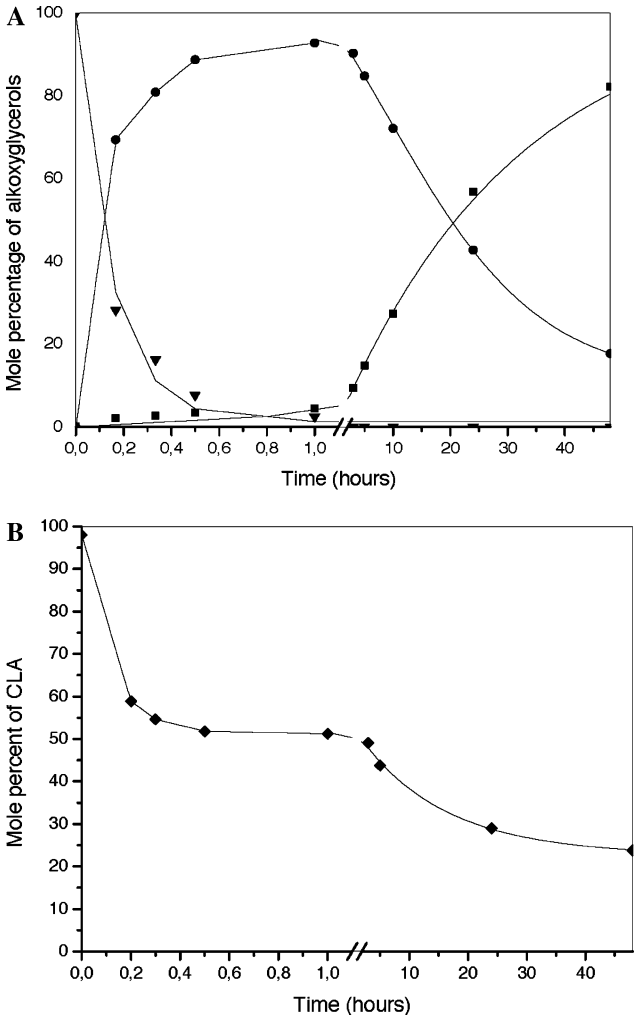

Fig. 3 Time course of the lipase-mediated esterification of CLA with the NEAG from the unsaponifiable residue. **a** *filled squares* DEAG; *circles* MEAG; *inverted triangles* NEAG. **b** *diamonds* CLA

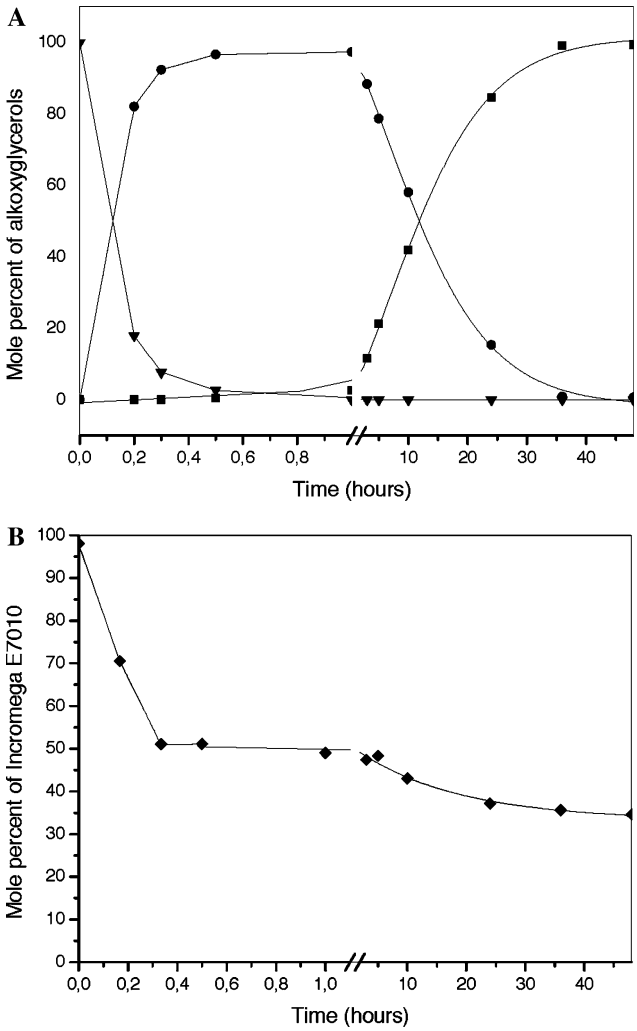

Fig. 4 Time course of the lipase-mediated transesterification of the eicosapentaenoic acid ethyl ester with the NEAG from the unsaponifiable residue. **a** *filled squares* DEAG; *circles* MEAG; *inverted triangles* NEAG. **b** *diamonds* EPAEE

consumption of NEAG is faster with CLA (first hour of reaction), although lower levels of DEAG were attained after 48 h of reaction. These results indicate that the removal of ethanol (in case of transesterification with CLAEE) or water (in case of esterification with CLA) in the second stage of the reaction could play a crucial role. Consequently, it can be speculated that the lower reaction rates observed could be caused by a less efficient elimination of water compared to that of ethanol.

Transesterification of NEAG with EPAEE

Finally, the transesterification reaction of NEAG was also carried out with a commercial mixture of EPA ethyl ester. The results are shown in Fig. 4. Similar trend to that obtained with CLAEE was attained.

These results indicate that immobilized *Candida antarctica* lipase B showed no discrimination between CLA or EPA ethyl esters for the acylation of alkoxyglycerols. Yields higher than 90% can be obtained with both compounds.

The present methodology describes a very efficient process for obtaining DEAG with CLA and EPA. Although further studies regarding the biological activities of these compounds need to be done, these compounds provide evidence of an enormous potential because they combine the beneficial properties of both alkoxyglycerols and CLA or EPA in a single molecule.

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