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# Enzymatic hydrolysis of conjugated linoleic acid-enriched anhydrous milk fat in supercritical carbon dioxide

Glaucia H.C. Prado, Mohamed Khan, Marleny D.A. Saldaña*, Feral Temelli

*Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, AB, Canada T6G 2P5*

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ABSTRACT

Consumption of conjugated linoleic acid (CLA) has been associated with numerous health benefits. CLA in its free form can be obtained by enzymatic hydrolysis of milk fat. In this study, enzymatic hydrolysis of CLA-enriched anhydrous milk fat (AMF) using supercritical $CO_2$ and Lipozyme TL IM were carried out to maximize the production of free fatty acids (FFAs). The effects of pressure (20–30 MPa), fat:water ratio (1:5–1:30 mol/mol) and their interaction in the FFA production were studied using response surface methodology. After performing the optimization, reactions at two different temperatures (45 and 65 °C) and then with three different enzymes (Lipozyme RM IM, Novozyme 435, and immobilized enzyme from *Candida rugosa*) were carried out to assess the optimum FFA production. CLA content in its free form and major lipid classes (FFA, monoglycerides (MG), diglycerides (DG), and triglycerides (TG)) in the reaction products were quantified by gas chromatography. An increase in FFA and intermediate reaction products (MG and DG), together with a decrease in TG compared to the starting material was observed at all experimental conditions studied. The maximum level of FFA (86.79%, w/w) was achieved using Lipozyme TL IM at 23 MPa, 1:5 fat:water ratio (mol/mol) and 55 °C. The maximum CLA content in FFA form (6.81 mg/g fat) was obtained using Lipozyme TL IM at 30 MPa, 1:30 fat:water ratio (mol/mol) and 55 °C that corresponds to 98% conversion of CLA in TG form to FFA form.



## 1. Introduction

Conjugated linoleic acid (CLA) is defined as a group of isomers of linoleic acid (octadecadienoic acid) for which the two double bonds have a conjugated arrangement [1]. The main isomer found naturally is *cis-9, trans-11*, which is produced by biohydrogenation of unsaturated fatty acids in ruminant animals [2]. Thus, main sources of CLA in nature are meat and dairy products [3]. Beneficial health effects of CLA such as anticancer, prevention of cardiovascular diseases, and positive immune and inflammatory responses have been reported based on animal studies [3]. Therefore, enrichment of CLA content of milk fat has been investigated extensively [4–7]. The main procedure to enhance CLA content of milk fat is by feeding cows with oils and oilseeds, rich in unsaturated fatty acids. Safflower, flax seed, rapeseed, corn, cottonseed, soybean and canola oils as well as fish oil are examples of supplements used to increase the CLA content of milk fat [4–7].

Anhydrous milk fat (AMF) is isolated from cream and has a complex structure due to the diversity of fatty acids (more than 400 fatty acids) found in its composition [8]. With such a diversity of fatty acids, AMF can be modified to provide a variety of functionalities for different food applications [9]. The production of free fatty acids (FFAs) by hydrolysis, which consists of the breakage of the ester linkage between a fatty acid and the glycerol backbone of a triacylglycerol [10], is one of the methods commonly used to modify AMF [9]. Enzymatic hydrolysis of milk fat has been conducted to achieve different products with desired properties such as specific structured lipids and tailor-made fats [9].

The use of supercritical fluid (SCF) technology in enzymatic reactions has expanded over the last two decades [11]. The main lipid reactions investigated using SCF include hydrolysis, alcoholysis, glycerolysis, esterification, interesterification, and transesterification [11,12]. The use of this technology in enzymatic reactions has received considerable attention mainly due to the solvation power of $CO_2$, which can be modified by small changes in pressure and temperature [12,13]. In addition, the solvent is easily removed from the system and the SCF has high rates of mass transfer because of its high diffusivity and low viscosity [13]. The use of enzymes in reactions performed in SCF media offers the advantage to operate at low reaction temperatures. Furthermore, enzymes in immobilized form are more stable in supercritical media compared to the enzymes in solution form and consequently, their activity remains almost unchanged [14]. Moreover, immobilized enzymes allow the reaction to be conducted in a continuous flow bed reactor, thus, catalyst removal is not necessary [11]. Several studies have focused on the hydrolysis of fats and oils using supercritical $CO_2$ (SC-$CO_2$),

* Corresponding author. Tel.: +1 780 492 8018; fax: +1 780 492 8914.
*E-mail address:* marleny@ualberta.ca (M.D.A. Saldaña).

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including enzymatic hydrolysis of canola oil [15–17], sunflower oil [18], blackcurrant oil [19], soy deodorized distillate [20], soybean oil [12], and glycerolysis–hydrolysis of canola oil in SC-$CO_2$ [21].

Response surface methodology (RSM) consists of the optimization of process variables based on a factorial design. RSM has been used in modeling of various industrial processes since it was introduced in 1950 by Box [22]. The objective is to achieve the optimal region of the response surface and calculate the model parameters by correlating the experimental data using linear or quadratic models [22]. Experiments are carried out to evaluate the effect of the most important factors. The selection of the factors and their levels is very important as inappropriate choices result in an unsuccessful optimization. RSM is used in food systems for product development, and optimization of functional and sensorial properties, evaluation of nutritional quality, shelf life, packaging performance and optimization of processing conditions.

The objective of this study was to optimize the enzymatic hydrolysis conditions for maximum production of FFA from CLA-enriched AMF in SC-$CO_2$ media. Response surface methodology and Lipozyme TL IM were utilized for the optimization by studying the effects of pressure and fat:water ratio on FFA formation. Then, reactions were carried out at different temperatures and finally using other sources of enzymes (Lipozyme RM IM, Novozyme 435, and immobilized enzyme from *Candida rugosa*). Quantification of CLA in its free form and major lipid classes in the reaction products was carried out at all conditions studied.

## 2. Materials and method

### 2.1. Materials

CLA-enriched milk was obtained using a special diet provided to dairy cattle at the University of Alberta Dairy Research and Technology Centre following the protocols described by Bell et al. [5]. Then, CLA-enriched AMF was produced using the methodology of Martínez-Monteagudo et al. [23]. Three different enzymes were used for the hydrolysis experiments: Lipozyme TL IM (immobilized from *Thermomyces lanuginosus*) was kindly provided by Novozymes North America Inc. (Franklinton, NC, USA) while Novozyme 435 (immobilized from *Candida antarctica B*), Lipozyme RM IM (immobilized from *Rhizomucor miehei*) and immobilized lipase from *C. rugosa* were purchased from Sigma–Aldrich Canada Ltd. (Oakville, ON, Canada). Carbon dioxide (bone dry, 99.8%) from Praxair Inc. (Mississauga, ON, Canada) was used for the reaction process. The chemicals obtained from Sigma–Aldrich Canada Ltd. (Oakville, ON, Canada) for analysis were phenolphthalein, petroleum ether (ACS reagent), hexane (anhydrous, 95%), benzene (ACS reagent, ≥99.0%), trimethylsilyl (TMS)-diazomethane (2 M in hexane), acetic acid (ACS reagent, ≥99.7%), anhydrous sodium sulfate (≥99%), sodium methoxide 0.5 M, pyridine (≥99%) and N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) solution. The lipid standards used for the analysis of reaction products and CLA quantification were purchased from Nu-Chek Prep. Inc. (Elysian, MN, USA). Chloroform (HPLC grade, 99.9%), sodium hydroxide (1 N standard solution) and methanol (ACS reagent) were obtained from Fischer Scientific Ltd. (Nepean, ON, Canada). Hydrogen and nitrogen ultra-high purity (99.999%) were purchased from Praxair Inc. (Mississauga, ON, Canada).

### 2.2. Analysis of CLA-enriched AMF

#### 2.2.1. Fat content determination

Fat content of CLA-enriched AMF was determined using the AOAC official method 938.06 [24]. A known amount of CLA-enriched AMF (1.5–2.5 g) was mixed with 15 mL of petroleum ether. This mixture was filtered using a weighed crucible and washed with 100 mL of petroleum ether. The crucible was dried at 100 °C to constant weight. Then, it was washed again with 25 mL of solvent and dried to a constant weight. The washing operation was repeated until there was no further loss in weight. The fat content was calculated using Eq. (1):

$$\%\text{Fat} = 100 - (\%\text{residue}) \quad (1)$$

#### 2.2.2. Determination of CLA content

The following method [25] was used for the methylation of fatty acids directly from the triglycerides (TG) present in AMF. Non-hydrolyzed CLA-enriched AMF (50 mg) was diluted in 0.5 mL of chloroform. Then, 50 μL of this solution was transferred to a screw-capped test tube and 2 mL of sodium methoxide (0.5 M) was added. The test tube was placed in an oven at 80 °C for 20 min. Afterwards, 2 mL of water, 2 mL of hexane and 0.5 mL of internal standard solution (methyl heptadecanoate, 1 mg/mL in hexane) were added, followed by the addition of a small amount of anhydrous sodium sulfate to remove water in the top layer. Finally, the solution was diluted in hexane to a concentration of 0.3 mg/mL for injection into the gas chromatograph (GC) as described later.

**Table 1**
Central composite face centered design with two variables ($x_1$: pressure, MPa and $x_2$: fat:water ratio, mol/mol) and experimental and predicted values of FFA after hydrolysis of triglycerides of CLA-enriched AMF in SC-$CO_2$ media.

| Run | Process variable | | $\%FFA_{Exp}$ | $\%FFA_{Pred}$ |
|---|---|---|---|---|
| | $x_1$ | $x_2$ | | |
| 1 | 25 | 1:30 | 62.23 | 62.77 |
| 2[a] | 25 | 1:17.5 | 73.17 | 70.65 |
| 3 | 30 | 1:17.5 | 57.33 | 65.16 |
| 4 | 20 | 1:17.5 | 56.87 | 50.11 |
| 5 | 30 | 1:5 | 60.22 | 56.20 |
| 6 | 20 | 1:30 | 21.89 | 25.37 |
| 7 | 25 | 1:5 | 77.59 | 78.54 |
| 8[a] | 25 | 1:17.5 | 70.75 | 70.65 |
| 9[a] | 25 | 1:17.5 | 69.53 | 70.65 |
| 10 | 20 | 1:5 | 71.57 | 74.85 |
| 11 | 30 | 1:30 | 77.94 | 74.13 |

[a] Mean ± standard deviation for the center point: 71.15 ± 1.51%.

### 2.3. Enzymatic reactions

#### 2.3.1. Experimental design

The experimental design consisted of three different sets of experiments. The first set was carried out using the statistical response surface methodology with central composite face centered design to evaluate the effects of pressure and oil:water ratio, with triplicate runs at the center point. The second set of experiments evaluated the effect of temperature while the third set focused on the use of different enzymes at the optimized conditions obtained in the first set. All experiments were carried out in duplicate for the second and third sets.

Central composite face-centered design and RSM were used to determine the conditions for maximum hydrolysis of triglycerides using Lipozyme TL IM in SC-$CO_2$ medium. The aim of the RSM technique is to determine which factors and their interaction have a statistically significant effect on the response after fitting the experimental results obtained to the selected model [22]. The parameters studied were pressure (20, 25 and 30 MPa) and fat:water ratio (1:5, 1:17.5 and 1:30 mol/mol) with three levels for each parameter. The experimental design with the levels of the variables, predicted and experimental results are presented in Table 1, including the triplicate runs for the center point (Runs 2, 8, and 9). The experimental data obtained were fitted to a second order polynomial