Effect of extrusion technology on hempseed (*Cannabis sativa L.*) oil cake: polyphenol profile and biological activities

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Abstract:

Effects of extrusion with varying barrel temperature, moisture content and screw speed on hempseed oil cake were studied for the first time. Extrusion at lower moisture (30 %) and higher screw speed (300 rpm) significantly increased the proportion of free polyphenols, flavonoids, and phenylpropionamide content, and α-glucosidase and acetylcholinesterase inhibition activities. Full factorial design confirmed the three-way interactions among all extrusion parameters for all chemical assays with the bound phenolic fraction, total flavonoid content and DPPH inhibition activity of the free phenolic fraction. HPLC-DAD-ESI-QTOF-MS/MS analysis tentatively identified 26 phenylpropionamides, and the contents of N-*trans*-caffeoyltyramine (66.26 µg/g) and total phenylpropionamides (85.77 µg/g) were significantly increased after extrusion at the lower moisture and higher screw speed extrusion conditions. The higher α-glucosidase inhibition activity at higher screw speed could be due to the N-

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trans-caffeoyltyramine ($r = 0.99$, $p < 0.01$), while the AChE inhibition activity appeared to be influenced more by the cannabisins A-C, M ($r > 0.8$, $p < 0.01$).

Keywords: acetylcholinesterase; glucosidase; hempseed oil cake; HPLC-DAD-ESI-QTOF-MS/MS; polyphenol.

Practical Application

Hempseed oil cake is a by-product of oil extraction, with high protein and high fibre contents. The results of this research could be used directly in food industry to improve the nutritional and commercial value of hempseed oil cake by extrusion technology.

1. Introduction

Seeds of hemp (*Cannabis sativa* L.) have been grown and consumed in Asian communities for around 10,000 years (Russo 2007). The last 20 years have seen the legalization to cultivate hempseed at low (< 0.3% w/w) tetrahydrocannabinol (THC) levels in Australia, New Zealand and the United States (Food Standards Australia New Zealand 2017; US Department of Agriculture 2018). However, only the highly unsaturated hempseed oil has been extensively utilized in the food industry. The demand for the by-product after dehulling process and oil press extraction, also known as the oil-press cake, is significantly low despite its richness in protein (~ 30 %), fibre (~ 46 %), and fibre-bounded polyphenols (Leonard et al. 2020a). The composition of oil-press cake allows its potential application in formulation of various food products, such as expanded snacks, bread and meat extender, or animal feed (Pojic et al. 2015). Thus, utilization of this by-product will reduce the waste generated from hempseed processing and provide value to the food industry. Our recent review has

highlighted the unique polyphenol profile of hempseed, of which are dominated by hydroxycinnamic acid amides and lignanamides (Leonard et al. 2020a).

Hempseed lignanamides were first identified by Sakakibara et al. (1991) in the form of cannabisin A, B and C. Past research indicated cannabisin B and N-*trans*-caffeoyltyramine as the major phenylpropionamides in hempseed polyphenol extract (Chen et al. 2012; Zhou et al. 2018). Cannabisin B demonstrated significant free radical scavenging activity and promoted autophagic cell death in liver hepatoblastoma (HepG2) cells (Chen et al. 2013). Other hemp lignanamides, including cannabisin A, C, D and M, exhibited strong antioxidant activities on DPPH assays similar to the positive control quercetin (Yan et al. 2015). Zhou et al. (2018) further isolated one new lignanamide, cannabisin Q, and eighteen other known compounds from hempseed, fourteen of which have significant inhibitory effects on the TNF-α release from LPS-induced BV2 microglia cells, suggesting a protective mechanism against neurodegenerative diseases. Collectively, these studies represent the growing interest in the unique hempseed polyphenol identity and its biological activities.

Extrusion is a continuous process in which the material undergoing mixing, heating, shearing and compression inside the extruder barrel and a sudden pressure change when the material exits the die (Dalbhagat et al. 2019). Since the 20th century, it has been extensively applied in the manufacture of cereal based products, snacks, pasta products, texturized vegetable proteins and meat analogues. Most food manufacturers prefer starch-dominated ingredients for extrusion, due to starch's expansion and gas holding property to make a desirably crunchy product. However, previous studies have demonstrated the successful application of extrusion for protein or fibre-rich food processing by-products, such as defatted almond powder in

puffed snack, flaxseed meal in cereal bars and sesame oil cake in corn grits (Leonard et al. 2020b).

Mixed findings were observed on effect of extrusion on the total phenolic content and antioxidant activity of extruded products. This may depend on the intrinsic nature of ingredients and process parameters. Xu et al. (2016) indicated that combination of low temperature and high moisture content accelerated the α-amylase activity in rice flour, thus the release of bound phenolic compounds. However, in another study to develop snacks from hazelnut and fruit processing by-products, subjecting the materials at higher temperatures of 150-175°C still ensure high retention of polyphenols (Yagci and Gogus 2009). Dlamini et al. (2007) reported significant reduction in total polyphenols and antioxidant capacity of whole and decorticated sorghum grains after extrusion. In contrast, extrusion of sorghum hull increases the total phenolic content (Salazar Lopez et al. 2016). The increase in polyphenols may be due to the release of bound form compounds from cell wall membrane, while the decrease could be explained by degradation or polymerization of phenolic acids at high temperature (Leonard et al. 2020b).

To the best of our knowledge, no past research has been conducted to compare the phenolic profile and biological activities between raw and extruded hempseed oil cake. In the previous part of our project, we investigated the effects of extrusion on the fibre-rich, hempseed hull (Leonard et al. 2021). However, as detailed in the succeeding sections, the effects of extrusion on the protein-rich, hempseed oil-cake is distinct to that of the hull, even after introducing more parameter changes (e.g. moisture). Although hempseed protein fractions (e.g. flour) have a lower phenolic content than hull and they have been applied in extruded products in the past, the focus is often on its physical, sensory and snack-like properties (e.g.

colour, expansion index, flavour, consumer acceptance). Studies on the impact of food processing technologies on hempseed polyphenol and antioxidant activities are extremely limited. Moreover, the literature detailing the effect of extrusion on α-glucosidase (linked to elevated risk of type 2 diabetes and its complications; Joshi et al. 2015) and acetylcholinesterase (linked to progression of Alzheimer's disease; Colović et al. 2013) inhibition activities of any plant material is nearly absent. Thus, the main objective of this study is to investigate the effects of extrusion technology on the phenolic profile, antioxidant capacities and enzyme inhibitory activities of hempseed oil cake. This may provide valuable information for food manufacturers on the combinations of extrusion parameters that led to the greatest phenolics retention. High performance liquid chromatography - diode array detector – coupled quadrupole time-of-flight mass spectrometry (HPLC-DAD-QTOF-MS/MS) was employed to analyse the polyphenols of the processed hempseed by-product. In addition, the effects of extrusion processing parameters, including moisture content, barrel temperature and screw speed, on the polyphenol profile and bioactivities were explored.

2. Materials and methods

2.1. Materials

Twenty kilograms of hempseed (*Cannabis sativa* L.) oil cake (particle size ~ 500 μm) was purchased from a commercial Australian hemp producer. The chemical composition of the cake, as detailed by the company information, is: 30.5 % protein, 9 % fats, and 45.7 % fiber (dominantly insoluble). The seeds were of CRS1 variety and grown in Westmoreland district, Tasmania (147.12°E, 41.60° S) in the season of 2019. The cake was packed in opaque aluminium bags and placed under cold (4°C) storage until extrusion.

2.2. Chemicals

All chemicals were purchased from Sigma-Aldrich (Castle Hill, New South Wales, Australia). The analytical standard N-trans-caffeoyltyramine was purchased from SynInnova Laboratories Inc. (Edmonton, Alberta, Canada).

2.3. Experimental design and extrusion operation

The hempseed oil cake was extruded using a co-rotating, twin-screw extruder (KDT30-II, Jinan Kredit Machinery Co Ltd., China) with settings provided in Table 1. Three operation parameters (independent variables) were varied during the extrusion cooking: barrel temperature (°C), moisture content (%) and screw speed (rpm). Minitab 19 software (Minitab, PA, USA) was used to generate several experimental runs in accordance to the full factorial design and re-arranged for presentation purposes (Table 1). All runs were carried out in triplicate (n=3)

The specifications of the extruder include a rectangular die with an area of 2 x 5 mm^2, screw length/ diameter ratio of 20:1 and a screw diameter of 20 mm. The barrel was divided into a feeding zone and four controlled temperature zones set at 40, 60, 80/100 and 80/100 °C. This was supported by an electric cartridge heating system and a water-cooling system. A control panel was used to set the final barrel temperature and screw speed, which also monitored the die pressure and motor electric current. Feed moisture was controlled using a piston dosing water pump (Lutz Jasco Piston Pump, Model: FEDOS E8) that could adjust the water feed

rate and calibrate according to the moisture content of hempseed oil cake (12 %). After preliminary trials, the extrusion barrel moisture working window for this material was very narrow. Lower (<30 %) feed moisture led to die blockage, while higher (>40 %) feed moisture led to structure-less extruded product. Additionally, the extruded product was burnt and extremely hard (due to extreme loss of moisture) after extrusion at 120 °C, thus the extrusion temperature window was set at 80 °C and 100 °C. The feed rate was kept constant at 5.6 kg/h. The extruded samples were stored in the dark at refrigerated temperatures (4 °C) until further analysis.

2.4. Extraction of hempseed oil cake phenolic compounds

2.4.1. Extraction of free and bound phenolic compounds

The methods developed by Wu et al. (2017) and Xiong et al. (2020), with minor modifications, were used to extract the phenolic compounds from hempseed oil cake. About 35 mL of 80 % (v/v) methanol was mixed with 4 g of hempseed oil cake samples, homogenised (8000 rpm for two min) and shaken for two hours at room temperature under nitrogen (N_2) gas. The resultant mixture was centrifuged (4500 rpm, 4 °C, 10 min) and supernatant collected as the free fraction. The resultant supernatant were evaporated to dryness using a rotary evaporator at 40 °C, dissolved in methanol and stored at -20 °C in the dark until analysis. The residue was further boiled with 2 M hydrochloric acid at 100 °C under nitrogen for one hour in a closed container. The bound, clear fraction was partitioned four rounds of 50 mL ethyl acetate. The resultant ethyl acetate fractions were pooled and evaporated to dryness using the rotary evaporator at 40°C. The dried sample was dissolved in methanol and stored at -20 °C in the dark until analysis.

2.5. Determination of total phenolic content (TPC) based on methanol-acid hydrolysis extraction method

The Folin-Ciocalteu method reported by Singleton & Rossi (1965) was used to determine the TPC of the sample extracts with minor modification. Briefly, 60 μL of free or bound phenolic extract was mixed thoroughly with 750 μL 10% Folin-ciocalteu reagent and 600 μL 7.5 % (w/v) sodium carbonate solution. The absorbance of the mixture was determined at 765 nm using a Thermo Scientific Multiskan GO Microplate Reader (Thermo Fisher Scientific, Victoria, Australia). Results were expressed as mg gallic acid equivalents (GAE) per g sample on a dry basis. The sum of free and bound phenolic content was determined as the TPC.

2.6. Determination of total flavonoid content (TFC) based on methanol-acid hydrolysis extraction method

The TFC of the sample extracts was determined in accordance to the method by Zhishen et al. (1999) with slight modification. An aliquot of 150 μL of free or bound phenolic extract was mixed together with 45 μL of 5 % sodium nitrite solution and 10 % of aluminium chloride solution, 600 μL of 0.5 M sodium hydroxide, then absorbance measured at 415 nm. Results were expressed as mg catechin equivalents (CAE) per g sample. The sum of free and bound flavonoid content was determined to be the TFC.

2.7. DPPH radical scavenging assay

The DPPH ((2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay was adopted (Thaipong et al. 2006) with minor changes. An aliquot of 100 μL of phenolic extract was mixed with 900 μL of DPPH working solution, shaken for two hours in the dark, and absorbance checked at 515 nm. The percentage DPPH inhibition rate was determined at 1 mg/mL sample extract using the equation:

$$DPPH\ Inhibition\ (\%) = \left(1 - \frac{A_{sample}}{A_{blank}}\right) x\ 100$$

Where A_{sample} is the absorbance of the sample and A_{blank} is the absorbance of the solution with the extract replaced by methanol. The DPPH inhibition percentage for each of the whole solution was acquired by combining equal volume of free and bound phenolic extract.

2.8. ABTS radical scavenging assay

The ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) radical scavenging assay was determined with the modified method of Thaipong et al. (2006). 100 μL of phenolic extract was mixed with 900 μL of ABTS working solution and the absorbance determined at 734 nm. The percentage ABTS inhibition rate at 1 mg/mL sample extract was determined using an equation similar to DPPH assay (Section 2.7).

2.9. α-glucosidase inhibitory activity

Determination of α-glucosidase inhibitory activity for the cake extract was carried out in accordance to the principle by Telagari and Hullatti (2015) with several modifications. In the 96-well microplate, 50 µL of 0.1 M phosphate buffer, 20 µL extract and 10 µL of α-glucosidase solution from rat intestinal powders (Sigma Aldrich I1630; 1 unit/ mL) were mixed. The mixture was allowed to incubate at 37 °C for 10 min. After incubation, 20 µL of 5 mM *p*-nitrophenyl-a-D-glucopyranoside solution (pNPG; pH 6.8) was added to each well. The mixture was incubated at 37 °C for 20 min, the reaction stopped by adding 50 µL of 0.1 M sodium carbonate, and absorbance measured at 405 nm. The α-glucosidase inhibitory activity was expressed as % inhibition at 1 mg/mL sample extract using the formula:

$$Inhibition\ \% = \left[1 - \frac{(A_{sample} - A_{sample\ background})}{(A_{control} - A_{control\ background})}\right]$$

Where A_{sample} is the absorbance of the test group, $A_{sample\ background}$ is the absorbance with pNPG replaced by buffer, $A_{control}$ is the absorbance with sample extract replaced by methanol and $A_{control\ background}$ is the absorbance with sample extract and pNPG replaced by methanol and buffer respectively. The α-glucosidase inhibition percentage for each of the whole solution was acquired by combining equal volume of free and bound phenolic extract. A commercial anti-diabetic drug acarbose at 0.1 mg/mL was used as the positive control.

2.10. Acetylcholinesterase (AChE) inhibitory activity

A modified Ellman's Method was used to determine acetylcholinesterase inhibitory activity in this study (Yan et al. 2015). An aliquot of 100 µL of 50 mM Trizma-hydrochloride buffer

(pH 7.8), 20 µl sample extract solution and 20 µL of AChE solution (from *Electrophorus electricus*, Sigma Aldrich C2888, 5 unit/ mL) was mixed and allowed to stand in room temperature for 30 min. Then, 40 µL of 3 mM 5,5'-dithiobis-2-nitrobenzoic acid and 20 µL of 15 mM acetylcholineiodide was added into each well, and absorbance recorded at 405 nm. Acetylcholinesterase inhibitory activity was expressed as % inhibition at 1 mg/mL sample extract and calculated using the same formula as α-glucosidase inhibitory activity assay. A commercial drug of treatment of cognitive decline in mild to moderate Alzheimer's disease, galanthamine, at 0.05 mg/mL was used as a positive control.

2.11. HPLC-DAD-ESI-QTOF-MS/MS identification of phenolic compounds

HPLC-DAD-ESI-QTOF-MS/MS analysis was carried out with an Agilent 1200 series high performance liquid chromatography (HPLC) (Agilent Technologies, Santa Clara, CA, USA) system attached to Agilent 6520 quadrupole time-of- flight (QTOF) MS/MS system with dual sprayer electrospray ionisation (ESI) with a diode array detector (DAD). Separation was executed through a Synergi^{TM} 4 µm Hydro-RP 80 A LC Column (4 µm particle size, 250 x 4.6 mm internal diameter, Phenomenex Inc., Lane Cove West, NSW, Australia) with a gradient of 0.1 % formic acid (A) and acetonitrile (B): 5 % B (0-10 min), 20 % B (10-45 min), 40 % B (45-60 min), 75 % B (60-61 min), 100 % B (61-66 min), 5 % B (66-70 min). A sample volume of 10 µL was injected with a flow rate of 500 µL/ min. The MS parameters were set as: nitrogen gas nebulization of 45 psi with a flow rate of 9 L/min at 325 °C, capillary voltage of 3500 V, fragmentor of 175 V, skimmer of 65 V, and dynamic range mode from m/z 70 to 1700, in both positive and negative ionisation modes. The MS/MS was set in auto mode, with scan range 60-1000 m/z and collision energy 15-30 eV. Every sample was analysed twice in positive and negative mode separately. The samples were monitored at

wavelengths 255, 280, 300, 320, 335, 370 nm by the DAD. The instrument control, data acquisition and processing were completed with MassHunter workstation software (Qualitative Analysis, version B.03.01) (Agilent Technologies, Santa Clara, CA, USA). The mass fragments and UV spectra (λmax) data of phenylpropionamides and lignanamides are derived from past research (Moccia et al., 2019; Nigro et al., 2020; Zhou et al., 2018), and identification was carried out using MassHunter workstation software to compare the MS, MS/MS fragments and UV spectra.

2.12. HPLC-DAD quantification of phenolic compounds

Measurement of individual phenylpropanoids was performed with an Agilent 1260 Infinity II Prime HPLC attached with Agilent 1260 series DAD (Agilent Technologies). The column and conditions were the same as in HPLC-DAD-ESI-QTOF-MS/MS identification. Phenylpropionamides were semi-quantified with N-*trans*-caffeoyltyramine on a linear regression of peak area against concentration ($R^2 > 0.99$), due to the limited availability of standards (Zhou et al. 2018). Only compounds with consistent peaks and confirmed in both positive and negative ionisation modes across all samples were quantified. Data was analysed using Agilent OpenLAB workstation (Agilent Technologies) and presented as a total sum of free and bound fractions.

2.13. Statistical analysis

Measurements were done in triplicate (n=3) and expressed as mean ± S.D. Means between treatments were calculated and distinguished with Fisher's least significant difference at 95 % confidence interval using the general linear model in Minitab 19 (Minitab) (One-way

ANOVA, two-sided). Pearson's correlation coefficient (*r* value) and significance were determined in Minitab 19.

3. Results and discussion

3.1. TPC and TFC

The TPC for hempseed oil cakes in this study was in the range of 0.385-0.906 mg GAE/g sample (Figure 1A; Table S1). It was within the range provided by Siano et al. (2018) for hempseed flour at 0.744 mg GAE/g, but considerably lower than the range of 3.9 to 15.6 mg GAE/g in defatted hempseed kernel, as reported by Chen et al. (2012). The variability in the observed results could be due to the differences in hempseed variety and extraction method. Extrusion, at all parameter combinations, significantly reduced (p <0.05) the total phenolic and flavonoid content of hempseed oil cake (Figure 1A). This effect was exacerbated at high moisture (40 %) extrusion condition due to the diluting effect of water. Similar finding was reported by Korkerd et al. (2015) in extruded snacks made from high protein defatted soybean meal and germinated brown rice meal. The high moisture and temperature during the extrusion process appears to accelerate degradation or polymerization among phenolic compounds, which lowers its extractability in Folin-ciocalteu chemical assays. However, increasing temperature by 20 ºC did not appear to further intensify the TPC loss in this study. Future studies may attempt to alter the extrusion configuration, expand the range of screw speed, moisture and temperature change, without sacrificing the structural integrity and acceptability of the food product. It should also be noted that although Folin's reagent has been widely used in TPC studies, it can interact with the protein in the sample. During extrusion, protein also undergoes transformation in its structure (e.g. denaturation and reaggregation into an insoluble complex) which could influence TPC values (Leonard et al.

2020b). Furthermore, we proposed that the effect of extrusion on the TPC also depends on the properties (e.g. polyphenol composition, material structures, rheology and heat absorption) of the raw materials, as an increased TPC after extrusion was observed in fibre-rich hempseed hull in our other study (2.85 mg GAE/g in raw hempseed hull vs. 2.96-3.84 mg GAE/g in extruded hull treatments; Leonard et al. 2021). This may also indicate that the polyphenols have a weaker bond with hemp protein than hempseed fibre.

Although the TPC of hempseed oil cake was lowered, relatively low moisture (30 %) extrusion increased the proportion of free polyphenols compared to the total phenolic content (Figure 1A). Evidence on the biological activities and health properties of phenolic compounds have been relentless in recent years. These phenolic compounds are mostly conjugated to macronutrients such as fibre or protein in plants. Therefore, liberation of polyphenols from the food matrix during extrusion may improve its bioavailability and re-balance its health properties between small intestine and colon (Ribas-Agustí et al. 2018; Tang et al. 2020). While the TFC results followed the trend of TPC, only two runs (Runs 2 LTHSLM and 7 HTHSLM), both characterized by the combination of low moisture and high screw speed, showed a higher proportion of free flavonoids relative to the total TFC compared to raw group (Figure 1A). This may suggest that flavonoids or its associated chemical assay is more sensitive to molecular changes induced by the extrusion process, or that flavonoids are more resistant to breakage from the matrix than simple phenolic acids. Details analysis on the changes in individual phenolic compounds are elaborated in section 3.7 and 3.8.

3.2. DPPH and ABTS radical scavenging assays

The antioxidant activity of hempseed oil cake was generally reduced after extrusion, as shown in the lower DPPH and ABTS inhibition activity in most extruded treatments compared to the unextruded one (Figure 1B; Table S2). This result might have the same explanation as the reduction of TPC and TFC after extrusion. It appears that combination of high screw speed and low barrel moisture alleviates the loss in antioxidant activity. Higher screw speed may promote the formation of antioxidative Maillard reaction products, such as glucose/fructose-lysine model, and release of polyphenols from food matrices (Gulati et al., 2016; Leonard et al., 2020b). This effect was amplified with the feed ingredients that are proportionately high in protein and carbohydrate. On the contrary, Altan, McCarthy and Maskan (2009) reported a trend of decreasing antioxidant activity with increasing screw speed (140-210 rpm) in barley-grape pomace extrudates. It is possible that the friction generated during extrusion increases the internal product's temperature, therefore leading to phenolics loss. These contrasting findings may be attributed to the different composition of raw material (i.e. higher pomace fibre in their study), extrusion parameters or antioxidant assays used.

3.3. In vitro enzyme (α-glucosidase and AChE) inhibitory assays

Inhibition of carbohydrate-hydrolysing enzymes, such as α-glucosidase, has been employed for treatment and delay of diabetes mellitus (Joshi et al. 2015). Unlike the trends displayed in phenolic and antioxidant assays, several extrusion runs recorded significantly ($p < 0.05$) higher α-glucosidase inhibition activity compared to raw hempseed oil cake (Figure 1B; Table S3). The inhibition rates at 1 mg/mL for the whole fraction of Treatment 2 (LTHSLM) and 7 (HTHSLM) samples were 29.53 $\pm$ 0.67 and 28.35 $\pm$ 2.46 %, respectively, as compared to the unextruded treatment at 24.26 $\pm$ 1.60 %. This gap was widened in the free polyphenol

fraction (22.22 ± 3.51 % in raw versus 30.56-34.66 % in extruded samples). It is likely that the higher content of phenylpropionamides (Table 4) and proportion of free polyphenols in these treatments led to the observed results, which will be further discussed in section 3.7 and 3.8. Therefore, additional studies are required to investigate the α-glucosidase inhibition by individual phenylpropionamides. Previous research about the effect of extrusion on enzyme inhibitory assays is very limited. There is one research reporting that extrusion (screw speed 120 rpm, moisture 12 %, barrel temperature 120 °C) improved α-glucosidase activity of whole and germinated grain corn (Gong et al. 2018). They attributed this activity to the free polyphenols, despite the lower phenolic count for the free fraction compared to the bound fraction. When the free fraction is combined with the bound fraction, an inhibition rate around the average of these two fractions is expected. This was observed in the present study, particularly in the high moisture extrusion samples where the inhibition activity exhibited by whole fractions were not close to that of either free or bound fractions. Thus, we proposed that the α-glucosidase inhibitory activity post-extrusion relies more on the availability of different phenolic compounds as shown in Section 2.5. At a significantly higher concentration (1 mg/mL), all hempseed oil cake samples still showed lower α-glucosidase inhibitory activity than positive control oral drug acarbose at 0.1 mg/mL (Figure 1B). Nevertheless, as the current findings suggest, optimization of extrusion parameters or the use of similar food processing technologies may enhance the anti-diabetic potential of hempseed oil cake. However, α-amylase inhibitory activities were nearly undetected across all tested samples (data not shown), which suggested that the potential anti-diabetic effect of hempseed oil cake need more evidence support, such as animal models.

AChE is an enzyme involved in the breakdown of the neurotransmitter acetylcholine, thus its link to the progression of neurodegenerative diseases such as Alzheimer's and Parkinson's (Colović et al. 2013). There are no records that display the AChE inhibitory activity of plant extracts post-extrusion, although some hempseed lignanamides have shown AChE inhibition *in vitro* (Yan et al., 2015). The results showed a more potent AChE inhibition by extruded hempseed oil cake extract at low moisture and high screw speed compared to raw samples (about 40 % versus 30 % at 1 mg/mL) (Figure 1B). The differences in AChE inhibition activity could be due to the different concentration of individual phenylpropionamides present in the sample, which will be further discussed in Section 3.8. As no previous studies have investigated the effect of extrusion on AChE inhibition, this represents an area for further research. The data also show that at the same concentration, free and bound fraction displayed similar AChE inhibition capacities, which is different to the α-glucosidase assay. In general, extrusion, at low moisture and high screw speed, showed a potential to enhance anti-neurodegeneration properties of hempseed oil cake. These activities were still far weaker compared to the positive control drug of galanthamine. The high abundance of phenolic compounds in the extract may interact and compromise its overall biological activities, in comparison to the pure compound (Figueiredo-González et al. 2018). Nonetheless, it still shows the prospect of extrusion to utilize and improve the anti-neurodegeneration activities of hempseed processing by-products. It should be further noticed that hempseed oil cake could be developed as a food product and its human consumption would be a much larger quantity comparing with the intake of a drug. The weaker anti-neurodegeneration properties of hempseed oil cake still have great potential to prevent the risk of the neurodegeneration disease when people consume it as a daily food product.

3.4. Contribution of extrusion parameters on the sample polyphenols and bioactivities

The full factorial design confirmed the general contribution (see F value) of each extrusion parameter on the responses (i.e. polyphenols and their bioactivities) of the samples, from the largest to the smallest: moisture content > screw speed > barrel temperature (Table 2). Higher phenolic content and biological activities were more likely to be achieved at lower moisture content and higher screw speed, suggesting a stronger interaction between these two parameters. In terms of two-way interactions, the strongest effect for moisture-screw speed was displayed in TPC/TFC/α-glucosidase inhibition in all fractions, temperature-screw speed in antioxidant assays of most fractions, and temperature-moisture in AChE inhibition whole and bound fractions (Table 2). The individual effect of changes in barrel temperature was not as significant to the other two factors, although it was still involved in three-way interactions with other parameters in several responses such as TFC, DPPH and ABTS whole fraction. Similarly, moisture content (20-50 %) appeared to be the more significant individual parameter compared to screw speed (200-400 rpm) in explaining the variances in phenolic content and antioxidant activities (DPPH/ABTS) of extruded green banana flour (Sarawong et al. 2014). It was proposed that higher screw speed lowers the filled length of extruder, torque and residence time, thus the reduced degradation of phenolic compounds. This effect, however, was completely nullified at high moisture content (50 %) due to enhanced shearing. Specific mechanical energy (SME) refers to the work that the motor generates during extrusion process, and calculation of SME has been performed to optimise the reaction efficiency, shear rate and residence time of biopolymer blends (Calderón et al. 2019). This is particularly useful in the context of food materials that contain high concentration of bioactive components (i.e. phenolics) prone to degradation and chemical reactions. Thus, determination of specific SME in future studies may be useful to prove this proposal. The present data was different to the conclusion made by Ma et al. (2018), who reported lower F

value (contribution) for screw speed than barrel temperature or moisture on TPC and DPPH inhibitory activity of extruded white ginseng root hair. It appears that formation of antioxidative Maillard reaction products is more likely at higher temperature range (110-140 ºC) and lower moisture (20-30 %) range used in their study. These differences are also expected in extrusion studies due to the variety of extruder type, extrusion parameters, the value range of the parameter and raw materials used.

3.5. HPLC-DAD-ESI-QTOF-MS/MS phenylpropionamides identification: hydroxycinnamic acid amides and lignanamides

Up to 26 phenylpropionamides (hydroxycinnamic acid amides and lignanamides) were tentatively identified using HPLC-DAD-ESI-QTOF-MS/MS in extruded hempseed oil cake (Table 3; Figure S1). Hydroxycinnamic acid amides were characterized by the linkage between hydroxycinnamoyl-CoA moeity and tyramine moeity. Lignanamides are derived from oxidative coupling of hydroxycinnamic acid amides, and have shown potent antioxidant, anticancer and anti-inflammatory activities (Leonard et al. 2020c; Leonard et al. 2020d).

N-*trans*-caffeoyltyramine has long been identified as the major phenylpropionamide in hempseed (Chen et al. 2012). Our present study confirmed the presence of N-*trans*-caffeoyltyramine with $[M-H]^-$ and $[M+H]^+$ ions at m/z 298.1079 and 300.1230, respectively, and matched the standard. Additionally, the $[M-H]^-$ MS^2 fragment at m/z 178 was likely derived from N-Cα bond cleavage, whereas the breakage of the tyramine moeity led to the MS^2 fragment at m/z 135 (Nigro et al., 2020). On the $[M+H]^+$ mode, it is possible that the

NH_3 loss after N-Cα bond cleavage led to the abundance of fragment ion at m/z 163. The arylnaphtalene-type lignanamide, cannabisin A, showed $[M-H]^-$ and $[M+H]^+$ ions at m/z 593.1962 and 595.2066, respectively. Similar to N-*trans*-caffeoyltyramine, the breakage of tyramine moiety might have given rise to the MS^2 fragment ions at $[M-H]^-$ m/z 430 and $[M+H]^+$ m/z 432. The mass spectra of cannabisin F and grossamide was very similar, though the additional UV λmax of grossamide at 300 nm allows for tentative differentiation between the two (Zhou et al. 2018). Rac-sativamide A, a nor-lignanamide (lignanamide derivative), was also tentatively identified with $[M+H]^+$ ion of 583.2061. The observed MS^2 fragments at m/z 446 and 418 could be due to cleavage of the tyramine moiety with (-165 Da) or without (-137 Da) the carbonyl group. Contrary to Moccia et al. (2019), $[M-H]^-$ ions for rac-sativamide A was fragmented in this study to present m/z 401 and 238, which could be due to the difference in collision energy used. Additionally, several unnamed lignanamides were detected that were likely to be dimers of N-*trans*-caffeoyltyramine or other tyramine-type hydroxycinnamic acid amides (Nigro et al. 2020). These findings represent the vast landscape of hemp polyphenols that are yet to be discovered and characterized.

3.6. HPLC-DAD quantification of phenylpropionamides in raw and extruded hempseed oil cake

HPLC-DAD quantification revealed N-*trans*-caffeoyltyramine as the most abundant phenylpropionamide in hempseed oil cake (Table 4). N-*trans*-caffeoyltyramine was also used to quantify or semi-quantify hempseed oil cake phenylpropionamides, because of the unavailability of other standards. The range of N-*trans*-caffeoyltyramine obtained in this study (9.9-85.77 µg/g) was comparable to the values reported in the hempseed whole meal at 0.0417-0.287 mg/g (Pojic et al. 2014), but significantly lower than whole hempseed at 25.08

mg/g reported in another study (Zhou et al. 2018). Despite the lower TPC, extrusion runs 2 (LTHSLM) and 7 (HTHSLM) recorded significantly ($p < 0.05$) higher phenylpropionamides compared to raw hempseed oil cake. It is likely that phenylpropionamides are not as sensitive to the Folin-Ciocalteu's reagent, more resistant to heat and shear stress than simple phenolic acids, or that the phenylpropionamides constitute a major part in the phenolic profile of hempseed, thus less affected by the changes in TPC assay. Other low-moisture runs (Runs 3-LTLSLM and 8-HTLSLM) also recorded statistically similar or higher content of several major lignanamides than raw control, such as cannabisin A, B and F. It may explain the higher AChE inhibitory values for some fractions of these runs (discussed in Section 3.3), as some lignanamides possess higher AChE inhibition activity than the others although it is still unclear if this effect is additive (Yan et al., 2015).

All phenylpropionamides displayed significant ($p < 0.05$) correlation in nearly all fractions for α-glucosidase and AChE assays, though at differing *r* value coefficient strength (Table S4). For instance, N-*trans*-caffeoyltyramine displayed positive correlation of 0.99 for α-glucosidase of whole fraction, which was the strongest of all compounds including the lignanamides cannabisin B (0.968), cannabisin C (0.94) and other hydroxycinnamic acid amides. This may suggest the higher contribution of N-*trans*-caffeoyltyramine in explaining α -glucosidase inhibition values compared to other compounds. However, N-*trans*-caffeoyltyramine and AChE inhibition values was not as highly correlated (0.714-0.783) as several lignanamides, such as cannabisin A (0.785-0.846), B (0.77-0.814), C (0.775-0.842) or M (0.721-0.8), which may justify the results from Runs 3 (LTLSLM) and 8 (HTLSLM) discussed earlier. It also appears that the overall correlation of phenylpropionamide-glucosidase is stronger compared to phenylpropionamide-AChE, indicating that the

phenylpropionamides may have a more dominant role in explaining the α-glucosidase inhibition of hempseed cake samples whereas other non-phenylpropionamide compounds may contribute to the AChE results. Moreover, the proportion of lignanamides is higher than hydroxycinnamic acid amides in samples with unfavourable extrusion conditions (high moisture, low screw speed), which was not observed in raw or run 2 (LTHSLM) and 7 (HTHSLM) samples (Table 4). This may suggest that under these conditions, hydroxycinnamic acid amides are more prone to degradation due to its simpler structure, or that a less likely polymerization of monomers has occurred.

4. Conclusion

The current study investigated the influences of extrusion technology on the polyphenol contents in hempseed oil cake using a full factorial design. The total phenolic content and antioxidant capacities of the hempseed oil cake extrudate were not enhanced in all treatment. Despite of this extrusion, at relatively lower moisture content (30 %) and higher screw speed (300 rpm), increased the proportion of free polyphenols/flavonoids, phenylpropionamide content, and α-glucosidase and AChE inhibitory activity of the hempseed oil cake. This added health-beneficial properties suggest the potential to utilize extrusion technology in the manufacturing of food products incorporated with hempseed oil cake, and provide valuable information for the industry on the retention of phenolics during extrusion. A total of 26 phenylpropionamides were identified and quantified/semi-quantified in the current study, where higher α-glucosidase inhibition in low moisture and high screw speed extruded samples could be due to the presence of N-*trans*-caffeoyltyramine ($r = 0.99$, $p < 0.01$, whole extract), while the lignanamides cannabisin A/B/C/M ($r > 0.8$, $p < 0.01$, whole extract) may explain the higher AChE inhibition activity. The current study also found several hempseed

lignanamides that are yet been identified, suggesting the need for further study. Given the potential health properties displayed by cannabisins and the phenylpropionamides they contain, there is a need for more in-depth investigation in this area.

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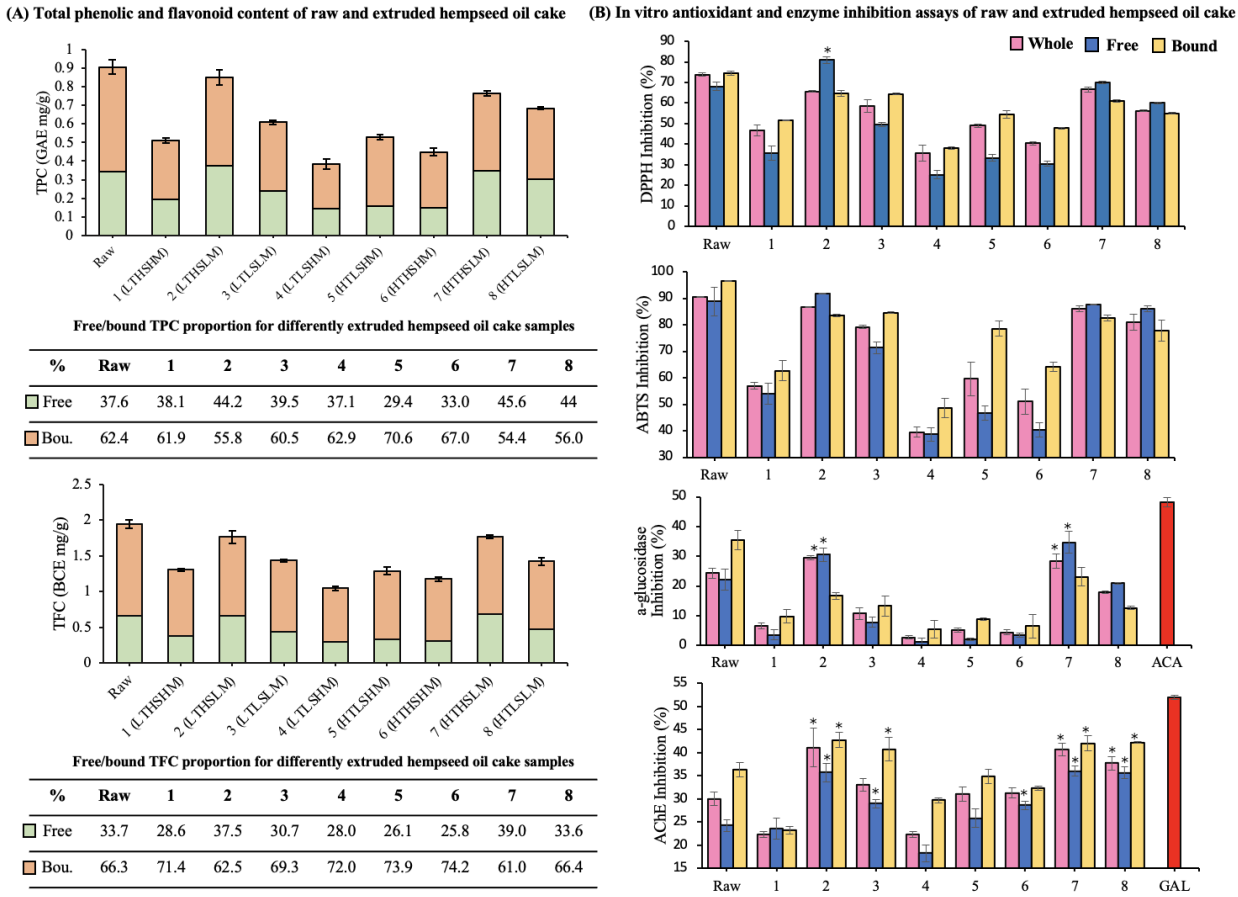

Figure 1. Phenolic content and biological activities of raw and extruded hempseed oil cake extract. The samples of raw and 1-8 refer to the samples in Table 1. All biological activities (right) displayed at 1 mg/mL sample extract. ACA: acarbose at 0.1 mg/mL. GAL: galanthamine at 0.05 mg/mL. All measurements done in triplicate (n=3). '*' denotes significantly ($p < 0.05$) higher value compared to raw, unextruded control.

Table 1. Full factorial design for extrusion of hempseed oil cake and its runs

Factors	Independent variables	Units	Actual values		Coded values	
			Min	Max	Min	Max
A	Maximum temperature	°C	80	100	-1	+1
B	Moisture content	%	30	40		
C	Screw speed	rpm	150	300		

Runs	Temperature	Screw speed	Moisture
Raw	-	-	-
1 (LTHSHM)	80	300	40
2 (LTHSLM)	80	300	30
3 (LTLSLM)	80	150	30
4 (LTLSHM)	80	150	40
5 (HTLSHM)	100	150	40
6 (HTHSHM)	100	300	40
7 (HTHSLM)	100	300	30
8 (HTLSLM)	100	150	30

H: high; L: low; T: temperature; S: screw speed; M: moisture content.

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Table 2. Full factorial model for the contribution of extrusion parameters on the responses (polyphenols and bioactivities) of the extruded hempseed oil cakes. F value reflects the degree of variance between groups by each individual factor or interaction factor. Only factors with p < 0.05 were displayed.

Response	Value	A: Maximum Temperature	B: Moisture content	C: Screw speed	A×B: Temperature x Moisture	A×C: Temperature x Screw speed	B×C: Moisture x Screw speed	A×B×C: Temperature x Moisture x Screw speed
Total TPC	F 165.28	-	862.79	109.14	6.88	109.93	62.28	-
	p < 0.0001	-	< 0.0001	< 0.0001	0.018	< 0.0001	< 0.0001	-
Free TPC	F 219.50	-	1226.91	163.32	14.51	68.06	61.37	-
	p <0.0001	-	< 0.0001	< 0.0001	0.002	< 0.0001	< 0.0001	-
Bound TPC	F 51.84	6.67	207.97	24.26	32.11	61.33	23.71	6.81
	p <0.0001	0.02	<0.001	<0.0001	<0.0001	<0.0001	<0.0001	0.019
Free/Bound TPC Distribution	F 35.99	6.80	175.33	16.47	48.60	-	-	-
	p <0.0001	0.02	<0.001	0.001	<0.0001	-	-	-
Total TFC	F 100.13	-	463.05	127.64	-	24.01	53.33	27.82
	p <0.0001	-	<0.0001	<0.0001	-	<0.0001	<0.0001	<0.0001
Free TFC	F 262.62	-	1267.31	318.71	11.80	20.54	203.61	14.23
	p <0.0001	-	<0.0001	<0.0001	0.003	<0.0001	<0.0001	0.002

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Bound TFC	F	31.52	-	117.48	37.13	14.61	17.54	6.90	25.39
	p	<0.0001	-	<0.0001	<0.0001	0.001	0.001	0.02	<0.0001
Free/Bound TFC Distribution	F	87.63	-	446.45	67.71	36.45	-	60.41	-
	p	<0.0001	-	<0.0001	<0.0001	<0.0001	-	<0.0001	-
DPPH Whole	F	81.08	-	448.13	29.72	4.82	19.27	15.44	46.68
	p	<0.0001	-	<0.0001	<0.0001	0.043	<0.0001	<0.0001	<0.0001
DPPH Free	F	396.57	-	2199.07	289.13	-	143.67	134.57	7.42
	p	<0.0001	-	<0.0001	<0.0001	-	<0.0001	<0.0001	0.02
DPPH Bound	F	409.44	-	1772.68	120.96	436.06	150.26	-	385.04
	p	<0.0001	-	<0.0001	<0.0001	<0.0001	<0.0001	-	<0.0001
ABTS Whole	F	100.45	9.17	615.63	17.81	6.48	31.68	-	21.84
	p	< 0.0001	0.01	< 0.0001	0.001	0.02	< 0.0001	-	<0.0001
ABTS Free	F	249.40	5.66	1570.12	43.10	28.47	91.64	-	-
	p	< 0.0001	0.03	< 0.0001	< 0.0001	< 0.0001	< 0.0001	-	-
ABTS Bound	F	80.82	30.90	350.51	4.69	82.22	27.51	5.37	64.54
	p	< 0.0001	< 0.0001	< 0.0001	0.046	< 0.0001	< 0.0001	0.03	< 0.0001
a-glucosidase Whole	F	215.27	9.15	1051.44	240.52	7.51	38.27	156.62	-
	p	<	0.008	<	<	0.015	< 0.0001	<	-

		0.0001		0.0001	0.0001			0.0001	
a-glucosidase Free	F	173.88	37.70	812.40	189.91	31.77	11.21	126.20	8.01
	p	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.004	< 0.0001	0.012
a-glucosidase Bound	F	14.91	-	68.43	13.68	-	-	8.01	10.64
	p	< 0.0001	-	< 0.0001	0.002	-	-	0.012	0.005
AChE Whole	F	46.95	52.22	224.89	13.39	19.96	-	12.49	-
	p	< 0.0001	< 0.0001	< 0.0001	0.002	< 0.0001	-	0.003	-
AChE Free	F	47.53	54.30	227.42	32.55	-	11.64	-	-
	p	< 0.0001	< 0.0001	< 0.0001	< 0.0001	-	0.004	-	-
AChE Bound	F	77.24	41.73	425.62	9.49	33.76	-	22.24	7.24
	p	< 0.0001	< 0.0001	< 0.0001	0.007	< 0.0001	-	< 0.0001	0.016

TPC: Total Phenolic Content; TFC: Total Flavonoid Content; DPPH: 2,2-diphenyl-1-picrylhydrazyl assay; ABTS: 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate); AChE: acetylcholinesterase.

Table 3. HPLC-DAD-ESI-QTOF-MS/MS identification of phenolic compounds from extruded hempseed oil cake

No	Phenolic Class	Tentative assignment	Molecular Formula	Retention Time (min)	UV λmax (nm)	Theoretical Mass	Experimental Mass	Error (ppm)	Mass Fragments	Ref.
Negative mode [M-H]⁻										
1	Lignanamides	Unnamed Lignanamide 1	$C_{34}H_{32}N_2O_9$	26.8	285, 335	612.2107	611.2074	7.59	595 (100); 432 (10); 322 (4); 485 (3)	(i)
2	Lignanamides	Unnamed Lignanamide 2	$C_{34}H_{34}N_2O_9$	28.9	285	614.2264	613.2124	9.88	596 (100)	(i)
3	Hydroxycinnamic acid amides	N-trans-caffeoylocetopamine	$C_{17}H_{17}NO_5$	26.9	322	315.1107	314.1058	9.68	152 (100); 135 (90); 179 (45)	(ii)
4	Nor-lignanamide	rac-sativamide A	$C_{33}H_{30}N_2O_8$	31.2	285, 360	582.2002	581.1980	9.88	401 (18); 238 (8)	N/A
5	Hydroxycinnamic acid amides	N-trans-caffeoyltyramine	$C_{17}H_{17}NO_4$	32.0	295; 320	299.1158	298.1079	0.13	135 (20); 178 (7); 148 (3); 162 (2)	(i)(ii)
6	Lignanamides	Cannabisin A	$C_{34}H_{30}N_2O_8$	34.6	252	594.2002	593.1962	6.64	430 (7); 485 (2)	(ii)
7	Lignanamides	Cannabisin B	$C_{34}H_{32}N_2O_8$	34.9	285, 335	596.2159	595.2127	7.96	433 (6); 486 (3); 269 (2); 434 (1); 323 (1)	(ii)
8	Lignanamides	Cannabisin H (Isomer 1)	$C_{28}H_{31}NO_8$	36.6	330	509.2050	508.2001	5.98	313 (100); 312 (93); 461 (67)	(i)
9	Lignanamides	Cannabisin H (Isomer 2)	$C_{28}H_{31}NO_8$	37.3	325	509.2050	508.2001	5.98	313 (100); 312 (60); 165 (19); 195 (16); 461 (16)	(i)
10	Hydroxycinnamic acid amides	N-trans-coumaroyltyramine	$C_{17}H_{17}NO_3$	37.8	290	283.1208	282.1159	10.78	119 (47); 162 (20); 145 (17)	(ii)
11	Hydroxycinnamic	N-feruloylty	$C_{18}H_{19}$	39.0	295sh;	313.1	312.12	10.7	178 (42); 148 (34); 298 (33);	(i

1	acid amides	ramine	NO_4		318	314	68	0	135 (17)	i)
12	Lignanamides	3-demethylcannabisin G	$C_{35}H_{34}N_2O_8$	39.4	285, 330	610.2315	609.2285	8.11	475 (4); 447 (4); 298 (2)	(i)
13	Lignanamides	Cannabisin C	$C_{35}H_{34}N_2O_9$	41.1	285sh, 330	610.2315	609.2291	9.09	447 (8); 298 (5); 323 (5); 486 (4)	(ii)
14	Lignanamides	Cannabisin D	$C_{36}H_{36}N_2O_8$	43.3	330	624.2472	623.2439	7.44	461 (9); 594 (2)	(i)
15	Lignanamides	Cannabisin G	$C_{36}H_{36}N_2O_8$	44.4	330	624.2472	623.2449	9.05	461 (21)	(i)
16	Lignanamides	3'3-demethylgrossamide	$C_{34}H_{32}N_2O_8$	44.6	340, 360	596.2159	595.2117	6.28	433 (32); 270 (5); 459 (4)	(i)
17	Lignanamides	Cannabisin E	$C_{36}H_{38}N_2O_9$	45.3	290, 320	642.2577	641.2564	10.35	490 (37); 328 (13); 152 (10); 312 (10)	(ii)
18	Lignanamides	cis-Cannabisin E	$C_{36}H_{38}N_2O_9$	46.7	325	642.2577	641.2540	6.61	151 (100); 136 (19); 313 (18) 152 (14)	(i)
19	Lignanamides	Grossamide K	$C_{28}H_{29}NO_7$	47.1	315	491.1944	490.1906	8.45	473 (100); 461 (68); 458 (28)	(i)
20	Lignanamides	Condensed unnamed trilignanamide		48.3	285	893.316	892.3125	4.98	595 (31)	(ii)
21	Lignanamides	Cannabisin M	$C_{34}H_{32}N_2O_8$	50.0	290, 318	596.2159	595.2124	7.46	299 (100)	(ii)
22	Lignanamides	Cannabisin Q	$C_{34}H_{32}N_2O_8$	51.8	285	596.2159	595.2130	8.47	298 (100); 596 (9)	(i)
23	Lignanamides	Isocannabisin N	$C_{35}H_{34}N_2O_9$	54.3	285, 330	610.2315	609.2276	6.63	313 (100); 418 (35); 296 (32); 178 (11)	(i)
24	Lignanamides	Cannabisin F	$C_{36}H_{36}N_2O_8$	55.0	285, 322	624.2472	623.2455	10.01	461 (19); 298 (4) 589 (3)	(iii)
25	Lignanamides	Grossamide	$C_{36}H_{36}N_2O_8$	55.4	285, 300sh, 322	624.2472	623.2452	9.53	592 (11); 433 (9); 461 (3)	(iii)

26	Lignanamides	Cannabisin O	$C_{54}H_{53}N_3O_{12}$	55.6	285, 330	935.3629	934.3483	7.13	636 (100); 623(20); 799 (8)	N/A
Positive mode $[M+H]^+$										
1	Hydroxybenzoic acid	Benzoic acid	$C_7H_6O_2$	24.0	290	122.0368	123.0437	8.45	77 (100); 95 (45); 82 (32)	(iv)
2	Lignanamides	Unnamed Lignanamide 1	$C_{34}H_{32}N_2O_9$	26.3	285, 335	612.2107	613.2175	1.86	460 (100); 432 (46); 595 (35); 295 (7); 187 (3)	N/A
3	Lignanamides	Unnamed Lignanamide 2	$C_{34}H_{34}N_2O_9$	28.4	335	614.2264	615.2308	5.75	460 (100); 597 (19); 432 (11); 367 (4); 295 (3)	N/A
4	Hydroxycinnamic acid amides	N-trans-caffeoylocetopamine	$C_{17}H_{17}NO_5$	26.4	294, 315	315.1107	316.1169	5.50	163 (100); 137 (15)	(ii)
5	Nor-lignanamide	rac-sativamide A	$C_{33}H_{30}N_2O_8$	30.7	285, 360	582.2002	583.2061	3.50	446 (30); 121 (16); 418 (12)	(ii)
6	Hydroxycinnamic acid amides	N-trans-caffeoyltyramine	$C_{17}H_{17}NO_4$	31.5	294, 320	299.1158	300.1230	2.47	163 (100); 121 (34); 145 (7)	(ii)
7	Lignanamides	Cannabisin A	$C_{34}H_{30}N_2O_8$	34.0	254	594.2002	595.2066	2.59	460 (100); 432 (16); 187 (4); 295 (2)	(ii)
8	Lignanamides	Cannabisin B	$C_{34}H_{32}N_2O_8$	34.1	284, 336	596.2159	597.2233	0.90	460 (100); 432 (18); 187 (5); 295 (4)	(ii)
9	Lignanamides	Cannabisin H. 1	$C_{28}H_{31}NO_8$	36.0	330	509.2005	510.2114	3.02	312 (100); 177 (80); 201 (59); 462 (46); 355 (43)	N/A
10	Lignanamides	Cannabisin H. 2	$C_{28}H_{31}NO_8$	36.6	330	509.2005	510.2113	3.21	177 (100); 312 (62); 201 (51); 355 (41)	N/A
11	Hydroxycinnamic acid amides	N-trans-coumaroyltyramine	$C_{17}H_{17}NO_3$	37.1	292	283.1208	284.1281	2.25	147 (100); 121 (26); 164 (1)	(ii)
12	Hydroxycinnamic acid	N-feruloyltyramine	$C_{18}H_{19}NO_4$	38.3	295sh, 313.1314		314.1382	3.63	177 (100); 121 (24); 145 (23)	(ii)

	amides	ramine			318					
13	Lignanamides	3-demethylcannabisin G	$C_{35}H_{34}N_2O_8$	38.7	285, 330	610.2315	611.2381	2.19	474 (100); 446 (18); 201 (5); 309 (3)	N/A
14	Lignanamides	Cannabisin C	$C_{35}H_{34}N_2O_9$	40.5	300sh, 330	610.2315	611.2377	2.85	474 (100); 311 (61); 446 (16);	(ii)
15	Lignanamides	Cannabisin D	$C_{36}H_{36}N_2O_8$	42.8	284, 336	624.2472	625.2542	1.50	488 (100); 460 (20); 201 (5); 364 (3)	N/A
16	Lignanamides	Cannabisin G	$C_{36}H_{36}N_2O_8$	43.8	290, 332	624.2472	625.255	0.22	488 (100); 201 (5); 460 (4); 121 (3)	N/A
17	Lignanamides	3'3-demethylgrossamide	$C_{34}H_{32}N_2O_8$	44.0	285, 325	596.2159	597.2219	3.25	297 (72); 434 (52); 323 (25) 460 (19)	N/A
18	Lignanamides	Cannabisin E	$C_{36}H_{38}N_2O_9$	44.8	285, 322	642.2577	643.265	0.99	462 (100); 325 (14); 177 (12); 201 (8)	(ii)
19	Lignanamides	cis-Cannabisin E	$C_{36}H_{38}N_2O_9$	46.0	285, 330	642.2577	643.2643	2.08	462 (100); 177 (7); 338 (6); 325 (5)	N/A
20	Lignanamides	Grossamide K	$C_{28}H_{29}NO_7$	46.4	320	491.1944	492.2009	2.93	325 (100); 337 (50); 462 (41); 201 (19)	N/A
21	Lignanamides	Unnamed condensed trilignanamide		47.5	285	893.316	894.3221	2.06	757 (70); 729 (4)	(ii)
22	Lignanamides	Cannabisin M	$C_{34}H_{32}N_2O_8$	49..4	285, 318	596.2159	597.2244	0.94	434 (33); 297 (32); 163 (6); 460 (6)	(ii)
23	Lignanamides	Cannabisin Q	$C_{34}H_{32}N_2O_8$	51.1	280	596.2159	597.2248	1.61	297 (63); 460 (31); 434 (18); 163 (6)	N/A
24	Lignanamides	Isocannabisin N	$C_{35}H_{34}N_2O_9$	53.7	285, 322	610.2315	611.2419	4.02	448 (43); 311 (22); 474 (8)	N/A
25	Lignanamides	Cannabisin F	$C_{36}H_{36}N_2O_8$	54.3	290, 322	624.2472	625.2581	4.73	462 (100); 325 (95); 351 (32); 488 (10)	(iii)

26	**Lignanamides**	**Grossamide**	**$C_{36}H_{36}N_2O_8$**	**54.6**	**288, 304sh, 318**	**624.2472**	**625.2576**	**3.93**	**488 (70); 462 (24); 351 (21)**	**(iii)**
27	**Lignanamides**	**Cannabisin O**	**$C_{54}H_{53}N_3O_{12}$**	**54.9**	**285, 325**	**935.3629**	**936.3749**	**4.34**	**799 (100); 771 (27); 538 (11)**	**(ii)**

References: (i) Nigro et al. (2020); (ii) Moccia et al. (2019); (iii) Zhou et al. (2018); (iv) MoNA MassBank; N/A: not available or other ionization mode is used.

Table 4. HPLC-DAD quantification of phenylpropionamides (hydroxycinnamic acid amides and lignanamides) in raw and extruded hempseed oil cake. All values expressed in µg caffeoyltyramine equivalents per g sample and presented as sum of free and bound fractions. Different letters in same row show significantly ($p < 0.05$) different values.

Compounds	Raw	1 (LTHSHM)	2 (LTHSLM)	3 (LTLSLM)	4 (LTLSHM)	5 (HTLSHM)	6 (HTHSHM)	7 (HTHSLM)	8 (HTLSLM)
Hydroxycinnamic acid amides									
N-*trans*-caffeoyltyramine	66.26 ± 0.26^{c}	18.85 ± 0.12^{f}	85.77 ± 0.41^{a}	34.08 ± 0.11^{e}	9.90 ± 0.07^{h}	18.64 ± 0.18^{f}	16.95 ± 0.18^{g}	74.68 ± 0.26^{b}	54.92 ± 0.83^{d}
N-*trans*-caffeoylocopamine	4.52 ± 0.13^{b}	1.16 ± 0.02^{f}	5.37 ± 0.08^{a}	1.82 ± 0.07^{e}	0.64 ± 0.00^{h}	0.91 ± 0.02^{g}	0.86 ± 0.03^{g}	4.21 ± 0.20^{c}	2.76 ± 0.24^{d}
N-*trans*-feruloyltyramine	26.60 ± 0.0	14.80 ± 0.13^{g}	36.02 ± 0.18^{a}	22.11 ± 0.06^{e}	8.12 ± 0.07^{i}	17.12 ± 0.11^{f}	13.41 ± 0.06^{h}	30.71 ± 0.30^{b}	24.46 ± 0.14^{d}

	6[c]								
N-*trans*-coumaroylytyramine	8.32 ± 0.11[d]	4.11 ± 0.07[f]	10.96 ± 0.04[b]	6.98 ± 0.08[e]	2.42 ± 0.04[i]	3.90 ± 0.10[g]	3.35 ± 0.04[h]	11.24 ± 0.07[a]	9.59 ± 0.22[c]
Total HCAA	**105.70 ± 0.17[c]**	**38.93 ± 0.33[g]**	**138.12 ± 0.58[a]**	**64.99 ± 0.12[e]**	**21.08 ± 0.18[i]**	**40.58 ± 0.24[f]**	**34.56 ± 0.18[h]**	**120.84 ± 0.21[b]**	**91.74 ± 1.37[d]**
Lignanamides									
Cannabisin A	5.69 ± 0.58[c]	4.30 ± 0.11[d]	7.21 ± 0.40[a]	5.50 ± 0.24[c]	2.77 ± 0.03[e]	4.25 ± 0.06[d]	3.81 ± 0.15[d]	6.58 ± 0.44[b]	7.50 ± 0.06[a]
Cannabisin B	10.37 ± 0.10[c]	4.98 ± 0.27[e]	15.80 ± 0.25[a]	7.96 ± 0.33[d]	3.28 ± 0.10[g]	4.27 ± 0.29[f]	4.30 ± 0.05[f]	13.60 ± 0.21[b]	10.28 ± 0.74[c]
Cannabisin C	5.28 ± 0.22[c]	3.35 ± 0.11[g]	6.95 ± 0.17[a]	4.50 ± 0.06[e]	2.12 ± 0.05[h]	3.86 ± 0.12[f]	3.14 ± 0.02[g]	6.27 ± 0.23[b]	5.02 ± 0.20[d]
Cannabisin D	11.76 ± 0.06[b]	7.39 ± 0.09[g]	12.17 ± 0.05[a]	8.98 ± 0.03[d]	4.51 ± 0.03[i]	8.59 ± 0.05[f]	7.01 ± 0.04[h]	10.52 ± 0.04[c]	8.74 ± 0.07[e]

Cannabisin E	5.58 ± 0.04[b]	3.97 ± 0.06[e]	6.36 ± 0.04[a]	4.65 ± 0.23[c]	2.59 ± 0.07[g]	4.44 ± 0.07[d]	3.66 ± 0.03[f]	5.73 ± 0.07[b]	4.80 ± 0.15[c]
cis-Cannabisin E	2.04 ± 0.02[d]	2.10 ± 0.12[d]	3.60 ± 0.10[a]	2.34 ± 0.07[c]	1.47 ± 0.07[f]	1.65 ± 0.10[e]	1.36 ± 0.07[f]	2.72 ± 0.19[b]	2.20 ± 0.12[cd]
Cannabisin F	7.53 ± 0.12[e]	7.41 ± 0.09[e]	12.32 ± 0.28[a]	8.31 ± 0.18[d]	5.81 ± 0.09[g]	6.62 ± 0.05[f]	5.91 ± 0.05[g]	10.42 ± 0.11[b]	8.60 ± 0.06[c]
Cannabisin G	4.54 ± 0.23[b]	3.06 ± 0.06[d]	5.41 ± 0.02[a]	3.90 ± 0.13[c]	2.08 ± 0.05[f]	3.01 ± 0.07[d]	2.73 ± 0.05[e]	4.45 ± 0.16[b]	3.77 ± 0.02[c]
Cannabisin M	8.80 ± 0.10[c]	7.32 ± 0.06[e]	12.56 ± 0.12[a]	8.49 ± 0.06[d]	5.51 ± 0.07[h]	6.93 ± 0.03[f]	6.00 ± 0.06[g]	11.06 ± 0.17[b]	8.88 ± 0.28[c]
Isocannabisin N	0.74 ± 0.04[c]	0.67 ± 0.03[c]	1.02 ± 0.05[a]	0.71 ± 0.02[c]	0.50 ± 0.02[d]	0.57 ± 0.07[d]	0.49 ± 0.03[d]	0.89 ± 0.08[b]	0.74 ± 0.04[c]
Cannabisin O	0.49 ± 0.04[de]	0.52 ± 0.02[d]	0.93 ± 0.08[a]	0.60 ± 0.02[c]	0.44 ± 0.03[ef]	0.44 ± 0.02[ef]	0.42 ± 0.02[f]	0.79 ± 0.04[b]	0.64 ± 0.03[c]
Cannabisin	2.06 ±	1.98 ±	3.58 ±	2.50 ±	1.08 ±	1.86 ±	1.31 ±	2.94 ±	2.16 ±

Q	0.09[d]	0.02[de]	0.16[a]	0.11[c]	0.01[g]	0.19[e]	0.02[f]	0.14[b]	0.05[d]
3-demethyl Cannabisin G	1.89 ± 0.04[c]	1.11 ± 0.08[e]	2.96 ± 0.09[a]	1.67 ± 0.07[d]	0.90 ± 0.03[g]	1.05 ± 0.08[ef]	0.98 ± 0.02[fg]	2.47 ± 0.03[b]	1.83 ± 0.10[c]
Grossamide	5.72 ± 0.07[d]	5.62 ± 0.07[d]	9.43 ± 0.27[a]	6.31 ± 0.01[c]	4.62 ± 0.09[f]	5.02 ± 0.03[e]	4.56 ± 0.08[f]	8.04 ± 0.05[b]	6.50 ± 0.12[c]
Grossamide K	4.02 ± 0.05[b]	2.64 ± 0.12[e]	3.63 ± 0.17[c]	3.56 ± 0.10[c]	2.10 ± 0.04[f]	3.17 ± 0.12[d]	3.06 ± 0.15[d]	4.67 ± 0.10[a]	4.14 ± 0.14[b]
3’3-demethyl grossamide	0.58 ± 0.02[b]	0.41 ± 0.02[de]	0.74 ± 0.02[a]	0.52 ± 0.09[bc]	0.22 ± 0.04[f]	0.40 ± 0.04[de]	0.36 ± 0.00[e]	0.70 ± 0.05[a]	0.47 ± 0.03[cd]
Rac-sativamide A	0.94 ± 0.03[a]	0.30 ± 0.03[f]	0.70 ± 0.05[c]	0.41 ± 0.00[e]	0.13 ± 0.01[g]	0.38 ± 0.02[e]	0.28 ± 0.04[f]	0.75 ± 0.03[b]	0.57 ± 0.03[d]
Total lignanamides	**78.03 ± 0.88[c]**	**57.14 ± 0.76[e]**	**105.39 ± 0.97[a]**	**70.91 ± 0.67[d]**	**40.13 ± 0.66[9]**	**56.50 ± 0.95[e]**	**49.40 ± 0.11[f]**	**92.60 ± 0.42[b]**	**76.83 ± 0.94[c]**
Total phenylprop	**183.74**	**96.07 ± 1.05[f]**	**243.50 ± 0.79[a]**	**135.90 ± **	**61.21 ± **	**97.08 ± 1.08[f]**	**83.96 ± 0.07[g]**	**213.44 ± **	**168.56 ± **

ionamides	**± 0.79[e]**	**0.66[e]**	**0.84[h]**		**0.49[b]**	**2.32[d]**

H: high; L: low; T: temperature; S: screw speed; M: moisture content