ENZYMATIC TRANSESTERIFICATION COUPLED TO PRODUCT EXTRACTION IN SUPERCRITICAL CARBON DIOXIDE

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ABSTRACT

A mixture of fatty acids ethyl esters (FAEE), rich in short chain fatty acids, was produced and extracted in a combined reaction – extraction system, integrated by the use of supercritical carbon dioxide ($scCO_2$). In this processing unit milk fat was partially converted to FAEE by enzymatic alcoholysis. Simultaneously, the lighter FAEE were extracted from the product mixture by flowing $scCO_2$. The integrated unit, consisting of a stirred reactor and a separation vessel, was operated in semi-continuous mode, allowing continuous feed of ethanol and CO_2. Process parameters such as the operation pressure and temperature and the molar ratio of reactants were investigated. The amount and composition of the FAEE extract obtained in the separator can be adjusted by manipulation of the mentioned process parameters. This design represents a novel and sustainable way to produce and isolate high value fatty acid derivatives from a natural source such as milk fat.

INTRODUCTION

Ethyl esters of fatty acids (FAEE) have a wide range of commercial uses, being also important intermediates in the production of a variety of oleochemicals [1, 2]. Short and medium chain fatty acids are especially interesting, since they do not abound in natural sources [1].
FAEE can be produced by transesterification of oils and fats with ethanol, reaction known as alcoholysis [3]. In this work milk fat is used as the fatty acid source to produce FAEE. Milk fat contains fatty acids of a wide range of chain lengths, being among the very few natural fats containing a significant amount of short-chain fatty acids (C4 to C8) and medium-chain fatty acids (C10 and C12), nearly 26% in molar basis [4].
Alcoholysis can be enzymatically catalyzed using lipases [1-3]. Lipases often show selectivity or specificity for a certain substrate type or molecular position [3], which is a feature of great interest when the purpose of the transesterification reaction is to synthesize structured triglycerides, or to enrich (one of) the reaction products in a certain fatty acid type [5]. Alcoholysis of milk fat using a short chain selective lipase can thus result in a FAEE product mixture that is enriched in the valuable short-chain fatty acid fraction.
Mixtures of FAEE can be fractionated using supercritical carbon dioxide ($scCO_2$) as extraction solvent. Carbon dioxide dissolves apolar compounds up to a moderate molecular weight, and solubility decreases with molecular weight at a given pressure and temperature [6]. Thus, light FAEE (shorter chains) will be preferably extracted by $scCO_2$ from a liquid mixture of FAEE. The work by Brunner [6] on concentration of omega-3 fatty acids from fish oil is a known example of this application.
Supercritical carbon dioxide has also been extensively studied as reaction solvent for lipase-catalyzed reactions [1, 3]. Comparable to organic solvents in terms of polarity, it is easily separated from reactants and products by depressurization, leaving virtually no residues. If CO_2 is dissolved in the reactants rather than the reactants in CO_2, a gas expanded liquid (GXL) is formed, in which CO_2 acts as co-solvent [7, 8]. The lower density and viscosity of the GXL in relation to the original mixture is

advantageous for mass transfer. When coincidentally it is possible to extract or fractionate the reaction products using supercritical carbon dioxide, its use as reaction solvent or co-solvent makes the integration of reaction and extraction feasible. This provides additional advantages: the in-situ removal of reaction products prevents them from the possibility of reacting back in the reaction mixture, and may contribute to enhance the reaction selectivity.
The possibilities of integrating an enzymatic alcoholysis reaction and the extraction of its lighter products are investigated in the present work. In the first stage, reaction and extraction were studied separately in order to determine their optimal conditions and assess their compatibility. Preliminary experiments in the integrated unit showed that simultaneous production and concentration of short-chain FAEE from milk fat can in fact be realized. Technical difficulties such as adjusting the kinetics of both tasks and compensating the concurrent extraction of reactants still need to be overcome. Yet, the first trials prove that the integration allows in-situ separation of the desired reaction products, and consequently the advantages of $scCO_2$ (enhanced mass transfer, easy recovery of products, lack of toxicity) can be exploited using it as both reaction and extraction solvent.

MATERIALS AND METHODS

Materials

Concentrated milk fat (98% fat) was obtained from Congress, Friesland Foods (Lochem, Netherlands) and stored at 4°C before use. The fatty acid composition of this milk fat, normalized to the nine major fatty acids, is shown in Table 1. In this work, butyric, hexanoic, octanoic, decanoic and lauric acids are considered short- and medium-chain fatty acids. The rest are considered long-chain fatty acids.

Table 1: Fatty acid composition of milk fat fraction used in the experiments.

Fatty acid	C number in fatty chain	Mol %
Butyric acid	4	7.3%
Hexanoic acid	6	4.0%
Octanoic acid	8	2.0%
Decanoic acid	10	3.8%
Lauric acid	12	4.1%
Myristic acid	14	10.1%
Palmitic acid	16	31.8%
Oleic acid	18 (monounsat.)	27.2%
Stearic acid	18	9.7%

Carbon dioxide was from Linde Gas Benelux (Schiedam, Netherlands). The immobilized enzyme Novozym 435 was obtained from Novozymes A/S (Bagsvaerd, Denmark). Absolute ethanol (99,9%) was purchased from Sigma-Aldrich (Zwijndrecht, Netherlands). Ethyl butyrate (99,5%), hexanoate (99,8%), octanoate (99,8%), decanoate (99,5%), myristate (98,5%), laurate (99,8%), palmitate (99,8%), oleate (99,5%) and stearate (99,5%), used as analytical standards, were also purchased from Sigma-Aldrich.

Enzymatic transesterification – extraction set-up

The experimental set-up is depicted in Figure 1. A jacketed stirred autoclave (Autoclave Engineers) was used for the transesterification under CO_2 pressure. The autoclave, designed for a pressure up to 300 bar, has a volume of 385 mL and is equipped with two sapphire windows, a bottom mesh filter and several inlet/outlet ports. In regular experiments, a certain amount of melted fat, enzyme and ethanol were introduced in the autoclave, which was then closed and pressurized. Temperature was kept at 45°C and the mixture was stirred using a 6-blade turbine-like impeller at 180 rpm. Samples from the reaction mixture, free of enzyme particles, were taken regularly by opening the bottom valve of the autoclave. After a certain reaction time, extraction of the reaction products was started by

initiating a continuous flow of $scCO_2$ through the reaction vessel (20-70 g/min). The stirring speed was then reduced to 120 rpm. The extracted material was condensed from the gas stream at ca. 25 bar and was collected in the separation vessel, maintained at 16°C by a thermostatic bath. During the extraction period, ethanol was fed to the reactor at a rate of 1 ml/min using a ISCO pump, in order to compensate its loss due to extraction in the carbon dioxide stream.

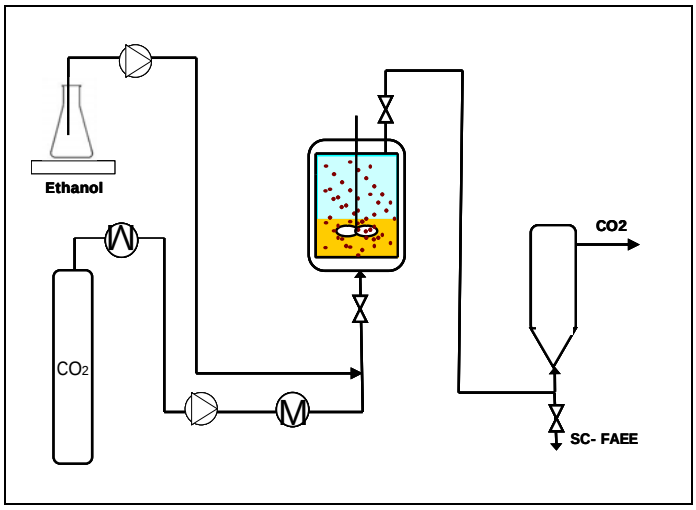


Figure 1: Reaction – separation set-up.

Analytical methods

Analyses of the FAEE and partial glyceride mixtures were carried out in a gas chromatograph Chrompack CP9002 (Varian Inc., Middelburg, Netherlands) equipped with flame ionization detector, using a VF5-ht GC-column (Varian Inc., Middelburg, Netherlands). Injection and detection temperature were 320°C. N_2 was the carrier gas at a flowrate of 1.5 mL/min. Oven temperature was kept at 50°C for 2 minutes, then raised at 20°C/minute to 350°C and kept for 2 minutes at the final temperature. This temperature profile allowed elution of all components and quantification of FAEE.

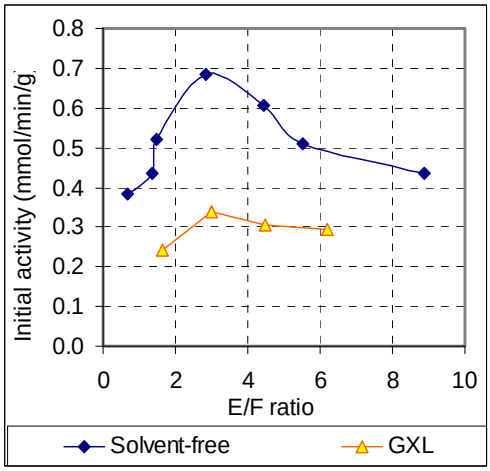
RESULTS AND DISCUSSION

Initially, reaction and separation were studied separately. Enzymatic alcoholysis of milk fat was carried out in CO_2-expanded oil (GXL) at 110 bar and 45°C. A certain amount of fat, ethanol and enzyme were introduced in the autoclave, which was then pressurized until only one phase (GXL phase) was observed, at around 100-110 bar. The selected immobilized enzyme, Novozym 435, has been reported in the literature to show selectivity for small substrates [9]. This was confirmed in our experiments: during the first hours of reaction Novozym 435 produced FAEE mixtures richer in short and medium chains than the original milk fat (up to 48 mol% vs 21 mol%). However, as conversion approached completion, the proportion of short-chain FAEE decreased to values close to that in the original milk fat, indicating that the enzyme catalyzes as well esterification of longer chain fatty acids, although at a lower rate.

The ratio of reactants had an effect on both the activity and selectivity of the enzyme. Initial activity was maximum at ethanol to fat molar ratios (E/F) around 3 mol ethanol/mol fat, as shown in Figure 1. This effect was observed both when the reaction was carried out in GXL and solvent-free conditions, being clearer in solvent-free conditions. Regarding enzyme selectivity, Figure 2 shows that, for a conversion of 40 mol%, increasing E/F from 1.5 to 4.5 resulted in a higher proportion of short- and medium-chain fatty acids in the FAEE product. A further increase of E/F to 6.2 resulted in a drop of enzyme selectivity. The optimal E/F considering both activity and selectivity appeared therefore to be in the range 3-4.5.

In order to have a first guess of the right process conditions (pressure and temperature) for the extraction of light FAEE, the solubility in $scCO_2$ of different FAEE was measured in a sapphire view

cell coupled to a gas chromatograph. Solubility measurements were made on multicomponent mixtures of ethyl butyrate (EB), ethyl oleate (EO), milk fat and carbon dioxide, in a pressure range of 60-180 bar and a temperature range of 40-70°C. As shown in Figure 3, solubility of light FAEE (ethyl butyrate) is much higher than that of heavier FAEE (ethyl oleate), especially above 80 bar. Thus, short-chain FAEE will preferably be extracted from the reaction mixture in the combined system.



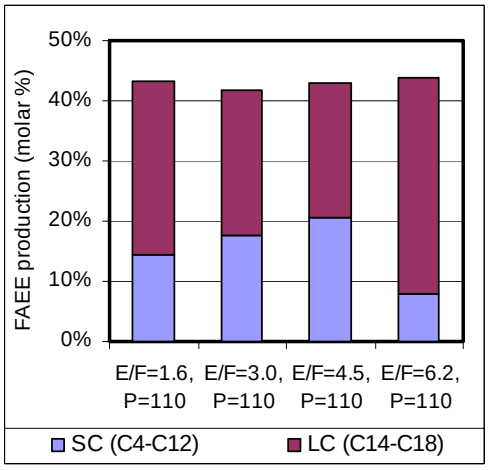


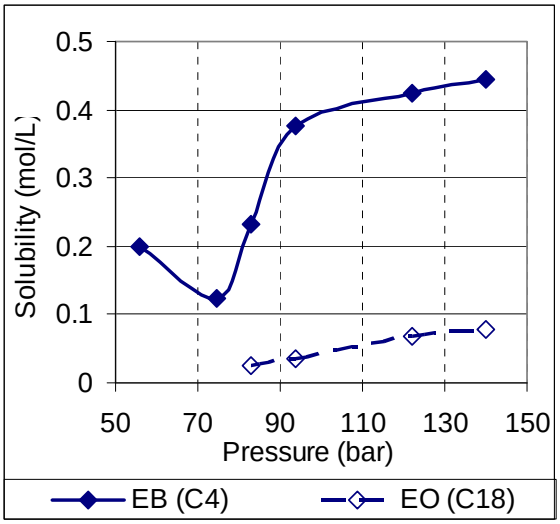
Figure 1 (left): Initial activity of Novozym 435 (mmol/min/g enzyme) in milk fat alcoholysis at varying E/F ratios, both in GXL and solvent-free. Figure 2 (right): FAEE product composition after approximately 9 hrs of reaction, for milk fat alcoholysis carried out with Novozym 435, at varying E/F ratios in GXL.

The fractionation of a FAEE mixture resembling the fatty acid composition of milk fat was then carried out in a supercritical carbon dioxide column. The fractionation was operated in semi-continuous mode, in a single equilibrium stage. The effect of CO_2 density on the yield and composition of the extract is shown in Figure 4, where the concentration factor of short-chain FAEE in the extract (CF_{SC}) is plotted versus the corresponding recovery (R_{SC}). CF_{SC} and R_{SC} are defined as indicated by Equations 1 and 2, where w stands for mass, x for mass fraction, E for extract and F for feed. As the operation proceeded, R_{SC} increased until eventually reaching one when all short-chain FAEE had been extracted. On the other hand, CF_{SC} logically decreased with the extraction time, due to concurrent extraction of long-chain FAEE. At low CO_2 density (220 g/L, experiment W), the solvent power of CO_2 was relatively low, and only 20% short-chain FAEE were recovered in the extract. At high CO_2 density (486 g/L, experiment Z), recovery of short-chain FAEE was practically completed at expense of low concentration factors, since both short and long FAEE were extracted. Moderate CO_2 densities in experiments X and Y (290 to 385 g/L) resulted in higher concentration factors for reasonable recoveries. Therefore, process values of pressure and temperature such that the density of carbon dioxide falls in the range 290-385 g/L are in principle advantageous for single-stage extraction of short-chain FAEE in the combined unit.

$$R_{SC} = \frac{w_{SC,E}}{w_{SC,F}}$$ **Equation 1: Short-chain FAEE (C4 to C12) recovery in the extract.**

$$CF_{SC} = \frac{x_{SC,E}}{x_{SC,F}}$$ **Equation 2: Short-chain FAEE (C4 to C12) concentration factor in the extract.**

First trials in the integrated unit were run at the process conditions shown in Table 2. The pressure and temperature applied were in a range such that the resulting CO_2 density was similar to that in the best fractionation runs (Figure 4). The alcoholysis proceeded in GXL for a certain time without CO_2 circulation, until a conversion of around 30-35% was reached. The extraction process was then started by initiating a CO_2 flow. Ethanol was fed during the course of the extraction, except in experiment C.



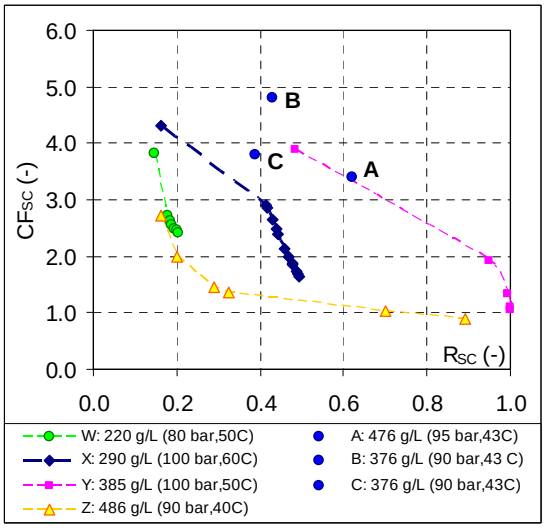


Figure 3 (left): Solubility of ethyl butyrate (EB) and ethyl oleate (EO) in $scCO_2$, at 40°C. Figure 4 (right): Concentration factor (CF_{SC}) vs. recovery (R_{SC}) of short-chain FAEE in single fractionation experiments.

Recovery and concentration factor of short-chain FAEE were used for comparing the different runs, as done for the above discussed fractionation experiments. In experiment A, a recovery R_{SC} of 62% and a concentration factor CF_{SC} of 3.4 were achieved. This result is practically coincident with the curve of experiment Y (single fractionation) as shown in Figure 4. However, experiments A and Y were run at different CO_2 densities. The coincidence of the result is probably due to the different initial composition of the material to extract, which was in experiment A certainly richer in short-chain FAEE than in experiment Y due to the lipase selectivity. In runs B and C, operated at lower CO_2 density, CF_{SC} increased to 4.8 and 3.8 respectively, although in contrast R_{SC} decreased to around 40%. These results are also in the vicinity of the curve Y. It seems an indication that the composition of the FAEE mixture at the start of the extraction is as important as the actual extraction conditions for determining the extract quantity and quality.

Table 2: Process conditions and main results in the combined reaction-extraction experiments. P_R, T_R: pressure and temperature in reactor. P_S, T_S: pressure and temperature in separation vessel. F_{CO2}, F_E: flowrate of CO_2 and ethanol. R_{SC}, C_{SC}: recovery and concentration factor of short-chain FAEE in extract.

	A	B	C
E/F (mol ethanol/mol fat)	4.4	3.2	3.3
P_R (bar)	95	90	90
T_R (°C)	43	43	43
ρ_{CO2} (g/L)	476	376	376
Conversion at t=0 (mol %)	29%	33%	34%
F_{CO2} (g/min)	60	62	68
F_{Eth} (g/min)	0.8	0.8	--
P_S (bar)	25	25	22
T_S (°C)	16.0	16.0	10.0
Total CO_2 consumed (kg)	7.3	9.3	10.0
R_{SC}	0.62	0.43	0.39
C_{SC}	3.4	4.8	3.8
Losses (wt. % of initial fat)	13.6%	13.8%	14.8%

Although it is shown that reaction and extraction can be effectively combined, the recovery of light FAEE in the extract was in overall low. This can be due to two reasons. Firstly, the reaction probably slowed down at the start of the CO_2 flow, due to concurrent extraction of the reactant ethanol from the

reaction vessel along with FAEE. Because ethanol is completely miscible with CO_2 at the applied experimental conditions, its removal from the reaction mixture is favored. Ethanol was in fact found in large quantities in the extract. The extra ethanol fed at 1 mL/min in experiments A and B seems to be not enough to compensate ethanol removal. On the other hand, the fact that the initial concentration of ethanol in experiment A was higher than that in experiments B and C can explain why R_{SC} was also the highest in A: most likely it took longer to extract all ethanol present, thus the reaction continued for longer time after the start of extraction and more FAEE were comparatively produced. A second reason for the low recovery is the mass loss during the experiments. A certain amount of the milk fat feed, around 15%, was not recovered in the extract or the raffinate. This material did presumably not condense in the separation vessel and escaped with the carbon dioxide off stream. Probably, the largest proportion of this lost material was constituted by light FAEE, which means that recovery (and possibly even concentration factor) would increase if this mass loss is avoided.
In summary, these three first runs show the potential of the integrated reaction-extraction unit for the production and concentration of a FAEE mixture which is enriched in short-chain fatty acids. However, technical problems such as the loss of fed material and removal of ethanol from the reaction mixture need to be solved. Subsequently, optimization of the process conditions can be carried out in order to maximize both recovery and concentration factor of short-chain FAEE in the extract.

CONCLUSIONS

Simultaneous production and extraction of fatty acid ethyl esters from milk fat was achieved in an integrated unit using supercritical carbon dioxide. Separately looking at the alcoholysis reaction, the optimal ethanol to fat ratio was found to lay around 3-4.5 mol ethanol/mol fat, considering both activity and short-chain fatty acid selectivity of the enzyme used. Solubility measurements and fractionation experiments on FAEE synthetic mixtures showed that a suitable CO_2 density for the extraction of short-chain FAEE is in the range 300-400 g/L.
When these process conditions were applied in the combined system, a short-chain FAEE mixture was recovered as the extract. Short-chain FAEE in the extract were up to 4.8 times more concentrated than in the original milk fat. The enzyme selectivity for short-chain fatty acids as well as the different solubility of short and long FAEE in CO_2 can be exploited in the integrated process. Recoveries were however still rather low, around 43%. Improved or alternative process configurations that avoid the extraction of ethanol along with the product or that allow to compensate its removal will help to increase the yield of FAEE. Another point of attention is the loss of extracted material with the carbon dioxide off stream. Future experiments will be oriented to improve the present design and overcome its limitations.

ACKNOWLEDGEMENTS

This project is financially supported by SenterNovem (Dutch Ministry of Economic Affairs, Netherlands). The authors would like to thank the industrial partners Kievit-Friesland Foods (Netherlands) for the useful discussions.

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