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Valentin Reungoat[a,b], Louis M. M. Mouterde[a]*, Morad Chadni[a], Julien Couvreur[a], Emilie Isidore[a], Florent Allais[a], Hélène Ducatel[b], Irina Ioannou[a]

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**Abstract**

The simultaneous extraction and enzymatic hydrolysis of sinapine from a mustard residue was designed for the recovery of sinapic acid, a high value-added phenolic acid. An initial screening allowed the identification of sinapoyl esterase activities in commercial enzymatic cocktails (Depol 740 L, Ultraflo XL, Deltazym VR AC-100, Pectinase-PL "Amano") and in a mono-enzymatic solution of rumen feruloyl esterase. These enzymatic cocktails were not very tolerant to ethanol with a diminution of 70–90% of the activity in presence of 10% (v/v) of ethanol. Different extraction processes on mustard bran were designed depending on the solvent compositions (ethanol 70% (v/v), water with or without sinapoyl esterase), pH (4.3, 7 or 12) and temperatures (50, 75 or 100 °C). Their respective efficiencies were discussed. The implementation of Depol 740 L in water allowed to recover 68% of the accessible sinapic acid (25.4 ± 0.1 μmol/g of bran dry matter) in 2h40 min under mild conditions (pH 7, 50 °C). This efficient biocatalytic production of sinapic acid from mustard feedstock using an enzymatic cocktail paves the way for new developments for the design of an industrial process.

**Keywords**

Mustard bran; enzymatic cocktails; feruloyl esterase; sinapic acid; phenolic acid extraction

# 1. Introduction

The transformation of mustard seeds generates a large quantity of by-products reaching up to 60% (w/w) of the tonnage processed (Sehwag and Das, 2015). To preserve the environment, these by-products (bran, meal, cake) have to be recycled (Tisserand, 2003). However, despite their high protein content – up to 40% (w/w) – (Sehwag and Das, 2015), their use as feed product is limited due to some of their anti-nutrient components such as phenolic compounds (Matthäus and Angelini, 2005; Milkowski and Strack, 2010) reported to be from 1 to 4% (w/w) of defatted mustard seeds (Thiyam-Holländer et al., 2014) and are mainly composed of sinapic acid derivatives (SAD).

Sinapine represents 90% (w/w) of these SAD (Mayengbam et al., 2014; Naczk et al., 1992) and is found in similar proportions in different Brassica species (Nićiforović and Abramovič, 2014). The sinapine content is reported to be 11 ± 8 mg/g of mustard seeds (Bouchereau et al., 1991) and similar values are observed in the different by-products (Mayengbam et al., 2014; Wang et al., 1998). The other SAD are found in lower amounts (< 1 mg/g) such as sugar derivatives (e.g., sinapoylhexoside) or flavonoid derivatives (e.g., kaempferol derivatives) (Arena et al., 2020; Engels et al., 2012). These SAD are sinapic ester equivalent (SAE) as they can release sinapic acid through a simple hydrolysis of the ester linkage.

Sinapic acid stirs more and more interest from researchers due to its many biological properties such as free radical scavenging, anti-inflammatory, anti-carcinogenic and anti-UV properties (Horbury et al., 2019; Nićiforović and Abramovič, 2014). Moreover, sinapic acid is particularly interesting as a platform molecule for the synthesis of various compounds such as anti-UV ingredients (Baker et al., 2016; Dean et al., 2014; Peyrot et al., 2020a; Rioux et al., 2020), non-endocrine disruptive antiradical additive (Jaufurally et al., 2016), bisphenol A substitute for polymer / resin synthesis (Janvier et al., 2017), and substrate for pre-polymers (Diot-Néant et al., 2017). However, the access to sustainable, affordable and high purity sinapic acid is currently scarce. It is obtained either through synthetic pathways (Mouterde and Allais, 2018; Peyrot et al., 2019; van Schijndel et al., 2016) or by an extraction and hydrolysis of SAD from Brassicaceae plant material, such as the seed by-products of rapeseed (Odinot et al., 2017; Thiel et al., 2015) and mustard (Achinivu et al., 2021; Chadni et al., 2021). Further research dedicated to the supply of biobased sinapic acid from Brassicaceae residues such as mustard bran could overcome the sustainability and cost issues.

The hydrolysis of the SAD from Brassicaceae can be conducted in alkaline conditions. However, such processing conditions lead to a loss of the product naturalness which could limit its use in several industries such as food or cosmetic (Khattab et al., 2014). Thus, in order to overcome this limitation and to develop a process that is in accordance with the principle of green extraction (Chemat et al., 2019), other hydrolysis approaches must be developed such as biocatalytic pathway (Figure 1).

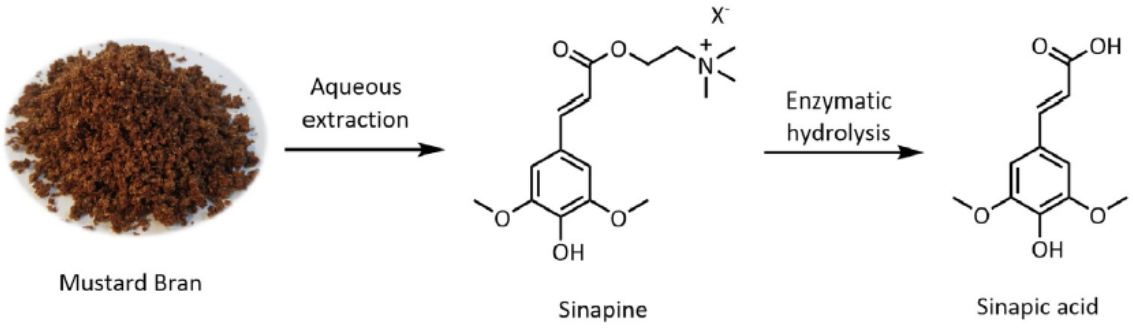


*Figure 1: Enzymatic hydrolysis of mustard bran for the recovery of sinapic acid.*

The hydrolysis of SAD was shown to be catalyzed by some feruloyl esterases (FAE) that also exhibit a sinapoyl esterase activity (Achinivu et al., 2021; Khattab et al., 2014; Laguna et al., 2019). This activity can be found in some commercial enzymatic cocktails of cellulases/hemicellulases/pectinase. They were showed to release sinapic acid either from phenolic extract of rapeseed (Vuorela et al., 2003) or directly from rapeseed meal (Thiel et al., 2015). These multi-enzymatic preparations are particularly interesting to increase the accessibility of phenolic compounds contained in the cytoplasm and bound to the plant cell wall (Gligor et al., 2019; Yuan, 2014). Furthermore, they are more affordable than purified FAE and readily accessible in high quantities. Thus, their use on the by-products of Brassicaceae seeds is of great interest to increase the recovery of sinapic acid. Compared to a previous study on mustard bran using a pure FAE from Clostridium thermocellum (Achinivu et al., 2021), the present study focused rather on the evaluation of commercial cellulolytic cocktails.

Herein, the objectives of this study were first to identify a sinapoyl esterase activity in commercial cellulolytic cocktails, and find their optimal operating conditions on a model SAD substrate (sinapine chloride). Then, the tolerance of the selected enzymatic solutions was assessed for different proportions of ethanol in water in order to maximize sinapic acid recovery (Achinivu et al., 2021; Reungoat et al., 2020). Finally, the coupling of the enzymatic hydrolysis with the extraction process was evaluated with a comparison between a pure FAE and a commercial enzymatic cocktail.

# 2. Materials and methods

## 2.1. Plant material

The mustard bran was provided by Charbonneaux-Brabant (Reims, France). This by-product is the result of the cold mechanical pressing of pickled mustard seeds (Brassica juncea) from Canada. The mustard bran is composed of seed hulls as well as other seed fractions. The material was not defatted, ground or dried. The mustard bran was stored in a cold room at 4 °C until use.

## 2.2. Chemicals

Pure synthetic sinapine chloride has been synthesized in-house and used for enzymatic hydrolysis as well as standard for HPLC analysis (Mouterde et al., 2020). Sinapic acid (98%) was purchased from Sigma Aldrich. The molecular masses of sinapic acid, sinapine chloride were 224.21 and 345.82 g/mol, respectively. The ethanol (purity >99%) used for the mustard extraction, formic acid and acetonitrile (LC–MS grade) for HPLC analysis were purchased from ThermoFisher. Sodium phosphate was purchased from Acros Organics. MilliQ water was produced with a Milli-Q Direct 8 from Merck Millipore (USA). Bradford's reagent was purchased from PanReac Applichem. The multi-enzymatic solutions Depol 740 L and Depol 670 L were kindly provided by Biocatalysts Limited (UK). Viscozyme L, Ultraflo XL, Pectinex Ultra SPL were purchased from Novozymes (Denmark). Deltazym VR AC-100 and Deltazym VR DP conc. were kindly provided by Weissbiotech (Germany). Pectinase PL "Amano" was kindly provided by Amano Enzyme Inc. (Japan). The mono-enzymatic solution of rumen FAE was purchased from Libios (France) with declared activity of 400 U/mL (1 U being defined as 1 µmol of ferulic acid ester converted per minute at pH 6.5 and at 40 °C).

## 2.3. Enzymatic screening on model substrate

The sinapoyl esterase activity of different enzymatic solutions was measured by spectrophotometry (BioTek Instruments, USA) on a model substrate in solution (0.029 µmol of sinapine chloride in 15 mM of sodium phosphate buffer).

### 2.3.1. Determination of sinapoyl esterase activity by spectrophotometry

Screening of sinapoyl esterase activity was performed by measuring the conversion rate of sinapine chloride to sinapic acid over a period of 10 min at temperature of 25, 35, 45, 50, 55 °C and at a pH of 6, 7, 8, adapted from Ralet et al. (1994). On a volume of 100 µL of the model substrate solution, the hydrolysis of sinapine chloride was evaluated at 335 nm over a period of 10 min (carried out in triplicate). 2.5 mL/g (1000 U/g) of sinapine chloride solution was used for the enzymatic reference (rumen FAE) assay. Only 250 mL/g of sinapine chloride was used for the assay of the enzyme cocktails because they are considered to be less concentrated than the rumen FAE.

The decrease in absorbance of the reaction medium during enzymatic hydrolysis was measured against a blank of pure sinapine chloride. The relationship between the absorbance and the concentration of sinapine chloride and sinapic acid was calculated according to Ralet et al. (1994). The molar extinction coefficients at 335 nm of sinapine chloride and sinapic acid at pH 6, 7, 8 using a 15 mM sodium phosphate buffer were measured at 10,760 ± 811 L/(mol.cm) and 6045 ± 286 L/(mol.cm), respectively. The concentrations of sinapine and sinapic acid in the reaction medium reach 290 µM and the Lambert-Beer law is respected. The conversion rate of sinapine chloride to sinapic acid ($X$) is calculated according to Eq.1:

$$X = \frac{A^f}{A_{max}} * 100 \qquad \textit{Eq.1}$$

Where $A^f$ is the relative absorbance, to the blank of sinapine chloride, of the aqueous reaction medium at the end of the measurement time and $A_{max}$ is the absorbance corresponding to a total conversion. It is calculated according to Eq.2.

$$A_{max} = 1.0296 * A_{sinapine}(pH) - 0.5099 + offset\ coefficient \qquad Eq.2$$

Where $A_{sinapine}(pH)$ is the absorbance of sinapine chloride as a function of the pH, naturally decreasing during the experiment due to the formation of sinapic acid. The difference in absorbance between the reaction medium and the blank was adjusted to zero with an $offset\ coefficient$ when necessary. The enzymatic activity for each solution was determined according to the conversion rate obtained over a period of 5 min (rumen FAE) or 10 min (enzymatic cocktails) under the determined optimal conditions (pH, temperature). Hereafter, one unit of sinapoyl esterase activity (1 U) is defined as the quantity of enzyme which hydrolyzes 1 µmol of sinapine chloride per minute under its optimal conditions.

### 2.3.2. Determination of the protein content of enzymatic cocktails

The protein content makes it possible to express the activity of the enzymatic cocktails in a more accepted and reproducible unit: U/mg of total protein in the cocktail. The protein content was measured according to a modified Bradford method (Bradford, 1976). Solutions of BSA (VWR) at concentrations of 60, 100, 200, 600, and 1000 mg/L and a dilution of commercial enzymatic cocktail were prepared. 75 µL of each of the previous solutions were added to a test tube with 1.5 mL of Bradford's reagent. After 30 min at room temperature and protected from light, the optical density (OD) at 595 and 450 nm were measured in 3 mL cuvettes against a water blank. The double measurement of absorbance ensures the control of potential drifts. Standard curve and protein content were obtained with the ratio between OD at 595 nm on OD at 450 nm of BSA solutions and enzymatic solution.

### 2.3.3. Enzymatic tolerance to ethanol

The tolerance to ethanol of the enzymatic solutions was assessed. 50 µL volumes of the model substrate solution containing ethanol were added to 50 µL diluted enzymatic solutions. The ethanol concentrations in the resulting reaction mediums tested are 0%, 10%, 20%, 30% (v/v). The relative conversion rate of sinapine chloride ($X'$) was chosen to represent the activity of the enzymatic solutions. This was calculated according to Eq.3.

$$X' = \frac{A^f_{EtOH}}{A^f} * 100 \qquad Eq.3$$

Where $A^f$ and $A^f_{EtOH}$ are the relative absorbances, to the blank of sinapine chloride at the end of the measurement time, of the aqueous and hydro-ethanolic reaction media, respectively. It was measured over a period of 30 min to increase the precision, at pH 7 and under the optimum temperatures for each enzymatic solution: 30 °C (rumen FAE), 50 °C (Depol 740L), 55 °C (Ultraflo XL, Deltazym VR AC-100, Pectinase PL "Amano"). The enzymatic dosages ($U/g_{sinapine}$) are respectively 500, 200, 150, 100 and 75 for rumen FAE, Depol740L, Ultraflo XL, Deltazym VR AC-100 and Pectinase PL "Amano". The experiments were carried out in triplicate.

## 2.4. Release of sinapic acid from mustard bran

### 2.4.1. Different extractions of sinapic acid equivalents by conventional solvent extractions

Samples of 1 mL were taken regularly from the mixture at 1, 10, 20, 40, 80 and 160 min, then analyzed by HPLC to determine when equilibrium is reached. Sinapic acid equivalents (SAE) content is considered to be the sum of sinapine and sinapic acid contents.

- ***Hydro-alcoholic extraction***

A quantity of 19.8 g of raw mustard bran (50.5% (w/w) of dry matter) was extracted by ethanol 70% (v/v) at 75 °C with a solvent to a matter ratio of 10 mL/g of bran dry matter (BDM) according to Reungoat et al. (2020).

- ***Aqueous extraction***

A quantity of 1.98 g of raw mustard bran was extracted with water at 100 °C, 100 mL/$g_{BDM}$ under the initial pH 4.3.

### 2.4.2. Alkaline hydrolysis of sinapine from mustard bran

Sinapic acid released by alkaline hydrolysis is considered to be the maximum content that can be obtained during the hydrolysis of sinapine to sinapic acid. The method used for the alkaline hydrolysis is adapted from Laguna et al. (2019). 1.98 g of raw mustard bran was mixed with water with a solvent to matter ratio of 100 mL/$g_{BDM}$. The pH was adjusted to 12 with 1M NaOH and controlled regularly during the hydrolysis. The mixture was heated at 30 °C and 1 mL samples were taken at 1, 10, 20, 40, 80 min and analyzed by HPLC.

### 2.4.3. Simultaneous extraction and enzymatic hydrolysis

According to the enzymatic screening on the model substrate, Depol 740L was the most efficient for the hydrolysis of sinapine to sinapic acid. It was therefore the only cocktail to be selected for the implementation of the enzymatic hydrolysis of mustard bran in water. Rumen FAE was also selected for a comparison of enzymatic cocktail with a mono-enzymatic solution. They were studied under their determined optimal operating conditions (50 °C, pH 7, 160 min). A control without enzyme was also included in the experiment. 1 g of mustard bran dry matter (BDM) was suspended in a volume of 100 mL of milliQ water contained in a 250-mL round bottom flask equipped with a condenser. Then, the pH was adjusted to 7.0 ± 0.3 using 450 µL of 1M sodium hydroxide. No buffer was used and only a variation up to 0.9 units of pH was observed at the end of the reaction which was also observed without enzymes. Depol 740L and rumen FAE (0.48 and 2.00 U/$g_{BDM}$ respectively) were added when the heating started from room temperature. The mixture was then incubated at 50 °C for 160 min. The enzymatic dosages had to be adjusted in order to observe the two maximum SAE yield obtained under this experimental condition.

The hydrolysis kinetics of sinapine to sinapic acid with enzymes was monitored by HPLC using 1 mL samples taken at 1, 10, 20, 40, 80, 160 min. Using the calibration curves established, the sinapine and sinapic acid contents were calculated according to the Eq.4.

$$Y = \frac{C * V_{solvent}}{m_{BDM}} \qquad Eq.4$$

Where $C$ is the concentration of either sinapine or sinapic acid in the extract (mg/L), $V_{solvent}$ (L) is the volume of the solvent and $m_{BDM}$ is the mass of the mustard bran dry matter (g).

### 2.5. Characterization of the mustard bran hydrolysate

#### 2.5.1. HPLC analysis of sinapine and sinapic acid

The 1 mL samples of the previous sections were filtered through a 0.20 µm Chromatofil filter, Xtra RC-20/25 before HPLC analysis. Sinapine and sinapic acid were quantified by reversed-phase UHPLC-DAD (Ultimate 3000; Dionex, ThermoFisher) equipped with a quadratic pump, autosampler, column furnace and diode array detector. A gradient elution was performed using water (solvent A), acetonitrile (solvent B), and formic acid 0.1% (v/v) in water (solvent C), on a C18 ThermoScientific™Accucore™aQ (100 × 3 mm with 2.6 µm particle size). Initial solvent (v/v) was 45% A, 5% B and 50% C. Solvent B gradient followed: 5% (0 min), 10% (0.990 min), 15% (3.190 min), 30% (7.440 min), 5% (8.510 min) while C remained constant. The column was maintained at 48 °C and run at a constant flow rate of 0.8 mL/min. Chromatograms were acquired at 320 nm and analyzed with Chromeleon software (version 6.8). Sinapine and sinapic acid were identified by comparing their relative retention times with their respective standards. Synthetic sinapine chloride and commercial sinapic acids were used in the preparation of the calibration curves. No significant differences ($p > 0.05$) were found for the HPLC responses either when sinapine or sinapic acid were dissolved in pure ethanolic, alkaline or aqueous solution.

#### 2.5.2. Other characterizations

The dry matter (DM) content was determined by drying the mustard bran into an air-flow oven (Binder) at 105 °C overnight. The crude protein content (N × 6.25) was determined by the Kjeldahl method, adapted from Bradstreet (1954). A distillation unit was used (Büchi K-350, Büchi Labortechnik AG, Switzerland). The total sugar content was determined by HPIC (Dionex CarboPac PA1, 4 × 250 mm, ThermoFisher Scientific) after an acidic hydrolysis with 4 N or 26 N sulfuric acid for 2.5 h. The ash content determination was adapted from ISO 749 (International Organization for Standardization (ISO), 1977). All results were expressed as percentage of the dry matter of the mustard bran or its extract.

### 2.6. Statistical analysis

All experiments were carried out in duplicate or in triplicate. The number of experiments performed is indicated by "n". The results are expressed as means ± standard deviations. Significant differences ($p < 0.05$) were determined by ANOVA followed by Tukey's test if necessary. The statistical analysis was carried out on the software R v4.0.2.

## 3. Results and discussion

### 3.1. Identification of sinapoyl esterase activity in different enzymatic solutions on model substrate

A screening was conducted at different pH and temperatures to identify and characterize several enzymatic cocktails that exhibit a sinapoyl esterase activity. The best cocktail was then used to hydrolyze sinapine from a raw mustard bran simultaneously with the extraction process.

The conversion rate of sinapine chloride to sinapic acid as a function of the pH and temperature with enzymatic solutions is presented in Figure 2. Only those with a conversion rate significantly different from zero were presented and considered to have a sinapoyl esterase activity.

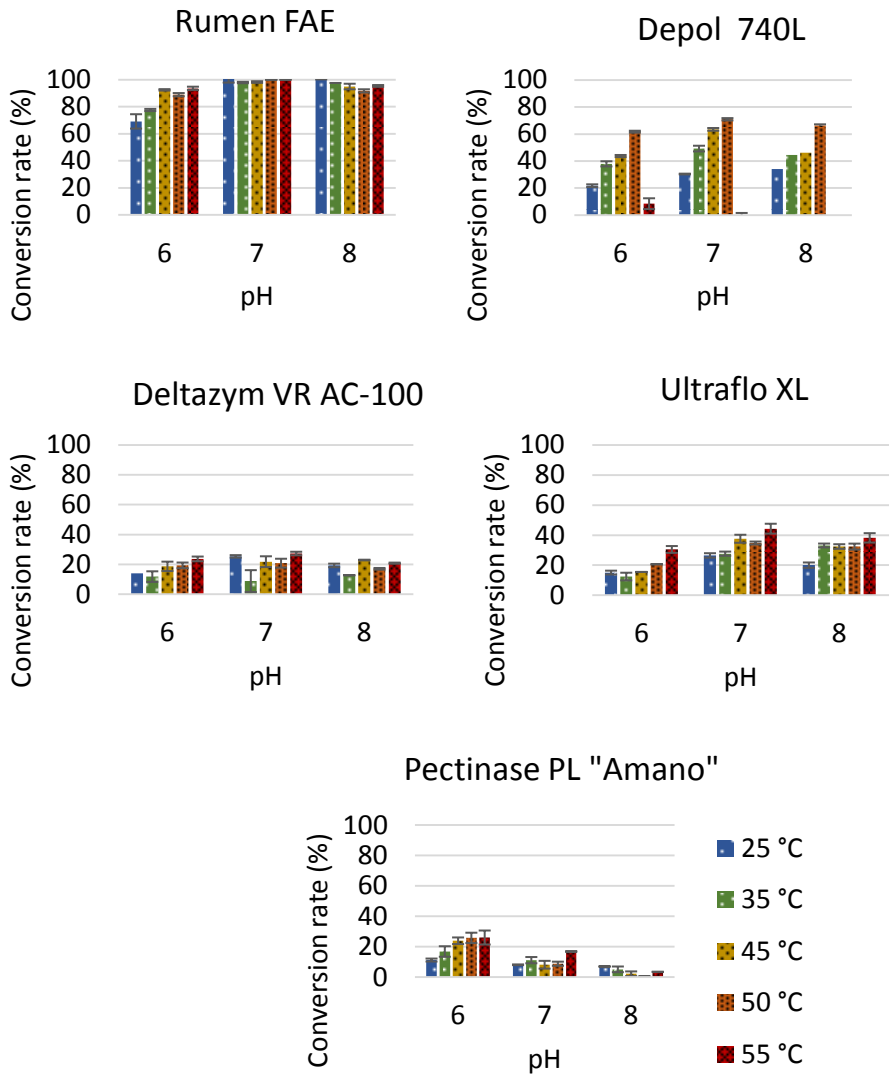


*Figure 2: Conversion rate of sinapine chloride into sinapic acid with enzymatic solutions for 10 min with n = 2.*

The screening showed that only rumen FAE, Depol 740L, Ultraflo XL, Deltazym VR AC-100 and Pectinase PL "Amano" solutions have a sinapoyl esterase activity. Other enzymatic solutions tested (Depol 670 L, Pectinex Ultra SPL, Viscozyme L and Deltazym VR DP conc.) were found to be ineffective for the hydrolysis of sinapine chloride for pH between 6 and 8 and temperatures between 25 and 55 °C. It is noteworthy to mention that the low activity of sinapoyl esterase found in Viscozyme L by Vuorela et al. (2003) was not detected in the present study.

According to Figure 2, the pure enzymatic solution of rumen FAE shows the best sinapoyl esterase activity among the enzymatic solutions evaluated. Total hydrolysis of sinapine to sinapic acid for 10 min using 1000 $U_{feruloyl\ esterase}$/g sinapine was obtained at pH 7 and 8 for temperatures between 25 and 55 °C. The activity measured was 200 ± 6 $U_{sinapoyl\ esterase}$/mL at 35 °C, pH 7. The activity towards sinapine is relatively high, even though it represents only half of the activity towards ethyl ferulate (400 $U_{feruloyl\ esterase}$/mL, according to the supplier). The sinapoyl esterase activity is considered as a secondary activity by the suppliers. Therefore, it is expected to be lower and depends of the type of SAD. Indeed, the activity was found even lower with ethyl sinapate as substrate (0.025 $U_{sinapoyl\ esterase}$/mL) (Achinivu et al., 2021).

The highest sinapoyl esterase activities for the enzymatic cocktails were found at pH 7 with a temperature of 50 °C (Depol 740L) and 55 °C (Ultraflo XL and Deltazym VR AC-100), whereas the optimum was found at pH 6 and 55 °C for Pectinase PL "Amano" (Figure 2). These results seem to indicate a diversity of sinapoyl esterases contained in the enzymatic cocktails.

Despite the high sinapoyl esterase activity, the mono-enzymatic rumen FAE cannot be used on an industrial scale as it is only available on an analytical scale and would be prohibitively expensive for higher scales. Therefore, it was only used as a reference in this study for the comparison with other enzymatic cocktails.

Table 1 summarizes, for each enzymatic solution, the optimum conditions of hydrolysis, the specific activity in U/mL of the cocktails and U/mg of total proteins in the cocktails.

*Table 1: Sinapoyl esterase activity measured in the active enzymatic solutions under their optimal conditions. Different superscript letters indicate a significant difference ($p < 0.05$).*

| Enzymatic solution | Protein content (mg/mL) | Specific activity (U/mL) | Specific activity [1] (U/mg) | pH | Temp. (°C) |
|---|---|---|---|---|---|
| Rumen FAE (reference) | 13.3 [2] | 200 ± 6 [a] | 15.0 ± 0.5 | 7 | 35 |
| Depol 740L | 15.4 ± 0.3 | 0.82 ± 0.02 [b] | 0.053 ± 0.001 | 7 | 50 |
| Ultraflo XL | 14.3 ± 0.8 | 0.59 ± 0.02 [c] | 0.041 ± 0.001 | 7 | 55 |
| Deltazym VR AC-100 | 21.3 ± 3.1 | 0.40 ± 0.01 [d] | 0.019 ± 0.001 | 7 | 55 |
| Pectinase PL ''Amano '' | 31.0 ± 3.1 | 0.3 ± 0.1 [d] | 0.009 ± 0.002 | 6 | 55 |

The sinapoyl esterase activities of Depol 740L, Ultraflo XL, Deltazym VR AC-100 and Pectinase PL were found to be of similar orders of magnitude. The activity of Depol 740L appeared significantly higher ($p < 0.05$) and was therefore selected for the rest of the study. Its present activity is similar to the one found by Thiel et al. (2015) (0.13 U/mL at pH 6, 50 °C on rapeseed meal). The denaturation of Depol 740L induced by the temperature seems to occur above 50 °C (Figure 2). This can be a limiting factor for the implementation of an extraction / hydrolysis process on plant matter for SAD recovery. The activity of Pectinase PL "Amano" was found to be slightly lower compared to the value obtained by Tsuchiyama et al., (2006) (2.0 U/mL at pH 5, 60 °C on methyl sinapate) certainly due to the nature of the substrates used in the different studies. It has to be noted that it is the first reported sinapoyl esterase side activity measured in Deltazym VR AC-100.

The use of enzymatic cocktails seems promising because they offer a dual effect: the degradation of the plant cell wall to ease the access to SAD, such as sinapine, and their concomitant hydrolysis. Another advantage of these enzymatic cocktails is their capacity to reduce the viscosity of the reaction medium (Mathlouthi et al., 2002). They are also commercially available in large quantities at a relatively low cost compared to pure enzymatic solution such as a rumen FAE.

## 3.2. Ethanol inhibition of the sinapoyl esterase activity

In order to improve the industrial process, the coupling of the solvent extraction step with the enzymatic hydrolysis must be addressed. It has been shown that, although enzyme tolerance to organic solvents is generally low, some natural enzymes tolerate certain levels of co-solvents. It is the case for

[1] The enzymatic concentration for each enzymatic solution represents the total quantity of enzymes present and not only the sinapoyl esterase (cellulase, hemicellulase, sinapoyl esterase, others).
[2] according to the supplier data.

the conversion of ethyl sinapate to sinapic acid by an FAE from *Clostridium thermocellum* in the presence of at least 32% (v/v) of ethanol (Achinivu et al., 2021). This is a characteristic that should not be overseen since one of the aims of the study is to evaluate the potential combination of hydrolysis by an enzymatic cocktail with a hydro-ethanolic extraction to produce sinapic acid.

The inhibition of the sinapoyl esterase activity of the active enzymatic solution was therefore determined. For this, the relative sinapine conversion rate (Eq.3) was plotted according to the ethanol concentration of the medium (Figure 3).

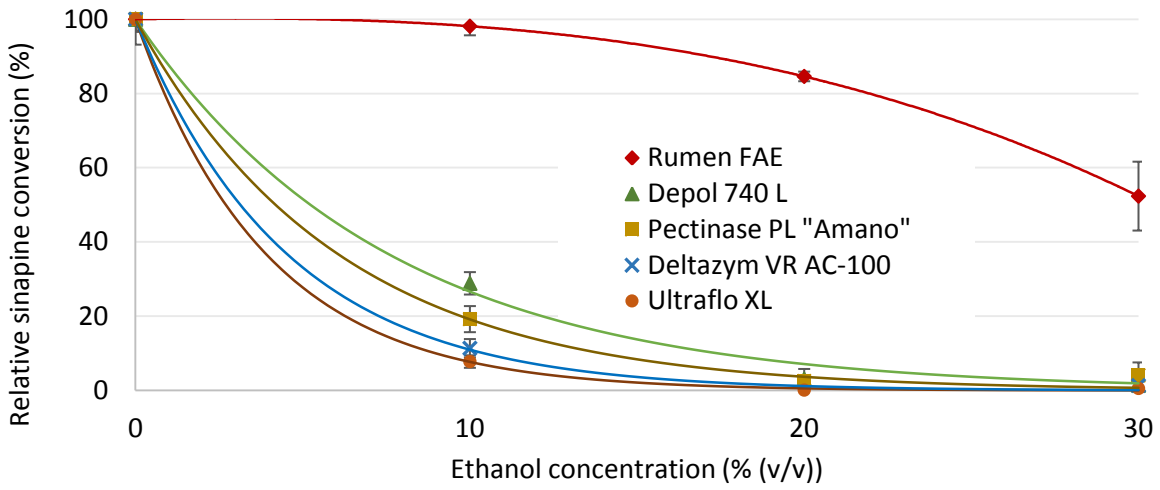


*Figure 3: Sinapine chloride conversion rate with enzymatic solutions in function of ethanol concentration for 30 min (n = 3).*

Figure 3 clearly shows that the sinapoyl esterase activity decreases when the ethanol concentration of the medium increases. This decrease is greater with enzymatic cocktails than with rumen FAE. The relative conversion rates achieved for all the enzymatic cocktails are between 8% and 29% with 10% (v/v) ethanol concentration and were completely inhibited beyond this concentration. Yet, the mono-enzymatic rumen FAE has a higher tolerance to ethanol with a relative conversion rate of 99% with 10% (v/v) ethanol and 50% with 30% (v/v) ethanol. A similar tendency was observed with pure feruloyl esterases from *Aspergillus niger, Talaromyces stipitatus and Clostridium thermocellum* (Achinivu et al., 2021; Faulds et al., 2011). These observations strengthen the idea that all the FAE do not have the same tolerance to ethanol. More studies are required to investigate their performances in the presence of ethanol. Nevertheless, it can be concluded that the combined use of the enzymatic cocktails in this study with a hydro-ethanolic solution would be ineffective for the hydrolysis of SAD into sinapic acid.

## 3.3. Release of sinapic acid from mustard bran by enzymatic hydrolysis

According part 3.1. , Depol 740L was selected to be applied on a mustard bran for the simultaneous extraction and hydrolysis to produce sinapic acid. Rumen FAE was also selected for a comparison with a mono-enzymatic solution. Their efficiencies were determined in several steps (Figure 4). For this, the maximum content of sinapic acid equivalents released from mustard bran was foremost quantified using hydro-ethanolic extraction and alkaline hydrolysis. The enzymatic hydrolysis was therefore combined with aqueous extraction process on mustard bran and the activity of Depol 740L and rumen FAE were measured.

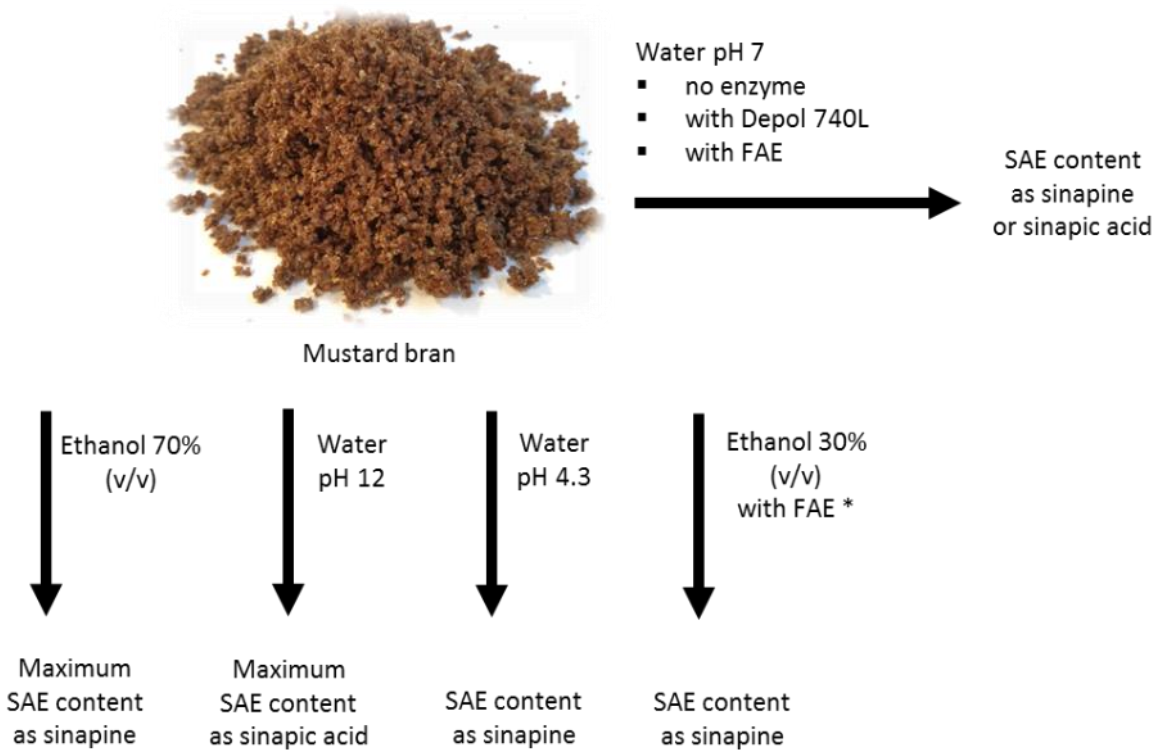


*Figure 4: Flowchart of the different extractions on mustard bran. * Details in annex materials.*

### 3.3.1. Characterization of the maximum SAE extractable from the mustard bran

The hydro-alcoholic extraction of mustard bran with 70% (v/v) ethanol released 37.2 ± 0.2 µmol/$g_{BDM}$ of sinapic acid equivalent (SAE) mainly as sinapine (> 99%). Moreover, the alkaline hydrolysis yielded a non-different quantity of SAE (36.9 ± 0.2 µmol/$g_{BDM}$) ($p$ > 0.05) but in the form of sinapic acid. Therefore, assuming that no degradation occurred (Laguna et al., 2019), it can be concluded that all the SAE were extracted with the hydro-ethanolic solvent. However, this extraction step is not compatible with the enzymatic hydrolysis (i.e., enzyme denaturation, see part 3.2.). As a consequence, an aqueous extraction was also carried out on the bran with a natural mixture pH of 4.3. This allows the release of 33.0 ± 0.3 µmol/$g_{BDM}$ of SAE in the form of sinapine, i.e. an efficiency of 89 ± 1 % compared to the hydro-alcoholic extraction. This content is consistent with other similar aqueous extraction on mustard seed (*B. juncea)* by-product (Dubie et al., 2013).

### 3.3.2. Enzymatic hydrolysis of mustard bran during aqueous extraction

The enzymatic cocktail Depol 740L was selected to release sinapic acid from mustard bran (50 °C, pH 7, 160 min) due to its higher activity observed on the model solution. Two controls were implemented: an aqueous extraction without enzymes and an aqueous extraction using rumen FAE. The sinapine and

(a) (b)

sinapic acid contents according to the extraction duration are presented in Figure 5.

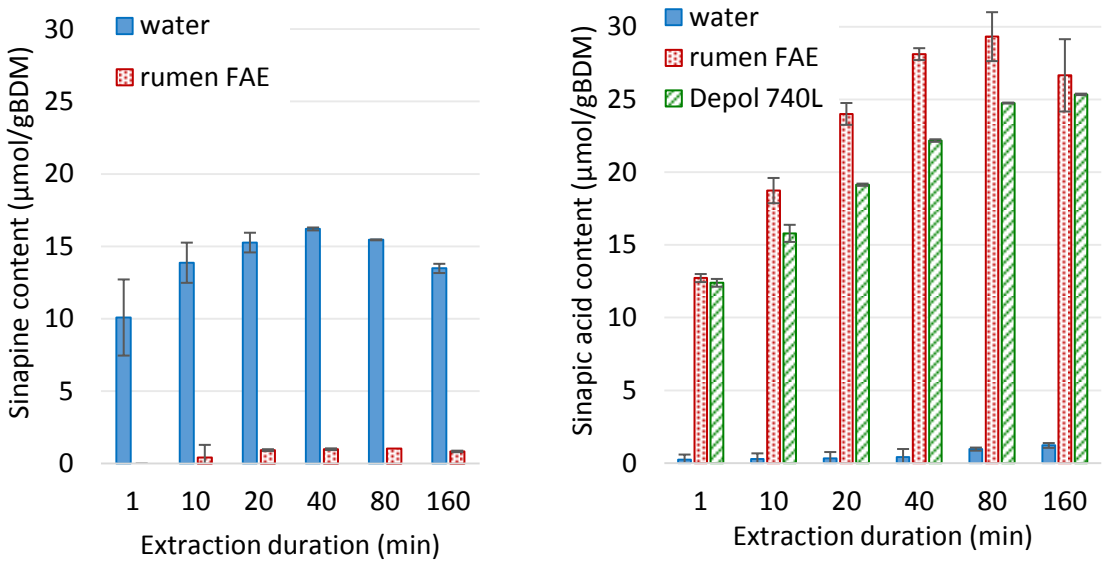


*Figure 5: Sinapine content (a) and sinapic acid content (b) obtained from mustard bran with Depol 740L, rumen FAE and water at 50 °C, pH 7 during 160 min with n = 2.*

According to Figure 5(a) and (b), the aqueous extraction without enzymes mainly releases sinapine. When adding rumen FAE and Depol 740L, sinapic acid becomes predominant in solution. The amount of enzymes added was calculated so that the hydrolysis lasts between 20 and 75 min. This calculation was based on the enzymatic activity on a model solution of sinapine as determined in part 2.3. . and on the sinapine content of mustard bran obtained in part 3.3.1. . However, Figure 5 shows a total hydrolysis after only 1 min for the two enzymatic solutions the hydrolysis of the sinapine extracted from the mustard bran is faster than that on the model solution of sinapine. This result can be explained by the fact that the medium would have a positive effect on the sinapoyl esterase activity of the enzymatic cocktails. This could be related to ionic strength, substrate concentration or cofactors (Bisswanger, 2014).

Moreover, the sinapic acid content in extract is increasing over time when sinapoyl esterases are used. However, the sinapine content is negligible while it continues to increase for aqueous extraction. Therefore, the diffusion of sinapine out of the mustard bran seems to be the rate limiting step of the overall extraction and hydrolysis process. By analogism, this limitation is observed on simultaneous saccharification and fermentation processes, when the production of ethanol by microorganisms is limited by the enzymatic production of glucose (Philippidis and Smith, 1995).

The content of SAE extracted from the mustard bran in presence of the rumen FAE and Depol 740L (29.9 ± 2.4 and 25.4 ± 0.1 µmol/$g_{BDM}$, respectively) is found higher than with the aqueous extraction (16.6 ± 0.5 µmol/$g_{BDM}$). This result could be explained by (i) the release of other SAD from the mustard bran by the action of the feruloyl esterase (Faulds, 2010), and/or (ii) the presence of ionic interactions with proteins (Jakobek, 2015). Indeed, during the hydrolysis at pH 7, sinapic acid is under the sinapate form ($RCO_2^-$) (Smyk and Drabent, 1989). With the presence of proteins in the extract (Figure 6), especially the positively charged napins (isoelectric point around 11 (Bérot et al., 2005; Neumann et al., 1996)), the sinapate might be dragged out of the mustard bran potentially due to ionic interactions with proteins. At the opposite, during aqueous extraction, the presence of the positively charged napins would not be favorable for the diffusion of the positively charged sinapine due to electrostatic repulsion. More studies are required to decipher these mechanisms.

Unlike rumen FAE that is a pure enzyme, Depol 740L is an enzymatic cocktail. The cellulases and hemicellulases contained in Depol 740L can then deconstruct parts of the mustard bran. Their effects on the total sugars, proteins and ash contents are thus presented in Figure 6.

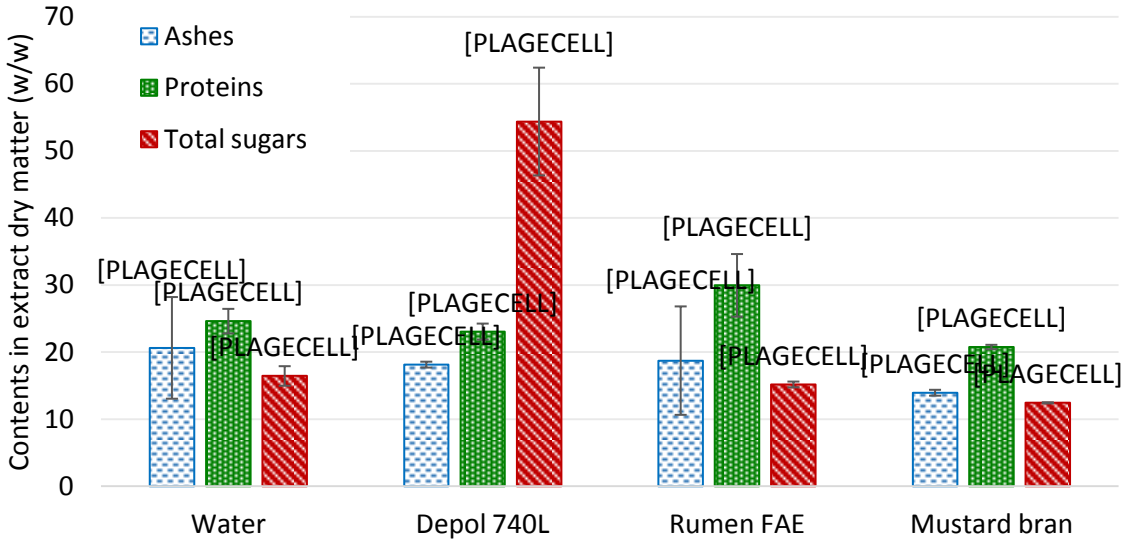


*Figure 6: Ash, protein and total sugar contents compared to the dry matter fraction of mustard bran. Different superscript letters indicate a significant difference between the media ($p < 0.05$).*

No significant differences were observed for the ash and protein contents between the three extracts (aqueous extraction, hydrolysis with rumen FAE and Depol 740L). Regarding the total sugar content in the extract dry matter (w/w), it is three times higher with Depol 740L (54 ± 8 %) than with the aqueous (16 ± 1 %) or with the rumen FAE extractions (15.2 ± 0.4 %). An increase of this sugar content was also reported by Yuan et al. (2014) using a pure hemicellulase on a mustard bran. This shows that a degradation of plant cell walls takes place with the use of the Depol 740L enzymatic cocktail. The higher release of sugar is associated with a higher release of dry matter (w/w) by Depol 740L (0.50 ± 0.06 %) compared to the two other extractions respectively (0.29 ± 0.02 % and 0.30 ± < 0.01 %). Nevertheless, the use of an enzymatic cocktail that deconstructs parts of the mustard bran is not conducive to increasing the SAE content as well as the rate of SAD release. Indeed, as can be seen on Figure 5, the maximum SAE content (29.9 ± 2.4 µmol/$g_{BDM}$) is observed at 80 min for hydrolysis with rumen FAE while it is 160 min for hydrolysis with Depol 740L (25.4 ± 0.1 µmol/$g_{BDM}$). This might be explained by the aggregation of the sinapate formed and the carbohydrates released through hydrogen bonds (Jakobek, 2015).

The highest sinapic acid content with Depol 740L was reached after 160 min (Figure 5(b)). In order to ensure that the sinapoyl esterase activity of the Depol 740L was not batch dependent, this activity was measured on four different batches of this enzymatic cocktail. An activity of 0.9 ± 0.1 U/mL was found on model sinapine chloride. This enzymatic cocktail can be considered a reliable source of sinapoyl esterase with minor needs for adjustments on the quantity implemented. For a same enzymatic loading, the time to reach the maximum sinapic acid content from mustard bran would be 143 ± 16 min. This is almost twice faster compared to the hydrolysis studied by Laguna et al. (2019) on rapeseed meal using a similar enzymatic loading (2.3 U/g meal).

The stability of sinapine and sinapic acid can also be observed on Figure 5. The SAE content increases until an equilibrium. Then, an extended reaction time decreases the sinapic acid or the sinapine contents. A drop of 8% in sinapic acid is observed 80 min after equilibrium with the rumen FAE. A drop of 10% in sinapine occurs 120 min after the equilibrium with aqueous extraction. The decrease of the sinapic acid content can be explained by its oxidative dimerization at pH 7 as reported by Rubino

et al. (1996). This phenomenon seems similar with sinapine and may be accelerated with increasing temperature (Figure S1).

### 3.3.3. Efficiency comparison of the different extraction processes of sinapic acid equivalents (SAE)

Different extraction processes were carried out in this study. Table 2 gathers the operating conditions as well as the efficiency of these different processes.

*Table 2: Extraction efficiency of different processes for the recovery of sinapic acid equivalent from mustard bran.*

| Extraction process | Operating conditions of the extraction | SAE content (µmol/$g_{BDM}$) | SAE extraction yield (%) | Major form of the SAE |
|---|---|---|---|---|
| Ethanol 70% (v/v) | pH = 4.3, 75 °C, 40 min, 10 mL/$g_{BDM}$ | 37.2 ± 0.2 [a] | Reference 100 | Sinapine |
| Aqueous | pH = 4.3, 100 °C, 40 min, 100 mL/$g_{BDM}$ | 33.0 ± 0.3 [b] | 89 ± 1 | Sinapine |
| Ethanol 30% (v/v) [1] with rumen FAE | pH = 7, 50 °C, 80 min, 100 mL/$g_{BDM}$ | 19.9 ± 0.5 [e] | 53 ± 2 | Sinapine |
| Aqueous | pH = 7, 50 °C, 40 min, 100 mL/$g_{BDM}$ | 16.6 ± 0.5 [f] | 45 ± 2 | Sinapine |
| Aqueous [2] | pH = 12, 30 °C, 40 min, 100 mL/$g_{BDM}$ | 36.9 ± 0.2 [a] | 99 ± 1 | Sinapic acid |
| Aqueous with rumen FAE | pH = 7, 50 °C, 80 min, 100 mL/$g_{BDM}$ | 29.9 ± 2.4 [b] | 80 ± 8 | Sinapic acid |
| Aqueous [1] with Depol 740L | pH = 7, 50 °C, 160 min, 100 mL/$g_{BDM}$ | 25.4 ± 0.1 [c] | 68 ± 1 | Sinapic acid |

1: with enzymatic hydrolysis. 2 with alkaline hydrolysis.

The hydro-ethanolic extraction process is taken as a reference (i.e., extraction efficiency of 100%). However, the extracted SAE are mainly in the form of sinapine. It is necessary to add a hydrolysis step to obtain sinapic acid for which strict aqueous conditions are needed. Thus, the aqueous extraction process coupled with alkaline hydrolysis has an efficiency equivalent to the reference process (99 ± 1 %). To substitute the alkaline hydrolysis by an eco-friendlier enzymatic hydrolysis, the coupling of enzymatic hydrolysis in a hydro-ethanolic medium was tested. However, among the enzymatic solutions in this study, only the rumen FAE retains part of its activity (Figure S2). Its implementation results in a yield of 53 ± 2 % under 30% (v/v) ethanol, twice lower than the reference process. Unfortunately, the main SAE remains under the sinapine form due to the sinapoyl esterase inhibition. The prospects for improvement relate to the use of sinapoyl esterase from another source of microorganism such as *Clostridium thermocellum.* Achinivu et al. (2021) showed that the pure enzyme applied on mustard bran could still produce sinapic acid in the presence of 25% (v/v) ethanol. However, the cost of pure enzyme may be limiting.

In order to combine the extraction and the enzymatic hydrolysis process, it is more suitable to switch to an extraction in aqueous medium. Certainly, the extraction efficiency is lower with water at 100 °C (89 ± 1 %) compared to the reference hydro-ethanolic process. However, when combined with sinapoyl esterases solutions, yields of 80 ± 8 % and 68 ± < 1 % as sinapic acid were achieved with rumen FAE and Depol 740 L, respectively. It is similar to the yields reported with a mono-enzymatic FAE solution on rapeseed meals of 64% (Laguna et al., 2019) and 77% (Vuorela et al., 2004). Some differences may be due to the variation of the SAE contents between Brassica species (Bouchereau et al., 1991). The sinapic acid content in extract obtained with Depol 740 L is 5.69 ± 0.01 mg/gBDM.

This is in the same order of magnitude observed by Achinivu et al. (2021) on mustard bran (9.64 ± 0.07 mg/gBDM) as they showed a variability in function of the seed batches. Finally, this study is part of a project to develop an integrated process. Further knowledge is required to determine the tolerance of enzymes and products during time under the operational conditions, and the best enzymatic dosage. Therefore, the present study opens the way for further optimization studies on the simultaneous extraction with enzymatic hydrolysis.

# 4. Conclusion

Sinapoyl esterase activity towards sinapine has been revealed for four commercial enzymatic cocktails. A same order of magnitude was found which allow to choose from several biocatalytic and accessible solutions and to not be dependent on only one manufacturer. It is particularly important for a viable development of an industrial process. Unfortunately, the enzymatic conversion using these cocktails was not effective above 10% (v/v) ethanol which hampers the simultaneous extraction and hydrolysis on mustard bran with ethanol. However, in aqueous condition (50 °C, pH 7), 68% of the sinapic acid equivalent contained in the mustard bran were successfully converted into sinapic acid in 2 h 40 min with Depol 740 L. Further optimization of the operating conditions should allow the development of an industrial process for the recovery of sinapic acid from mustard bran.

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# ANNEX MATERIALS

## Annex S1

Introduction

Enzymatic hydrolysis of mustard bran was conducted with Depol 740L at a temperature higher than its optimum condition in order to enhance the extraction yield of sinapic acid equivalents.

Material and method

1 g of mustard bran dry matter (BDM) was suspended in a volume of 100 mL of milliQ water contained in a 250-mL round bottom flask equipped with a condenser. Then, the pH was adjusted to 7.0 ± 0.3 using 450 µL of 1M sodium hydroxide. No buffer was used and only a variation up to 0.9 pH unit was observed at the end of the reaction which was also observed without enzymes. Amounts of 2 $U/g_{BDM}$ for Depol 740L were added when heating began. The mixture was then incubated at 75 °C for 160 min. The hydrolysis kinetics of sinapine to sinapic acid with Depol 740L was monitored by HPLC using samples of 1 mL taken at 1, 10, 20, 40, 80 and 160 min. The aqueous extraction without enzymes was also conducted under the same conditions as a control.

Results

Figure S1 presents the sinapine and sinapic acid contents during the enzymatic hydrolysis in water. The is a competition between the extraction of sinapine from the bran, its enzymatic hydrolysis to sinapic acid and both compounds degradation overtime. Therefore, the combination of high temperature (75 °C) under pH 7 seems to have a huge impact on the stability of sinapic acid and sinapine, as their contents decrease during the reaction time.

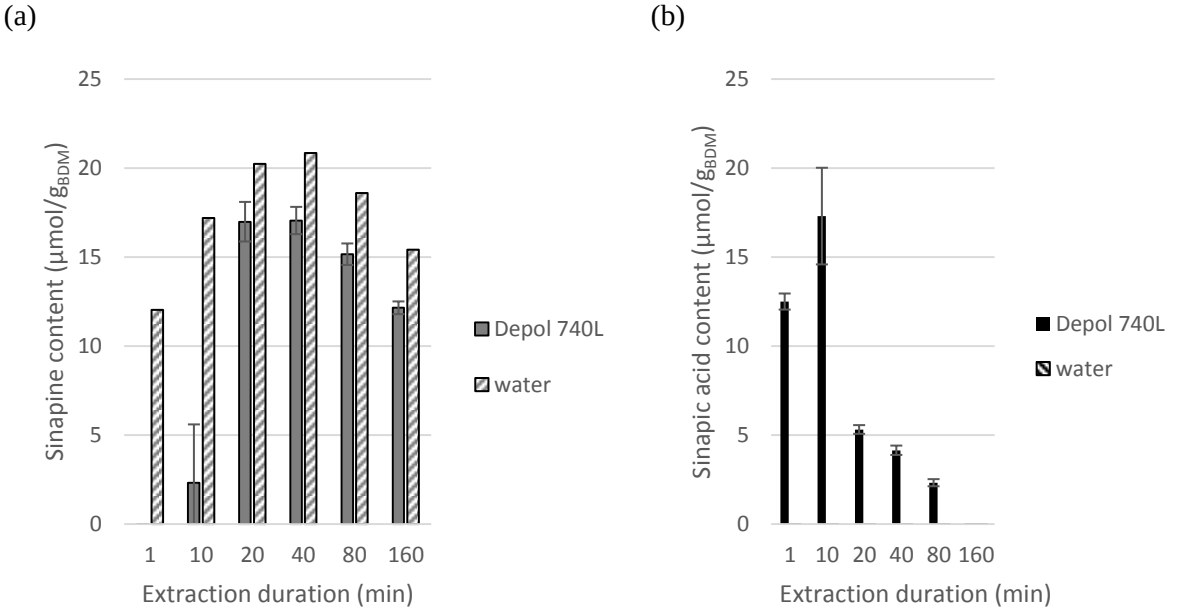


Fig. S1. Monitoring of hydrolysis kinetics during extraction of sinapic acid equivalents from mustard bran with Depol 740L and without enzymes at 75 °C, pH 7, n = 2.

# Annex S2

Introduction

The enzymatic hydrolysis of mustard bran was conducted using a hydro-ethanolic mixture to enhance the extraction yield of sinapic acid equivalents.

Material and method

The hydrolysis of mustard bran using a mixture of hydro-ethanolic solvent and rumen FAE was conducted at 50 °C and pH 7 in a ratio of 100 $mL_{solvent}/g_{BDM}$. The ethanol concentrations of the mixture (%v) were 0, 10, 20 and 30%. A 1 mL sample of the extracts at equilibrium (80 min) was analyzed by HPLC.

Results

Figure S2 presents the content of sinapic acid equivalents obtained during the extraction with the rumen FAE under different ethanol concentration in water. The proportion of converted sinapine to sinapic acid decreases as ethanol concentration in the media increases. The highest content of SAE extracted remains without ethanol in the mixture. The increasing quantity of sinapine represents the deactivation or denaturation of the rumen FAE on one side, and the higher extraction efficiency of the hydro-ethanolic solvent on the other.

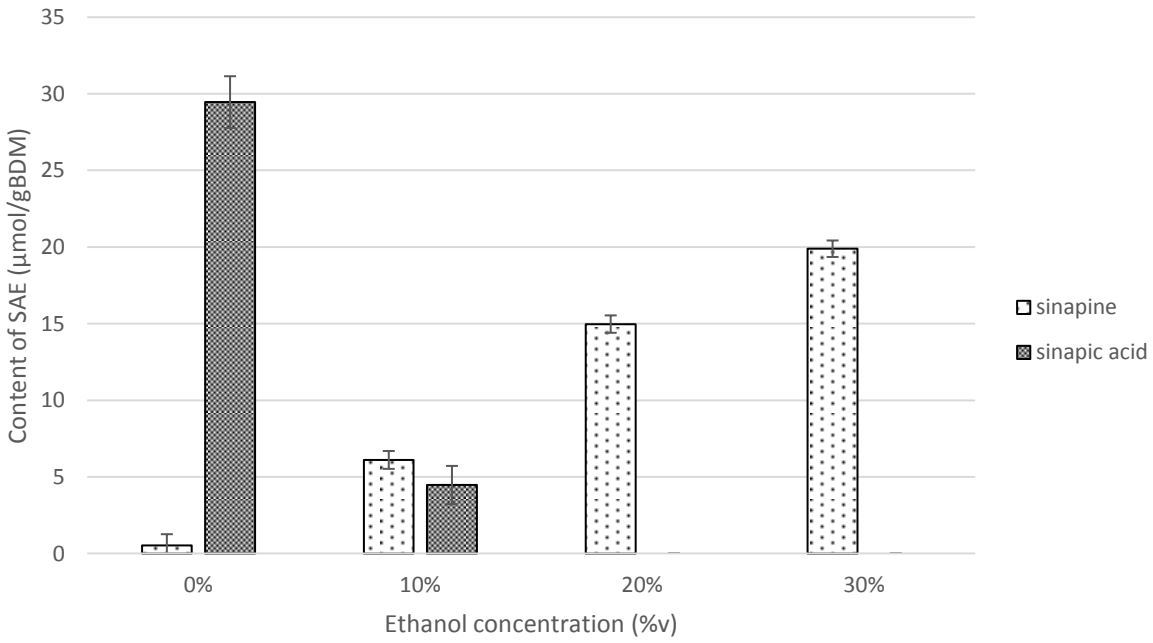


Fig. S2. Sinapic acid equivalent content for the simultaneous extraction and hydrolysis with rumen FAE under different ethanol concentration at 50 °C, pH 7 with n = 2