Attenuation of Sinapic Acid and Sinapine-Derived Flavor-Active Compounds Using A Factorial-Based Pressurized High-Temperature Processing

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Abstract

De-oiled canola meals are sources of protein-containing flavor-active phenolic compounds. Conventional canola oil processing utilizes an excess amount of solvents and is associated with the release of high-intensity bitter flavor-active phenolic compounds, limiting the use of the canola meal. Recent advances in the extraction and isolation of the bitter favor-active phenolic compounds from canola by-products produce protein isolates, however, would benefit the industry by producing a side-stream ingredient rich in phenolics. High temperature and pressure-aided processing, namely the accelerated solvent extraction (ASE) was investigated to extract the flavor-active bitter molecules from the canola meal. The extractability of flavor-active phenolic compounds including the major sinapates, kaempferol derivatives, and other thermo-generative compounds including thomasidioc acid (TA) was evaluated. The effects of temperature, solvent extractant and concentration, and the particle size of the meal, were examined on the extraction efficiency of these phenolic compounds. Extraction temperature (180oC) was the primary determinant ($p<0.05$) for the attenuation of major sinapates including sinapine and sinapic acid. Both ethanol and methanol extractants at a concentration of 70% (v/v) significantly ($p<0.05$) extracted the flavor-active phenolic compounds. The pressurized high temperature through optimized ASE conditions attenuated the bitter undesirable flavor-active phenolic molecules from canola meal thereby facilitating a potential value-added phenolic-rich by-product.

Keywords – accelerated solvent extraction (ASE), high temperature, de-oiled canola, bitter compounds, processing, sinapine

1. Introduction

Currently, up to 60% of the world's dietary protein is provided by plant-based sources (Gorissen & Witard, 2018). With the current emphasis on sustainable ingredients, plant-based protein has garnered interest by the food and feed protein industry to meet consumer demands for new and alternative sources. Both canola and pea protein blends are reported to exceed the protein quality of meat and dairy and would play a key aspect in fulfilling the future protein demand for humans (Gläser et al., 2020; Hald et al. 2019). However, the presence of undesirable bitter complexes initiated by compounds such as glucosinolates, phytates, tannins, phenolics, and its high fibre content limits the use of canola meal in food sources (Khattab et al., 2010; Naczk et al., 1998). Moreover, the associations between the proteins and the tannins further contributes to the bitter taste in the protein products (Naczk et al. 1998). Recent advancements in canola industry have led to produce valuable protein isolates and other protein ingredients from canola meal. Hence, the residual meal after isolation of the protein fractions may impart as a value-added by product to produce bitter flavor-active phenolic compounds to introduce in the nutraceutical industry.

The phenolic compounds in canola can be categorized as free, esterified, and insoluble bound (with benzoic and/or cinnamic acid) (Alu'datt et al., 2017; Li & Guo, 2016b; Quinn et al., 2017). Kozlowska *et al.*, (1983) reported the content of insoluble and bound phenolic compounds in canola meal ranged from 32-50 mg/kg. The predominant free phenolic compounds in rapeseed meal were sinapic acid, vanillic acid, protocatechuic acid, syringic acid, *p*-coumaric acid, ferulic acid, caffeic acid, and chlorogenic acid (Kozlowska et al., 1990). The predominant phenolic compounds in canola by-products are esterified, with sinapine accounting for over 80%, and

sinapic acid occurring as the major free form (Li & Guo, 2016b; Quinn et al., 2017). The traditional processing methods require large amount of extraction solvents (for example 1 g meal requires 70 mL ethanol). This is considered environmentally undesirable even though up to 85% of the phenolics can be removed (Li & Guo, 2016b; Quinn et al., 2017). The abundance of sinapates and kaempferol derivatives present in the meal before and after solvent extraction warrants further investigation. Moreover, these bitter-flavoring phenolic compounds conjugate with other food ingredients including proteins, peptides, and lipids (Alu'datt et al., 2017). Consequently, the amount, bonding, and structure can have a profound effect on the extraction of these complex phenolic compounds; for example, their initial concentration determines the tannin-protein, protein-phenolic and lipid-phenolic-protein complexes (Alu'datt et al., 2017; Mišan et al., 2010).

The targeted removal and co-extraction of these bitter flavor-contributing compounds, especially sinapine, and kaempferol derivatives will contribute to further innovative processing of canola by-products. Furthermore, these value-added by products could be introduced as a source of nutraceuticals with high antioxidant activity (Alu'datt et al., 2017; Li & Guo, 2016b). Apart from sinapine, both sinapic acid and canolol are both reported as strong antioxidative, anti-radical and anti-mutagenic molecules (Cao et al., 2015; Chen, 2016; Morley et al., 2013). The formation of canolol is closely associated with high temperature processing as temperature-dependent parameters are necessary to improve the functional properties of canolol (Li & Guo, 2016a; Nandasiri et al., 2019). Hence, the isolation and purification of these flavor-active phenolic compounds and other antioxidative compounds would be an asset to the industry. Thus, a targeted efficient extraction method capable of releasing or separating the bitter-flavor active phenolic compounds from proteinaceous matter would be advantageous to the industry.

Both pressurized solvent extraction (PSE) and accelerated solvent extraction (ASE) have recently been applied by the natural product industry to extract phenolic compounds at a relatively high temperature (~200°C), and pressure (~2000 psi) (Li & Guo, 2016a; Nandasiri et al., 2019). The higher phenolic extraction efficiency associated with these methods facilitate attenuation of the bitter-flavoring compounds in the meal, by impacting the extraction of the major sinapic acid derivatives, primarily sinapine and kaempferol derivatives (Li & Guo, 2016a, 2016b; Nandasiri et al., 2019). Thermal processing and the high pressure associated with ASE have many advantages including reduction in the surface tension and viscosity of the extracting solvents, which improves the solubility and mass transfer of targeted phenolics (Li & Guo, 2016a). ASE is also equipped with a closed chamber so that an inert supply with N_2 ensures the stability of the crude extracts with a higher yield of phenolic compounds (Nandasiri et al., 2019).

Previous research reported that structural alterations of phenolics resulted from the application of high pressure, and high temperature (Nandasiri et al., 2019), which generated canolol and flavor-active novel dimers and trimers (Harbaum-Piayda et al., 2010; Kraljić et al., 2015). These previous works discussed extraction yields and instability of these flavor-active phenolic compounds, however on a lab-scale, and further investigation is yet to be considered. A potential major drawback in converting them at both bench-top and industrial scale is absent so far. Consequently, targeted extraction of bitter flavor-active phenolic co-stream ingredients from canola meal should substantially increase its value as a source of nutraceuticals. The present study investigated the pressurized temperature processing (ASE) as method of extraction of flavor-active phenolic compounds. Two different particle sizes (0.5 mm and 1.0 mm) and two extractants (methanol and ethanol) at different concentrations (30%, 40%, 60%, and 70% v/v) under high pressure (1500 psi) at three different temperatures (140, 160, and 180°C) were

examined in the current study. The present study investigated important parameters for extracting the bitter compounds, sinapine, sinapic acid, thomasidioc acid (TA), and major flavor-active kaempferol derivatives. Furthermore, the application of pressurized temperature processing *via* ASE with the targeted extraction of canolol was investigated. The targeted extraction has implications in co-processing of the canola meal to produce value-added phenolic compounds.

2. Materials

Mechanical crushed (double expeller pressed) canola meal containing an oil content of 4-6% (*Brassica napus* L.) was used in this study. All the raw materials were obtained from the Viterra group, St. Agathe, Manitoba. Sinapic acid (purity > 98%) were purchased from Fisher scientific Canada Ltd (Ottawa, ON, Canada). Sinapine (purity > 97%) was purchased from ChemFaces Biochemical Co., Ltd (Wuhan, Hubei, China) Canolol was synthesized in the lab (purity > 97%) and its purity confirmed via HPLC. Cellulose filter papers were purchased from Thermo Scientific Canada Ltd (Mississauga, ON, Canada). All the extraction solvents were purchased from Fisher scientific Canada Ltd (Ottawa, ON, Canada).

3. Methods

3.1 Sample preparation

Canola meal was sieved (Mesh sieve size of 0.5 and 1.0 mm, Ro-Tap Testing Sieve Shaker Model B, WS Tyler, Mentor, Ohio, USA) to obtain two different particle sizes. A Mastersizer 2000 (Malvern Instruments Ltd, Malvern, United Kingdom) was used to confirm the particle size. Samples were defatted using the Soxtec 2050 (Foss-Tecator, Foss North America, MN, USA) and stored at -20^{o}C until further analyzed (Khattab, et al., 2010).

3.2 Synthesis and purification of canolol

The synthesis of canolol was carried out as described by Simpson *et al.* (2005) and Zago *et al.* (2015) by Knoevenagel condensation. In a 200 mL flask, syringaldehyde (3,5-dimethoxy-4-hydroxybenzaldehyde) (8.23 μmol, 1.5 mg), malonic acid (12.35 μmol, 1.3 mg) and piperidine

(41.17 μmol, 4.07 mL) was dissolved in toluene (21.0 mL). The reaction mixture was heated to reflux (115°C) with continuous magnetic stirring (200 rpm). Traces of piperidine were eliminated by adding 20 mL of toluene to the precipitate under vacuum evaporation (Zago et al., 2015). The remaining precipitate was purified using a glass column filled with silica gel 60 Å as the stationery and n-hexane/ethyl acetate (70/30, v/v) as the mobile phase. Fraction separation was followed by applying drops of each collector tube to TLC (EMD Millipore Silica Gel 60 F254) plates which were developed with n-hexane/ethyl acetate/formic acid (70/30/1, v/v/v), dried and directly analyzed using a UV lamp (Zago et al., 2015).

3.3 Extraction of bitter compounds using accelerated solvent extraction (ASE)

Extraction of bitter compounds was performed using aqueous methanol and ethanol at different concentrations (30%, 40%, 60%, and 70% v/v) under high pressure (1500 psi) and at three different temperatures (140, 160, and 180°C) using ASE (ASE 300, Dionex, NY, USA). Sieved samples were mixed with Ottawa sand in a ratio of 1:5 to optimize the yield of the compounds (**Figure 1**). Extracts were concentrated using the rotary evaporator (BÜCHI Rotavapor® R-100, BÜCHI Labortechnik AG, Flawil, Switzerland) and freeze-dried in a freeze dryer (6 Freezone, Labconco Corporation, Kansas City, MO, USA) at -50°C for 36 to 48 hours. All freeze-dried samples were reconstituted with 100% methanol to a final volume of 30.0 mL and diluted up to 10- times prior to HPLC analysis (**Figure 1**).

3.4 Effect of acidification on bitter flavor compounds

Canola meal matrix was acidified with 1.5% (v/v) *O*-phosphoric acid solution and extracted at 160°C with three different extractants (100% (v/v) water, 70% (v/v) methanol, and 70% (v/v) ethanol) as described in **3.3** and subjected to HPLC as described in **3.5**.

149 **3.5 Identification of flavor-active bitter phenolics by HPLC-MS/MS**

150 HPLC analysis was adapted and carried out on a Kinetex® Biphenyl C_{18} 100 Å RP
151 column (2.6 mm, 150 x 4.6 mm, Phenomenex, Canada) maintained at 30°C with 0.4 mL/min
152 flow rate, and 10 µL injection volume as Harbaum-Piayda *et al.* (2010) as described in Nandasiri
153 *et al.* (2019). The mobile phase was consisted of 0.1% formic acid in water (A) and 0.1% formic
154 acid in methanol (B). Chromatograms were acquired at 270 and 330 nm in triplicate by
155 Chromeleon software Version 7.2 SR4 (Dionex Canada Ltd, Oakville, ON Canada). Calibration
156 curves of sinapine, sinapic acid, and canolol were obtained from a series of standard solutions in
157 methanol from 1.0 to 100 µg/mL (n = 11) with R^2 = 0.998 for sinapic acid, R^2 = 0.999 for
158 canolol and R^2 = 0.999 for sinapine with detection limit of each compound at 0.001 mg/mL.

159 Structural elucidation of kaempferol-3-*O*-(2‴-*O*-sinapoyl-*β*-sophoroside), kaempferol-3-
160 *O*-sophoroside, thomasidioic acid (TA) were tentatively identified by liquid chromatography
161 with mass spectrometry and tandem mass spectrometry (LC-MS) using the HPLC method
162 described above. Fractions were collected at one-minute intervals, and were dried (N_2) and
163 analyzed by ESI-MS-MS/MS. Positive ion mode (ESI^+) was used, and spectra recorded on a
164 Bruker Compact high resolution quadrupole time of flight mass spectrometer (Q-TOF-MS)
165 (Bruker Daltonics, Billerica, Massachusetts, USA). MS mode was applied during the formula
166 generation and the mass range was from 50 m/z to 2500 m/z was used. The elute pump was
167 operated at a maximum pressure of 10150 psi, with a capillary voltage of 3500V at a dry gas
168 flow rate of 4.0 L/min with a drying temperature of 200°C. MS/MS tuning was carried out with
169 5.0 eV (ion energy) and 10.0 eV (collision energy). The obtained fragments were compared with
170 the literature values in confirming the phenolic structures (Cai et al. 1999; Hald et al. 2019;
171 Rubino et al. 1996).

3.6 Statistical analysis

All the experiments were carried out in triplicates. Results were presented as mean ± standard deviation of triplicate analysis. Data points were checked for their normality and required transformations were carried out to obtain normalized data (Pallant, 2011). For the current experiment, logarithmic and square root transformations were conducted accordingly to obtain normalized data (Pallant, 2011). A factorial design consists with four independent factors including particle size (0.5 and 1.0 mm), type of extraction solvent (ethanol and methanol), concentration (v/v) of the solvent (30%, 40%, 60%, and 70%, v/v) and extraction temperature (140, 160 and 180ºC). Data analysis was carried out using the general linear multiple regression model using the two-way analysis of variance (ANOVA). Multiple mean comparison was performed using Tukey's test at the level of significant of 0.05 ($p < 0.05$) (Pallant, 2011). To identify the correlation between each phenolic compound partial correlation analysis and a regression analysis was conducted for the major phenolic compounds to elucidate the structure-function relationship. All the data analysis tests were assessed by SPSS statistical software version 22 (IBM, New York, USA).

4. Results & Discussion

4.1 Extraction efficiency of major sinapates

The hydrolysis of sinapine to sinapic acid is considered the major structural-alteration pathway contributing to the flavor-active properties present in canola meal (Li & Guo, 2016a; Nandasiri et al., 2019; Siger et al., 2013). Apart from sinapine, other sinapate derivatives including sinapic acid and canolol also contributes to the flavor properties of the canola meal (Morley et al., 2013; Thiyam et al., 2009; Thiyam et al., 2006). Furthermore, the decarboxylation of sinapic acid to canolol takes place at higher processing temperatures (Zago et al., 2015). Hence, the higher processing temperatures (>100°C) are associated with the improved extractability of the bitter flavor-active phenolic compounds (Nandasiri et al. 2020). Thus, our findings demonstrated that both extraction temperature and extractant concentration appears to be the most important parameters for attenuating the major sinapates from the canola meal. Statistical analysis further illustrated that the extraction efficiency of these sinapates including sinapine, sinapic acid and canolol, were influenced by concentration of the extractant, type of solvent, and extraction temperature (**Table S1 a, b, c, d, e,** and **f**). It was previously reported that solvent concentration is an important factor affecting the rate and the degree of decarboxylation of sinapic acid (Li & Guo, 2016a; Nandasiri et al., 2019; Siger et al., 2013). Current study further confirmed that both the extractant concentration and the extraction temperature are the dominant factors attenuating the major sinapates. However, the particle size of the meal was the least important factor in extracting the flavor-active bitter molecules including the sinapates.

The extractability of sinapine, the major flavor-active phenolic compound present in canola meal (Thiyam et al., 2009) was primarily dependent on the extractant concentration and

209 the extraction temperature. According to the model fit statistics both particle size ($p = 0.12$) and
210 type of solvent ($p = 0.15$), had no significant effect on the extractability of sinapine (**Table S1a**).
211 This further confirms that the removal of sinapine was much less affected by the particle size of
212 the dried canola meal compared to the type of solvent extractant (methanol, ethanol). A similar
213 trend was observed for the extractability of sinapic acid, another flavor-active phenolic acid
214 present in canola meal by-products. Except for particle size ($p = 0.81$), type of solvent ($p = 0.30$)
215 and size*concentration interaction ($p = 0.24$), all other independent variables were significant (p
216 < 0.05) (**Table S1b**) for extracting sinapic acid using the pressurized temperature processing.
217 However, the extractability of canolol was mainly dependent on both the extractant
218 concentration and type of extractant including the extraction temperature (**Table S1c**). The size
219 of canola meal particles ($p = 0.11$) had a negligible effect on extractability of the canolol. The
220 statistical analysis of the model accuracy was further conformed with the higher co-efficiencies
221 of variances for all the major sinapates (sinapine - $R^2 = 0.998$, sinapic acid - $R^2 = 0.990$ and
222 canolol - $R^2 = 0.982$).

223 The polarity of the extractant solvent could affect the extractability of phenolic
224 compounds and its antioxidant properties (Teh & Birch, 2013). Furthermore, Li & Guo (2016a)
225 reported that different polarities of the extractant solvents yield different distributions of major
226 sinapates. The application of pressurized heat *via* ASE further facilitates the concurrent
227 extraction of phenolic compounds and their transformations (Li & Guo, 2016a; Nandasiri et al.,
228 2019). It was reported that the application of pressurized heat improves the H-bonding donor and
229 accepting ability (Li & Guo, 2016a). Furthermore, the pressurized heat would further eliminate
230 the number of hydroxyl groups and other attachments attached to the phenolic structure thereby
231 improving the extractability of the phenolic compounds (Gaspar et al., 2008). The current study

validated 70% (v/v) of both ethanol and methanol aqueous extractants as the optimum concentration for extracting the major sinapates compared to their corresponding concentrations. Hence, the extractability of phenolic compounds increases with a decrease in the polarity index of the type of extractant (Terpinc et al., 2012). Considering the polarity index of both methanol (0.762) and ethanol (0.654) with having similar polarities confirms the current research findings. These results agree with previous reports where major sinapates including canolol was extracted at higher temperatures and when the optimum aqueous solvent concentration was 70% (v/v) (Li & Guo, 2016b, 2016a; Nandasiri et al., 2019; Thiyam et al., 2004; Zago et al., 2015).

The above results confirmed that the extractability of these three flavor-active sinapates were minimally affected by particle size ($p > 0.05$). Generally, the higher extraction efficiency of hydroxycinnamic acids is solely attributed to thermal degradation. For example, the generation of aroma compounds such as 4-vinylguaiacol (the product of the decarboxylation of ferulic acid), guaiacol and vanillin from ferulic acid and the bitter series *O*-caffeoyl-, *O*-feruloyl-, *O*-dicaffeoyl- and quinide derivatives derived from chlorogenic and quinic acids (Rahman et al., 2020).

4.2 Extraction efficiency of other flavor-active minor compounds

Apart from the major flavor-active sinapates, other classes of phenolics also serve as active bitter flavoring compounds such as kaempferol 3-*O*-*β*-sophoroside (KS) and, kaempferol 3-*O*-(2‴-*O*-sinapoyl-*β*-sophoroside) (KSS) (Hald et al., 2019; Yang et al., 2015). A sensory study conducted by Hald *et al.* (2019) demonstrated that protein isolates of rapeseed (canola) containing kaempferol 3-*O*-*β*-sophoroside (KS) exhibited a bitter taste above the low threshold concentration of 3.4 µmol/L confirming the as the key flavor-active molecule of the protein

254 isolates. Kaempferol 3-*O*-*β*-sophoroside was also reported as a flavor-active phenolic compound
255 found to be present in *Brassica* family (Yang et al., 2015). Extraction, identification, and
256 quantification of these unique minor compounds would advance the avenues for biorefinery
257 approach as well as feed formulations targeting the removal of off flavors. Liquid
258 chromatography (LC) coupled with mass spectrometry and tandem mass spectrometry (MS/MS-
259 MS) identified this unique flavor-active molecule to be present in our extracts. The quantification
260 of this molecule was done based on sinapic acid equivalents (SAE) to understand the impact of
261 extraction parameters including concentration of the extractant, type of solvent, extraction
262 temperature and the particle size.

263 The statistical analysis indicated that extractability of KSS was impacted by all the
264 extraction parameters including concentration of the extractant, type of solvent, extraction
265 temperature and the particle size, indicating the stability of this unique flavor active molecule
266 (**Table S1e**). Nevertheless, post-hoc analysis using Tukey's test indicated that both 60% (v/v)
267 and 70% (v/v) for both methanol and ethanol extractants had a minimal impact on the
268 extractability of KSS (**Table 1a**). This further confirms that lower solvent polarities enable the
269 extraction of this unique flavor active molecule, thereby attenuating the bitter off flavors from
270 the meal. The application of less organic solvents and other harmful chemicals are often
271 rewarded by the industries and the government, and often provide many economic benefits
272 (Chen et al., 2014). However, the other kaempferol derivative, KS showed a different
273 extractability compared to KSS. The extractability of KS was mainly depended on both solvent
274 concentration and the particle size. Interestingly, both solvent type ($p = 0.26$) and extraction
275 temperature ($p = 0.50$) had a minimal impact on its extractability (**Table S1d**). The results
276 further indicated that this minor compound showed relatively higher thermal stability than the

277 other flavor-active compounds. Further, post-hoc analysis indicated that each concentration level
278 had a significant impact on the extractability of KS (**Table 1a**). Hence, the use of smaller particle
279 size meal with higher polarity aided a relatively higher concentration of KS. The above results
280 confirmed that the extractability of these two unique flavor active minor compounds (KSS and
281 KS) differed considerably. Thus, the results further confirmed the structural alterations in the
282 phenolic compounds would affect the extractability parameters and may impact its flavor profile.

283 Thomasidioic acid (TA) is another flavor-active molecule but the structural alteration due
284 to processing and extraction has not received much attention in recent years. Both Rubino *et al.*
285 (1996) and Cai *et al.* (1999) reported that TA was not a natural phenolic compound but formed
286 during the high temperature processing in the presence of oxygen at both acidic and alkaline pH.
287 The formation of TA takes place in the acidic medium with the precursor sinapic acid with
288 dehydrosinapic acid lactone as its intermediary product (Rubino, Arntfield, & Charlton, 1995).
289 TA is categorized under the phenolic group of lignans. These lignans were reported to convert
290 into hormone like compounds by the gut microflora inside the body, which protects the body
291 against hormone dependent cancers (Ward, 1993). The quantification of this thermo-generative
292 compound was conducted to understand the impact of each extraction parameter.

293 The statistical analysis indicated that extractability of TA was primarily depended on
294 both extraction temperature and the concentration of the extractant (**Table S1f**). Both the size of
295 the canola meal particles ($p = 0.48$) and the type of solvent ($p = 0.14$) had a minimum impact on
296 the extractability of TA agreeing with the previous reports. On the contrary, at higher extraction
297 temperatures, these lignan compounds further converts to other complex phenolic compounds
298 including its dimers, trimers, and oligomers (Harbaum-Piayda et al., 2010; Morley et al., 2013;

299 Oehlke et al., 2017; Siger et al., 2013). Consequently, the concentration of free TA would
300 decrease with the formation of these phenolic derivatives. This was further confirmed via the
301 statistical analysis showing that both 140 and 180°C processing temperatures had no significant
302 differences on the extractability of TA (**Table 1c**).

303 **4.3 Impact of pressurized heat on flavor-active phenolic compounds**

304 The literature generally supported that thermal processing affected sinapates. The high
305 temperature (up to 200°C) and pressure (~1500 psi) of ASE facilitates the removal of the
306 aglycone moieties attached to phenolic compounds by hydrolysis with minimal interference on
307 its original composition (Yang et al., 2015). The application of ASE yielded comparatively
308 higher amounts of phenolic compounds compared to conventional methods as well as ultrasound
309 extraction (Li & Guo, 2016a; Nandasiri et al., 2019). This was attributed to the high pressure of
310 ASE which increased the solubility of the targeted compounds and the diffusion rates as well as
311 the mass transfer rates of the solutes (Li & Guo, 2016a). The concurrent extraction of ASE also
312 facilitated the structural transformations of sinapine to sinapic acid and canolol, at elevated
313 temperatures (Li & Guo, 2016a).

314 These transformations would enable the attenuation of bitter flavor-active phenolic
315 compounds while improving its co-processing. For example, the decreasing content of sinapine,
316 largely impacted by the increase in temperature (**Table 2**) is attributed to the decomposition or
317 hydrolysis pathway (Khattab, et al., 2014; Oehlke et al., 2017). Results indicated that the
318 concentration of sinapine decreased significantly ($p > 0.05$) from 9.75 mg/g DW to 5.12 mg/g
319 DW with the increase in temperature from 140°C to 180°C with 70% (v/v) ethanol whereas, the
320 concentration of sinapine further decreased from 12.1 mg/g DW to 5.12 mg/g DW with increase

321 in temperature from 160°C to 180°C (**Table 2**). This confirms the transformation of sinapine at
322 higher temperatures, either from the bound and free forms (Chen, et al., 2014; Khattab, et al.,
323 2010). The thermal decomposition order of the phenolic compounds showed the following
324 decreasing pattern; sinapine > sinapic acid > canolol (Khattab, et al., 2010).

325 High temperature pre-conditioning and thermal processing treatments can also
326 significantly ($p < 0.05$) influence the structure of phenolic compounds besides sinapine, as well
327 as sinapic acid and canolol (Siger et al., 2013; Siger et al., 2015; Thiyam et al., 2009; Wroniak et
328 al., 2016). Temperatures namely, 160°C and 180°C with a high pressure induced the hydrolysis
329 of sinapine into sinapic acid which is consequently produces canolol by decarboxylation (Li &
330 Guo, 2016a; Morley et al., 2013; Zago et al., 2015). In this study, the higher concentrations of
331 sinapic acid and canolol produced by ASE confirm the conversion of sinapine to sinapic acid and
332 canolol at the higher temperatures (**Table 2**). Thus, the combined treatment of ASE with
333 microwave improved the quantity of phenolic compounds at relatively higher processing
334 temperatures ranging from 160 to 180°C (Li & Guo, 2016a; Siger & Józefiak, 2016; Wroniak et
335 al., 2016). The visually apparent darker brown/black colored extracts obtained at higher
336 processing temperatures (180°C) by ASE was indicative of the presence of higher amounts of
337 Maillard reaction products apart from the phenolics (Chen et al., 2014; Rubino et al., 1996).

338 The highest concentration of sinapic acid was attained at 160°C for both organic
339 extractants (70% (v/v) methanol - 0.55 mg/g DW and 70% (v/v) ethanol - 0.63 mg/g DW)
340 compared to 180°C (**Table 2**). A reduction in total sinapic acid and canolol content observed at
341 temperatures above 160°C may be due to the loss of the *cis*-isomer of sinapic acid at
342 temperatures higher than 140°C (Siger et al., 2015). Above 140°C, the *cis*-sinapic acid content

343 decreased rapidly, and was undetectable at temperatures of 160ºC and 180ºC. Furthermore, both
344 Harbaum-Piayda *et al.* (2010) and Kraljić *et al.* (2015) reported that canolol at high temperatures
345 (>180ºC) is converted into its other forms including dimers, trimers and oligomers. Therefore, a
346 reduction in both sinapic acid and canolol is observed under higher processing temperatures.
347 Spielmeyer *et al.* (2009) noted that the optimal temperature for extracting canolol was 160ºC.
348 Moreover, Morley *et al.* (2013) also reported that optimum roasting temperature for the
349 formation of canolol is at the extraction temperature of 160°C. These findings are in agreement
350 with our results, which also found that the highest level of canolol formation was at 160ºC. In
351 addition, Zago *et al.* (2015) reported 2-hour hydration of the meal before the treatment of super-
352 heated steam (160ºC) increased both the antioxidant activity and its total phenolic content (TPC)
353 by 12% (22 mg SAEg/DM) compared to the non-hydrated meal further in agreement with our
354 current findings. The authors suggest that the increase in its TPC may be due to the release of the
355 bound phenolic compounds via the partial breakdown of the plant cell walls during the super-
356 heated steam. The extraction conditions of ASE would facilitate similar properties yielding
357 higher phenolic composition.

358 Khattab *et al.* (2014) reported that over 95% of sinapine was converted to sinapic acid
359 using 70% (v/v) methanol:water by microwave extraction from canola meal. However,
360 approximately 55% of sinapic acid was then decarboxylated to canolol with a yield of 4.2 g/kg.
361 Thus, the relatively lower conversion rate of sinapic acid to canolol can be explained with the
362 formation of other intermediaries of sinapic acid at higher extraction temperatures. The
363 formation of TA at high temperature at acidic pH conditions with the precursor sinapic acid is a
364 good example for the lower conversion rate of sinapic acid to canolol (Rubino et al., 1995).

In another note, Cai *et al.* (1999) reported that autoclaving of sinapic acid at 121°C for 15 minutes at 0.1 MPa pressure would also produce TA, which further confirms the processing conditions applied in ASE (200°C and 1500 psi) is ideal for the formation of TA at relatively higher temperature and pressure levels. The formation of these lignan derivatives and the Maillard reaction products at higher temperatures could directly influence the antioxidant activity as well as the total phenolic and flavonoid content of the extracts (Chen et al., 2014; Rubino et al., 1996). These lignans directly impact the flavor-profile at the higher processing temperatures and pressure conditions although the existing literature have not discussed this aspect. The use of high pressure and temperature on the other hand is ideal for a short-time treatment to obtain these flavor-active compounds. Thus, shorter extraction time (~10-20 minutes) associated with the ASE provides the ideal environment for extracting these flavor-active minor compounds.

A recent sensory analysis conducted by Hald *et al.* (2019) further confirmed that the bitter flavor of canola meal by-products was due to the presence of kaempferol 3-*O*-(2‴-*O*-sinapoyl-*β*-sophoroside). They further reported that of these esterified products, KSS and KS were the most influential bitter compounds affecting the flavor profile (Hald et al., 2019; Yang et al., 2015). Further work by Siger *et al.* (2013) reported that other kaempferol derivatives are present in canola extracts including kaempferol 3-dihexoside-7-sinapoyl-hexoside (30 mg/100 g of DW). They further reported that the concentration of these kaempferol derivatives increased with acid hydrolysis (Siger et al., 2013), which is relevant to the production and precipitation of protein concentrates.

386 On the contrary, most flavonoids are easily oxidized under aerated conditions, so the
387 presence of an inert gas (N_2) is important to attenuate the oxidation (Nandasiri et al., 2019).
388 Thus, an oxygen-free environment is essential for the extraction and the co-processing of the
389 flavonoid-based flavor-active phenolic compounds. Apart from wet heat and high pressure,
390 ASE's closed system equipped with inert gas (N_2) could facilitate the preservation of phenolic
391 compounds and their antioxidant properties, which otherwise will be detrimental at such high
392 and pressured conditions. Furthermore, this technique can readily recover highly reactive
393 phenolic compounds and prevent their auto-oxidation. Frolov *et al.* (2013) reported that a closed
394 system equipped with inert gas during ASE extraction minimized the rate of oxidative
395 degradation by the complete evacuation of air from the extractants. Moreover, Li & Guo (2016a)
396 stated that the formation and stability of canolol may be affected by shorter extraction times and
397 the method of cooling after each extraction. The centrifugation of the extractants at 4°C
398 immediately after each extraction step, in our method facilitated the higher recovery of phenolic
399 compounds including canolol. Thus, an efficient cooling procedure is recommended soon after
400 the thermal extraction to produce higher yields of flavor-active phenolic compounds including
401 canolol, after ASE extraction. These extraction conditions correspondingly disfavor the Wessely-
402 Moser regrouping thereby improving the extraction efficiency of flavor-active bitter-phenolics
403 (Wang, 2010).

404 **4.4 Relationship between the major phenolic compounds**

405 Understanding the relationship between major sinapates and other flavor-active bitter
406 phenolic compounds would help to clarify questions regarding the extraction of these
407 compounds. A partial correlation analysis was conducted to evaluate the degree and direction of
408 these major flavor-active phenolic compounds (**Table 3**). The results confirmed a moderate

409 positive correlation between sinapine (r = 0.50) and sinapic acid (r = 0.59) with canolol (**Table**
410 **3**). Similarly, sinapic acid had a moderate to strong correlation of 0.57 with sinapine (**Table 3**).
411 All the correlation values were significant for sinapine, sinapic acid and canolol.

412 In terms of other flavor-active phenolic compounds, a poor correlation was observed
413 between KSS and all the other phenolic compounds except KS (r = 0.59). On the contrary, KS
414 demonstrated a moderate correlation among all the other phenolic compounds with having the
415 highest correlation with sinapic acid (r = 0.66) (**Table 3**). Although, the statistical correlation
416 between sinapic acid and TA was insignificant, the negative correlation proved that sinapic acid
417 is a likely precursor for the production of TA (Rubino et al., 1996).

418 Apart from the partial correlation analysis, linear regression model was used to evaluate
419 the relationship between major flavor-active phenolic compounds. Our results indicated a linear
420 relationship for the conversion of sinapine to sinapic acid (R^2 = 0.77) (**Table 4**). A linear
421 response further demonstrated that at higher temperatures alteration of the sinapine structure
422 could occur with sinapic acid formed by elimination of the choline-ester (Khattab et al., 2010;
423 Thiyam et al., 2009). The higher extraction temperatures would enable the dissociation of the
424 choline ester from the sinapine, which increases the yield of sinapic acid at higher extraction
425 temperatures (Khattab et al., 2010; Thiyam et al., 2009). Li & Guo (2016b) further suggested that
426 the higher level of sinapic acid associated with increase in temperature, could be due to either its
427 greater extraction from the meal at higher temperatures or the concurrent conversion of sinapine
428 to sinapic acid. Thus, high pressure further promotes the cleavage of hydrogen bonds in the
429 water molecules thereby increasing the concentration of protons (H^+) in the medium making it

more acidic with lower pH. This phenomenon would also facilitate the conversion of sinapine to sinapic acid during the ASE extraction (Nandasiri et al., 2019).

The lower linearity between sinapic acid and canolol (R^2 = 0.42) suggests that the formation of canolol could be associated with other factors besides temperature (**Table 4**). The lipophilic nature of canolol and its attachment to the cell and other biological membranes, was thought to make it unavailable for other types of reactions (Khattab et al., 2014). Furthermore, the lower polarity and higher reactivity of canolol may further lowers the availability of canolol thereby reducing the linearity between sinapic acid and canolol (Chen et al., 2014; Khattab et al., 2010). Likewise, the linearity between the sinapic acid and TA was low (R^2 = 0.35) (**Table 4**). Both lower linearity relationships between the above phenolic compounds directs the structure-based activity of sinapic acid between TA and canolol. Furthermore, both Spielmeyer *et al.* (2009) and Morley *et al.* (2013) reported that the optimal temperature for the extraction of canolol was 160°C. At processing temperatures above 180°C, canolol forms dimers and trimers which will affect its concentration (Harbaum-Piayda et al., 2010; Kraljić et al. 2019; Kraljić et al., 2015). Thus, improving the extractability of these flavor-active minor compounds *via* pressurized temperature processing would aid the production of value-added by-products including phenolic antioxidants. These by-products may hold promising results in the nutraceutical industry.

The effect of acidification was found to have a minimal effect on the phenolic composition (**Table 5**). The amounts of sinapine, sinapic acid and canolol did not significantly increase with acidification of the medium. Harbaum-Piayda *et al.* (2010) pretreated canola oil distillate with phosphoric acid followed by methanol extraction and reported that that

452 acidification/protonation together with high temperature, produced higher yields of phenolics
453 including canolol and its derivatives. The extraction of flavor compounds from the
454 corresponding canola meal in this study, however, did not to show any significant differences
455 among phenolic compounds at 160ºC using either 70% (v/v) ethanol/water or 70% (v/v)
456 methanol/water as extractants (**Table 5**).

457 ## 5. Conclusions

458 The occurrence of major sinapates, namely sinapine, sinapic acid, and canolol and other
459 active molecules including TA and kaempferol derivatives imparts flavor to canola meal. The
460 targeted extraction and co-processing using ASE proved to be an efficient method for extracting
461 these flavor-active molecules while attenuating the bitter molecules from the canola meal. The
462 use of shorter extraction times (20 minutes), and lower solvent usage, improved concurrent and
463 targeted extractability of flavor-active phenolic molecules. Therefore, the use of ASE could
464 enable the creation of co-streams of phenolic rich antioxidants. These phenolic rich antioxidative
465 compounds from the meal characterize an additional potential source for use in the food and
466 nutraceutical industries. These new co-streams can be piloted with canola protein industries to
467 benefit the ongoing strong demand for alternative plant-based natural preservatives and shelf-life
468 improving agents.

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