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Production of Functional Food Ingredients Using Sub-critical Water

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ABSTRACT

Subcritical water utilized in both and extraction and reaction mode has been utilized to produce functional food ingredients as reported below. Polyphenols extracted from blueberry (*Vaccinium corymbosum*) pomace were extracted with subcritical water under pressure at 120°C using both batch and continuous systems. After extraction, both dead end and batch nanofiltration membranes were used to concentrate the polyphenol-laden extracts. The extract contained total anthocyanin concentrations in the range 85–125 mg/100mL. High-performance liquid chromatography was used for the determination of total anthocyanins, flavanols and chlorogenic acid in the hot water extract. Nanofiltration membranes (NF245 and NF270) showed complete rejection of phenolic compounds at good permeances, whereas crossflow mode of filtration was found to reduce membrane fouling considerably. Furthermore, a suitable protocol was developed for clean-in-place of the used membranes. After repeated filtrations followed by the cleaning protocol, the relative permeance was recovered up to 73% for NF245 membrane and 99% for the NF270 membrane.

Similarly, subcritical water was used for the hydrolysis of hemp seed oil to produce *omega*-free fatty acids via hydrolysis over the temperature range of 150-275°C and appropriate pressures. A "micro" continuous flow reactor system was constructed for the hydrolysis experiments consisting of an oven and back pressure regulator permitting experiments at temperature (150-275°C), reaction times (40-120 min), and variable oil/water ratios (0.125-0.250). These conditions were selected and optimized using an ANOVA analysis and surface response plots. The free fatty acid yield ranged between 20 and 91% (w/w) with the highest degree of hydrolysis took place at temperatures above 200°C and retention times in the reactor coil over 60 minutes. Similar fatty acid profiles were observed in the unhydrolyzed and hydrolyzed oils, which indicates minimal or no fatty acid degradation; linoleic acid (C18:2) being the predominant fatty acid. However, FTIR-ATR analysis showed that higher reaction temperatures caused the formation of aldehydes and fatty acid dimers, significantly above 200°C. This was confirmed by the GC-MS analysis based on the retention time correlations and corresponding spectra identified the presence of long chain

aldehydes such as cis,cis,cis-7,10,13-hexadecatrienal, 9,12,15-octadecatrienal and dimers of C16:0 and C18:2 fatty acids as derived from the hemp seed oil.

INTRODUCTION

Subcritical water is a versatile medium for extracting food ingredients from natural products and similarly using its hydrolysis properties to produce value-added ingredients [1]. Both modes for using subcritical water depend on the adjustment of its density under requisite pressures to yield conditions compatible with the extraction or suitable for performing a reaction on the target substrate. Such processing yields aqueous diluted product-extracts that require product concentration by removing water for their further use as functional food ingredients [2]. We report here research on two such applications of subcritical water, namely (1) the subcritical water extraction of blueberry pomace followed by membrane processing to yield concentrates containing functional food ingredients exhibiting high anti-oxidant capacities [3], and (2) the hydrolysis of hemp seed oil primarily to nutritionally functional *omega*-fatty acids desired by the food industry.

Blueberries (*Vaccinium corymbosum L.*) contain large amounts of polyphenols[3]. It has been suggested that consumption of blueberries can help suppress inflammation [4,5], display anti-cancer properties [6], improve human gut microbiome [7], reduce the risk of coronary heart disease [8,9] and scavenge oxidative radicals [10]. Most of these benefits are attributed to the high content of monomeric and polymeric anthocyanins, a class of polyphenols. These belong to a wide variety of arabinosides, galactosides and glucosides of cyanidin, delphinidin, malvidin, peonidin and petunidin [11] possessing orange-red, purple and blue plant pigments that have significant importance in the food industry as they determine color, taste and health benefits of marketed products.

Hemp (*Cannabis sativa L.*) is a versatile plant which can yield a variety of use products including fibers [12], a nutritional-valuable edible oil [13], and medical-beneficial cannabinoid compounds [14]. Hemp seed oil is somewhat unique in that approximately 75% of its triglyceride-based oil is unsaturated, consisting mainly of omega-3 and -6 fatty acids [15]. The health-beneficial properties of omega-3 and -6 fatty acids are well known [16] particularly with regard to beneficial health effects. Here we also report on the results of employing a small-scale continuous hydrolysis system based on subcritical water under pressure for the hydrolysis of commercially-available hemp seed oil.

MATERIALS AND METHODS

Blueberry (*Vaccinium corymbosum L.*) pomace was obtained by processing of non-clarified juice, which was carried out in accordance with a previous established protocol. NF245 and NF270 polyamide thin-film composite nanofiltration membranes with nominal molecular weight cut-off of 200-400 Da were obtained in form of flat sheets from Filmtec™ (Dow, Minneapolis, MN). Prior to insertion in the dead-end filtration vessel, the membranes were cut by hand and then soaked in deionized water for at least 24 hours. The active separation areas were 14.6 cm^2 for dead-end setup and 42.0 cm^2 for crossflow setup. Disposable filters (0.22 µm and 0.45 µm) were purchased from

GE Healthcare Life Sciences (Whatman®, Pittsburgh, PA) and used to prefilter large particles. Sodium hydroxide (analytical grade) was purchased from Macron Fine Chemicals (Avantor Performance Materials, Center Valley, PA). Hydrochloric acid (37% v/v) was purchased from EMD Millipore (Billerica, MA). Deionized water was produced with Thermo Scientific, model Smart2Pure 12 UV/UF (Waltham, MA), 18.0 MΩ·cm. Frozen blueberry pomace was allowed to thaw to 21°C prior to extraction. A Dionex model 200 accelerated solvent extractor (ASE) system interfaced with a solvent controller (Dionex Corp., Sunnyvale, CA) was used to extract anthocyanins from blueberry pomace. Samples (0.5 g) were loaded into 22 mL stainless steel extraction cells with a cellulose paper filter inserted at the bottom of the cells. The ASE extraction was carried out using water as solvent; 68 bar pressure, 120°C temperature, five extraction cycles, 70% flush volume, 90 sec nitrogen purge time (no static time and no preheat time). For each extraction cycle it took approximately 5-6 min for the water to heat to 120°C for a total run time of 25-30 min. Approximately 22 mL of extract from each extraction cycle was pooled after passing through a large microporous sieve. Pressurized hot water extracts were stored at -20°C prior to total anthocyanin analysis and nanofiltration testing. For the dead-end filtration, a starting volume of 200 mL feed was loaded in a stainless-steel pressure vessel (Sterlitech, Kent, WA), which was continuously stirred on a magnetic stirrer plate (OptiChem, Vineland, NJ). The feed side was pressurized with nitrogen at pressures between 10-17 bar. The flow through the membrane was quantified by collecting the solution on an electronic balance (Mettler Toledo PL602-S, Columbus, OH) connected to a computer. Membrane permeance was calculated from:

$$P = \frac{V}{A \cdot \Delta t \cdot p} \quad (1)$$

where *V* is the volume of permeate, Δt is the time of permeation, *A* is membrane area, and *p* the is applied pressure. A crossflow system was custom built as shown in **Figure 1**. An initial volume of 600 mL was loaded into the stainless steel vessel and then placed on a magnetic stirrer plate (Corning PC-210, Corning, NY) at 200 rpm. The feed was pumped through the pressurized system with a twin piston pump (Milton Roy Company, Houston, TX) at a constant crossflow rate of 57 mL/min. The transmembrane pressure was kept constant at 3 bar. The flow through the membrane was quantified in a similar manner as explained for dead-end mode. The temperature of feed, permeate and concentrate was measured before and after filtration and it was found not to change by more than ±1.6°C. Membrane permeance was calculated from Eqns. (2) and (3) as:

$$P = \frac{V}{A \cdot \Delta t \cdot TMP} \quad (2)$$

and

$$TMP = \frac{p_{inlet} + p_{outlet}}{2} - p_{permeane} \quad (3)$$

where *V* is the volume of permeate, Δt is the time of permeation, *A* is membrane area, and *TMP* is the transmembrane pressure calculated from the pressures read at inlet, outlet and permeate (0 bar).

Blueberry ASE extracts were screened for the determination of total anthocyanins and total flavanols content using a method adapted from Cho et al. [17]. For rejection analysis, samples from feed, retentate and permeate were evaluated for total monomeric anthocyanin content by the pH differential assay using a Hewlett Packard Model 8452A Diode Array Spectrophotometer (Palo Alto, CA.

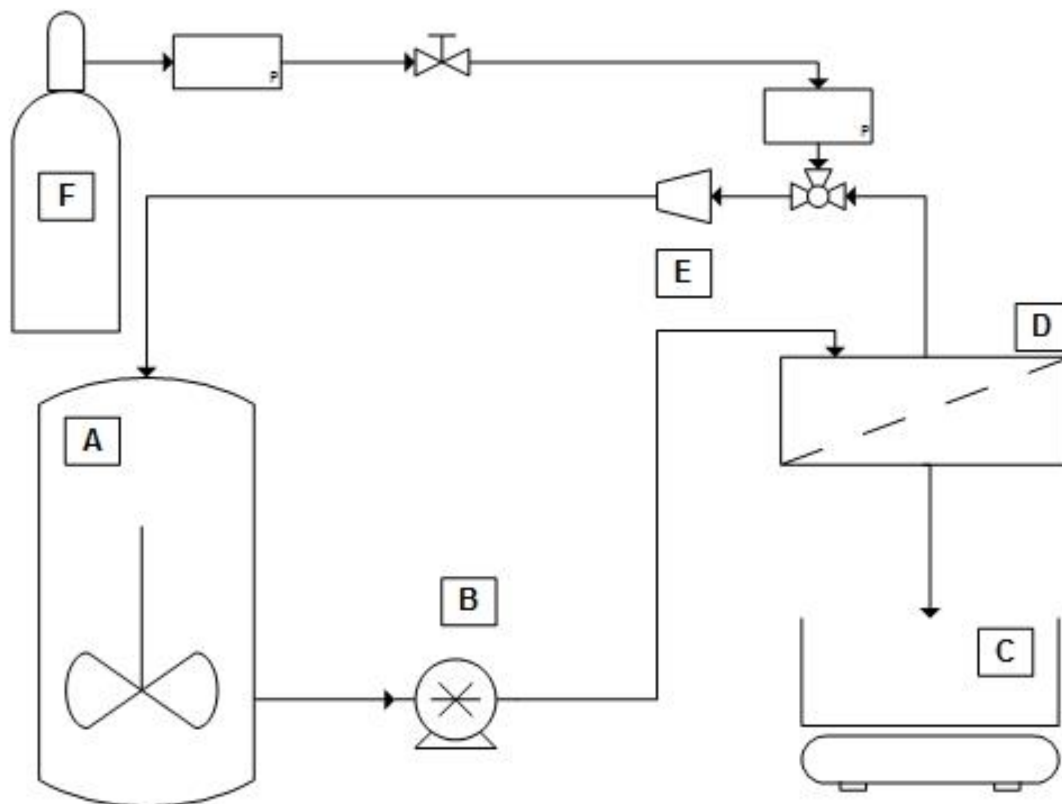

Figure 1: Process flow of experimental setup for nanofiltration in crossflow mode. A: Feed stirred vessel; B: Piston pump; C: Permeate collection and balance; D: Crossflow cell; E: Pressure regulator and F: gas supply.

Hemp seed oil and deionized wared were poured separately in 2L glass Buchner flasks, then, a magnetic bar was placed in each flask. Later, both containers were covered with a rubber block, placed on a stirring plate, and connected to a vacuum pump with a liquid trap (both provided by VWR International, Rednor, PA) for degassing. Rotation speed was set to 1100 rpm and the applied vacuum was 200mm Hg. Degassing time was 4h. After degassing water and oil were taken to the hydrolysis stage. For batch hydrolysis, degassed oil and water were mixed at specific ratios and poured into a PARR 4530 1-liter bench stirred reactor (Parr Instruments, Moline, IL, USA). The remaining headspace in the reactor was then filled with ultra-high purity compressed nitrogen to establish a 6 Bar base pressure. The temperature was increased to the desired level and maintained using a Model 4848 PARR Instruments controller. Due to that temperature increase, the internal pressure inside the reactor increased. After reaching the process temperatures, the agitator rotation speed was set, and agitation started. Temperature and agitation were maintained for the specific reaction time; and after this time, the reactor cooled to decrease the temperature. After reaching 40°C, the pressure was released by opening the manual relief valve. Then, the vessel

was opened and the hydrolyzate mixture removed from the reactor vessel. Samples were poured into 50ml tubes and immediately centrifuged at 3900 x g for 30 minutes in a Beckman-Coulter Allegra™ X-22R benchtop centrifuge (Brea, CA, United States). After centrifugation, the resultant two phases were separated. The oil layer was separated by pipetting and stored under freezing conditions for further analytical tests. Process conditions were established using a central composite design with reaction temperature, oil fraction (ml/ml), reaction time and agitation speed being used as the processing variables. For the continuous
hydrolysis was done in a home-built system consisting of two glass containers, two-TELEDYNE ISCO 260D syringe pumps (Lincoln, NE, United States). Two 1/16" nominal diameter stainless steel tubing coils. A gas chromatography oven -Hewlett Packard- HP5890 Series II (Avondale, PA, United States) was used for temperature control. One GO Regulator (Spartanburg, SC) and a backpressure valve was used to maintain the internal pressure

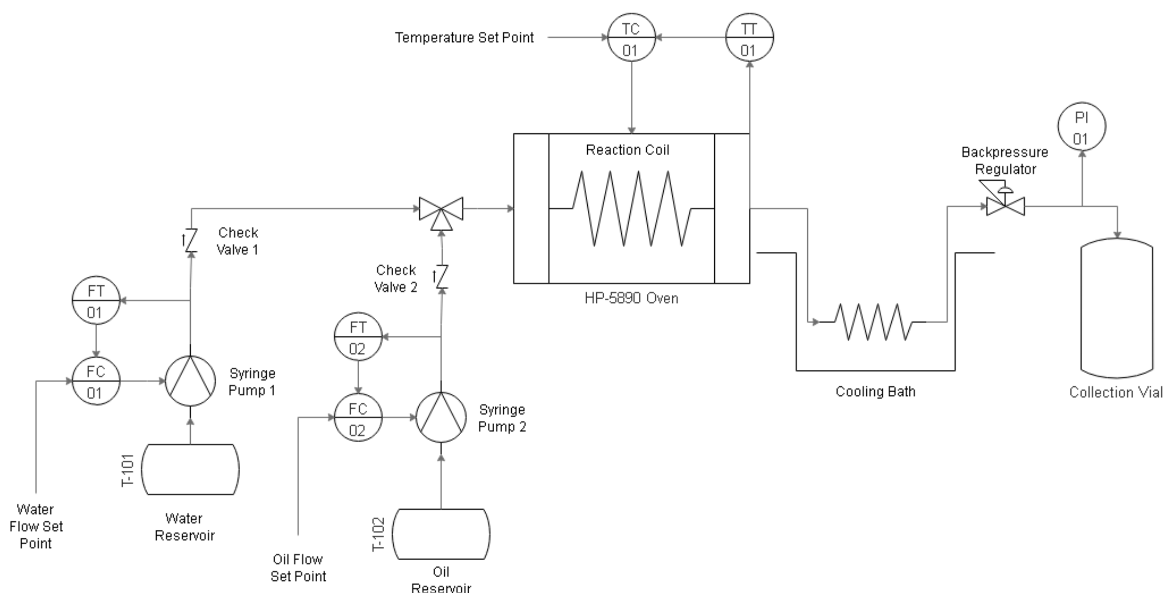

Figure 2: Continuous hydrolysis experimental apparatus flow diagram.

RESULTS

Total and specific phenolic compounds, as well as sugar retention from HPLC in the ASE extract, retentate and permeate are summarized in **Table1**. The study revealed that the highest quantity of polyphenols was due to total anthocyanins (with over 82% wt.), while the highest amount of sugars was due to fructose (with over 70% wt.) in the feed (ASE extract). Both the membranes NF270 and NF245 showed complete retention of total polyphenols, total chlorogenic acid and sucrose. Only small amounts of glucose and fructose were found in the permeate fractions so that the rejections were higher than 97%. For dead-end filtration, the concentration of polyphenols from blueberry pomace extract was tested using two commercially available nanofiltration membranes and then optimized experimentally based on (1) mixing speed, (2) prefiltration and (3) filtration

time. Due to their low molecular weight cut-off, both membranes showed complete rejection of polyphenols regardless of the experimental parameter. Membrane performance based on permeance using pomace extract was tested at 0 rpm, 200 rpm and 400 rpm while holding the filtration volume constant. The permeance was found to change drastically as a function of mixing speed; especially when no stirring is applied the permeance reaches very slow values. At 400 rpm, mechanical mixing has been found to increase the starting permeance 6.8 times (NF270) and 2.1 times (NF245) compared to no-stirring conditions, while at 200 rpm it was 4.0 times (NF270) and 2.0 times (NF245) higher. The appearance of the used membranes showed a dark purple coloration for the non-stirred experiments, while the other membranes remained just slightly tainted when stirring was applied. This leads to hypothesize that fouling due to polyphenol agglomeration or adsorption can be efficiently disrupted if mechanical stirring is applied. The rejection of total polyphenols was complete and independent of stirring speed.

Table1: HPLC analysis results for ASE extract and for retentate and permeate fractions after rejection in dead-end mode.

Sample	Total ACY[b)]	Total FLA[b)]	CLA[b)]	Sucrose[c)]	Glucose[c)]	Fructose[c)]
ASE extract (feed)	61.0	7.9	4.7	0.05	0.60	1.54
NF270 retentate[a)]	73.9	9.9	6.1	0.07	0.72	2.12
NF270 permeate	n.d.[d)]	n.d.	n.d.	n.d.	0.01	0.02
NF245 retentate[a)]	84.7	10.7	6.6	0.09	1.14	2.39
NF245 permeate	n.d.	n.d.	n.d.	n.d.	0.01	0.02

[a)] dead-end filtration; [b)] mg / 100 mL; [c)] mg / mL; [d)] n.d. – not detected.

For crossflow filtration, the filtration performance in dead-end mode was tested under different experimental conditions. The setup was constructed to keep the feed continuously stirred and the crossflow rate was set at a maximum flowrate of 57 mL/min. Then, the feed was passed through a 0.22 μm prefilter to remove larger aggregated particles. Total polyphenols were rejected completely and no polyphenol degradation was observed during analysis. The permeance was considerably higher with NF270, which started at 3.5 L m-2 h-1 bar-1 and then reached approximately 2.0 L m-2 h-1 bar-1 after 3 hours of filtration. For NF245 the permeance started at 1.5 L m-2 h-1 bar-1 and then decreased to 0.6 L m-2 h-1 bar-1 after the same filtration time. Table 2.

Table 2: Volume reduction-concentration factor for dead-end filtration at various times.

Membrane, filtration time	Feed volume, mL	Volume reduction, % v/v	Concentration factor
NF270, 19 hrs	250	80	4.59
NF245, 19 hrs	250	60	2.19
NF245, 29 hrs	250	78	3.14

After 3 hours of filtration time with NF270, 15% v/v of total volume was removed and the polyphenol concentration factor was 1.24. With NF245 approximately 7% v/v were removed and the polyphenol concentration factor was 1.11.
For the hemp seed oil hydrolysis using the continuous flow system, it was found that reaction temperature and retention time have a significant effect over the free fatty acid level of the treated oil samples. Equation 4 quantifies these effects:

$$FFA_{yield} = e^{0.5756+0.0136T+0.0047t} \tag{4}$$

where: FFA_{yield} is the free fatty acid level of the treated oil samples as mass percentage of oleic acid equivalent, T is the reaction temperature in °C and T is the retention time in the reactor in minutes. The surface response plot for these trends is show in **Figure 3** below:

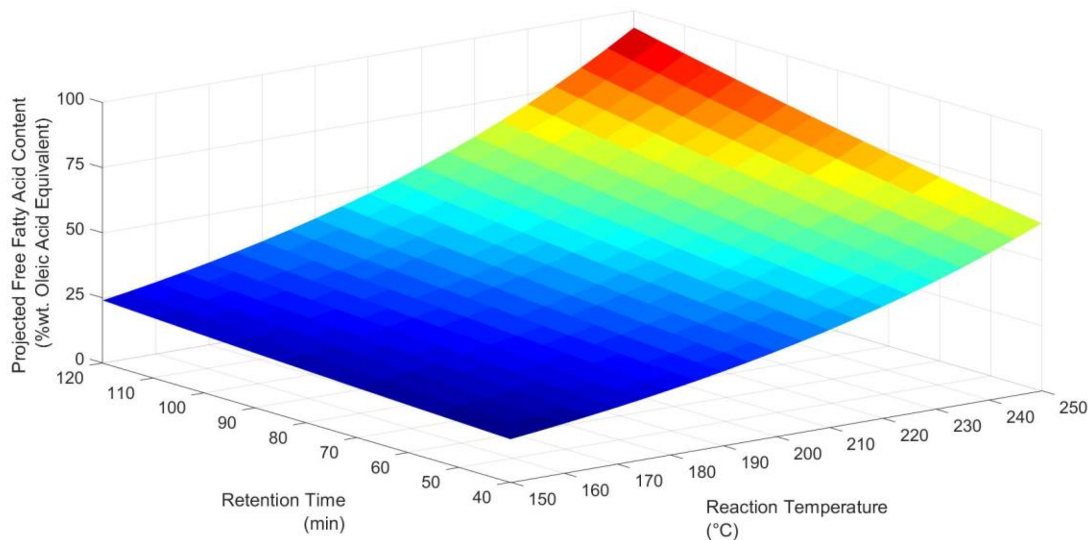

Figure 3. Obtained response surface for the free fatty acid content of the processed oil samples and its relation with reaction temperature (°C) and retention time (min).

To determine the presence of trans isomerization phenomenon, FTIR-ATR spectra of the original oil and the processed samples were analyzed. And it was found that elevated reaction temperature caused an increase of the absorbance in the trans-isomer region. To estimate the impact the absorbance ration between the 722 cm^{-1} (cis) and 966 cm^{-1} (trans) wavenumbers was calculated.

Larger ratios were indicative of a low trans isomer presence. Equation 2 shows the spectroscopic relation:

$$Abs_{\frac{cis}{trans}ratio} = 1{,}5981 + 0{,}0101T - 3.642 \times 10^{-5}T^2 \quad (2)$$

where T is the reaction temperature in °C.

It was observed that the cis/trans ratio dramatically decreases with higher temperatures which is an indicator of an increased trans isomer in the samples. GC and GC-MS were used to determine the fatty acid content of the non-processed and hydrolyzed samples as follows:

- **Palmitic acid (C16:0):** Average 5.19%, Minimum value 4.51%, Maximum value 6.98%.
- **Stearic acid (C18:0):** Average 3.20%, Minimum value 1.64%, Maximum value 6.70%.
- **Oleic acid (C18:1):** Average 5.19%, Minimum value 4.51%, Maximum value 6.98%.
- **Linoleic acid (C18:2):** Average 54.49%, Minimum value 46.06%, Maximum value 58.07%.
- **γ-Linolenic acid (C18:3):** Average 4.97%, Minimum value 4.08%, Maximum value 7.42%.
- **α-Linolenic acid (C18:3):** Average 16.43%, Minimum value 14.33%, Maximum value 18.02%.
- **Arachidic acid (C20:0):** Average 1.94%, Minimum value 1.29%, Maximum value 3.89%.

The proportions of specific fatty acids varied among hydrolysate samples and it was identified that high FFA levels in the hydrolyzates were related to a decrease on the unsaturated species. **Figure 4** below shows the obtained species profiles.

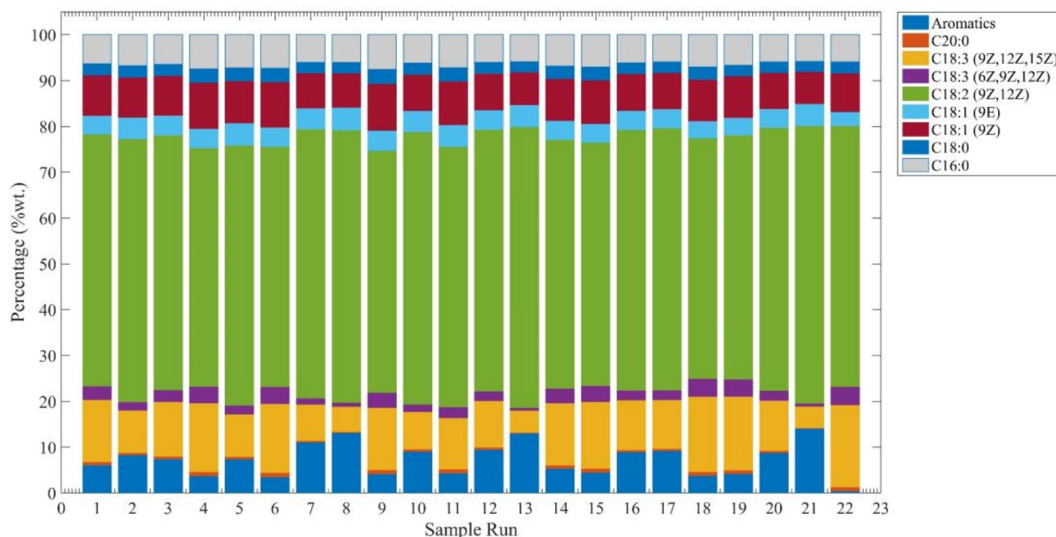

Figure 4. Compound proportions on every obtained sample. Run 22 corresponds to non-processed NUTIVA® cold pressed hemp seed oil.

It could be seen that unsaturated species tend to decrease, and aromatics appear on all samples which could lead to infer that a thermal oxidation phenomenon appears. After performing species identification using NIST library search (NIST, Gaithersburg, MD), octadecadienal and octadecatrienal appeared. This could be an indicative of C18:3 oxidation. Finally, polymerization of all the fatty acid species was identified. This could be caused due to thermal cracking of the triglyceride molecules.

CONCLUSIONS

For the application of nanofiltration to the subcritical water extracts both dead-end and crossflow modes were used to concentrate polyphenol content from blueberry pomace. The most prevalent polyphenols were identified as total anthocyanins, total flavonols and chlorogenic acid. The sugar content analyzed using HPLC revealed that fructose was the predominant sugar. Both nanofiltration membranes showed complete rejection of total anthocyanins, total flavonols, chlorogenic acid, sucrose and more than 95% rejection for glucose and fructose. The rejection performance was unaffected by the experimental parameter of the filtration mode. In the dead-end mode 80% of water volume was removed in 19 hours using NF270, while only 60% of water volume was removed with NF245. Stirring was found crucial for obtaining good permeances and crossflow mode was found to improve membrane fouling considerably. The membrane reconditioning protocol delivered almost complete recovery of water permeance for NF270 used in crossflow mode and up to 73% recovery for NF245.
For the hemp seed oil hydrolysis, the free fatty acid yield ranged between 20 and 91% (w/w). The highest degree of hydrolysis took place at temperatures above 200ºC and retention times over 60 minutes. Similar fatty acid profiles were observed in the unhydrolyzed and hydrolyzed oils, which indicates minimal or no fatty acid degradation. Linoleic acid (C18:2) was the predominant fatty acid. FTIR-ATR analysis showed that elevation of reaction temperatures favored the formation of aldehydes and fatty acid dimers as judged by the intensity of absorbances at 1700 and 900 cm-1 wavenumbers respectively. Which significantly increased above 200°C. This was confirmed by the GC-MS analysis that based on the retention time correlations and spectra identified probable presence of long chain aldehydes such as cis,cis,cis-7,10,13-hexadecatrienal, 9,12,15-octadecatrienal and dimers of C16:0 and C18:2 are decomposition and polymerization products of the hydrolyzed hemp seed oil.

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