Industrial Crops & Products 139 (2019) 111579

Contents lists available at ScienceDirect

Industrial Crops & Products

journal homepage: www.elsevier.com/locate/indcrop

Release of phenolic acids from sunflower and rapeseed meals using different carboxylic esters hydrolases from *Aspergillus niger*

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ARTICLE INFO

Keywords:
Rapeseed and sunflower meals
Aspergillus niger
Chlorogenate esterase
Feruloyl esterase
Sinapic acid
Caffeic acid

ABSTRACT

Sunflower and rapeseed meals are agro-industrial coproducts that contain high amount of phenolics (1–4 % dry matter), mostly as esters of caffeic acid (CA) and sinapic acid (SA), respectively. The enzymatic hydrolysis of the ester bonds enables to recover the corresponding free phenolic acids that are bioactive compounds and platform molecules for various applications in green chemistry. Here we aimed to find the best route for producing free CA and SA by applying various fungal carboxylic ester hydrolases from recombinant *Aspergillus niger* strains either directly on crude meal or on their phenolic extracts obtained by methanolic extraction. Two types of meals were studied: (i) industrial (commercial) meals (I-meals), produced by a process that includes cooking at 95–100 °C and steam desolventizing at 105–107 °C, and (ii) non-industrial meals (NI-meals) obtained at pilot-scale with much milder heat treatment, that offer a higher total phenolic content. CA release through hydrolysis of sunflower meal (SFM) was successfully achieved with *A. niger* type-B feruloyl esterase (AnFaeB) and chlorogenic acid esterase (ChlE). Maximal amount of free CA released was of 54.0 ± 1.1 to 59.8 ± 2.1 µmol/g defatted dry matter (DDM) from I-SFM (94–100% hydrolysis yield) against 42.0 ± 1.1 to 52.3 ± 0.2 µmol/g DDM (59–73% hydrolysis yield) from NI- SFM in which CA release was hampered by a phenolic oxidation side-reaction, seemingly due to meal endogenous polyphenol oxidase activities. AnFaeB and ChlE hydrolysis of phenolic extracts from NI-SFM increased the CA amount obtained to 55.0–68.1 µmol/g DDM (77–95% hydrolysis yield). In all cases, AnFaeB showed broader specificity towards SFM caffeoyl quinic acid isomers than ChlE. In particular, ChlE did not hydrolyze 3-*O*-caffeoylquinic acid. The maximal amount of free SA released by AnFaeA hydrolysis was 41.3 ± 0.3 µmol/g DDM from NI-SFM (50% hydrolysis yield) and 32.3 ± 0.4 µmol/g DDM from the phenolic extract (64% hydrolysis yield), with AnFaeA also having sinapine transesterification activity that led to the synthesis of 1,2-di-*O*-sinapoyl-β-D-glucose. Finally, of all the substrates tested for enzymatic hydrolysis in our conditions, I-RSM and NI-SFM extract showed the best compromise between initial total phenolic content, hydrolysis yields and amounts of CA/SA released.

Abbreviations: 1,2-diSG, 1,2-di-*O*-sinapoyl-β-D-glucose; 3-CQA, 3-*O*-Caffeoylquinic acid; 3,4-diCQA, 3,4-di-*O*-caffeoylquinic acid; 3,5-diCQA, 3,5-di-*O*-caffeoylquinic acid; 4-CQA, 4-*O*-Caffeoylquinic acid; 4,5-diCQA, 4,5-di-*O*-caffeoylquinic acid; 5-CQA, 5-*O*-Caffeoylquinic acid; *A. niger*, *Aspergillus niger*; ACE, angiotensin-I converting enzyme; AnFaeA, *A. niger* type-A feruloyl esterase; AnFaeB, *A. niger* type-B feruloyl esterase; CA, caffeic acid; CAE, caffeic acid equivalents; CAEsters, caffeic acid esters; ChlE, chlorogenic acid esterase; DDM, defatted dry matter; DM, dry matter; HPLC, high performance liquid chromatography; I, industrial; I-meals, industrial meals; I-RSM, industrial rapeseed meal; I-SFM, industrial sunflower meal; MBTH, 3-methyl-2-benzothiazolinonehydrazone; MOPS, 3-(*N*-morpholino) propanesulfonic acid; nkat, nanokatal; NI, non-industrial; NI-meals, non-industrial meals; NI-RSM, non-industrial rapeseed meal; NI-SFM, non-industrial sunflower meal; PPO, polyphenol oxidase; RSM, rapeseed meal; SA, sinapic acid; SAE, sinapic acid equivalents; SAEsters, sinapic acid esters; SFM, sunflower meal; SNP, sinapine; TCAD, total caffeic acid derivatives; TPC, total phenolic compounds; TSAD, total sinapic acid derivatives

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https://doi.org/10.1016/j.indcrop.2019.111579
Received 3 April 2019; Received in revised form 23 June 2019; Accepted 16 July 2019
Available online 25 July 2019

1. Introduction

Rapeseed (*Brassica napus* L.) and sunflower (*Helianthus annuus* L.) are two of the most important oilseed crops in the world (Lomascolo et al., 2012) and their oils are mainly used for human consumption or for biofuel production (Borredon et al., 2011; Fine et al., 2015). Rapeseed (RSM) and sunflower (SFM) meals are the main co-products of the seed pressing and de-oiling processes. In 2018, worldwide production of RSM and SFM was estimated at 39.2 Mt and 21.0 Mt respectively (http://www.indexmundi.com/agriculture/). They are mostly dedicated to animal feed because of their high protein content with about 38 g/100 g dry matter (DM) and 28 g/100 g DM for RSM and SFM respectively (González-Pérez and Vereijken, 2007; Ivanova et al., 2016; Ugolini et al., 2015). In addition, studies have shown that these raw materials can also serve as a source of protein isolates with bioactive properties, such as Angiotensin-I Converting Enzyme (ACE) inhibitory activities, antioxidant or antithrombotic activities, and bile acid-binding capacities (Aider and Barbana, 2011; Campbell et al., 2016; Ugolini et al., 2015). These protein isolates also possess emulsification and foaming properties and are considered as promising ingredients for industrial non-food applications such as adhesives and detergents, among others (Fetzer et al., 2018, 2019). Moreover, RSM and SFM can also be used as substrates in microbial fermentative processes like saccharification and production of enzymes, antibiotics and other bioproducts (for a review, see Lomascolo et al., 2012). Also, as outlined in the work of Licata et al. (2018), these meals represent a potential source of energy production through combustion processes or biogas production and could be considered as economically convenient in a short agro-energy chain.

RSM and SFM also have relatively high total phenolic compound (TPC) contents, ranging from 1 to 2 g/100 g DM for RSM and 2–4 g/100 g DM for SFM. These phenolics are almost exclusively sinapic acid esters (SAEsters) in RSM, with sinapine as the main derivative (80% of the TPC content) and the remaining 20% composed of mono-, di- and tri-sinapoyl esters of sugars and/or kaempferol (Baumert et al., 2005; Milkowski and Strack, 2010; Siger et al., 2013). In SFM, phenolics are mostly found as caffeic acid esters (CAEsters). 5-Caffeoylquinic acid (chlorogenic acid) is the major phenolic compound accounting for 70% of the TPC content, while the remaining 30% are other mono- and di-caffeoylquinic acids, as well as coumaroyl- and feruloylquinic acids. Free caffeic (CA) acid is also found in SFM but accounts for just 0.5% of the TPC content (Guo et al., 2017; Szydłowska-Czerniak et al., 2011; Weisz et al., 2009).

Phenolic acids, particularly hydroxycinnamic acids, are bioactive molecules with interesting properties that include antioxidant (Gullón et al., 2018; Shahidi and Ambigaipalan, 2015), anti-inflammatory (Oueslati et al., 2012) and antimicrobial (Ouerghemmi et al., 2017) activities among others. These bioactive properties can be enhanced by modifying the hydrophilic/hydrophobic balance of the molecules (Laguerre et al., 2013; Lecomte et al., 2010; Szydłowska-Czerniak et al., 2018). For example, esterification with aliphatic alcohols of adequate chain-length improved their antioxidant activity in liposome suspensions, oil-in-water emulsions (Laguerre et al., 2010; Sørensen et al., 2015) and even in cultured cells, as well as their bactericidal activity on selected bacteria (Suriyarak et al., 2013). This phenomenon, related to the so-called “cut-off effect”, was attributed to a better location and concentration of esters at the interfacial membrane where lipid oxidation occurs (Laguerre et al., 2011; McClements and Decker, 2018), and to their ability to cross or interact with membranes of living cells and microorganisms. Moreover, owing to their particular structure and reactivity, phenolic acids (*i.e.* hydroxybenzoic and hydroxycinnamic acids), as their corresponding vinyl phenols, have gained much importance during the last decade for the synthesis of new sustainable polymers and pre-polymers (Aouf et al., 2012; Diot-Néant et al., 2017; Pion et al., 2014; Reano et al., 2015; Wang et al., 2011; Zago et al., 2015a). So any raw material and process enabling large-scale production and recovery of phenolic acids would positively facilitate the development of these sustainable active platform molecules and polymers. In this context, SFM and RSM, which are cheap, abundant, and rich in phenolics, make perfectly suitable candidates. It is worth noting that only few studies have reported the use of these two raw materials for the production of phenolic acids and derivatives like vinyl phenols (Kreps et al., 2014; Odinot et al., 2017).

Microbial cinnamoyl and feruloyl esterases are carboxylic ester hydrolase enzymes (EC 3.1.1) that are able to hydrolyze ester linkages between hydroxycinnamic acids and sugars present in plant cell-walls (Faulds, 2010) with various *in vivo* specificities according to type of enzyme and origin of microorganism. For instance, the type-A feruloyl esterase from the filamentous fungus *Aspergillus niger* (called AnFaeA) is highly active on sinapic and ferulic acid methyl esters and on some 1,5-esterified feruloylated oligomers from cereals. Type-B feruloyl esterase from *A. niger* (so called AnFaeB) is rather active on caffeic and *p*-coumaric acid methyl esters and on some 1,2- and 1,6-esterified feruloylated oligomers from sugar-beet pulp (Brézillon et al., 1996; Faulds and Williamson, 1994). Another type of *A. niger* carboxylic ester hydrolase, called chlorogenic acid hydrolase (or chlorogenate esterase, ChlE) was primarily found to be highly specific to chlorogenic acid (5-*O*-caffeoylquinic acid) hydrolysis (Benoit et al., 2007). Historically, pure AnFaes were shown to be essentially inactive when used directly on natural raw substrates. However, we recently studied the enzymatic hydrolysis of an industrial rapeseed meal (I-RSM) using a recombinant AnFaeA to produce sinapic acid (Odinot et al., 2017) and demonstrated that this enzyme was highly effective on the raw RSM. In the best conditions, AnFaeA hydrolyzed most of the SAEsters present in the RSM, with an overall yield of 68–76 % (100% hydrolysis of sinapine).

Taking this previous work forward, here we aimed to find the best route and operating conditions for producing free CA and SA by applying various fungal carboxylic ester hydrolases from recombinant *Aspergillus niger* strains either directly on crude meal or on their phenolic extracts obtained by methanolic extraction. Two types of meals were studied: (i) I-meals (industrial meals), produced by a process that includes cooking at 95–100 °C and steam desolventizing at 105–107 °C, and (ii) NI-meals (custom-made non-industrial meals), obtained at pilot-scale with much milder heat treatment. In a general way, the NI-meals have a net higher phenolic content than the corresponding I-meals (Zago et al., 2015b). We first studied the hydrolysis of sunflower meals (NI-SFM and I-SFM) with two types of fungal ester hydrolases and determined the best conditions for caffeic acid (CA) production. Exploiting their hydrolytic activity on CAEsters (Faulds, 2010; Gopalan et al., 2015; Ramos-de-la-Peña and Contreras-Esquivel, 2016), we tested an *A. niger* type-B recombinant feruloyl esterase (AnFaeB) and a recombinant *A. niger* chlorogenate esterase (ChlE) on the SFMs. To the best of our knowledge, these two enzymes have never been used on oilseed meals. Enzymatic hydrolysis was also performed on methanolic extracts from NI-SFM which are mainly composed of phenolics, minerals, and carbohydrates (Sripad and Narasinga Rao, 1987) but are devoid of the proteins and other meal constituents. Then, the same approach was applied to a non-industrial rapeseed meal (NI-RSM) and the corresponding phenolic extract, by using AnFaeA to release free SA. The results (hydrolysis yield and highest released SA amount) were compared with our previous data obtained on an I-RSM (Odinot et al., 2017). All cases were compared and discussed based on the following parameters: hydrolysis yields, qualitative and quantitative composition of hydrolyzed hydroxycinnamoyl esters, and free CA or SA amount released per gram of substrate.

2. Materials and methods

2.1. Chemicals

Sinapine thiocyanate (99.0% HPLC) was isolated from rapeseed meal according to the method outlined by Mailer (2008). 3,4-di-*O*-

caffeoylquinic, 3,5-di-*O*-caffeoylquinic and 4,5-di-*O*-caffeoylquinic acids (> 99.0% HPLC) were isolated from yerba mate leaves, following the procedure previously described in Tong et al. (2015). Sinapic acid (98%) was from AlfaAesar (Karlsruhe, Germany). 4- and 5-caffeoylquinic (chlorogenic) acids (95%), caffeic acid (> 98%), methanol and water (both HPLC grade, > 99.9%), NaOH (> 97%) and MOPS (3-(N-morpholino)propanesulfonic acid) buffer were purchased from Sigma–Aldrich (Saint-Quentin-Fallavier, France). ACS grade ethyl acetate (> 995%) was purchased from Honeywell Riedel-de-Haën (Seelze, Germany)

2.2. Plant materials

2.2.1. Preparation of the non-industrial sunflower and rapeseed meals (NI-SFM and NI-RSM)

NI-SFM and NI-RSM were prepared at OLEAD oilseed–protein crops technology platform's pilot oilseed plant (Pessac, France), according to the following methods. Whole sunflower (*Helianthus annuus* L.) seeds (75 kg) were first cold flaked in a flaker (Damman-Croes N.V., Roeselare, Belgium) equipped with two smooth 500 mm-diameter contra-rotating cylinders spaced at 0.3 mm (capacity: 200 $kg.h^{-1}$). Whole rapeseed (*Brassica napus* L.) seeds (120 kg) were first cold pressed on a MBU20 screw press (OLEXA®, Feuchy, France), fed at 75 $kg.h^{-1}$ of seeds, to remove 75% of the initial oil amount. The residual oil from the sunflower or rapeseed press cakes was further extracted by steeping in hexane at 50 °C, in a 480 L-capacity Guedu Pilot Agitated Filter Dryer (De Dietrich Process Systems, Semur, France). Extraction was performed on 73 kg of each press cake by immersion in hexane for 15 min followed by filtration. Five or six successive immersion-filtration steps were required to remove the oil from the SFM and RSM, respectively. The defatted meals were then desolventized in the same device, under vacuum at 60 °C and without steam injection. Then, the meals obtained were finely ground by a high-shear impact mill (Hosokawa-alpine, type UPZ100, Augsburg, Germany) using a 0.1-mm (for NI-RSM) or a 0.5-mm (for NI-SFM) grid operated at room temperature at a speed of 18,000 rpm, and a feeder speed of 4 $kg.h^{-1}$.

2.2.2. Industrial sunflower and rapeseed meals (I-SFM and I-RSM)

I-SFM and I-RSM were provided by the Technical Centre for Oilseed Crops, Grain Legumes and Industrial Hemp (Terres Inovia, Pessac, France). I-SFM and I-RSM were obtained from seed pressing and hexane solvent oil extraction. Further industrial processing steps included: preconditioning at about 45 °C, cooking at 95–100 °C for 60 min, then steam desolventizing at 107 ± 2 °C for 80 ± 5 min in the case of I-RSM or 105 ± 2 °C for 50 ± 5 min in the case of I-SFM.

2.3. Enzyme production

The extracellular recombinant feruloyl esterase A (AnFaeA), feruloyl esterase B (AnFaeB) and chlorogenate esterase (ChlE) were produced, respectively, by the recombinant strains *A. niger* BRFM451, *A. niger* BRFM766 and *A. niger* BRFM891, formerly obtained in our Laboratory of Fungal Biodiversity and Biotechnology (UMR1163 BBF, Marseille, France), by genetic engineering of the host strain *A. niger* D15#26, as previously described (Benoit et al., 2007; Levasseur et al., 2004; Record et al., 2003). Briefly, in this study, these strains were used to produce batches of AnFaeA, AnFaeB and ChlE, respectively, in a culture medium containing (per liter contents): glucose 50 g, $NaNO_3$ 5.95 g, KCl 0.52 g, $MgSO_4{\cdot}7H_2O$ 0.49 g, and 1 ml of trace elements (1000× stock, per liter: $ZnSO_4{\cdot}7H_2O$ 21.85 g, H_3BO_3 11 g, $MnCl_2{\cdot}4H_2O$ 4.95 g, $FeSO_4{\cdot}7H_2O$ 5 g, $CoCl_2{\cdot}6H_2O$ 1.69 g, $CuSO_4{\cdot}5H_2O$ 1.6 g, $Na_2MoO_4{\cdot}2H_2O$ 1.5 g, EDTA-$Na_2{\cdot}2H_2O$ 64.76 g) in a 0.1 M citrate-sodium phosphate buffer at pH5 (Levasseur et al., 2004).

2.4. Accession number of protein sequences

The protein sequences of AnFaeA, AnFaeB and ChlE were registered in the NCBI database under accession numbers CAA70510, AJ309807, and ABK62698, respectively.

2.5. Enzyme activity assay

Esterase activity was assayed spectrophotometrically at either 37 °C and 55 °C for AnFaeA or 37 °C and 50 °C for AnFaeB and ChlE, as previously described (Odinot et al., 2017), by monitoring the A_{335} with respect to the rate of hydrolysis of 0.032 mM of the enzyme substrate in 88 mM of a 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer (pH 5.5 for AnFaeA and pH6 for AnFaeB and ChlE). The synthetic substrates used were methyl sinapate for AnFaeA, methyl caffeate and methyl *p*-coumarate for AnFaeB, and chlorogenic acid for ChlE. The extinction coefficients at 335 nm were (in $L.mol^{-1}.cm^{-1}$): 13,318 for methyl sinapate, 5,500 for sinapic acid, 19,524 for methyl ferulate, 4,409 for ferulic acid, 12,560 for methyl caffeate, 6,060 for caffeic acid, 821 for methyl *p*-coumarate, 673 for *p*-coumaric acid and 15,423 for chlorogenic acid.

Endogenous polyphenol oxidase (monophenol, *o*-diphenol:oxygen oxidoreductase, EC 1.14.18.1) activity was determined in SFM as per a procedure adapted from Halaouli et al. (2005). Five grams of meal were homogenized in 50 ml sodium phosphate buffer (100 mM, pH 6.8) at 37 °C for 3 h under stirring at 200 rpm. After centrifugation at 9500 × *g* for 40 min, the supernatant was used to assay monophenol oxidase (or cresolase) and diphenol oxidase (or catecholase) activity (Halaouli et al., 2005). Briefly, monophenolase activity was determined in meal extract by monitoring the A_{484} (extinction coefficient 22,300 $L.mol^{-1}.cm^{-1}$) with respect to rate of oxidation of 1.25 mM L-tyrosine in sodium phosphate buffer (50 mM, pH6.8) at 30 °C, in the presence of 3-methyl-2-benzothiazolinonehydrazone (MBTH, 5 mM) and L-DOPA using a molar L-DOPA-to-L-tyrosine ratio of 0.057. Diphenolase activity was determined by monitoring A_{484} (extinction coefficient 22,300 $L.mol^{-1}.cm^{-1}$) with respect to rate of oxidation of 4.2 mM L-DOPA in sodium phosphate buffer (50 mM, pH6.8) at 30 °C, in the presence of 5 mM MBTH.

All enzymatic activities were expressed in nanokatals. One nanokatal of activity is defined as the quantity of enzyme that hydrolyzed 1 nmol of substrate per second. Experiments were performed in triplicate, and the standard deviation was lower than 1% of the mean.

2.6. Determination of meal dry matter and lipid contents

Dry matter content of the meals was determined as follow: around two grams (exactly weighed at +/−0.01 mg) of meal were placed into an aluminium capsule (previously weighed dry with the same precision scale) and the whole was dried at 105 °C until constant weight (difference between two weighings < 1%). Then, capsule and meal were cooled in a desiccator at room temperature for 30 min and finally weighed. The dried matter content was calculated from the difference between the weight of the sample (meal and capsule) before and after drying to constant weight.

Lipid content of the meals was determined by the method previously outlined in the work of Laguna et al. (2018) : Around two grams (exactly weighed at +/−0.01 mg) of each fraction were placed into a filter paper envelope previously dried and weighed and the whole was dried at 105 °C until constant weight. Then, envelopes were extracted with n-hexane for 5 h using a Soxhlet apparatus. After extraction, envelopes were desolventized at room temperature for 12 h, dried at 105 °C, then cooled in a desiccator at room temperature for 30 min and finally weighed. The oil content was calculated from the difference between the dry weight of the sample (meal and envelope) before and after defatting. Values were given as percentage of oil per gram of dry matter.

These two values were taken into account to express other results per gram of dry defatted matter. All experiments were done in triplicate.

2.7. Methanolic extraction of phenolic compounds from meals

The extraction of TPC from meal samples was carried out in closed tubes at 75 °C for 20 min using a meal-to-methanol ratio of 1:100 (w/v) following the procedure previously described in Laguna et al. (2018). After drying under a nitrogen stream, extracts were kept at −20 °C until being processed. All experiments were done in triplicate.

2.8. Determination of the potentially releasable phenolic acid content in meals by alkaline hydrolysis

Fifty milligrams of meal were mixed with 1.5 ml of methanol. Then, 6 ml of 2 M NaOH were added to the mixture and incubated at 30 °C for different times (15, 30, 60 and 120 min). The mixture was then acidified to pH 2.0 using 4 M HCl with addition of 300 mg NaCl and further extracted three times with ethyl acetate (2 mL) by stirring for 2 min at room temperature. After each extraction, samples were centrifugated (3000 × *g*, 5 min) and supernatants were collected and pooled. The organic phase was dried under nitrogen stream and the residue was kept at −20 °C until HPLC analysis. All experiments were performed in triplicate. The amount of free phenolic acid released from meals after alkaline hydrolysis was used, when possible, as the 100% baseline reference for the calculations of enzymatic hydrolysis yields.

2.9. Enzymatic hydrolysis of caffeic acid derivatives in sunflower meals and in the corresponding phenolic extracts with AnFaeB and ChlE

Fifty milligrams of the meal were weighed into a brown flask with a screw cap, and 5 ml of 100 mM MOPS buffer at pH 6 were added. The closed flasks were incubated in an orbital shaker (350 rpm) at 50 °C run for 30 min for homogenization. After this pre-incubation step, two samples (mixture of meal and MOPS buffer) were cooled to −80 °C and freeze-dried. Phenolics were then extracted as described in Section 2.7, and their content determined and set as t = 0. In parallel, different amounts of ChlE and AnFaeB, from 0 to 30 nkat enzyme/g meal, were added to other pre-incubated samples. The hydrolysis assay was run for 10 to 240 min, at 50 °C and 350 rpm. An enzyme-free control was also performed in the same conditions. After the desired treatment duration, the reaction mixture was cooled to −80 °C and freeze-dried. Phenolics were then extracted as described in Section 2.7. After drying under a nitrogen stream, extracts were kept at −20 °C until HPLC analysis. Two independent trials were performed per experiment.

The enzymatic treatment was also applied to a dry methanolic extract obtained from 50 mg of NI-SFM as described in Section 2.7. Hydrolysis was performed at 50 °C, as described above, on the dry phenolic extract solubilized in 5 ml MOPS buffer containing 30 nkat/g meal for AnFaeB or 10 nkat/g meal for ChlE, respectively. After treatment, the reaction medium was cooled to −80 °C, then freeze-dried and finally extracted as described in Section 2.7. After drying under a nitrogen stream, the dry residue was kept at −20 °C until HPLC analysis. Two independent trials were performed per experiment.

2.10. Enzymatic hydrolysis of sinapic acid derivatives in rapeseed meal and in the corresponding phenolic extracts with AnFaeA

The enzymatic hydrolysis of NI-RSM with AnFaeA was performed in the optimized conditions previously determined on I-RSM by Odinot et al. (2017), with some modifications. Briefly, 50 mg of the meal were weighed into a brown flask with a screw cap and 5 ml of 100 mM MOPS buffer at pH 5.5 were added. The closed flasks were stirred in an orbital shaker (350 rpm) at 55 °C run for 30 min for homogenization. After this pre-incubation step, two samples (mixture of meal and MOPS buffer) were cooled to −80 °C and freeze-dried. Phenolics were then extracted as described in Section 2.7 and their content determined and set as t = 0. In parallel, 39 nkat AnFaeA/g meal was added to other pre-incubated samples. The enzymatic hydrolysis assay was run at 55 °C, 350 rpm for 10 to 240 min. An enzyme-free control was also performed in the same conditions. After treatment, the reaction mixture was cooled to −80 °C and the mixture was freeze-dried. The methanolic extraction of phenolics was done as described in Section 2.7. After drying under a nitrogen stream, extracts were kept at −20 °C until HPLC analysis. Two independent trials were performed per experiment. Enzymatic hydrolysis was also applied to a dry NI-RSM methanolic extract obtained from 50 mg of meal as described in Section 2.7. Hydrolysis was performed at 55 °C, as described above, on this dry phenolic extract solubilized in 5 ml MOPS buffer containing 39 nkat AnFaeA/g meal. After treatment, the reaction medium was cooled to −80 °C, then freeze-dried and finally extracted as described in Section 2.7. After drying under a nitrogen stream, the dry residue was kept at −20 °C until HPLC analysis. Two independent trials were performed per experiment.

2.11. High Performance Liquid Chromatography (HPLC) analysis of phenolics from hydrolyzed meals

The dry phenolic extracts, obtained as described in the previous sections, were solubilized in 5 ml methanol/water (2/1, v/v) and filtered (0.45-µm nylon filter) before HPLC analysis. Phenolic compounds were quantified with an XR UFLC Shimadzu liquid-phase chromatograph equipped with an SPD-M20A diode array detector (Kyoto, Japan). Separation was carried out with an ACE C18 reversed-phase column (5 µm, 250 mm × 4.6 mm, Phenomenex, Le Pecq, France). Injection volume, flow rate, and oven temperature were set at 20 µL, 1 mL.min^{-1} and 30 °C respectively. The gradient elution was performed using HPLC-grade water with 0.1% (v/v) acetic acid (A) and methanol with 0.1% (v/v) acetic acid (B) in the following conditions: 15% B (0–5 min), 15–80% B (5–30 min), 80–100% B (30–31 min), 100% B (31–35 min), 100-15% B (35–40 min).

2.11.1. Sunflower samples

Sunflower phenolics were identified according to retention times and quantified at their maximum detection wavelength, as previously described in Laguna et al. (2018). Total caffeic acid derivative (TCAD) content was calculated by summing peak area of all caffeoyl-containing molecules after normalization (*i.e.* each peak area was divided by the number of caffeoyl moiety contained in the corresponding molecule) and expressed in µmol of caffeic acid equivalent (CAE)/g DDM. CA was selected as external standard and the calibration curve was obtained from series of standard methanolic solutions from 12.5 to 800 µM (R^2 = 0.9991).

2.11.2. Rapeseed samples

SA and sinapine thiocyanate were chosen as external standards for the calibration curves. Their maximum detection wavelength and retention time were 323 nm – 17.8 min and 328 nm – 11.5 min, respectively. Total sinapic acid derivative (TSAD) content was calculated by summing peak area of all sinapoyl-containing molecules after normalization (*i.e.* each peak area was divided by the number of sinapoyl moiety in the corresponding molecule) and expressed in µmol of sinapic acid equivalent (SAE)/g DDM. Calibration curves were obtained from series of standard methanolic solutions from 12.5 to 200 µM with R^2 = 0.9983 for sinapic acid and R^2 = 0.9987 for sinapine.

2.12. Statistical analyses

Means and standard deviations (SD) were calculated using Minitab Statistical Software v.18. The means were then compared using one-way ANOVA analysis, with level of significance set to $\alpha = 0.05$.

Table 1
Lipid and moisture content of SFM and RSM samples[#].

	Lipid content (% DM)	Moisture (% DM)
NI-SFM	1.5 ± 0.0	7.3 ± 0.8
I-SFM	1.9 ± 0.3	10.5 ± 0.7
NI-RSM	1.4 ± 0.0	13.1 ± 0.3
I-RSM	1.5 ± 0.1	10 ± 0.1

[#] Values are mean ± SD (n = 3).

3. Results and discussion

3.1. Characterization of raw materials

3.1.1. Moisture, lipid and total phenolic compound contents of the meals

The moisture and lipid contents of the meals used in this study are shown in Table 1, with values of 7–13% DM and 1.4–1.9% DM, respectively. These data were consistent with the values available in the literature (Lomascolo et al., 2012).

The TPC initially present in SFM and RSM samples, as determined after methanolic extraction, are shown in Tables 2 and 3 respectively. The TPC content of SFM samples, which was expressed as TCAD content as 95% of SFM phenolics were found as caffeic acid esters (Karamać et al., 2012; Weisz et al., 2009), was 71.7 ± 0.5 µmol TCAD/g DDM for NI-SFM and 57.4 ± 1.5 µmol TCAD/g DDM for I-SFM. These data were consistent with the values available in the literature (Baumert et al., 2005; Cai and Arntfield, 2001; Weisz et al., 2009). The TPC content of RSM samples, which was expressed as TSAD content as 98% of their phenolics were shown to be sinapic acid esters (Khattab et al., 2010; Siger et al., 2013), was 64.2 ± 1.2 µmol TSAD/g DDM for NI-RSM and 44.8 ± 0.2 µmol TSAD/g DDM for I-RSM. Thus, TPC content was 1.2- to 1.4-fold higher in NI-meals than I-meals, in our conditions.

A mild alkaline treatment (NaOH 2 M, 30 °C, 30 min) was applied to NI-SFM and I-SFM samples in order to determine their respective total releasable CA content (CAEsters in soluble form and potentially bound to the lignocellulosic matrix (Shahidi and Yeo, 2016)), to be taken as reference. Unfortunately, no CA was recovered (Table 2) from either NI-SFM or I-SFM despite the mild alkaline treatment used. Indeed, the high chemical reactivity of CA make it prone to autoxidation at high pH due to its catechol function that rapidly reacts with NaOH and oxidizes in the presence of reactive oxygen species (Wildermuth et al., 2016). Thus, as alkaline hydrolysis was not suitable for assessing the total potentially releasable CA from SFM, all calculations and comparisons between various enzymatic hydrolysis conditions were made on the basis of TCAD content after methanolic extraction, expressed as µmol CAE/g DDM, and determined from the values of 71.7 ± 0.5 µmol CAE/g DDM for NI-SFM and 57.4 ± 1.5 µmol CAE/g DDM for I-SFM (Table 2). These values were taken as 100% reference for the calculations of enzymatic hydrolysis yields.

As done for SFM samples, the total amount of SA initially present in rapeseed meals as soluble conjugates and potentially releasable forms was determined after alkaline hydrolysis. The result was 62.5 ± 1.8 µmol/g DDM for NI-RSM and 46.8 ± 3.0 µmol/g DDM for I-RSM (Table 3). These values were not significantly different from those of the initial TSAD content of each meal (64.2 ± 1.2 µmol SAE/g DDM for NI-RSM and 44.8 ± 0.2 µmol SAE/g DDM for I-RSM), indicating that all the sinapic esters from the meal were hydrolyzed and recovered after our alkaline hydrolysis protocol. The latter values, which represented the total amount of potentially releasable SA per gram of DDM, were set as the 100% reference and used for the calculations of enzymatic hydrolysis yields. Unlike the significant loss of phenolics observed by Vuorela et al. after alkaline hydrolysis of RSM with a 4 M NaOH solution at 37 °C for 4 h (Vuorela et al., 2003), the milder conditions (NaOH 2 M, 30 °C, 30 min) implemented here managed to not degrade the SA and its derivatives.

3.1.2. Characterization of meal phenolic composition

This study identified the phenolic compounds present in the I-SFM by HPLC-DAD. As shown by HPLC analysis of I-SFM methanolic extract (Fig. 1), the main phenolic compounds were CAEsters such as mono- and di-caffeoylquinic acids, the most abundant being 4- *O*-caffeoylquinic acid (4-CQA) and 5-*O*-caffeoylquinic acid (5-CQA). These two compounds co-eluted and were named chlorogenic acid. Other minor phenolics were 3-*O*-caffeoylquinic acid (3-CQA), two di-caffeoylquinic acid (di-CQA) isomers (3,5- and 4,5-di-*O*-caffeoylquinic acids), 5-*O*-*p*-coumaroylquinic acid, 5-*O*-feruloylquinic acid, and CA. These results were in good agreement with other studies (Karamać et al., 2012; Weisz et al., 2009), and were comparable to the NI-SFM composition previously determined by Laguna et al. (2018).

Two of our previous studies characterizing the phenolic composition of NI-RSM and I-RSM (Laguna et al., 2018; Odinot et al., 2017) showed that RSM phenolics comprised: sinapine, sinapoyl glucopyranoside, sinapic acid, di- and tri-sinapoyl gentiobiosides, di-sinapoyl glucopyranoside isomer, and sinapoyl keampferol derivatives.

3.2. Release of free caffeic acid from sunflower meals by A. niger AnFaeB and ChlE

3.2.1. Enzymatic hydrolysis of I-SFM

This study selected AnFaeB and ChlE for the hydrolysis of phenolic esters present in SFMs. Numerous studies have already shown that these enzymes can hydrolyze CAEsters in other raw materials such as coffee pulp, apple marc, autoclaved maize bran and sugar-beet pulp (Benoit et al., 2006, 2007; Faulds, 2010; Gopalan et al., 2015; Ramos-de-la-Peña and Contreras-Esquivel, 2016), but SFM has never yet been investigated. The optimum pH and temperature of these enzymes established in previous work were set to 6.0 and 50 °C for synthetic substrates (*e.g.* methyl sinapate and 5-CQA) (Asther et al., 2005; Benoit et al., 2006).

Given the drastic heat treatment undergone during the production process, it is reasonable to posit that I-SFM was somewhat recalcitrant to enzymatic degradation. We therefore traced the kinetics of hydrolysis of the major phenolic esters in I-SFM and the release of free CA as a function of enzyme amount (Fig. 2). In the presence of 20–30 nkat AnFaeB/g meal, all the CAEesters, initially present in I-SFM, were completely hydrolyzed after 1 h of incubation (Figs. 2 and Supplementary data S1). An amount of 5 nkat AnFaeB/g meal failed to completely hydrolyze the phenolics. An amount of 10 nkat AnFaeB/g meal

Table 2
Phenolic content of Non-Industrial and Industrial SFM[#].

	NI-SFM		I-SFM	
	Before alkaline hydrolysis	After alkaline hydrolysis	Before alkaline hydrolysis	After alkaline hydrolysis
Chlorogenic acid (µmol CAE/g DDM)	55.8 ± 0.9[b]	n.d.	41.4 ± 2.5[c]	n.d.
Caffeic acid (µmol/g DDM)	1.2 ± 0.0[e]	n.d.	2.1 ± 0.5[d]	n.d.
TCAD[*] (µmol CAE/g DDM)	71.7 ± 0.5[a]	n.d.	57.4 ± 1.5[b]	n.d.

[#] Values are mean ± SD (n = 3). Values followed by same superscript letters are not significantly different, $p \leq 0.05$.
[*] TCAD: total caffeic acid derivatives in SFM, expressed in µmol CAE/g DDM. CAE: caffeic acid equivalents. DDM: defatted dry matter.

Table 3
Phenolic content of Non-Industrial and Industrial RSM[#].

	NI-RSM		I-RSM	
	Before alkaline hydrolysis	After alkaline hydrolysis	Before alkaline hydrolysis	After alkaline hydrolysis
Sinapine (μmol SAE/g DDM)	31.7 ± 0.7[c]	–	21.6 ± 0.2[d]	–
Sinapic acid (μmol/g DDM)	3.9 ± 0.1[e]	62.5 ± 1.8[a]	2.2 ± 0.0[f]	46.8 ± 3.0[b]
TSAD[*] (μmol SAE/g DDM)	64.2 ± 1.2[a]	62.5 ± 1.8[a]	44.8 ± 0.2[b]	46.8 ± 3.0[b]

[#] Values are mean ± SD (n = 3). Values followed by same superscript letters are not significantly different, $p \leq 0.05$.
[*] TSAD: total sinapic acid derivatives in RSM, expressed in μmol SAE/g DDM. SAE: sinapic acid equivalents. DDM: defatted dry matter.

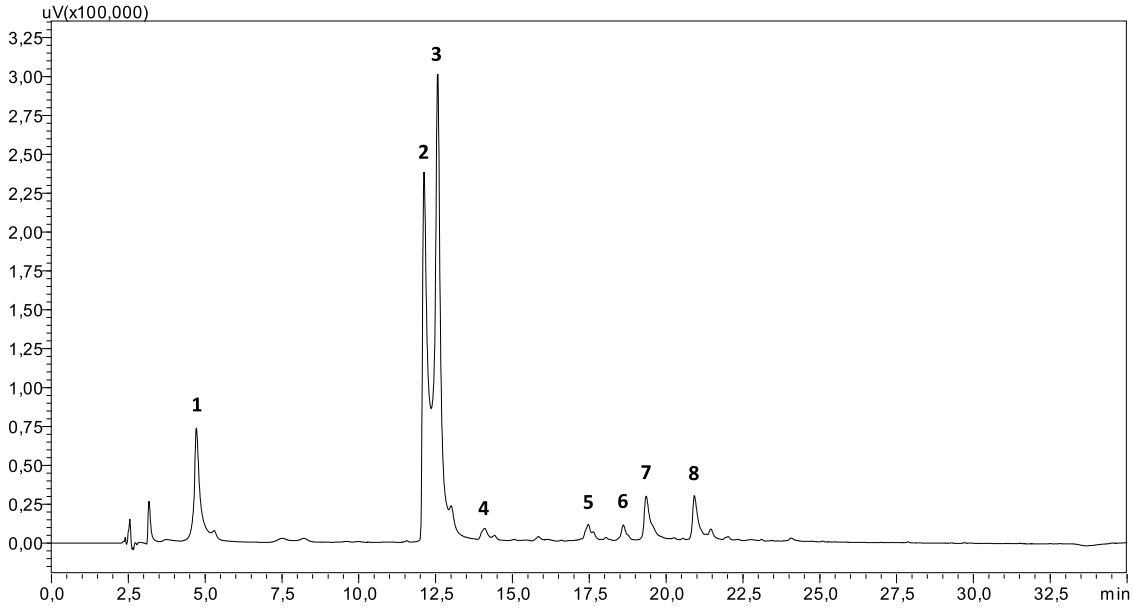

Fig. 1. HPLC chromatogram of industrial SFM methanolic extract at 326 nm. Main identified phenolics: 3-*O*-caffeoylquinic acid (3-CQA) (**1**), 4-*O*-caffeoylquinic acid (4-CQA) (**2**), 5-*O*-caffeoylquinic acid (5-CQA) (**3**), caffeic acid (CA) (**4**), 5-*O*-coumaroylquinic acid (5-CoQA) (**5**), 5-*O*-feruloylquinic acid (5-FQA), (**6**), 3,5-di-*O*-caffeoylquinic acid (3,5-diCQA) (**7**) and 4,5-di-*O*-caffeoylquinic acid (4,5-diCQA) (**8**).

was able to hydrolyze all CAEesters except 3-CQA (Fig. 2). A minimum concentration of 20 nkat AnFaeB/g meal was thus required for complete hydrolysis of all phenolics, and the highest free CA content was obtained with 30 nkat AnFaeB/g meal after a 3 h incubation (59.8 ± 2.1 μmol/g DDM), which corresponded to a 100% hydrolysis yield. AnFaeB thus showed high catalytic efficiency on all the mono- and dicaffeoylquinic acid isomers present in I-SFM (Fig. 2), together with the *p*-coumaroyl and feruloyl derivatives (Supplementary data, Fig. S1).

In the presence of ChlE, the hydrolysis of 3,5-diCQA and 4,5-diCQA was complete after 3 h of incubation whatever the amount of enzyme. Conversely, 3-CQA was not hydrolyzed at all in any condition, even though 4-CQA and 5-CQA were 90% hydrolyzed after 2 h of incubation with ChlE (Fig. 2). The highest free CA content of obtained from I-SFM with ChlE was 54.0 ± 1.1 μmol/g DDM after 2–4 h incubation with 5–30 nkat enzyme/g meal. Compared to AnFaeB, this slightly lower value of free CA released was mainly due to the inability of ChlE to hydrolyze 3-CQA, which was still found at 6.1 ± 0.3 μmol CAE/g DDM whatever the enzyme concentration (Figs. 2 and supplementary data S1).

AnFaeB has already been shown to have a broader specificity than ChlE for hydrolyzing chlorogenic acids from agricultural by-products such as maize bran and sugar-beet pulp (Benoit et al., 2006). For instance, unlike ChlE, AnFaeB was found to hydrolyze 5-*O*-*p*-coumaroylquinic acid and feruloylated oligomers (Benoit et al., 2007). This confirmed the decisive role of the moieties adjacent to the ester bond for correct alignment of the substrate in the active site of Faes. To the best of our knowledge, there is no literature data on the relative activity of AnFaeB and ChlE against the different mono- and di-caffeoylquinic acids. Here, we demonstrated that ChlE showed selectivity for different caffeoylquinic acid isomers in the SFM compared to AnFaeB. The concentrations of 30 nkat AnFaeB/g meal and 10 nkat ChlE/g meal were then chosen to be further applied on NI-SFM

3.2.2. Enzymatic hydrolysis of NI-SFM

Fig. 3A and Fig. 3B shows the results of enzymatic hydrolysis performed on NI-SFM with AnFaeB and ChlE, respectively. When AnFaeB was used, all the chlorogenic acid (4- and 5-CQA) was hydrolyzed after 10 min. When ChlE was used, 4- and 5-CQA were 95% hydrolyzed in our conditions. The highest concentration of free CA released in the reaction medium was observed after 30 min with AnFaeB (52.3 ± 0.2 μmol/g DDM) and ChlE (42.0 ± 1.1 μmol/g DDM). However, these values remained lower than the initial concentration of chlorogenic acid of 55.8 ± 0.9 μmol CAE/g DDM in the meal (Table 2). It is worth noting that there was a strong decrease in TCAD content over time (Fig. 3), with or without enzyme (control), resulting in a loss of TCAD compared to the initial content in the meal (Table 2). While the effect of other factors (including pH and temperature) cannot be totally ruled out, the most likely explanation was the presence of endogenous enzymes in the NI-SFM that degraded CA and its derivatives during the pre-incubation and incubation steps. Studies have already demonstrated the presence of polyphenol oxidase (PPO) in sunflower meal and its high activity on chlorogenic acid (Wildermuth et al., 2016). Therefore, although our hydrolysis conditions (pH 6.0, 50 °C) did not exactly correspond to PPO optimum pH and temperature (7.5 and 45 °C respectively; Wildermuth et al., 2016), we found it reasonable to posit

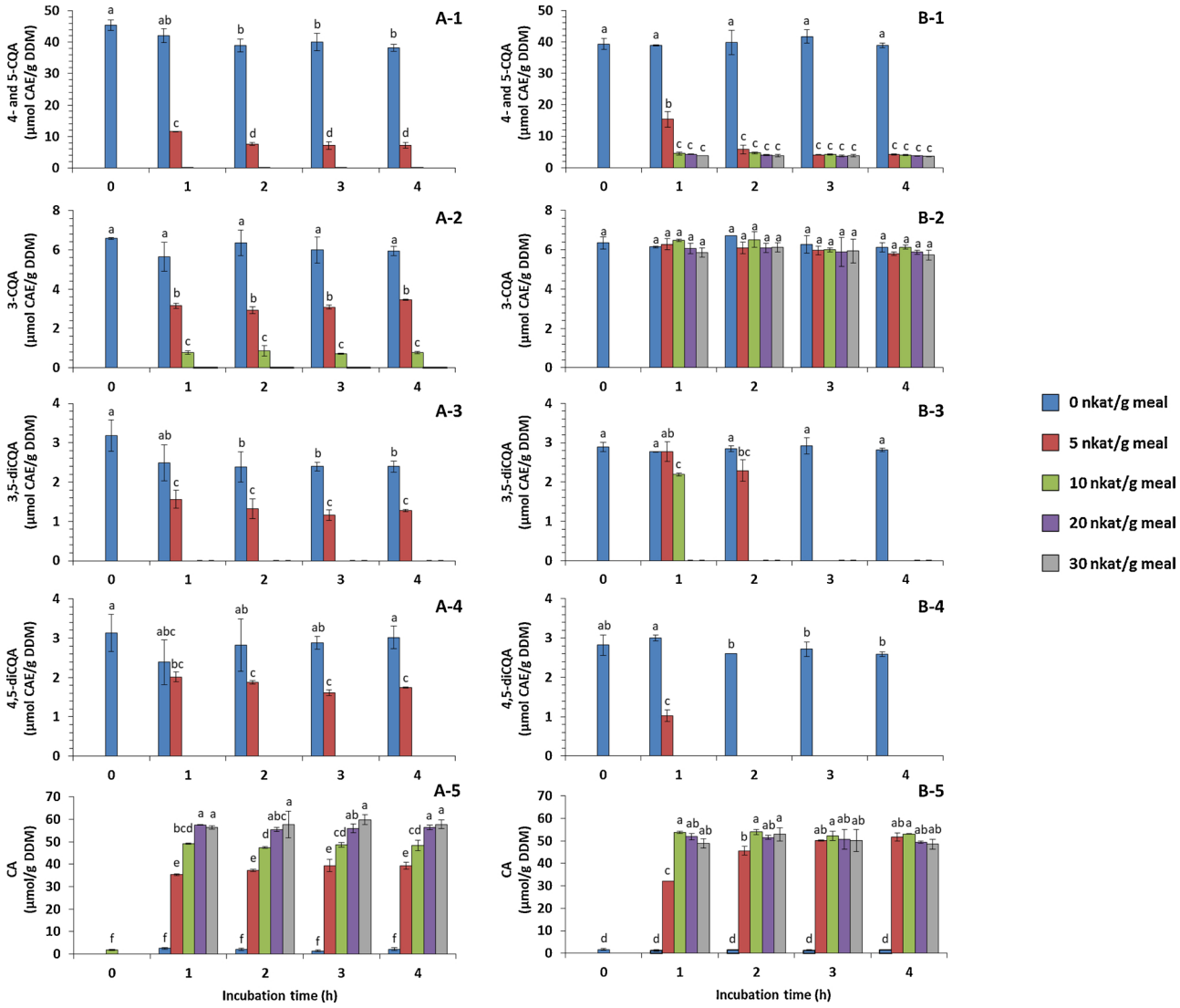

Fig. 2. Hydrolysis kinetics of the main CAEsters in industrial SFM, as a function of enzyme type: AnFaeB (**A**); ChlE (**B**), and enzyme concentration. CAEsters: 4- and 5-*O*-caffeoylquinic acid (4- and 5-CQA, **1**); 3-*O*-caffeoylquinic acid (3-CQA, **2**); 3,5-di-*O*-caffeoylquinic acid (3,5-diCQA, **3**), 4,5-di-*O*-caffeoylquinic acid (4,5-diCQA, **4**) and caffeic acid (CA, **5**). Hydrolysis conditions: pH6, 50 °C. Before starting the enzymatic hydrolysis, the reaction mixture was pre−incubated for 30 min for homogenization. Values are mean ± SD (n = 2) and expressed as caffeic acid equivalents (CAE). For each series, values followed by same superscript letters are not significantly different, $p \leq 0.05$.

that the residual PPO activity was sufficient to continuously oxidize chlorogenic acid and related compounds from the pre-incubation step. Here we measured PPO activity in endogenous SFM meal extracts and found 1.02 (± 0.08) nkat/mL diphenol oxidase activity and 0.43 (± 0.02) nkat/mL monophenol oxidase activity, which are characteristic of a tyrosinase-type activity (Halaouli et al., 2005). In addition, it is noteworthy that 15 putative PPO-encoding gene sequences were predicted in the publicly-available *Helianthus annuus* genome from the NCBI Database (https://www.ncbi.nlm.nih.gov/search/all/?term=polyphenol+oxidase+helianthus+annuus).

Owing to this degradation phenomenon, and regardless of enzyme (AnFaeB or ChlE), the maximal amount of CA released from NI-SFM remained lower than that released from I-SFM (52.3 ± 0.2 µmol/g DDM *versus* 59.8 ± 2.1 µmol/g DDM for AnFaeB, and 42.0 ± 1.1 µmol/g DDM *versus* for 54.0 ± 1.1 µmol/g DDM for ChlE). Thus, it seemed that the heat treatment (cooking at 95–100 °C followed by steam desolventizing at about 105 °C) applied to I-SFM, but not NI-SFM, led to inactivation of endogenous enzymes in the SFM even if this heat treatment was also responsible for a significant decrease in TPC (Table 2). This constituted a major drawback that greatly limited the value of non-thermally-treated NI-SFM as a substrate for *in situ* enzymatic production of CA.

3.3. AnFaeB and ChlE hydrolysis of NI-SFM methanolic extract

Enzymatic hydrolysis was also performed on phenolic extracts from NI-SFM after a sequence of methanolic extraction, drying, and solubilization in reaction buffer. Methanol mainly extracted phenolic compounds, minerals and carbohydrates from the meal (Sripad and Narasinga Rao, 1987), but not the proteins, including the possible endogenous enzymes responsible for the degradation of phenolics. Fig. 4A shows the fast hydrolysis of CAEsters by AnFaeB in the methanolic extract from NI-SFM at 50 °C. After only 10 min, all the phenolic esters were hydrolyzed into free CA, which gave rise to a concentration of 68.1 ± 1.7 µmol/g DDM with 95% hydrolysis yield (Table 4).

When hydrolysis was carried out with ChlE at the same temperature (50 °C), chlorogenic acid was hydrolyzed more slowly (30 min instead of 10 min) and was not completely converted even after 240 min (Fig. 4B), and only a small fraction of 3-CQA got hydrolyzed (Table 4, Supplementary data Fig. S2). This typical feature of ChlE, already

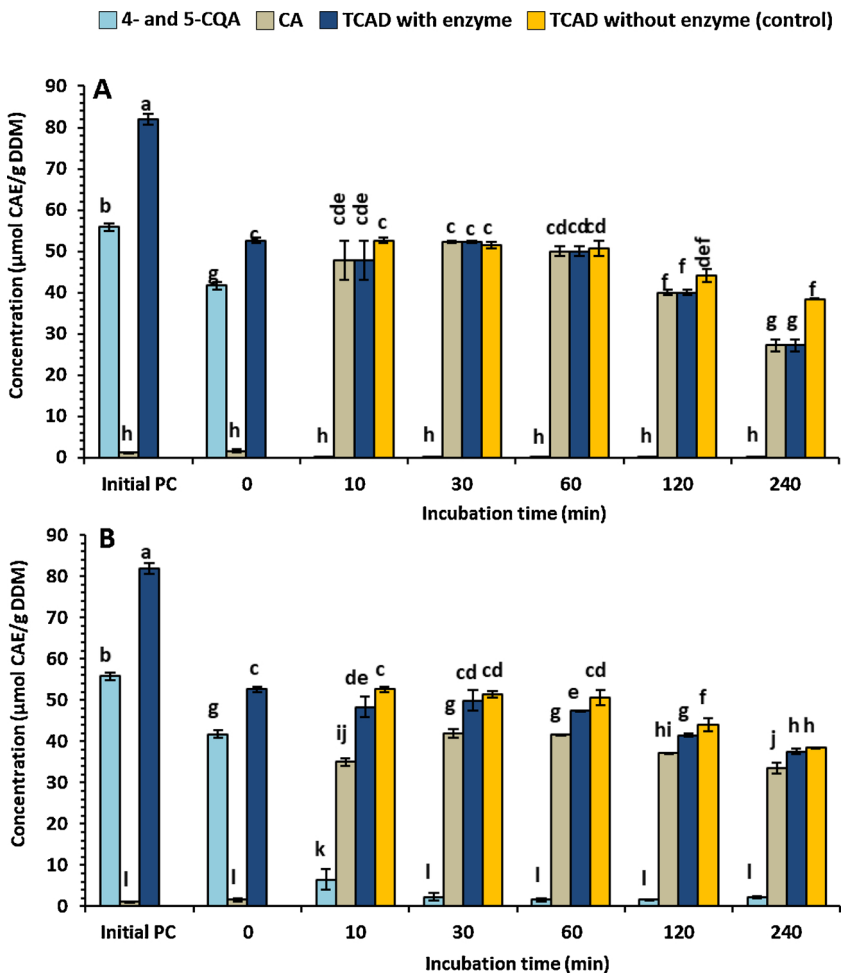

Fig. 3. Time course of 4- and 5-*O*-caffeoylquinic acid (4- and 5-CQA), caffeic acid (CA) and total caffeic acid derivatives (TCAD) concentrations during enzymatic hydrolysis with **AnFaeB** (pH6, 30 nkat/g meal) (**A**) and **ChlE** (pH6, 10 nkat/g meal) (**B**), applied to non-industrial SFM at 50 °C. Before starting the enzymatic hydrolysis, the reaction mixture was pre−incubated for 30 min for homogenization. Initial PC: Initial phenolic concentration in non−industrial SFM. Values are mean ± SD (n = 2) and expressed as caffeic acid equivalents (CAE). Values followed by same superscript letters are not significantly different, $p \leq 0.05$.

observed during I-SFM hydrolysis (see Section 3.2.1), could explain why the highest concentration of CA was only 55.4 ± 0.3 µmol/g DDM after 60 min incubation, which corresponded to a hydrolysis yield of only 77% (Table 4). It is worth noting that, in both cases (AnFaeB and ChlE), no decrease in the TCAD content was observed over enzymatic incubation time on methanolic extracts (Fig. 4), conversely to the crude NI meal (Fig. 3). This might indirectly confirm the presence of phenolic-degrading enzymes (*i.e.* PPO for instance) in the meal that were removed after phenolic extraction with methanol.

On NI-SFM, the best CA yields were achieved through enzymatic hydrolysis of methanolic extract, particularly with AnFaeB that allowed fast, selective, and quantitative production of CA (68.1 ± 1.7 µmol/DDM from methanolic extract hydrolysis against 52.3 ± 0.2 µmol/g DDM from crude NI-meal hydrolysis).

3.4. Enzymatic hydrolysis of sinapic acid esters in NI-RSM and in the corresponding methanolic extract with AnFaeA

Hydrolysis conditions (pH, temperature and enzyme concentration) for the enzymatic hydrolysis of sinapic esters present in an I-RSM (a commercial RSM thermally treated by cooking at 95–100 °C followed by steam desolventizing at about 107 °C) was previously established by Odinot et al. (2017). These conditions were implemented on NI-RSM and on the corresponding methanolic extract. NI-RSM was chosen for its higher total sinapic acid derivatives (TSAD) content compared to the I-RSM (Table 1).

3.4.1. AnFaeA hydrolysis of NI-RSM

The evolution of the concentration of sinapine (SNP), sinapic acid (SA) and TSAD during enzymatic hydrolysis of NI-RSM with AnFaeA (39 nkat/g meal, 55 °C, pH 5.5) is charted in Fig. 5A. The maximum amount of SA released was 41.3 ± 0.3 µmol/g DDM after 240 min of incubation with AnFaeA. Almost all the SNP initially present in the meal (31.7 ± 0.6 µmol SAE/g DDM) (Fig. 5A) and the mono- and di-sinapoyl glucose isomers (Supplementary data Fig. S3A) were hydrolyzed at the end of incubation.

However, kaempferol-based sinapic acid derivatives, as well as di-sinapoyl gentiobiosides and some tri-sinapoyl gentiobiosides were not (or partially) hydrolyzed by the enzyme (supplementary data, Fig.S3A). These results were in agreement with our previous work on I-RSM (Odinot et al., 2017). TSAD content measured after the 30-min pre-incubation step (Fig. 5A) was decreased by about 16% compared to the initial content in the unprocessed meal (64.2 ± 1.2 µmol SAE/g DDM), possibly due to the activity of endogenous enzymes (possibly PPO) present in the crude meal. In the case of NI-RSM, no PPO-type activity was detectable in the meal in our assay conditions. However, it is noteworthy that a laccase-encoding gene (*AtTT10* gene) was identified in the genome of *Brassica napus* (Zhang et al., 2013). This PPO was involved in proanthocyanidin polymerization and lignin biosynthesis as well as seed coat pigmentation. Conversely, this decrease was not

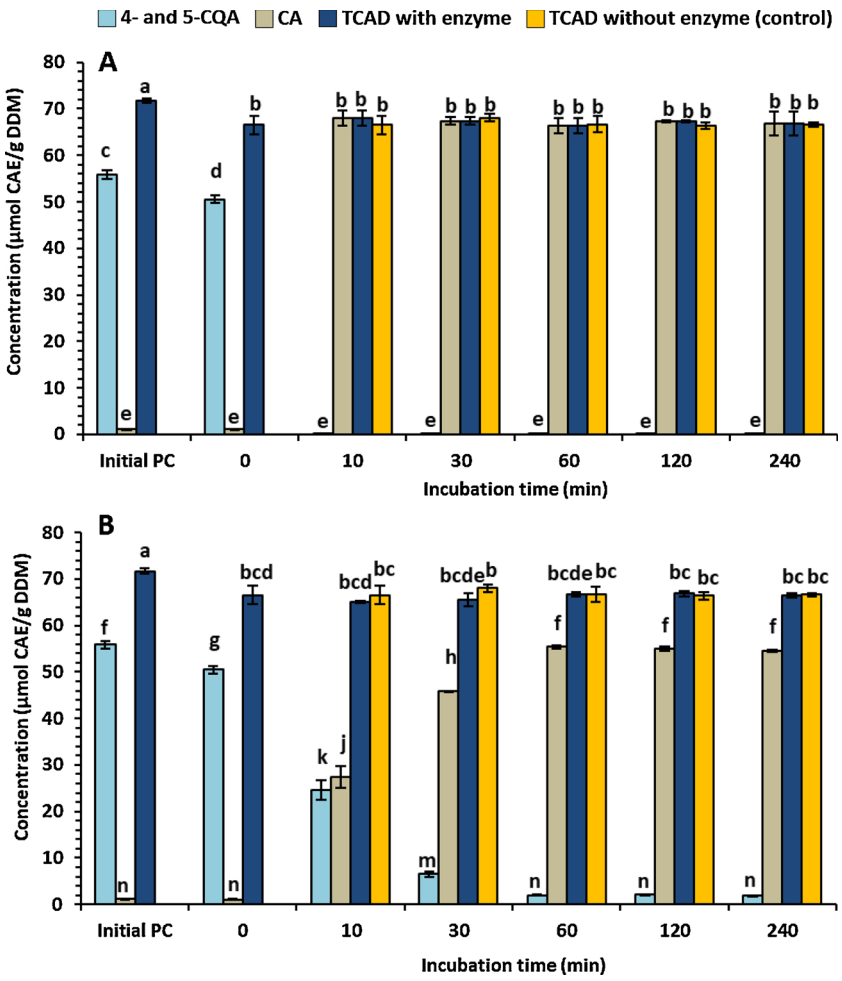

Fig. 4. Time course of 4- and 5-*O*-caffeoylquinic acid (4- and 5-CQA), caffeic acid (CA) and total caffeic acid derivatives (TCAD) concentrations during enzymatic hydrolysis with **AnFaeB** (pH6, 30 nkat/g meal) (**A**) and **ChlE** (pH6, 10 nkat/g meal) (**B**), applied to non-industrial SFM **methanolic extract** at 50 °C. Before starting the enzymatic hydrolysis, the reaction mixture was pre−incubated for 30 min for homogenization. Initial PC: Initial phenolic concentration in non−industrial SFM methanolic extracts. Values are mean ± SD (n = 2) and expressed as caffeic acid equivalents (CAE). Values followed by same superscript letters are not significantly different, $p \leq 0.05$.

observed in enzymatic hydrolysis applied to an I-RSM (Odinot et al., 2017). Like for I-SFM, but in contrast to NI-RSM, the thermal treatments (95–107 °C) applied to I-RSM seemed to inactivate endogenous phenolic-degrading enzymes.

3.4.2. *AnFaeA hydrolysis of the NI-RSM methanolic extract*

Like for NI-SFM, enzymatic hydrolysis was also performed on phenolic extracts from NI-RSM obtained after a sequence of methanolic extraction, drying, then solubilization in reaction buffer. The time-concentrations of SNP, SA and TSAD during enzymatic hydrolysis of NI-RSM dry methanolic extract with AnFaeA (39 nkat/g meal, 55 °C, pH 5.5) is shown in Fig. 5B. Unexpectedly, the maximum amount of SA released (32.3 ± 0.4 µmol/g DDM) was lower than the amount obtained from NI-RSM (41.3 ± 0.3 µmol/g DDM) (Table 5), concomitantly with the appearance of a new phenolic compound in the reaction medium (Fig. 5B, green bar) that corresponded to a peak with a retention time of 22.25 min in our HPLC conditions (Supplementary data Fig. S3B).

Based on its retention time in the HPLC chromatogram (Compound 9, Supplementary data Fig. S3B), its maximum wavelength absorption of 326 nm, and its mass and NMR (^{1}H, ^{13}C) spectra (Supplementary data Figs. S4 and S5), the new compound was 1,2-di-*O*-sinapoyl-β-D-glucose. Therefore, given the presence of significant amounts of glucose (Naczk and Shahidi, 1990), as well as mono- and di-sinapoyl glucose

Table 4
Concentration of caffeic acid (CA), CAEsters and total caffeic acid derivatives (TCAD) before and after hydrolysis of NI-SFM methanolic extract with AnFaeB and ChlE at 50 °C[#].

	Initial content in the meal	AnFaeB	ChlE
4- and 5-CQA (µmol CAE/g DDM)	55.8 ± 0.9^{c}	–	2.0 ± 0.1^{h}
3-CQA (µmol CAE/g DDM)	8.9 ± 0.4^{e}	–	7.8 ± 0.1^{f}
3,5-di-CQA (µmol CAE/g DDM)	4.0 ± 0.2^{g}	–	0.6 ± 0.0^{j}
4,5-di-CQA (µmol CAE/g DDM)	1.8 ± 0.1^{g}	–	–
CA (µmol/g DDM)	1.2 ± 0.0^{i}	68.1 ± 1.7^{ab}	55.4 ± 0.3^{cd}
TCAD* (µmol CAE/g DDM)	71.7 ± 0.5^{a}	68.1 ± 1.7^{ab}	65.8 ± 0.4^{b}
CA yield (%) based on initial TCAD in meal	–	95.0	77.2
CA yield (%) based on TCAD at t = 0 of enzyme incubation[§]	–	100.4	84.2

* TCAD: total caffeic acid derivatives; CAE: caffeic acid equivalent. DDM: defatted dry matter. Values are mean ± SD (n = 2). Values followed by same superscript letters are not significantly different, $p \leq 0.05$.

[#] 10 min incubation for AnFaeB, 60 min incubation for ChlE.

[§] The substrate was pre-incubated for 30 min in MOPS buffer before adding enzyme (t = 0 of enzyme incubation).

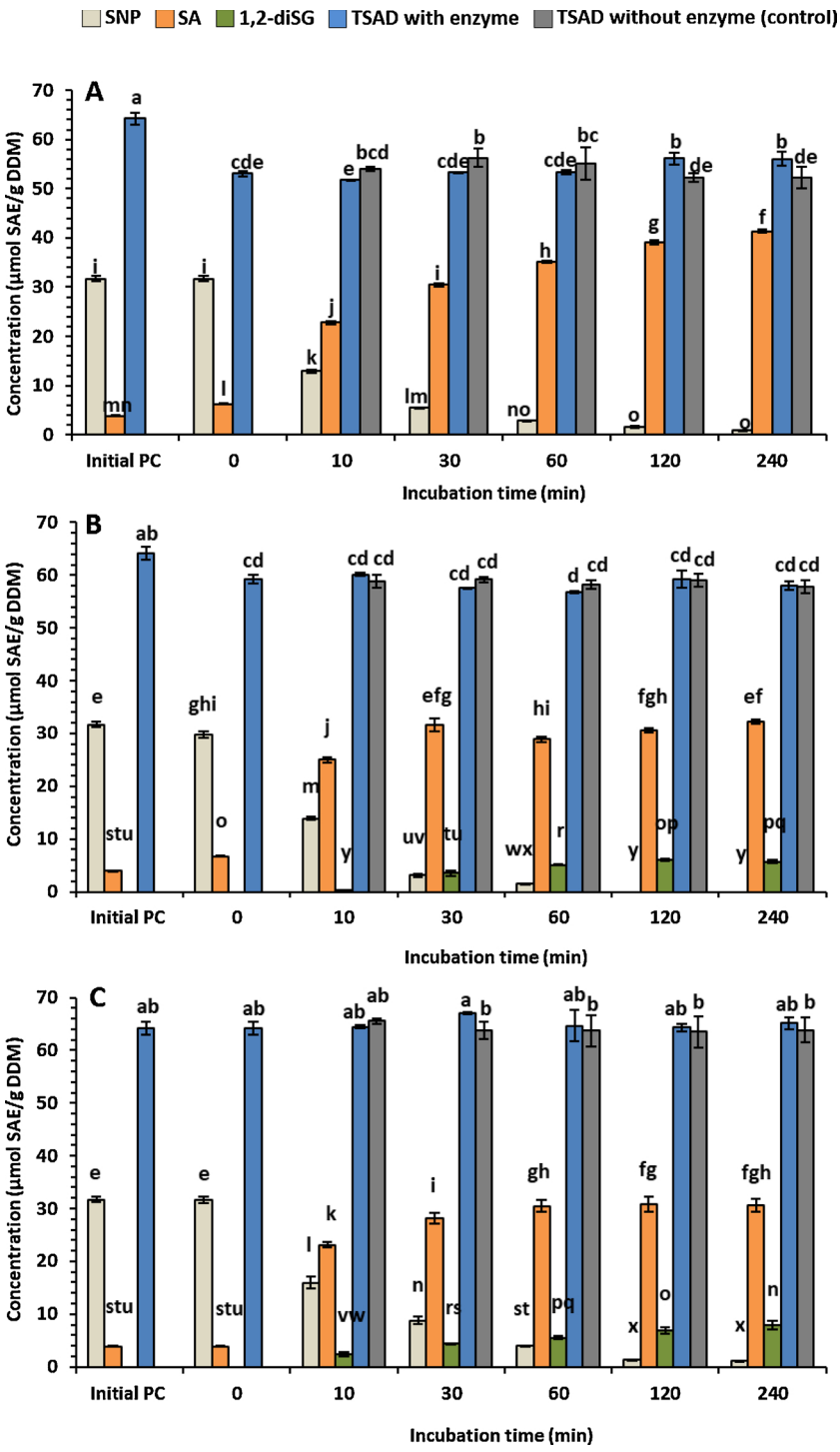

Fig. 5. Time course of sinapine (SNP), sinapic acid (SA), 1,2-di-*O*-sinapoyl-β-D-glucose (1,2-diSG) and total sinapic acid derivatives (TSAD) concentrations during enzymatic hydrolysis with **AnFaeA** (pH5.5, 39 nkat/g meal) applied to non-industrial RSM at 55 °C (**A**), and to non-industrial RSM methanolic extract at either 55 °C (**B**) or 37 °C (**C**). Before starting the enzymatic hydrolysis, the reaction mixture was pre−-incubated for 30 min for homogenization. Initial PC: Initial phenolic concentration in non−industrial RSM (A) or in non−industrial RSM methanolic extract (B, C). Values are mean ± SD (n = 2) and expressed as sinapic acid equivalents (SAE). Values followed by same superscript letters are not significantly different, $p \leq 0.05$.

Table 5
Maximal amount and yield of sinapic acid (SA) after enzymatic treatment of NI-RSM and the corresponding methanolic extract with AnFaeA.

	NI-RSM, 55 °C	NI-RSM methanolic extract, 55 °C	NI-RSM methanolic extract, 37 °C
TSAD* at t = 0 of enzyme incubation§ (µmol SAE/g DDM)	53.1 ± 1.0^{c}	59.2 ± 2.3^{b}	65.6 ± 2.3^{a}
Maximum SA released after 240 min incubation with enzyme (µmol/g DDM)	41.3 ± 0.3^{d}	32.3 ± 0.4^{e}	30.6 ± 1.2^{e}
SA yield (%) based on initial TSAD in meal	64.3	50.3	47.7
SA yield (%) based on TSAD at t = 0 of enzyme incubation§	77.7	54.6	46.6

Values are mean ± SD (n = 2). Values followed by same superscript letters are not significantly different, $p \leq 0.05$.

* TSAD: total sinapic acid derivatives; SAE: sinapic acid equivalent. DDM: defatted dry matter.

§ The substrate was pre-incubated for 30 min in MOPS buffer before adding enzyme (t = 0 of enzyme incubation).

isomers in the NI-RSM extract (Laguna et al., 2018), it can be reasonably assumed that this new compound resulted from transesterification of sinapine with the aforementioned molecules extracted from the meal. If so, these results would confirm the well-known acyl transferase activity of AnFaeA (Hatzakis and Smonou, 2005; Hüttner et al., 2017; Vafiadi et al., 2009). However, the reason why such a reaction only occurred in the NI-RSM methanolic extract remains to elucidate.

TSAD content measured during enzymatic hydrolysis of the methanolic extract (59.2 ± 2.3 µmol SAE/DDM) remained higher than TSAD content measured during meal hydrolysis (53.1 ± 1.0 µmol SAE/ DDM). This might corroborate the presence of phenolic-degrading endogenous enzymes in the crude meal.

Hydrolysis of the NI-RSM methanolic extract was also performed at 37 °C in order to assess the possible influence of temperature on yield of 1,2-di-*O*-sinapoyl-β-D-glucose synthesis (Fig. 5C). At 37 °C, TSAD concentration (65.6 ± 2.3 µmol/ DDM) measured during hydrolysis remained stable throughout the incubation time at an equivalent level to the initial content in the unprocessed meal (64.2 ± 1.2 µmol SAE/g DDM). The decrease over time of SNP was slightly slower at 37 °C than at 55 °C (Fig. 5C): hydrolysis was complete in less than 120 min at 55 °C whereas 3% of SNP remained in the medium even after 240 min at 37 °C. Production of 1,2-di-*O*-sinapoyl-β-D-glucose was higher overall (*e.g.* 8.0 ± 0.8 µmol SAE/g DDM at 37 °C against 5.7 ± 0.3 µmol SAE/ g DDM at 55 °C after 240 min incubation), which may suggest that lower temperature facilitated acyl transfer more than sinapine hydrolysis, which occurs in both conditions.

Taken together, the results presented in Section 3.4 converged to show the best yield of SA came from hydrolysis performed directly in the crude NI-RSM with AnFaeA compared to hydrolysis of the corresponding methanolic extract. The enzymatic treatment of the NI-RSM methanolic extract seemed to be characterized by acyl transfer reactions that reduce the overall yield of free SA release.

4. Conclusion

It is possible to obtain free CA from sunflower meal and free SA from rapeseed meal by alkaline hydrolysis and solvent extraction (Vuorela et al., 2003; Zago et al., 2015b), but in high temperature, strong base and toxic solvent conditions, which runs counter to a 'green' process. Here we showed that *A. niger* AnFaeB, ChlE and AnFaeA were very efficient catalysts for the enzymatic release of CA and SA from both industrial and non-industrial meals. To the best of our knowledge, this is the first time that AnFaeB and ChlE were applied on sunflower meals as raw natural substrates. AnFaeB was able to hydrolyze all the mono- and di-caffeoylquinic acid isomers, together with the coumaroylquinic and feruloylquinic acid isomers identified in SFMs, whereas ChlE was unable to hydrolyze 3-CQA. The non-industrial meals processed under mild thermal conditions presented the advantage of higher initial phenolics contents. However, phenolic acid release was hampered by phenolic oxidation side-reactions, seemingly due to meal endogenous PPO-type activities, which is a major drawback for industrial applications. Here we found evidence that using an intermediary step of phenolic extraction with methanol, before enzymatic hydrolysis, could overcome this drawback for NI-SFM but not NI-RSM due to AnFaeA transesterification side-reactions. Nevertheless, this acyl transfer activity of AnFaeA could prove beneficial for the enzymatic synthesis of novel sinapic and ferulic acid esters with sugars or polyols. Of all the substrates tested for enzymatic hydrolysis, industrial rapeseed meal and non-industrial sunflower meal extract showed the best compromise between the three following parameters: initial TPC, hydrolysis yields, and amounts of CA and SA released. The proof of concept of such an enzymatic hydrolysis process was already successfully scaled-up in a 10-L bioreactor in the case of FaeA and raw industrial RSM (Odinot et al., 2017). In addition, regarding enzymatic hydrolysis applied to meal methanolic extract, the extraction of phenolics by ethanol (less toxic and biosourced solvent, almost as efficient as methanol) followed by concentration/desolventization steps could be easily implemented at industrial scale as it is already done with hexane for instance. In the case of sunflower meal extract, this process may be advantageous since phenolic concentration as well as enzyme load would be better controlled, limiting matrix effect in the same time. This work opens new perspectives on the biotechnological applications of fungal Faes and ChlEs to other hydroxycinnamic acid-containing raw materials, including green coffee bean wastes, co-products of rapeseed and sunflower crops (*e.g.* stems, straws, seed shells, sunflower capitula) as well as other oilseed plants (mustard, flax).

Author contributions

Jérôme Lecomte, Oscar Laguna and Anne Lomascolo conceived, designed, supervised the experiments, and wrote the paper. Oscar Laguna, Elise Odinot, Alexandra Bisotto and Bruno Baréa performed the experiments. Frédéric Fine, Jean-Claude Sigoillot and Pierre Villeneuve contributed to the conception of the work and help to write the final draft of the paper. Craig B. Faulds and Laurence Lesage-Meessen brought expertise on feruloyl esterases. Eric Record is at the origin of *Aspergillus niger* recombinant strains. All the authors contributed to finalize the manuscript.

Declaration of Competing Interest

The authors have declared no conflict of interest.

Acknowledgements

This work was funded by the Technical Centre for Oilseed Crops, Grain Legumes and Industrial Hemp (TERRES INOVIA, Pessac, France) and the Inter-Branch Organization for Vegetable Oils and Proteins (TERRES UNIVIA, Paris, France). This work was also funded and performed, in partnership with the SAS PIVERT, within the frame of the French Institute for the Energy Transition (Institut pour la Transition Energétique (ITE) P.I.V.E.R.T. (www.institut-pivert.com) selected as an Investment for the Future ("Investissements d'Avenir"). The French Government under the reference ANR-001-01 supported this work, as part of the Investments for the Future.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.indcrop.2019.111579.

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