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EFFECT OF SUBCRITICAL WATER AND STEAM EXPLOSION PRETREATMENTS ON THE RECOVERY OF STEROLS, PHENOLS AND OIL FROM OLIVE POMACE

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Running Title: The total recovery of olive pomace using hydrothermal pretreatments

ABSTRACT

Hydrothermal pretreatments including steam explosion and subcritical water (SCW) support hydrolyses of plant cell wall materials and supply environmentally friendly extraction solvents to recover value added compounds. In this study, the effect of steam and SCW and temperature (160, 180 and 200 °C, 5 min) on the yield and β -sitosterol (BS) content of pomace oil and phenolics for value added utilization of olive pomace. Compared to acid hydrolysis, hydrothermal pretreatments yielded similar oil recovery. 54-76% of the bound oil and 18-32% of the bound BS of the pomace were recovered by hydrothermal treatments. Steam pretreatment was more effective than SCW pretreatment at lower

temperatures, however SCW shows increasing trend related to steam pretreatment. As a further research, use of sequential temperature might be applied, starting with steam explosion (<180 °C) and separated SCW pretreatments (>200 °C) of aqueous and deoiled meal part for process development studies for total valorization of olive pomace.

Keywords: Subcritical water, steam explosion, olive pomace, β -sitosterol, phenolic compounds

1. Introduction

Disposal of olive oil processing waste streams, olive pomace and olive mill waste water (OMW), presents a challenging environmental problem due to their high organic content, which has not been solved completely in spite of research on new techniques for their value added utilization: as fertilizer/soil conditioner, herbicide/pesticide and animal feed; for the recovery of residual oil and organic compounds (pectins, antioxidants and enzymes); for the production of alcohols, biosurfactants, biopolymers, activated carbons and for generation of energy (Niaounakis & Halvadakis, 2006; Albuquerque et al., 2004).

The two phase system (alperujo) was introduced in olive oil processing for the first time in Spain during the 1991-1992 harvesting season to minimize water consumption resulting in environmental and economic advantages (Niaounakis & Halvadakis, 2006). According to world olive data from 2010-2016, 11 778 000 tonnes of alperujo (calculated according to 20% oil yield) was generated in the world annually (Vlyssides et al. 2004; IOC, 2016). It is composed of 56-75 % moisture, oil (8-20 %) containing higher β -sitosterol (339-430 mg/100 g) compared to virgin olive oil (VOO: 181-226 mg/100 g) (Seçmeler & Üstündağ, 2017c), cell wall constituents such as cellulose (14-25 %), lignin (32-56 %) and hemicellulose (27-42 %), protein (4-12 %), water-soluble carbohydrates (1-16 %) and phenolic compounds (1-2 %) on a dry weight basis (Albuquerque et al., 2004).

The extent of loss of bioactive compounds during olive oil processing and their composition in the waste streams have important implications for the valorization of olive pomace. Our previous studies have shown significant processing loss for β -sitosterol and α -tocopherol (32%, 31%, respectively), while squalene was quantitatively recovered in the oil stream (Seçmeler & Üstündağ, 2017b-c). Comparison of these values with the loss value for oil (6%), and the relative distribution of these compounds in the olive matrix

point to differences in the extraction and degradation behavior of these compounds. Recovery of sterols from plant matrices might be increased by minimizing their interactions with matrix components such as phospholipids, proteins and cellulose (Read & Bacic, 2002; Peng et al., 2002).

Hydrothermal processes/pretreatments involving hydrolysis, liquefaction, and/or extraction using steam, subcritical water (SCW) and supercritical water offer an innovative green approach for olive waste processing. Hydrothermal pretreatments have been used for the hydrolysis of hemicellulose, cellulose and lignin in olive pomace, olive stone and OMW for biofuel production (Möller et al., 2011; Brunner, 2009; Garrote et al., 1999; Fernández-Bolaños, Felizón, Heredia, Guillén & Jiménez, 1999; Fernández-Bolaños, Felizón, Brenes, Guillén & Heredia, 2001; Abu Tayeh et al., 2016; Kumar et al., 2009) and for the recovery/extraction of phenolics (Fernández-Bolaños, Felizón, Heredia, Guillén & Jiménez, 1999). Gasification of biomass such as OMW using supercritical water (550 °C, 30 s, 100-300 bar) was also investigated for the production of hydrogen and other gases (Kıpçak, Söğüt & Akgün, 2011).

Steam pretreatment or steam explosion (steam pretreatment followed by rapid decompression) (160-260 °C, 0.69-4.83 MPa) of olive pomace is used to breakdown the lignocellulosic matrix. While steam treatment affects the lignocellulosic materials via autohydrolysis reactions (i.e. hydrolysis of acetyl groups present in hemicellulose), mechanical forces also contribute during steam explosion (Manorach, 2014). Steam explosion of olive stones and alperujo was used for the recovery of the major phenols (hydroxytyrosol and tyrosol), to increase digestibility and to recover dietary fiber by converting hemicelluloses into soluble carbohydrates (monosaccharides xylose, arabinose, and glucose; mannitol and oligosaccharides) and to enhance enzymatic hydrolysis of cellulose (Fernández-Bolaños, Felizón, Heredia, Guillén & Jiménez, 1999; Fernández-

Bolaños, Rodríguez, Gómez, Guillén, Jiménez et al., 2004; Rodríguez, Lama-Muñoz, Rodríguez, Jiménez et al., 2008; Rodríguez-Gutiérrez, Rubio-Senent, Lama- Muñoz, et al., 2014; Fernández-Bolaños, Felizón, Brenes, Guillén & Heredia, 1998; Fernández-Bolaños, Rodríguez-Gutiérrez, Rodríguez, Guillén, et al., 2002). Olive stone and alperujo were pretreated with steam explosion in a 2 L reactor at 200-236 °C for 2-4 min with/without acid (Fernández-Bolaños, Felizón, Heredia, Guillén & Jiménez, 1999; Fernández-Bolaños, Felizón, Brenes, Guillén & Heredia, 2001; Fernández-Bolaños, Felizón, Brenes, Guillén & Heredia, 1998) and at 160-240 °C for 2-10 min with acid (Fernández-Bolaños, Rodríguez, Gómez, Guillén, Jiménez et al., 2004; Fernández-Bolaños, Rodríguez-Gutiérrez, Rodríguez, Guillén, et al., 2002), respectively. Steam explosion of alperujo at 200 °C for 5 min resulted in reduced hemicellulose (75-88%), lignin and protein content (50%), and a separate liquid fraction containing hydroxytyrosol, oligosaccharides, glucose, mannitol by auto-hydrolysis (Rodríguez-Gutiérrez, Rodríguez, Jiménez, Guillén & Fernández-Bolaños, 2007). Solubilization of lignin fragments resulted from de-polymerization of polysaccharides (mainly hemicellulose) and breaking of the lignin-carbohydrate bonds (Rodríguez-Gutiérrez, Lama- Muñoz, Rodríguez, Jiménez, Guillén & Fernández-Bolaños, 2008). Further degradation of monosaccharides lead to hydroxymethylfurfural (HMF) and furfural formation at higher temperatures.

In another reactor (100 L reactor), steam pretreatment of alperujo without explosion (150-170 °C; 15-90 min) resulted in a reduction in solids (up to 35.6-47.6 %) and an increase in oil yield and sterols (up to 97 % and 33 %, respectively) due to solubilization of cell wall material and release of bound oil and sterols (Lama-Muñoz, Rodríguez-Gutiérrez, Rubio-Senent, Gómez-Carretero & Fernández-Bolaños, 2011). Even at 160°C after 15 min, a significant increase in the level of β -sitosterol was observed (Lama-Muñoz, Rodríguez-Gutiérrez, Rubio-Senent, Gómez-Carretero & Fernández-Bolaños, 2011). In another

study, after steam pretreatment (170°C for 15 min), acid hydrolysis, ultrafiltration and column chromatography were applied to obtain phenols and oligosaccharides (Rubia-Senent, Lama-Muñoz, Rodríguez-Gutiérrez & Fernández-Bolaños, 2013).

From ambient temperature (>100 °C and 0.1 MPa) to supercritical conditions (374 °C at 22.1 MPa), solvent power of water is modified (as it changes from an ionic to non-ionic solvent), its polarity and pH value decrease while its reactivity increases (Möller et al., 2011; Brunner, 2009). SCW is used as a solvent for extraction of compounds having a wide range of polarity. High dielectric constant ($\epsilon=80$ at RT) of water can be significantly decreased to values close to 35 (similar to those of organic solvents such as acetonitrile and methanol) when water is heated up to 200 °C while maintaining it in liquid state by applying pressure. Thus SCW can be used for the extraction of less-polar compounds such as oils from natural materials (Teo et al., 2010; Abdelmoez, Abdelfatah & Tayeb, 2011; Pourali et al., 2009), in addition to phenolics (He et al., 2012; Roudsari, 2007; Yu et al., 2014). SCW extraction of olive pulp (160°C, 30 min) was found to be more efficient than methanol extraction (2 hr) for the recovery of chlorogenic acid, homovanillic acid, gallic acid, hydroxytyrosol, quercetin, and syringic acid (Yu et al., 2014). SCW oil extraction studies have been carried out to obtain optimized conditions with solid:water ratios: cotton seed oil at 270°C for 30 min (1:2) (Abdelmoez, Abdelfatah & Tayeb, 2011), soybean oil and protein at 150°C and 66°C (1:11.7) for extruded and not extruded soybean flakes, respectively (Ndlela et al., 2012), sunflower oil (with high antioxidant capacity) at 130°C for 30 min (1:20) (Ravber et al., 2015). SCW extracted oil however needs to be refined due to high free fatty acid content (FFA) (>2 %) (Ravber et al., 2015).

In addition to being an environmentally friendly extraction solvent, SCW is a unique and sustainable reaction/pretreatment medium (Möller et al., 2011; Kruse & Dinjus, 2007; Akiya & Savage, 2002). The decreased dielectric constant and the increased ionic strength

of SCW ($K_w > 10^{-14}$) lead to generation of hydronium ions, which are involved in ionic reactions, such as hydrolyses of cell wall materials (hemicellulose, lignin and cellulose) into sugar-monomers and polar lipids (phospholipids and glycolipids), and further degradation of HMF to carboxylic acids, as a reactant (Garote et al., 1999; Ndlela et al., 2012). In the subcritical region the density of water remains constant between 100°C to 374°C so the pressure effect on physical properties of SCW is minimal (Möller et al., 2011; Teo et al., 2010).

Hydrolysis of corn oil and hemicellulose was carried out in the range of 150-280°C and 150-230°C, respectively (Pinto, & Lanças, 2006 and Garrote, Dominguez & Parajo, 1999). Significant increase was observed in reaction rate of oil hydrolysis above 200°C and for cellulose degradation above 210-220°C, respectively. Optimum SCW pretreatment conditions for recovery of protein, sugar and lignin from deoiled olive pomace was reported as 200°C, 30 min with 2 mL/min flow at 220 bar where sugar (65.9 %) and lignin (66.3 %) were recovered partially (Kazan, Çeliktas, Sargin, & Yeşil-Çeliktas, 2015). Maillard and caramelization reactions, which occur at temperatures higher than 180°C, and biofuel production are limited if the samples are rich in phenolic compounds (Plaza et al., 2010) and sterol glucosides (Songtawee, Ratanawilai & Tongurai, 2014), respectively. Therefore temperature and time of SCW treatment need to be optimized based on the target compound(s) and the end use of the pretreated pomace. SCW can thus be an effective reaction medium/solvent for the total valorization of olive pomace, which offers the advantage of semi-continuous processing and the recovery of multiple fractions.

While previous studies have shown the effects of steam and SCW on the components of biomass and their potential for the recovery of oil, phenols and sterols from olive pomace, process development studies for the recovery of multiple value added products aiming at total valorization are lacking. Therefore, the main objective of this

study was to use hydrothermal pretreatments for value added utilization of olive pomace to obtain sterol enriched oil and phenols. The pretreated biomass, which would be free of sterol glucosides and phenols, could further be utilized for biofuel production. Specific objectives of this study were to investigate the effect of pretreatment method (steam and SCW), and temperature on oil, β -sitosterol (BS) and phenol recovery from olive pomace.

2. Materials and Methods

2.1. Samples

Olive pomace (alperujo) samples of picual type olives were obtained from the pilot VOO production plant (including a hammer crusher, two serial malaxers (25.2°C and 26.3°C), a decanter (2-phase, 3000 rpm), and separator) at the Instituto de la Grasa in Sevilla, Spain (November, 2015). Fresh pomace samples were stored in cooling cabinets for short period (4°C) and long period (−20 °C), protected from light.

Fresh pomace samples were homogenized, freeze dried and stored in a desiccator. Moisture and oil content (by Soxhlet) of pomace samples were determined in triplicate (IOC, COI/OH/Doc. No 1., 2011).

After hydrothermal treatment processed samples, which included pomace and process water, were centrifuged (13180 g, 20 min) to separate aqueous and meal fractions. Aqueous fraction was concentrated by vacuum evaporator at 50°C. Aqueous and meal fractions were also freeze dried and then homogenized and stored in a desiccator until further analysis (Fig. 1). Dried meal fraction and pomace were milled and sieved (1 mm) to separate pit (& cuticle).

2.2. *Chemicals and reagents*

β -sitosterol (100 μ g/mL in chloroform, analytical standard) and triolein (Glyceryl trioleate, 61%) were obtained from Supelco (PA and WI, US). Chlorotrimethylsilane (TMCS) (GC Grade) was obtained from Sigma-Aldrich (Damstadt, Germany). Chloroform (GPR Rectapur) and n-hexane (HPLC Grade) were obtained from VWR (Fotenay-sous-Bois, France). 5 α -cholestane (99.57%, GC Grade) and pyrogallol (HPLC) were obtained from Sigma-Aldrich (Jerusalem, Israel and Dorset, UK). n-hexane (99%, GC grade) was obtained from Sigma-Aldrich (Israel). Sodium chloride (ACS grade) was obtained from Panreac (Barcelona, Spain). β -sitosterol (75.8% β -sitosterol, 10.3% beta-sitostanol, 7.7% campesterol), pyridine (extra dry, 99.5%) and hexamethyldisilazane (HMDS) (98%, reagent plus) were obtained from Acros Organics (France, Geel, Belgium and Germany). Hydrochloric acid (37%) and diethyl ether were obtained from Carlo Erba (Rodano, Italy and Val- de-Reuil, France). Potassium hydroxide (90%, flakes, pure) and ethanol (absolute) were obtained from Scharlau (Sentmenat, Spain). 5-hydroxymethylfurfural, hydroxytyrosol 4- β -d-Glucoside, vanillic acid, p-coumaric acid and 3,4-dihydroxyphenylglycol were obtained from Sigma-Aldrich (Deisenhofer, Germany). Tyrosol was obtained from Fluka (Buchs, Switzerland). Hydroxytyrosol, oleuropein and apigenin were obtained from Extrasynthese (Lyon Nord, Geney, France).

2.3. *Sample Preparation*

Oil extraction and acid hydrolysis procedure of olive pomace was modified for the determination of free and total BS (including free β -sitosterol glucoside (BSG) and bound BS-BSG) based on the method developed for oil seeds and other food matrices (Toivo et al., 2001; Aguirre et al., 2012). Firstly, total lipid and total BS content (including free,

esterified, and glycosidic BS with bound BS analyzed as free BS) were determined by acid hydrolysis and subsequent oil extraction with hexane:diethyl ether. Secondly, oil content and free BS were determined after hexane:diethyl ether oil extraction without acid hydrolysis and compared with Soxhlet extraction by hexane. Finally, the difference between the results obtained with and without acid hydrolysis was calculated to obtain total BSG content (bound and free). However, the differences in oil contents were not an exact indication of bound oil as acid hydrolysis results in the hydrolysis of triglycerides and phospholipids producing FFA not all of which can be recovered by the extraction solvent. It was reported by Philips et al. (1997) that more polar shorter chain fatty acids were not recovered by extraction after acid hydrolysis using hexane:diethyl ether and thus total lipid recovery was lower, acid hydrolysis leads to extraction of nonfat materials additionally. Extract obtained from fresh pomace without acid hydrolysis included total triglycerides and phospholipids but FFA content of hydrothermally pretreated samples was expected to be higher than fresh pomace.

Total BS content of acid hydrolyzed extract (including free, esterified, and glycosidic BS with bound BS analyzed as free BS) was determined by the method of Toivo et al. (2001), which was modified for olive pomace. 0.5 g (1 g for samples with pit) of dry sample was weighed into a 20 mL vial with screw cap. After adding 3.5 mL absolute ethanol, the tube was vortexed. 5 mL of 6 M HCl was added and mixed again. Then, vials were incubated at 80 °C in a water bath for 60 min (t_w) by shaking every 15 minutes. After heating, samples were cooled by cold tap water and stored for 5 minutes in the freezer. Five mL absolute ethanol was then added and mixed. Samples were poured into a 50 mL Falcon® tube for total lipid extraction. 20 mL hexane:diethyl ether (1:1, v/v) was added and mixed by rotator for 15 minutes (x2). Tubes were centrifuged at 1000 rpm (169 g) for 5 minutes to achieve phase separation. Supernatant phase was transferred to 12 mL glass

tubes and dried under fume hood and subsequent nitrogen flush. After extracted lipid content was determined, the oil sample was stored at -20 °C for determination of total BS.

Low repeatability values (1-8 % RSD in acid hydrolyzed extracts with pit) obtained for oil content was attributed to the nonhomogeneous distribution of pit in the dry pomace, which contained 43 % pit. To remove the pit, samples were sieved with mesh size of 1 mm and 0.5 mm, and suitable mesh size was determined according to the % oil content and BS content of sieved samples (Table S1). To modify sample preparation for pomace, t_w (60, 90, 120 min) was tested in duplicate for higher oil content and BS concentration (Table S1).

2.4. GC-FID Analysis of β -sitosterol

GC-FID method of Seçmeler and Güçlü Üstündağ (2017a), which was developed for the determination of lipophilic bioactives in VOO, was modified for the determination of BS of oils obtained from dried olive pomace and processed samples due to its complex composition. Oil preparation steps were modified for saponification parameters and derivatization time. GC program was modified by increasing final temperature of oven (330 °C), detector temperature (360 °C), and decreasing split ratio (15:1).

Hexane:diethyl ether extracted fresh pomace oil was expected to contain high amount of wax esters, fatty acid alkyl esters, propyl and butyl esters, esterified BS (BSE) and BSG (BSGE) related to VOO (Antonopoulos et al., 2006; Pérez-Camino et al., 2002). To break the ester bonds (for higher level unsaponifiable matter), 60 gr KOH in 40 mL water was used instead of 50% KOH solution (AOAC Official Method Ca 6b-53). Saponification temperature and time was increased from 58 °C to 80 °C and 10 min to 60 min, respectively. Solvent extraction after saponification was carried out by hexane (4 mL x 3) for free BS. 30 °C and 120 min was preferred for complete derivatization. Effectiveness of

saponification and derivatization was tested by GCMS detection of preliminary samples for the presence of esterified compounds.

GC analyses were carried out using a HP 6890 series GC system (Hewlett Packard, Waldbronn, Germany) with a FID detector using an HP-5MS (Crosslinked 5%Ph Me Silicone) capillary column (30 m x 0.25 mm id, 0.25 μ m film thickness, Hewlett Packard, USA). FID gases (air and hydrogen) and make up gas (nitrogen) flow rates were 136 mL/min, 35 mL/min, and 45 mL/min, respectively. Helium was used as the carrier gas (1.0 mL/min) with split injection (15:1). The analyses were carried out in the programmed temperature mode from 270 to 330 °C with a ramp rate of 4 °C/min followed by 10 min hold at 270 °C, 5 minutes hold at 330 °C. The detector temperature was 360 °C and the injector temperature was 300 °C. Peak identification was done by comparison of the retention times of sample peaks with those of individual standards (cholestane and β -sitosterol).

Stock standards of 5 α -cholestane (4 mg/mL), β -sitosterol (0.80 mg/mL) were prepared in chloroform and quality control (QC) standard mixture was prepared from stock standards by mixing 0.05; 0.50; 0.20 mL, respectively. Dried QC standards were stored at -20°C for 1 month (Schrack et al., 2004). Calibration standards were prepared in different amounts in the range of β -sitosterol amounts in pomace olive oil (Lama-Muñoz, Rodríguez-Gutiérrez, Rubio-Senent, Gómez-Carretero & Fernández-Bolaños, 2011) by spiking triolein (blank matrix) and dried under nitrogen at RT before derivatization.

Quantification was performed (Agilent Technologies GC ChemStation) using external calibration method by plotting calibration curves (Peak area versus concentration (mg/100 g)). Internal standards (50 μ l) were added to the sample at the beginning of the

saponification and hydrolysis procedure, respectively. For this case, calibration curves were plotted using concentration of analyte (mg/100 g) versus $\text{Area}_{\text{analyte}}/\text{Area}_{\text{IS}}$.

2.5. Determination of Phenolic Profile

10 mL methanol:water (80:20) solvent was added to 1 g freeze-dried sample (fresh pomace, aqueous and meal part of processed samples) in a 10 mL glass tube with screw cap. All tubes were mixed for 30 minutes by a rotator, then incubated in a water bath at 70°C for 1 hour and vortexed again and finally stored at -20°C until HPLC analysis.

Total phenol (TP) concentration was determined according to the Folin–Ciocalteu spectrophotometric method, using gallic acid as a reference standard compound and expressed as mg Gallic Acid Equivalent (GAE)/100g dry weight (Cicco et al., 2009). Total phenol concentration was determined by triplicate readings and extracts of all samples.

The individual phenolic compounds were quantified using a Hewlett-Packard 1100 liquid chromatography system with a C-18 column (Mediterranea SEA 18, Teknokroma (Barcelona, Spain), 250 mm x 4.6 mm i.d. 5 μm). The system was equipped with a diode array detector (DAD; the wavelengths used for quantification were 280 and 340 nm) and Rheodyne injection valves (20 μL loop). The mobile phases were 0.01 % trichloroacetic acid in water (A) and acetonitrile (B) utilizing the following gradient over a total run time of 55 min: 95 % A initially and 5 % B, 75 % A and 25 % B in 30 min, 50 % A and B in 45 min, 100 % B in 47 min, 75 % A and 25 % B in 95 min, and 95 % A and 5 % B in 52 min until completion of the run. Phenolic standards used for calibration were 5-hydroxymethylfurfural, hydroxytyrosol 4- β -d-Glucoside, vanillic acid, p-coumaric acid and 3,4-dihydroxyphenylglycol, tyrosol, hydroxytyrosol, oleuropein and apigenin. Content of total phenol and individual phenolic compounds in the aqueous and meal fractions were reported as mg/100g on a dry basis.

2.6. Hydrothermal Pretreatments

Hydrothermal experiments using steam and SCW were carried out in the flash hydrolysis laboratory pilot unit at the Instituto de la Grasa (Sevilla, Spain), which had previously been used only for steam treatment (Fernández-Bolaños, Rodríguez, Gómez, Guillén, Jiménez et al., 2004; Fernández-Bolaños, Rodríguez, Rodríguez, Guillén & Jiménez, 2002). The pilot unit included a steam generator, accumulator, 2 L stainless steel reaction chamber (maximum operating pressure 42 Kg/cm²) and stainless steel steam expansion chamber.

Three temperatures (160°C, 180°C, 200°C) were chosen based on previous studies of steam treatment (Lama-Muñoz, Rodríguez-Gutiérrez, Rubio-Senent, Gómez-Carretero & Fernández-Bolaños, 2011; Fernández-Bolaños, Rodríguez-Gutiérrez, Gómez, Guillén, Jiménez, Heredia & Rodríguez, 2004) to compare the effects of direct steam and SCW pretreatments for 5 minutes. To obtain SCW conditions, additional water was added (up to 1.5 L) to 2 L reaction chamber and after the temperature reached the target temperature (160 °C, 180 °C, 200 °C), the pressure was increased suddenly from 5, 10, 16 to 25 Kg/cm² (Table 1). In SCW treatment, lower set temperature 160 °C could not be reached due to design limitations of system, therefore SCW experiments were carried out at 150 °C. Treatments were applied to fresh moist pomace (300 g) in duplicates. Pressure (y1) and temperature (y2) values in the reaction chamber were recorded every 10 seconds for 5 minutes (steam) or 6 minutes (SCW) due to rapid pressure rise after approximately one minute in SCW pretreatment (Table 1).

Oil in fresh and processed pomace samples were extracted by hexane: diethyl ether (1:1) with and without acid hydrolysis and their oil content and BS content were

determined (n=3) (Table 2). Thus, total BS and free BS were determined, respectively, where their difference shows the bound BS and total BSG content in samples.

2.7. Statistical Analysis

Data were expressed as mean $\pm$ standard deviation. Microsoft Excel and SPSS software were used for statistical analysis. In method modification part of the study, one way ANOVA with replication and Tukey test for multiple comparisons were performed to compare the oil content and BS content of different samples. The statistical analysis of processed samples was carried out by two way ANOVA (GLM procedure) with replication including interaction terms to determine the effect of temperature and treatment type on the solid content (%), oil content (%) and BS content (mg/100g) of dried aqueous and meal fractions, separately ($\alpha=0.05$). When interaction was significant, simple main effect tests was carried out to compare the effect of the temperature and treatment at each level, otherwise Tukey's test was used.

3. Results and Discussion

3.1. Sample Preparation

In order to determine total oil and total BS content of pomace, pomace oil was extracted by hexane:diethyl ether after acid hydrolysis. This method was previously applied to different types of food samples including flour, refined rapeseed oil (not containing sterol glycosides), corn meal (containing high concentration of bound carbohydrates and steryl conjugates), onion (vegetable matrix) and sunflower kernel (having high lipid content) with 60 min hydrolysis time, t_w (Toivo et al., 2001).

Considering the heterogeneous nature of the sample, a mesh size of 1 mm was chosen to remove the pit by sieving. Removing the pit and cuticle caused 20% BS and 7.1% oil

loss. This loss was lower for processed samples where cuticle passed to the sieved (<1 mm) part as smaller fragments. The matrix difference between fresh pomace and processed samples were considered when comparing their results.

As hydrolysis time (t_w : 60, 90, 120 min) did not have a significant effect on the oil content and BS concentration of fresh sieved pomace (Table S1, $p>0.05$), 60 min was used for the hydrolysis of 0.5 g freeze-dried and sieved sample (< 1 mm), as previously done for flour samples (Toivo et al., 2001).

There was no significant difference between free BS content and oil content determined by Soxhlet extraction and hexane:diethyl ether extraction (Table S1, $p>0.05$). Therefore, hexane:diethyl ether extraction without acid hydrolysis was used in further analysis for solvent economy in this study.

The use of acid hydrolysis before hexane:diethyl ether extraction increased the oil yield significantly ($p<0.05$) from 15.7 % to 19.4 % as expected as bound lipids and hexane:diethyl ether soluble hydrolyzed products of polar lipids (phospholipids and glycolipids) (Karantonis et al., 2008) could be extracted using acid hydrolysis.

Application of acid hydrolysis increased BS concentration of pomace oil by 33 % (from 339 mg/100 g oil to 451 mg/100 g oil) and BS recovery from pomace significantly by 64 % (from 53 mg/100 g to 87 mg/100 g pomace) ($p<0.05$). This effect on BS is similar to the results of literature studies that reported around 33 % (Toivo et al., 2001) and 22-42 % (Jonker et al., 1985) increase in sterol content upon hydrolysis from plant foods such as cauliflower, wax beans, lettuce, cucumber, banana, potato, peanuts and whole wheat flour. These results were attributed to the liberation of bound sterols from cell membrane structure and free plant sterols from glycosylated sterols (such as BSG) by cleavage of the acid-labile acetal bond (not only ester bond of sterol esters such as BSE or BSGE).

Therefore BS content of acid hydrolyzed samples includes not only bound BS, also bound BSG and free BSG.

3.2. Effect of Hydrothermal Pretreatments

3.2.1. Effect on Solid Distribution

Distribution of pomace solids between aqueous and meal fractions after the pretreatments are shown in Table 1 as % ratio of aqueous and meal fractions (sieved and pit parts) to total dry pomace (94.6 g from 300 g fresh pomace).

28-33 % of total solids was recovered in aqueous fraction during hydrothermal treatments. No significant effect of pretreatment and temperature was found in aqueous fractions ($p>0.05$). As this fraction contained carry over from the meal fraction in addition to water soluble components solubilization of pomace could not be determined quantitatively.

Reduction in solid content of pomace by hydrothermal treatments (by 40-50 %) can be explained by the broken cell wall structure, hydrolysis of cell wall polysaccharides (hemicellulose and pectin) and lignin into water soluble components (such as phenolics, oligosaccharides and monosaccharides) and lost solids (9-17 %, mainly pit) during sample transfer.

Fragments of pit and cuticle (>1 mm) in the pomace sample were broken into small pieces during hydrothermal treatment. Thus, matrix of meal fraction was composed of more cuticle fragments (containing triterpenic acids, wax) and pit fragments (cellulosic compounds) than that of fresh pomace, which were able to pass through the sieve (1 mm).

In meal fraction, temperature and pretreatment effects were significant but their interaction was not significant ($p>0.05$). After SCW pretreatment, percent ratio of sieved fraction to total dry pomace were lower (by 15-23 %) than that of steam pretreatment,

while its pit part (>1 mm) were higher (by 13-22 %) than that of steam pretreatment at all temperatures. This can be attributed to the fact that physical effect of steam explosion on size reduction was more effective than SCW, in spite of higher pressure of SCW pretreatment (25 Kg/cm^2). It can be said that explosion (rapid decompression), resulted in disruption of the bundles to individual fibers (Stelte, 2016), is the determining factor for physical disruption and accordingly size reduction of pits. At 200°C for both pretreatments, % ratio of pit part to total dry pomace was significantly lower and in contrast sieved fraction ratio (% dry basis) was significantly higher than that at 160°C (5 Kg/cm^2) due to higher temperature and higher initial pressure at 200°C (16 Kg/cm^2) for both process.

3.2.2. *Effect on Color and Odor*

Hydrothermal pretreatments changed the color of pomace samples and increased color intensity, which might be due to browning reactions (Maillard and/or caramelization) and pigment degradation. As the temperature increased and acidic environment obtained, together with the degradation of chlorophylls and carotenoids, formation of anthocyanins via oxidative cleavage of proanthocyanidin take place (Wallace, 2010). Under acid hydrolysis conditions, anthocyanins (red color) are converted to their aglycones, anthocyanidins. Proanthocyanidins, major antioxidants found in OMW (El-Abbassi et al., 2012), are expected to be also present in two phase pomace.

Dried aqueous fractions, having high sugar content, were dark brown because of Maillard and/or caramelization reactions that occurred during hydrothermal treatments. However, it was observed that aqueous fractions of SCW samples were more reddish than those of steam treated samples before freeze-drying. This might be attributed to increased solubility due to improved diffusion through cell walls of the SCW samples, resulting in

higher anthocyanin stability. Effect of SCW was shown by Ju and Howard (2005) in extraction of anthocyanidins from grape skin that SCW extraction (at 160°C for 40 s) was 38% higher than conventional methanol extraction (at 50°C for 1 h). Besides 50% higher total anthocyanins were extracted at 160°C by sulfured SCW related to SCW which reduces the dielectric constant of the water. The smell of SCW pretreated meal fractions was more intense and their color was darker than that of steam samples, especially for the 200°C SCW sample.

3.2.3. *Effect on Oil Yield*

Mean dry matter oil content (DMO%) of pomace and its pretreated fractions (aqueous and meal) extracted with/without acid hydrolysis are presented in Table 2. The use of acid hydrolysis increased the oil yield of fresh pomace by 33% (14.6% - 19.4%, dry basis; 2.4% -3.2%, wet basis). The effect of acid hydrolysis and hydrothermal treatment (19.4% and 19.1-20.9%, respectively) on oil yield of pomace were similar ($p>0.05$). This is attributed to the effectiveness of hydrothermal treatments due to hydrolysis of cell wall structure during the process in combination with physical effect of explosion. In spite of 5 min process time, the oil yields of the treated samples were higher than those of a previous steam pretreatment study with longer process time (150-170°C, 15-90 min) (11.8-16.0%) (Damarchi, Savage & Dutta, 2005). Using hydrothermal pretreatment, 54-76 % of the bound oil was recovered (Table 2) where significantly higher recoveries were obtained by steam pretreatment than by SCW pretreatment ($p<0.05$).

In aqueous fraction, effect of temperature and treatment, and interaction were significant ($p<0.05$). Oil contents of SCW pretreated aqueous fractions were significantly higher than those of steam pretreated samples by 17% and 30% at 160°C and 180°C, however at 200°C it was significantly lower (by 8%) ($p<0.05$) (Table 2). As temperature

increased, oil content of SCW pretreated samples decreased significantly (up to 20%, $p<0.05$). However, in steam pretreatment a significant increase was observed from 180 °C to 200 °C by 23%. This increase in the oil content of aqueous fraction obtained by steam pretreatment can be attributed to higher carry over due to smaller size particles as explained in section 3.2.1. Considering hydrolytic degradation of oil during pretreatments, composition of extracted oil might include FFA, diglycerides, monoglycerides in addition to triglycerides, while glycerol, low molecular weight FFAs might be solubilized in the aqueous fraction. Degradation at 200°C might be start in SCW pretreatment. Consequently fractions of treated samples should be examined.

Only pretreatment effect was significant on the total oil yield of aqueous fraction, which was determined by acid hydrolysis ($p<0.05$), such that yields of SCW treated samples were significantly higher than those of steam treated samples similar to yields determined without acid hydrolysis (Table 2). In spite of higher recoveries obtained by steam pretreatment in aqueous fraction, opposite trend in their acid hydrolyzed results support the hydrolytic degradation assumption during SCW pretreatment due to complete oil hydrolysis and considering all glycerol, low molecular weight FFAs for all samples.

In the meal fraction, effects of pretreatment and interaction on oil content were significant ($p<0.05$). Effect of pretreatment was significant at only 160°C where oil content of steam pretreated meal was higher than that of SCW (by 15%) (Fig. 2a). This might be attributed to the 10°C (150/160°C) temperature difference between both pretreatments. Oil recovery of SCW pretreated meal samples increased with temperature (by 9%) however that of steam pretreatment decreased (by 7%) (Fig. 2b). Although total oil yield of meal fractions were similar for all process conditions ($p>0.05$), opposite trend of steam and SCW pretreatments with temperature can be attributed to their different heat, mass transfer and reaction mechanisms.

Steam, which is a more efficient heat transfer fluid than SCW due to its low density and high free convective heat transfer coefficient, penetrates every part of the matrix easily with convective heating in its gas form. Density of saturated steam increased from 3.1115 Kg/m³ (at 158 °C, 6 Kg/cm²) to 7.9315 Kg/m³ (at 200 °C, 16 Kg/cm²). Thus, convective heat transfer of saturated steam decreased with increase in its density. On the contrary, density of SCW decreases from 918.13 Kg/m³ (at 150 °C) to 865.34 Kg/m³ (at 200 °C) for 25 Kg/cm² with temperature.

Autohydrolysis of acetyl groups present in lignocellulosic material is catalyzed by high temperature (Kumar, P. et al., 2009; Manorach, 2014). If water, in the liquid state at elevated temperatures, pass through lignocellulosics, such a pretreatment has been termed as hydrothermolysis (Kumar, P. et al., 2009). Autohydrolysis depend on mainly molecular collusion, convective heat transfer of steam but hydrothermolysis depend on mainly molecular diffusion, conductive heat transfer of liquid which increase as the temperature difference between the SCW and pomace sample increase. When temperature increases the convective heat transfer coefficient of steam decreases, whereas that of SCW increases. Therefore their opposite trend of oil yield might be attributed to the opposite trend in autohydrolysis related to convective heat transfer and increase in hydrothermolysis related to conductive heat transfer of SCW.

Differences in the mass transfer behavior of steam and SCW depend mainly on behavior of SCW as a reactant and a solvent. Ionic product of water (K_w) increases (from 10⁻¹⁴ mol/L at 25°C to 10⁻¹¹ mol/L at 300°C) and dielectric permittivity decreases with temperature (Möller et al., 2011). Thus SCW gets involved in all ionic reactions (such as hydrolyses of cell wall materials and polar lipids (phospholipids and glycolipids)) as a reactant. In addition to water ionization, pH of the SCW reaction medium decreases by the generation of organic acids via reaction pathways of glucose and fructose.

However, steam does not act as a solvent and reactant. Lack of solubilization was reported as the limitations of steam explosion of aspen wood (Kumar et al., 2009). Other limitations of steam for biomass pretreatment are due to incomplete destruction of the xylan fraction, incomplete disruption of the lignin-carbohydrate matrix and unremoved reaction products of hydrolysis and polymerization by water (Kumar, P. et al., 2009). Under SCW conditions, as formic acid and acetic acid, endogenous intermediates of hemiacetyl splitting, are generated, xylan (cell wall polysaccharide) solubilization starts (Abu Tayeh et al., 2016). Additionally, as temperature of SCW pretreatment increases, higher amounts of polar lipids might be released from cell wall constituents and solubilization of all oils might increase due to lower dielectric constant ($\epsilon = 35$ at 200°C).

3.2.4. *Effect on BS*

Total BS and free BS content of samples were determined by extraction with and without acid hydrolysis, respectively (Table 2). BS content of pretreated pomace was calculated by multiplying BS content of fractions with % solid content. The difference between total BS and free BS was indicated as bound BS and BSG (free and bound) content of the samples.

The use of acid hydrolysis increased BS content of pomace by 74% (50 - 87 mg/100 g) which is higher than oil yield increase. Unlike oil yield results, the effect of acid hydrolysis on BS content of pomace was significantly higher than that of hydrothermal pretreatment (87 and 58-65 mg/100 g, respectively) ($p < 0.05$), and maximum BS recovery (95 mg/100 g) was obtained by acid hydrolysis of SCW pretreated sample at 200°C , which was significantly higher ($p < 0.05$) than that of fresh pomace. Similar to oil yield results, combined effect of hydrothermal treatment and acid hydrolysis indicate that the

effectiveness of acid hydrolysis was increased by hydrothermal pretreatment due to additional hydrolysis required for complete recovery.

Using hydrothermal pretreatment, 18-32 % of the bound BS was recovered ($p < 0.05$, Table 2). Although BS recoveries of steam and SCW pretreatments were similar ($p > 0.05$), total BS content of SCW pretreated whole samples was significantly higher (by 2-14 %) than that of steam pretreated ones ($p < 0.05$). This might be due to reactivity of SCW reaction medium resulting in release of bound BSG, which was determined as BS after acid hydrolysis ($p < 0.05$).

In the aqueous fraction, effect of temperature, treatment, and their interaction were significant ($p < 0.05$). BS content of SCW pretreated samples were higher than that of steam pretreated samples by 52 % and 57 % at 160°C and 180°C, however at 200°C it was significantly lower (by 11 %) than that of steam pretreated sample. Similar to oil recovery results, BS recovery in aqueous fractions of pretreatments showed opposite trend with temperature and a significant increase from 180°C to 200°C in steam pretreated samples was observed.

Effect of pretreatment and interaction on BS content of meal fraction were significant ($p < 0.05$). Effect of pretreatment was significant at only lowest operational temperature ($p < 0.05$) where BS content of steam pretreated sample at 160°C was higher (by 27 %) than that of SCW pretreated samples at 150°C. This might be attributed to temperature difference (10°C). Significant increase from 150°C to 180°C (by 20 %) and to 200°C (by 26 %) was determined in SCW pretreated meal samples ($p < 0.05$) (Figure 2c). However, a significant decrease (by 13 %) was obtained from 160°C to 200°C by steam pretreatment (Figure 2d).

According to BS content of aqueous and meal fractions after acid hydrolysis, pretreatment effect was significant ($p < 0.05$). BS content of SCW pretreated samples were

significantly higher than that of steam pretreated samples ($p < 0.05$) (by 10-44 % and 3-17%, respectively) (Table 2). The highest BS content in aqueous (44 mg/100 g) and meal (144 mg/100 g) fraction was determined by acid hydrolysis of SCW pretreated samples at 150 and 200 °C, respectively. More BSG might be liberated and solubilized in the oil during SCW treatment at 150 °C. In meal fraction, bound BS/BSG could be liberated at higher temperatures and solubilized, especially at 200°C, during SCW treatment. However as BSG could not be extracted by hexane:diethyl ether due to its polar nature, the BSG content could not be analyzed without acid hydrolysis.

3.2.5. *Effect on Phenolic Compounds*

Total phenolic content (TP) (4020.7 mg/100 g untreated sample) of the pomace increased (by 11 %) at only 200°C by steam and SCW pretreatment (4512.4 and 4453.0 mg/100 g total treated sample, respectively) (calculated from Table 3), which might be attributed to decomposition of lignin. Similarly maximum phenolic recovery (20.87 mg/g) was obtained at 200 °C by Gameiro (2016) when olive pomace was extracted with SCW at 140-200 °C (10 ml/min, 61.2 Kg/cm²). Although SCW at 200°C is a suitable solvent for olive pomace phenolics with dielectric constant (35) close to those of acetonitrile (37.5) and methanol (32.7), TP content of steam pretreated pomace samples were significantly higher than those of SCW samples (1-24 %) ($p < 0.05$). After hydrothermal pretreatments, the majority of the phenolics were concentrated in the aqueous fraction (76-89 % TP content).

Phenolic profiles of fresh and pretreated pomace samples (aqueous and meal fractions) were analyzed by HPLC (Table 3). Hydroxytyrosol (HT), 3,4-dihydroxyphenylglycol (DHPG), hydroxytyrosol 4- β -D-glucoside (HTG), tyrosol (Ty), vanilic acid, vanillin, ferulic acid, oleuropein derivate, p-coumaric acid and apigenin were detected in aqueous

fractions obtained after hydrothermal treatments. Tyrosol and apigenin were not detected in steam pretreated meal and oleuropein derivative was not detected in SCW pretreated meal fraction.

No significant effect of temperature or pretreatment was observed on HTG content of aqueous and meal fractions and total recovery values ($p>0.05$). Temperature effect was significant for all other phenolics in the aqueous fraction ($p<0.05$). Similar to TP results, a significant increase ($p<0.05$) was observed in HT and oleuropein derivatives, vanillic acid and vanillin, concentration of the aqueous fractions as temperature increased. Production of phenolic compounds such as vanillic acid, syringic acid, vanillin, and syringaldehyde by thermal decomposition of lignin was reported (Fernández-Bolaños et al. (2002); Yu et al. (2014).) Therefore it can be said that in this study lignin decomposition started at temperatures higher than 160°C, particularly at 200°C considering concentration of vanillic acid and vanillin.

HT and tyrosol are the characteristic phenolics in olive fruit. HT concentration of aqueous fraction (0.82 % w/w d.b.) obtained by steam treatment at 200°C was higher than the amount previously reported (0.56 % w/w d.b.) for steam pretreated 3-phase pomace at the same conditions (200°C, 5 min) by 46 % (Fernández-Bolaños, Rodríguez-Gutiérrez, Rodríguez, Guillén & Jiménez, 2002). This difference can be partly attributed to the use of destoning and defatting steps in the other study, which decreased HT content of the 3-phase pomace sample. Concentration of HT in mean of both pretreatments for sum of both fractions (465.8 mg/100 g at 200°C) was three times higher than its concentration in the fresh pomace (160.8 mg/100 g). This result might be attributed to increase in HT recovery by hydrothermal treatments due to hydrolysis of the oleuropein and other secoiridoids like EA-HT (3,4-dihydroxyphenyl-ethanol) linked to elenolic acid or EDA-HT (p-hydroxyphenylethanol) linked to dialdehydic form of elenolic acid (Ghanbari et al., 2012;

Brenes et al., 2001). Only temperature effect was significant on the HT concentration of aqueous fraction ($p<0.05$), HT was significantly higher at 200 °C. Tyrosol, which was not detected in fresh and meal samples, increased significantly with temperature ($p<0.05$) (by 3 times from 160 °C to 200 °C).

Concentration of DHPG in aqueous sample was significantly higher at 180°C than at 160°C and 200°C ($p<0.05$). Lower concentration of DHPG in aqueous fraction at 200 °C might be due to decomposition of DHPG. Notable decrease (from 1.3 to 0.3 g/Kg) in DHPG content of olive pomace was reported as the temperature increased from 180 to 240 °C in a previous steam pretreatment study (over 5 minutes) without catalyst due to a decrease in pH resulting from autohydrolysis (Rodríguez-Gutiérrez, Lama-Muñoz, Trujillo, Espartero & Fernández-Bolaños, 2009). Pretreatment effect was significant for oleuropein derivative, DHPG, vanilic acid and ferulic acid where SCW pretreatment was more effective than steam ($p<0.05$). Significant interaction was present in concentration of ferulic acid and SCW pretreatment effect was significantly higher (by 67 %) than that of steam at 200°C ($p<0.05$).

Considering the meal fraction, both treatment and temperature significantly affected only vanillin concentration of meal samples ($p<0.05$). Highest concentration was obtained by SCW pretreatment at 180 °C. Pretreatment effect was significant for concentration of vanillin where SCW pretreatment was more effective than steam ($p<0.05$). Temperature effect was significant for oleuropein derivatives, vanillin, vanilic acid and ferulic acid, such that their concentration increased with temperature.

3.2.6. *Effect on HMF*

HMF was not detected in aqueous fractions obtained at 150-160°C, but it was detected at 180°C (217.2-557.0 mg/100 g) and 200°C (4006.7-4273.7 mg/100g) (Table 3). HMF

could be formed by Maillard and/or caramelization reactions depending on amino acid and a reducing sugar content such as glucose and fructose of pomace (Plaza et al., 2010; Nie et al., 2013). HMF was completely solubilized in the aqueous part due to its water solubility. It was reported by Kazan, et al. (2015) that reducing sugar content of olive pomace increased with temperature from 180°C to 200°C during supercritical water extraction, however decreased at temperatures higher than 200°C due to decomposition of sugars. In SCW treatment of rice bran, the start of browning reaction was reported as 180°C by measuring furfural content (Wiboonsirikul et al., 2008). These findings supports our results, which showed formation of HMF at 180 °C. This can be explained based on two mechanism of HMF formation: thermal degradation of reducing sugars and Maillard involving the reaction of a specific amino acid with a reducing sugar depend on temepreature and pH (Nie et al., 2013). Namely, in SCW, the increased Kw and high temperature triggers significant glucose conversion to fructose even at neutral pH values. 5 % conversion was reported at 180°C for 2 minutes and this ratio increased with temperature (Möller et al., 2011). D-fructose further degrades to HMF during browning reactions, and then HMF and intermediate degradation products of biomass monomers degrades to carboxylic acids such as acetic acid and formic acid, which decreases the pH of the medium (Möller et al., 2011). Thus, pH dependent reactions, such as Maillard, are catalyzed accordingly.

4. Conclusions

Hydrothermal pretreatments, steam explosion and SCW treatment, were applied to olive pomace to produce value added compounds such as phenolic compounds, sterols including their polar conjugates and oil by decomposition of the cell wall structure and hydrolysis reactions. 54-76% of the bound oil of the pomace sample was recovered by hydrothermal

treatments (5 min). Steam pretreatment was more effective than SCW pretreatment for oil recovery at lower temperatures. However, SCW shows increasing trend on oil yield related to steam pretreatment due to their different heat transfer, mass transfer and reaction mechanisms. Compared to acid hydrolysis, a chemical pretreatment, hydrothermal pretreatments yielded similar oil recovery however were not efficient for the recovery of BS even at 200°C (for 5 minutes) such that only 18-32% of the bound BS of the pomace sample could be recovered by hydrothermal treatments.

Recovery of BS, particularly BSG, can be increased by pretreating olive pomace at temperatures higher than 200 °C and using longer reaction times. The use of high temperatures might also increase recovery of phenolics and result in the formation of new antioxidant compounds by degradation reactions such as Maillard reactions. Further research at higher temperatures should include determination of BSG, FFA, polar lipids composition of extracts and their antioxidant activity. However, high amount of HMF was generated at 180-200 °C, which is a toxic, unwanted compound for food industry but is used in automotive industry as a prospective precursor for “green fuels” (Möller et al., 2011).

These results can form the basis for process development studies for total valorization of olive pomace, which might include use of sequential temperature (low, medium and high) in a continuous process. After steam pretreatment of pomace at low temperature (160°C), oil and phenolics, sugars might be recovered through meal and aqueous part, respectively. After SCW pretreatment of aqueous part at medium temperature (>200 °C), HMF might be generated from sugars as another by-product. Oil might be removed by steam pretreated meal part. After SCW pretreatment of deoiled meal part at high temperature (210-230°C), complete destruction of cell wall constituents (cellulose and

lignin) and separation of BSG and appropriate biomass (BSG free) for biofuel production might be possible.

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Figures

Figure 1. Flow chart of overall methodology for BS determination

(a)

(b)

(c)

(d)

Figure 2. Oil and BS content of meal fractions obtained using different (a,c) temperatures, and (b, d) pretreatments

Table 1. Temperature and time data of hydrothermal treatments and Solid-liquid distribution after centrifugation and freeze drying for two replicates (Rep)

Op. Runs		Measured Values		Total solid in aqueous fraction	Meal Fraction		Lost solid
T_{set}	Rep.	$\overline{T}_{act}$	t_{rxn}		Sieved part <1 mm	pit & cuticle part	
°C		°C	sec	%**	%**	%**	%**
Fresh pomace		-	-	-	53%	47%	-
Direct steam pretreatment							
160	1	163 ± 5	290	31%	34%	26%	9%
	2	164 ± 4	290	31%	33%	26%	10%
180	1	184 ± 4	290	31%	37%	21%	12%
	2	180 ± 4	290	28%	34%	25%	13%
200	1	204 ± 3	290	31%	36%	16%	17%
	2	203 ± 2	290	32%	39%	16%	13%
Subcritical water pretreatment							
150	1	144 ± 1	280	32%	28%	28%	12%
	2	153 ± 2	300	28%	24%	31%	16%
180	1	178 ± 2	260	29%	30%	28%	13%
	2	179 ± 3	340	29%	27%	27%	17%
200	1	200 ± 3	350	32%	31%	19%	17%
	2	199 ± 2	340	33%	33%	20%	14%

* t_{rxn} : Time where actual reaction take place at set temperature or subcritical water phase

**Fractions are based on total dry solid

Table 2. Distribution of mean dry matter oil content (DMO %)* and β -sitosterol content (mg/100 g, d. b.) in fresh and pretreated pomace samples

T°C	Oil Content%						Bound oil/BS Recovery
	Acid hydrolyzed			Without acid hydrolysis			
	Aqueous	Meal**	Whole Sample	Aqueous	Meal**	Whole Sample	
Fresh pomace	-	-	19.4±0.7	-	-	14.6±0.5	
Steam pretreatment***							
160	14.5 ± 2.90	29.0 ± 2.19	22.0 ± 2.47	13.7 ± 0.28	27.6 ± 1.20	20.9 ± 0.71	76%
180	13.3 ± 0.85	26.2 ± 0.49	20.3 ± 0.14	10.6 ± 0.14	26.5 ± 1.13	19.3 ± 0.64	56%
200	14.5 ± 1.13	28.4 ± 0.85	22.1 ± 0.07	14.0 ± 0.49	25.6 ± 0.28	20.3 ± 0.35	68%
Subcritical water pretreatment***							
150	19.1 ± 0.28	27.4 ± 0.92	22.9 ± 0.28	16.0 ± 0.00	23.5 ± 0.28	19.5 ± 0.07	58%
180	16.2 ± 0.35	29.2 ± 0.14	22.7 ± 0.49	13.8 ± 0.35	24.6 ± 0.14	19.1 ± 0.14	54%
200	13.3 ± 0.42	29.5 ± 0.64	21.4 ± 0.64	12.8 ± 0.85	25.7 ± 1.20	19.2 ± 1.06	55%
BS Content (mg/100g)							
Fresh pomace	-	-	87 ± 6.5	-	-	50 ± 0.8	
Steam pretreatment***							
160	35 ± 4.2	120 ± 6.4	84 ± 4.9	31 ± 1.4	89 ± 2.1	65 ± 0.7	32%
180	29 ± 6.4	114 ± 2.8	79 ± 1.4	24 ± 2.1	84 ± 2.8	60 ± 0.7	21%
200	34 ± 2.1	123 ± 6.4	87 ± 4.9	36 ± 0.7	77 ± 2.8	61 ± 1.4	24%
Subcritical water pretreatment***							
150	44 ± 1.4	123 ± 9.2	85 ± 5.7	47 ± 1.4	65 ± 1.4	58 ± 1.4	18%
180	41 ± 5.7	131 ± 4.9	90 ± 2.8	37 ± 0.0	78 ± 9.9	61 ± 4.9	23%
200	37 ± 2.8	144 ± 11	95 ± 2.8	32 ± 2.1	82 ± 0.0	60 ± 1.4	22%

* In order to compare with DMO of fresh pomace, DMO of pretreated pomace was calculated by multiplying DMO of fractions with % solid content (g dried fraction/ g total solid of pomace).

**Meal: sieved meal fraction

***Mean values and standard deviations were calculated from process replicates (p=2) determined from mean concentrations of extraction replicates (n=3)

Table 3. Total phenolic content of pomace samples and concentration of individual phenolic compounds in aqueous and meal part of pretreated pomace (mg/100g)

Individual phenolic compounds, mg/100g*												Total Phenolic Contents
T°C	DHPG ***	HMF**	HTG	HT	Ty**	V.acid	Vanilin	Fer. acid	Oleu. Derv.	p-cmr. acid	Apigenin **	(mg /100g GAE)
Fresh P.	33.7	-	222.3	215.8	nd	7.2	204.7	5.1	164.8	24.4	60.4	4020.7
Pit**	12.5	-	134.4	105.7	nd	nd	nd	nd	125.7	7.2	nd	1478.7
Aqueous part												
Steam pretreatment												
160	425.4	tr	370.0	536.3	51.9	60.9	256.9	20.0	282.6	37.4	58.6	6335.3 ± 323.1
180	516.1	557.0	382.4	671.3	104.8	101.5	229.5	38.9	306.4	22.8	nd	6030.0 ± 331.0
200	312.3	4273.7	354.7	818.0	184.3	77.3	368.9	43.7	314.3	9.5	nd	7327.5 ± 573.8
Subcritical water pretreatment												
150	417.1	nd	370.2	622.2	63.0	77.3	224.0	17.7	254.7	46.8	58.6	5576.2 ± 298.8
180	645.6	217.2	368.6	703.2	97.2	149	294.0	43.0	291.7	23.2	42.4	4296.6 ± 129.2
200	452.8	4006.7	377.9	874.4	194.8	98.8	440.8	72.9	302.9	12.7	nd	6966.2 ± 587.0
Meal part												
Steam pretreatment												
160	11.2	nd	106.0	62.3	nd	13.8	215.2	17.7	135.1	4.9	nd	998.1 ± 98.03
180	13.2	nd	107.0	64.0	nd	18.8	321.6	7.1	127.1	3.7	nd	1296.9 ± 129.9
200	nd	nd	107.6	67.8	nd	17.5	190.9	13.1	127.8	10.8	nd	1723.1 ± 232.1
Subcritical water pretreatment												
150	19.6	nd	106.7	66.9	nd	10.5	255.3	13.3	nd	4.9	nd	870.4 ± 24.12
180	15.8	tr	105.3	59.8	nd	19.7	331.7	5.2	nd	10.0	nd	909.8 ± 135.3
200	5.0	tr	105.9	61.1	nd	16.4	208.0	9.2	nd	9.9	nd	1385.2 ± 276.3

* Mean values and standard deviations were calculated from process replicates (n=2) determined from mean concentrations of extraction (n=3) and injection replicates (n=3)

**Tr: concentration < 0,1 mg/L, nd: non detected

***DHPG: 3,4-dihydroxyphenylglycol, Fer. Acid: ferulic acid, HMF: 5-hydroxymethylfurfural, HT: hydroxytyrosol, HTG: hydroxytyrosol 4- β -d-Glucoside, Oleu. Derv.: oleuropein derivative, p-cmr. acid: p-coumaric acid, Ty: tyrosol, V. acid: vanilic acid.