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## ► To cite this version:

Ezinne C. Achinivu, Amandine Flourat, Fanny Brunissen, Florent Allais. Valorization of waste biomass from oleaginous “oil-bearing” seeds through the biocatalytic production of sinapic acid from mustard bran. *Biomass and Bioenergy*, 2021, 145, pp.105940. 10.1016/j.biombioe.2020.105940 . hal-03492936

**HAL Id: hal-03492936**

**<https://hal.science/hal-03492936>**

Submitted on 7 Mar 2022

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1 **Valorization of Waste Biomass from Oleaginous “Oil-Bearing”**  
2 **Seeds through the Biocatalytic Production of Sinapic Acid from**  
3 **Mustard Bran**

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## 9 ABSTRACT

10 This paper focuses on developing a sustainable and integrated process for the **biocatalytic**  
11 **extraction of sinapic acid from the waste biomass of oleaginous “oil-bearing” seeds**. Using an  
12 optimized chemo-enzymatic technique, sinapine was effectively liberated **from mustard bran** and  
13 then completely hydrolyzed into sinapic acid. Several parameters were optimized to release  
14 sinapic acid (*e.g.*, mustard species, extraction methods, mustard production dates, ethanol  
15 concentration, solids loading, extraction time and enzyme type or concentration). **Subsequently,**  
16 **an integrated bioprocess was developed and scaled up guided**. The results revealed that the  
17 biomass type or source and the enzyme used can substantially impact the release of sinapic acid,  
18 and overall cost, respectively. For the Canadian lot of crude mustard bran, ~10 mg of sinapic  
19 acid per g mustard bran was successfully extracted, along with 43 mg total phenolics per g  
20 mustard bran and 71 mg sugars per g mustard bran. Lastly, supplementary portions of the  
21 biomass (*i.e.*, lipids) were also discussed for their applicability as value added products.

22 **KEYWORDS**

23 biocatalysis; sinapic acid; waste biomass valorization; mustard bran; bioprocessing; green  
24 chemistry

## 25 1. INTRODUCTION

26 In recent years, the use of biomass to produce bioproducts (such as bio-based chemicals) has  
27 grown tremendously around the world—potentially displacing significant amounts of petroleum  
28 consumption [1]. Scientists and engineers have been able to demonstrate that non-food biomass,  
29 e.g., the whole plant matter of agricultural/food residues, can be converted into key bio-based  
30 products such as plastics, chemicals, and fertilizers [2]. Nevertheless, to develop a cost-  
31 competitive bioeconomy, it is essential to thoroughly explore complete biomass/resource  
32 utilization for the sustainable production of bio-based chemicals that could replace petro-derived  
33 alternatives.

34 In addition to their lignocellulosic fraction, most biomass can contain small amounts of  
35 phenolic compounds that are often targeted due to their aromatic functionality and their  
36 numerous biological activities [3]. In particular, the oleaginous biomass that is generated from  
37 the industrial by-products of rapeseeds such as mustard and canola (known as mustard bran and  
38 canola meal respectively) contains ~1-5 wt. % of sinapic acid derivatives [4]. Hitherto these  
39 compounds have been largely ignored, lost in waste streams, underutilized, and have not been  
40 valorized within the context of a bioproduction facility. These bioactive compounds could  
41 potentially be a stream of value-added products that can be used to supplement and sustain the  
42 **commercial capability** of the biorefinery.

43 Sinapic acid is a naturally occurring *p*-hydroxycinnamic acid, a member of the  
44 phenylpropanoids family [3–6]. This compound generally exhibits antioxidant, antimicrobial,  
45 anti-inflammatory, anticancer, and anti-anxiety activities [3,5], and therefore, has been suggested  
46 for possible use in food processing, cosmetics, and the pharmaceutical industry. More recently, it  
47 has been used as a platform chemical for the syntheses of (1) key additives that can function as

48 non-endocrine disruptive anti-UV and antiradical agents [7–11], and (2) syringaresinol, a  
49 bisphenol A substitute for polymers/resins synthesis [12,13]. This research activity has the  
50 potential to be impactful given that the top chemicals used as anti-UV agents and antioxidants  
51 have several issues associated with developmental and reproductive toxicity, hormone disruption  
52 [14], carcinogenicity [15], and environmental persistence [16,17]. Despite the promise associated  
53 with the applications for sinapic acid, it is currently produced from the petroleum-derived  
54 syringaldehyde *via* a Knoevenagel-Doebner condensation [18]. It is, therefore, imperative to  
55 explore the release of this compound from natural sources (such as waste mustard bran) to  
56 improve the sustainability index for the use of this compound.

57 Over 1 billion ton of cereals and oilseeds are produced yearly, hence, generating various by-  
58 products (*e.g.*, straw, bran, press cake) that contain sinapic acid [19]. A small amount of these  
59 resources are presently employed for animal feed [20] or biogas (methanization) [21], however,  
60 this is a vast underutilization of resources that have the potential to produce bioproducts for  
61 direct revenue generation [22,23]. Supported by a collaboration with a local mustard producer in  
62 Reims, France (Charbonneaux-Brabant), this paper explores the possibility of releasing sinapic  
63 acid from the residue recovered after the mustard production process (mustard bran), along with,  
64 additional product streams that can be generated for additional valorization.

65 Various methods have been reported to extract phenolics from different parts of the mustard,  
66 and the most common techniques utilize a mixture of water and an organic solvent often coupled  
67 with thermal and physical methods [24–32]. In a previous study, a very simple and eco-friendly  
68 process was developed to obtain the phenolic extract using only one extraction with ethanol and  
69 water without any pre-treatment [33]. Nevertheless, the phenolic compounds typically recovered  
70 are mainly sinapic acid derivatives. The most common sinapic acid derivative is sinapine (~50-

71 80%), the choline ester of sinapic acid, followed by other analogues of sinapic acid (*e.g.*,  
72 glucosidic ester, glucopyranosyl sinapate), leaving only a small amount of freely existing sinapic  
73 acid [34–37]. Although phenolic esters can be hydrolyzed with sodium hydroxide [38–40], the  
74 liberated phenolics are prone to chemical degradation under such conditions. Consequently, the  
75 enzymatic release of sinapic acid was the preferred route in this study. Enzymes with esterase  
76 activity have been successfully used for hydrolyzing phenolic esters in various biomass  
77 feedstock including rapeseed meal and therefore, they will be applied to this study [37,41–43].

78 Therefore, this paper primarily focuses on developing an integrated process for the extraction  
79 and conversion of the sinapic acid derivatives directly into sinapic acid—the leading bioactive  
80 and chemically relevant form of this molecule. This research study demonstrates that sinapic acid  
81 can be entirely extracted biocatalytically from mustard bran, while also investigating process  
82 optimization, improvement, and scale up. Several parameters were optimized to release the  
83 maximum amount of sinapic acid (mustard species, extraction methods, mustard production  
84 dates, ethanol concentration, solids loading, extraction time, enzyme type and enzyme loading).  
85 Subsequently, an integrated process was developed (combining phenolic extraction with the  
86 enzymatic hydrolyses) and scaled up to determine the **practicability** at larger scales. Lastly,  
87 supplementary portions of the biomass (lipids and sugars), that are readily extracted during this  
88 process, were also discussed for their applicability as value-added products.

## 89 **2. MATERIALS/METHODS**

### 90 **2.1. Materials**

91 Industrial mustard residue was kindly furnished by Charbonneaux-Brabant (Reims, France)  
92 and used as-received. Charbonneaux-Brabant uses mostly seeds of *Brassica juncea* (90 wt. %)  
93 but also *Sinapis alba* from Canadian and French suppliers. Several lots were furnished from

94 different regions (France and Canada) and different production years (2015, 2016, 2017) and  
95 different processing conditions (NEW Sieve, OLD Sieve). Based on prior analyses [33], the  
96 mustard residue has a representative composition of about 53 wt. % moisture, a residual fat  
97 proportion of 5 wt. %, corresponding to 11 wt. % with regards to dry matter (Figure 1).  
98 Therefore, to accurately report our raw matter to what is often described (*i.e.*, dried and defatted  
99 mustard), one can thus consider that 2.3 g of crude product is equivalent to 1 g of dried and  
100 defatted material (DDM) [33].

101 All reagents were provided by Sigma Aldrich, solvents were provided by VWR (Ethanol,  
102 methanol, hexane and sodium carbonate). Folin-Ciocalteu reagent was purchased from Merck.  
103 Formic acid and acetonitrile LC-MS grade was purchased from ThermoFisher Scientific. Milli Q  
104 water was produced by Milli-Q Direct 8 from Merck Millipore (Burlington, USA). Ultraflo L  
105 (source: *Humicola insolens*), was obtained from Novozymes (Bagsvaerd, Denmark). Feruloyl  
106 esterases labelled as FAE1 (source: *Clostridium thermocellum* with activity 0.6 U mg<sup>-1</sup>) and  
107 FAE2 (source: rumen microorganism with activity ~25 U mg<sup>-1</sup>) were obtained from Megazyme  
108 (Chicago, USA). *trans*-Ethyl (ES) sinapate was synthesized in house in two steps from  
109 syringaldehyde (Knoevenagel-Doebner condensation followed by an esterification with ethanol).  
110 NMR spectra were recorded on Fourier 300, Bruker, 300 MHz <sup>1</sup>H spectra and 75 MHz <sup>13</sup>C  
111 spectra were calibrated on CDCl<sub>3</sub> residual peak,  $\delta = 7.26$  and 77.16 ppm, respectively (See  
112 supporting information Figure S1-2).

## 113 **2.2. Experimental Methods**

### 114 **2.2.1. Defatting and Drying**

115 The mustard residue was placed in a Soxhlet apparatus (accompanied with a cellulose  
116 Whatman™ extraction thimble) for complete defatting. The extraction was then carried out for at



117 least 12 hours using hexane as a solvent. After the extraction was complete, the resultant hexane  
118 phase was recovered and evaporated to dryness first with a rotary evaporator and then with a  
119 high vacuum pump (Vacuubrand PC3-RZ6). The resultant lipids were stored in a sealed  
120 container and later analyzed for the presence of phenolics (using  $^1\text{H}$  NMR on a Fourier 300,  
121 Bruker) and for the fatty acid content (see below). The moisture content of the mustard residue  
122 was determined using a Mettler Toledo Moisture Analyzer HB43-S.

### 123 **2.2.2 Fatty acids Transmethylation and Gas Chromatography**

124 The fatty acid composition of the recovered lipids (from above) was determined after the  
125 transmethylation of the lipids with  $\text{BF}_3/\text{CH}_3\text{OH}$  in the presence of an internal standard (C17:0)  
126 [44]. For each sample, an aliquot (about 10-120  $\mu\text{L}$  containing about 1 mg) of the lipid extract  
127 was dried with a nitrogen flow in a water bath at 40  $^\circ\text{C}$  and then transmethylated using a mixture  
128 of 1.2 mL of  $\text{MeOH-BF}_3$  (14 wt. %) and 10  $\mu\text{g}$  of the C17:0 internal standard. The reaction  
129 mixture was then placed in a water bath at 90  $^\circ\text{C}$  for 10 min to transform the lipid molecules into  
130 fatty acid methyl esters (FAME). Next, the samples were cooled to room temperature, dissolved  
131 in 1.5 mL of hexane, and then the organic phase containing the FAME was washed three times  
132 with 2 mL of water. The final organic phase containing lipids was then stored at -20  $^\circ\text{C}$  until GC  
133 analysis.

134 Transmethylated samples were analyzed using gas chromatography coupled to a mass  
135 spectrometer (GC-MS) using an Agilent 7890A/5975C system equipped with a split/splitless  
136 injector (injected quantity: 1  $\mu\text{L}$ , split set at 10:1). The separation column used was DB-FatWax  
137 UI (30 m x 0.25 mm x 0.25  $\mu\text{m}$ ) containing a polar phase of polyethylene glycol (PEG). The  
138 carrier gas (hydrogen) flow rate was constant at 2  $\text{mL min}^{-1}$ . The gas was produced using a  
139 hydrogen generator (WM-H2, F-DGSi, Evry, France). Temperature was 250  $^\circ\text{C}$  for the injector

140 and 250 °C for the external auxiliary. The oven was at 70 °C for 1 min, then temperature  
141 increased at 4 °C min<sup>-1</sup> to 175 °C, followed by a second rise of 1 °C min<sup>-1</sup> to 195 °C, and finally  
142 at 4 °C min<sup>-1</sup> to 250 °C for 5 min. Individual fatty acids were identified by comparison of their  
143 retention times and mass spectra with those of standards (FAME mix; Supelco, Sigma Saint  
144 Quentin Fallavier, France), while quantification was done using ChemStation, Openlab software  
145 and a comparison between the respective peak area and the one obtained for the internal standard  
146 C17:0 added during the transmethylation stage. Results were expressed as percentage of each  
147 fatty acid (FA) to the total fatty acids (TFA).

### 148 **2.2.3. Extraction of Total Phenolics**

149 Phenolics were extracted from the mustard residue using a predetermined condition based on  
150 our previously described methods [33]. This method is a simple and eco-friendly process that  
151 involves the use of ethanol and water without any pre-treatment. This research study extensively  
152 evaluated the process for the optimal conditions that are required for total phenolic using a  
153 mustard residue with similar phenotypical characteristics (see above). Therefore, this served as a  
154 starting point for our tests. For a typical phenolic extraction, the mustard (crude or defatted) was  
155 loaded into the extraction vessel (based on the dry defatted equivalent-DDE) and then charged  
156 with a mixture of 50 vol. % ethanol/water at a rate of 1 g 30 mL<sup>-1</sup> solids loading (on a 1 g  
157 biomass scale). The extractions were then carried out in a Radley Tornado<sup>TM</sup> (United Kingdom)  
158 equipped with an overhead stirrer and baffled wide-neck flask at 60 °C for 4 h. After the  
159 extraction, the mixture was centrifuged to recover the supernatant (phenolic liquor) for the  
160 determination of total phenolics content (TPC), while the residual solid (extractive-free mustard)  
161 was air-dried (3-5 days) for further tests downstream. Additional process optimizations were

162 performed as a part of this study to account for different mustard species, extraction methods,  
163 mustard production dates, ethanol concentration, solids loading and extraction time.

#### 164 **2.2.4. Biocatalytic (Enzymatic) Release of Sinapic Acid**

165 Following the extraction, the phenolic liquor is further processed to hydrolyze the esters into  
166 acids. For the enzymatic reactions, the buffering solution (along with its pH and concentration),  
167 the reaction temperature and the enzyme loadings were all determined based on the  
168 recommendations furnished by the enzyme producer for a theoretical maximum yield (see Table  
169 S1). These were the recommended conditions required to yield the optimum enzymatic activity  
170 and served as a starting point for the enzymatic reactions. However, these reactions are substrate  
171 dependent and further optimization was necessary to reach the suitable conditions for the  
172 hydrolysis of sinapine and/or ethyl sinapate.

173 In a typical enzymatic reaction, the phenolic liquor (containing ethanol) was directly combined  
174 with a pH buffering solution (50 vol. %) and incubated for 30 minutes at the activation  
175 temperature. Subsequently, the mixture was loaded with a prepared enzyme cocktail marking the  
176 start of the reaction. The amount of the enzyme was loaded based on the total number of moles  
177 of the phenolic ester present and the activity (U) of the enzyme (see Table S1). Once the reaction  
178 was completed (after the 30 minutes), the hydrolysis was terminated by freeze shocking the  
179 enzymes (cooling in a -20 °C freezer for 10 minutes) followed by immediate HPLC and UV-Vis  
180 analyses for sinapic acid content and TPC, respectively. For kinetic studies, identical solutions  
181 were created, and each reaction was terminated at a predetermined time [0, 0.5, 1, 2, 4, 8, 24] h.  
182 Note: Due to variations in different esters (functionality and their molecular weights), further  
183 optimizations were performed to improve the enzymatic yield of the recovered phenolic acids  
184 (higher enzyme loading, longer reaction time).

185 For the investigation of the optimal reaction conditions, a model phenolic liquor was created  
186 using a typical phenolic ester (ethyl sinapate) in a solution with similar characteristics as the  
187 mustard phenolic liquor based on approximate TPC and ethanol/water ratio. The reaction was  
188 studied kinetically and then, based on the results, specific conditions were selected to be utilized  
189 in the actual release of sinapic acid from the mustard residue where the phenolic liquor derived  
190 above was used in place of the model solution. Then, these results were employed in the process  
191 integration and scale up tests. After the enzymatic treatment, the released phenolics were  
192 extracted three times with diethyl ether: ethyl acetate (50 vol. %). The organic phases were  
193 collected, evaporated to dryness and re-dissolved in ethanol.

#### 194 **2.2.5. Process Integration and Process Scale Up**

195 As part of this study, different process configurations were explored to enable the successful  
196 scale up and eventual commercialization of this process. In a typical configuration (A), the  
197 phenolic extract is separated (*via* centrifugation) from the residual mustard bran before  
198 performing the enzymatic hydrolysis. An alternative approach is (B) a one-pot technique where  
199 the enzymatic hydrolysis is performed immediately after phenolic extraction in the same reaction  
200 vessel, thereby, eliminating the need for solid-liquid separations. To enable the one pot  
201 technique, scale-up tests were performed looking at the effect of linear scale-up going from a 0.5  
202 mL scale to a 50 mL (100X) and then to a 5 L scale (10,000X) using a jacketed round bottom  
203 reactor (Heidolph™ Radley Reactor Ready System, United Kingdom) (see Figure S4). For each  
204 test, the reaction was monitored over time to determine the minimal conditions required for full  
205 conversion.

### 206 **2.3. Analytical Methods**

#### 207 **2.3.1. Determination of Total Phenolic Content (TPC): Bathochromic Method**

208 Phenolic compounds and their derivatives can exist in different forms under acidic and basic  
209 conditions. Increase in pH is known to lead to a bathochromic shift of the position of the  
210 maximum absorption wavelength ( $\lambda_{\max}$ ), and sometimes increases in the molar extinction  
211 coefficient ( $\epsilon_{\max}$ ). Therefore, using acidic (acetate) and basic (carbonate) buffers (pH 4.0 and  
212 pH 10.5, respectively) the phenolic liquors were studied with UV-analyses to determine the total  
213 phenolic content present in the solution [45]. In a typical experiment, the phenolic liquor was  
214 combined with the acidic and basic buffers separately (11.1 vol %) using a 96-well plate. The  
215 plate was shaken for complete homogenization for 30 mins after which the full UV - absorption  
216 spectrum was measured [200-800 nm]. For analyses, the wavelength of maximum separation  
217 between acidic and basic solutions was determined. Using this wavelength, a standard curve was  
218 created using sinapic acid solutions with concentrations ranging from [600 to 25] mg L<sup>-1</sup> in a  
219 mixture of ethanol and water (50:50) as the base solution. NOTE: This is to match the solution  
220 media that was chosen for the phenolic extraction. This method was used to determine the total  
221 phenolic content and will be supported with data from the Folin-Ciocalteu method, along with  
222 chromatographic methods like HPLC (see below).

### 223 **2.3.2. Determination of Total Phenolic Content (TPC): Folin-Ciocalteu Method**

224 The total phenolic content of can also be determined spectrophotometrically using a Folin-  
225 Ciocalteu assay [46,47]. This will be utilized to supplement the bathochromic method for the  
226 determination of TPC. The phenolic liquor was diluted (0.44 vol %) and then combined with the  
227 Folin-Ciocalteu reagent (20 vol %) and homogenized for a total volume of 1 mL. Subsequently,  
228 1 mL of a solution of 19 wt. % sodium carbonate (Na<sub>2</sub>CO<sub>3</sub>) was added, and the mixture was  
229 vortexed and incubated for 30 min at 40 °C before reading of the absorbance at 765 nm (Cary 60  
230 UV spectrophotometer, Agilent, CA, USA) Using this same wavelength, a standard curve was

231 created using sinapic acid solutions with concentrations ranging from [600 to 25] mg L<sup>-1</sup> in a  
232 mixture of ethanol and water (50 vol. %) as the base solution. Once the standard curve was  
233 established, then the analyses was carried out for the samples with unknown concentrations.

### 234 **2.3.3. Sinapic Acid Characterization (HPLC)**

235 Phenolic extracts (phenolic liquor) were analyzed using a reversed-phase HPLC-DAD (HPLC,  
236 Ultimate 3000; Dionex, Sunnyvale, CA, USA) equipped with an online degasser, binary pump,  
237 autosampler column, column heater and diode array detector. A gradient elution was performed  
238 using eluents (A) MilliQ water, (B) Acetonitrile and (D) 0.1 vol. % formic acid using one C18  
239 column (Thermo Scientific™ Accucore™ aQ, 2.6 μm particle size and 100 x 3 mm i.d.). For the  
240 gradient, solvent was 50% component D, while Component B was varied to achieve an isocratic  
241 flow: [5% (0 min), 10% (0.990 min), 15% (3.190 min), 30% (7.440 min), 0% (8.510 min)]. The  
242 column was maintained at 48 °C and the flow rate of the mobile phase was 0.8 mL min<sup>-1</sup>. All  
243 solvents as well as phenolic extracts were filtered through 0.2-micron filters. Chromatograms  
244 were acquired at 210, 254, 285 and 320 nm and the data were analyzed using the Chromeleon  
245 software (Version 6.8). Peaks were identified by comparing their relative retention times with  
246 those of the authentic standards.

### 247 **2.3.4. Determination of Total Reducing Sugars: DNS Assay**

248 DNS reagent was synthesized as follows using a previously established procedure [48]. Using  
249 a 1 L volumetric flask, 10 g of 3,4-dinitrosalicylic acid was dissolved in 200 mL DI water,  
250 followed by continuous stirring and the slow addition of a solution of NaOH (16 g) dissolved in  
251 150 mL distilled water. Next, the mixture was incubated at 50 °C with stirring for complete  
252 dissolution. Afterward, about 403 g of potassium sodium tartrate tetrahydrate was added in small  
253 portions until the mixture was homogeneous. Lastly, the solution was filtered, and the volume

254 completed with water to make up to 1 L. The solution was stored in dark glass bottle at  
255 temperature below 20 °C. For sugar analyses, 1 mL of each sample (standards or phenolic liquor)  
256 was combined with 1 mL of a 0.05 M acetate buffer (pH 4.8) and made uniform. Subsequently, 3  
257 mL of the DNS reagent was added followed by heating in boiling water for 5 minutes. After the  
258 samples were cooled to room temperature the absorbance at 540 nm was measured. NOTE: This  
259 method will be supported with chromatographic method such as HPLC to determine the amount  
260 of each sugar in the unknown samples.

### 261 **2.3.5. Sugar Characterization (HPLC)**

262 The sugars were analyzed using high performance liquid chromatography, HPLC-RI (HPLC,  
263 Ultimate 3000; Dionex, Sunnyvale, CA, USA)) equipped with a degasser online, a binary pump,  
264 an automatic sampling column, a column heater and a refractive index detector. Elution was  
265 performed with 8 mM sulfuric acid and using a column (Aminex HPX87H, 300 mmx7.8 mm).  
266 The column was maintained at 35 °C and the flow rate of the mobile phase was 0.5 mL min<sup>-1</sup>.  
267 The solvent, as well as the extracts, were filtered through 0.2-micron filters. The data was  
268 analyzed by Chromeleon software (Version 6.8).

### 269 **2.4. Statistical Analysis**

270 All experiments were performed in triplicate. The data displayed are the mean ± standard  
271 deviation, and statistical analysis was performed using Student's t test, while the p values were  
272 calculated and assigned (see supporting information Section S2 and Table S2).

## 273 **3. RESULTS/DISCUSSION**

### 274 **3.1. Effect of Process Variables on Total Phenolic Extraction**

275 For this study, the extraction of total phenolics was first optimized, followed by the conversion  
276 of the phenolic esters to sinapic acid. Using the methods described above, the total phenolic  
277 compounds were extracted from the mustard bran. The starting conditions for the extraction were  
278 selected based on the results from a previous study (Table 1) [33]. Subsequently, optimizations  
279 were carried out to study the effect of different mustard species, extraction methods, mustard  
280 production dates, solids loading and extraction time on the TPC. The results show that both  
281 crude and defatted biomass will generate a similar amount of total phenolics yielding an average  
282 of 8.71 mg phenolics per g mustard (Table 1, S2). This suggests that harsh pretreatments are not  
283 necessary to remove the fats/lipids from the biomass before phenolic extraction. Additionally,  
284 the extraction method (thermal, microwave, or Soxhlet) was not an important factor for the  
285 release of phenolics (Table 1). The temperature for each of these methods were controlled (50  
286 °C), therefore, there were no major variations in the TPC. Considering that the thermal method is  
287 a batch process, it is simpler and easier to scale up, hence, the microwave and Soxhlet extraction  
288 were not recommended for this study.

289 The results also reveal that the biomass type and the mustard production dates played a  
290 substantial role on the TPC. Canadian sourced mustard has over 4 times (4X) the phenolics as  
291 the French counterpart (Table 1). Additionally, the year the mustard was processed also affected  
292 the TPC—mainly because the mustard bran was stored at room temperature. It is likely that the  
293 phenolic content reduces over time due to oxidative-mediated oligomerization of phenolics [49].  
294 For future tests, the mustard residues should be stored in a temperature-controlled device at low  
295 temperature (> 4 °C). The extraction time also positively affected the TPC, plateauing at 4 h.  
296 Lastly, the product yield for different solids loading remained constant until ~5 g per 30 mL  
297 (0.167 g mL<sup>-1</sup>), where it plummeted (Table 2).



298 Various reports in the literature have described phenolic extraction from different parts of the  
299 mustard such as seed, flour, leaf, hull and cotyledon, or meal [38]. The biomass in this study is  
300 similar to meal, it is important to note that each biomass is very specific (e.g., botanical species,  
301 growth conditions, treatment, type of extraction), therefore, equating our results directly to  
302 previously reported studies is rather inadequate. Nevertheless, reports from Flourat *et. al.*  
303 studying the ethanolic extraction of *Brassica juncea* (90%) mixed with *Sinapis alba* from both  
304 Canadian and French yielded ~ 9.0 mg TPC per g dried mustard [33]. Additionally, Dubie *et. al.*  
305 also determined that the meal of *Brassica juncea* generated about 6.0 to 13.8 mg TPC per g  
306 mustard depending on the extraction conditions [50]. Thiyam-Holländer *et. al.* reported TPC  
307 between 11.5 and 42.3 mg per g after methanolic (70%) extraction on mustard seeds flour and  
308 defatted mustard flour (*Brassica juncea*, *Sinapis alba*, yellow and oriental mustard) procured in  
309 Canada [25].

### 310 **3.2. Enzyme Screening: Selection of Enzymes, Optimal Reaction Conditions**

311 Following the phenolic extraction (particularly the phenolic esters), the recovered liquor was  
312 enzymatically hydrolyzed to produce sinapic acid. Figure 2 shows the general pathway for the  
313 conversion of phenolic esters into phenolic acids. For a preliminary screening, an analogous  
314 model compound that can be easily synthesized was utilized - ethyl sinapate (Figure 2, 3A).  
315 Indeed, although sinapine and sinapoyl glucose are naturally occurring phenolic esters, they are  
316 very difficult to isolate in large quantity and high purity. There has hitherto been an  
317 industrial/commercial process for extracting these compounds on a large scale from waste  
318 biomass. Not only do these compounds exist in very low quantities in the biomass (see Figure 1),  
319 but they also require extensive chemical manipulation to be effectively extracted at high yields.  
320 Generating quantitative results requires a counterion to balance the charge on sinapine, therefore,

321 sinapine is typically extracted as sinapine bisulfate, although in nature sinapine exists as sinapine  
322 chloride [51]. While there are several new methods being developed to address this problem  
323 [52], the large-scale processing and production of sinapine from biomass have not been fully  
324 investigated. With the model compound, various reaction conditions can be surveyed (such as  
325 the ideal reaction time, temperature, or enzyme loadings), which allows for the design of a  
326 rigorous study with extensive research and development.

327 For each enzymatic reaction, identical amounts of enzyme (U per g phenolic ester) were added  
328 based on the activity suggested by the commercial provider (Table S1). Using a 1 mL solution  
329 containing 0.2 mg of ethyl sinapate ( $200 \text{ mg L}^{-1}$ ), the relative activity was monitored to aid in  
330 screening the enzymes and optimizing the reaction conditions (Figure 3). The enzyme loadings  
331 recommended by the commercial provider revealed to be insufficient for attaining complete  
332 conversion for all enzyme types. Therefore, further tests were carried out to optimize the reaction  
333 for each enzyme type. In figure 3B, the results show that only FAE 1 can achieve complete  
334 conversion of ethyl sinapate. To probe for the rate limiting factors (with FAE 2 and Ultraflo) the  
335 enzyme loading was doubled (2X) and the reaction time tripled (3X). The response revealed that  
336 the reaction time is the rate limiting factor—yielding >300% increase in the resultant yield  
337 (Figure 3B). Subsequently, a kinetic study was carried out and the reaction time was studied in  
338 detail and extended to 24 hours (Figure 3C). With these conditions, all enzymes finally attained  
339 the full conversion (Figure 3C).

340 Comparatively, the outcomes demonstrated that FAE 1 can achieve the fastest release of  
341 sinapic acid, however, the enzyme is more expensive (U Euro<sup>-1</sup>) and might not be suitable for  
342 implementing large scale processing (Table 2). FAE 2, on the other hand, is more affordable,  
343 although, complete conversion required an enzyme loading supplement, and a longer reaction

344 time (Figure 3B, 3C, Table 2). Further review of the datasheets reveals that the enzymatic  
345 activity for FAE2 is substrate dependent. The rate of hydrolysis for ethyl sinapate (compared to  
346 other phenolic esters) is only 5% of the published activity (Table S1). Enzymes can often exhibit  
347 substrate specificity amongst a group of chemically similar compounds [53]. It is often necessary  
348 to have a satisfactory structural and conformational match to enable effective binding between  
349 enzyme and substrate. This is clearly depicted in the case of FAE2. Lastly, Ultraflo® (ULT) is a  
350  $\beta$ -glucanase with mixed cellulase, xylanase and esterase activity. This enzyme is robust in  
351 activity, however, the time required to reach conversion is much longer and necessitated an  
352 increase in the recommended enzyme loading (Figure 3B, 3C, Table 2).

353 Lastly, the amount of ethanol present in the phenolic liquor used for the enzymatic reaction  
354 was studied in preparation for the process integration tests (Figure 3D). It is well known that  
355 enzymes can have reduced activity in the presence of organic solvents, however, optimal  
356 phenolic release occurs at about 45-55 vol. % ethanol-based on previous work of Flourat *et. al.*  
357 [33]. Prior to enzymatic hydrolysis, the ethanol is usually evaporated after phenolic extraction  
358 and then replaced with water. This is an energy intensive process that is worth avoiding.  
359 Although ethanol concentration slightly reduces the yield of sinapic acid released (Figure 3D),  
360 the latter can be further improved by increasing the reaction time (which we have determined to  
361 be the rate limiting factor). Thus, a compromise of 50 vol. % ethanol was selected for the  
362 remaining phenolic extraction.

### 363 **3.3. Process Integration and Scale Up**

364 After optimizing both the phenolic extraction and the enzymatic release of sinapic acid, the  
365 process was carried out sequentially and simultaneously to explore the impact of process  
366 consolidation. Additionally, the scalability of the process was studied using a simple linear scale

367 up model. As a reminder, the preferred conditions are crude Canadian (NEW) biomass, produced  
368 in 2017, extracted thermally for 4 h in a solution containing 2.0 g mustard per 30 mL (50%  
369 ethanol/water), and hydrolyzed with the FAE 1 enzyme (Figure 4). **Despite the higher cost of the**  
370 **FAE 1 enzyme, it was selected for the scale-up study due to the faster kinetics (i.e., maximal**  
371 **yield is obtained more quickly) that minimizing reactor residence time, as well as the relatively**  
372 **small amounts required to achieve maximum sinapine conversion.**

373 With the sequential approach (configuration A) in Figure 4, the release of sinapic acid from the  
374 phenolic extract produced about 1 to 10 mg of sinapic acid per g of mustard bran (Figure 5).  
375 Similar to the results obtained from the phenolic extraction, the biomass origin was the most  
376 important factor affecting the sinapic acid release, while the year of production and processing  
377 conditions contributed to a lesser extent (Figure 5). For the biomass with the highest phenolic  
378 content, the sinapic acid released was exactly 9.64 mg sinapic acid per g mustard. Based on the  
379 30 mL reaction scale (and 2 g of mustard), this is equivalent to  $\sim 643 \text{ mg L}^{-1}$  of sinapic acid in  
380 solution. **The mass and species balance (Figure S7) indicate a complete conversion of sinapine,**  
381 **along with 102% yield for sinapic acid. The slightly higher yield is likely due to other sinapate**  
382 **esters presents in very small amounts in the mustard bran that can be also hydrolyzed (Figure 1)**  
383 **(see section S7 in supporting information). Vuorela *et. al.* also demonstrated that over 90% of**  
384 **sinapic acid derivatives were hydrolyzed to yield sinapic acid using both enzymes and sodium**  
385 **hydroxide [37]. However, their results also substantiate that base hydrolysis, compared to**  
386 **enzyme treatment, was slightly detrimental to the liberated phenolics (at least 20% of the**  
387 **phenolic yield was lost during base hydrolysis, and new peaks were identified for unknown**  
388 **phenolic compounds) [37].**

389        Thereafter, the effect of process integration on the sinapic acid release (Figure 4) was studied.  
390        In this scenario (configuration B), the enzyme is added to the phenolic liquor immediately after  
391        extraction. Although the presence of ethanol and the residual biomass in the extract is generally  
392        well known to impede the effectiveness of the enzyme, in our case, based on the optimized  
393        conditions, the amount of sinapic acid released did not drastically change when this  
394        configuration B was applied (Table 3). This was, therefore, a promising approach for process  
395        implementation and this was immediately studied on a larger scale.

396        Using a linear scaling factor (for simplicity), the reaction was scaled up to 100X the original  
397        size keeping all the process conditions constant (Table 3). A linear scale up model was suited  
398        since the reaction kinetics for the conversion is very fast. With the FAE 1 enzyme and the  
399        conditions chosen from the optimized process, the enzyme completes conversion within 10-30  
400        minutes (Figure 3B). However, as the reaction was scaled up, this time was no longer enough for  
401        the conversion to take place and had to be increased up to 8 h. The final sinapic acid release was  
402        only 95% of the theoretical release. As the time increased, the conversion finally stabilized  
403        although slightly lower than the theoretical. Several factors can be responsible for this difference  
404        in reaction time/efficiency. Sometimes it is not always practical to use a linear scale model. Most  
405        biochemical reactions often have exponentially modeled reactions, perhaps at larger scales the  
406        reaction is not as fast and cannot be linearly scaled up. Additionally, at larger scales, mixing and  
407        mass transfer are more difficult and therefore, the enzymatic reaction is inherently slower.

#### 408        **3.4. Free Sugars Released and Fatty Acid Content of Residual Oils**

409        During the extraction of phenolics, it was observed that free monomeric sugars were also  
410        recovered. In fact, ~5-10% of the mustard bran is comprised of free sugars that can be easily  
411        extracted with water or organic solvents. Sugars are key building blocks in the development of

412 the bioeconomy; therefore, this fraction of the biomass is worth evaluating for the valorization of  
413 the mustard bran. The results show that ~70-90 mg g<sup>-1</sup> of reducing sugars were extracted along  
414 with the sinapic acid (Figure 5), comprising mainly of glucose and xylose. Additionally, the  
415 sugar released can be improved using commercially available enzymes such as cellulases to  
416 further deconstruct the other polysaccharidic components of the biomass into sugars.

417 The last main portion of the mustard extract worth considering is the oil content (Figure 6). In  
418 addition to serving as an edible oil or heating oil, mustard oil can be transformed into biodiesel  
419 [22]. Biodiesel produced from mustard oil has been shown to have comparable fuel properties  
420 with the conventional fossil diesel (~42 MJ Kg<sup>-1</sup> heating value) [54,55]. In the present study, the  
421 residual oils in the mustard bran were extracted and characterized for their fatty acid profiles. As  
422 previously mentioned, the mustard bran contained a residual fat proportion of about 5 wt. %,  
423 which corresponded to 11 wt. % with regards to dry matter [33]. This lipid extract was found to  
424 contain predominately C18 fatty acids (10 wt. % stearic acid, 37 wt. % linoleic acid and 22 wt. %  
425  $\alpha$ -linolenic acid), as well as, 18 wt. % palmitic acid (C16) and trace amounts (<1 wt. %) of  
426 various fatty acids with concentrations below the detection limit of the GC-MS [C18:3n3 ( $\alpha$ -  
427 linolenic), C20:2 (*cis*-11,14-eicosadienoic), C20:3n6 (*cis*-8,11,14-Eicosatrienoic)] (Figure 6).  
428 This composition is analogous to other reports for mustard seeds of the *Brassica juncea* species  
429 [56]. However, the lipids recovered do not contain erucic acid (C22:1), which is often found in  
430 large quantities in mustard oils [56,57]. This could potentially affect the heating value if this oil  
431 is used for biodiesel. However, the presence of high erucic acid in oil is considered anti-  
432 nutritional, as it has been reported to cause certain diseases in children and monkeys [57,58].

433 NMR analysis of the mustard oil (Figures S5-S6), reveal that no phenolic compounds were  
434 present in the mustard oils. The extracted lipids are pure and will not affect the phenolic content

435 (another fraction of high interest discussed). <sup>1</sup>H NMR spectra were analyzed to determine the  
436 percentage and concentration of triglycerol esters (TAG), methyl ester of fatty acids (FAME) and  
437 free fatty acids (FFA), and appear to be similar for all the mustard residues received (Figure S5-  
438 S6). Amongst the NMR spectra, the minor change that was observed was due the presence of  
439 FAMEs in the lipid extract ( $\delta \sim 3.70$  ppm). In particular, the mustard processed with OLD sieve  
440 (Figure S5) vs. NEW sieve (Figure S6) depicted about 2-3% of free FAMEs in the lipid fraction.  
441 This has been shown to be associated with immature development stages in the mustard embryo  
442 [59]. It is not clearly understood why this lot shows a small fraction of free FAMEs since they  
443 are from the same source. This could be due to the difference in field sources (associated with  
444 harvesting dates) for separate batches that could contain small amounts of prematurely developed  
445 seeds. Nevertheless, this fraction is not significant and should not drastically affect the lipids'  
446 properties.

447 Based on the results of this study and the technology readiness level (aka TRL) of some of the  
448 valorization options, the second option (starred), is the most realistic approach that can be  
449 adopted immediately. Figure 7 shows all the possible conversion pathways that the mustard  
450 residue could have gone through to release various products. In this scenario, sinapic acid can be  
451 released from the biomass (along with free sugars) using the developed process (above). The  
452 residual biomass is still intact and can still be used for methanization or animal feed. Due to the  
453 mild extraction process adopted, the mustard bran does not lose any of its major components  
454 (*e.g.*, carbohydrates/proteins) during the extraction process and is still viable after phenolic  
455 extraction. For instance, from Canadian (NEW) lot of crude mustard bran, ~10 mg of sinapic  
456 acid per g mustard bran were successfully extracted, representing ~ 1 wt. % of the mustard bran.  
457 This same extract also contained 43 mg total phenolics per g mustard bran (~ 4 wt. %) and 71 mg

458 sugars per g mustard bran (~7 wt. %). Additionally, if the mustard is defatted, ~5% of lipids can  
459 also be generated (or 11 wt. %. Based on DDM) This yields ~76 wt. % of the mustard bran still  
460 available for methanization or animal feed. As more technologies are being developed within the  
461 biorefinery space, further fractionation techniques can be employed to recover some of the other  
462 components of the biomass for valorization which could enable a detailed cost analysis (Figure  
463 7).

#### 464 4. CONCLUSION

465 The continued integration of bio-based chemicals and materials along the biofuel-production  
466 pathway is a necessary milestone towards the successful commercialization of  
467 bioenergy/bioproducts. Development of these processes can lead to new technology  
468 developments, and economic opportunities, which can, in turn, enable the cost-effective  
469 production of advanced biofuels, improve energy security, reduce greenhouse gas emissions, and  
470 contribute to job growth. This study was able to demonstrate a relatively green and sustainable  
471 approach to valorize several key components of mustard residue. In particular, the focus was on  
472 demonstrating an **integrated** and scalable means for biocatalytically releasing sinapic acid from  
473 mustard waste. The results revealed that the biomass type/source can significantly impact the  
474 release of sinapic acid, additionally, the enzyme used can impact the process efficiency and  
475 overall cost. With this optimized process, ~10 mg of sinapic acid per g of mustard bran was  
476 successfully extracted, representing ~ 1 wt. % of the mustard bran along with phenolics and  
477 sugars. The residues also contain ~11 wt. % of lipids, which allows for ~76 wt. % of the mustard  
478 bran to be available for further methanization or animal feed. Through this work, we have  
479 successfully elucidated several different opportunities and approaches for valorizing mustard  
480 bran that can be applied at any “*moutarderie*” or any processing facility working with



481 oilseeds/rapeseeds. Following this study, it will be necessary to explore different separation and  
482 recovery approaches for the sinapic acid (either using organic solvents and/or membrane-based  
483 techniques) and the enzymes. It might also be useful to perform more detailed techno-economic  
484 analyses and/or life-cycle analyses to explore the feasibility of other valorization approaches.

485 **ASSOCIATED CONTENT**

486 **Supporting Information**

487 The following files are available free of charge.

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497 **Author Contributions**

498 The manuscript was written through contributions of all authors. All authors have given approval  
499 to the final version of the manuscript.

500 **ACKNOWLEDGMENT**

501 We would like to thank the Region Grand Est, the Conseil Départemental de la Marne and the  
502 Grand Reims for their financial support, as well as Charbonneaux-Brabant for providing the  
503 mustard residue. URD ABI also want to acknowledge the US-France Fulbright Commission and  
504 Grand Est Region for granting Ezinne Achinivu with a Fulbright-Grand Est Fellowship along  
505 with the AAUW for the 2018/9 Research Publication Grant

506 **CONFLICT OF INTEREST**

507 The authors declare that the research was conducted in the absence of any commercial or  
508 financial relationships that could be construed as a potential conflict of interest.

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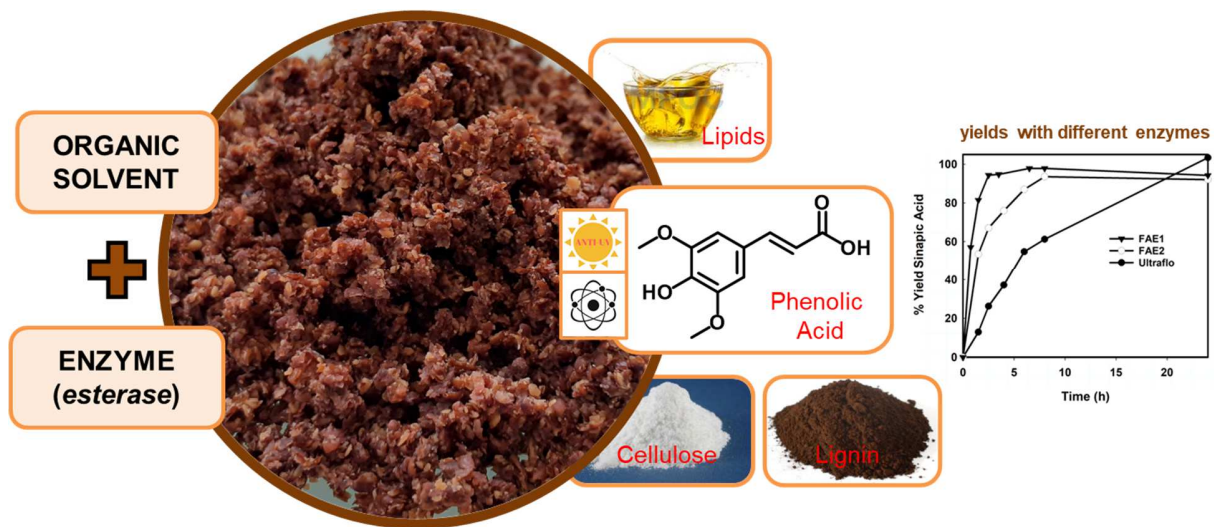
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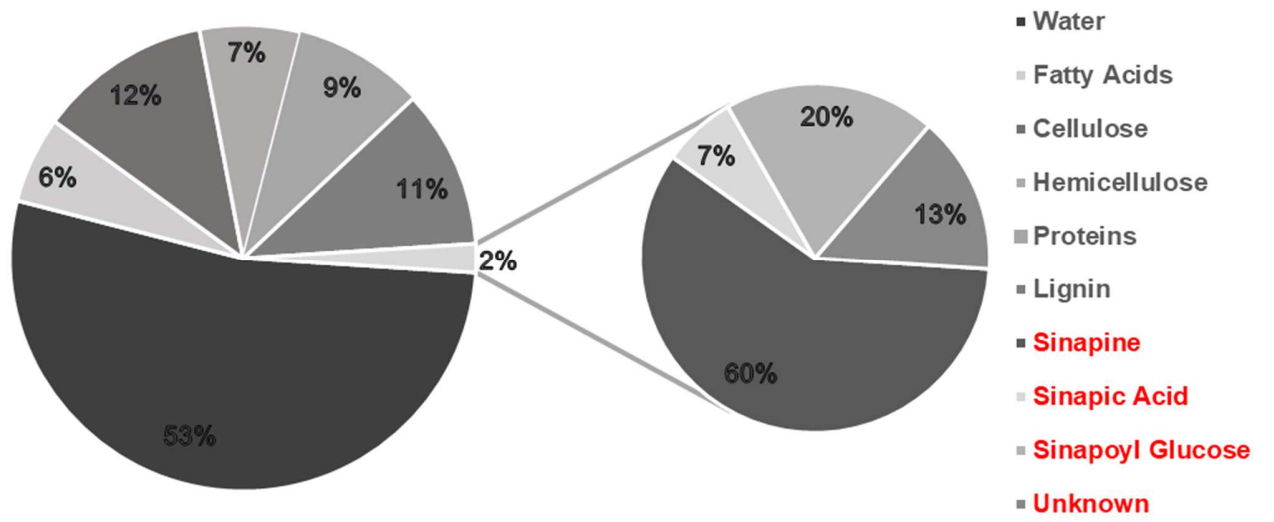
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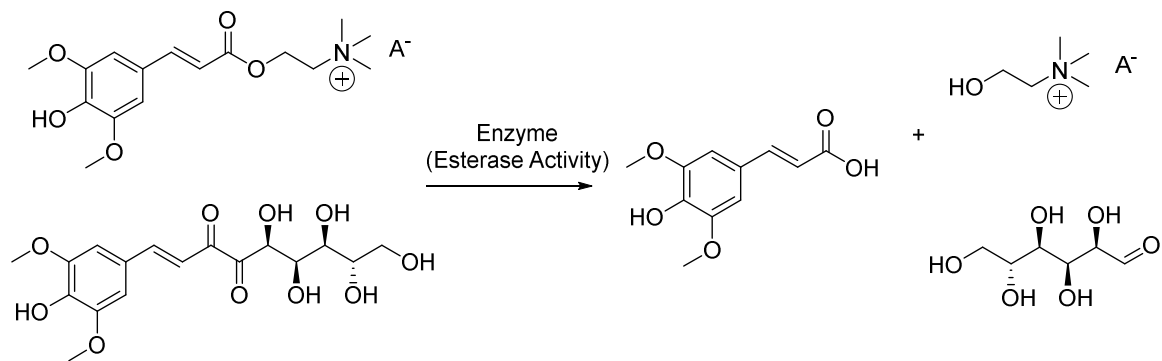
691 **SYNOPSIS**

692 Naturally occurring bioactive molecule (sinapic acid) was extracted from mustard bran using a  
693 sustainable chemo-enzymatic method

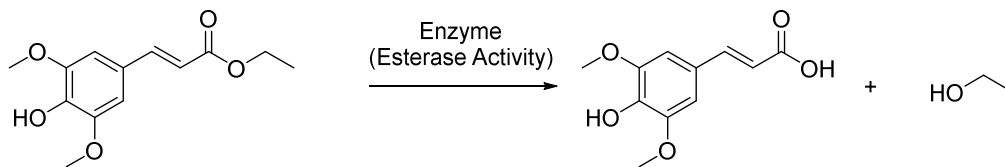




**Figure 1.** Compositional analyses of the crude/fresh mustard bran used in the study.



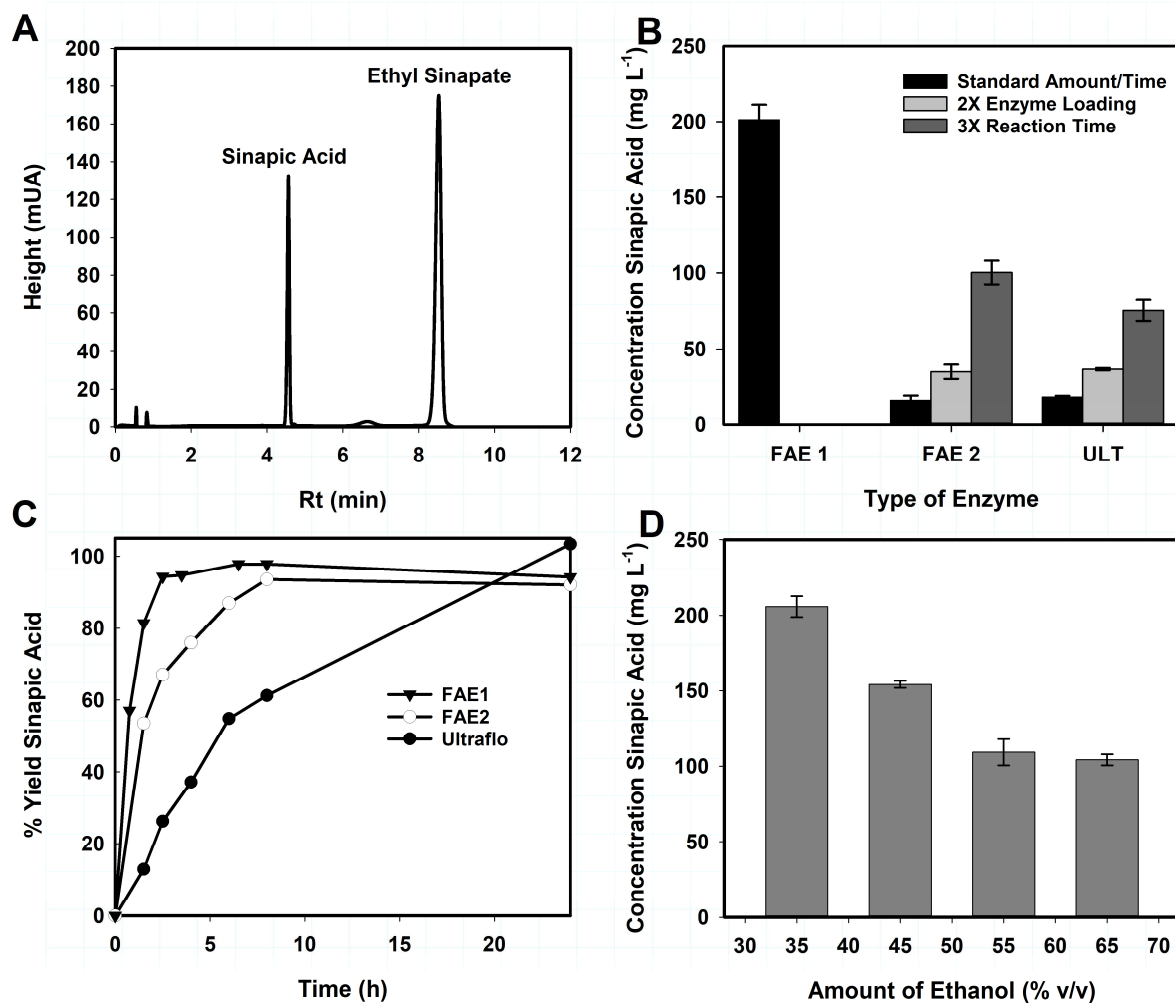
Sinapine (top) and glucosyl sinapate (bottom) can be found in mustard extract



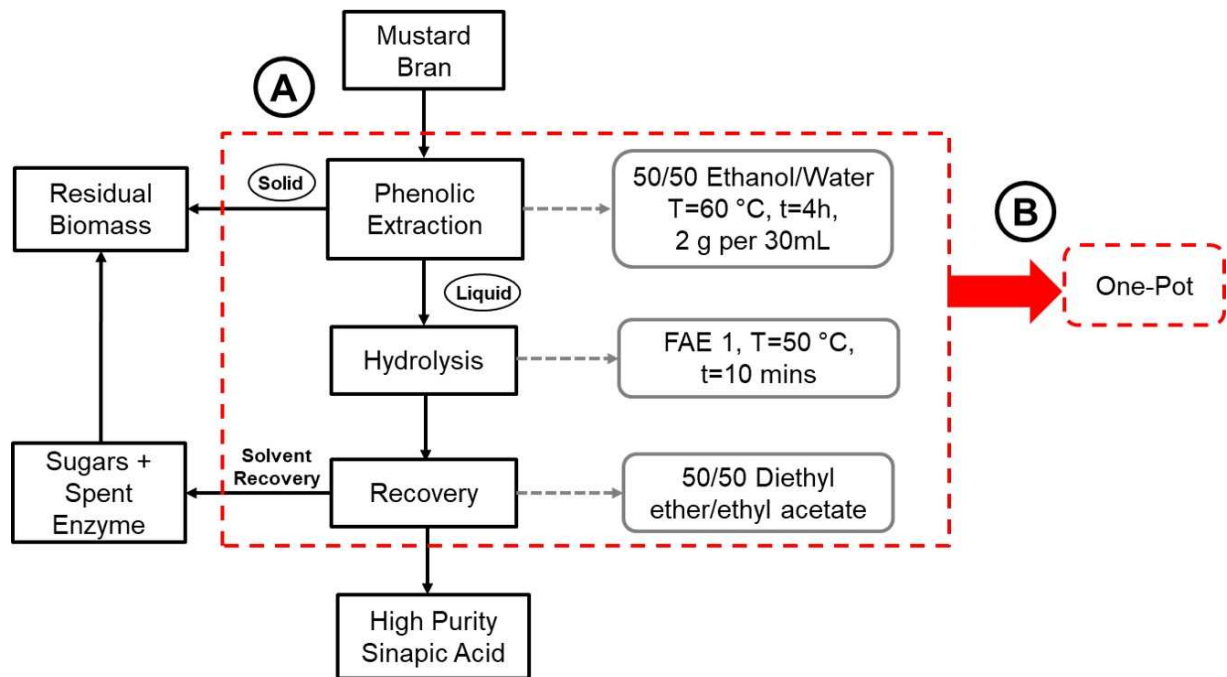
Ethyl sinapate = model compound

**Figure 2.** Conversion of phenolic esters (sinapine, glucosyl sinapate, ethyl sinapate) to sinapic acid (Note: A<sup>-</sup> stands for Anion which is typically Cl<sup>-</sup> for naturally occurring sinapine)

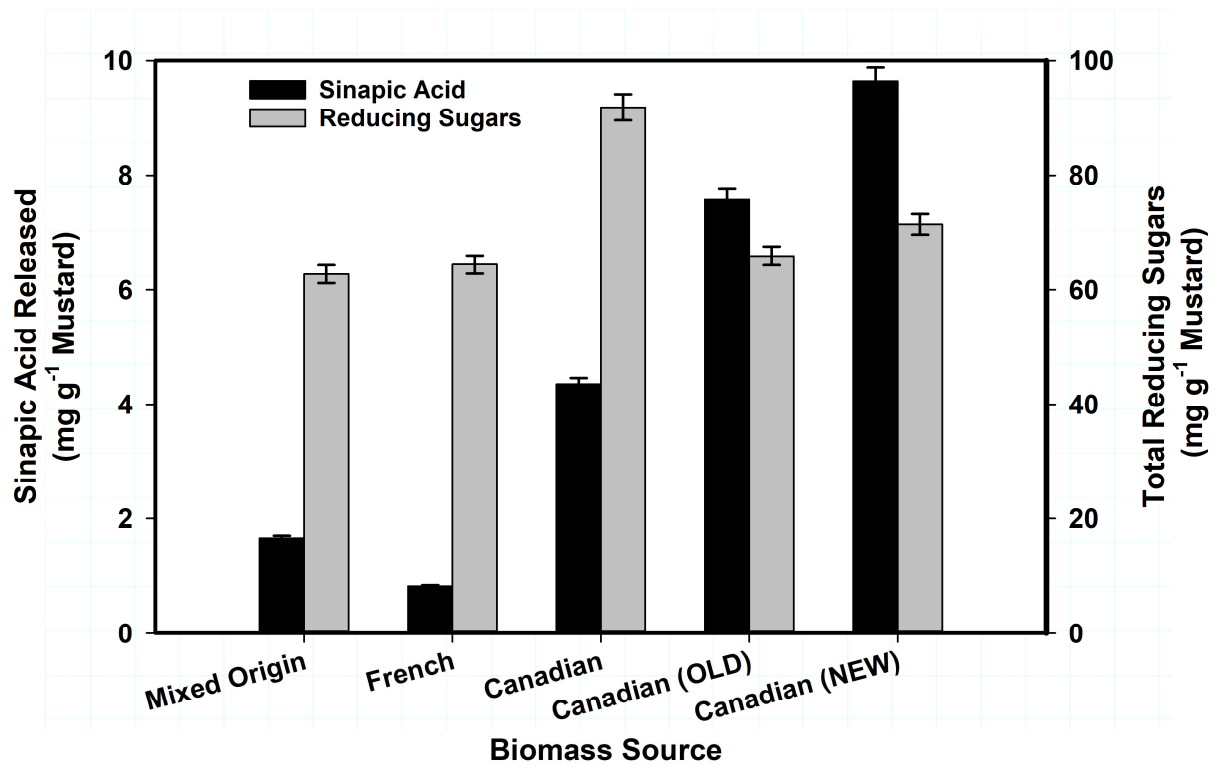




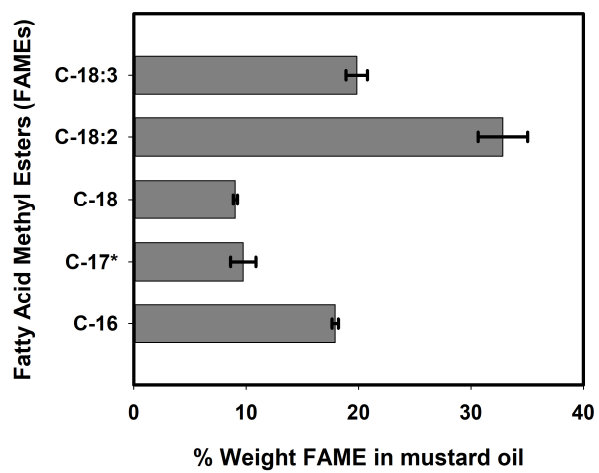
**Figure 3.** (A) HPLC Chromatogram showing separation between sinapic acid (SA) and ethyl sinapate (ES), (B) Different reaction times/enzyme loadings, (C) Rate of conversion of ES to SA using three different enzymes and (D) Effect of ethanol on the release of sinapic acid through enzymatic hydrolysis.



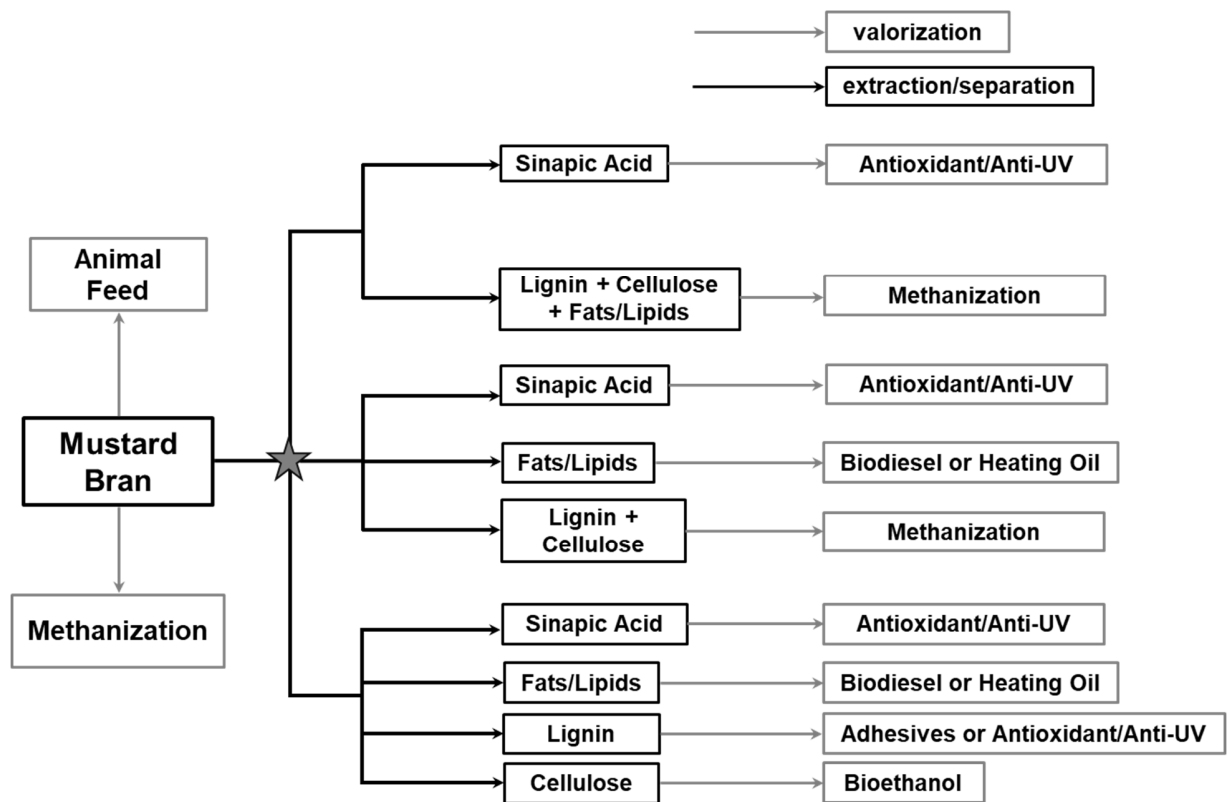
**Figure 4.** Block Flow Diagram comparing the process conditions.



**Figure 5.** Sinapic acid release from mustard bran based on a sequential approach (phenolic extraction then enzymatic hydrolyses with FAE1).



**Figure 6.** Fatty acid profile of mustard oil extract. (\*internal standard).



**Figure 7.** Different fractionation strategies/conversion pathways for biomass valorization.

**Table 1. Total phenolic content (\*mg g<sup>-1</sup> mustard DDM equivalent) from mustard bran (\*\*starting conditions)**

<b>Biomass Treatment</b>	<b>Extraction Method</b>	<b>Extraction Time</b>	<b>Biomass Loading</b>	<b>Biomass Type</b>	<b>Year</b>	<b>Total Phenolic Content*</b>
Defatted	Thermal	1h	1g 30mL <sup>-1</sup>	Mixed	2015	8.40 ± 0.17 →
**Crude	Thermal	1h	1g 30mL <sup>-1</sup>	Mixed	2015	9.01 ± 0.07 →
Crude	Microwave	1h	1g 30mL <sup>-1</sup>	Mixed	2015	8.61 ± 0.15 →
Crude	Soxhlet	1h	1g 30mL <sup>-1</sup>	Mixed	2015	8.99 ± 0.50 →
Crude	Thermal	2h	1g 30mL <sup>-1</sup>	Mixed	2015	13.58 ± 0.11 ↑
Crude	Thermal	4h	1g 30mL <sup>-1</sup>	Mixed	2015	17.99 ± 1.00 ↑
Crude	Thermal	4h	2g 30mL <sup>-1</sup>	Mixed	2015	17.94 ± 0.99 →
Crude	Thermal	4h	5g 30mL <sup>-1</sup>	Mixed	2015	11.75 ± 0.41 ↓
Crude	Thermal	4h	7.5g 30mL <sup>-1</sup>	Mixed	2015	10.78 ± 0.59 ↓
Crude	Thermal	4h	2g 30mL <sup>-1</sup>	French	2016	13.61 ± 0.10 ↓
Crude	Thermal	4h	2g 30mL <sup>-1</sup>	Canadian	2016	33.06 ± 0.21 ↑
Crude	Thermal	4h	2g 30mL <sup>-1</sup>	Canadian (OLD)	2017	40.19 ± 1.13 ↑
Crude	Thermal	4h	2g 30mL <sup>-1</sup>	Canadian (NEW)	2017	42.99 ± 0.20 →

**Table 2. Comparison of enzyme activity, efficiency and cost to attain the complete conversion (for the hydrolysis of 0.2 mg ethyl sinapate in 1 mL).**

Enzyme	Total activity (as purchased)	Cost	Actual amount used	Time for full conversion	Cost for full conversion
FAE 1 ( <i>Clostridium thermocellum</i> )	10 U Feruloyl esterase	€ 16/U	0.5 U	~ 2.5 h	€ 8
FAE 2 (from rumen microorganism)	1000 U Feruloyl esterase	€ 0.16/U	26 U	~ 8 h	€ 4.16
Ultraflo L®	Cellulase, xylanase	Varies, commercially available	~26 mL/mg ES	~ 10-24 h	N/A *

\*N/A – not available

**Table 3. Summary of process conditions and efficiencies for different scale and process integration.**

<b>Scale (biomass)</b>	<b>Process Integration</b>	<b>Sinapic Acid Release (mg g<sup>-1</sup>)</b>
2 g	Sequential	9.64 ± 0.07
2 g	Simultaneous	9.01 ± 0.10
200 g	Simultaneous	8.56 ± 0.06