J Am Oil Chem Soc (2019) **96:** 1327–1335
DOI 10.1002/aocs.12297

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ORIGINAL ARTICLE

Enrichment of Erucic and Gondoic Fatty Acids from *Crambe* and *Camelina* Oils Catalyzed by *Geotrichum candidum* Lipases I and II

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Received: 22 July 2019 / Revised: 16 September 2019 / Accepted: 17 September 2019
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Abstract Erucic (22:1, *cis*Δ13) and gondoic acids (20:1, *cis*Δ11) are building blocks obtained from renewable sources for the oleochemical industry. Different biocatalytic strategies for the enrichment of these compounds with high recovery yields were developed in our group. *Geotrichum candidum* lipases (GCL) strongly discriminate against fatty acids longer than 18 carbon atoms. Thus, GCL-I and -II were investigated using hydrolysis or ethanolysis reactions with *Crambe* and *Camelina* oils. Hydrolysis was also studied using fatty acid ethyl esters (FAEE) derived from the corresponding oil. Both isoforms were highly selective; however, interesting differences were observed. Although it has been reported that GCL-I displays a higher preference toward 18 *cis*Δ9, which is present in the studied oils at high levels, GCL-II showed higher enrichment values during hydrolysis independent of the substrate used. Hence, enrichments of 87% (*Crambe* oil) and 82% (*Crambe* FAEE) for erucic acid and 50% (*Camelina* oil) and 45% (*Camelina* FAEE) for gondoic acid, with recovery values between 89% and 99%, were achieved. On the contrary, the best enzyme for ethanolysis was GCL-I (82% and 41% for erucic and gondoic acid, respectively). In this case, although GCL-II also displayed good enrichment and recovery levels (77% and 28%, respectively), they were lower compared to the former reactions. In both ethanolysis reactions, the FAEE fraction contained between 92% and 97% of 18 unsaturated fatty acids.

Keywords *Geotrichum candidum* lipase · Erucic acid · Gondoic acid · *Crambe* oil · *Camelina* oil · Fatty acid enrichment

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Supporting information Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Introduction

The development of sustainable energy and chemical compound production processes is one of the major challenges currently faced by the chemical industry. To satisfy these environmental, economic, and social concerns while matching the increasing demand for chemical products, new ecofriendly approaches must be adopted, such as the use of renewable material sources and the application of greener reaction technologies. In this sense, vegetable oils can be used as an important alternative to traditional petrol-derived oil. The fatty acids (FA) obtained from these oils are important building blocks for the production of different kinds of relevant molecules (Biermann et al., 2011; Liu et al., 2014; Schörken and Kempers, 2009). In particular, long-chain FA, such as gondoic (20:1, *cis*Δ11) and erucic

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acid (22:1, *cis*Δ13), have been used in industry for the production of surfactants, erucamide as a polymer building block, and lubricants (Leonard, 1994). Plants of the genera *Cruciferae* can be used to produce oils with a high concentration of these compounds. For instance, *Crambe* oil represents an outstanding starting material as it is mainly composed of erucic acid (59%) (Dubois et al., 2007), while *Camelina* oil contains approximately 15% of gondoic acid (Murphy, 2016). However, despite the high amounts of the desired FA present in these oils, further enrichment is often necessary to meet the purity required by the oleochemical industry (Leonard, 1994; Murphy, 2016; Zhu, 2016). A promising alternative to traditional separation technologies, which usually entail harsh conditions and/or moderate efficiency, is the development and application of more sustainable technologies, such as the implementation of biocatalytic processes. Predictably, lipases are the most obvious choice to achieve this task as they naturally catalyze the hydrolysis of triacylglycerides (TAG) into glycerol and free FA (FFA). Moreover, this class of enzymes displays other interesting features from an industrial point of view, such as: (1) broad substrate spectrum; (2) excellent chemo-, regio-, and stereoselectivity; (3) high stability in harsh reaction conditions; (4) independence of cofactors; (5) enabling efficient transesterifications; and, furthermore, (6) a wide variety of lipases is commercially available (Oroz-Guinea et al., 2018; Zorn et al., 2016). Consequently, several studies analyzing the selectivity of lipases toward different FA and their potential use for the enrichment of gondoic and erucic acids were described. Recently, the creation of *Candida antarctica* lipase A (CAL-A) variants was reported by our group as being able to enrich long-chain mono-unsaturated FA (MUFA) by either (1) enhanced selectivity toward the hydrolysis of FA shorter than 20 carbon atoms (Zorn et al., 2018a, b) or (2) increased tendency for their hydrolysis (Oroz-Guinea et al., 2019). Thus, these approaches led to the enrichment of the longer FA in the esterified FA (EFA) fraction or in the FFA fraction, respectively. Prior to these studies, some wild-type lipases, such as *Candida rugosa* lipase (CRL) or *Geotrichum candidum* lipase (GCL), showed their ability to sharply discern between FA with 18 and 20 carbon atoms during hydrolysis reactions (Holmquist, 1998). Therefore, several approaches for the production of different enriched oils were described using GCL (Diks and Lee, 1999; McNeill et al., 1996; McNeill and Sonnet, 1995; Shimada et al., 1994, 1995; Sonnet et al., 1993), including the enrichment of erucic acid (McNeill and Sonnet, 1995; Sonnet et al., 1993). However, in those cases involving 22:1 accumulation, the biocatalyst was added as a crude extract (Weber et al., 1995). Unfortunately, utilization of such preparations can lead to low reproducibility of the results due to the change in composition from batch to batch of enzymes as a consequence of the production of varying amounts of lipase isoforms depending on the cultivation conditions. In particular, GCL-I and GCL-II possess distinct patterns of substrate specificity despite their 86% identical primary protein sequence. Thus, GCL-I prefers ester substrates with long-chain *cis*Δ9 unsaturated FA moieties, whereas GCL-II also accepts medium-length (8–14 carbon atoms) FA as substrate (Bertolini et al., 1995; Holmquist et al., 1997; Veeraragavan et al., 1990). Furthermore, to the best of our knowledge, the biocatalytic enrichment of gondoic acid has never been studied with these enzymes (McNeill and Sonnet, 1995; Sonnet et al., 1993).

Hence, the aim of this work was to explore and compare the capability of the recombinantly overexpressed GCL-I and -II to enrich gondoic or erucic acid from *Camelina* or *Crambe* oils. For this purpose, the suitability of both lipase isoforms to access enriched FA fractions was investigated in hydrolysis and ethanolysis reactions. In addition, as it was previously observed that the lipase selectivity can be affected by the type of ester substrate used (Oroz-Guinea et al., 2019; Zorn et al., 2018a, b), during the hydrolysis reactions, two formulations were used, the corresponding TAG and the FAEE derivatives.

Materials and Methods

Materials

Unless stated otherwise, all chemicals were purchased from Fluka (Buchs, Switzerland), Sigma (Steinheim, Germany), Merck (Darmstadt, Germany), VWR (Hannover, Germany), or Carl Roth (Karlsruhe, Germany).

Methods

The following assays were performed as previously described: (1) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), (2) protein concentration quantification, (3) determination of the melting temperature, and (4) selection of the best *P. pastoris* clones (Oroz-Guinea et al., 2019; Zorn et al., 2018a, b).

GCL-I and GCL-II Cloning and Overexpression in P. pastoris

Plasmid pGAPZα bearing either the GCL-I (GenBank accession code: AAA03429.1) or the GCL-II (GenBank accession code: AJT57367.1) encoding genes were digested using *Avr*II restriction enzyme to obtain the pure linearized plasmid. Subsequently, 5–10 μg were used to transform *P. pastoris* GS115-competent cells by

electroporation as described by Invitrogen. From each transformation, 20 colonies were picked, and the best *P. pastoris* clones were selected by screening the Zeocin resistance and the hydrolytic activity of the secreted lipases on indicator plates as previously described (Kouker and Jaeger, 1987; Zorn et al., 2018a, b).

Lipase overexpression was achieved by high cell-density culture of the previously chosen clones. The fed-batch process started by adding a freshly plated colony of the corresponding *P. pastoris* clone to 50 mL of BMGY medium in a 250 mL Erlenmeyer baffled flask. This preculture was incubated for ~48 hours (OD_{600} = ~50) at 30 °C on an orbital shaker at 180 rpm. Subsequently, 0.6 L of Basal Salts Medium (BSM, made as described by Invitrogen in "*Pichia* Fermentation Process Guidelines") contained in a 1 L fermenter vessel were inoculated with 30 mL of the corresponding preculture. In approximately 24 hours, the culture was grown until the glycerol was completely consumed (glycerol batch phase). Afterward, the cultures were fed with 50% glycerol containing 1.2% PTM1 for 5 days (glycerol fed-batch phase). The temperature was kept constant at 30 °C, and the pH was maintained between 5.0 and 6.0 using NH_4OH (30%) and H_3PO_4 (50%). The airflow and the stirrer speed were adjusted to maintain the dissolved oxygen (DO) above 20%. The fermentation process was followed by measuring the cell growth (OD_{600} and wet cell weight) of 10 mL samples taken at least once a day. In addition, protein overexpression (SDS-PAGE) and hydrolytic activity of the samples collected before culture harvesting were analyzed. Fermentation was completed by centrifuging the cultures at 4200 g for 1 hours. Supernatants were collected, frozen at −80 °C, and lyophilized.

Measurement of the Hydrolytic Activity

The hydrolytic activity of GCL-I and GCL-II was measured using a pH-stat (Titrino Plus 877, Metrohm GmbH & Co. KG, Filderstadt, Germany). The reaction medium was prepared by emulsifying 5 g of triolein and 2 g of gum Arabic in 100 mL of $CaCl_2$ (10 mM) solution at room temperature at 24,000 rpm (Ultra Turrax T25 basic, IKA Labortechnik, Staufen, Germany) for 2 min. Reactions were started by the addition of the enzyme to 10 mL of the emulsion and incubation at 25 °C for 10 min. In all cases, the reactions were performed in triplicates by titration using 10 mM NaOH of pH 7.5. One unit of lipase activity was defined as the release of 1 μmol FA per minute.

FA Enrichment Reactions

Erucic and gondoic FA enrichment was studied using two approaches: hydrolysis and ethanolysis.

In the first case, hydrolysis reactions were performed in a 2 mL polypropylene tube containing 250 μL of the corresponding substrate emulsion, 0.1 U of GCL-I or GCL-II, and distilled water for a final volume of 400 μL. The substrate emulsion was prepared by shear mixing 5 g of the desired starting oil substrate, i.e. *Crambe* oil, *Crambe* ethyl esters, *Camelina* oil, or *Camelina* ethyl esters (Solutex, Zaragoza, Spain) (Table S1); 2 g gum Arabic; and 100 mL buffer Tris/HCl (100 mM) plus $CaCl_2$ (10 mM) at pH of 7.5. Before the addition of the corresponding enzyme to the reaction, the lyophilized supernatant was dissolved in water and dialyzed, and their activity toward triolein was determined as described above. Reactions were stopped by adding 25 μL of HCl (4 N), followed by vortexing and storage at −20 °C until extraction. FA composition of the EFA and FFA fractions was monitored by analyzing time point samples taken after 0, 0.5, 1, 2, 4, 8, 24, and 48 hours. All reactions were carried out in triplicates, incubated at 25 °C, and shaken at 1400 rpm.

Ethanolysis reactions were carried out in a similar way as described above, only varying the reaction composition. In this case, the reaction mixture contained 375 μL of substrate emulsion, 75 μL of EtOH, and 1 U of enzyme (460 μL final volume).

The initial estimation of the amount of lipase to be added, sample extraction, and derivatization, as well as the analysis and quantification of the FA composition, in the different oil fractions was performed as previously described (Zorn et al., 2018a, b; see the Supplementary Information section). Chemical composition of the starting oil substrates is detailed in Table S1.

Results and Discussion

In order to investigate the lipase-catalyzed enrichment of erucic and gondoic acids, the genes encoding lipases GCL-I and GCL-II were cloned into *P. pastoris*, and lipases were overexpressed in high cell-density cultures. Proper overexpression of both proteins was verified by SDS-PAGE (Fig. S1), hydrolytic activity measurement, and determination of their melting temperature (Table S2).

Enrichment of Erucic Acid by Hydrolysis of *Crambe* Oil and *Crambe* Ethyl Ester Derivatives

As mentioned previously, in order to enrich a desired FA, plant oils can be used in their original TAG form or as FA methyl/FAEE. The choice of substrate formulation might influence not only the enrichment achieved but also the recovery yield of the required FA. Thus, lipases I and II from *G. candidum* were studied for the enrichment of erucic acid using as a substrate either *Crambe* oil, i.e. TAG, or its FAEE derivatives.

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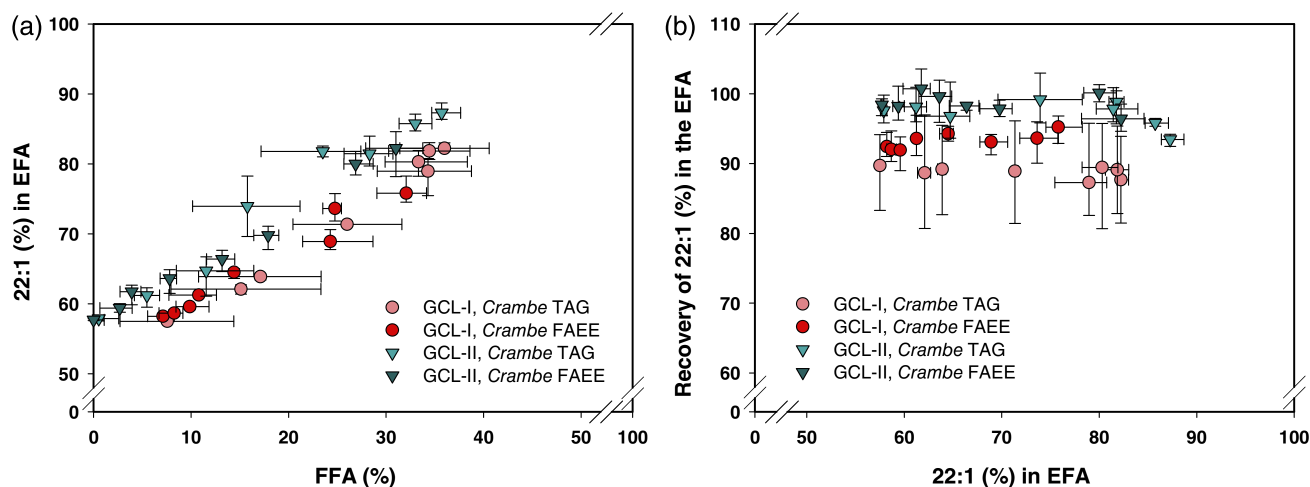

Fig. 1 Analysis of the erucic acid enrichment using either GCL-I or GCL-II as biocatalysts and *Crambe* oil or its FAEE derivatives as starting substrate for 48 hours (time point samples taken after 0, 0.5, 1, 2, 4, 8, 24, and 48 hours). (a) Content of 22:1 in the EFA fraction during the hydrolysis reaction and (b) theoretical recovery percentage of 22:1 that could be achieved with a determined 22:1 enrichment in the EFA fraction. The recovery is defined as the amount of 22:1 present in the enriched fraction in comparison to the total amount of 22:1 in the sample. Please note that 100% is the maximum recovery, and values given in panel b take into account the deviation of data from three independent measurements

As can be observed from Fig. 1a, after 24 hours of reaction, the hydrolysis level (i.e. the percentage of hydrolyzed FA over the total amount of FA present) achieved was approximately 30% of the total content of FA, independent of the substrate or enzyme added. These values did not significantly increase after 48 hours, indicating that the equilibrium threshold was reached. The concentration of 22:1 in the EFA fraction increased from approximately 60% of the starting material (Table S1) to 76–96% as the hydrolysis of the substrates proceeded. When acting on the TAG, the enrichment values (i.e. the percentage of a specific FA present in a determined oil fraction over the total amount of FA in that oil fraction) were slightly better, albeit noticeable, for both isoenzymes. Hence, when the TAG oil was used, the maximum erucic acid enrichment values were 82% and 87% for GCL-I and GCL-II, respectively. On the other hand, the highest concentrations in the FAEE derivatives reaction were 76% and 82%. Given that the initial FA composition and the hydrolysis degree at the maximum enrichment value were similar for both substrates, the variation in purity achieved can only be explained by the different selectivities displayed by the lipase isoenzymes depending on the structure of the ester molecule hydrolyzed. It is worth noting that GCL-II allowed the highest concentration values, despite having a lower specific activity and preference of this enzyme toward *cis*Δ9 unsaturated FA when compared to GCL-I (Holmquist, 1998; Holmquist et al., 1997). Theoretical recovery yields accomplished were also excellent. As can be seen in Fig. 1b, the recovery values remained the same during the reaction time, representing between 90% and 96% of the total erucic acid present in the substrates.

Comparison of the results achieved in this work with those previously reported in the literature using GCL as biocatalysts (McNeill and Sonnet, 1995; Sonnet et al., 1993) is difficult because, in those studies, the reactions were not extensively monitored along time, and only one or two time point samples were analyzed. For instance, Sonnet et al. reported the GCL-mediated enrichment of erucic acid from rapeseed oil (37.5% initial 22:1 content), obtaining up to 79% of 22:1 enrichment in the EFA fraction after 70% of oil hydrolysis (Sonnet et al., 1993). In a similar example, McNeill and Sonnet reported that the maximum accumulation of erucic acid was achieved in the form of diacylglycerides (DAG) were the enrichment reached 85% of the total composition. This implies that the 22:1 enrichment of the total EFA fraction, i.e. the addition TAG, DAG, and monoacylglycerides (MAG), was lower (McNeill and Sonnet, 1995). These enrichment values greatly resemble the ones obtained in this study for GCL-I. This is consistent with the fact that, in both cases, crude preparations of enzymes were used for biocatalysis as *G. candidum* produces four kinds of lipase isoenzymes (GCL I-IV), with GCL-I as the major component (Sugihara et al., 1991).

Generally, when comparing these results with those previously obtained for the best 22:1-enriching CAL-A variants during the hydrolysis of *Crambe* oil or FAEE (Table S3), it can be observed that higher enrichment and recovery yields could be achieved with both GCLs. This difference was especially acute when using the oil as the initial substrate. In particular, the 22:1 concentration achieved in the enriched fraction with GCL-II was 11% higher than in the reactions with CAL-A V238L/V290L

and 14% higher than in the reactions with CAL-A V286N. Recovery values were increased by 21% and 14% compared to these variants, respectively. Hence, GCL-II represents the best alternative to enrich erucic acid from both *Crambe* oil and its FAEE derivatives.

Enrichment of Gondoic Acid by Hydrolysis of *Camelina* Oil and *Camelina* Ethyl Ester Derivatives

In a similar manner, the enrichment of gondoic acid was studied using *Camelina* oil and its ethyl ester derivatives.

When the biocatalysis reactions were performed with GCL-I, it could be observed that, after 48 hours, the hydrolysis values had reached the equilibrium, at 57% and 36% for the oil and the FAEE derivatives, respectively (Fig. 2a). Maximum enrichment values were achieved after 8 and 48 hours when the desired FA represented 33% and 25% of the ester bound fraction, respectively. At that point, in both cases, the recovery yields were above 94% and did not significantly change during the whole course of the reaction (Fig. 2b).

Concerning GCL-II, the hydrolysis values found after 48 hours were much higher than those obtained with GCL-I, constituting 80% and 59% for *Camelina* oil and FAEE, respectively (Fig. 2a). At that point, the 20:1 concentration represented 52% and 45% for each of the substrates. The recovery yield in the reaction with GCL-II and *Camelina* FAEE remained stable (>99%) along the reaction time. However, in the reactions involving *Camelina* oil as substrate, the high level of accumulation entailed a decrease of the recovery value to 62% of the total gondoic acid present in the sample at the maximum enrichment. Fortunately, a recovery yield of 88% could be achieved, thus obtaining an EFA containing 50% of 20:1 (Fig. 2b).

Enrichment of this FA represents a major challenge due to two non-exclusive reasons that must be taken into account to explain the lower levels achieved for gondoic acid: (1) its chain length only differs by two carbon atoms, 20, from the chain length of the majority of FA that should be hydrolyzed, 18, and (2) the initial concentration of gondoic acid in the oil is much lower compared to erucic acid, being approximately 15% (Table S1).

It is worth noting that, in this case as well, GCL-II displayed better enrichment values, ~20%, with both substrates (50% and 45%) than GCL-I (33% and 25%), although lower levels of recovery for the GCL-II plus *Camelina* oil combination (88% vs. 95%) were obtained. Moreover, to the best of our knowledge, these are the highest values described until now for the biocatalytic enrichment of gondoic acid. A previous attempt by our group to enrich 20:1 from *Camelina* FAEE utilizing CAL-A V290W led to enrichment and recovery values of 34% and 52%, respectively (Zorn et al., 2018b).

Ethanolysis of *Crambe* and *Camelina* Oils

Esterification and transesterification reactions catalyzed by lipases might entail a change in selectivity compared to the FA hydrolysis (Zorn et al., 2018a). A plausible reason for this may be that the intermediate acylated enzymes, when reacting with alcohols, are exposed to nucleophiles larger than water, which may offer an added basis for discrimination. For that reason, the behavior of both *G. candidum*

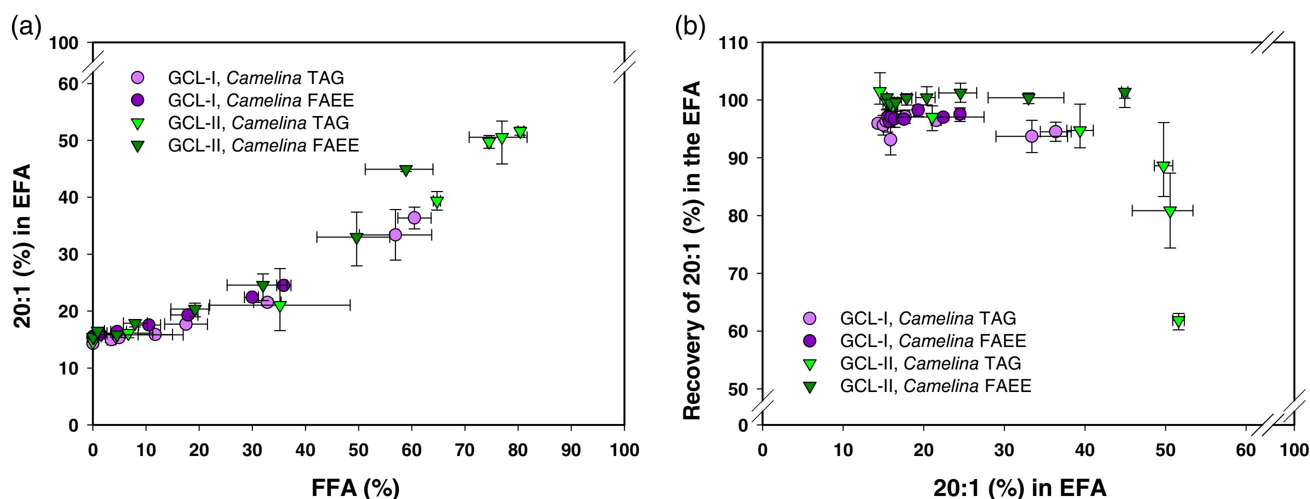

Fig. 2 Analysis of the gondoic acid enrichment using either GCL-I or GCL-II as biocatalysts and *Camelina* oil or its FAEE derivatives as starting substrate for 48 hours (time point samples taken after 0, 0.5, 1, 2, 4, 8, 24, and 48 hours). (a) Content of 20:1 in EFA fraction during the hydrolysis reaction and (b) theoretical recovery percentage of 20:1 that could be achieved with a determined 20:1 enrichment in the EFA fraction. The recovery is defined as the amount of 20:1 present in the enriched fraction in comparison to the total amount of 20:1 in the sample. Please note that 100% is the maximum recovery, and values given in panel b take into account the deviation of data from three independent measurements

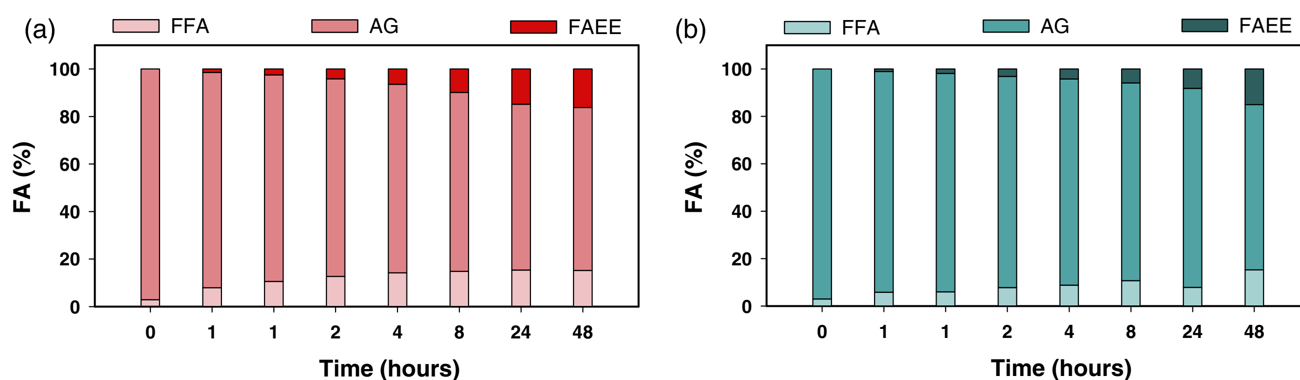

Fig. 3 Each plot shows the total amount of FA in fractions of FAEE, AG, and FFA determined during the biocatalytic ethanolysis of *Crambe* oil with (a) GCL-I and (b) GCL-II

isoforms was studied for the enrichment of 22:1 from *Crambe* oil and 20:1 from *Camelina* oil using ethanolysis reactions.

Ethanolysis of Crambe *Oil*

GCL-I and GCL-II ethanolysis with *Crambe* oil as substrate produced similar reaction mixtures. In both cases, after 48 hours of reaction, the FFA, acylglyceride (AG, which comprises TAG, DAG, and MAG), and FAEE fractions accounted for approximately 15, 69, and 15% of the total FA mixture, respectively (Fig. 3), thus indicating the existence of hydrolysis as a side reaction, which was not totally unexpected as the biotransformations were performed in an aqueous medium.

The composition of these three fractions was analyzed. The data retrieved for the reactions involving GCL-I concerning the enrichment of 22:1 in the AG fraction were similar to those observed during the hydrolysis reaction (Fig. 4). Hence, an 82% enrichment of 22:1 was achieved while maintaining the same value of recovery yield present at the beginning of the reaction (94%). On the other hand, when GCL-II was used, the maximum enrichment reached was 77% with a recovery of 90%. In spite of the excellent nature of these values, they were lower than those described above for the hydrolytic reaction using this lipase (87% and 93%, respectively).

These results are remarkably better than those obtained before involving CAL-A wt or its best variant in terms of enrichment and recovery (Zorn et al., 2018a). CAL-A wt was enriched to a maximum of 77% in the FAEE fraction, achieving a very low theoretical recovery (25%). Likewise, CAL-A V238L/V290L exhibited 71% 22:1 in the most enriched AG fraction with a theoretical recovery of 40%.

Interestingly, in both cases, concomitant with the enrichment of 22:1 in the AG fraction, an enrichment of oleic (18:1, *cis*Δ9), linoleic (18:2, *cis*Δ9, *cis*Δ12), and linolenic (18:3, *cis*Δ9, *cis*Δ12, *cis*Δ15) acids was observed in the FAEE fraction. Hence, these 18*cis*Δ9 unsaturated FA (18_{UFA}) were accumulated as ethyl esters, representing 99% of the FAEE fraction in the reactions catalyzed by GCL-I and 92% in the reactions performed with GCL-II. This translated into recoveries of 56% and 48% of the total content of these FA in the whole sample (Fig. 4).

When comparing the enrichment values of 18_{UFA} in the ethanolysis FAEE fraction after 48 hours to those obtained for the FFA fraction during the hydrolysis of *Crambe* oil, it can be appreciated that, during the hydrolysis, the enrichment values decreased to 81% (89% recovery) and 69% (98% recovery) for GCL-I and GCL-II, respectively.

Ethanolysis of Camelina *oil*

In a similar manner as that described for *Crambe* oil, *Camelina* TAG were subjected to ethanolysis with each GCL isoform.

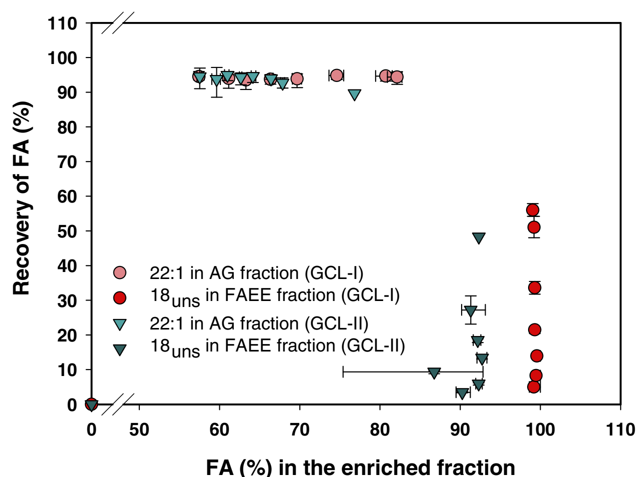

Fig. 4 Recovery percentage of 22:1 and 18_{UFA} achieved in their corresponding enriched fractions, i.e. the AG fraction and the FAEE fraction, respectively, during the ethanolysis reaction of *Crambe* oil using GCL-I and GCL-II as biocatalysts

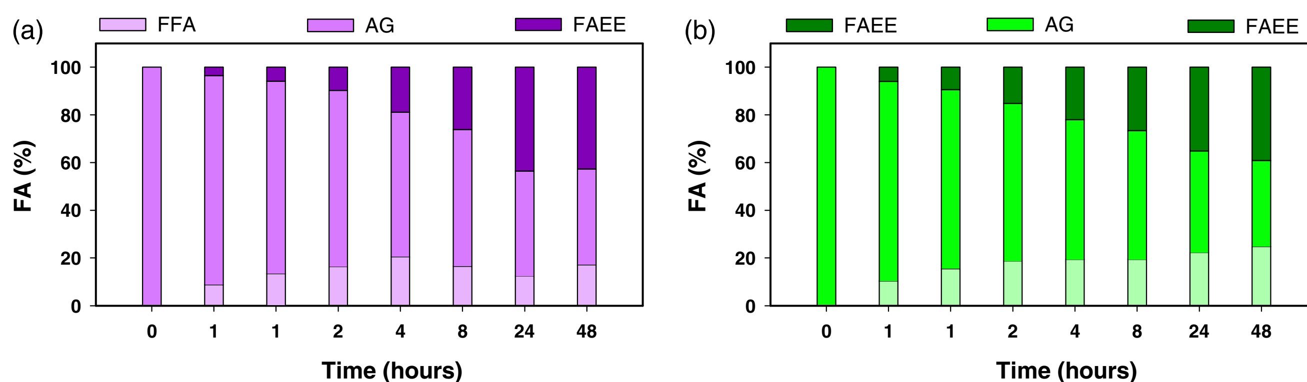

Fig. 5 Each plot shows the total amount of FA in fractions of FAEE, AG, and FFA determined during the biocatalytic ethanolysis of *Camelina* oil with (a) GCL-I and (b) GCL-II

In this case, the percentage of each one of the oil fractions differed from the *Crambe* oil reactions due to the lower concentration of FA longer than 18 carbon atoms present in the *Camelina* oil (Fig. 5). At the end of the reactions, the percentage of AG was lower (40% for GCL-I and 36% for GCL-II), while the FAEE was noticeably increased (43% for GCL-I and 39% for GCL-II). The amount of FFA derived from the hydrolysis side reaction also increased, although not proportionally (17% for GCL-I and 24% for GCL-II).

Regarding the composition of the AG fraction, a maximum enrichment of 44% of 20:1 was achieved in the reactions with GCL-I after 48 hours. This percentage diminished to 28% for GCL-II in the same amount of time. Compared to the results observed during the hydrolysis, 20:1 enrichment in the AG fraction remained stable and above 97% along the reaction with GCL-I but decreased to 60% when GCL-II acted on the oil. Nevertheless, in the latter case, the reaction could be stopped after 4 hours to increase the recovery up to 92% with a 24% enrichment of 20:1 (Fig. 6).

Compared to the *Camelina* oil hydrolysis biocatalysis, GCL-I displayed improved enrichment values of 20:1 (44% for the ethanolysis and 36% for the hydrolysis), exhibiting similar recovery values. In contrast, GCL-II 20:1 accumulation during the ethanolysis (24%) was half of that observed during hydrolysis (50%), although the recovery was slightly better (92% and 88% for the ethanolysis and hydrolysis, respectively) (Fig. 6).

Concomitantly, enrichment of 18_{UFA} was also accomplished in these reactions. Once again, the FAEE produced by GCL-I were almost completely formed by oleic, linoleic, and linolenic FA, containing less than 2% of other FA. GCL-II also presented enrichment values above 90%, although it was slightly inferior (91%). In terms of recovery, 65% and 55% of the total 18_{UFA} could be found in the FAEE fraction for GCL-I and GCL-II, respectively (Fig. 6). As mentioned above for *Crambe* oil, the 18_{UFA} enrichment levels of the FAEE during ethanolysis were considerably higher than those obtained in the FFA fraction of the hydrolysis reactions, being 55% (84% recovery) and 83% (93% recovery) for GCL-I and GCL-II in the latter case, respectively.

These results demonstrate that it is necessary to take into account the exact enrichment reaction when selecting the best biocatalyst. In this sense, on the one hand, GCL-II was the best alternative when performing the enrichment reaction through hydrolysis of the substrate, while on the other hand, GCL-I should be the preferred biocatalyst for the ethanolysis approach. This is because, while GCL-I retained its selectivity independent of the reaction type, for GCL-II, the ethanolysis implied a reduction in its discrimination against FA longer than 18 carbon atoms.

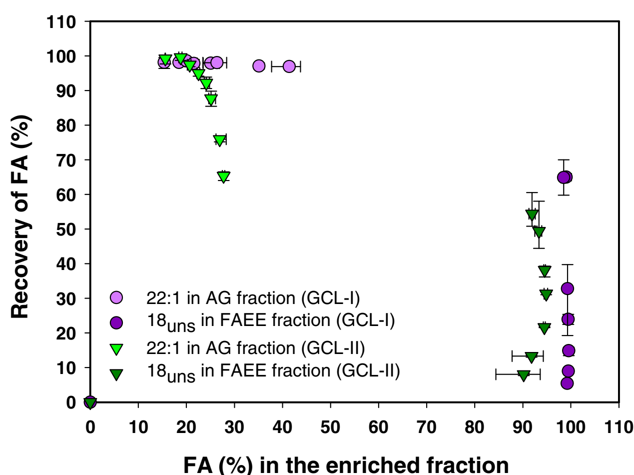

Fig. 6 Recovery percentage of 20:1 and 18_{UFA} achieved in their corresponding enriched fractions, i.e. the AG fraction and the FAEE fraction, respectively, during the ethanolysis reaction of *Camelina* oil using GCL-I and GCL-II as biocatalysts

It is worth noting that both enzymes demonstrated their ability to create an FAEE fraction composed of more than 90% 18_{UFA} while simultaneously enriching the AG fraction with 22:1 or 20:1 FA. To the best of our knowledge, there were no prior studies reporting the use of ethanolysis catalyzed by these lipases to produce this double enrichment. This is particularly surprising as the discrimination of these enzymes against FA longer than 18 carbon atoms is well known (and has been for several decades), as well as the predilection of GCL-I, and to a lesser extent GCL-II, toward FA containing a *cis*Δ9 double bond during hydrolysis and esterification reactions (Bertolini et al., 1995; Haas et al., 1999; Holmquist, 1998; Holmquist et al., 1997; Veeraragavan et al., 1990). Moreover, the production of such a highly enriched 18_{UFA} FAEE fraction could find different applications in industry.

Conclusions

In summary, the research described here demonstrates that lipase selectivity is a powerful tool to replace conventional separation technologies for purification of distinct FA from natural oils by a more environment-friendly process. In particular, the specific behavior of two GCL isoforms, GCL-I and -II, was investigated in the discrimination of erucic and gondoic FA. In this sense, both lipases constitute suitable alternatives for the enrichment of the aforementioned FA. Nevertheless, interesting particularities arose during their application in the different biocatalytic reactions, i.e. hydrolysis and ethanolysis. GCL-II achieved higher accumulation values during the hydrolysis reactions independent of the FA to be enriched or the substrate formulation used. On the other hand, the best values for the ethanolysis processes were exhibited by GCL-I. This highlights the relevance of selecting the appropriate biocatalyst depending on the biotransformation and the convenience of avoiding the use of isoenzyme mixtures.

Furthermore, to the best of our knowledge, the values reported in this study are the best ones regarding enrichment and recovery for both erucic and gondoic FA by means of a lipase-catalyzed process. In addition, when using GCL-I and GCL-II in ethanolysis reactions with both oils, a highly enriched 18_{UFA} FAEE fraction could be obtained without decreasing the enrichment or recovery of the FA with a longer chain length. Hence, this resulted in a double FA enrichment in a single step that could be very valuable from the oleochemical point of view.

Acknowledgments The COSMOS project has received funding from the European Union's Horizon 2020 Research and Innovation Program under grant agreement No. 635405.

Conflict of Interest The authors declare that they have no conflict of interest.

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