RECOVERY OF STEROLS FROM PLANT BASED FOOD WASTE FOR THE DEVELOPMENT OF FUNCTIONAL FOOD INGREDIENTS

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To my parents

with all my thanks and respect…

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ABSTRACT

RECOVERY OF STEROLS FROM PLANT BASED FOOD WASTE FOR THE DEVELOPMENT OF FUNCTIONAL FOOD INGREDIENTS

Bioactive compounds recovered from food processing waste can be used for the development of functional food ingredients providing value added products and an alternative solution for environmental problems. The general objective of the thesis is to recover sterols from olive pomace using hydrothermal pretreatments, which also enable recovery of phenolics producing sterol rich pomace oil to be used as a functional food ingredient source and phenolic extract. An in-depth review of the literature data and an overall partitioning study (samples taken from an integral olive milling plant) of pre-dominant lipophilic bioactives (squalene, α-tocopherol and β-sitosterol) were carried out to understand distribution of these bioactives between oil and waste streams during virgin olive oil (VOO) processing. To be used in this study, a rapid in-house GC-FID method was developed to determine lipophilic bioactives (squalene, α-tocopherol and β-sitosterol (BS)) simultaneously, and validated covering; linearity (16.0-700; 12.5; 8.4-500 mg/100g, respectively), recovery (93-95; 93-110; 92-97 per cent) and repeatability. Loss of these lipophilic compounds during VOO processing was determined by analyzing oil in olive fruit, olive oil, pomace and olive mill wastewater samples. The loss of BS (44 per cent) and α-tocopherol (42 per cent) were higher than that of squalene (12 per cent) and oil (14 per cent) due to non-recovered bound forms. To recover sterols and phenolics and to increase oil yield, pretreatments hydrolyzing plant cell wall, steam and subcritical water reaction medium was applied to olive pomace at different temperature (160, 180 and 200°C) in a pilot system. While oil recovery was similar to that obtained with acid hydrolysis, 18-32 per cent of the bound BS of the pomace was recovered as free form by hydrothermal pretreatments. Maximum BS recovery was obtained using hydrothermal pretreatment in combination with acid hydrolysis, which might be due to release of bound BS as glycosides from cell membrane structure. Significant increase in phenolic compounds at 200°C and formation of 5-hydroxymethylfurfural (HMF) were indication of lignin decomposition and Maillard/caramelization reactions, respectively.

ÖZET

FONKSIYONEL GIDA KATKI MADDESI GELIŞTIRMEK IÇIN BITKISEL GIDA PROSES ATIKLARINDAN STEROLLERIN GERI KAZANIMI

Gıda üretim atıklarından geri kazanılan biyoaktif maddelerin gıda katkı maddesi olarak kullanılması katma değer sağlarken çevresel problemlere de alternatif bir çözüm sunmaktadır. Bu çalışmanın ana hedefi ise sterollerin zeytinyağı üretiminde açığa çıkan sulu prinadan geri kazanımını için fenolik maddelerin geri kazanımında kullanılan hidrotermal ön işlemlerin kullanılması ve gıda katkı maddesi olarak kullanılmak üzere, sterolce zengin yağ ve aynı zamanda fenolikçe zengin ekstrakt elde etmektir. Dominant lipofilik biyoaktif maddelerinin (skualen, α-tokoferol ve β-sitosterol) proses sırasında yağ ve atıklar arasındaki dağılımı, literatür taraması ve entegre zeytinyağı işleme tesisinden alınan numunelerle gerçekleştirilen deneysel çalışma ile incelenmiştir. Bu çalışma kullanılmak üzere zeytinyağı içerisindeki dominant lipofilik biyoaktif maddelerin (skualen, α-tokoferol ve β-sitosterol (BS)) aynı anda tespitini mümkün kılan hızlı bir metot geliştirilmiş, ve doğrusallık, geri kazanım ve tekrarlanabilirlik parametreleri kapsamında metot geçerli kılınmıştır. Zeytinyağı üretimi sırasında ilgili lipofilik biyoaktif maddelerin kayıp oranları zeytin, zeytinyağı, prina ve karasu yağı analiz edilerek hesaplanmıştır. Bağlı formları nedeni ile geri kazanımı sağlanamayan BS (yüzde 44) ve α-tokoferol (yüzde 42) kaybının, skualen (yüzde 12) ve yağ (yüzde 14) kaybından daha fazla olduğu tespit edilmiştir. Sterollerin, prinadan geri kazanımını sağlamak ve ham prina yağı verimini artırmak için hücre duvarını parçalayan ön işlemlerden buhar ve subkritik su reaksiyon ortamı pilot sistemde farklı sıcaklıklarda sıcaklığın (160, 180 and 200°C) uygulanmıştır. Asit hidroliz ve hidrotermal ön işlemlerin yağ geri kazanımına etkisinin benzer olduğu ve bağlı BS'in yüzde 18-32'si serbest BS cinsinden hidrotermal ön işlemlerle geri kazanımı sağlanmıştır. Maksimum BS geri kazanımı hidrotermal ön işlem sonrası asit hidroliz uygulandığında elde edilmesi ile hücre zarında bağlı bulunan glikozit formdaki BS'in serbest hale geçmiş olabileceği düşünülmektedir. 200°C'de her iki ön işlemde de fenoliklerin geri kazanımının ve HMF miktarının artması, hücre duvarı yapısında bulunan ligninin ayrıştığının ve maillard/karamelizasyon reaksiyonlarının gerçekleştiğinin bir göstergesidir.

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LIST OF SYMBOLS/ABBREVIATIONS

A	Derivatization temperature, T_d
a_x	Coefficients of equation
B	Derivatization time, t_d
C	Saponification temperature, T_s
C	Mass fraction (measured mass of the analyte in the sample/mg sample)
c_s	Measured mass fraction of the analyte in spiked sample (mg/mg)
c_b	Measured mass fraction of the analyte in unspiked matrix blank (mg/mg)
c_a	Calculated mass fraction of the analyte added in unspiked sample (mg/mg)
D	Saponification time, t_s
L	Processing loss
m	Slope estimated from calibration curve/mass of the unspiked sample (mg)
n	The number of calibration points/the number of replicates
s_r	Standard deviation of mass fraction (mg/mg)
s(r)	Residual standard deviation
T	Temperature
t	Time
W'_{total}	Total weight of sample in the process (kg)
Y	Standardized Peak Area (Area/mg sample)
y_i	The observed value of y for a given value of x_i
$\hat{y}_i$	The value of y predicted by the equation of the calibration line for a given value of x_i
x_i	Amount of analyte, mg
?	Unaccounted value
AOCS	American Oil Chemists' Society
AHL	Acid hydrolysis/ Acid hydrolyzed
BS	β-sitosterol

BSG	β-sitosterol glucoside
COPO	Crude olive pomace oil
DHPG	3,4-dihydroxyphenylglycol
DMO	Oil content dry matter basis (per cent)
EUROCHEM	A network of organizations in Europe (focus on analytical chemistry)
FMO	Oil content fresh matter basis (per cent)
GC-FID	Gas chromatography with flame ionization detector
HHL	Hydrothermally treated
HMDS	Hexamethyldisilazane
HMF	5-hydroxymethylfurfural,
HORRAT	The Horwitz ratio
Hyty	hydroxytyrosol,
HytyG	hydroxytyrosol 4-β-d-Glucoside,
IOC	International Olive Council
IS	Internal standard
LOD	Limit of detection
LOQ	Limit of quantification
O	Olive sample
OFDM	Oil free dry matter (per cent)
OMW	Olive mill waste water
P	Pomace Sample
PRSD	Predicted relative standard deviation
PTV	Programmed temperature vaporizer
PY	Process yield
R	Operational run
R’	Olive stone removal
R’’	Second centrifugation/extraction run
ROO	Refined olive oil
RSD	Relative standard deviation
RSM	Response surface methodology
RT	Room temperature
SCOO	Second centrifugation olive oil
SCD	Sitosterol cyclodextrin

SCW	Subcritical water treatment
sito	β-sitosterol
SPE	Solid phase extraction
sq	Squalene
TLC	Thin layer chromatography
TMCS	Trimethylchlorosilane
TP	Total phenol content
toco	α-tocopherol
tr	trace concentration < 0,1 mg/L
Tyr	tyrosol,
VOO	Virgin olive oil
W/OAHL	Without acid hydrolysis

1. INTRODUCTION

In the last few years, much attention has been paid to health effects of bioactive compounds in food products, how bioactive compounds behave during processing: their retention in the final product or loss through the waste stream, and accordingly optimization of food production design, and valorization of waste streams. One such food is olive oil which contains hydrophilic, lipophilic and amphiphilic bioactive compounds (phenolics, squalene, phytosterols, tocopherols etc.) having anticancer, anticholesterol and antioxidant activities. In olive oil production, these bioactives are distributed between the product or waste streams according to their solubility and mass transfer behavior and stability against thermal treatment.

First part of the thesis (section 2.1-2.4) focuses on the behavior of lipophilic bioactives, squalene, sterols and tocopherols, during olive oil processing using a systematic approach. Herein their content in the olive fruit, distribution between its anatomic parts, and the effect of process methods and parameters on the lipophilic bioactive content of olive oil were reviewed. To test the findings of section 2.4, loss of predominant lipophilic bioactives present in olive fruit (squalene, α-tocopherol and β-sitosterol), was determined (chapter 4) using samples taken from a single operational run at an integrated olive milling plant producing VOO and second centrifugation olive oil (SCOO). The lipophilic content of the olives, oil (VOO, SCOO) and waste samples (olive mill waste water (OMW) and pomace-alperujo and orujo) were determined using the developed method.

The analytical methodologies utilizing HPLC or GC techniques for the determination of these bioactives in olive oil may include a number of sample pretreatment steps (such as clean up (using TLC and SPE), saponification and derivatization) making them rather lengthy and impractical. Thus, a rapid and in-house GC-FID method was developed and validated for the determination of these bioactives in VOO using response surface methodology for optimization of critical parameters (time and temperature of saponification and derivatization) (chapter 3). The developed method was fully validated covering all parameters; linearity, LOQ, LOD, recovery and repeatability.

Finally, in chapter 5, hydrothermal treatments was applied for value added utilization of olive pomace and to obtain sterol enriched oil and phenolics from olive pomace. A

methodology for the determination of free and total β-sitosterol (including unbound β-sitosterol and β-sitosterol glucoside (BSG) in olive pomace with/without acid hydrolysis using GC-FID was developed. The effect of hydrothermal (steam and subcritical water) pretreatments and pretreatment temperature (160°C, 180°C and 200°C) on the yield, β-sitosterol, phenolics and 5-hydroxymethylfurfural (HMF) content of pomace oil were determined.

2. LITERATURE REVIEW

2.1. OLIVE LIPOPHILIC BIOACTIVES

2.1.1. Composition and Lipophilic Bioactive Content of the Olive Fruit

While olive composition varies depending on factors such as cultivar and ripeness, olive fruit used for oil extraction on average contains 22 per cent oil and 50 per cent water. The oil is concentrated mainly in the pulp, which makes up 70-90 per cent of the olive fruit, while the remaining oil is distributed in the seed and stone. Based on 100 g of olive fruit containing 70 g pulp, 27 g stone, and three gram seed, >95 per cent of the oil is present in the pulp, while the seed and stone contains aproximately four and one per cent oil, respectively (Table 2.1) [1,2]. As most of the oil is present in the pulp, composition of pulp oil is the determining factor for the nutritional value and stability of the extracted oil.

Table 2.1. Composition of whole olive fruit and its anatomic parts (per cent, w/w)

	Whole olive fruit*	**Pulp** (Mesocarp)	**Seed** (Kernel)	**Stone** (Endocarp)
Weight, %	100	70-90	2-3**	9-27**
Oil, %	22	30**	27**	1**
Water, %	50	60**	30**	10**
Sugar, %*	12.5	3-7.5	27	41
Cellulose%*	5.8	3-6	2	38
Protein, %*	1.6	3	10	3
Minerals/ash, %*	1.5	1-2	1.5	4
Other compounds*	1-3	2-3	2.5	3.5

*[1,3,4], **[2]

Carbohydrates, which make up 19 per cent of the olive fruit, include pectin, cellulose (six per cent) and hemicelluloses (mainly xyloglucan and xylan) [1,5]. In addition to lignin,

proteins (1.6 per cent), minerals (min. 1.5 per cent) and volatile compounds (aldehydes, alcohols, esters, hydrocarbons, ketones, furans), olive fruit contains minor bioactive compounds including phenolics (between one to three per cent), lipophilic bioactive compounds (<one per cent), and pigments (chlorophyll and carotenoids) [1,3] (Table 2.1). Lipophilic olive bioactives include sterols (mainly β-sitosterol, and campesterol, delta-5-avenasterol, stigmasterol), hydrocarbons (mainly squalene and carotenoids, β-carotene and lutein), tocopherols (chiefly α-tocopherol and others (β-, γ-, and δ-)), triterpenic acids (ursolic, oleanolic and maslinic acids) and triterpenic dialcohols (erythrodiol and uvaol), and aliphatic alcohols [6-8].

Although there is a wealth of data on the bioactive content of VOO or olive fruit, data on distribution of bioactives in the olive fruit is rather scarce. Based on available literature data, summarized in Table 2.2, β-sitosterol concentration of the seed (~4000 mg/kg oil) can exceed twice the concentration in the pulp (~2000 mg/kg oil) [8,9]. In another study, the composition of pulp oil and stone oil of three different cultivars showed that the stone oil had between two to four times higher total sterol content [10]. A similar pattern was also observed for total sterols and also tocopherol concentration (216 mg/kg oil) in seed was higher than in the pulp (122 mg/kg oil) [11]. On the other hand, squalene was mainly concentrated in pulp oil (10000 mg/kg oil) [12].

In addition to their distribution in the anatomic parts of the olive fruit, the extraction behavior of olive oil and bioactives can be affected by their distribution within the plant cell and interaction with other cellular components. The oil within the cells is partly located in the vacuole (approximately 76 per cent), where it is free. The other portion lies within the cytoplasm (approximately 24 per cent) as a dispersion stabilized by a lipoprotein membrane. This bound portion is difficult to extract and is mainly lost in the waste unless the interactions between oil and colloids are broken up and microgel formation is prevented during processing by the use of enyzmes to aid recovery of bound oil [13,14].

Table 2.2. Lipophilic bioactive profiles of whole olive fruit and its anatomic parts (mg/kg oil)

	whole olive fruit	**skin** (epicarp)	**pulp** (mesocarp)	**seed** (kernel)
squalene	no data	nd[12]	10000[12]	Tr[12]
	4359[15]	no data	no data	no data
β-sitosterol	2705[9]	1648[9]	2331[9]	4407[9]
	1496[8]	no data	1481[8]	3556[8]
Total sterol	no data	1840[11]	1906[11]	2945[11]
	1847[17]	no data	1832[17]	4128[17]
	1043[16]	no data	no data	4939[16]
α-tocopherol	no data	193[11]	122[11]	216[11]
	55[15]	no data	no data	no data

a. tr: trace amount, b. nd: not detected, c. The underlined values were calculated according to specified references and oil content of 22 per cent [3].

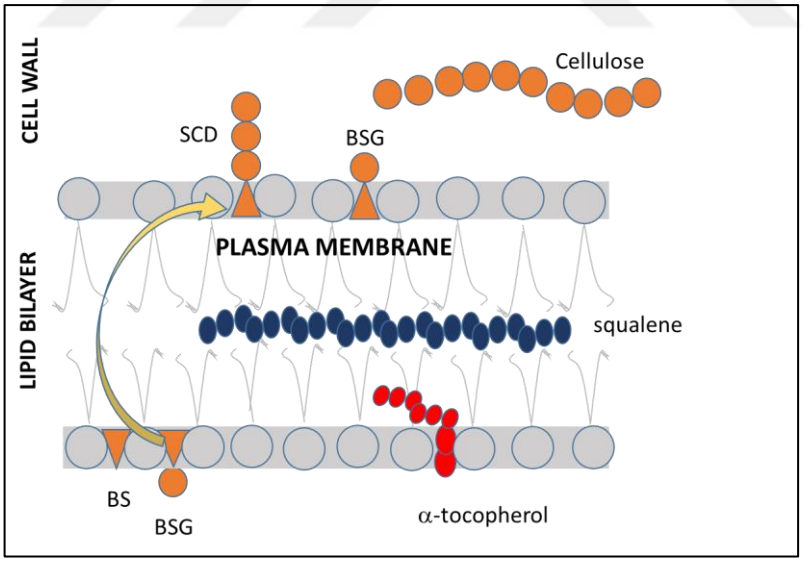


Figure 2.1. Schematic representation of olive fruit cell membrane illustrating the proposed positions of squalene, α-tocopherol and β-sitosterol in the cell membrane and during cellulose synthesis [30,31,32,33] (BS: β-sitosterol, BSG: β-sitosterol glucoside, SCD: sitosterol cyclodextrin)

A schematic representation of olive fruit cell membrane illustrating the positions of squalene, α-tocopherol and β-sitosterol based on studies on olive and plant cell membranes and proposed models of cellulose synthesis in plant cells is provided in Figure 2.1 [18-21]. Squalene is in free form and in the midplane of the lipid bilayer [19], but β-sitosterol [20,21], and α-tocopherol [18] are bound to phospholipids and proteins in the plasma membrane. While the 3β-OH group of sterols face the water interface, the side chain extends into the hydrophobic core of the cell membrane to interact with fatty acyl chains of phospholipids and proteins [22]. β-sitosterol might further interact with cellulose in the cell wall structure.

2.1.2. Lipophilic Bioactives in Virgin Olive Oil

The lipophilic bioactives squalene, sterols and tocopherols are present in the unsaponifiable fraction of olive oil (0.4–five per cent) together with phenolic compounds [6]. They are insoluble in water and soluble in oils, fats and fat solvents (hexane, ether etc.) while most of the phenolic compounds are amphiphilic [6,23,24].

Squalene is a polyunsaturated hydrocarbon of the triterpene type (Figure 2.2a), which is liquid at room temperature. Squalene, at up to 0.1-0.8 per cent (w/w), is uniquely high in VOO as compared to other fats and oils [25]. Due to its strong hydrophobic nature and its unsaturated structure, squalene is not very stable and gets easily oxidized [26]. Moreover, it protects polyunsaturated fatty acids against temperature-dependent autoxidation and UVA-mediated (320 nm – 380 nm) lipid peroxidation in olive oil, squalene acts mainly as peroxyl radical scavenger [27-29]. Decomposition of squalene in olive oil was reported as 26–47 per cent after 6 months storage in the dark and at room temperature [15].

Phytosterols having triterpenoid structure are crystalline solid at room temperature. They are present in olive oil between 1000 and 2300 ppm [30]. β-sitosterol (Figure 2.2b) is the most abundant sterol in olive oil (≥93 per cent), existing in three crystal forms: anhydrate, hemihydrate, and monohydrate [31]. Sterol composition of olive oil as specified in the International Olive Council Trade Standard, includes apparent β-sitosterol (β-sitosterol, Δ-5-avenasterol, Δ-5-23-stigmastadienol, clerosterol, sitostanol, Δ-5-24-stigmastadienol) (≥93 per cent of total sterols, w/w), brassicasterol, stigmasterol, campesterol, delta-7-stigmastenol, cholesterol [32].

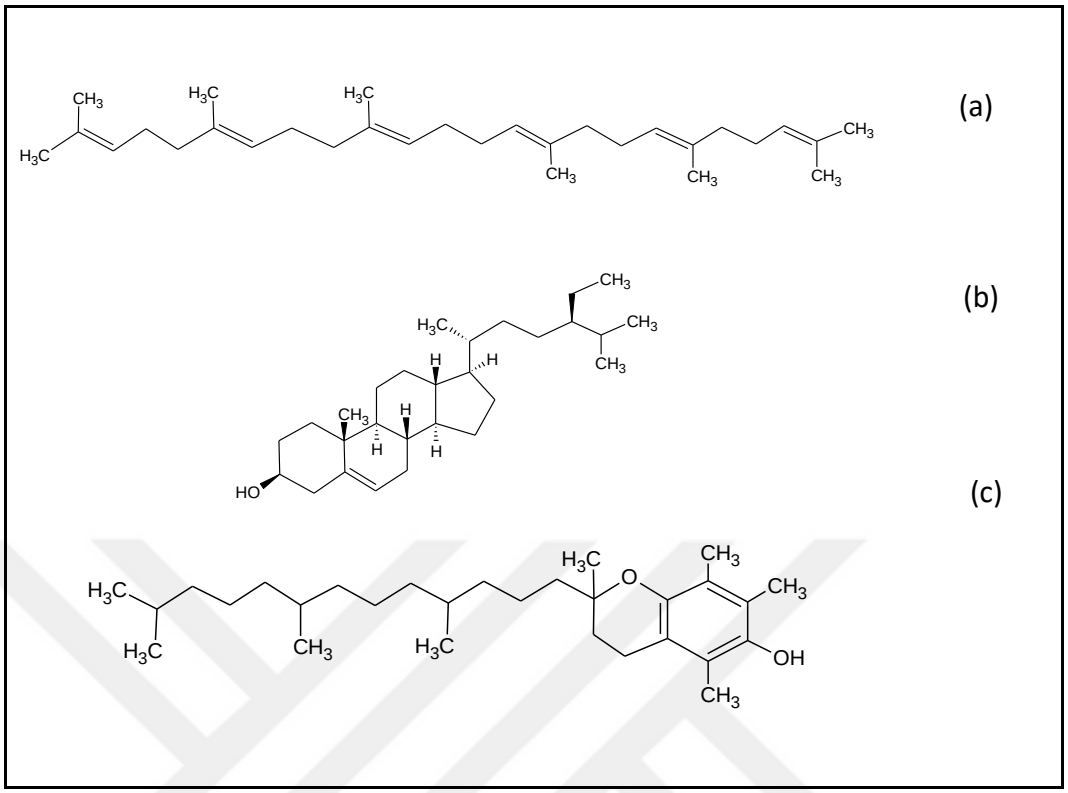


Figure 2.2. Structure of (a) squalene, $C_{30}H_{50}$; (b) β-sitosterol, $C_{29}H_{50}O$; (c) α-tocopherol, $C_{29}H_{50}O_2$

Phytosterols and their fatty acid esters are quite stable compounds, which undergo only limited degradation during heating. The phytosterol content of olive oil was not significantly affected by heating at 50°C for several weeks and at 100 °C for one hour [33]. Only harsh conditions, such as high temperatures (>100°C) in the presence of oxygen increase their oxidation rate [33, 34]. Phytosterols are mono-unsaturated compounds (double bond in the B-ring), which are much more stable than the mono-unsaturated fatty acids (e.g. oleic acid), as the steric hindrance by the ring structure prevents chemical reactions. Phytosterol esters were found to be more susceptible to oxidation at elevated temperatures than free phytosterols [35], and to have higher solubility in fats and oils and higher cholesterol-lowering activity compared to the free forms [36]. Additionally, phytosterols exhibited anticancer properties in vivo on prostate, lung, stomach, colon, ovarian and breast cancer [37, 38]. In an in-vitro study, tumor growth of a human colon cancer cell line was effectively inhibited by β-sitosterol [39]. Prostatic hyperplasia (the enlargement of the prostate) is treated clinically with β-sitosterol-containing products in Europe [40].

Tocopherols (α-, β-, δ- and γ-), which belong to vitamin E family, are particularly important bioactive constituents in vegetable oils mainly due to their antioxidative effects. Tocopherols consist of a chroman ring and a long saturated phytyl chain (Figure 2.2c). Tocopherols are viscous oils at room temperature and are slowly oxidized by atmospheric oxygen [24], they are sensitive to light, heat, alkali, and metals; therefore, they are easily oxidized to tocoquinones, which no longer have antioxidant properties [23]. In VOO, 90 per cent of tocopherols is α-tocopherol with up to 300 mg/kg concentration while others (β-, γ-, and δ-) are present in low amounts, up to 25 ppm [3].

2.2. OLIVE OIL PRODUCTION

There are different grades of olive oils based on their quality attributes and process types employed for their production; VOO, crude olive pomace oil (COPO) and refined olive oil (ROO). VOO is obtained by a mechanical process including aqueous extraction, washing, decantation, centrifugation and filtration. COPO is the oil obtained by treating olive pomace with solvents or other physical treatments (aqueous extraction). ROO and refined olive pomace oil are obtained from VOO and COPO by refining [32].

2.2.1. Virgin Olive Oil Processing

The aim of VOO processing is to extract the oil from a semisolid matrix formed by particles of skin, pulp and seed [30]. Fundamentally, VOO processing is a physical extraction process including the unit operations of milling (crushing), homogenization with thermal treatment (malaxation), phase separation (centrifugation), filtration and sedimentation. During VOO processing complex set of enzymatic and physicochemical processes occur simultaneously with extraction and separation of cellular oil.

After the washing and milling steps, physical separation of VOO is done either using the classical press system or the modern continuous two-phase or three-phase centrifugation system (Figure 2.3). The classical discontinuous pressing process was initially replaced by continuous centrifugation, using a three-phase system and later a two-phase system was introduced to minimize water consumption due to environmental, economic and quality considerations [41,42]. In addition to OO, pressing or the three-phase system also generates

solid waste (olive cake or “orujo”, containing skin, pulp, stone and olive kernel) and aqueous liquor, which comes from the vegetation water and the soft tissues of the olive fruits, and water added during processing (so-called “alpechin” or “olive mill waste water”). The use of a modern two-phase processing technique to which no water is added except washing step generates oil and a new by-product that is a combination of liquid and solid waste, called “alperujo” or “two-phase olive mill waste” [43].

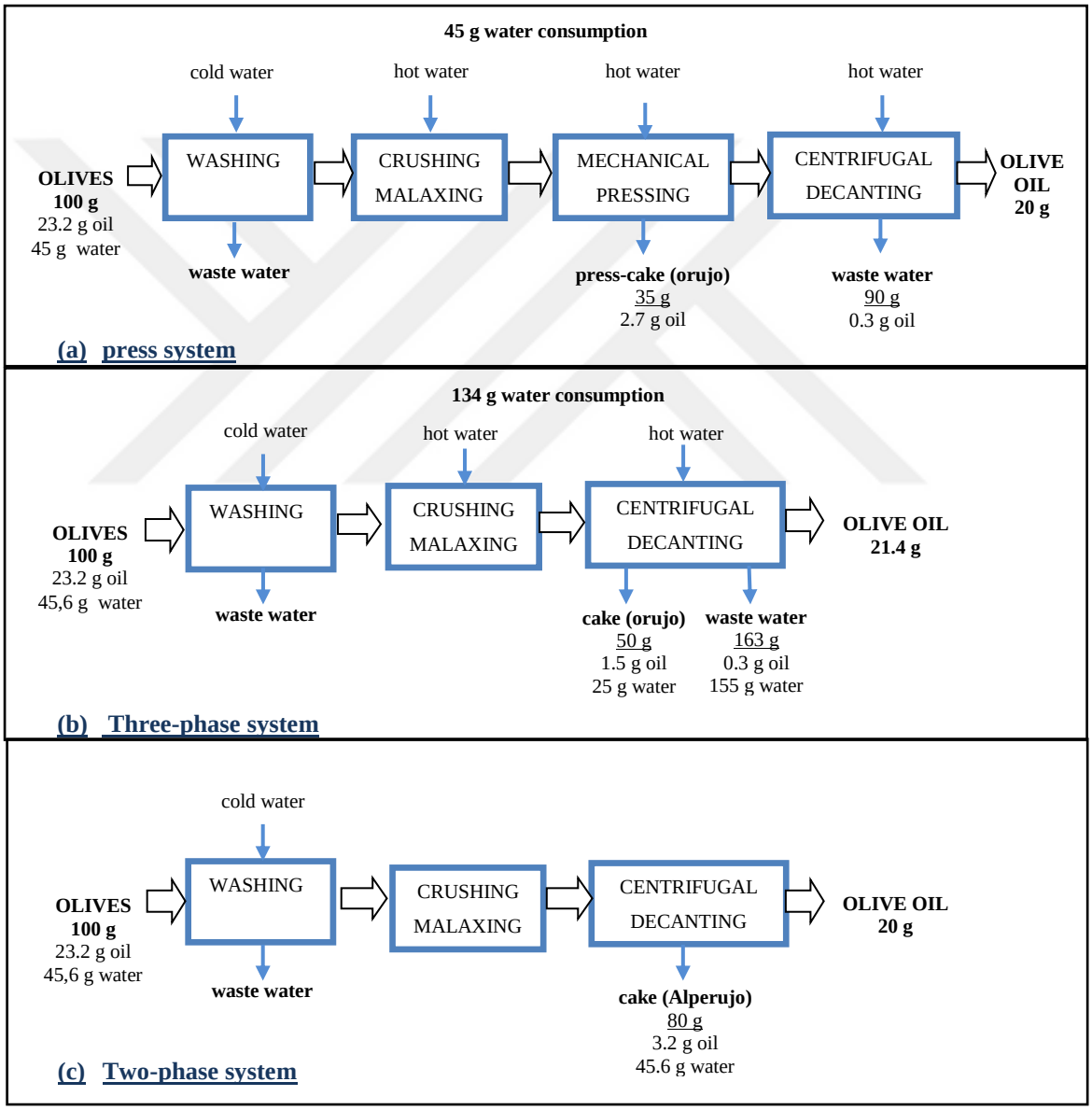


Figure 2.3. Flow chart of virgin olive oil extraction: (a) press, (b) three-phase, (c) two-phase system. Calculations based on literature, weight distribution (total, oil and water) data of all process streams [44]

2.2.2. Olive Pomace Processing

Olive pomace (orujo or alperujo) is the solid or semi solid by-product of VOO processing. Residual oil phase in the oil pomace is recovered as COPO by treating olive pomace with solvent (hexane) or physical (aqueous) extraction [32]. To differentiate oils processed by solvent extraction of orujo and second centrifugation/aqueous extraction of the fresh or stored alperujo, “second centrifugation oil” (SCOO) (40-60 per cent of the residual oil) term is used additionally. Both pomace oils have to be refined for edible use [45]. Orujo from press or three-phase system requires different preconditioning procedures prior to solvent extraction than alperujo coming from two-phase systems (Figure 2.4) due to its lower water content. three-phase pomace is dried (to approximately 8 per cent moisture) and then pitted before solvent extraction but two-phase pomace is pitted and then second physical extraction is applied before drying and solvent extraction [46]. Water contents of olive pomace from press, three-phase and two-phase system are 25-30, 45 and 55-70 per cent, respectively [42,46].

Due to its biologically active minor constituents, currently there is a growing interest in COPO [43]. Alperujo, the waste stream produced in the two-phase systems, corresponds to about 800 kg per ton of processed olives and contains 2.5-3.5 per cent of residual oil and 55-70 per cent water [42,47]. As only 40-60 per cent of residual oil can be recovered in a single centrifugation step, nowadays alperujo is treated with a second or third centrifugation step to decrease the oil content of the final waste stream to one to 1.5 per cent. Due to the low oil content, high humidity and organic load the industrial extraction of the remaining oil is not very viable. Currently, olive pomace oil is commercially sold as edible oil after refining process, which includes settling/degumming, neutralization, bleaching and deodorization steps. It should be noted that a significant portion of the vegetable oil lipophilics are lost during the refining process (especially deodorization step), which makes by-products of refining (eg. deodorizer distillates) attractive raw materials for their recovery [23,48-51].

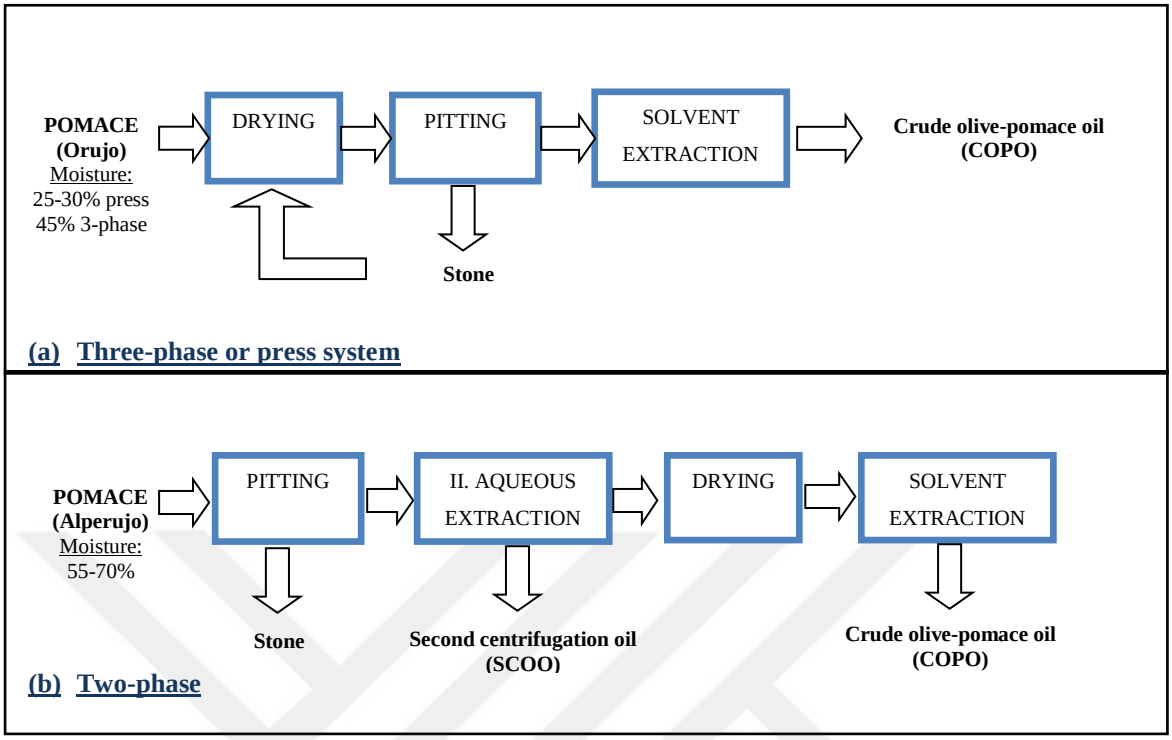


Figure 2.4. Flow chart of oil production from pomace: (a) press & 3-phase, (b) 2-phase system. [45,46]

2.3. EFFECT OF OLIVE OIL PROCESSING ON LIPOPHILIC BIOACTIVES

In order to understand the behavior of lipophilic bioactives during VOO processing, processing parameters that affect yield of VOO and lipophilic bioactives (such as malaxation time, temperature, decantation type) were reviewed.

2.3.1. Crushing

The objective of crushing (fruit milling) is to break the olive tissue in order to liberate the oil drops contained in the mesocarp cells using a crusher. Crushing is performed by stone-mills in traditional press systems and by hammer-crushers or toothed disk-crushers in continuous systems.

To obtain good process yields, appropriate size of sieves should be selected considering olive characteristics [52]. Compounds are mixed and distributed according to their affinity and

concentration between different phases of olive paste (free oil, free olive vegetation water, flexible and laminar fragments, pseudo-colloidal gel and woody endocarp fragments), which contain anatomic parts of olive fruit (epicarp (skin), mesocarp (pulp), endocarp (stone and seed)) [53]. So, a hypothetical, simplified olive paste structure, after crushing, contains: free oil, free olive vegetation water, fragments of woody endocarp and pulp [53]. Squalene is expected to be higher in free oil of olive paste while α-tocopherol and β-sitosterol mainly remain in the cell membrane fragments or complexed to the cell wall.

The crusher type affects the oil yield through its effect on the extraction mechanism. While oil diffuses across clearly cut, opened cells in hammer crushers, oil is extracted from broken and damaged cell walls due to a strong mechanical effect in stone mills. Because of this, stone mills had lower oil yields and lower total phenolic concentration in the VOO, while hammer crusher resulted in a higher total phenolic concentration (by 35 per cent) [54]. Additionally, the size of the stone fragments produced by the hammer-crushers was smaller than that obtained by the disk-crushers, which were used to obtain uncrushed olive stone together with the pulp [55,56]. Use of a hammer crusher resulted in a higher oil yield (by 20-50; 39-67 per cent, respectively) and α-tocopherol concentration (by 6.5-10 per cent; 17-20 per cent, respectively) than the blade cutter (as a disk-crusher) and mortar (as a stone mill) for two olive varieties [55].

It is reported that stronger the crushing conditions of hammer crusher (i.e. smaller grid holes and a higher rotation speed), the higher the oil yield, α-tocopherol and phenolic content in the oil due to better cell cuts [55,57]. A similar trend was observed when different crusher types (hammer mills, blade cutter and mortar) were compared with higher oil yield and α-tocopherol obtained with stronger crushing conditions [55].

2.3.2. Malaxation (Kneading)

Malaxation, which involves continuous kneading of the olive paste at a carefully monitored temperature, prepares the paste for separation of the oil. It is very relevant to the quality, yield and chemical composition of the final product as it affects the partitioning of olive components between oil and water phases and activity of enzymes, which are released during the crushing step owing to disruption of the cell tissues [58]. The efficiency of malaxation

depends mainly on malaxation time and temperature, which should never exceed 35°C and 90 min, respectively, for good manufacturing practices [59]. Other factors are oxygen concentration, and addition of coadjuvants (water, talc, salt and enzymes) for better rheological characteristics [58,60].

The malaxation time and temperature employed in the olive oil industry range from 45 to 60 min and 27 °C to 35 °C, respectively, depending on olive characteristics [58]. Oil yield increases with malaxation temperature and time. An increase in malaxation time (from 30-60 min) or malaxation temperature (from 27-35 °C) increased VOO extraction recovery (by up to 2.5 per cent) [58,61], as the small oil droplets merge into large drops due to a reduction in the oil viscosity with increasing temperature [62]. In their study on the effect of malaxation temperature (15, 30, 45, 60 °C) and time (30, 60, 90, 120 min) on oil yield, Kalua et al. (2006) obtained the lowest (33.8 per cent) and highest yield (44.3 per cent) at 45 °C, 30 min and 30 °C, 90 min, respectively [63].

Malaxation conditions affect extraction of bioactive compounds to a lesser extent. Malaxation at 30°C and 45°C yielded similar β-sitosterol, stigmasterol, Δ5-avenasterol concentrations and campesterol/stigmasterol ratio in VOO (75.6 and 75.9 per cent, 0.8 and 0.9 per cent, 15.5 and 15.1 per cent, five and 4.8 per cent, respectively) with the highest effect observed for stigmasterol (13 per cent increase with temperature) [64]. In another study, malaxation temperature (18, 28, 38 °C) and time (15, 30, 60 min) did not have much of an effect on β-sitosterol per cent of total sterols in VOO (84.21, 84.05, 84.33 per cent), but as the temperature increased from 18 to 38 °C for 30 min total sterol content increased by 15 per cent (from 1670 to 1924 ppm) [9]. Similarly, squalene content of three extra virgin olive oil varieties was not affected by malaxation temperature (20, 25, 30, 35 °C) for 60 min malaxation [65]. α-tocopherol content in three VOO varieties increased approximately by 27 per cent with malaxation temperature in the range of 20-35°C [62]. Additionally, highest concentration of total phenols (170 mg/kg) was obtained at 27 and 30 °C (for 45 min) in the temperature range of 20-36°C [62,66].

2.3.3. Centrifugation

During VOO processing separation of liquid (oil) and solid/semisolid phase (olive paste) takes place during the centrifugation step (Figure 2.3). Two-phase and three-phase continuous centrifugation systems are progressively replacing the classical-press system and percolation (selective filtration by metal discs/plates dipped into the paste).

While pressing produces pomace with lower moisture content and lower amount of vegetation water, it has several drawbacks such as high labor requirements, cost of the filtering diaphragms and the discontinuous manner of processing [42]. Three-phase processing with continuous centrifugation requires addition of warm water into the olive paste (Figure 2.3), resulting in production of high amount of wastewater and loss of phenols posing a great environmental problem [41,42]. Technological progress led to the development of a two-phase centrifugation system minimizing wastewater [67]. The two-phase system has a comparable oil yield to the three-phase system [44], yet has the disadvantages of difficulty in recovery of residual oil, economic problems with waste utilization associated with high moisture content of the solid waste and increased transportation costs [42].

The study that compared indirect centrifugation after percolation and direct centrifugation with horizontal centrifuge by processing three olive types reported higher total sterols (by six to 19 per cent), total tocopherols (by 18-75 per cent) and total phenolics (by five to 62 per cent) contents in VOO for direct centrifugation, but oil yields were not statistically different ($p \leq 0.05$) [68]. Addition of water (300 L/h) after percolation also resulted in a higher amount of wastewater (0.72 vs 0.10 m^3/ton olives average).

Classic (press and vertical centrifuge), two- and three-phase systems produced oils with small but reportedly significant changes in the amount of β-sitosterol (76.0, 75.7 and 75.5 pre cent total sterols) and campesterol/stigmasterol ratio (4.5, 5 and 5.1) [64]. In another study, stigmasterol content was higher in three-phase system extracted oils, whereas higher apparent β-sitosterol concentration was obtained using the press system [69]. Similar values of β-sitosterol content (as per cent of total sterols) were reported for two- and three- phase systems for three varieties of olives but total sterol concentrations (mg/100 g) varied by five, six and -10 per cent depending on olive variety [70].

Although extraction system affected the level and nature of other quality characteristics (such as aroma, total phenolic content), no observable differences were found between α-tocopherol levels of VOO obtained using two-phase, three-phase and classical-cold press extraction systems [71]. These results are in agreement with those reported for Italian oils by Ranalli and Angerosa (1996) [70]. Other studies reported higher α–tocopherol contents for two-phase decanter than a laboratory mill and press system (by eight and 33 per cent, respectively), however both higher and lower values (by 11 and 4 per cent) were reported compared to a three-phase decanter [69,72].

2.3.4. Process Improvements

Processes and applications such as enzyme-assisted extraction, nitrogen application, ultrasound, microwave and hydrothermal treatment of pomace, which are being investigated to improve oil yield and quality, might also influence the bioactive content of olive oil.

2.3.4.1. Enzyme Assisted Extraction

The use of enzyme preparations in olive oil processing has been widely investigated to improve oil yield. In this technique, enzyme solutions are usually added before malaxation mostly in liquid form, and as they are water soluble, they are completely removed in the vegetation water without leaving any residue [73]. It should be noted that industrial utilization of enzymes is not permitted in VOO processing [74].

During physical extraction of olive oil, water and solid phases need to be separated more thoroughly to increase the oil extraction yield. Often the crusher does not break all the cells, so minor compounds such as phenols and tocopherols cannot be extracted and remain in the olive pomace [75]. The enzyme formulations containing pectinase, cellulolytic and hemicellulolytic enzymes degrade the walls of the oil bearing cells that remain after crushing, and break down the colloidal particles such as pectins, hemicelluloses, proteins that retain the oil droplets. The enzyme breaks up the liquid/solid and liquid/liquid emulsions improving the rheological characteristics of the paste. Oil droplets are thus released and gradually merge into larger free droplets, which are favorable for mechanical extraction [74,76,77].

The enzymatic formulations used in the olive oil studies are Cytolase® 0, Rapidase adex D®, Bioliva® and Olivex®, Glucanex®, Novoferm® 12, Viscozyme®, Pectinex® Ultra SP–L (nowadays concentrated form used Pectinex® Ultra Olio) [74,78,79].

Effect of enzymatic treatment on cell wall structure and accordingly the oil yield depends on the enzymes used. Oil yield increases of five to 12; 11-15; one to two per cent were reported for different enzyme formulations (cytolase (pectolytic); formulation of Olivex (rich in pectinolytic, hemicellulolytic and cellulolytic side activities) and Glucanex (β-glucanase) in two concentrations; and Cytolase 0 (pectolytic), respectively) [13,77,80,81].

Studies on the effects of enzyme treatment on bioactive compounds in olive oil showed an increase in the concentration of tocopherols and and phenolics in addition to oil yield, whereas squalene and sterols contents were not affected. For example, cytolase enzyme applied to different olive varieties in a two-phase system increased the oil yield by five to 12 per cent w/w, phenolic compounds by 11-17 per cent w/v, and α-tocopherol by one to 14 per cent w/w [77]. However there was no trend for the effect on total sterols and β-sitosterol content of olive oil (within six and two per cent). In another study [80], mixture of the enzyme formulations Olivex and Glucanex improved oil yield by 15 per cent w/w and 66 per cent w/w increase was observed for total phenolic content. On the other hand, the sterol and β-sitosterol content was not affected by enzyme treatment. Exogenous "Cytolase 0" enzyme complex used in de-stoned olives resulted in one to two per cent increase in oil yield, significant increase in tocopherol content (15-23 per cent), but had no significant change on squalene content ($p<0.05$) [80].

According to these results, enzyme assisted extraction has an impact mainly on oil yield and concentration of phenolics and tocopherols rather than squalene and sterols. These results might be attributed to the differences between location of these compounds at the cellular level and their interactions with other components as described in Section 2.1.1.

The use of enyzmes for pomace oil extraction was also investigated [82]. In a study by Novozyme, an enzyme manufacturer in Switzerland, enzymatic oil extraction from olive pomace was carried out using commercial enzymes, Viscozyme® L and Celluclast® 1.5 L [82]. Enzyme preparation (Viscozyme® L (150-300 ml/t pomace) or Viscozyme® L (100-200 ml/t pomace) in combination with Celluclast 1.5 L (40-80 ml/t pomace)) was diluted by 10 per cent in cold clean tap water, to add in specified dosages. Recommended temperature

was approximately 45°C and holding time was one to two hour. The oil yield increased (by 15-38 per cent) and pomace oil quality improved due to decrease in free acidity accompanied by an increase in the content of polyphenols and tocopherols accordingly reducing rancidity.

2.3.4.2. De-stoning

In the last few years, much attention has been paid to de-stoned olive processing to improve quality of VOO; and obtain higher levels of green aromas, tocopherol content, phenolic content and improved oxidative stability [80,83,84]. De-stoning decreased the oil yields by approximately 1.5 per cent due to the absence of the draining effect of pit pieces during malaxation and absence of seed oil [11,16,80].

Studies on phytochemical composition of VOO processed from whole olive fruit and de-stoned olive fruit showed differences according to the olive seed/pit content [80,85,86]. De-stoned oils had higher contents of biophenols and pleasant volatile compounds especially trans-2-hexenal, hexanal and cis-3-hexen-1-ol [80,85,86].

Although effects of de-stoning on tocopherol content have been reported, no trend could be established: four to 27 per cent increase [80,85], and one to 12 per cent reduction [83,87,88] were observed in the concentration of total tocopherols after de-stoning. Combining nitrogen flushing with destoning lead to a 16 per cent increase in tocopherol concentration instead of a six per cent decrease obtained with destoning alone [82]. Destoning did not have an effect on squalene concentration of olive oil [80].

Removal of olive seeds, which are richer in phytosterols than olive pulp as discussed in Section 2.1.1, by destoning did not affect the concentration of sterols, mainly β-sitosterol, in olive oil significantly [82,84,88] as olive seeds constitute a small proportion (~two to three per cent) of the whole fruit compared to pulp (78-85 per cent) [8].

2.3.4.3. Nitrogen Application

Inert gases like nitrogen have been used during malaxation to preserve the olive paste from oxygen contact in order to limit oxidation reactions extending shelf life of VOO [81,83,89]. Although some of the enzymes present in the olive fruit are deactivated during the oil

extraction process or crushing step, enzymes such as lipoxyganase and polyphenol oxidase retain detectable level of activity in olive paste and in VOO [76]. These are the enzymes that catalyze the oxidation of fatty acids, volatiles and antioxidant bioactive compounds (such as tocopherols and phenolic compounds) under atmospheric conditions, in the presence of oxygen [57,83,90]. Additionally, autoxidation of fatty acids leads to olive oil deterioration in the presence of oxygen and light during storage. The use of a nitrogen flush during malaxation to remove oxygen leads to higher resistance to oxidation during processing, and storage and extend shelf life of VOO [57,83,90].

Malaxation process under inert gases, nitrogen and argon, increased the tocopherol concentration of olive oil (by 11 per cent and by 37 per cent -when destoning applied) due to protective effect of nitrogen against oxidation [83]. However, malaxation under nitrogen had no significant effect on sterol concentration of VOO ($p<0.05$) [83]. Thus nitrogen application mainly affects the antioxidants in VOO such as tocopherols and phenolic compounds, which are susceptible to oxidation, whereas sterols, which are relatively stable during processing, are not affected.

2.3.4.4. Ultrasound Assisted Extraction

In addition to enzymes, ultrasound is also used as a processing aid for extraction from edible oils [91]. The mechanical effect of ultrasound improves mass transfer by enabling the release of soluble compounds from the olive tissues by disrupting cell walls [75].

Ultrasound use in aqueous extraction of olive oil was reported [92]. In this method, malaxation was performed using ultrasound devices with indirect and direct sonication, which provided quick-heating of the olive paste (at 25 and 24 kHz, 30ºC for 30 min, respectively). This method, which increased the oil yield by two to seven per cent, did not alter the quality parameters (fatty acid composition, free acidity value, peroxide value, K270 and K232) of the oil that was produced. Moreover, there was no trend in tocopherol content of samples from different harvest periods and off-flavor volatiles were not detected in oils, however, total phenolic content decreased [92].

Ultrasound-assisted VOO extraction processes was tested against the traditional method, by applying sonication treatment on olives submerged in a water bath (before crushing) and on

olive paste (after crushing). Better extractability (from 84 per cent untreated to 89 per cent than 87 per cent) and higher content of minor compounds (total tocopherols, phenols, chlorophylls and caretenoids) were obtained by sonicating the olives submerged in a water bath than by sonicating olive paste [75].

2.4. DISTRIBUTION OF LIPOPHILIC BIOACTIVES IN OLIVE OIL AND WASTE STREAMS

While oil yield data provides a useful measure of the extent of oil loss during olive oil processing, data on the lipophilic content of input and output streams are required to evaluate the distribution of lipophilic bioactives. Literature studies having lipophilic composition data of oil and waste samples taken from the same processing run were used for calculation of lipophilic bioactive loss (Table 2.3). However such literature data was rather limited for β-sitosterol, α-tocopherol and squalene with only one set of data available for each compound [9,15].

Table 2.3. Loss of lipophilic bioactives during VOO processing

	Squalene[15]	**α-tocopherol**[15]	**β–sitosterol**[9]
Olive Fruit (mg/kg fruit)	959	55	568**
VOO (mg/kg oil)	4083	184	1550
System*	Press	press	Abecor®***
% loss	19	36	48
% recovery	81	64	52

*oil extracted by specified industrial system with 0.19 kg oil/ kg fruit oil yield
**Calculated by using 0.21 kg yield determined using Soxhlet,
***Abencor system has 1.5-2 per cent lower oil yield (0.19 kg oil/ kg fruit oil) than an industrial process (0.21 kg oil/ kg fruit for 3-phase) [93]

The recovery of VOO is around 80-98 per cent (w/w) with the remaining oil being lost in the waste streams mainly in the pomace [42,44,94]. While oil loss in the two-phase system and three-phase system is similar (three to four per cent), highest amount of oil loss occurs using the press system (7.6 per cent) (Figure 2.3) [44].

Loss of β-sitosterol, α-tocopherol and squalene (per cent) during VOO processing was calculated by determining the per cent of the lipophilic content of the olive fruit that was recovered in VOO, as 48; 36; 19 per cent, respectively (Table 2.3). These values mainly represent the amounts lost in the waste streams, yet degradation during processing should also be considered. Unlike phenolics, a significant portion of which is lost with the wastewater (53 per cent) during crushing and malaxation [95,96], these bioactives are expected to be mainly retained in the pomace due to their lipophilic nature. The calculated loss values are also supported by comparably higher bioactive concentration of oil extracted from fresh alperujo (3092 mg/kg squalene, 2495 mg/kg β-sitosterol, 328 mg/kg α-tocopherol, 2866 mg/kg aliphatic alcohols, and 436 mg/kg triterpenic alcohols) [97]. After steam treatment of fresh or stored alperujo, pomace oil contained 4860 mg/kg squalene, 5812 mg/kg β-sitosterol, 407 mg/kg α-tocopherol, 7065 mg/kg aliphatic alcohols, 1220 mg/kg triterpenic alcohols as a maximum values. β-sitosterol and α-tocopherol values were higher than VOO values by 153 per cent and 36 percent, respectively. In another study, tocopherols and sterols were reported as 250 mg/kg and 1500 mg/kg in olive oil and 290 mg/kg and 3500 mg/kg in olive pomace oil, respectively [48]. The bioactives lost during VOO production remain in the pomace, which is further extracted for residual oil in pomace plants.

Comparison of the yield of VOO (81 per cent, w/w) and lipophilic bioactives (52, 64, and 81 per cent) shows that while the yield of squalene, which is mainly present in olive pulp in free form, was similar to that of VOO, oil yield was not predictive of the recovery of β-sitosterol and α-tocopherol. The concentration of these compounds in the seeds of the olive fruit (Table 2.2) and their interaction with other compounds such as proteins and phospholipids might limit their extractability using mechanical means resulting in lower yield/higher loss values. Recovery of these compounds, particularly β-sitosterol, can thus be improved by facilitating their release from the solid matrix mainly focusing on their interactions with other matrix components. Interactions with cellulose might be further limiting recovery of β-sitosterol and requires further attention. Degradation behavior of tocopherols as affected by malaxation conditions should also be considered when interpreting processing loss of tocopherols.

Loss of squalene, β-sitosterol and α-tocopherol during conventional olive pomace processing (oil of fresh, stored, dried and solvent extracted pomace) could not be assessed

due to lack of literature data on lipophilic bioactive content of the related process streams. The waste stream of two-phase VOO production, alperujo, is composed of 55-70 per cent moisture and ground olive stones and pulp, containing lignocellulosic material [1]. According to a study about olive stone [43], which is around five per cent of the olive fruit [1], it contains hemicellulose (22–28 g/100 g dry matter), cellulose (30–34 g/100 g dry matter) and lignin (21–25 g/100 g dry matter) as main components.

2.5. DETERMINATION OF LIPOPHILIC BIOACTIVES IN OLIVE OIL: SQUALENE, α-TOCOPHEROL AND β-SITOSTEROL

The analytical methodologies utilizing HPLC or GC techniques for the detection and determination of predominant lipophilic bioactives in olive oil (β-sitosterol, squalene and α-tocopherol) may include a number of sample pretreatment steps (such as cleanup using (TLC or SPE, saponification and derivatization) making them rather lengthy and impractical [98-109].

2.5.1. Current Methods

The sterol content of olive oil is typically determined by saponification, isolation of unsaponifiable fraction, TLC, which is the conventional method to separate the sterols, and/or GC-flame ionization detector (GC-FID) [98, 99]. Alternative to the time consuming TLC method, SPE applications were also studied [100, 101]. LC-MS [102, 103], GC-MS [104] and LC-GC [105] methods have also been used for the analysis of sterols in olive oil.

Sterols in olive oil are also analyzed in multicomponent analysis by various detection techniques such as with erythrodiol and uvaol by GC-SPE [106], with tocopherols and triterpenic alcohols by LC-MS [107], with tocopherols by GLC–FID [108] and by GC-FID [109], with their free and esterified forms by GCMS-SPE [100] and with triterpene alcohols by GLC-TLC [110]. These chromatographic methods include different sample pretreatment steps such as saponification by ethanolic KOH at different concentrations (0.35 N, one or two N) [100, 106, 109] and temperature time combinations (at 80ºC for 40 min [100, 106] or for 30 min [100, 107], at 90ºC for 10 min [108] and at 50ºC for 60 min [109], extraction by organic solvents (ethylether (3x6 mL) [106, 107], ethyl acetate [106], cyclohexane [108],

hexane [109]), and phase separation by centrifugation [108] or using a separation funnel [106, 107, 109]. Several washing steps with additional saponification by KOH (0.5 N) [100, 107], antioxidant addition for tocopherols (pyrogallol [108], vitamin C [109]), drying of organic solvent by anhydrous sodium sulphate [100, 107, 108], and additionally SPE [100, 106] or TLC [110] and membrane filtration prior to LC analysis [107], were also used. Various derivatization reagents and conditions such as TMS silylation by 9:3:1 pyridine/hexamethyldisilazine(HMDS)/ chlorotrimethylsilane (TMCS) (v/v/v) at RT [123], by 1:1 pyridine and the mixture of N,O-bis(trimethylsilyl)acetamide–trimethyl-silylchlorosilane–trimethylsilylimidazole (3:2:3, v/v/v) at RT for 15 min [108] and by Sylon BFT (99 per cent BSTFA +1 per cent TMCS) at 50ºC for 60 min, at RT for overnight [109] and 70ºC for 25 min [100] were also used for GC analysis.

Squalene is commonly analyzed by GC without derivatization [99]. Tocopherols are typically analyzed by HPLC due to their low thermal stability [111-114].

Multicomponent analysis of olive oil and other vegetable oils for the determination of sterols, squalene and tocopherols was studied mainly using separate methods (such as GC, GCMS, GLC, HPLC, LCMS, APCI-MS) [98-115] except for a few studies on simultaneous determination techniques; by GC-FID [116], by HPLC [117] and by LC-GC in olive oil [118]. In sample pretreatment of the GC-FID method [116], the unsaponifiable fraction was directly derivatized (silylation reaction) eliminating TLC fractionation, but only relative standard deviations (RSDs) of the analytes (n=3) were reported (sterols; 2.89-3.59 per cent, squalene; 1.90 per cent, α-tocopherol; 3.63 per cent). In the rapid LC-GC method for edible oils [118], sample preparation was integrated into the GC by on-line coupling of reversed-phase liquid chromatography (RPLC- GC) for direct injection using a programmed temperature vaporizer (PTV) as interface. But only RSD (n=3, six per cent from peak areas) and limit of detection (LOD) (0.3 ppm) of squalene were reported for olive oil [118]. These methods were not fully validated covering all parameters; linearity, limit of quantification (LOQ), LOD, trueness and precision.

2.5.2. Method Validation

Performance parameters that must be established for in-house developed quantitative methods for the analysis of main components and trace amount components are selectivity,

LOD, LOQ, working range including linearity, trueness (bias) and precision (repeatability and intermediate precision) [119]. Blank samples, routine test samples, spiked materials, measurement standards, incurred materials can be used as a validation tool according to purpose of these performance parameters and the method.

Selectivity shows the method can be used to determine analyte(s) in mixtures or matrices without interferences from other components with similar behaviour [119]. Selectivity of the method can be checked using standard mixture and routine samples to determine wheter or not detection and quantification are inhibited by any interferences.

Several approaches for determining the LOD and LOQ are possible depending on wheter or not an instrument ise used in the procedure. LOD and LOQ can be determined based on calibration curve using samples containing an analyte in the range of detection limit and the residual standard deviation s(r) of a regression line may be used as the standard deviation [120]. LOD and LOQ may be expressed as:

$$LOD = 3.3\ x\ \frac{s(r)}{m} \tag{2.1}$$

$$LOD = 10\ x\ \frac{s(r)}{m} \tag{2.2}$$

$$s(r) = \sqrt{\frac{\sum_{i=1}^{n}(y_i - \hat{y}_i)^2}{n-2}} \tag{2.3}$$

where

s(r): residual standard deviation
m: slope estimated from calibration curve
y_i : the observed value of y for a given value of x_i
$\hat{y}_i$: the value of y predicted by the equation of the calibration line for a given value of x_i
n : the number of calibration points.

The ‘working range’ is the range providing an acceptable uncertainity for the method. The lower end of the working range is bounded by the LOQ [119]. The upper end of the working

range should be the concentration where analytical sensitivity is obtained by showing accuracy of the method at that level.

Linear range might be different from working range. Linearity can be evaluated by drawing the calibration graphs and analyzing the coefficient of determination ($r^2 \geq 0.9$) and p value ($p<0.05$) from regression statistics [121]. Calibration range should be determined considering concentration range of analyte in the routine sample.

Accuracy of a measurement depends on trueness and precision of the method. Trueness can be determined by calculating bias from a reference sample or by calculation of recovery from a spiked sample [119]. Recovery (per cent) is calculated as:

$$Recovery = \frac{(c_s - c_b)x\ m}{c_a}\ x\ 100 \tag{2.4}$$

where,

c_s: measured mass fraction of the analyte in spiked sample (mg/mg)
c_b: measured mass fraction of the analyte in unspiked matrix blank (mg/mg)
c_a: calculated mass fraction of the analyte added in unspiked sample (mg/mg)
m: mass of the unspiked sample (mg)

Precision of a method can be measured by repeatability, which is a measure of the variability in results produced by a single analyst using the same equipment over a short timescale. Repeatability is calculated as the relative standard deviation (RSDr) of replicate results produced by a single analyst. RSDr can be evaluated according to the HORRAT(r) ratio. The empirical acceptable range for HORRAT(r) ratio should be between 0.3-1.3, which is calculated by per cent RSDr divided by per cent predicted relative standard deviation (PRSDr) (Eq. 2.6) [122, 123].

$$HORRAT_r = \frac{RSD_r}{PRSD_r} \tag{2.5}$$

$$PRSD_r = C^{-0.15} \quad (2.6)$$

$$RSD_r = \frac{s_r}{C} x100 \quad (2.7)$$

where,

sr: standard deviation of mass fraction (mg/mg)
C: mass fraction (measured mass of the analyte in the sample/mg sample)

For reporting HORRAT values, the data must be reported as mass fraction where the units of the numerator and denominator are the same: e.g., for 100 per cent (pure materials), the mass fraction C = 1.00; for one μg/g (ppm), C = 0.000001 [122].

2.6. USE OF HYDROTHERMAL PRETREATMENTS FOR THE RECOVERY OF β-SITOSTEROL FROM OLIVE POMACE

Hydrothermal pretreatments including auto-hydrolysis, aqueous liquefaction or extraction, steam pretreatment or steam extraction, subcritical water (SCW) and supercritical water are innovative green technologies based on change of the properties of water by temperature increase and recently used for olive waste processing. From ambient temperature (>100°C and 0.1MPa) to supercritical conditions (374°C at 22.1MPa), characteristics of water changes from an ionic to non-ionic solvent, its polarity and pH value decreases and its reactivity increases [124,125]. Accordingly reactions; carbonization (100-200°C), liquefaction (200–350°C; 5–28 MPa) and gasification (350-750°C) takes place [124].

Steam explosion (160-260°C, 0.69-4.83 MPa) and subcritical water (SCW) (100-374°C and 0.1-22.1MPa) have been used for hydrolysis of hemicellulose, alteration of properties of cellulose and lignin from alperujo and OMW [124-130] and recovery/extraction of phenolic compounds [127] and supercritical water can be used to produce hydrogen and other gases by gasification of biomass such as OMW (7.71 mL/mL OMW, 550°C, 30 s, 100-300 bar) [131].

2.6.1. Steam Pretreatment

Steam pretreatment of olive stones and olive cake followed by rapid decompression (steam explosion) 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 [127,132-136]. In these studies, olive stone or alperujo was pretreated with steam explosion in a two liter reactor to recover hydroxytyrosol and soluble carbohydrates; from olive stone at 200-236 ºC for 2-4 min with/without acid [127,128,135] and from alperujo at 160-240 °C for 2-10 min with acid [132,136]. Steam explosion at 200 °C for five min resulted in reduced hemicellulose (75-88 per cent), lignin and protein content (50 per cent), and a separate liquid fraction containing hydroxytyrosol, oligosaccharides, glucose, mannitol by auto-hydrolysis [47]. Solubilization of lignin fragments resulted from de-polymerization of polysaccharides (mainly hemicellulose) and breaking of the lignin-carbohydrate bonds [133]. Further degradation of monosaccharides lead to hydroxymethylfurfural (HMF) formation at higher temperatures.

In another reactor (100 L reactor), steam treatment of alperujo (150-170 ºC; 15-90 min) resulted in a reduction in solids (up to 35.6-47.6 per cent) and an increase in oil yield and sterols (up to 97 per cent and 33 per cent, respectively) due to solubilization of cell wall material and release of bound oil and sterols [97]. Even at 160°C after 15 min, a significant increase in the level of β-sitosterol was observed [97]. After steam treatment (170°C for 15 min) [137], acid hydrolysis, ultrafiltration and column chromatography were applied to obtain phenols and oligosaccharides.

2.6.2. Subcritical Water Pretreatment

In addition to being an environmentally friendly extraction solvent, subcritical water is a unique and sustainable reaction/pretreatment medium (100-374°C and 0.1-22.1MPa) [124]. An increase in process temperature reduces the reaction time and changes the reaction mechanisms. While ionic reactions prevail at low temperature, high temperatures promote homolytic bond cleavage and thus the formation of radicals. Thus radical reactions occurs that hydronium ions generated from water auto-ionization are involving all ionic reactions

as a reactant 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 which decreases the pH [126, 138].

In the subcritical region the density of water remains constant between 100°C to 374°C so the pressure effect is minimal [124,139]. Hydrolysis reactions take place at 150-230°C (for cellulose degradation above 210-220°C, for significant increase in reaction rate of oil hydrolysis above 200°C) [126, 140]. SCW pretreatment (140-170°C, 10-13 atm) with formic acid addition, was reported as increasing the saccarification yield of OMW for bioethanol production [129].

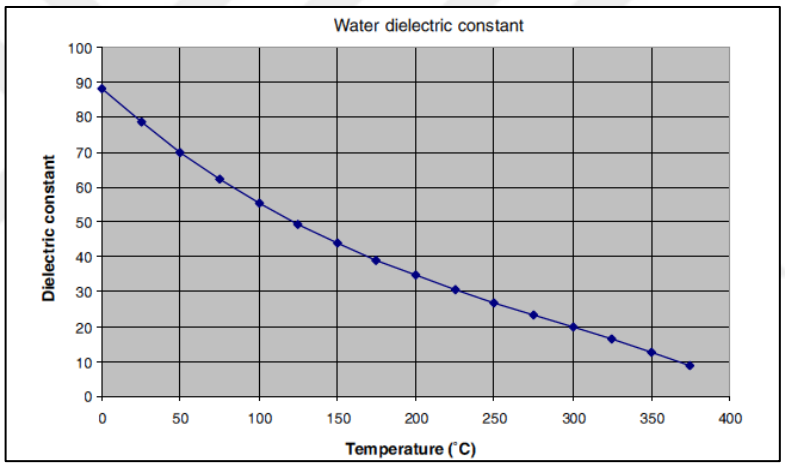


Figure 2.5. Graphical representation of dielectric constant of water versus temperature [141]

High dielectric constant (ε = 80 at RT) of water can be significantly decreased to values close to 35 (close to those of acetonitrile and methanol) when water is heated up to 200 ºC (Figure 2.5) 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 [125, 138, 141-150], in addition to phenolics. SCW extraction of olive pulp (160°C, 30 min) was more efficient than methanol extraction (two hour) for the recovery of chlorogenic acid, homovanillic acid, gallic acid, hydroxytyrosol, quercetin, and syringic acid [150]. Cotton seed oil was extracted (180-280°C, 5-60 min) with optimum yield at 270°C for 30 min (1:2) [142]. Soybean oil and protein were extracted with optimum yield at 150°C without

solid:liquid ratio effect and 66°C (1:11.7) depend on using extruded or not extruded soybean flakes, respectively [138]. Sunflower oil with high antioxidant capacity was extracted with highest yield at 130°C for 30 min (1:20) but refining was needed after SCW extraction due to higher free fatty acids (FFA) (>2 per cent) [151].

Recovery of sterols from plant matrices might be increased by cleaving the bonds and interactions with other cellular components such as phospholipids and proteins bound to plasma membrane and cellulose microfibrils using hydrothermal treatments, alternatives to acid hydrolysis [20,21,124]. Based on previous steam pretreatment studies of alperujo, SCW and steam explosion might be used to obtain sterol enriched oil and recover phenolics at the same time.

The general objective of the thesis is to recover sterols from olive pomace using hydrothermal pretreatments which enable recovery of phenolics, to produce sterol rich pomace oil to be used as a functional food ingredient source and phenolic extract. The specific objectives are:

- develop a simultaneous method for lipophilic bioactives (squalene, α-tocopherol and β-sitosterol) in olive oil by improving sample preparation steps and optimizing the critical factors using response surface methodology (RSM), and to carry out in house method validation based on method performance parameters.
- to determine the distribution of pre-dominant lipophilic bioactives (squalene, β-sitosterol and α-tocopherol) between olive oil and waste streams, and to calculate loss of these lipophilic bioactives during industrial VOO production using simultaneous GC-FID analysis of samples (olive fruit, VOO, SCOO, Pomace, OMW) taken from a two-step milling integrated plant.
- to investigate the effect of hydrothermal pretreatment (steam and SCW) and pretreatment temperature on the yield and BS content of pomace oil and phenolics.

3. A RAPID IN-HOUSE VALIDATED GC-FID METHOD FOR SIMULTANEOUS DETERMINATION OF LIPOPHILIC BIOACTIVES IN OLIVE OIL: SQUALENE, α-TOCOPHEROL AND β-SITOSTEROL

3.1. INTRODUCTION

In recent years, bioactive compounds, their health benefits and their composition and content in raw materials, products and by-products of the food industry, have been widely investigated [152, 1, 29, 153-155]. In this context, development of rapid and accurate analytical techniques to be used in research and quality assessment in the industry is an important area of research. Lipophilic bioactive compounds in olive oil (such as sterols, squalene and tocopherols) have received increased interest due to their health benefits [152, 29, 155] and contributions to product quality and shelf life [115,156].

The objectives of this study were to a) develop a simultaneous method for lipophilic bioactives (squalene, α-tocopherol and β-sitosterol) in olive oil by improving sample preparation steps (derivatization, saponification and phase separation) and optimizing the critical factors (time and temperature of saponification and derivatizaton) using response surface methodology (RSM), and b) to carry out in house method validation based on method performance parameters (LOQ, LOD, trueness and precision [120-123]).

3.2. MATERIALS AND METHODS

3.2.1. Samples

Virgin olive oil sample used for preliminary experiments were obtained from a commercial store in Istanbul, Turkey and virgin olive oil sample used for RSM experiments and method validation was obtained from an olive oil plant in Ayvalık, Turkey.

3.2.2. Reagents

Squalene (99.0 per cent) and β-sitosterol (100 μg/ml in chloroform, analytical standard) were obtained from Supelco (PA, US). (±)-α-tocopherol (97 per cent, HPLC grade), pyridine (anhydrous, GC Grade, 99.8 per cent), ethanol (absolute, GC grade) and chloroform (GC grade) were obtained from Sigma-Aldrich (Damstadt, Germany). 5α-cholestane (99.5 per cent, GC Grade) and 5α-cholestan-3β-ol (98 per cent) were obtained from Sigma-Aldrich (Rehovot, Israel and Dorset, UK). Triolein (Glyceryl trioleate, 61 per cent), hexamethyldisilazane (HMDS) (99.9 per cent, reagent plus) and n-hexane (chromasolv®, GC grade) were obtained from Sigma-Aldrich (MO, US). Trimethylchlorosilane (TMCS) (GC Grade), potassium hydroxide (pellets), pyrogallol and sodium chloride (ACS grade) were obtained from Merck (Damstadt, Germany). β-sitosterol (ca 10 per cent campesterol, ca. 75 per cent β-sitosterol) was obtained from Acros Organics (Geel, Belgium).

3.2.3. Method Development

The methodological framework, which was based on literature [106,109,116], was modified to increase method performance (Figure 3.1). Then, critical parameters (derivatization temperature (T_d), derivatization time (t_d), saponification temperature (T_s) and saponification time (t_s)) were optimized using RSM.

3.2.3.1. Saponification and Phase Separation

Firstly, the effects of KOH concentration (two M and 0.35 M) and antioxidant addition (Figure A.1-A.2) before saponification reaction (pyrogallol (three per cent) in ethanol and vitamin C (0.05 mL, 20 per cent) in water) on all analytes were investigated using saponification conditions of 40ºC and 40 min [116]. Different concentrations of the selected antioxidant, pyrogallol, (one per cent [157], three per cent [158, 159] and 6 per cent [101,160]) were tested to determine the most effective concentration. Phase separation method was optimized by testing the effect of duration of phase separation (three, five and 16 h) and the use of multistep centrifugation with two mL n-hexane at 6000 rpm for five min (Figure A.3). Means of results were compared using t-test or ANOVA (α=0.05). As main

parameters, saponification temperature (50, 70 and 90ºC) and time (10, 30 and 60 min) were tested in five combinations (Figure 3.2) and analyzed using ANOVA and Tukey test. During these preliminary tests, derivatization conditions were kept at 50°C for 15 min.

Final procedure, before optimization of critical parameters, was as follows: 0.1 to 0.2 g of olive oil was accurately weighed into a five mL screw-cap borosilicate glass tube. Two mL ethanol with selected antioxidant (to prevent oxidation of tocopherols during saponification) was then added followed by KOH water solution (0.6 mL and 50 per cent (w/v)) using nitrogen flush. The samples were mixed for five min using a rotator and then saponified in a water bath at Ts ºC for ts min, then mixed again by vortex two times during and after incubation. After cooling in a freezer for five min, one mL deionized distilled water with 1 per cent NaCl and hexane (4x2 mL) were added and mixing by rotator was carried out before each centrifugation step for 5 minutes. The supernatant hexane phase was transferred to a screw borosilicate glass tube (nine mL) with a teflon lined cap and dried under nitrogen flow at 40ºC.

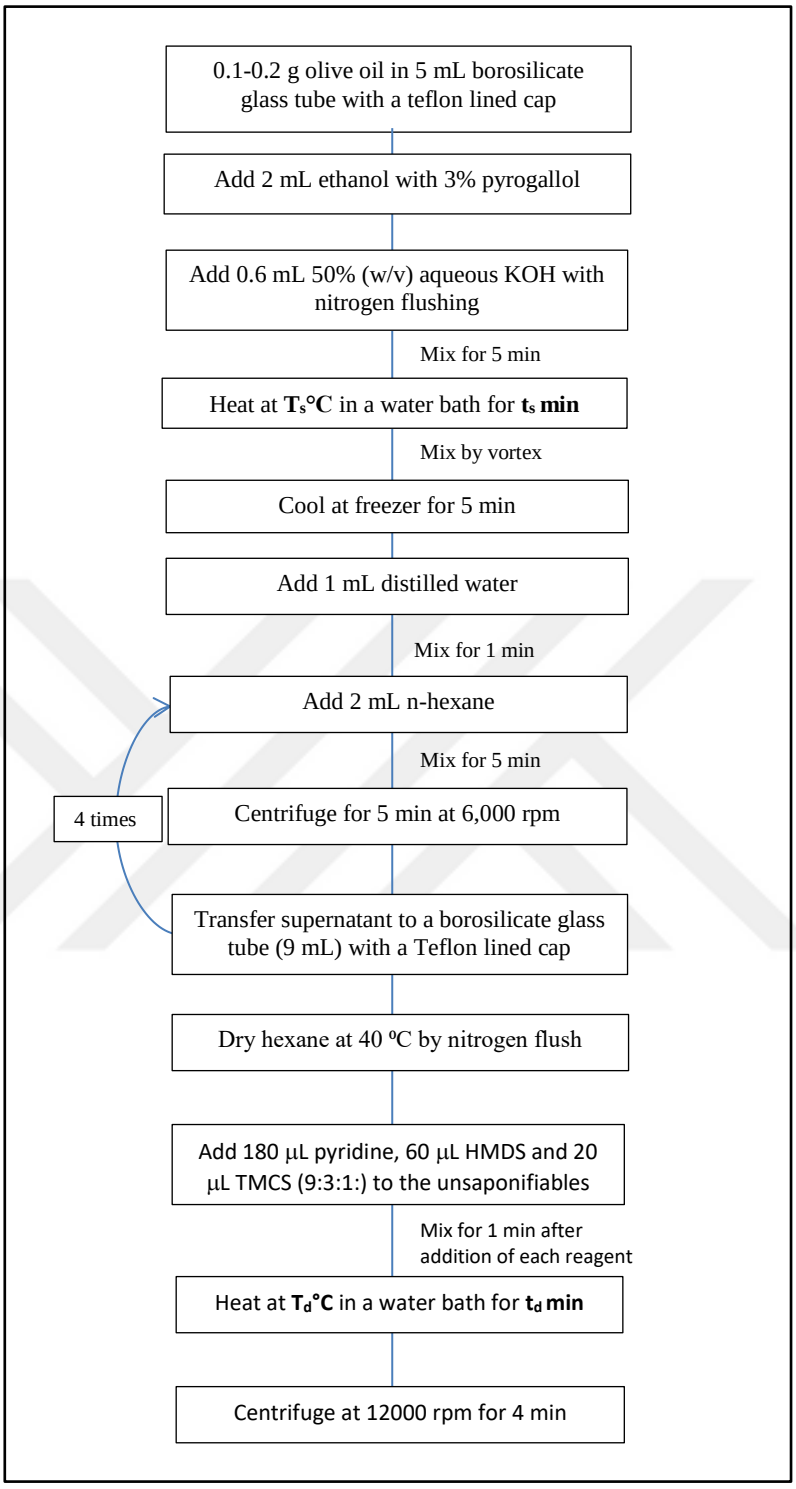


Figure 3.1. Flow chart of overall procedure

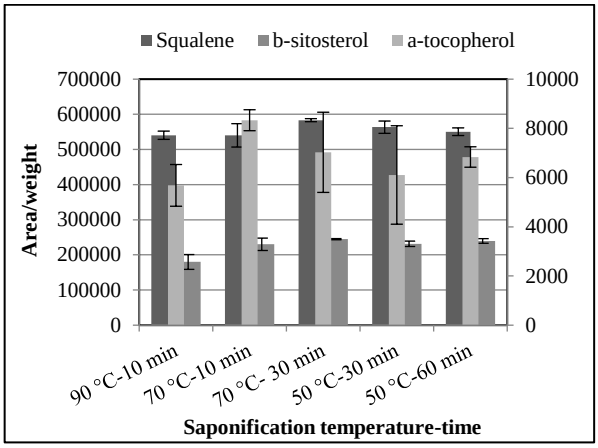


Figure 3.2. The effect of saponification temperature and time (n=3) on squalene (y1), β-sitosterol (y1) and α-tocopherol (y2)

3.2.3.2. Derivatization

Silylation (simple reactions that involve replacement of active hydrogen (-OH, -COOH) by a silyl group reducing hydrogen bonding and the polarity of the compound), which is one of the most prevalent derivatization methods [161], is commonly used for determination of sterols. It is also preferred for tocopherol determination rather than alkylation and acylation, which contain difficult esterification reactions and procedures (heating at 100–140ºC) [162].

The silylation mixture, hexamethyldisilazane (HMDS, a weak silylating reagent): TMCS (catalyst): pyridine (polar solvent), was added (260 µl, 3:1:9, v/v) to dry unsaponifiable matter of olive oil (<1 per cent) [99, 116]. Reagents were added under fume hood, separately (60 µL HMDS, 20 µL TMCS and 180 µL pyridine) by mixing at the time of the derivatization reaction to avoid floc formation due to moisture adsorption or deterioration of the reagent. After the derivatization reaction was completed at T_d for t_d min, the mixture was centrifuged at 12000 rpm for four min to precipitate and discard ammonium chloride salt, a by-product of silylation reaction, which causes decomposition and extraneous peaks [163]. The clear supernatant phase (150 µL) was transferred to a GC vial with insert and one µL sample was injected into GC-FID.

In the preliminary experiments different combinations of derivatization time and temperature were tested (15 min and 5, 10, 15 hr at RT and 50ºC (Figure A.4) and 70ºC for

15 and 30 min (Figure 3.3.) using saponification conditions of 40ºC and 40 min [116] and peak area/weight ratios. The results were evaluated by ANOVA ($p>0.05$).

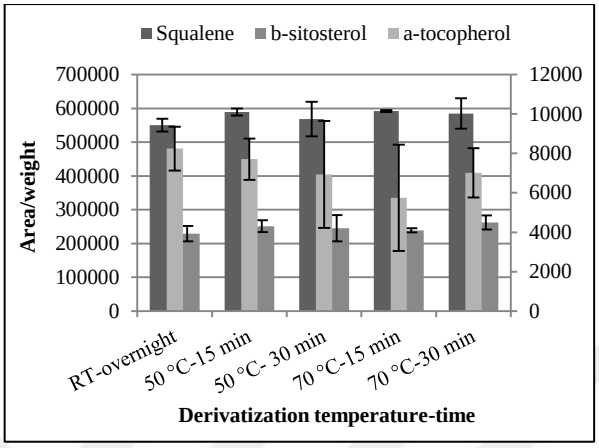


Figure 3.3. The effect of derivatization temperature and time (n=3) on squalene (y1), β-sitosterol (y1) and α-tocopherol (y2)

3.2.3.3. GC-FID Method

GC analyses were carried out using a Trace GC Ultra (Thermo Scientific, Rodano-Milan, Italy) with a FID detector using an Optima-5 capillary column (30 m x 0.25 mm id, 0.25 um film thickness, Macherey-Nagel, Neumann-Neander-Str., Düren, Germany). 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 (60:1). The analyses were carried out in the programmed temperature mode from 270 to 290 ºC with a ramp rate of 4ºC/min followed by 23 min hold at 270ºC and 12 min hold at 290ºC. The detector temperature was 320ºC 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 (squalene, α-tocopherol and β-sitosterol).

Stock standards of squalene (one mg/mL), 5α-cholestane (4 mg/mL), α-tocopherol (0.5 mg/mL), 5α-cholestanol (one mg/mL) and β-sitosterol (0.75 mg/mL) were prepared in chloroform and quality control (QC) standard mixture was prepared from stock standards by mixing 0.40; 0.10; 0.05; 0.05; 0.20 mL, respectively. Dried QC standards were stored at -20ºC (one month stability was checked). Calibration standards were prepared in different

amounts in the range of β-sitosterol, squalene and α-tocopherol amounts in olive oils [7, 111,164] by spiking triolein (blank matrix) and dried under nitrogen at RT before derivatization. A sample chromatogram is given in Figure 3.4.

The peak areas were calculated with Thermo Fisher Scientific ChromQuest, and quantification was performed using external calibration method by plotting calibration curves (Peak area versus concentration (mg/100 g)). The use of internal standard (IS: 5α-cholestane [165] (IS1) (four mg/mL) and 5α-cholestanol [99] (IS2) (one mg/mL)) for quantification was also evaluated in the method validation part (in repeatability experiments). Internal standards (IS1; IS2, 100 µl; 50 µl) were added to the sample at the beginning of the saponification procedure to compensate for losses at each step of the sample preparation. For this case, calibration curves were plotted using concentration of analyte (mg/100 g) versus Areaanalyte/AreaIS. Instrument performance was checked in each sequence by injection of QC standard mixture between every five samples.

3.2.3.4. RSM

After modification of the sample preparation parameters, the critical parameters (derivatization temperature (T_d), derivatization time (t_d), saponification temperature (T_s) and saponification time (t_s)) were optimized by RSM using Design Expert (DE) 9.0.3.1. software (Sat-Ease Inc. Minneapolis). The levels of these independent variables (Table 3.1) were selected according to preliminary tests at different levels (t_s: 10, 30, 60 min, T_s: 50, 70, 90ºC, and t_d: 15, 30, 60 min, T_d: RT, 50, 70ºC) by comparing peak area/weight ratios of analytes in the same sequence/run. Means of results were compared using t-test and ANOVA (α=0.05, Tukey's test for multiple comparisons).

A 3-level Box-Behnken experimental design with five central points (Table 3.2) was carried out in determined ranges (30ºC - 70ºC; 10 min - 60 min) at three different levels (–1, 0, 1). A total of 29 experiments was carried out to evaluate the effects of the four critical independent parameters on area/weight ratio of squalene, α-tocopherol and β-sitosterol.

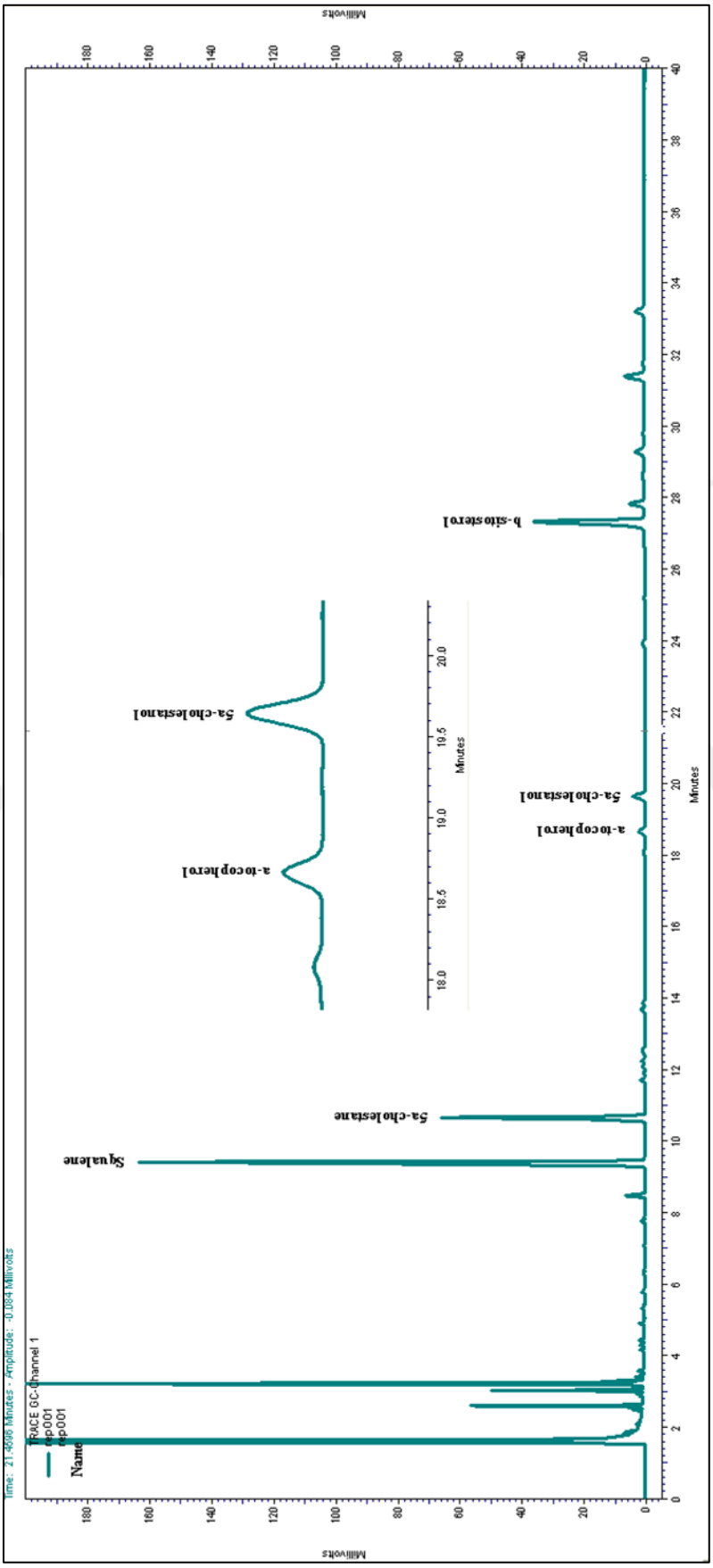


Figure 3.4. GC chromatogram of olive oil

Table 3.1. Levels of independent variables used in Box-Behnken design

Variable	Unit	Factor	-1	0	1
T_d	ºC	A	30	50	70
t_d	min	B	10	35	60
T_s	ºC	C	30	50	70
t_s	min	D	10	35	60

Alternative models (linear, quadratic, cubic, two-factor interaction) were evaluated and best model, having insignificant lack of fit test ($p>0.05$) and significant model for the analyte ($p<0.05$), was suggested by DE. Lack of fit test compares the residual error to the pure error from replicated design points to check for systematic variations unaccounted by the hypothesized model. Additionally, predicted R^2, a measure of how good the model predicts a response value, and adjusted R^2, R^2 which is adjusted for the number of terms in the model relative to the number of points in the design, were evaluated.

For the quadratic model, the results were fit with a second order polynomial Equation (3.1):

$$Y= a_0+a_1A+a_2B+a_3C+a_4D+a_{11}A^2+a_{22}B^2+a_{33}C^2+a_{44}D^2+ a_{12}AB+a_{13}AC+a_{14}AD+a_{23}BC+a_{24}BD+a_{34}CD \quad (3.1)$$

In this equation, Y is the predicted response, a_0 is the intercept, a_1, a_2, a_3 and a_4 are the linear coefficients; a_{11}, a_{22}, a_{33} and a_{44} are the squared coefficients; a_{12}, a_{13}, a_{14}, a_{23}, a_{24} and a_{34} are the interaction coefficients. A, B, C and D are the coded independent variables. Significant factors ($p<0.05$) were analyzed on 3D surface plots for interactions.

Factors were optimized using the desirability function. The goal was set as maximum response (standardized peak area of analytes) for four factors (A, B, C, D).

Table 3.2. The Box-Behnken design matrix of four test variables with the observed responses

Exp.	Factors				Standardized Peak Area* (Area/mg sample)		
No	**A**	**B**	**C**	**D**	**Y_1 x 10^8**	**Y_2 x 10^6**	**Y_3 x 10^7**
1	0	0	1	-1	1.20239	1.41660	3.48024
2	0	0	0	0	1.22259	2.74166	3.69095
3	0	0	-1	1	1.10175	2.36666	2.95691
4	1	1	0	0	1.27538	0.90278	4.08181
5	-1	-1	0	0	1.20254	1.40146	3.67182
6	0	0	1	1	1.18817	2.12763	3.63750
7	1	-1	0	0	1.29779	2.14959	3.91336
8	0	0	-1	-1	1.13807	2.88870	2.82281
9	-1	1	0	0	1.22459	1.31030	3.49056
10	1	0	-1	0	1.16690	2.54486	3.08404
11	0	1	0	-1	1.15306	2.18264	3.09124
12	0	1	0	1	1.35384	2.96726	4.24788
13	0	-1	0	1	1.23332	1.23373	3.93043
14	1	0	1	0	1.16607	1.27857	3.49729
15	-1	0	1	0	1.17004	1.24889	3.64284
16	-1	0	-1	0	1.18024	2.64407	3.05880
17	0	-1	0	-1	1.51283	3.52125	4.13997
18	0	0	0	0	1.19634	1.15553	3.59395
19	0	1	1	0	1.21646	2.42177	3.72456
20	1	0	0	1	1.26996	2.53207	3.90002
21	0	1	-1	0	1.19203	2.72887	2.99583
22	0	-1	-1	0	1.18596	2.60025	3.00816
23	0	0	0	0	1.25900	2.80600	3.82215
24	0	-1	1	0	1.25138	1.60704	3.84090
25	-1	0	0	-1	1.29345	3.09121	3.49855
26	-1	0	0	1	1.21757	2.34043	3.53899
27	1	0	0	-1	1.23352	1.48974	3.25970
28	0	0	0	0	1.26016	1.94736	3.69859
29	0	0	0	0	1.25198	1.02447	3.87095

*Y_1: squalene, Y_2: α-tocopherol, Y_3: β-sitosterol,
A: Derivatization temperature, B: Derivatization time,
C: Saponification temperature, D: Saponification time

3.2.4. Method Validation

Method validation based on method performance parameters (linearity, LOD, LOQ, repeatability and recovery) was carried out using optimized method parameters. Linearity was evaluated by drawing the calibration graphs and analyzing the coefficient of determination ($r^2 \geq 0.9$) and p value ($p<0.05$) from regression statistics [121].

Calibration range was determined considering β-sitosterol, squalene and α-tocopherol concentrations in olive oils [7,111,164]. Calibration of squalene and sterols was done at seven to eight levels, evenly spaced across the range of interest, by spiking triolein, in duplicate. The concentration of analyte in the sample (mg/100 g sample) was calculated using the calibration curve.

Triolein was spiked with the analytes at four concentration levels at the low range (4.1-28.8 mg/100 g) and these samples and blank matrix were analyzed. LOD and LOQ were then calculated using Equations 2.1-2.3, using the residual standard deviation, s(r), of the calibration curve at low mass ratio levels [120].

Trueness was determined by calculatiing recovery from a spiked sample using Equation 2.4. Triolein was used to decrease the sample analyte content. Analysis was performed in triplicate at three concentrations.

Repeatability was determined by analyzing unspiked blank matrix (olive oil: triolein, 1:4) in triplicate and evaluated according to the HORRAT(r) ratio using Equations 2.5-7. The use of internal standards (IS1 and IS2) on repeatability was also evaluated.

3.3. RESULTS AND DISCUSSION

Method development was conducted in three parts: modification of the sample preparation procedure to improve method performance, preliminary experiments to determine the level of critical parameters (time and temperature of derivatization and saponification) and optimization of critical parameters by RSM. The developed method was validated based on method performance parameters (linearity, LOD, LOQ, repeatability and recovery).

Selectivity of the method was checked for standard mixture and olive oil samples (Figure 3.4). Detection and quantification in these matrices were not inhibited by any interferences.

3.3.1. Saponification

The investigated saponification parameters included concentration of KOH and addition of an antioxidant to prevent tocopherol oxidation. In literature, two M KOH is commonly used for the saponification of triacylglycerols in methods for the determination of sterols, squalene and tocopherols in oils [106, 107, 116]. Additionally a concentration of 0.35 M was also reported by Du and Ahn (2002) for tocopherol analysis [109], because tocopherols are sensitive to alkali and therefore, they are easily oxidized to tocoquinones [26]. Therefore, two M and 0.35 M KOH concentration were tested using t-test and a significant concentration effect ($p<0.05$, $n=2$) was observed for β-sitosterol and α-tocopherol. β-sitosterol peak area increased by 38 per cent as concentration of KOH increased from 0.35 M to two M (data not shown). However, peak area of α-tocopherol decreased by 59 per cent with increasing KOH concentration. As a result, two M KOH concentration was chosen for saponification but antioxidant addition was studied in order to prevent oxidation of α-tocopherol [166].

Aqueous vitamin C solution (0.5 mL 20 per cent (w/v)) was added to prevent oxidation of tocopherols during saponification according to the study of Du and Ahn (2002) [109]. While α-tocopherol concentration increased significantly ($p<0.05$) by 136 per cent with vitamin C addition (Figure A.1), there was no significant change in β-sitosterol and squalene concentration ($p>0.05$, $n=2$). However, a blurred phase line between hexane and water-ethanol phase was observed due to high aqueous and low ethanol solubility of vitamin C making phase separation difficult [167].

Pyrogallol, which is soluble in pure ethanol, was tested as an alternative antioxidant. Comparing antioxidant effects of pyrogallol in pure ethanol (three per cent (w/v)) and aqueous vitamin C (0.5 mL of 20 per cent (w/v)), a significant difference ($p<0.05$) was observed for α-tocopherol such that pyrogallol value was higher than vitamin C value by 145 per cent (Figure A.2). This might be partly attributed to solubility behavior of pyrogallol compared to hydrophilic vitamin C [167]. Additionally, the oxidation of

pyrogallol resulted in a dark color in the saponified phase, which made it possible to see the phase line easily during the phase separation step compared to the blurred phase line observed with vitamin C addition. Therefore, pyrogallol was chosen as an antioxidant.

Three concentration values of pyrogallol were investigated: one per cent [166], three per cent [167, 168] and six per cent [101, 169] by comparing mean peak area/weight ratios of analytes (n=2). There was no significant difference between the tested concentrations ($p>0.05$) for any of the analytes (data not shown). Three per cent pyrogallol concentration was chosen for future analyses.

Based on these results, after nitrogen flush to remove oxygen, two mL ethanolic pyrogallol (three per cent, w/v) solution, prepared daily, was mixed with 0.6 mL concentrated aqueous KOH (50 per cent, w/v) solution (2.06 M).

3.3.2. Phase Separation

The effect of phase separation duration (three, six and 15 hours) and method (with and without centrifugation) on the recovery of analytes in the organic phase was also studied. A multi-step centrifugation was used for better recovery. No significant difference ($p>0.05$, n=3) was observed between phase separation for three, six and 15 hours in the dark and with centrifugation (3x2 mL, at 6000 rpm for five minutes with mixing before each step) (Figure A.3). Therefore a multistep centrifugation step was used for phase separation. Further modifications were made (use of four steps centrifugation, 4x2mL, and the use of additional mixing (five min) at the start, middle and end of saponification) to ensure high recovery. Compared to long phase separation applications [109] multistep centrifugation decreased the duration of phase separation to 40 min (4x10 min).

3.3.3. RSM for Critical Parameters

Completion of the derivatization reaction in a reasonable time is a critical factor to develop a rapid method. At RT, derivatization time (15 min, five, 10 and 15 hours) did not have a significant effect ($p>0.05$, n=2) on the amount of analytes (peak area/weight) (Figure A.4). No significant effect ($p>0.05$, n=2) was observed for higher derivatization temperatures

(50ºC and 70ºC for 15 and 30 min) (Figure 3.3). Based on these results, ranges of derivatization factors were determined as 30ºC-70ºC; 10 min and 60 min to ensure maximum derivatization and recovery of each compound (Table 3.1).

The main parameters of saponification; temperature (50, 70 and 90ºC) and time (10, 30 and 60 min) were also tested in five combinations (Figure 3.2) using derivatization at 50 °C for 15 min and analyzed using ANOVA and Tukey test. Saponification at 70ºC for 30 min provided highest peak area/weight for squalene and β-sitosterol but for α-tocopherol saponification at 70ºC for 10 min provided highest values. However only the decrease in β-sitosterol content observed at 90ºC for 10 min was significant ($p<0.05$) which was concluded from the pairwise analysis of all combinations. Based on these results, ranges of saponification factors were selected as 30ºC - 70ºC; 10 min and 60 min (Table 3.1).

In ANOVA results of α-tocopherol, within group variation was higher than between group variation. A higher variation of α-tocopherol content (Figure 3.2-3.3) might be due to the low concentration of α-tocopherol in the oil or stability issues. To minimize variation, extra care was taken at each step of sample preparation to protect the sample from light.

RSM using a three-level Box-Behnken design with five central points was used for the optimization of derivatization and saponification parameters (time and temperature of saponification and derivatization) to ensure maximum recovery of each analyte (Table 3.1). In model fitting, quadratic model for squalene and β-sitosterol, and two factor interaction (2FI) model for α-tocopherol were suggested by DE based on the p-value and lack of fit values (Table 3.3). ANOVA results for three responses (area of squalene, β-sitosterol and α-tocopherol) are presented in Table 3.3. The suggested model for squalene and β-sitosterol (i.e. the quadratic model) had p-value below 0.05. However, the model suggested for α-tocopherol (2FI) was not significant relative to variation ($p=0.1909$). In this study, method validation was carried out for squalene, β-sitosterol and α-tocopherol using factors optimized for squalene and β-sitosterol content (Figure 3.5). α-tocopherol was included in method validation to assess the suitability of the developed method for α-tocopherol analysis.

The lack of fit p-values for squalene and β-sitosterol were higher than 0.05 implying that the lack of fit was not significant relative to the pure error, which means the model was valid (Table 3.3).

The R^2 values of the model were 0.8428 for squalene and 0.9039 for β-sitosterol, respectively (Table 3.3). Model reduction was applied by excluding insignificant model terms and outlier data. The ANOVA results of the reduced model are given in Table 3.4. All model p-values were found significant (<0.0001) and lack of fit values were insignificant (p>0.05). The coefficients of significant model terms of squalene (B, C, BD, B^2 and C^2) and β-sitosterol (C, D, BD and C^2) are given in Equations 3.9-3.10 for squalene and β-sitosterol, respectively. After modification, R^2 values of the models were 0.8728 for squalene and 0.8084 for β-sitosterol (Table 3.4), indicating a good fit between predicted values and experimental data.

Table 3.3. ANOVA results for RSM

	Squalene (Quadratic)		**β-sitosterol (Quadratic)**		**α-tocopherol (2FI)**	
Source	**F Value**	**p-value (Prob > F)**	**F Value**	**p-value (Prob > F)**	**F Value**	**p-value (Prob > F)**
Model	5.36	0.0017	9.40	< 0.0001	1.58	0.1909
A-A	0.67	0.4277	2.05	0.1745	0.25	0.6242
B-B	3.27	0.0919	2.24	0.1569	$1.81x10^{-8}$	0.9999
C-C	2.39	0.1441	44.61	< 0.0001	6.17	0.0231
D-D	1.29	0.2746	10.82	0.0054	0.20	0.6598
AB	0.27	0.6117	1.08	0.3168	0.77	0.3924
AC	0.012	0.9145	0.26	0.6200	$9.55x10^{-3}$	0.9232
AD	1.72	0.2108	3.17	0.0967	1.85	0.1907
BC	0.23	0.6397	0.095	0.7621	0.27	0.6092
BD	31.44	< 0.0001	16.45	0.0012	5.43	0.0317
CD	0.067	0.8002	$4.73x10^{-3}$	0.9462	0.87	0.3622
A^2	0.22	0.6462	0.81	0.3846		
B^2	6.31	0.0249	3.74	0.0734		
C^2	20.00	0.0005	40.40	< 0.0001		
D^2	1.03	0.3266	1.32	0.2694		
Lack of Fit	2.91	0.1572	2.83	0.1643	0.50	0.8502
R^2		0.8428		0.9039		0.4677
Pred R^2		0.1743		0.4962		-0.3133
Adj R^2		0.6856		0.8077		0.1719

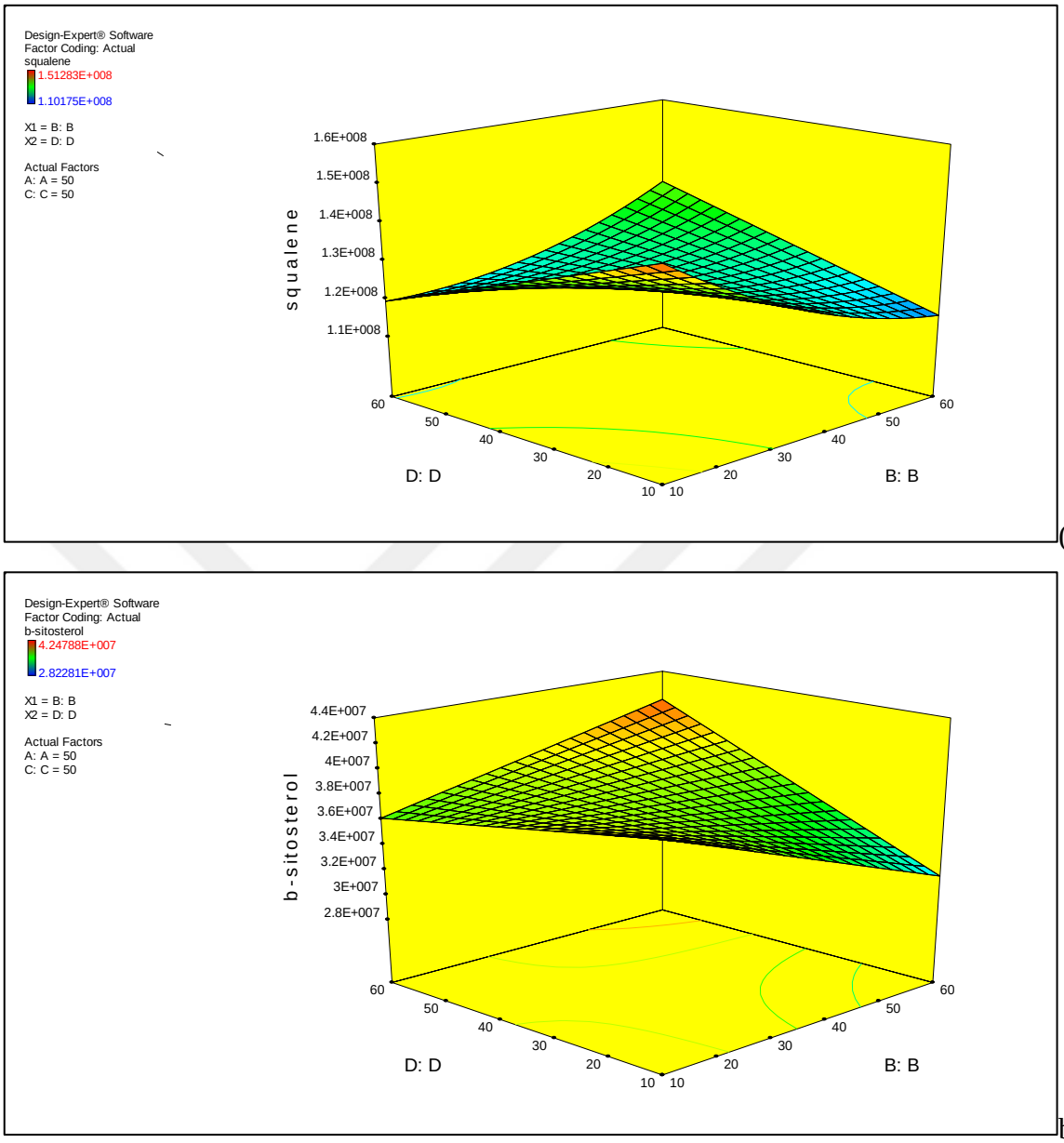
Table 3.4. ANOVA results for RSM of squalene and β-sitosterol after model reduction

	Squalene (Quadratic)		β-sitosterol (Quadratic)	
Source	**F Value**	**p-value (Prob > F)**	**F Value**	**p-value (Prob > F)**
Model	24.01	< 0.0001	19.41	< 0.0001
B-B	11.99	0.0023	1.84	0.1875
C-C	4.46	0.0470	36.78	< 0.0001
D-D	2.41	0.1358	8.92	0.0066
BD	58.51	< 0.0001	13.56	0.0012
B^2	17.02	0.0005		
C^2	47.08	< 0.0001	35.95	< 0.0001
Lack of Fit	1.34	0.4277	2.83	0.1643
R^2		0.8728		0.8084
Pred R^2		0.7072		0.6584
Adj R^2		0.8364		0.7668

$$Y_{squalene} = 1.25 \times 108 - 3.29 \times 106 \times B + 1.91 \times 106 \times C - 1.41 \times 106 \times D + 5.05 \times 106 \times B^2 - 8.28 \times 106 \times C^2 + 1.20 \times 107 \times BD \quad (3.2)$$

$$Y_{sitosterol} = 3.73 \times 107 - 7.27 \times 105 \times B + 3.25 \times 106 \times C + 1.60 \times 106 \times D - 4.19 \times 106 \times C^2 + 3.42 \times 106 \times BD \quad (3.3)$$

A positive coefficient value in the model represents a positive relationship between the factor and the response, while a negative value indicates an inverse relationship. Saponification temperature (C) was a significant factor for β-sitosterol and squalene, and affected their contents positively. Other significant factors for β-sitosterol and squalene were saponification time (D) and derivatization time (B), respectively. β-sitosterol was positively affected by saponification time, whereas there was an inverse relationship between squalene content and derivatization time (B).



(a)

b)

Figure 3.5. Response surface plots for standardized peak area of: (a) squalene (b) β-sitosterol for BD intereaction (other variables were kept constant at center points for each graph, 50°C and 35 min) (B: Derivatization time C: Saponification temperature D: Saponification time)

BD, which is an interaction term for “time of derivatization and saponification”, was a significant model term with positive coefficient for both responses. The effect of this interaction was investigated on the 3D surface plot. Response surface plots of the second-order polynomial equation, keeping one variable (C) constant and varying the other two (B, D) within the experimental range, are given in Figure 3.5. Based on derivatization and

saponification time interaction (BD), it can be concluded that maximum and minimum levels of B and D resulted in higher responses than moderate levels for squalene and β-sitosterol.

Reduced quadratic models for β-sitosterol and squalene were used for optimization. Maximum standardized peak area of β-sitosterol and squalene was chosen as a goal to establish an optimized output value for three factors (B, C, D). Importance of responses were chosen 5-plus for two responses. Due to insignificance of derivatization temperature (A) in each model, this parameter was not included in numerical optimization.

The desirability function approach is a technique for the simultaneous determination of optimum performance levels of factors for one or more responses. The levels of the independent variables that simultaneously produce the most desirable predicted responses/goals (maximum, minimum, target response, the range of response or none) are set and so the overall desirability ($0 \leq d_i \leq 1$) is maximized with respect to the controllable factors [168]. Optimum levels are thus determined to obtain maximum desirability.

Optimum conditions for β-sitosterol and squalene were determined as 10 min derivatization time for 30ºC derivatization temperature, 56ºC saponification temperature and 10 min saponification time with desirability of 0.867 (Figure 3.6).

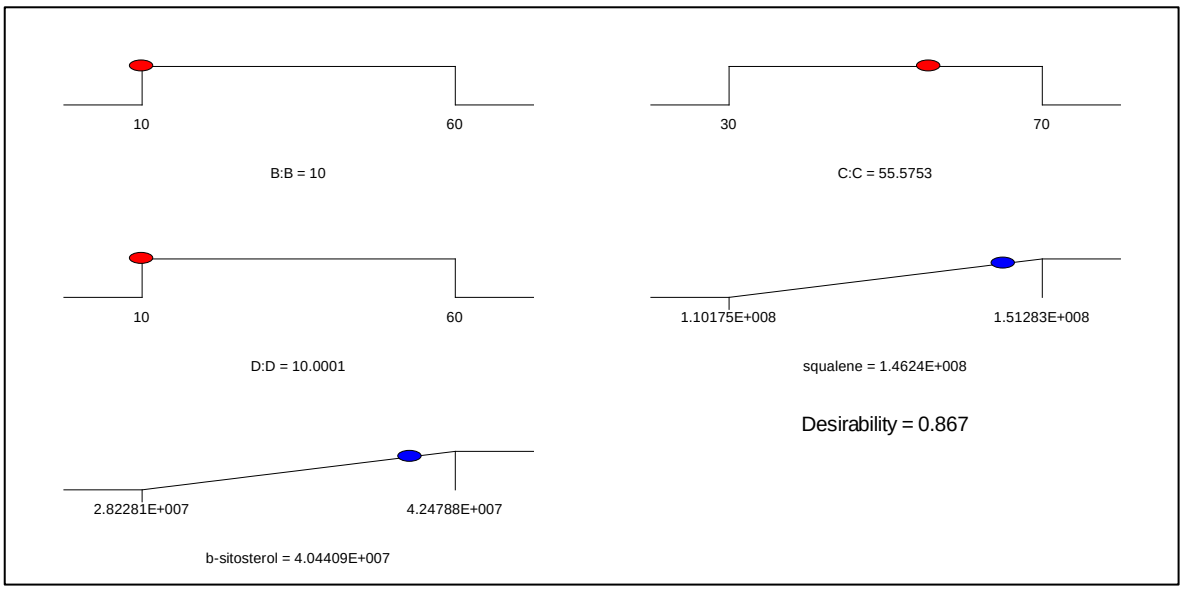


Figure 3.6. Desirability ramp for optimization of squalene and β-sitosterol

3.3.4. Method Validation

Method validation based on method performance parameters (linearity, LOD, LOQ, repeatability and recovery) was carried out using optimized method parameters (T_d: 30ºC, t_d= 10 min, T_s= 56ºC, t_s=10 min).

Linearity of the method was checked by regression analysis of calibration curves in the working range. To determine the working range of the method for each analyte by determining LOQ and upper end (if needed), calibration curves (Peak area vs concentration of analyte in the standard spiked sample (mg/100g)) were evaluated using regression analysis (regression lines and residual plot) [121]. Coefficient of determination (R^2) for all analytes were higher than 0.9 (0.9791, 0.9869 and 0.9680 for squalene, β-sitosterol and α-tocopherol, respectively) and residual plots had random distribution.

Table 3.5. LOD, LOQ and recovery results of squalene, β-sitosterol and α-tocopherol calculated using spiked samples*

Analytes	**Squalene**			**β-sitosterol**			**α-tocopherol**		
LOD mg/100g	5.3			2.8			4.1		
LOQ mg/100g	16.0			8.4			12.5		
Upper end mg/100g	700			500			-		
Recovery									
Concentration (mg/100g)**	245.6	361.1	421.5	136.7	225.4	289.7	16.2	38.4	58.4
% Recovery	93	98	95	92	96	97	93	105	110

*Blank matrix: 133.0 mg/100g squalene, 53.3 mg/100g β-sitosterol, 7.4 mg/100g α-tocopherol
**Average values of three replicates

LOD and LOQ values (Table 3.5) were determined using Equations 1-3. To determine LOD and LOQ, calibration curve of each analyte were drawn in the low mass range (5-30; 4-22 and 2-10 mg/100 g, respectively) (n=3, at four levels). The working range of β-sitosterol, squalene and α-tocopherol was determined to be between LOQ value (8.4; 16.0; 12.5 mg/100 g, respectively) and upper end (500; 700; 75 mg/100 g). Below LOQ, samples might be analyzed by spiking to acceptable levels, and near LOQ level sample replication might

be increased. For the analysis of samples having out of range concentrations (i.e. above upper end level) sample weight might be decreased.

LOD value of RPLC- GC method for olive oil was reported as 0.03 mg/100 g which was calculated from three times the background noise [118]. Residual standard deviation usage for LOD-LOQ determination including all steps of the method results in more realistic values than the calculation using background noise.

Recovery values of spiked samples for squalene, β-sitosterol and α-tocopherol were 93-98; 92-97; 93-110 per cent (Table 3.5), respectively, which are within acceptable recovery limits (90-108; 90-108; 85-110 per cent) for the concentration range of squalene, β-sitosterol and α-tocopherol used (0.1; 0.1; 0.01 per cent (w/w), respectively) [19]. Spike recovery results of α-tocopherol obtained in this study (93-110 per cent) were close to the recovery data (98-100 per cent) in the method of Kohler et al. [169] (modified AOCS Ce 7-87 (2009)), which focuses on determination of tocopherols by GC.

Mass fraction of the analyte (mg analyte/mg blank matrix) was calculated for each replicate (n=6) and PRSD was calculated using mean mass fraction of replicates (Equation 3.3). Then, HORRAT values (RSD per cent/PRSD) were calculated for squalene, β-sitosterol and α-tocopherol using both internal standards and without internal standard (Table 3.6). HORRAT values should be between 0.3-1.3 for acceptable repeatability [123].

Table 3.6. Repeatability results (n=6) of olive oil with internal standard (IS1 and IS2) addition to blank matrix and without IS (IS1: 5α-cholestane, IS2: 5α-cholestanol)

Analytes	**Squalene**			**β-sitosterol**			**α-tocopherol**		
Quantification method	**W/out IS**	**IS1**	**IS2**	**W/out IS**	**IS1**	**IS2**	**W/out IS**	**IS1**	**IS2**
Concentration (mg/100g)	525.7	458.0	505.2	183.1	166.3	199.8	23.8	21.2	21.1
RSD (per cent)	4.61	1.22	6.49	5.76	2.52	5.24	8.25	3.23	3.86
PRSD	2.20	2.24	2.21	2.57	2.61	2.54	3.50	3.56	3.56
HORRAT	2.1	0.5	2.9	2.2	1.0	2.1	2.4	0.9	1.1

Higher RSD per cent values lead to higher HORRAT values (above 1.3) in results quantified without IS. Lower HORRAT values were obtained in IS1 added olive oil samples suggesting that repeatability (n=6) of the method can be improved using 5α-cholestane as an internal standard with acceptable RSD per cent values of squalene, β-sitosterol and α-tocopherol (1;3;3 per cent, respectively). Higher HORRAT values were obtained in IS2 added olive oil samples for squalene and β-sitosterol. The different behavior of IS2, squalene and β-sitosterol in the derivatization step might be the limiting factor. While IS2 (5α-cholestanol) is derivatized, squalene does not participate in the derivatization reaction. Moreover, the lower concentration of IS2 might have resulted in higher per cent RSD. However, its concentration could not be increased in order not to overload the derivatization capacity. Based on these results, 5α-cholestane appears to a better choice as an internal standard for the developed method, while 5α-cholestanol might be used as a surrogate standard for β-sitosterol by adding it before the saponification step to monitor recovery as affected by the entire analytical process from sample preparation to instrument performance.

The per cent RSD value of the developed method for squalene (one per cent, n=6) was lower than that of the RPLC-GC method [118] (six per cent, n=3). The per cent RSD value of the developed method for α-tocopherol (three per cent, n=6) was close to the per cent RSD value of the GC method developed by Kohler et al. [169] (one per cent, n=20, 90 per cent mixed tocopherols-oil sample) and lower than the value of IUPAC method (2.432) [113] (5 per cent, HPLC). In the direct GC-FID method [116], RSDs (n=3) of sterols (2.89-3.59 per cent), squalene (1.90 per cent) and α-tocopherol (3.63 per cent) were close to per cent RSD values of developed method (Table 3.5).

3.4. CONCLUSIONS

A rapid GC-FID in-house method was developed to determine squalene, β-sitosterol and α-tocopherol content in olive oil, simultaneously. Optimum method parameters were determined using RSM as 10 min derivatization time for 30ºC derivatization temperature, 56ºC saponification temperature and 10 min saponification time. Time of analysis was reduced to approximately five hour for every six samples compared to ~20 hours specified in published methods [109, 116]. The method was fully validated based on method performance parameters (linearity, LOD, LOQ, repeatability and recovery). In order to

determine the linear range of the method, the LOQ value of β-sitosterol, squalene and α-tocopherol were determined as 8.4; 16.0; 12.5 mg/100 g, respectively, and upper end of β-sitosterol and squalene were determined as 500; 700 mg/100 g, respectively. Recovery values for β-sitosterol, squalene and α-tocopherol were obtained within the acceptable range (92-97; 93-95; 93-110 per cent, respectively) with an acceptable repeatability.

4. DISTRIBUTION OF PREDOMINANT LIPOPHILIC BIOACTIVES (SQUALENE, α-TOCOPHEROL AND β-SITOSTEROL) DURING OLIVE OIL PROCESSING

4.1. INTRODUCTION

Squalene, sterols and tocopherols are the pre-dominant lipophilic bioactives in olive oil having anticancer, anticholesterol and antioxidant activities [170,171]. While squalene, tocopherol and sterol content of olive oil have been widely reported in literature [9,15,55,62], the distribution of these compounds between oil and waste streams during olive oil processing has not been addressed. Few studies reported the content of these compounds in the input and output streams of olive oil processing [9,15], yet no attempt has been made to integrate this information to assess their distribution between these streams and calculate processing loss. In literature review, we investigated the distribution of these compounds between oil and waste streams during olive oil processing (section 2.4.). The loss of squalene, α-tocopherol and β-sitosterol were calculated based on available literature process data, which was limited to press and abencor® lab systems (19, 36 and 48 per cent, respectively) [9,15]. As there was no literature data on the content of these compounds in the input and output streams in a single operational run of industrial VOO production, their partitioning during industrial VOO processing could not be addressed. Therefore the objectives of this study were a) to determine the distribution of pre-dominant lipophilic bioactives (squalene, β-sitosterol and α-tocopherol) between olive oil (VOO and SCOO) and waste streams (Olive mill waste water-OMW and pomace), and b) to calculate loss of these lipophilic bioactives during industrial VOO production using simultaneous GC-FID analysis of samples (olive fruit-O, VOO, SCOO, Pomace-P, OMW) taken from a two-step milling integrated plant.

4.2. MATERIALS AND METHODS

4.2.1. Sampling

Samples (Table 4.1) were obtained from a single operational run at an olive milling plant in Burhaniye, Turkey in November 2013. The design of the system, which included production of VOO and SCOO in an integrated manner utilizing a two-phase and a three-phase continuous system, respectively, (Figure 4.1) enabled calculation of loss throughout olive oil and pomace processing.

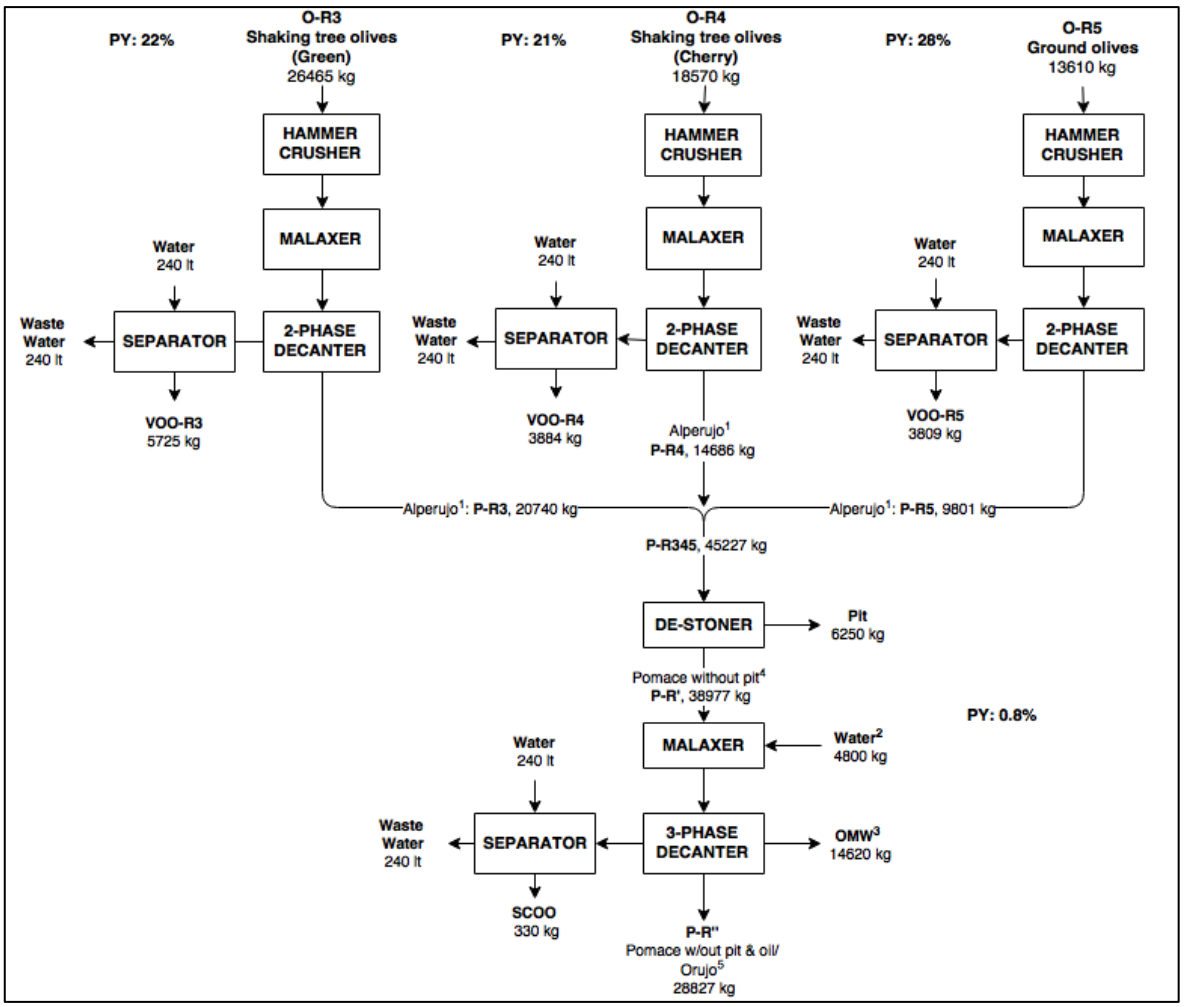
Table 4.1. Types of samples and operational conditions of the sampled processing lines at the OO production plant

Types and codes of samples	
Olive fruit (O) (5 kg)**	O-R3-4-5
Olive oil (VOO/SCOO) (2 L)	VOO-R3/ VOO-R4/ VOO-R5/ SCOO
Pomace (P) (5 kg)	P-R3/P-R4/P-R5/P-R345/ P-R'/ P-R''
Olive mill waste water (OMW) (5 L)	OMW-R''
Operational conditions	
Harvest period and year	November 2013
Olive type and origin	Edremit-Ayvalık
Type of crusher	Hammer
Runs	R3; R4; R5 (VOO)/ 2nd extraction (SCOO)
Malaxation temperature (^{0}C)	37.4; *; 34.9 / 40
Malaxation time (min)	70 /40-50
Type of decanter	2 phase for VOO, 3 phase for SCOO according to Figure 4.1
Revolution of decanter (rpm)	3000
Yield (kg oil/kg olive fruit)	1st ext.: 5625/26465 2nd ext.: 8/1000

*Not recorded
**O-R3: Green olives, O-R4: Cherry olives, O-R5: Ground olives
Abbreviations: Olive (O), Pomace (P), Virgin olive oil (VOO), Second centrifugation olive oil (SCOO), Olive mill waste water (OMW), Run (R), Olive stone removal (R'), second extraction run (R'')

Three different Edremit type olive samples (green (shaking tree)/cherry (shaking tree)/ground) were processed in three different lines/runs (R) including a hammer crusher, malaxer, decanter (two-phase), and separator using operational conditions specified in Table 4.1. Olive pomace (alperujo) of the three lines (P-R3-5) were combined (P-R345) and then

extracted continuously using a three-phase system. Total oil yield of first extraction lines (13418 kg oil /58645 kg O) and 2nd extraction line (330 kg oil /38977 kg pomace without pit (P-R')) were obtained from the plant for an eight hour operation.



Abbreviations: Olive (O), Pomace (P), Virgin olive oil (VOO), Second centrifugation olive oil (SCOO), Olive mill waste water (OMW), Run (R), Olive stone removal (R'), second extraction run (R''), process yield (PY)

[1]P-R3, P-R4 & P-R5: Calculated by subtracting olive oil from olive amount

[2] Water added: 400-800 L/h for eight hour operation, estimated by the plant according to moisture content of olive paste for water content material balance. It was taken as 600 L/h for eight hour operation.

[3] OMW: Calculated as the difference between total waste water (based on annual waste water data: 890 tonnes OMW/ 3350 tonnes olive fruit.year) and separator washing water (assumed as 30 L/h for eight hour operation from another production plant).

[4] Pomace without pit, calculated by subtracting pit amount from combined pomace amount.

[5] Pomace without pit and oil, calculated by subtracting pit, OMW and SCOO amount from combined pomace amount.

Figure 4.1. Flow chart of the sampled olive oil processing lines (Samples are coded as in Table 4.1 and values are provided on an eight hour operation basis.)

Oil samples (VOO and SCOO), O and P were stored at 4 °C, -80 °C and −20 °C, respectively. O were ground and all solid samples were homogenized using a blender (two min at medium speed) under liquid nitrogen and freeze-dried prior to analysis. Samples were protected as much as possible from light at every step.

4.2.2. Reagents

Squalene (99.0 per cent) and β-sitosterol (100 μg/ml in chloroform, analytical standard) were obtained from Supelco (PA, US). (±)-α-tocopherol (97 per cent, HPLC grade), pyridine (anhydrous, GC Grade, 99.8 per cent), ethanol (absolute, GC grade) and chloroform (GC grade) were obtained from Sigma-Aldrich (Damstadt, Germany). 5α-cholestane (99.5 per cent, GC Grade) and 5α–cholestan-3β–ol (98 per cent) were obtained from Sigma-Aldrich (Rehovot, Israel and Dorset, UK). Triolein (Glyceryl trioleate, 61 per cent), hexamethyldisilazane (HMDS) (99.9 per cent, reagent plus) and n-hexane (chromasolv®, GC grade) were obtained from Sigma-Aldrich (MO, US). Trimethylchlorosilane (TMCS) (GC Grade), potassium hydroxide (pellets), pyrogallol and sodium chloride (ACS grade) were obtained from Merck (Damstadt, Germany). β-sitosterol (ca 10 per cent campesterol, ca. 75 per cent β-sitosterol) was obtained from Acros Organics (Geel, Belgium).

4.2.3. Determination of Oil Content and Oil Yield

Oil extraction from freeze-dried samples (~20 gr) (O, P, OMW) was carried out in triplicate using Soxhlet method with n-hexane at 60-70°C for six hours [24]. Oil samples, collected in amber glass balloons, were obtained after evaporation of hexane using a vacuum evaporator (at 35°C) followed by nitrogen flush, and stored in tubes protected from light. Oil content (per cent) was determined on a dry matter basis (DMO) and then calculated on a fresh matter basis (FMO) (Table 4.2). Moisture content was determined according to the “Guide for the determination of the characteristics of oil-olives” [93] in triplicate (Table 4.2). Oil free dry matter (OFDM) was determined by subtracting moisture content (per cent) and FMO (per cent) from total (100 per cent). Oil samples extracted in triplicates were pooled for the analysis of lipophilic bioactive content of O and P samples. Process yield was calculated using process data (kg oil/kg of olives crushed) (Figure 4.1).

Table 4.2. Water, oil and solid contents (per cent) of the olive and waste (pomace and wastewater) samples*

Sample codes		Water (%)	DMO (%)	FMO (%)*	OFDM (%)*
Olive	O-R3	43	38	21	36
	O-R4	43	43	24	33
	O-R5	20	41	33	47
Pomace	P-R3	60	6.7	2.7	37
	P-R4	65	8.1	2.8	32
	P-R5	62	6.4	2.4	36
	P-	64	9.4	3.4	33
	P-R'	69	11	3.4	28
	P-R''	57	7.7	3.3	40
	OMW	95	11	0.5	4.5

* Data presented as mean (RSD(%)) (n=3)
Abbreviations: DMO: per cent oil content dry matter basis, experimental value, FMO: per cent oil content fresh matter basis and OFDM: oil free dry matter, calculated values

4.2.4. Determination of Lipophilic Bioactive Content using GC-FID

The concentration of lipophilic bioactive compounds (squalene, α-tocopherol and β-sitosterol) in the oil samples (O, P, VOO and SCOO) was determined (mg/100 g) using the GC-FID method developed and validated by Seçmeler and Güçlü Üstündağ (2017) (Table 4.3) [172]. To summarize the method (Chapter 3), oil sample was saponified by adding 50 per cent KOH in the presence of ethanol solution with six per cent pyragallol as an antioxidant. After saponification (56°C, 10 min), four step hexane extraction was carried out and hexane was dried under nitrogen at 40°C. Unsaponifiable residue was derivatized by 260 µl TMS reagent (HMDS: TMCS: pyridine, 3:1:9, v/v) for GC-FID determination. Quantification was performed using external calibration method (Peak area versus concentration (mg/100 g)) and internal standard (5α-cholestane, four mg/mL).

Table 4.3. Concentration of squalene (sq), α-tocopherol (toco) and β-sitosterol (sito) in extracted oil samples

Sample code			Concentration, C			Average Concentration			RSD%		
			sq mg/100g	toco mg/100g	sito mg/100g	sq mg/100g	toco mg/100g	sito mg/100g	sq %	toco %	sito %
Olive oil	VOO_R3	1*	544	26.6	261	438[a]	23.8[a]	207[a]	4.3	4.9	3.8
		2	452	23,0	212						
		3	425	24,6	201						
	VOO_R4	1	508	13,2	182	508[b]	13,5[b]	181[b]	0,3	12,3	0,6
		2	507	12,0	179						
		3	510	15,3	180						
	VOO_R5	1	308	7,2	228	307[c]	<LOQ[c]*	226[c]	0,5	3,6	1,6
		2	308	7,7	221						
		3	305	7,7	228						
	SCOO	1	398	20,5	253	405	19,4	266	1,5	5,6	4,3
		2	408	18,3	271						
		3	409	19,3	275						
Olive fruit***	O-R3	1	521	40,0	279	502[a]	38,8[a]	270[a]	9,7	16,4	9,5
		2	538	44,4	289						
		3	447	31,9	241						
	O-R4	1	365	20,6	256	398[b]	19,9[b]	279[a]	10,3	4,8	9,0
		2	386	18,8	277						
		3	444	20,2	306						
	O-R5	1	251	3,7	311	250[c]	<LOQ[c]*	307[a]	1,0	15,2	1,9
		2	251	2,9	300						
		3	247	2,9	309						
Pomace	P-R3	1	254	34,2	359	240[a]	32.5	339[a]	8.5	7.2	8.2
		2*	187	26,7	284						
		3	225	30,9	319						
	P-R4	1	393	61,3	444	387[b]	62,2[b]	430[b]	5,8	1,5	3,8
		2	362	63,2	412						
		3	406	62,1	434						
	P-R5	1	237	29,6	352	244[a]	31,6[a]	362[a]	2,4	5,4	2,3
		2	247	32,6	366						
		3	246	32,6	366						
	P-R345	1*	470	63.3	480	365	42.6	356	2.5	26.0	3.4
		2	372	34,8	347						
		3	358	50,5	364						
	P-R'	1	307	67,7	395	303	63,6	372	2,0	11,0	5,5
		2	296	67,6	364						
		3	305	55,6	357						
	P-R"	1	256	39,7	462	289	33,2	454	10,5	16,9	2,9
		2	316	29,8	461						
		3	295	30,2	438						
	OMW	1	337	37,4	303	336	34,4	307	1,4	13,0	1,1
		2	331	36,6	309						
		3	340	29,3	309						

*Data was excluded due to outlier of IS.
**Lower than LOQ= 12.5 mg/100g
***O-R3: Green olives, O-R4: Cherry olives, O-R5: Ground olives

4.2.5. Calculation of Processing Loss

Processing loss of the lipophilic bioactives, L (per cent), was calculated as:

$$W'_{oil}(kg) = FMO(\%)\ x\ W'_{total}\ (kg) \tag{4.1}$$

$$W'_{sq}\ (kg) = W'_{oil}(kg)\ x\ c_{sq}\ \left(\frac{mg}{100g}\right) x \frac{1}{100000} \tag{4.2}$$

$$L_{sq}(\%) = \frac{W_{sq}^{olive}\ -\ W_{sq}^{VOO/SCOO}}{W_{sq}^{olive}} x100 \tag{4.3}$$

$$P_{sq}(\%) = \frac{W_{sq}^{P}}{W_{sq}^{olive}} x100 \tag{4.4}$$

$$?\ (\%) = L_{sq}(\%) - P_{sq} - OMW_{sq} \tag{4.5}$$

The total oil content of the sample, W'_{oil}, (kg, Table 4.4, Equation 4.1), was calculated by multiplying per cent oil content fresh matter basis (FMO, Table 4.2) and total weight of sample in the process, W'_{total} (kg, Figure 4.1). The lipophilic content of the samples, W'_{x} (kg, Table 4.4, Equation 4.2), was calculated by multiplying the amount of total oil W'_{oil} (kg, Table 4.4) by concentration of lipophilics c_{sq} in the oil extracted from the sample (mg/100g, Table 4.3). Processing loss, L_x (per cent, Table 4.5, Equation 4.3) of squalene (sq), β-sitosterol (sito) and α-tocopherol (toco) was then calculated by subtracting amounts in oil outputs, $W_x^{VOO/SCOO}$ (kg, VOO and SCOO), from amounts in olive inputs, W_x^{olive} (kg, Table 4.4), divided by amounts in olive (Table 4.4).

The difference between the processing loss (per cent) and the recovered amount in the final waste streams (per cent), P_{sq} and OMW_{sq} (OMW and P, Equation 4.4), was the "unaccounted-for" amount, ? (per cent), which might be lost due to degradation or poor extraction from paste and pomace (Table 4.5, Equation 4.5).

Table 4.4. Distribution of oil, water, solid and lipophilic bioactives (squalene (sq), α-tocopherol (toco) and β-sitosterol (sito)) during VOO and SCOO processing

Input/ Output	Sample codes	W'_{total} (kg)	W'_{oil} (kg)	W'_{water} (kg)	W'_{solid} (kg)	W'_{sq} (kg)	W'_{toco} (kg)	W'_{sito} (kg)
First extraction								
Olive inputs	O-R3	26465	5672	11380	9413	28.5	2.2	15.3
	O-R4	18570	4520	7985	6065	18.0	0.9	12.6
	O-R5	13610	4453	2722	6435	11.1	0,1	13.7
	Total O	**58645**	**14645**	**22087**	**21913**	**57.6**	**3.2**	**41.6**
VOO outputs	VOO-R3	5725	5725	-	-	25.1	1.4	11.8
	VOO-R4	3884	3884	-	-	19.7	0.5	7.0
	VOO-R5	3809	3809	-	-	11.7	0.3	8.6
	Total VOO	**13418**	**13418**	**-**	**-**	**56.5**	**2.2**	**27.4**
Pomace samples	P-R3	20740	556	12444	7740	1.2	0.2	1.9
	P-R4	14686	416	9546	4724	1.6	0.3	1.8
	P-R5	9801	238	6077	3486	0.6	0.1	0.9
	Total P	**45227**	**1211**	**28067**	**15950**	**3.4**	**0.6**	**4.5**
De-stoning								
Input	P-R345	45227	1530	28945	14751	5.6	0.7	5.4
Outputs	Pit	6250	-	875	5375	-	-	-
	P-R'*	38977	1341	26894	10742	4.1	0.9	5.0
Second extraction								
Input	Water	4800	-	4800	-	-	-	-
Outputs	SCOO	330	330	-	-	1.3	0.1	0.9
	P-R"	28827	954	16431	11441	2.8	0.3	4.3
	OMW	14620	149	13231	1240	0.5	0.1	0.5
	Total	**43777**	**1433**	**29663**	**12681**	**4.6**	**0.5**	**5.7**

*Input of second extraction

4.2.6. Statistical Analysis

One-way analysis of variance (ANOVA) using the Tukey test was performed to evaluate the significance of differences between mean values of triplicate analysis among VOO, O and P samples to compare their concentration results in three operational runs (α=0.05). T-test (paired two sample for means) was applied to determine the difference between inputs (olive or pomace without pit) and their outputs (VOO or SCOO). Microsoft Excel software was used for statistical analysis of squalene, α-tocopherol and β-sitosterol.

Table 4.5. Process loss of lipophilic bioactives (squalene (sq), α-tocopherol (toco) and β-sitosterol (sito)) and their distribution in the waste streams during VOO and SCOO processing

Run/Sample codes	Oil (%)	sq (%)	toco (%)	sito (%)
1st extraction				
L-R3	-1	12	38	23
L-R4	14	-9	42	44
L-R5	14	-5	-	37
P-R3	10	4	8	12
P-R4	9	9	29	14
P-R5	5	5	53	6
?-R3	-11	8	30	11
?-R4	5	-18	13	30
?-R5	9	-11	-	31
De-stoning				
L-R'	12	27	-31	8
2nd extraction				
L-R"	75	67	92	82
P-R"	71	68	37	87
OMW	11	12	6	9
?-R"	-6.8	-13	49	-14
Overall Process				
L-total	6	0	31	32
VOO	92	98	67	66
SCOO	2	2	2	2
P-R"	7	5	10	10
OMW	1	1	2	1
?	-1	-6	19	21

* Loss values were calculated based on data in Table 4.4.
**L: Processing loss; ?: Unaccounted value

4.3. RESULTS AND DISCUSSION

In order to determine the loss of lipophilics during industrial olive oil processing, sampling (O, P, OMW, VOO & SCOO) was done at a plant in Burhaniye, Turkey. The plant was processing VOO in three separate processing lines of a two-phase system (VOO-R3-5). The combined pomace of these lines (P-R345) was then de-stoned (P-R') and extracted to obtain SCOO using a three-phase system. The mass balance of the overall process, which was

calculated on the basis of the production data of O, VOO, SCOO, OMW and pit provided by the plant, is given in Figure 4.1.

Water, solid and oil contents of O, P and OMW samples are provided in Table 4.2. Water content of ground olive fruits (O-R5, 20 per cent) was lower than that of shaking tree olives (43 per cent) as expected. Accordingly, ground olive fruits had higher oil content (33 per cent) than others (21 and 24 per cent) on a fresh matter basis (FMO) in spite of similar DMO values (38, 43, and 41 per cent, respectively) (Table 4.2). The oil contents of the pomace samples of the three VOO processing lines (FMO, 2.7, 2.8 and 2.4 per cent, respectively) were lower than the maximum level permitted for pomace of two-phase centrifugation (four per cent) [93] and 0.5 per cent oil was determined in OMW, which had 4.5 per cent solid content (OFDM).

4.3.1. Lipophilic Concentration of the Samples

Concentrations of lipophilic bioactives of the samples are given in Table 4.3. Squalene, α-tocopherol and β-sitosterol concentration of the oils obtained from the olives using Soxhlet extraction were in the range of 250-502 mg/100 g, up to 39 mg/100 g and 270-307 mg/100 g, respectively (Table 4.3). Considering squalene and α-tocopherol concentrations, three different olive samples were significantly different from each other in the order of ground olive < cherry olive < green olive ($p<0.05$). Contrary to squalene and α-tocopherol, the highest β-sitosterol concentration was obtained for ground olives, yet the differences between β-sitosterol concentrations were not significant ($p>0.05$).

4.3.1.1. Olive Oil

Lipophilic concentrations of VOO produced from three different olive types were in the literature range (200-925 mg/100 g; 5-549 mg/100 g; 14.8-24.0 mg/100 g for squalene; β-sitosterol; α-tocopherol, respectively [7,100,164,173-175]), except α-tocopherol concentration of VOO-R5 from ground olives, which was lower than LOQ (limit of quantification) value of the method.

β-sitosterol and α-tocopherol concentrations of VOO were lower than that of oils obtained from olive fruits by 23-35 per cent and 32-39 per cent, whereas VOO obtained from cherry and ground olives had higher squalene concentrations than olive fruits (23-28 per cent). A significant difference was observed in β-sitosterol content of VOO samples, with cherry olives (VOO-R4) having the lowest β-sitosterol concentration. Contrary to β-sitosterol, α-tocopherol and squalene concentrations of VOO obtained from ground olives were the lowest (<LOQ and 307 mg/100 g, respectively) similar to olive oil samples. Order determined for α-tocopherol concentration of VOO samples was the same as α-tocopherol and squalene concentration of olive samples (ground olive < cherry olive < green olive). However, squalene concentration of VOO obtained from cherry olives was higher than that of green olives.

4.3.1.2. Pomace Samples

Oil obtained from three replicate extractions of pomace and olive samples were pooled and analyzed as one sample, the RSD values of replicate (n=3) determinations from pooled samples were in the range of 0.3-10.5 per cent, 0.6-9.5 per cent and 1.4-26.0 per cent for concentration of squalene, β-sitosterol and α-tocopherol, respectively. High RSD value of α-tocopherol was due to its lower concentrations.

Lipophilic bioactive content of the pomace samples of VOO processing of three different olives (P-R3; 4; 5), and oil (SCOO) and pomace samples of second extraction (three-phase) (P-R´´) are given in Table 4.3. Squalene, β-sitosterol and α-tocopherol concentration of pomace (P-R3; 4; 5), were in the range of 240-387 mg/100g, 339-430 mg/100 g and 32-62 mg/100 g, respectively (Table 4.3). While squalene and tocopherol concentrations compared well with values previously reported for fresh pomace in the literature (309 mg/100 g and 33 mg/100 g, respectively [97]) concentration of β-sitosterol was 35 per cent higher than the literature value (250 mg/100 g [97]).

Highest bioactive concentration was obtained in the pomace of cherry olives (P-R4) (387 mg/100 g, 62 mg/100 g and 430 mg/100 g squalene, α-tocopherol and β-sitosterol respectively). α-tocopherol and β-sitosterol concentrations of unrecovered oil in pomace samples were higher than those of VOO samples. This concentration effect was most

pronounced for mature cherry olives with tocopherol and sterol concentration in pomace oil 2.4 and 4.6 times higher than those in VOO samples. As olive cell ripens, the cell wall is weakened due to partial solubilization of pectic, hemicellulosic and cellulosic polysaccharides [176,177]. This might decrease extraction efficiency as the weakened cell wall structure was shown to prevent cell disruption during crushing [176]. Squalene concentrations of pomace oils, however, were lower than those of VOO.

In second extraction, β-sitosterol and α-tocopherol concentration of SCOO (266 mg/100 g and 19.4 mg /100g) was significantly ($p<0.05$) lower (by 28 and 70 per cent) than those in oil of P-R′, which might be attributed to their incomplete recovery by physical extraction as supported by β-sitosterol and α-tocopherol concentration in the last orujo (P-R": 454 mg/100 g and 33.2 mg/100 g). A similar trend was also observed for the oil yield of SCOO and P-R' samples (0.8 per cent for SCOO and 3.4 per cent for P-R'). On the other hand, squalene concentration of SCOO (405 mg/100g) was significantly higher ($p<0.05$) than pomace without pit (P-R': 303 mg/100 g) by 34 per cent while oil extracted from P-R" contained 289 mg squalene/100g. Squalene, β-sitosterol and α-tocopherol concentration of OMW were 336 mg/100 g, 307 mg/100 g and 34.4 mg/100 g, respectively.

The increase in the concentrations of β−sitosterol and α−tocopherol in pomace oils, and the differences in the extraction behavior of squalene and these compounds can be attributed to their distribution in the olive fruit and within the plant cell and interactions with other components (section 2.1.1.). While squalene, a polyisoprene hydrocarbon without polar groups, is present in free form in the midplane of the lipid bilayer [19], β−sitosterol and α−tocopherol, which contain hydroxyl groups, are bound to the cell membrane.

4.3.2. Calculation of Loss Ratios

The distribution of oil and lipophilic compounds between process streams (Table 4.4) and their loss of during VOO and SCOO processing (per cent loss, Table 4.5) were determined based on mass balance using production data and lipophilic concentration of the process streams (Figure 4.1, Table 4.3) as described in Section 4.2.5. In addition to loss values, discrepancies in the mass balance were calculated as unaccounted values (?) (Table 4.5) and

are discussed below considering possible inaccuracies in production data provided by the plant.

4.3.2.1. Oil Loss

The oil yields of VOO processing (R3, R4, R5) were calculated as 22, 21, and 28 per cent respectively (Figure 4.1) indicating oil loss values in the range of zero to 14 per cent during VOO processing based on oil content (FMO) of the olive samples determined using Soxhlet extraction (21, 24, 33 per cent respectively, Table 4.2). These loss values mainly correspond to the amount that could not be recovered by first physical extraction from the olive matrix remaining in the pomace streams (P). Two per cent of the oil was recovered by second extraction as SCOO and total oil loss was calculated as 6 per cent. However seven and one per cent of the oil was recovered in the final waste streams, P_{oil} and OMW_{oil}, which is higher than total oil loss (six per cent) (Table 4.5). Unaccounted value (minus one per cent) can be attributed to the inaccuracy of amount data taken from the plant.

In R4 and R5 of the VOO process, 14 per cent of the oil in the cherry and ground olives was lost while nine per cent and five per cent of the oil in olive remained in pomace, respectively (Table 4.5). However, in R3 of the VOO process, calculations based on oil content of green olives and VOO-R3 indicated no process loss, yet 10 per cent of the oil in O-R3 remained in P-R3. It is possible that oil of green olives (O-R3) could not be extracted efficiently using Soxhlet. Unaccounted values of oil in three runs were -11, five and nine per cent, respectively. The minus value is because of the lower oil value of O-R3 than expected.

Oil loss during pomace processing (second extraction) was considerably higher with only 25 per cent of the oil content of the combined pomace sample without pit (P-R') recovered as pomace oil (SCOO) by three-phase physical extraction where 71 and 11 per cent of oil of P-R' remained in last orujo (P-R'', three per cent FMO) and OMW (one per cent FMO), respectively. As pomace mainly includes bound oil, which is difficult to extract, loss in pomace processing was higher than olive processing.

Oil loss values calculated as the difference between the oil content of input olive and output oil streams of VOO (R3) and SCOO processing underestimated the actual process loss as evidenced by the higher oil content of the waste streams (-11 and minus seven per cent,

respectively). As mentioned above, it is possible that oil of green olives (O-R3) could not be extracted efficiently using Soxhlet. Second extraction with higher malaxation temperature and second crushing could be more efficient for depitted matrix (P-R') than Soxhlet where two per cent of the oil was recovered by second extraction.

4.3.2.2. Lipophilic loss

The loss of β-sitosterol during VOO processing (R3, R4 and R5) was calculated as 23, 44, and 37 per cent, respectively (Table 4.5). Based on literature data of sterol concentration of Spanish olive varieties processed by Abencor system, we previously calculated process loss of β-sitosterol as 48 per cent (section 2.4), which is comparable to values obtained for cherry and ground olives. The recovery of β-sitosterol and oil in pomace samples were similar with 12, 14 and six per cent of β-sitosterol and 10, nine and 5.4 per cent of oil remaining in the pomace samples (P-R3; R4; R5), respectively. These values correspond to 11, 30 and 31 per cent unacounted values for β-sitosterol, which might be attributed to limited extractability of β–sitosterol from pomace. Unaccounted values of β-sitosterol for three runs (R3-4-5) were higher than those of oil yield (-11, five and nine per cent, respectively). This might be due to different mass transfer behavior of oil and β-sitosterol during extractions. It can be said that β-sitosterol content of VOO does not represent the actual content of the olive fruit. To recover remaining oil, second extraction was applied using the three-phase method in the plant. In second extraction, process loss of β-sitosterol calculated from the difference between the amount of β-sitosterol in the pomace without pit and SCOO was 82 per cent, and 87 per cent of the β-sitosterol in P-R' remained in the last pomace (P-R'') and nine per cent was in OMW (Table 4.5). Similar to oil loss, β-sitosterol content of the waste streams was 14 per cent higher than the calculated process loss due to efficiency of second extraction where two per cent of the β-sitosterol was recovered. Overall process loss of β-sitosterol was 32 per cent and 10 per cent of the β-sitosterol in olive remained in the last pomace (P-R'') and one per cent was in OMW with 21 per cent unaccounted value.

It should be noted that only free β-sitosterol and esterified β-sitosterol were quantified in olive, pomace, VOO and SCOO samples as β-sitosterol glucosides could not be recovered by hexane due to its low polarity [159]. Olive oil contains up to three mg/kg β-sitosterol

glucoside [178]. Further research is required to determine total loss of β−sitosterol including β-sitosterol glucosides and other bound or conjugated forms in last pomace and olives using acid hydrolysis. Higher β-sitosterol recovery could be obtained (about 34 per cent according to Toivo et al., (2001) [159] and about 22-42 per cent according to Jonker et al. (1985) [179]) from plant foods such as cauliflower, wax beans, lettuce, cucumber, apple, banana, potato, peanuts and whole wheat due to liberation of free plant sterols from (acylated) steryl glycosides by cleavage of the acid-labile acetal bond.

Loss of α-tocopherol during two-phase VOO processing of green and cherry olives (R3 and R4) was 38 per cent and 42 per cent, respectively, which were similar to the value calculated using literature data in section 2.4 (36 per cent). However, loss in R5 could not be calculated because α-tocopherol concentrations in ground olives, VOO and pomace samples (VOO-R5, O-R5 and P-R5) were lower than LOQ of the GC-FID method used. For runs three and four, eight per cent and 29 per cent of α-tocopherol remained in the pomace samples, whereas 30 per cent, and 13 per cent of α-tocopherol were unaccounted for. Similar to β-sitosterol, this might be attributed to limited extractability of α-tocopherol from pomace, degradation of α-tocopherol during processing should also be considered. In second extraction process (R"), which had a higher malaxation temperature (40 °C, 40-50 min) and three-phase decantation, the loss was 92 per cent of α-tocopherol in P-R', but only 37 per cent and six per cent of α-tocopherol in olives remained in pomace and OMW, respectively (Table 4.5). 49 per cent of α-tocopherol was unaccounted for during second extraction where two per cent of the α-tocopherol recovered. This might be attributed to degradation of α-tocopherol during processing. Similar to β-sitosterol, overall process loss of α-tocopherol was 31 and 10 per cent of the α-tocopherol in olive remained in the last pomace (P-R'') and two per cent was in OMW with 19 per cent unaccounted value.

Loss of squalene during VOO processing (R3, R4 and R5) was calculated as 12, minus nine, and minus five per cent, respectively (Table 4.5). We have previously determined squalene loss to be 19 per cent based on literature data taken from mean concentration of squalene in Italian olives with different stage of ripeness and varieties (Gentile (Larino and Colletorto), Coratina, Peranzana and Leccino) and in their olive oil products (section 2.4). In the second extraction, the process loss of squalene (67 per cent) was recovered in pomace (68 per cent) and OMW (12 per cent) with accounted value (-13 per cent), similar to oil content.

There was no loss (zero per cent) of squalene during the overall process. This points to higher extractability of squalene than α-tocopherol and β-sitosterol from olive and pomace samples. Squalene, a nonpolar compound, is mainly concentrated in the oil of the pulp and is present in free form in the cell membrane as described in section 4.3.1.2, whereas α-tocopherol and β-sitosterol are concentrated in the seed of the olive fruit (inside the stone wall) and are bound to the cell membrane. Five and one per cent of total squalene content remained in P-R'' and OMW in the overall process. Similar to the oil contents, unaccounted value (minus six per cent) can be attributed to the inaccuracy of the production amount data provided by the plant.

To calculate loss during pitting process (L-R′), combined pomace (P-R345) was taken as input and pomace without pit was taken as product. 1.3 per cent of total oil was lost during this process. Per cent loss of squalene, α-tocopherol and β-sitosterol were calculated as 27, -31 and eight per cent, which is included in the unaccounted (?) values of the overall process by three, minus six and one per cent (Table 4.5). α-tocopherol content of pitted pomace (P-R') was higher than combined pomace indicating that α-tocopherol might be liberated from the pomace matrix during the pitting process.

4.4. CONCLUSIONS

In this study, the distribution of pre-dominant lipophilic bioactives (squalene, β-sitosterol and α-tocopherol) between olive oil (VOO and SCOO) and waste streams (OMW and pomace) was determined and loss of these lipophilic bioactives during VOO production was calculated using simultaneous GC-FID analysis of samples (O, VOO, SCOO, P, OMW) taken from a two-step integrated milling plant.

In the overall process, 92 per cent of oil, 98 per cent of squalene, 67 per cent of α-tocopherol and 66 per cent of β-sitosterol were recovered in VOO and two per cent of oil and lipophilics were recovered in SCOO. The presence of lipophilic bioactives (squalene, β-sitosterol and α-tocopherol) in SCOO or pomace oil was an indication of loss of these compounds in during VOO processing.

Squalene, which is present in free form in the pulp of the olive fruit, was recovered completely in the product streams (VOO and SCOO), but the loss of α-tocopherol and β-sitosterol was 31 and 32 per cent, respectively which are higher than the loss of oil (six per cent). While negligible amounts of oil and lipophilics (one to two per cent) were lost in OMW, seven per cent, five, 10 and 10 per cent of oil, squalene, α-tocopherol and β-sitosterol remained in the last pomace (P-R''), respectively, as determined by Soxhlet extraction.

β-sitosterol and α-tocopherol, which are mainly concentrated in the seed of the olive fruit, were recovered in VOO to a lesser extent (66 and 67 per cent). But 19 per cent of the α-tocopherol and 21 per cent of the β-sitosterol loss was unaccounted for, which can be attributed to degradation of α-tocopherol and incomplete recovery of sterols from the olive and pomace matrices. Lower unaccounted values of oil content (minus one per cent) and squalene (minu six per cent) were attributed to the inaccuracy of production amount data provided by the plant.

According to distribution data of oil and lipophilics, squalene, which is nonpolar and is present in free form in plasma membrane, and olive oil showed similar behavior (zero and six per cent processing loss) during processing, whereas the recovery of α-tocopherol and β-sitosterol in VOO were lower (31 and 32 per cent processing loss). The results of this study point to anatomic and cellular distribution of these compounds and their interactions with other cell components to be important factors in determining their distribution/loss behavior. Acid hydrolysis of the olive and pomace samples will enable the determination of the total loss of oil including polar and bound lipids and β-sitosterol and α-tocopherol including bound forms, providing a more complete picture of their extraction behavior. New extraction and pretreatment techniques could be developed to achieve quantitative recovery of tocopherols and sterols from olive fruit and can be applied to other plant based waste streams.

5. USE OF HYDROTHERMAL PRETREATMENTS FOR THE RECOVERY OF β-SITOSTEROL FROM OLIVE POMACE

5.1. INTRODUCTION

Olive oil processing waste of a two-phase olive mill (alperujo, 11 355 200 tonnes/year in the World, 80 per cent of olive used based on 2009-2015 data) [44,180], is composed of 56-75 per cent moisture and main components of its organic fraction are oil (8-20 per cent) and cell wall constituents such as cellulose (14-25 per cent), lignin (32-56 per cent) and hemicellulose (27-42 per cent), protein (4-12 per cent), water-soluble carbohydrates (1-16 per cent) and water-soluble phenols (1-2 per cent) on a dry weight basis [181]. Additionally, olive pomace is rich in β-sitosterol with pomace oil containing 339-430 mg β-sitosterol /100 g (compared to 181-226 mg/100 g in VOO) (Chapter 4). Disposal of olive wastes, which is an environmental problem, has not been solved completely in spite of research on new techniques for value added utilization of olive wastes 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 [1,181].

Phytosterols, which are well known as cholesterol lowering agents, have many other health effects (anti-inflammatory, anti-atherogenicity, anti-cancer and anti-oxidative activities) and are used in pharmaceuticals, food, nutrition and cosmetics area [182]. Major source of phytosterols are vegetable oils, they are especially rich in maize oil (763.4 mg/100g) and rapeseed oil (704.4 mg/100g) [183], because of their low concentration in crude oils, they are recovered mainly from deodorization distillate and tall oil pitch [35, 182]. However, these recovery techniques are limited with sterol content of oils.

The main objective of this study was to use hydrothermal pretreatments for value added utilization of olive pomace to obtain sterol enriched oil and phenolics. Specific objectives of this study were to investigate the effect of hydrothermal pretreatment (steam and SCW) and pretreatment temperature on the yield and BS content of pomace oil and phenolics.

5.2. MATERIALS AND METHODS

5.2.1. Samples

Pomace (alperujo) samples (˜100 kg) were obtained from the pilot VOO production plant (two-phase) at the Instituto de la Grasa in Sevilla, Spain (November, 2015). Pomace samples were stored in cooling cabinets for short period (4°C) and long period (−20 °C), protected from light. In the plant, picual type olives were processed in a production line including a hammer crusher, two serial malaxers (25.2°C and 26.3°C), a decanter (two-phase, 3000 rpm), and separator.

Fresh pomace samples were homogenized and freeze dried and stored in a desiccator. Moisture and oil content (by Soxhlet) of pomace samples were determined according to the "Guide for the determination of the characteristics of oil-olives" (COI/OH/Doc. No 1 November 2011) in triplicate [93].

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 freeze dried, and then homogenized and stored in a desiccator until further analysis (Figure 5.1).

5.2.2. Reagents

β-sitosterol (100 μg/ml in chloroform, analytical standard) and triolein (Glyceryl trioleate, 61 per cent) 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). 5α-cholestane (99.57 per cent, GC Grade) and pyrogallol (HPLC) were obtained from Sigma-Aldrich (Jerusalem, Israel and Dorset, UK). n-hexane (99 per cent, GC grade) was obtained from Sigma-Aldrich (Israel). Sodium chloride (ACS grade) was obtained from Panreac (Barcelona, Spain). β-sitosterol (75.8 per cent β-sitosterol, 10.3 per cent beta-sitostanol, 7.7 per cent campesterol), pyridine (extra dry, 99.5 per cent) and hexamethyldisilazane (HMDS) (98 per cent, reagent plus) were obtained from Acros

Organics (France, Geel, Belgium and Germany). Hydrochloric acid (37 per cent) and diethyl ether were obtained from Carlo Erba (Rodano, Italy and Val- de-Reuil, France). Potassium hydroxide (90 per cent, 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).

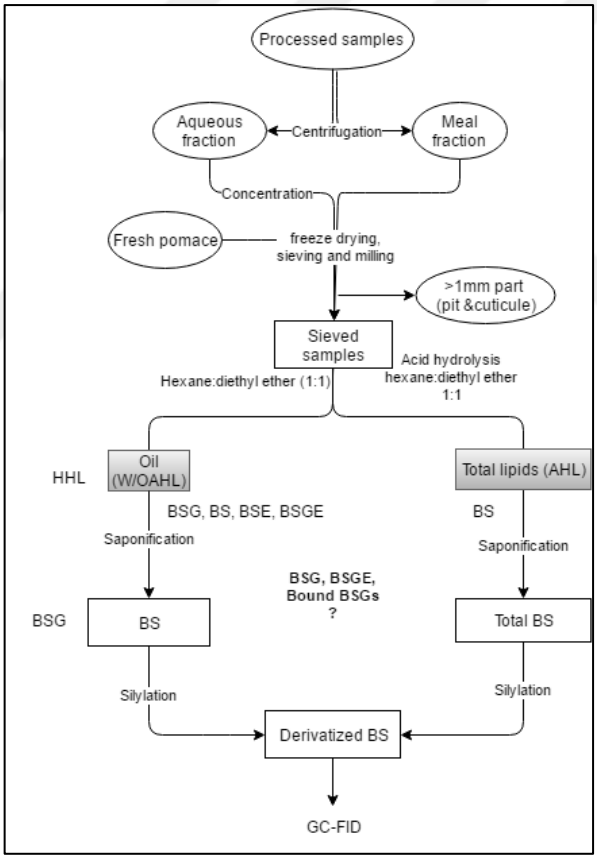


Figure 5.1. Flow chart of overall methodology

5.2.3. Sample Preparation

A methodological framework for the determination of free (BS) and total β-sitosterol (including bound BS-BSG and free BSG) in olive pomace was developed by modifying

sample preparation methods used previously for oil seeds and other food matrices [159,184]. Oil extraction and sample preparation of GC-FID method (Chapter 3) were modified as follows. The scheme for developed sample preparation steps is given in Figure 5.1.

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 (AHL) and subsequent oil extraction with hexane:diethyl ether [159]. 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 AHL and without AHL was calculated to obtain bound BS-BSG and free BSG. However, the differences in oil contents were not exact indication of bound oil as triglycerides and phospholipids extracted from acid hydrolyzed samples were recovered as FFA, not intact. It was reported by Philips et al. (1997) that more polar shorter chain fatty acids were not recovered by AHL extraction using hexane:diethyl ether and thus total lipid recovery was lower, AHL leads to extraction of nonfat materials additionally [185]. Extract obtained from fresh pomace without AHL included total triglycerides and phospholipids but FFA content of hydrothermally pretreated (HHL) samples was expected to be higher than fresh pomace.

5.2.3.1. Extraction of Total Lipids and Total BS

Total BS content of AHL (including free, esterified, and glycosidic BS with bound BS analyzed as free BS) was determined by the method of Toivo et al. (2001) [159], which was modified for olive pomace (Figure 5.2). 0.5 g (one gram for samples with pit) of dry sample was weighed into a 20 ml vial with screw cap. After adding 3 mL absolute ethanol and IS2 (5α-cholestanol) in 0.5 mL absolute ethanol (100 μg/mL), 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). The vials were shaken every 15 minutes. After heating, samples were cooled by cold tap water and stored for five minutes in the freezer. Five milliliter 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 five minutes to achieve phase separation. Supernatant phase was transferred to 12 mL glass tubes using a Pasteur pipette

while solvent evaporation was done by air drying under fume hood and subsequent nitrogen drying in block heated concentrator. The tube was weighed to determine extracted lipid content and then stored at -20 °C for determination of total BS including bound and unbound forms.

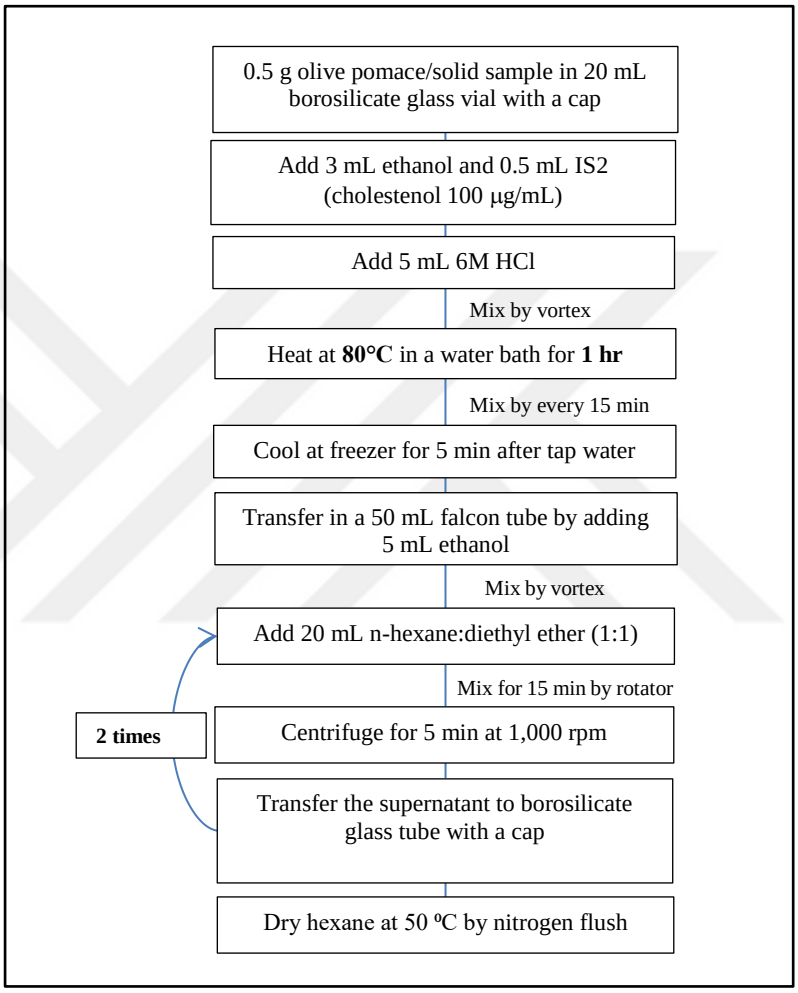


Figure 5.2. Flow chart of modified acid hydrolysis (AHL) procedure [159]

Low repeatability values (1-8 per cent RSD in AHL with pit) obtained for oil content was attributed to the nonhomogeneous distribution of pit in the dry pomace, which contained 43 per cent pit (Figure A.5). 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 per cent oil content and BS content of sieved samples (Table 5.1). To modify sample preparation for pomace, t_w (60, 90, 120 min) was tested in duplicate for higher oil content and BS concentration (Table 5.2).

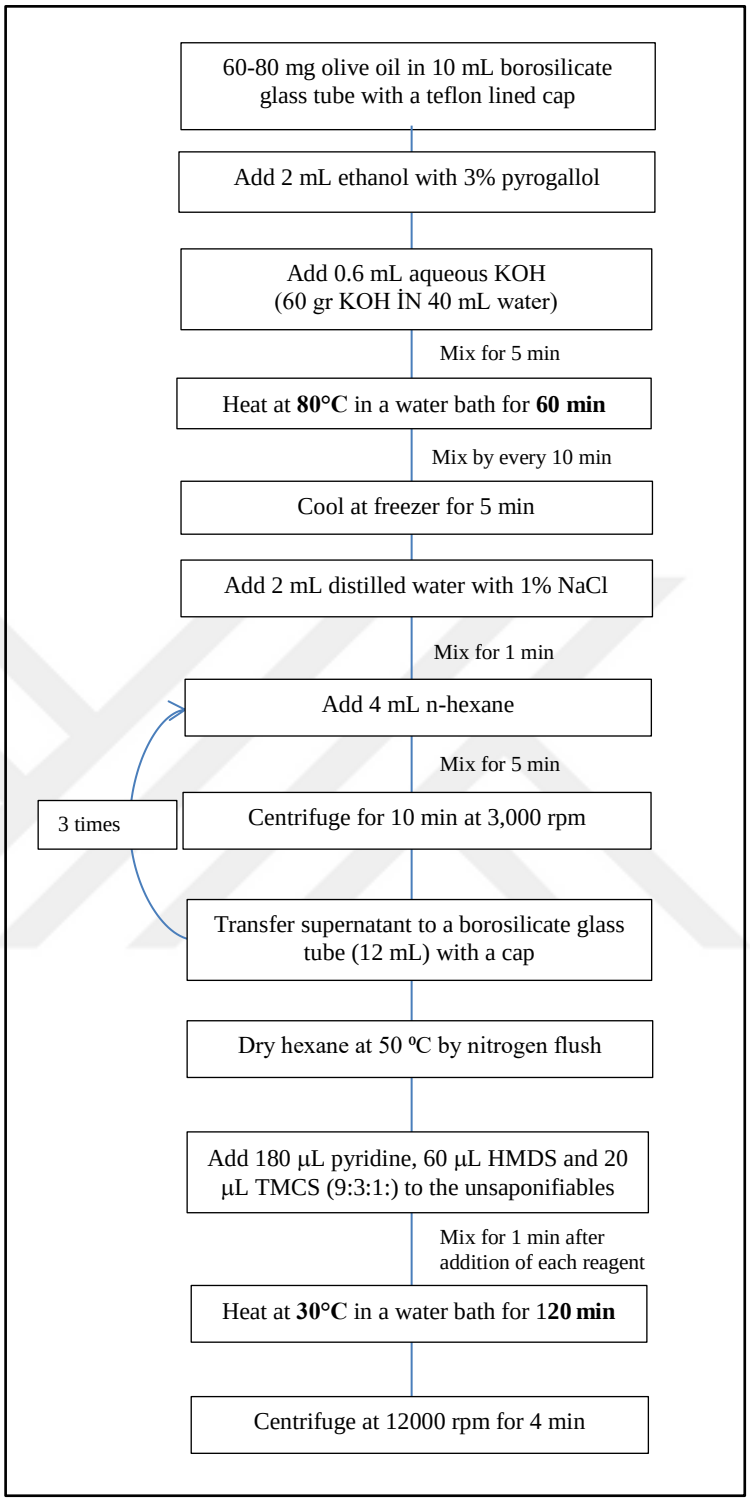


Figure 5.3. Flow chart of GC-FID procedure

Table 5.1. Distribution of total lipid and BS through the sieved pomace fractions at different mesh sizes (n=2)*

Parameters	Weight Distribution %	Oil yield %	Total BS conc. in ext. oil sample (mg/100g)	Total BS conc. in sieved sample (mg/100g)	Dist. of BS through whole sample (%)
Dusty part (< 0.5 mm)	42	18.4 ± 0.14^{b}	499 ± 11 (2.1%)^{a}	92	60
Small fragments of pit & cuticle (1-0.5 mm)	16	17.9 ± 0.39^{b}	452 ± 6 (1.4%)^{a}	81	20
pit and cuticle particles (>1 mm)	43	7.1 ± 0.42^{a}	421 ± 65 (15.5%)^{a}	30	20

*Average values of 2 replicates
**AHL time: 120 min

Table 5.2. Determination of hydrolysis time at 80 °C for sieved pomace sample (n=2) and effect of extraction type and acid hydrolysis (n=3)***

Parameters	Oil yield %	Total BS conc. in ext. oil sample (mg/100g)	BS conc. in dried pomace (mg/100g)
AHL time at 80 °C, t_w			
60 min	18.0 ± 0.16^{a}	475 ± 18^{a}	86 ± 4
90 min	18.0 ± 0.29^{a}	432 ± 1^{a}	78 ± 1
120 min	18.7 ± 0.25^{a}	465 ± 2^{a}	87 ± 1
Extraction type and acid hydrolysis			
AHL (60 min)	19.4 ± 0.72^{b}	451 ± 41^{b}	87 ± 7^{b}
Soxhlet	15.7 ± 0.70^{a}	297 ± 46^{a}	46 ± 5^{a}
W/OAHL	15.7 ± 0.15^{a}	339 ± 8^{a}	53 ± 2^{a}

*HL: hexane-diethyl ether extraction after hydrolysis,
W/OAHL: hexane-diethyl ether extraction without hydrolysis
**One-way ANOVA with replication was used.
***Sieved samples (>1 mm)

5.2.4. GC-FID Analysis of β-sitosterol

GC-FID method of Seçmeler and Güçlü Üstündağ (2016) (section 3), 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 (Figure 5.3). 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) due to complex composition of pomace oil.

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 [48,186]. To break the ester bonds, according to AOAC Official Method Ca 6b-53 (for higher level unsaponifiable matter), 60 gr KOH in 40 ml water was used instead of 50 per cent KOH solution. 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. According to our previous method's optimization data (section 3.3), derivatization time and temperature were not significant when only considering BS response. To obtain maximum BS response, derivatization time (for saponification at 70°C and 60 min) needed to be increased to 60 min due to significant saponification time interaction and 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 um 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, five minutes hold at 330°C. The detector temperature was 360ºC 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 5α-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 -20ºC for one month [124]. Calibration standards were prepared in different amounts in the range of β-sitosterol amounts in pomace olive oil [97] by spiking triolein (blank matrix) and dried under nitrogen at RT before derivatization. A sample chromatogram is given in Figure 5.4.

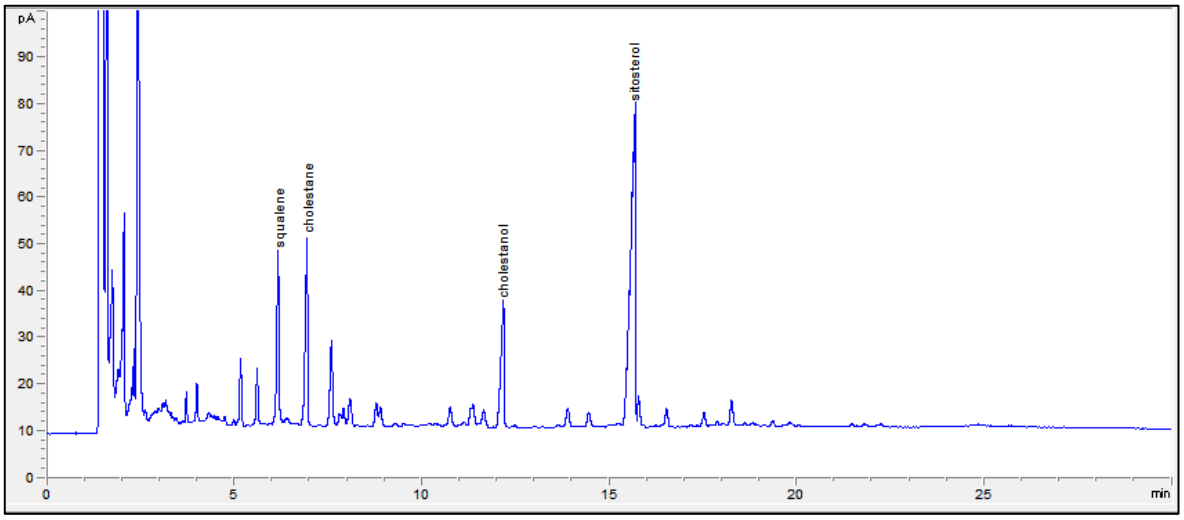


Figure 5.4. GC chromatogram of β-sitosterol in fresh pomace sample

The peak areas were calculated with Agilent Technologies GC ChemStation, and quantification was performed using external calibration method by plotting calibration curves (Peak area versus concentration (mg/100 g)). Internal standards (50 µl) 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 $Area_{analyte}/Area_{IS}$. Instrument performance was checked in each sequence by injection of QC standard mixture between every five samples.

5.2.5. Determination of Phenolic Profile

10 ml methanol:water (80:20) solvent was added to one gram 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 rotator, then incubated in water bath at 70°C for one hour and mixed by vortex 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 [187]. Total phenol concentration was determined by three replicate reading (up to 17 per cent RSD) for three replicate extracts (up to 15 per cent RSD) of all samples (aqueous, meal fractions of processed samples, fresh pomace and pit). Then, TP contents were expressed as mg Gallic Acid Equivalent (GAE)/100g dry weight.

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 per cent trichloroacetic acid in water and acetonitrile utilizing the following gradient over a total run time of 55 min: 95 per cent A initially, 75 per cent A in 30 min, 50 per cent A in 45 min, 0 per cent A in 47 min, 75 per cent A in 95 min, and 95 per cent A 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.

Amount of total phenol and individual phenolic compounds were reported as mg/100g in dry base for aqueous and meal fraction.

5.2.6. Hydrothermal Treatments

Hydrothermal experiments (steam and subcritical water treatments) were carried out in the flash hydrolysis laboratory pilot unit at the Instituto de la Grasa (Sevilla, Spain) (Figure A.6), which had previously been used only for steam treatment [132,136].

The pilot unit included a steam generator, accumulator, two liters stainless steel reaction chamber (maximum operating pressure 42 kgf/cm^2) and stainless steel steam expansion chamber.

Generated steam was fed to the reaction chamber containing the sample by the help of a pneumatic valve until the set saturation pressure (kgf/cm^2) was maintained. The target temperatures were obtained by setting the steam pressure on the accumulator. To reach 180°C, accumulator pressure was adjusted to 10 kgf/cm^2 (from steam table). When the two temperature probes in the reaction chamber read the same temperature as the probe of the steam generator, the steam entrance valves were closed and reaction started (It was also possible to start reaction timer from the controller, manually). Reaction took place during the set reaction time (five minutes) in the reaction chamber. After the reaction was finished, exit valve was opened and steam expansion created an explosion through the steam

expansion chamber, which was cooled using water (50°C) in the jacket. While the aqueous part of the processed sample was drained by a valve under the chamber, the meal part, which stuck to the wall of the chamber, was recovered by washing manually with water. Before each treatment, the chamber was pre-heated by steam flushing. Duration (sec) of five phases of the run are shown on the screen of the controller; F1: Pre-heating (without sample), F2: Heating, F3: Compression, F4: Reaction, F5: Expansion.

In order to obtain subcritical conditions, nine preliminary runs were carried out and the pressure and temperature profile of aqueous solution in the reaction chamber were observed. The standard deviation of temperature and pressure and temperature difference between upside and downside of chamber was observed for each condition for homogeneity and stability of temperature and pressure (Table 5.3). Three volumes of aqueous pomace mixture (1 L, 1.5 L, 1.7 L) and three temperatures (150°C, 180°C, 220°C) were tested preliminarily. Pressure and temperature data were taken from the PC controller.

Three temperatures (160°C, 180°C, 200°C) were chosen based on previous studies of steam treatment [97,132] to compare the effects of direct steam and subcritical water hydrothermal treatments for five minutes. To obtain subcritical water conditions, additional water was added (up to 1.5 L) to two liters 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 kgf/cm^2 (Table 5.3). 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 (Figure A.7-8) for five minutes (steam) or six minutes (subcritical) (Table 5.3).

In subcritical water conditions, Lower set temperature 160°C could not be reached due to design limitations of system. That's why 150°C (144 -153°C) could be applied in the reactor while water was added. While comparing the steam treatment at 160°C, the temperature difference was considered (Figure A.7-8).

Table 5.3. Temperature and time data of hydrothermal treatments and Solid-liquid distribution after centrifugation and freeze drying

Op. Runs		Measured Values		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

Oil in fresh and processed pomace samples were extracted by hexane: diethyl ether (1:1) with (AHL) and without acid hydrolysis (HHL). Oil content and BS content were determined in triplicates (Table 5.4-5.5). Free BS and total BS were determined by the analysis of HHL and AHL, respectively. The bound BS and total BSG content in samples were calculated as the difference between content of BS determined from AHL and HHL (Figure 5.2).

5.2.7. Statistical Analysis

Data were expressed as mean ± standard deviation. Microsoft Excel and SPSS software was 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 (per cent), oil content (per cent) and BS content (mg/100g) of dried aqueous and meal fractions, separately (α=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.

5.3. RESULTS AND DISCUSSION

In order to use hydrothermal treatments for value added utilization of olive pomace by obtaining sterol enriched oil and phenolics, the study was carried out in three steps. Firstly, a methodology for sample preparation for the determination of free and total BS (including free and bound BS and β-sitosterol glucoside (BSG)) in olive pomace using acid hydrolysis was developed. Secondly, the effect of hydrothermal pretreatment method (steam and subcritical water) and temperature on the oil content and recovery of sterol and phenolic was investigated.

5.3.1. 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 SGs), 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 [159]. For this study, t_w (60, 90, 120 min) was optimized for fresh pomace sample (one gram) considering its lignocellulosic content (n=2). Oil content (per cent) was determined as 10.9, 12.6 and 13.2 per cent with one, eight and seven per cent RSD values, respectively.

Considering the heterogeneous nature of the sample, to decrease per cent RSD of results and to decrease sample size, a mesh size of one mm was chosen to remove the pit by sieving based on the weight distribution, oil content per cent and BS content of sieved samples (Table 5.1, Figure A.5). Pit & cuticle part (> 1 mm) contained only 7.1 per cent oil, oil content of other parts (0.5-1 mm and <0.5 mm) containing cuticle and pulp were 17.9 and 18.4 per cent, respectively. There was no significant difference between BS concentration of pit and cuticle (421 mg/100 g) and sieved parts (452 mg/100 g and 499 mg/100 g) (n=2, $p>0.05$) (Table 5.1). By multiplying weight distribution ratio of pomace sample (300 g) and BS concentration of sieved sample, it was shown that removing pit and cuticle caused 20 per cent BS loss, the remaining parts, small fragments of pit & cuticle (1-0.5 mm) and dusty part (< 0.5 mm), containing 20 per cent and 60 per cent BS, respectively. 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.

After removal of pit and cuticle fraction and reducing the amount of sample from one gram to 0.5 g, hydrolysis time study (t_w: 60, 90, 120 min) was repeated (Table 5.2, n=2). Precision was improved in oil content results of 90 min and 120 min hydrolysis by removing pit and cuticle: per cent RSD values were decreased from 8-7 per cent to 2-1 per cent. There was no significant difference in oil contents (18.0 per cent, 18.0 per cent and 18.7 per cent, respectively) and BS concentrations (475 mg/100 g, 432 mg/100 g and 465 mg/100 g, respectively) (n=2, $p>0.05$). Therefore, 0.5 g freeze-dried and sieved sample (< 1 mm) was used and 60 min hydrolysis was applied as previously done for flour samples [159].

Oil content determined by Soxhlet (SL) extraction (15.7 per cent) was the same as that of hexane:diethyl ether extraction (15.7 per cent) (Table 5.2, n=3). Moreover, there was no significant difference between free BS content of hexane:diethyl ether (53 mg/100g) and Soxhlet (46 mg/100 g) extracts (n=3, $p>0.05$). Therefore, hexane:diethyl ether extraction without acid hydrolysis (HHL) was used in further analysis for solvent economy in this study.

The use of acid hydrolysis before hexane:diethyl ether extraction (AHL) increased the oil yield significantly ($p<0.05$) from 15.7 per cent to 19.4 per cent as expected as bound lipids and hexane: diethyl ether soluble hydrolyzed products of polar lipids (phospholipids and

glycolipids) such as glycerylether-sn-2-acetyl glycolipid [188] could be extracted using acid hydrolysis.

Application of acid hydrolysis increased BS concentration of pomace oil by 33 per cent (from 339 mg/100g oil to 451 mg/100g oil) and BS recovery from pomace significantly by 64 per cent (from 53 mg/100g to 87 mg/100 g pomace) ($p<0.05$). It is higher than the results of literature studies that reported around 33 per cent [159] and 22-42 per cent [179] increase in sterol content upon hydrolysis from plant foods such as cauliflower, wax beans, lettuce, cucumber, banana, potato, peanuts and whole wheat. 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). This means that BS content of acid hydrolyzed samples includes not only bound BS, also bound BSG and free BSG.

5.3.2. Effect of Hydrothermal Pretreatments on Oil Yield and BS Content of Pomace Oil

5.3.2.1. Preliminary Studies

In this study, SCW pretreatment was applied to pomace for the first time. Preliminary experiments (nine runs) were carried out to develop a procedure to obtain SCW conditions as the pilot unit was not operated at SCW conditions previously. Subcritical water conditions could be reached at various temperatures (150-220°C) when extra water was added to the pomace and pressure was increased suddenly. Actual pressure measured in the reaction chamber was higher than the saturation pressure at the mean temperature (mean of T_{up} and T_{down} measured). A pressure higher than the saturation value could be achieved in this manner.

Practical rules were obtained for the operation of the system under subcritical conditions.

- Pressure should be increased after the target temperature is obtained. Additionally, pre-heating the reaction chamber is critical to reach target temperature quickly.
- For lower temperatures (<150°C) subcritical conditions could be obtained using an isobaric process but for higher temperatures gradual pressure increase is needed.

- In order to get temperature homogeneity, it was decided to use 1.5 L total volume (water and sample) for further experiments.
- Set pressure should be nearly lower than the saturation pressure (24 kgf/cm^2) of target temperature (220°C) and pressure should be increased suddenly after measured temperature reaches the target temperature.
- Time passing after the pre-heating process (F1) and initial set pressure is critical to get the target temperature in order to apply SCW pretreatment in this system.

5.3.2.2. Effect on Solid Distribution

Solid particle distribution of the pomace samples before and after the pretreatments are shown as per cent ratio of total dry pomace (94.6 gr of 300 gr fresh pomace), in Table 5.3. After centrifugation of processed sample, meal and aqueous fractions were obtained and freeze-dried (Figure 5.1). Dried meal fraction and pomace were milled and sieved (1 mm) to separate pit (& cuticle) fraction as mentioned in section 3.1. In spite of removal of dry pit part, its ratio was also calculated in addition to aqueous and meal fractions.

28-33 per cent of total solid was recovered in aqueous fraction and no significant effect of pretreatment and temperature was found in aqueous fractions ($p>0.05$). As this fraction contained carry over from meal fraction in addition to water soluble components solubilization of pomace could not be determined quantitatively.

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

Fragments of pit and cuticle (>1 mm) were broken into small pieces and they were passed to meal fraction (<1 mm). 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 from the sieve (1 mm).

In meal fraction and pit part, temperature and pretreatment effect were significant but their interaction was not significant ($\alpha=0.05$). Per cent ratio of SCW pretreated meal fractions

related to total solid content of pomace were lower (by 15-23 per cent) than that of steam pretreatment, while its pit fraction (>1 mm) were higher (by 13-22 per cent) 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 pretreatments. Water in SCW pretreatments might lower the effect of explosion as compared to steam. It can be said that explosion (rapid decompression) is the determining factor for physical disruption and accordingly size reduction of pits. In wood pellet production using steam explosion, it was reported that explosion effect resulted in disruption of the solid residues from bundles to individual fibers [189].

At 200°C for both pretreatments, per cent ratio of pit part related to total solid content of pomace was significantly lower and ratio of meal was significantly higher than that at 160 °C. Physical disruption was high at 200 °C due to higher temperature and high pressure.

5.3.2.3. Effect on Color and Odor

Hydrothermal pretreatments increased color intensity. This color change might be due to browning reactions (Maillard and/or caramelization) and pigment degradation.

The main olive pigments are chlorophylls (green) and carotenoids (red) and their composition change during ripening. All of the oil pigments were not released from the pomace matrix during oil extraction, most remained occluded in the pomace. Although chlorophyll was partly converted to pheophytin (dark bluish) due to the release of acids during VOO processing, ratio of green fraction to carotenoid in pomace was greater than that in olive fruit [190]. Antocyanins in olive fruit are Cyanidin-3-glucoside, Cyanidin-3-rutinoside, Cyanidin-3-caffeyglucoside, Cyanidin-3-caffeylrutinoside and Delphinidin 3-rhamosylglucoside-7-xyloside [191]. Together with the degradation of chlorophylls and carotenoids, anthocyanins were formed via the oxidative cleavage of the proanthocyanidin, the polymeric chains of which are major antioxidants found in OMW [192,193]. Under acid hydrolysis conditions, anthocyanins were degraded to their aglycone, anthocyanidins [193].

Dried aqueous fractions, having high sugar content, were dark brown color because of Maillard and/or caramelization reactions during processing. However, it was observed that aqueous fraction of SCW samples was more reddish than that of steam samples before

freeze-drying. This might be attributed to lower pH of the medium. Anthocyanins are stable only at low pH values. However, combined effect of pH and temperature on cyanidin-3-glucoside and Cyanidin-3-rutinoside in black rice was reported as at 165°C even at pH 2.2 as these compounds were degraded in less than five minutes [194]. As a degradation intermediate product, cyanidin was produced by deglycosylation of cyanidin-3-glucoside and then, cleavage as protocatechuic acid and phloroglucinaldehyde [195].

In the meal fraction, the smell of SCW pretreated samples were more intense and their color was darker than that of steam samples, especially for the 200°C SCW sample (Figure A.9).

5.3.2.4. Effect on Oil Yield

Mean dry matter oil content (per cent DMO) of pomace and its pretreated fractions (aqueous and meal) extracted with/without acid hydrolysis were determined (Table 5.4). In order to compare with DMO of fresh pomace, DMO of pretreated pomace was calculated by multiplying DMO of fractions with per cent solid content (g dried fraction/ g total solid of pomace).

5.3.2.4.1. Fresh and Processed Sample

The use of acid hydrolysis increased the oil yield of fresh pomace from 14.6 per cent to 19.4 per cent (2.4 per cent -3.2 per cent, wet basis) as discussed in section 3.1. The effect of acid hydrolysis and hydrothermal treatment (19.4 per cent and 19.1-20.9 per cent, 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 spite of 5 min process time, oil yield values were higher than that of a previous steam pretreatment study with longer process time (150-170°C, 15-90 min) (11.8-16.0 per cent) [104]. Maximum oil recovery (22.9 per cent), which was obtained by acid hydrolysis of SCW pretreated sample at 150°C, was significantly higher ($p<0.05$) than that of fresh pomace indicating incomplete hydrolysis of cell wall structure by acid hydrolysis due to lower surface area.

Using hydrothermal pretreatment, 54-76 per cent of the bound oil was recovered (Table 5.4) where significantly higher recoveries were obtained by steam pretreatment than by SCW pretreatment ($p<0.05$).

Table 5.4. Distribution of dry matter oil content (per cent) in fresh and pretreated pomace samples

	Oil Content%						
	Acid hydrolyzed			**Without acid hydrolysis**			**Bound oil**
T	**Aqueous**	**Meal***	**Total**	**Aqueous**	**Meal***	**Total**	**Recovery**
°C	**%**	**%**	**%**	**%**	**%**	**%**	**%**
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%**

*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)

Considering hydrolytic degradation of oil during pretreatments, composition of extracted oil might include FFA, diglycerides, monoglycerides in addition to triglycerides, but glycerol, low molecular weight FFAs might be solubilized in the aqueous fraction.

5.3.2.4.2. Fractional Analysis of Processed Samples

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 steam pretreated samples by 17 per cent and 30 per cent at 160°C and 180°C, however at 200°C it was significantly lower (by 8 per cent) than those of steam pretreated fractions ($p<0.05$) (Figure 5.5a and Table 5.4). As temperature increased, oil content of SCW pretreated samples decreased significantly (up to 20 per cent, $p<0.05$). However, in steam pretreatment a significant increase was observed from 180°C to 200 C by 23 per cent (Figure 5.5b). This increase in the oil content of aqueous fraction obtained by steam pretreatment can be attributed to polar lipids passed to aqueous fraction and higher carry over due to smaller size particles as explained in section 3.2.2.

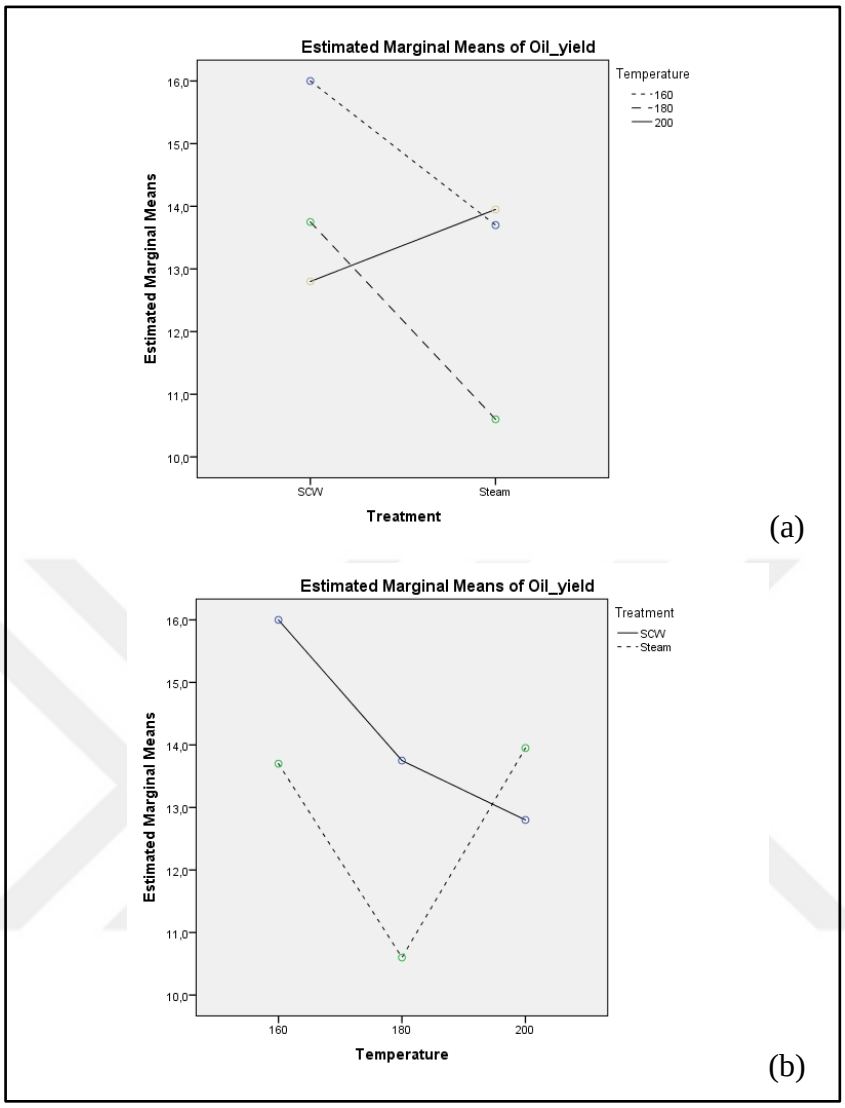


Figure 5.5. Oil content of aqueous fractions obtained using different (a) temperatures, and (b) pretreatments

According to total oil yield of aqueous fraction, obtained by acid hydrolysis, only pretreatment effect was significant ($p<0.05$). Like HHL extracts of aqueous fraction, total oil content of SCW treated samples were significantly higher than that of steam treated samples (Table 5.4).

In meal fraction, effect of pretreatment and interaction on oil content were significant ($p<0.05$). Effect of pretreatments was significant at only 160°C where oil content of steam pretreated meal was higher than that of SCW (by 15 per cent) (Figure 5.6a). Significant increase (by 26 per cent) from 160°C to 200°C was observed in SCW pretreated meal samples ($p<0.05$) (Figure 5.6b). Oil recovery of SCW pretreated meal samples increased

with temperature however that of steam pretreatment decreased (Figure 5.6b). Although total oil yield (AHL) 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 different heat and mass transfer mechanisms of steam (autohydrolysis) and SCW (hydrothermolysis) and behavior of SCW as a reactant.

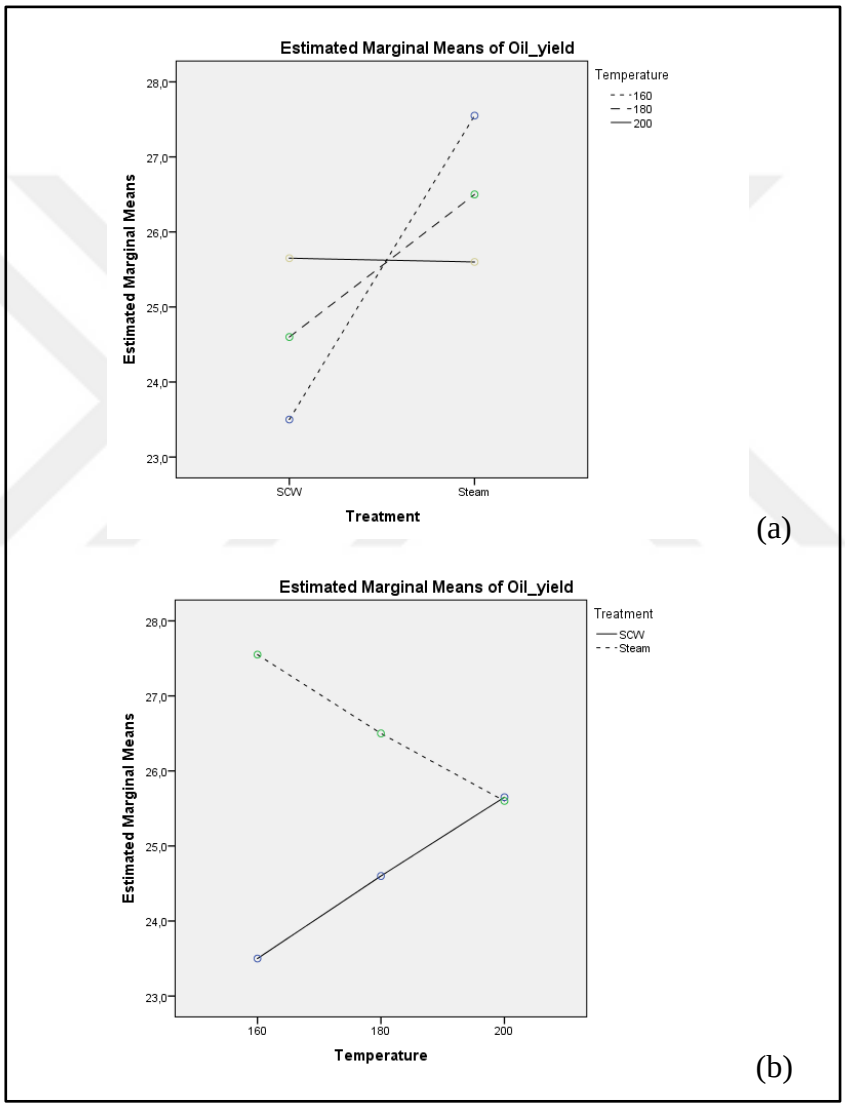


Figure 5.6. Oil content of meal fractions obtained using different (a) temperatures, and (b) pretreatments

Steam is a more efficient heat transfer fluid than SCW due to its low density, penetrates easily to every part of the matrix. Steam explosion which involves the combination of physical and chemical effects leads to hydrolysis of acetyl groups of hemicellulose. In spite

of these advantages, the decrease in oil yield at temperatures higher than 160°C can be attributed to the limitations of steam explosion while advantages of SCW become visible.

SCW reacts with cell wall materials and polar lipids (phospholipids and glycolipids) based on solid reduction and oil content (HHL) results. It was reported that as the ionic product of water (K_w) (10^{-14} mol/L at 25°C to 10^{-11} mol/L at 300°C) increases with temperature, degradation of monosaccharides increases and their degradation products, carboxylic acids are produced and decreases the pH. Increased ionic strength and decreased dielectric permittivity leads to water getting involved in all ionic reactions (such as hydrolyses) as a reactant [124]. However, steam which is not a reactant to involve hydrolysis reactions and not a solvent to remove compounds produced from depolymerization reactions. Not enough solubilization and then repolymerization of lignin during condensation were reported as the limitations of steam explosion in aspen wood [130]. The other limitations are incomplete destruction of xylan fraction and incomplete disruption of the lignin-carbohydrate matrix [130]. In SCW conditions, as the formic acid and acetic acid, endogenous intermediates of hemiacetyl splitting, are generated, xylan solubilization starts [129].

Opposite trends in oil recovery of SCW and steam pretreated samples by increasing temperature can be attributed to compositional differences of oil extracts. While polar lipids might be released by SCW and hydrolytic degradation might start in steam pretreatment. In their study on steam pretreatment application at 160 °C for 15 min, Lama-Munoz et al. (2011) [97] determined per cent of oxidized triglycerides, FFA and diglycerides by high pressure size exclusion chromatography as per cent of total polar compounds. A significant increase of FFA by 100 per cent and diglycerides by 129 per cent ($p<0.05$) was observed indicating hydrolytic degradation, however no significant increase was determined in the content of oxidized triglyceride, which are indicative of oxidative degradation [97, 196]. However, in a SCW study about hydrolysis of corn oil (150-280°C), hydrolysis started at temperatures, higher than 200 °C (40 min) [140]. Accordingly, while oil yield of steam pretreatment decreased with temperature due to oil hydrolysis and came close to that of SCW which increased with temperature, the difference between the pretreatments was not significant at 200°C. Glycolipid, FFA and diglyceride content of HHL extracts can be determined for exact evaluation as a further research.

As a conclusion, 54-76 per cent 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.

5.3.2.5. Effect on BS

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

5.3.2.5.1. Fresh and Processed Sample

The use of acid hydrolysis increased BS content of pomace from 50 mg/100g to 87 mg/100g. 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) ($p<0.05$). Maximum BS recovery (95 mg/100g) was obtained by acid hydrolysis of steam pretreated sample at 160°C which was significantly higher ($p<0.05$) than that of fresh pomace due to the incomplete hydrolysis of cell wall structure by acid hydrolysis. Combined effect of hydrothermal treatment and acid hydrolysis indicate that acid hydrolysis was not effective for determination of total BS content of pomace due to lower surface area.

Using hydrothermal pretreatment, 18-32 per cent of the bound BS was recovered ($p<0.05$, Table 5.5). Although BS recoveries by of steam and SCW pretreatments were similar ($p>0.05$), total BS content of SCW pretreated samples was significantly higher (by 2-14 per cent) than that of steam pretreated samples ($p<0.05$). This might be due to reactivity of SCW reaction medium resulting in release of bound BSG, which was determined as BS after acid hydrolysis. ($p<0.05$).

Table 5.5. Distribution of β-sitosterol content (mg/100 g, d. b.) in fresh and pretreated pomace samples

	BS Content (mg/100g)						
	Acid hydrolyzed			**Without acid hydrolyzed**			**Bound BS**
T	**Aqueous**	**Meal***	**Total**	**Aqueous**	**Meal***	**Total**	**Recovery**
°C	**mg/100g**	**mg/100g**	**mg/100g**	**mg/100g**	**mg/100g**	**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%**

*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)

5.3.2.5.2. Fractional Analysis of Processed Sample

In aqueous fraction, effect of temperature and treatment, and interaction were significant ($p<0.05$). BS content of SCW pretreated samples were higher than that of steam pretreated by 52 per cent and 57 per cent at 160°C and 180°C, however at 200°C it was significantly lower (by 11 per cent) than that of steam pretreated sample (Figure 5.7a). Similar with oil recovery analysis, BS recovery of 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 (Figure 5.7a-b). Similar trend shows that BS recovery depended on oil recovery.

According to BS content of aqueous fractions after acid hydrolysis, only 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 per cent) (Table 5.5). The highest BS content in aqueous fraction (44 mg/100 g) was determined by acid hydrolysis of SCW pretreated samples at 150°C. Recovered bound BS content might be the BS content coming from mostly polar BSG (free and bound) centrifuged through the aqueous fraction.

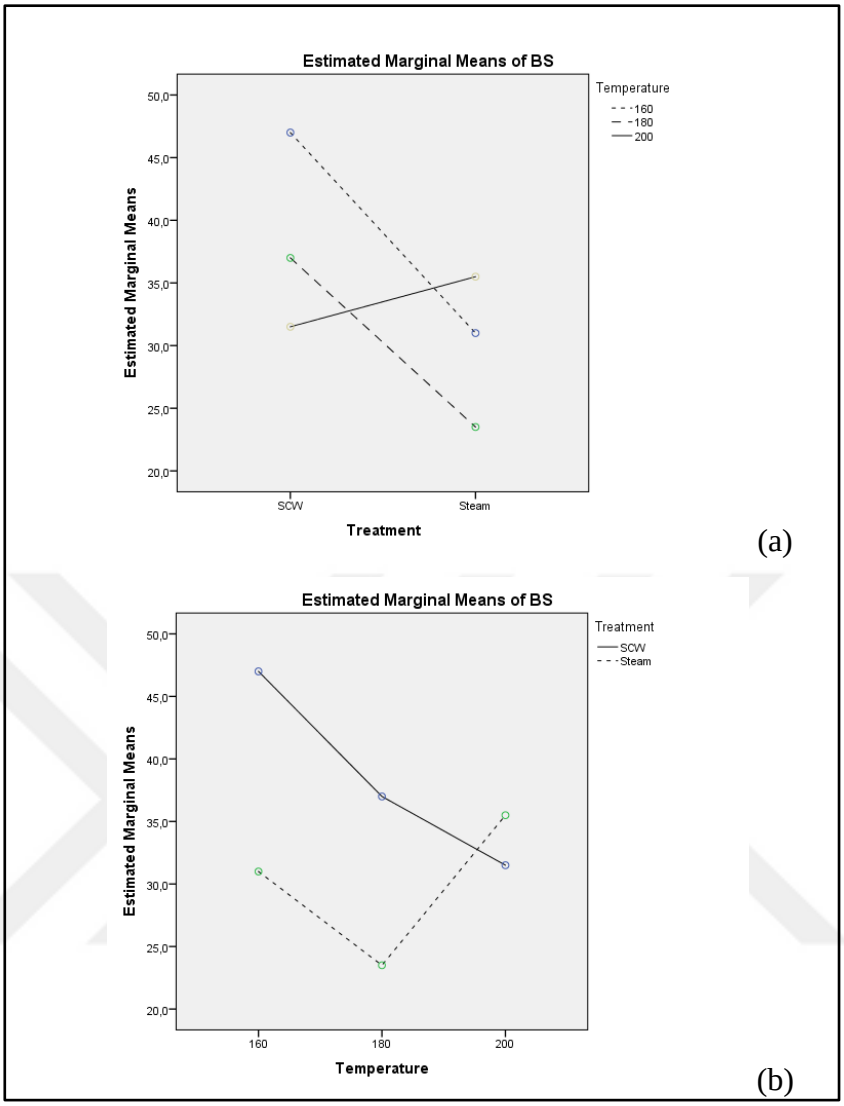


Figure 5.7. BS content of aqueous fractions obtained using different (a) temperatures, and (b) pretreatments

Effect of pretreatment and interaction on BS content of meal fraction were significant ($p<0.05$). Effect of pretreatments was significant at only 160°C ($p<0.05$) where BS content of steam pretreated sample was higher (by 27 per cent) than that of SCW pretreated samples. Significant increase from 160°C to 180°C (by 20 per cent) and 200°C (by 26 per cent) was determined in SCW pretreated meal samples ($p<0.05$) (Figure 5-9b). However, a significant decrease (by 13 per cent) was obtained from 160°C to 200°C by steam pretreatment (Figure 5.8b).

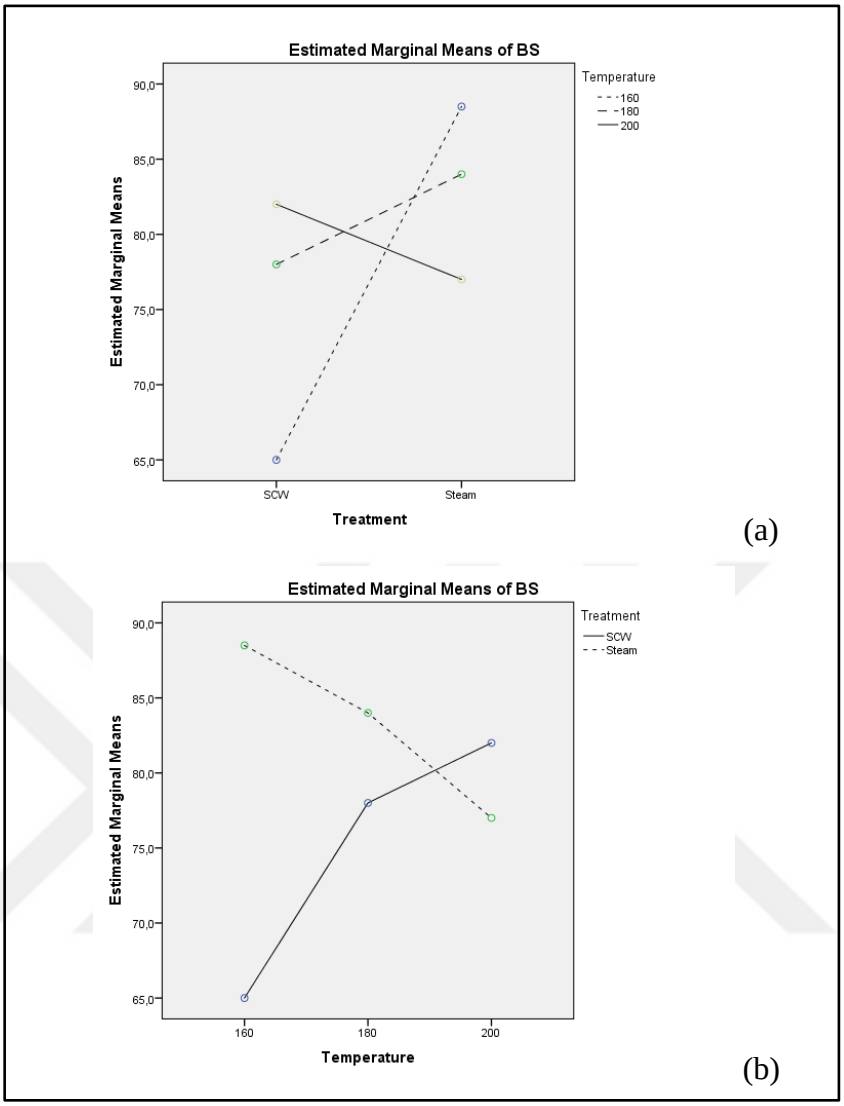


Figure 5.8. BS content of meal fractions obtained using different (a) temperatures, and (b) pretreatments

BS content of SCW pretreated meal sample obtained by acid hydrolysis was significantly higher than that of steam pretreated and acid hydrolyzed samples by 3-17 per cent ($p<0.05$). More BSG was liberated and solubilized in the oil during SCW treatment. Bound BS/BSG was liberated at higher temperatures, especially at 200°C, during SCW treatment, but this could not be observed without acid hydrolysis because BSG content could not be extracted by hexane:diethyl ether due to its polar nature. As mentioned above, SCW treatment increased BSG content coming from cell wall/membrane structure. As a further research, to determine BSG content of samples, chloroform:methanol extraction could be used.

As a conclusion, only 18-32 per cent of the bound BS of the pomace sample was recovered by hydrothermal treatments (~5 min). Extend of reaction time might increase the recovery of BSG. Deoiled olive pomace might be pretreated using SCW at higher temperature than 200°C for more BSG recovery. Olive pomace which contain 89-95 mg/100g BS, is a rich source of phenolic compounds. Phenolic recovery data at high temperature should be considered to decide appropriate industrial process for utility of the technique for valorization of pomace.

5.3.2.6. Effect on Phenolic Compounds

5.3.2.6.1. Total Phenolic Contents

Total phenolic content (TP) (4020.7 mg/100g) of the fresh sample increased (by 11 per cent) at only 200°C (4512.4 and 4453.0 mg/100 g) by steam and SCW pretreatments, respectively (Table 5.6). A significant increase was observed in the TP content of pretreated samples from 160°C to 200°C. Decomposition of lignin at 200ºC might increase the recovery of phenolic compounds. TP content of pit part (25 per cent) removed from whole pomace (2987 mg/100g) should be considered and additionally degradation of its lignin during pretreatments might be source of this increase. Although water at 200ºC was a suitable solvent for phenolics with dielectric constant (35) close to those of acetonitrile (37.5) and methanol (32.7), TP content of steam pretreated samples were significantly higher than those of SCW samples (1-24 per cent) ($p<0.05$). Most of them were concentrated in the aqueous fraction (76-89 per cent).

5.3.2.6.2. Individual Phenols

Phenolic profiles of fresh and pretreated pomace samples (aqueous and meal fractions) were analyzed by HPLC (Table 5.7-8). Hydroxytyrosol (3,4-dihydroxy-phenylethanol, Hyty), 3,4-dihydroxyphenylglycol (DHPG), hydroxytyrosol 4-β-d-glucoside (HytyG), tyrosol, 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.

Table 5.6. Total phenolic content of pomace samples

	Total phenolic contents (mg total phenol/100g)**		
T°C	**Aqueous**	**Meal**	**Total**
Steam pretreatment			
160	6335.3 ± 323.1	998.1 ± 98.03	3756.7 ± 41.39
180	6030.0 ± 331.0	1296.9 ± 129.9	3625.7 ± 77.98
200	7327.5 ± 573.8	1723.1 ± 232.1	4512.4 ± 445.7
Subcritical water pretreatment			
150	5576.2 ± 298.8	870.4 ± 24.12	3602.0 ± 206.6
180	4296.6 ± 129.2	909.8 ± 135.3	2767.8 ± 60.99
200	6966.2 ± 587.0	1385.2 ± 276.3	4453.0 ± 490.1
Fresh pomace (without pit)			4020.7
Pit			1478.7

***** Meal: sieved meal fraction
**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)

No significant effect of temperature or pretreatment was observed on HytyG, the conjugated form of Hyty, content of aqueous and meal fractions and total recovery values (369.0, 106.9 and 240.8 mg/100g, respectively) ($p>0.05$). Temperature effect was significant for all other individual phenolics in aqueous fraction ($p<0.05$).

Similar to TP results, a significant increase ($p<0.05$) was observed in Hyty and vanillin concentration of the aqueous samples as temperature increased to 200°C. Thermal decomposition of lignin produces phenolic compounds such as vanillic acid, syringic acid, vanillin, and syringaldehyde [135,150]. Concentration of oleuropein derivatives and vanillic acid increased significantly with temperature from 160°C to 180°C and 200°C ($p<0.05$). According to concentrations of vanillic acid, it can be said that reaction of lignin decomposition started at temperatures higher than 160°C, especially at 200°C. After formation of sugar monomers by lignin decomposition, formation of HMF via browning reactions started at temperature higher than 160°C, also (Table 5.7).

Table 5.7. Concentration of individual phenolic compounds in aqueous part of pretreated pomace (mg/100g)

	Individual phenolic compounds, mg/100g*											
T°C	**run**	**DHPG *****	**HMF****	**HytyG**	**Hyty**	**Tyr****	**V.acid**	**Vanilin**	**Fer. acid**	**Oleu. Derv.**	**p-cmr. acid**	**Apigenin ****
Steam water pretreatment												
160	**1**	349.8	tr	376.4	544.2	62.2	34.3	328.4	19.7	289.6	42.4	58.7
	2	501.0	tr	363.5	528.4	41.5	87.4	185.3	20.2	275.5	32.3	58.4
180	**1**	512.0	727.6	374.3	700.6	111.2	98.6	237.6	40.5	294.7	19.8	nd
	2	520.1	386.3	390.5	641.9	98.3	104.3	221.3	37.2	318.0	25.7	58.8
200	**1**	314.4	3593.3	362.2	791.3	187.7	88.8	355.8	49.4	322.7	10.7	nd
	2	310.2	4954.1	347.2	844.7	180.9	65.7	381.9	37.9	305.8	8.2	nd
Subcritical pretreatment												
150	**1**	352.8	nd	376.3	602.1	56.5	63.9	223.3	16.6	244.9	62.3	58.6
	2	481.4	tr	364.0	642.3	69.5	90.7	224.7	18.8	264.5	31.3	58.6
180	**1**	655.7	71.8	360.6	638.4	90.9	148.7	300.9	39.0	284.0	21.6	58.9
	2	635.5	362.6	376.5	767.9	103.5	149.3	287.1	46.9	299.3	24.7	25.8
200	**1**	445.3	4028.3	372.2	830.5	192.9	103.2	512.5	70.2	294.9	11.5	nd
	2	460.2	3985.1	383.5	918.2	196.7	94.4	369.0	75.5	310.8	13.9	20.7
Fresh P.		33.7	-	222.3	215.8	nd	7.2	204.7	5.1	164.8	24.4	60.4
Pit**		12.5	-	134.4	105.7	nd	nd	nd	nd	125.7	7.2	nd

***** Average concentrations of extraction (n=3) and injection (n=3) replicates
**Tr: concetration < 0,1 mg/L, nd: non detected
***DHPG: 3,4-dihydroxyphenylglycol, Fer. Acid: ferulic acid, HMF: 5-hydroxymethylfurfural, Hyty: hydroxytyrosol, HytyG: hydroxytyrosol 4-β-d-Glucoside, Oleu. Derv.: oleuropein derivative, p-cmr. acid: p-coumaric acid, Tyr: tyrosol, V. acid: vanilic acid.

Hyty and tyrosol are the characteristic phenolics in olive fruit. Hyty concentration of aqueous fraction (0.87 per cent) obtained by steam treatment at 200°C was higher than the amount previously reported (0.56 per cent) for steam pretreated orujo at the same conditions (200°C, 5 min) by 55 per cent [136]. This difference can be partly attributed to the use of destoning and defatting steps (second extraction), which decreased Hyty content of orujo sample. Concentration of Hyty in total of aqueous and meal fractions (464.2 mg/100 g at 200°C) was ten times higher than its concentration in the fresh pomace (46.2 mg/100 g). This means hydrothermal treatment increased the recovery of Hyty (3,4-dihydroxyphenyl-ethanol) (3,4-DHPEA) by the hydrolysis of the oleuropein and other secoiridoids like EA-Hyty (3,4-dihydroxyphenyl-ethanol linked to elenolic acid) or EDA-Hyty (p-hydroxyphenylethanol linked to dialdehydic form of elenolic acid) [6, 197].

Table 5.8. Concentration of individual phenolic compounds in meal fraction of pretreated pomace samples (mg/100g)

	Individual phenolic compounds, mg/100g*											
T°C	**run**	**DHPG *****	**HMF****	**HytyG**	**Hyty**	**Tyr****	**V.acid**	**Vanilin**	**Fer. acid**	**Oleu. Derv.****	**p-cmr. acid**	**Apigenin ****
Steam treatment												
160	**1**	35.0	nd	104.2	59.6	nd	12.9	213.0	17.8	135.1	4.4	nd
	2	11.2	nd	107.8	65.0	nd	14.7	217.3	17.5	128.8	5.3	nd
180	**1**	10.0	nd	107.9	66.6	nd	18.3	318.7	8.8	125.9	3.9	nd
	2	16.3	nd	106.1	61.3	nd	19.2	324.4	5.3	128.3	3.5	nd
200	**1**	7.6	nd	106.0	59.9	nd	17.5	196.7	9.0	124.3	15.1	nd
	2	nd	nd	109.2	75.7	nd	19.0	185.1	13.1	131.2	6.4	61.1
Subcritical water treatment												
150	**1**	14.0	nd	108.4	66.9	tr	10.3	245.9	13.9	133.9	4.3	nd
	2	19.6	nd	105.0	61.6	nd	10.7	264.7	12.7	nd	5.5	nd
180	**1**	23.0	tr	105.6	59.8	nd	19.3	342.3	4.9	nd	9.8	nd
	3	15.8	tr	104.9	59.8	nd	20.0	321.1	5.4	nd	10.1	nd
200	**1**	2.8	tr	106.1	62.9	nd	17.6	194.0	11.9	nd	9.5	nd
	2	7.2	tr	105.6	59.2	tr	15.1	222.0	6.5	nd	10.3	nd
Fresh P.		33.7	nd	222.3	215.8	tr	7.2	204.7	5.1	164.8	24.4	60.4
Pit**		12.5	nd	134.4	105.7	tr	nd	nd	nd	125.7	7.2	nd

* Average concentrations of extraction (n=3) and injection (n=3) replicates
**Tr: concentration < 0,1 mg/L, nd: non detected
***DHPG: 3,4-dihydroxyphenylglycol, Fer. Acid: ferulic acid, HMF: 5-hydroxymethylfurfural, Hyty: hydroxytyrosol, HytyG: hydroxytyrosol 4-β-d-Glucoside, Oleu. Derv.: oleuropein derivatives, p-cmr. acid: p-coumaric acid, Tyr: tyrosol, V. acid: vanilic acid.

Tyrosol, which was not detected in fresh and meal samples, increased significantly with temperature ($p<0.05$) (by 3-4 times from 160 °C to 200 °C).

Concentration of DHPG was significantly higher when pretreated at 180°C than at 160°C and 200°C ($p<0.05$). Lower concentration of DHPG in pretreated samples at 200 °C might be due to decomposition of DHPG. Notable decrease (1.3 to 0.3 g/kg) in DHPG content of pomace was reported as the temperature increased from 180 to 240 °C (over five minutes) in a previous steam pretreatment study without catalyst due to decrease in pH resulting from autohydrolysis [198].

Treatment effect was significant for oleuropein derivative, DHPG, vanilic acid and ferulic acid where steam treatment was more effective than SCW ($p<0.05$).

Significant interaction was present in concentration of ferulic acid and steam pretreatment effect was significantly higher (by 40 per cent) than that of SCW at 200°C ($p<0.05$).

Considering the meal fraction, both treatment and temperature significantly affected only vanilin concentration of meal samples ($p<0.05$). Highest concentration was obtained by steam pretreatment at 180°C. Pretreatment effect was significant for concentration of oleuropein derivatives such that they are detected only in steam pretreated meal fractions. Temperature effect was significant for vanilic acid and ferulic acid.

5.3.2.7. Effect on HMF

HMF could be formed by Maillard and/or caramelization reactions depending on amino acid and glucose content of pomace [199]. HMF was not detected in aqueous fractions obtained at 150-160°C, but it was detected at 180°C (387.1 mg/100 g) and 200°C (4140.2 mg/100g) (Table 5.7). HMF, which is formed by thermal degradation of hexoses (mainly fructose), was completely solubilized in the aqueous part due to its water solubility. According to reaction pathway of glucose and fructose in SCW, the increased Kw and high temperature triggers significant glucose conversion to fructose in spite of neutral pH. Five per cent conversion was reported at 180°C for two minutes and this ratio increased with temperature [124]. As a further reaction, D-fructose degrades to HMF, 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 [124]. Thus, pH dependent reactions, such as degradation of fructose, are catalyzed accordingly. In SCW treatment of rice bran, the start of browning reaction was reported as 180°C by measuring furfural content [200]. This finding supports our results, which showed formation of HMF at 180 °C.

Optimum SCW pretreatment conditions for recovery of protein, sugar and lignin from deoiled olive pomace was reported as 200°C, 30 min with two mL/min flow at 220 bar where sugar (65.9 per cent) and lignin (66.3 per cent) were recovered partially [201]. It was reported that reducing sugar content increased with temperature from 180°C to 200°C, however decreased at temperatures higher than 200°C due to decomposition of sugars [201].

Occurrence of Maillard and caramelization reactions are limited if samples are rich in phenolic compounds [199]. It was also demonstrated by same group that Maillard reaction results in formation of neoantioxidants.

5.4. CONCLUSIONS

Hydrothermal pretreatments, steam explosion and subcritical water reaction medium, were applied to pomace to produce value added compounds such as phenolic compounds, sterols including their polar conjugates and oil by decomposition of cell wall structure. Compared to acid hydrolysis, a chemical pretreatment, hydrothermal pretreatments were similar for oil recovery however not efficient for the recovery of BS even at 200°C (for five minutes) such that only 18-32 per cent of the bound BS of the pomace sample was recovered by hydrothermal treatments. Calculated bound BS content include BSG which are polar conjugates of BS having glycosidic bond. To recover more BSG, deoiled olive pomace might be pretreated at temperatures higher than 200°C and reaction time might be increased. 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” [124]. Considering significantly higher recovery of phenolics and neoantioxidants formed by Maillard reactions, further research at higher temperatures could include determination of BSG, FFA, polar lipids composition of extracts and their antioxidant activity for neoantioxidants additionally.

6. GENERAL CONCLUSION AND RECOMMENDATIONS

Behavior and distribution of predominant lipophilic bioactives (squalene, α-tocopherol and β-sitosterol) during virgin olive oil processing was reviewed and investigated in a partitioning study. A rapid in-house validated method for simultaneous determination of these lipophilic bioactives in virgin olive oil was developed, and modified for the determination of β-sitosterol (total and free) in olive pomace with/without acid hydrolysis. Hydrothermal treatments (steam and subcritical water) were carried out to increase recovery of oil and β-sitosterol, in addition to phenolics.

Based on available literature process data, loss of β-sitosterol (48 per cent) and α-tocopherol (36 per cent) were shown to be higher than that of oil (2-20 per cent) and squalene (19 per cent) due to their molecular interactions and cellular distribution. These findings were verified by an experimental study, which showed the loss of β-sitosterol (44 per cent) and α-tocopherol (42 percent) to be higher than that of squalene (12 per cent) and oil (14 per cent) due to non-recovered bound forms. This is the first study reporting the partitioning of these lipophilic bioactives during industrial olive oil processing using samples from an integrated olive milling plant. In the overall integrated process, 7 per cent of the oil, present in the olives was lost in the final pomace. Squalene, which is present in free form in the midplane of the lipid bilayer and pulp of the olive fruit, was recovered completely in the product streams. 10 per cent of β-sitosterol and α-tocopherol present in the olives, which are mainly concentrated in the seed of the olive fruit and bound form in the plasma membrane, were lost in final pomace. 19 per cent of the α-tocopherol and 21 per cent of the β-sitosterol loss was unaccounted for, which can be attributed to degradation of α-tocopherol and incomplete recovery of sterols from the olive and pomace matrices. Application of acid hydrolysis increased β-sitosterol recovery of olive pomace significantly by 64 per cent. Findings of literature (section 2.4) and the partitioning study (chapter 4), particularly information on distribution of these bioactive compounds between olive oil and waste streams as affected by process parameters can be used to maximize their retention in olive oil and their recovery from waste streams. It was shown that olive pomace might be a good source of β-sitosterol with potential applications in food, nutraceutical and pharmaceutical sectors owing to its anticancer and cholesterol-lowering activity. By considering possible

mechanisms of processing loss of β-sitosterol and α-tocopherol, new recovery techniques could be developed for these compounds. Applying additional pretreatment steps before oil extraction can enable the quantitative recovery of β-sitosterol and α-tocopherol including bound forms. This framework can be extended to other olive oil plants and to the study of other plant based matrices to investigate potential sources of β-sitosterol and α-tocopherol and provide a basis for process development studies.

The in-house GC-FID method developed for determination of lipophilic bioactives in virgin olive oil (chapter 3) offers a rapid, accurate and validated method alternative to current official methods and simultaneous methods recently published in the literature. Time of analysis was reduced to approximately five hours for every six samples compared to ~20 h specified in official methods. The developed method was fully validated covering all parameters; linearity, LOQ, LOD, recovery, and repeatability. This method can be incorporated into quality control programs for olive oil and its application areas can be expanded to include other plant oils, such as sunflower oil, after further validation studies. A methodological framework for the determination of free and total β-sitosterol in olive pomace was developed by modifying sample preparation method based on literature. As a further research, the method for pomace oil could be modified for determination of β-sitosterol glucoside and validated for pomace oil and other matrices such as fruits/vegetables, and plant based food wastes.

Subcritical water reaction medium commonly used for recovery of phenolics was applied first time for the recovery of sterols as a hydrothermal pretreatment. Pretreatments hydrolyzing plant cell wall, steam and subcritical water reaction medium was applied to pomace at different temperatures (160, 180 and 200°C) and evaluated by comparing with acid hydrolysis. It was understood from combined effect of hydrothermal pretreatment and acid hydrolysis that acid hydrolysis was not effective for determination of total BS content of pomace. This shows a new perspective for determination of total sterol in plant matrices. By hydrothermal pretreatments, 54-76 per cent of the bound oil and 18-32 per cent of the bound β-sitosterol of the pomace were recovered. Considering increase in phenolic compounds and Maillard/caramelization reactions during hydrothermal pretreatments, this study can be developed by working with different reaction times and by determining β-sitosterol glucoside content, oil degradation compounds including free fatty acids, oxidized

triglycerides and glycerides, and sugar degradation compounds including neoantioxidants. Addition of ethanol to SCW in different ratios might increase extraction of polar compounds such as β-sitosterol glucoside, triterpenic acids and phenolics. Two step SCW pretreatment with lower temperature application for phenolics and oil recovery and further higher temperature application for sterol recovery and cellulose hydrolysis to be used as a suitable biomass for biotechnological applications such as biohydrogen production could be studied to make the process more feasible and to increase value added usage of olive pomace. This green technique can be applied to olive stone (seed) and different plant matrices and plant based food wastes such as sunflower oil industry.

REFERENCES

1. D. M. Niaounakis and C.P. Halvadakis. Waste Management Series 5 Olive Processing Waste Management. Elsevier Ltd, Italy, 2006.

2. D. Connor and E. Fereres, The Physiology of Adaptation and Yield Expression in Olive. *Horticultural Reviews*, 31: 1–69, 2005.

3. D. Boskou, G. Blekas and M. Tsimidou. Olive Oil Composition. In: D. Boskou, editor, *Olive Oil Chemistry, Properties, Health Effects*, pages 41-72. AOCS Press, Thessaloniki 2006.

4. H. Jemai, M. Bouaziz and S. Sayadi. Phenolic Composition, Sugar Contents and Antioxidant Activity of Tunisian Sweet Olive Cultivar with Regard to Fruit Ripening. *Journal of Agriculture and Food Chemistry*, 57: 2961-2968, 2009.

5. E. Vierhuis, M. Servili, M. Baldioli, H. A. Schols, A. G. Voragen and G.F. Montedoro. Effect of Enzyme Treatment During Mechanical Extraction of Olive Oil On Phenolic Compounds and Polysaccharides. *Journal of Agricultural and Food Chemistry*, 49: 1218–23, 2001.

6. R. Ghanbari, F. Anwar, K. M., Alkharfy, A.H. Gilani and N. Saari, Valuable Nutrients and Functional Bioactives in Different Parts of Olive (*Olea Europaea L.*) -A Review. *International Journal of Molecular Sciences*, 13: 3291–340, 2012.

7. H. Schwartz, V. Ollilainen, V. Piironen and A-M. Lampi. Tocopherol, Tocotrienol and Plant Sterol Contents of Vegetable Oils and Industrial Fats. *Journal of Food Composition and Analysis*, 21: 152-161, 2008.

8. A. Ranalli, L. Pollastri, S. Contento, G. Di Loreto, E. Lannucci, L. Lucera and F. Russi. Sterol and Alcohol Components of Seed, Pulp and Whole Olive Fruit Oils. Their Use to Characterize Olive Fruit Variety by Multivariate. *Journal of the Science of Food and Agriculture*, 82: 854–859, 2002.

9. C. Guillaume, L. Ravettei, D.L. Ray and J. Johnson. Technological Factors Affecting Sterols in Australian Olive Oils. *Journal of American Oil Chemists' Society*, 89: 29–39, 2011.

10. S. Bornaz, A. Sahli, M. Maaoui, S. Sifi and M. Aiachi. Effect of Stoning on Chemical Composition of Olive Oils from Three Tunisian Olive Cultivars (Olea Europaea L.). *6th IS on Olive Growing, Acta Horticulture, ISHS*, 949: 495–504, 2012.

11. N. Cortesi, P. Rovellini and P. Fiorino. The Role of Drupe Different Anatomic Parts on Chemical Olive Oil. *3rd International Symposium on Olive Growing, ISHS, Acta Horticulture*, 474: 643–644, 1999.

12. Bianchi, G. and Vlahov, G. Composition of Lipid Classes in the Morphologically Different Parts of the Olive Fruit, cv. Corafina (Olea europaea Linn.). *European Journal of Lipid Science and Technology*, 96: 72–77, 1994.

13. A. Ranalli, G. De Mattia and M. L. Ferrante. The Characteristics of Percolation Olive Oils Produced with a New Processing Enzyme Aid. *International Journal of Food Science and Technology*, 33: 247–258, 1998.

14. C. Petrakis. Olive Oil Extraction. In: D. Boskou. Editor, *Olive Oil: Chemistry and Technology*, pages 196-197. AOCS Press Illinois, 2006.

15. P. Manzi, G. Panfili, M. Esti and L. Pizzoferrato. Natural Antioxidants in the Unsaponifiable Fraction of Virgin Olive Oils from Different Cultivars. *Journal of the Science of Food and Agriculture*, 77: 115–120, 1998.

16. N. Mulinacci, C. Giaccherini, M. Innocenti, A. Romani, F. F. Vincieri, F. Moratta and A. Mattei, Analysis of Extra Virgin Olive Oils From Stoned Olives. *Journal of the Science of the Food and Agriculture*. 85: 662–670, 2005.

17. A. Ranalli and S. Contento. Analytical Assessment of Destoned and Organic-Destoned Extra-Virgin Olive Oil. *European Food Research Technology*, 230: 965–971, 2010.

18. S. Lopez, B. Bermudez, S. Montserrat-de la Paz, S. Jaramillo, L. M. Varela, A. Ortega-Gomez, R. Abia and F. J. G. Muriana. Membrane Composition and Dynamics: A Target of Bioactive Virgin Olive Oil Constituents. *Biochimica et Biophysica Acta*. 1838: 1638-1656, 2014.

19. T. Haub, S. Dante, N.A. Dencher and T.H. Haines. Squalane is in the Midplane of the Lipid Bilayer: Implications for its Function as a Proton Permeability Barrier. *Biochimica et Biophysica Acta- Bioenergetics*. 1556: 149-154, 2002.

20. S. M. Read and T. Bacic. Plant Biology. Prime Time for Cellulose. *Science*, 295: 59-60, 2002.

21. L. Peng, Y. Kawagoe, P. Hogan and D. Delmer. Sitosterol-beta-glucosidase as Primer for Cellulose Sythesis in Plants. *Science*, 295: 147-150, 2002.

22. M. Hartmann. Plant Sterols and the Membrane Environment. *Trends in Plant Science*, 3: 170–175, 1998.

23. R. A. Ferrari, E. Schulte, W. Esteves and L. Bruhl, K.D. Mukherjee. Minor Constituents of Vegetable Oils During Industrial Processing. *Journal of the American Oil Chemists' Society*, 73: 587–592, 1996.

24. Merck Index, Online 2012. Merck Sharp and Dohme Corp., subsidiary of Merck and Cooperates, Inc., Whitehouse Station, N.J., U.S.A., 2006.

25. J. M. Lou-Bonafonte, C. Arnal, M.A. Navarro and J. Osada. Efficacy of Bioactive Compounds from Extra Virgin Olive Oil to Modulate Atherosclerosis Development. *Molecular Nutrition and Food Research*, 56: 1043–1057, 2012.

26. M. Spanova and G. Daum. Squalene - Biochemistry, Molecular Biology, Process Biotechnology, and Applications. *European Journal of Lipid Science and Technology*, 113: 1299–1320, 2011.

27. M.A. Dessì, M. Delana, B. W. Day, A. Rosa, B. Sebastiano and F. P. Corongiu. Oxidative Stability of Polyunsaturated Fatty Acids: Effect of Squalene. *European Journal of Lipid Science and Technology*, 104: 506–512, 2002.

28. Y. Kohno, Y. Egawa, S. Itoh, S. Nagaoka, M. Takahashi and K. Mukai. Kinetic Study of Quenching Reaction of Singlet Oxygen and Scavenging Reaction of Free Radical by Squalene in n-butanol, *Biochimica et Biophysica Acta,* 1256: 52-56, 1995.

29. D. Boskou. Olive Oil: Minor Constituents and Health. CRC Press, New York, 2009.

30. P. L. Benitez-Sánchez, L. M. Camacho and R. A. Aparicio. Comprehensive Study of Hazelnut Oil Composition with Comparisons to Other Vegetable Oils, Particularly Olive Oil. *European Food Research and Technology*, 218: 13–19, 2003.

31. A. Von Bonsdorff-Nikander, S. Lievonen, L. Christiansen, M. Karjalainen, J. Rantanen, and Y. Jouko. Physical Changes of Beta-Sitosterol Crystals in Oily Suspensions During Heating. *The AAPS Journal*, 6: E413–20, 2005.

32. International Olive Oil Council. COI/T.15/NC No 3/Rev.10 Nov. (2015), Trade Standard Applying to Olive Oils and Olive-pomace Oils, http://www.internationaloliveoil.org/estaticos/view/222-standards [retrieved 4 June 2016].

33. T.T. Thanh, M. F. Vergnes, J. Kaloustian, T.F. El-Moselhy, M. J. Amiot-Carlin and H. Portugal. Effect of Storage and Heating on Phytosterol Concentrations in Vegetable Oils Determined by GC/MS. *Journal of the Science of Food and Agriculture*, 86: 220–225, 2006.

34. M. Rudzińska, R. Przybylski, and E. Wąsowicz. Products Formed During Thermo-oxidative Degradation of Phytosterols. *Journal of the American Oil Chemists' Society*, 86, 7: 651–662, 2009.

35. R. Cantrill and Y. Kawamura. Phytosterols, Phytostanols and Their Esters, Chemical and Technical Assessment (CTA). *69th JECFA*. 1-13, 2008.

36. D. Quianchun, Z. Pin, H. Qingde, H. Fenghong, W. Fang, Z. Mingming, Y. Xiao, Z. Qi and Z. Chang. Chemical Synthesis of Phytosterol Esters of Polyunsaturated Fatty Acids with Ideal Oxidative Stability. *European Journal of Lipid Science and Technology*, 113: 441–449, 2011.

37. T. A. Woyengo, V. R. Ramprasath and P. J. H. Jones. Anticancer Effects of Phytosterols. *European Journal of Clinical Nutrition*. 63: 813–20, 2009.

38. P.J. Jones and S.S. AbuMweis. Phytosterols as Functional Food Ingredients: Linkages to Cardiovascular Disease and Cancer. *Current Opinion Clinical Nutrition and Metabolic Care*. 12: 147–151, 2009.

39. A.B. Awada and C.S. Fink. Phytosterols as Anticancer Dietary Components: Evidence and Mechanism of Action. *The Journal of Nutrition*, 130: 2127–2130, 2000.

40. R.R. Berges, J. Windeler and H.J. Trampisch. Randomised, Placebo-controlled, Double-Blind Clinical Trial of Beta-Sitosterol in Patients with Benign Prostatic Hyperplasia. *The Lancet*, 345: 1529–1532, 1995.

41. H. Zbakh and A. El Abbassi. Potential Use of Olive Mill Wastewater in the Preparation of Functional Beverages: A Review. *Journal of Functional Foods*, 4: 53-65, 2012.

42. A. Ranalli and N. Martinelli. Integral Centrifuges for Olive Oil Extraction, at the Third Millenium Threshold. Transformation Yields. *Grasas y Aceites*, 46: 255–263, 1995.

43. G. Rodriguez-Gutierrez, A. Lama-Munoz, M.V. Ruiz-Mendez, F. Rubio-Senent and J. Fernandez-Bolanos. New olive-pomace oil improved by hydrothermal pretreatments. In: B. Dimitrios. 250 *Olive Oil – Constituents, Quality, Health Properties and Bioconversions*, pages 250-266, Intech Org, Crotia, 2012.

44. A. Vlyssides, M. Loizides and P. Karlis. Integrated Strategic Approach for Reusing Olive Oil Extraction By-Products. *Journal of Cleaner Production*, 12: 603–611, 2004.

45. M. Brenes, C. Romero, A. Garcia, J. Hidalgo and M. V. Ruiz-Mendez. Phenolic Compounds in Olive Oils Intended for Refining: Formation of 4-Ethylphenol During Olive Paste Storage. *Journal of Agriculture and Food Chemistry*, 52: 8177–81, 2004.

46. P.S. Moral and M.V.R. Méndez. Production of Pomace Olive Oil. *Grasas y Aceites*, 57: 47–55, 2006.

47. G. Rodriguez-Gutierrez, R. Rodrigues, A. Jimenez, R. Guillen and J. Fernandez-Bolanos. Effect of Steam Treatment of Alperujo on the Composition, Enzymatic Saccharification, and in Vitro Digestibility of Alperujo. *Journal of Agricultural and Food Chemistry*, 55, 1: 136–42, 2007.

48. K. Antonopoulos, N. Valet, D. Spiratos and G. Siragakis. Olive Oil and Pomace Olive Oil Processing. *Grasas y Aceites*, 57: 56-67, 2006.

49. M. V. Ruiz-Mendez, A. Lopez-Lopez and A. Garrido-Fernandez. Characterization and Chemometric Study of Crude and Refined Oils from Table Olive by-products. *European Journal of Lipid Science and Technology*, 110: 537-546, 2008.

50. M. V. Ruiz-Mendez, J. Velasco and G. Marquez-Ruiz. Recovery of Phytosterols from Refining by Products of Vegetable Oils. In: D. Garner, editor, *Phytosterols*. pages 43-61, Nova publishers, New York, 2015.

51. A. Lanzon, T. Albi, A. Cert and J. Gracian, The Hydrocarbon Fraction of Virgin Olive Oil and Changes Resulting from Refining. *Journal of American Oil Chemists' Society*, 71: 285-291, 1994.

52. M. Uceda, A. Jiménez and G. Beltrán. Trends in Olive Oil Production Olive Oil Extraction and Quality. *Grasas y Aceites*, 57: 25–31, 2006.

53. C.G. Herrera. Matter Transfer During Virgin Olive Oil Elaboration. *Grasas y Aceites*, 58: 194–205, 2007.

54. S. Veillet, V. Tomao, I. Bornard, K. Ruiz and F. Chemat. Chemical Changes in Virgin Olive Oils as A Function of Crushing Systems: Stone Mill and Hammer Crusher. *Comptes Rendus Chimie*, 12: 895–904, 2009.

55. A.M. Inarejos-García, G. Fregapane and M. D. Salvador. Effect of Crushing on Olive Paste and Virgin Olive Oil Minor Components. *European Food Research and Technology*, 232: 441–451, 2010.

56. F. Caponio and P. Catalano. Hammer Crushers vs Disk Crushers: the Influence of Working Temperature on the Quality and Preservation of Virgin Olive Oil. *European Food Research and Technology*, 213: 219-224, 2001.

57. L. Di Giovacchino, S. Sestili and D. Di Vincenzo. Influence of Olive Processing on Virgin Olive Oil Quality. *European Journal of Lipid Science and Technology*, 104: 587–601, 2002.

58. M. L. Clodoveo. Malaxation: Influence on Virgin Olive Oil Quality. Past, Present and Future – An Overview. *Trends in Food Science and Technology*, 25: 13–23, 2012.

59. C. Petrakis. Good Manufacturing Practice (GMP) Guidelines for Virgin Olive Oil Production. *Grasas y Aceites*. 45: 53–54, 1994.

60. A. Cert, J. Alba, M. Leon-Camacho, W. Moreda and M.C. Perez-Camino. Effects of Talc Addition and Operating Mode on the Quality and Oxidative Stability of Virgin Olive Oils Obtained by Centrifugation. *Journal of Agriculture and Food Chemistry*, 44: 3930–3934, 1996.

61. R. Amirante, E. Cini, G. L. and Montel, A. Pasqualone. Influence of Mixing and Extraction Parameters on Virgin Olive Oil Quality. *Grasas y Aceites*. 52: 198–201, 2001.

62. A. Ranalli, S. Contento, C. Schiavone and N. Simone. Malaxing Temperature Affects Volatile and Phenol Composition as well as Other Analytical Features of Virgin Olive Oil. *European Journal Lipid Science Technology*, 103: 228–238, 2001.

63. C.M. Kalua, R. Bedgood, A.G. Bishop and P.D. Prenzler, Changes in Volatile and Pphenolic Compounds with Malaxation Time and Temperature During Virgin Olive Oil Production. *Journal of Agriculture and Food Chemistry*, 54: 7641–51, 2006.

64. A. Koutsaftakis, F. Kotsifaki and E. Stefanoudaki. Effect of Extraction System, Stage of Ripeness, and Kneading Temperature on the Sterol Composition of Virgin Olive Oils. *Journal of American Oil Chemists' Society,* 76: 1477–1481, 1999.

65. A., Ranalli, A. Malfatti, L. Lucera, S. Contento and E. Sotiriou. Effects of Processing Techniques on the Natural Colorings and the Other Functional Constituents in Virgin Olive Oil. *Food Research International*, 38: 873-878, 2005.

66. A. Parenti, P. Spugnoli, P. Masella and L. Calamai. The Effect of Malaxation Temperature on the Virgin Olive Oil Phenolic Profile Under Laboratory-Scale Conditions. *European Journal of Lipid Science and Technology*, 110: 735–741, 2008.

67. J. Fernandez-Bolanos, G. Rodriguez, R. Rodriguez and R. Guillen and A. Jimenez. Potential Use of Olive by-products Extraction of Interesting Organic Compounds from Olive Oil Waste. *Grasas y Aceites*, 57: 95-106, 2006.

68. A. Ranalli, N. Costantini, G. Mattia, M. L. Ferrante and S. Angelo. Evaluating Two Kinds of Centrifuged Virgin Oils Arising from Continuous Olive Processing. *Journal of the Science of the Food and Agriculture*, 80: 673-683, 2000.

69. M. D. Salvador, F. Aranda, S. Gomez-Alonso and G. Fregapane. Influence of Extraction System, Production Year and Area on Cornicabra Virgin Olive Oil: a Study of Five Crop Seasons. *Food Chemistry*, 80: 359-366, 2003.

70. A. Ranalli and F. Angerosa, Integral Centrifuges for Olive Oil Extraction. The Qualitative Characteristics of Products. *Journal of American Oil Chemists' Society*, 73: 417–422, 1996.

71. E. Psomiadou, M. Tsimidou and D. Boskou. Alpha-tocopherol Content of Greek Virgin Olive Oils. *Journal of Agriculture and Food Chemistry*, 48: 1770–5, 2000.

72. E. Stefanoudaki and A. Koutsaftakis and J. L. Harwood. Influence of Malaxation Conditions on Characteristic Qualities of Olive Oil. *Food Chemistry,* 127: 1481–1486, 2011.

73. A. Ranalli, G. De Mattia and M. L. Ferrante. Comparative Evaluation of the Olive Oil Given by a New Processing System. *International Journal of Food Science and Technology*, 32: 289–297, 1997.

74. E. Chiacchierini, G. Mele, D. Restuccia and G. Vinci. Impact Evaluation of Innovative and Sustainable Extraction Technologies on Olive Oil Quality. *Trends in Food Science and Technology,* 18: 299–305, 2007.

75. M.L. Clodoveo, V. Durante and D. La Notte. Working Towards the Development of Innovative Ultrasound Equipment for the Extraction of Virgin Olive Oil. *Ultrasonics Sonochemistry*, 20: 1261–70, 2013.

76. B. Aliakbarian, D. De Faveri, A. Converti and P. Perego. Optimization of Olive Oil Extraction by Means of Enzyme Processing Aids Using Response Surface Methodology. *Biochemical Engineering Journal*, 42: 34–40, 2008.

77. A. Ranalli and G. De Mattia. Characterization of Olive Oil Produced with a New Enzyme Processing Aid. *Journal of American Oil Chemistry Society*, 74: 1105–1113, 1997.

78. H. J. Chih, A. P. James, V. Jayasena and S. S. Dhaliwal. Addition of Enzymes Complex During Olive Oil Extraction Improves Oil Recovery and Bioactivity of Western Australian Frantoio Olive Oil. *International Journal of Food Science and Technology,* 47: 1222-1228, 2012.

79. L. Najafian, A. Ghodsvali, M.H. H. Khodaparast and L.L. Diosady, Aqueous extraction of Virgin Olive Oil Using Industrial Enzymes. *Food Research International*, 42: 171–175, 2009.

80. A. Ranalli, D. Marchegiani, D. Pardi, S. Contento, D. Pardi, F. Girardi and F. Kotti. Evaluation of Functional Phytochemicals in De-Stoned Virgin Olive Oil. *Food and Bioprocess Technology*, 2: 322–327, 2009.

81. B. Iconomou, D. Arapoglou and C. Israilides. Improvement of Phenolic Antioxidants and Quality Characteristics of Virgin Olive Oil with the Addition of Enzymes and Nitrogen During Olive Paste Processing. *Grasas y Aceites*, 61: 303–311, 2010.

82. Novozymes. Enzymatic Oil Extraction from Olive Pomace, http://www.theolivecentre.com/Olive-Equipment/olive-oil-PROCESSING-AIDS/Enzyme-Pectinex-Ultra-SPL-1L-3.html# [retrieved 5 May 2016].

83. A. Yorulmaz, A. Tekin and S. Turan. Improving Olive Oil Quality with Double Protection: Destoning and Malaxation in Nitrogen Atmosphere. *European Journal of Lipid Science and Technology*, 113: 637–643, 2011.

84. M. Servili, A. Taticchi, S. Esposto, S. Urbani, R. Selvaggini and G.F. Montedoro. Effect of Olive Stoning on the Volatile and Phenolic Composition of Virgin Olive Oil. *Journal of Agriculture and Food Chemistry*, 55: 7028–35, 2007.

85. A. Del Caro, V. Vacca, M. Poiana, P. Fenu and A. Piga. Influence of Technology, Storage and Exposure on Components of Extra Virgin Olive Oil (*Bosana cv*) from Whole and De-Stoned Fruits. *Food Chemistry*, 98: 311–316, 2006.

86. P. Amirante, M. Clodoveo, G. Dugo, A. Leone and A. Tamborrino. Advance Technology in Virgin Olive Oil Production from Traditional and De-Stoned Pastes: Influence of the Introduction of a Heat Exchanger on Oil Quality. *Food Chemistry*, 98: 797–805, 2006.

87. V. Lavelli and L. Bondesan. Secoiridoids, Tocopherols, and Antioxidant Activity of Monovarietal Extra Virgin Olive Oils Extracted from Destoned Fruits. *Journal of Agriculture and Food Chemistry*, 53: 1102–7, 2005.

88. G. Gambacorta, M. Faccia, M. A. Previtali, S. Pati, E. La Notte and A. Baiano. Effects of Olive Maturation and Stoning on Quality Indices and Antioxidant Content of Extra Virgin Oils (*Cv. Coratina*) During Storage. *Journal of Food Science*, 75: C229–35, 2010.

89. L. Di Giovacchino, M.R. Mucciarella, N. Costantini, M.L. Ferrante and G. Surricchio. Use of Nitrogen to Improve Stability of Virgin Olive Oil During Storage. *Journal of American Oil Chemists' Society*, 79: 339–344. 2002.

90. E. Vierhuis, M. Servili, M. Baldioli, H. A. Schols, A. G. J. Voragen and G.F. Montedoro. Effect of Enzyme Treatment During Mechanical Extraction of Olive Oil on Phenolic Compounds and Polysaccharides. *Journal of Agriculture and Food Chemistry*. 49: 1218–23, 2001.

91. N.A. Febrianto and T.A. Yang. Producing High Quality Edible Oil by Using Eco-Friendly Technology: A Review. *Journal of Food Science*, 3: 317–326, 2011.

92. A. Jiménez, G. Beltrán and M. Uceda. High-power Ultrasound in Olive Paste Pretreatment. Effect on Process Yield and Virgin Olive Oil Characteristics. *Ultrasonics Sonochemistry*, 14: 725–31, 2007.

93. International Olive Oil Council. COI/OH/Doc. No 1. 2011, Guide for the Determination of the Characteristics of Oil-olives, http://www.internationaloliveoil.org/estaticos/view/222-standards [retrieved 4 June 2016].

94. C. Pattara, G.M. Cappelletti and A. Cichelli, Recovery and Use of Olive Stones: Commodity, Environmental and Economic Assessment. *Renewable Sustainable Energy Reviews*, 14: 1484–1489, 2010.

95. P.El-Abbassi S. Rodis, V. T. Karathanos and A. Mantzavinou. Partitioning of Olive Oil Antioxidants Between Oil and Water Phases. *Journal of Agriculture and Food Chemistry,* 50: 596–601, 2002.

96. T. J. Klen and B.M. Vodopivec, The Fate of Olive Fruit Phenols During Commercial Olive Oil Processing: Traditional Press Versus Continuous Two- and Three-Phase Centrifuge. *LWT--Food Science and Technology*, 49: 267-274, 2012.

97. A. Lama-Muñoz, G. Rodriguez-Gutierrez, F. Rubio-Senent, Gomez-Carretero and J. Fernandez-Bolanos. New Hydrothermal Treatment of Alperujo Enhances the Content of Bioactive Minor Components in Crude Pomace Olive Oil. *Journal of Agriculture Food Chemistry,* 59: 1115–23, 2011.

98. International Olive Oil Council. IOC/T.20/No30. 2013, Determination of the Composition and Content of Sterols and Triterpene Dialcohols by Capillary Column Gas Chromatography, http://www.internationaloliveoil.org/estaticos/view/224-testing-methods [retrieved 2 February 2017].

99. EU Commission. EEC 2568/91. 1991, EU Commission Regulation on the Characteristics of Olive Oil and Olive-Residue Oil and on the Relevant Methods of Analysis, http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A01991R2568-20151016 [retrieved 2 February 2017].

100. S. S. Cunha and J. O. Fernandez. Quantification of Free and Esterified Sterols in Portuguese Olive Oils by Solid-Phase Extraction and Gas Chromatography-Mass Spectrometry. *Journal of Chromatography A*, 1128: 220-227, 2006.

101. D. Grigoriadou, A. Androulaki, E. Psomiadou and M. Tsimidou. Solid Phase Extraction in the Analysis of Squalene and Tocopherols in Olive Oil. *Food Chemistry*, 105: 675–680, 2007.

102. J. L. Martínez-Vidal, A. Garrido-Frenich, M. A. Escobar-García and R. Romero-González. LC–MS Determination of Sterols in Olive Oil. *Chromatographia*, 65: 695–699, 2007.

103. A., S. Carretero, A. Carrasco-Pancorbo, S. Cortacero, A. Gori, L. Cerretani and A. Fernández-Gutiérrez. A simplified Method for HPLC-MS Analysis of Sterols in Vegetable Oil. *European Journal of Lipid Science and Technology*, 110: 1142-1149, 2008.

104. S. A. Damarchi, G. P. Savage and P. C. Dutta. Sterol Fractions in Hazelnut and Virgin Olive Oils and 4,4′ -Dimethylsterols as Possible Markers for Detection of Adulteration of Virgin Olive Oil. *Journal of American Oil Chemists' Society*, 82: 717-725, 2005.

105. I. Bohaèenko and Z. Kopicová. Detection of Olive Oils Authenticity by Determination of their Sterol Content Using LC/GC. *Czech Journal of Food Sciences*, 19: 97–103, 2001.

106. B. Mathison and D. A. Holstege. Rapid Method to Determine Sterol, Erythrodiol, and Uvaol Concentrations in Olive Oil. *Journal of Agricultural and Food Chemistry*, 61: 4506-13, 2013.

107. W. Zarrouk, A. Carrasco-Pancorbo, M. Zarrouk, A. Segura-Carretero and A. Fernández-Gutiérrez. Multi-component Analysis (sterols, tocopherols and triterpenic dialcohols) of the Unsaponifiable Fraction of Vegetable Oils by Liquid Chromatography-atmospheric Pressure Chemical Ionization-ion Trap Mass Spectrometry. *Talanta*, 80: 924-34, 2009.

108. J. Parcerisa, I. Casals, J. Boatella, R. Codony and M. Rafecas. Analysis of Olive And Hazelnut Oil Mixtures By High-Performance Liquid Chromatography-Atmospheric Pressure Chemical Ionisation Mass Spectrometry of Triacylglycerols and Gas-Liquid Chromatography of Non-Saponifiable Compounds (Tocopherols and Sterols). *Journal of Chromatography A*, 881: 149–58, 2000.

109. M. Du and D. U. Ahn. Simultaneous Analysis of Tocopherols, Cholesterol, and Phytosterols Using Gas Chromatography. *Food and Chemical Toxicology*, 67: 1696-1700, 2002.

110. T. Itoh, K. Yoshida, T. Yatsu, T. Tamura and T. Matsumoto. Triterpene alcohols and sterols of Spanish olive oil. *Journal of the American Oil Chemists' Society*. 58: 545-550, 1981.

111. S. C. Cunha, J. S. Amaral, J. O. Fernandes, M. Beatriz and P. P. Oliveira. Quantification of Tocopherols and Tocotrienols in Portuguese Olive Oils Using HPLC with Three Different Detection Systems. *Journal of Agricultural and Food Chemistry*, 54: 3351-6, 2006.

112. A. Gliszczyńska-świgło, E. Sikorska, I. Khmelinskii and M. Sikorski. Tocopherol Content in Edible Plant Oils. *Polish Journal of Food And Nutrition Sciences*, 57: 157-161, 2007.

113. BS EN ISO 9936:2006 +A1. Animal and Vegetable Fats and Oils. Determination of Tocopherol and Tocotrienol Contents by High-performance Liquid Chromatography, BSI, 2011.

114. International Union of Pure and Applied Chemistry. IUPAC 2.432/1992. Determination of tocopherols and tocotrienols in vegetable oils and fats by high performance liquid chromatography. In *Standard Methods for the Analysis of Oils*, Fats and Derivatives, 7th ed.,
old.iupac.org/publications/books/ISBN0632033371_compress.pdf [retrieved 2 February 2017].

115. G. Blekas, M. Tsimidou, D. Boskou. Contribution of Alpa-tocopherol to Olive Oil Stability. *Food Chemistry*, 52: 289-294, 1995.

116. J. Giacometti, Determination of Aliphatic Alcohols, Squalene, α-Tocopherol and Sterols in Olive Oils: Direct Method Involving Gas Chromatography of the Unsaponifiable Fraction Following Silylation. *Analyst*, 126: 472-475, 2001.

117. C. L. Ortiz, M. P. Moya and V. B. Navarro. A Rapid Chromatographic Method for Simultaneous Determination of β-sitosterol and Tocopherol Homologues in Vegetable Oils. *Journal of Food Composition and Analysis*, 19: 141-149, 2006.

118. J. Villen, G. P. Blanch, M. L. Luisa Ruiz del Castillo and M. A. Herraiz, Rapid and Simultaneous Analysis of Free Sterols, Tocopherols, and Squalene in Edible Oils by Coupled Reversed-Phase Liquid Chromatography - Gas Chromatography. *Journal of Agricultural and Food Chemistry*. 8561: 1–4, 1998.

119. EUROCHEM: The Fitness for Purpose of Analytical Methods, A Laboratory Guide to Method Validation and Related Topics. 2nd ed., EUROCHEM, 2014.

120. The International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH). Q2 (R1), 2005, Version 4, Validation of Analytical Procedures: Text and Methodology, http://www.ich.org/products/guidelines/quality/quality-single/article/validation-of-analytical-procedures-text-and-methodology.html [retrieved 2 February 2017].

121. V. Barwick, Preparation of calibration curves: A guide to best practice, LGC/VAM/2003/032, LGC, 2003 (www.lgcgroup.com/nmi)

122. W. Horwitz and R. Albert. The Horwitz ratio (HorRat): A Useful Index of Method Performance with Respect to Precision. *Journal of AOAC International*, 89: 1095-1109, 2006.

123. Association of Analytical Communities (AOAC). Guideline for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals, 2002, http://www.aoac.org/aoac_prod_imis/AOAC_Docs/StandardsDevelopment/SLV_Guidelines_Dietary_Supplements.pdf [retrieved 2 February 2017].

124. M. Möller, P. Nilges, F. Harnisch and U. Schröder. Subcritical Water as Reaction Environment: Fundamentals of Hydrothermal Biomass Transformation. *ChemSusChem*, 4: 566–79, 2011.

125. G. Brunner. Near Critical and Supercritical Water. Part I. Hydrolytic and Hydrothermal Processes. *The Journal of Supercritical Fluids*, 47: 373–381, 2009.

126. G. Garrote, H. Dominguez and J.C. Parajo. Hydrothermal Processing of Lignocellulosic Materials. *European Journal of Wood and Wood Products*, 57: 191–202, 1999.

127. J. Fernández-Bolaños, B. Felizón, A. Heredia, R. Guillén and A. Jiménez. Characterization of the Lignin Obtained by Alkaline Delignification and of the Cellulose Residue from Steam-exploded Olive Stones. *Bioresource Technology*. 68: 121-132, 1999.

128. J. Fernandez-Bolanos, B. Felizon, M. Brenes, R. Guillen and A. Heredia. Steam-explosion of Olive Stones: Hemicellulose Solubilization and Enhancement of Enzymatic Hydrolysis of Cellulose. *Bioresource Technology*, 79: 53-61, 2001.

129. H. Abu Tayeh, O. Levy-Shalev, H. Azaizeh and C. G. Dosoretz. Subcritical Hydrothermal Pretreatment of Olive Mill Solid Waste for Biofuel Production. *Bioresource Technology*, 199:164-172, 2016.

130. P. Kumar, D. M. Barrett, M. J. Delwiche and P. Stroeve. Methods for Pretreatment of Lignocellulosic Biomass for Efficient Hydrolysis and Biofuel Production. *Industrial and Engineering Chemistry*, 48: 3713-3729, 2009.

131. E. Kıpçak, O. Ö. Söğüt and M. Akgün. Hydrothermal Gasification of Olive Mill Wastewater as A Biomass Source in Supercritical Water. *The Journal of Supercritical Fluids*, 57:50-57, 2011.

132. J. Fernandez-Bolanos, G. Rodriguez, E. Gomez, R. Guillen, A. Jimenez, A. Heredia and R. Rodrigues. Total Recovery of the Waste of Two-phase Olive Oil Processing: Isolation

of Added-Value Compounds. *Journal of Agricultural and Food Chemistry*, 52: 5849-55, 2004.

133. G. Rodríguez, A. Lama, R. Rodrigues, A. Jimenez, R. Guillen and J. Fernandez-Bolanos. Olive Stone an Attractive Source of Bioactive and Valuable Compounds. *Bioresource Technology*, 99: 5261–9, 2008.

134. G. Rodríguez-Gutiérrez, F. Rubio-Senent, A. Lama-Muñoz, A. García and J. Fernández-Bolaños. Properties of Lignin, Cellulose, and Hemicelluloses Isolated from Olive Cake and Olive Stones: Binding of Water, Oil, Bile Acids, and Glucose. *Journal of Agricultural and Food Chemistry*, 62: 8973-8981, 2014.

135. J. Fernandez-Bolanos, B. Felizon, M. Brenes, R. Guillen and A. Heredia. Hydroxytyrosol and Tyrosol as the Main Compounds Found in the Phenolic Fraction of Steam-exploded Olive Stones. *Journal of the American Oil Chemists' Society*, 75: 1643-1649, 1998.

136. J. Fernandez-Bolanos, G. Rodriguez, R. Rodriguez, R. Guillen and A. Jimenez. Production in Large Quantities of Highly Purified Hydroxytyrosol from Liquid-Solid Waste of Two-Phase Olive Oil Processing or "Alperujo". *Journal of Agricultural and Food Chemistry*, 50: 6804-11, 2002.

137. F. Rubia-Senent, A. Lama-Munoz, G. Rodrigues-Gutierrez and J. Fernandes-Bolanos. Isolation and Identification of Phenolic Glucosides from Thermally Treated Olive Oil Byproducts. *Journal of Agricultural and Food Chemistry*, 61: 1235-1248, 2013.

138. S. C. Ndlela, J. M. L. N. de Moura, N. K. Olson and L. A. Johnson. Aqueous Extraction of Oil and Protein from Soybeans with Subcritical Water. *Journal of the American Oil Chemists' Society*, 89: 1145-1153, 2012.

139. C. C. Teo, S. N. Tan, J. W. H. Yong, C. S. Hew and E. S. Ong. Pressurized Hot Water Extraction (PHWE). *Journal of Chromatography A*, 1217: 2484-2494, 2010.

140. J. S. S. Pinto and F. M. Lanças. Hydrolysis of Corn Oil Using Subcritical Water. *Journal of the Brazilian Chemical Society*, 17: 85-89, 2006.

141. M. Herrero, A. Cifuentes and E. Ibanez. Sub- and Supercritical Fluid Extraction of Functional Ingredients from Different Natural Sources: Plants, Food-by-products, Algae and Microalgae A Review. *Food Chemistry*, 98: 136–148, 2006.

142. W. Abdelmoez, R. Abdelfatah and A. Tayeb. Extraction of Cottonseed Oil Using Subcritical Water Technology. *AIChE Journal*, 57: 2353–2359, 2011.

143. O. Pourali, F.S. Asghari and H. Yoshida. Simultaneous Rice Bran Oil Stabilization and Extraction Using Sub-Critical Water Medium. *Journal of Food Engineering*, 95: 510–516, 2009.

144. S. Lee, J. Kim, Y. Ishida and T. Hasegawa. Application of Far-Infrared and Subcritical Water for Recovering Antioxidant Compounds from Rice Hulls. *International Symposium on EcoTopia Science, ISETS07,* 2007.

145. L. He, X. Zhang, H. Xu, C. Xu, F. Yuan, Ž. Knez, Z. Novak and Y. Gao. Subcritical Water Extraction of Phenolic Compounds from Pomegranate (Punica Granatum L.) Seed Residues and Investigation into their Antioxidant Activities with HPLC–ABTS+ Assay, *Food and Bioproducts Processing*, 90: 215-223, 2012.

146. M. H. Roudsari. Master Thesis: Subcritical Water Extraction of Antioxidant Compounds from Canola Meal, Canada, 2007.

147. W. Abdelmoez, S. M. Nage, A. Bastawess, A. Ihab and H. Yoshida. Subcritical Water Technology for Wheat Straw Hydrolysis to Produce Value Added Products. *Journal of Cleaner Production*, 70: 68-77, 2014.

148. J. M. Prado, T. Forster-Carneiro, M. A. Rostagno, L. A. Follegatti-Romero, F. Maugeri Filho and M. A. A. Meireles. Obtaining Sugars from Coconut Husk, Defatted Grape Seed,

and Pressed Palm Fiber by Hydrolysis with Subcritical Water. *Journal of Supercritical Fluids*, 89: 89-98, 2014.

149. N. Lebovka, E. Voroniev and F. Chemat. Enhancing Extraction Processes in the Food Industry. CRC Press, Taylor and Francis Group, New York, 2011.

150. X. Yu, P. Zhu, Q. Zhong, M. Li and H. Ma. Subcritical Water Extraction of Antioxidant Phenolic Compounds from Xilan Olive Fruit Dreg. *Journal of Food Science and Technology*, 52:1-9, 2014.

151. M. Ravber, Z. Knez and M. Škerget. Simultaneous Extraction of Oil- and Water-soluble Phase from Sunflower Seeds with Subcritical Water. *Food Chemistry*, 166: 316-323, 2015.

152. R. Watson and V. Preedy. *Bioactive Foods and Extracts Cancer Treatment and Prevention*, CRC Press Taylor and Francis Group, New York, 2011.

153. M. J. Dumont and S. S. Narine, Soapstock and Deodorizer Distillates from North American Vegetable Oils: Review on their Characterization, Extraction and Utilization. *Food Research International*, 40: 957-974. 2007.

154. C. M. Galanakis. Recovery of High Added-Value Components from Food Wastes: Conventional, Emerging Technologies and Commercialized Applications. *Trends in Food Science Technology*, 26: 68-87, 2012.

155. D. Boskou. Olive Oil Constituents, Quality, Health Properties and Bioconversions, InTech, Croatia, 2012, http://www.intechopen.com/books/olive-oil-constituents-quality-health-properties-and-bioconversions [retrieved 2 February 2017].

156. E. Psomiadou and M. Tsimidou. On the Role of Squalene in Olive Oil Stability. *Journal of Agriculture and Food Chemistry*, 47: 4025-4032, 1999.

157. L. S. Maguire, S. M. O'Sullivan, K. Galvin, T. P. O'Connor and N. M. O'Brien. Fatty Acid Profile, Tocopherol, Squalene and Phytosterol Content of Walnuts, Almonds, Peanuts,

Hazelnuts and the Macadamia Nut. *International Journal of Food Sciences and Nutrition*, 55: 171–8, 2004.

158. J. Parcerisa, I. Casals, J. Boatella, R. Codony and M. Rafecas. Analysis of Olive and Hazelnut Oil Mixtures by High-performance Liquid Chromatography-atmospheric Pressure Chemical Ionisation Mass Spectrometry of Triacylglycerols and Gas-liquid Chromatography of Non-saponifiable Compounds (tocopherols and sterols). *Journal of Chromatography A*. 881: 149–58, 2000.

159. J. Toivo, K. Phillips, A.-M. Lampi and V. Piironen. Determination of Sterols in Foods: Recovery of Free, Esterified, and Glycosidic Sterols. *Journal of Food Composition and Analysis*, 14: 631–643, 2001.

160. R. Gasior, M. Pieszka and F. Brzóska. Validation of a Method for Simultaneous Determination of Tocopherols and Tocotrienols in Cereals Using Normal Phase HPLC. *Journal of Animal and Feed Sciences*, 18: 173-192, 2009.

161. F. Orata. Derivatization Reactions and Reagents for Gas Chromatography Analysis. In: M. A. Mohd, editor, *Advanced Gas Chromatography – Progress in Agricultural, Biomedical and Industrial Applications*, pages 83–108, InTech, Croatia, 2012.

162. W. F. De Greyt, V. Petrauskaite, M. J. Kellens and A. D. Huyghebaert. Analysis of Tocopherols by Gas-liquid and High-performance Liquid Chromatography: A Comparative Study. *Lipid*, 100: 503-507, 1998.

163. W. Talent and R. Kleiman, Bis(trimethylsily1) acetamide in the Silylation of Lipolysis Products for Gas-liquid Chromatography. *The Journal of Lipid Research*, 9; 146-148, 1968.

164. T. Verleyen, M. Forcades, R. Verhe, K. Dewettinck, A. Huyghebaert and W. Greyt. Analysis of Free and Esterified Sterols in Vegetable Oils. *Journal of the American Oil Chemists' Society*, 79: 117-122, 2002.

165. AOAC 976/26. Cholesterol in Multicomponent Foods-Gas Chromatographic. *AOAC* 45.4.06, 1977.

166. R. E. Wrolstad, R. E. Decker, S. J. Schwartz and P. Sporns. Handbook of Food Analytical Chemistry, Water, Proteins, Enzymes, Lipids, and Carbohydrates. John Wiley and Sons, Canada, 2005.

167. A. Shalmashi and A. Eliassi, Solubility of L-(+)-ascorbic Acid in Water, Ethanol, Methanol, Propan-2-ol, Acetone, Acetonitrile, Ethyl Acetate, and Tetrahydrofuran. *Journal of Chemical and Engineering Data*, 53: 1332-1334, 2008.

168. G. C. Derringer. A Balancing Act: Optimizing a Product's Properties. *Quality Progress*. 27: 51-58, 1994.

169. K. Kohler, B. Michell and W. Ellefson. Determination of Tocopherols in Nutritional Products Containing Greater than 30 per cent Total Tocopherols by Gas Chromatography. *AOAC International, P-T-072*,

170. M. I. Covas. Olive Oil and the Cardiovascular System. *Pharmacological Research,* 55: 175–86, 2007.

171. M. I. Covas, V. Ruiz-Gutierrez, R. de la Torre, A. Kafatos, R. M. Lamuela-Raventos, J. Osada, R. W. Owen and F. Visioli. Minor Components of Olive oil: Evidence to Date of Health. *Nutrition Reviews*, 64:20–30, 2006.

172. Ö. Seçmeler and Ö. G. Üstündağ. A rapid in-house Validated GC-FID Method for Simultaneous Determination of Lipophilic Bioactives in Olive Oil: Squalene, α-tocopherol, and β-sitosterol. *European Journal of Lipid Science and Technology*, 119: 1500420, 2017.

173. R W. Owen, A. Giacosa, W.E. Hull, R. Haubner, G. Würtele, B. Spiegelhalder, H. Bartsch. Olive-oil Consumption and Health: the Possible Role of Antioxidants. *Lancet Oncology*. 1: 107–12, 2000.

174. C. López Ortíz, M. Prats Moya and V. Berenguer Navarro. A Rapid Chromatographic Method for Simultaneous Determination of β-sitosterol and Tocopherol Homologues in Vegetable Oils. *Journal of Food Composition and Analysis*, 19: 141-149, 2006.

175. G. Sivakumar, C. B. Bati, E. Perri and E. Uccella. Gas Chromatography Screening of Bioactive Phytosterols from Mono-cultivar Olive Oils. *Food Chemistry*, 95: 525-528, 2006.

176. I. Mafra, B. Lanza, A. Reis, V. Marsilio, C. Campestre, M. Angelis, M. A. Coimbra. Effect of Ripening on Texture, Microstructure and Cell Wall Polysaccharide Composition of Olive Fruit (*Olea europaea*). *Physiologia Plantarum*, 111: 439-447, 2001.

177. A. Jimenez, R. Rodriguez, I. Fernandez-Caro, R. Guillen, J. Fernandez-Bolanos and A. Heredia. Olive Fruit Cell Wall: Degradation of Pectic Polysaccharides During Ripening. *Journal of Agriculture and Food Chemistry*, 49: 409-415, 2001.

178. R. B. Gomez-Coca, M. Perez-Camino and W. Moreda. Specific Procedure for Analysing Steryl Glucosides in Olive Oil. *European Journal of Lipid Science and Technology*. 114: 1417-1426, 2012.

179. D. Jonker, G.D. van der Hoek, J. F. C. Glatz, C. Homan, M. A. Posthumus and M. B. Katan. Combined Determinatıon of Free, Esterified and Glycosylated Plant Sterols in Foods. *Nutrition Reports International*, 32: 943-951, 1985.

180. International Olive Oil Council. World Olive Oil Figures, Production, 2015, http://www.internationaloliveoil.org/estaticos/view/131-world-olive-oil-figures [retrieved 18 October 2016].

181. J. A. Alburquerque, J. Gonzalvez, D. Garcia and J. Cegarra. Agrochemical Characterisation of "alperujo", A Solid by-product of the Two-phase Centrifugation Method for Olive Oil Extraction. *Bioresource Technology*, 91: 195-200, 2004.

182. P. Fernandes and J. M. S. Cabral. Phytosterols: Applications and Recovery Methods. *Bioresource Technology*, 98: 2335-2350, 2007.

183. J. Plumb, M.J.C. Rhodes, A. M. Lampi, M. Buchgraber and P. Kroon. Phytosterols in Plant Foods: Exploring Contents, Data Distribution and Aggregated Values Using an Online Bioactives Database. *Journal of Food Composition and Analysis*, 24: 1024-1031, 2011.

184. M. R. Aguirre, M. V. Ruiz-Mendez, L. Velasco and M. C. Dobarganes. Free Sterols and Steryl Glycosides in Sunflower Seeds with High Phytosterol Contents. *European Journal of Lipid Science and Technology*, 114: 1212-1216, 2012.

185. K. M. Philips, M.T. Tarrago-Trani, T. M. Grove, I. Grün, R. Lugogo, R. F. Harris and K. K. Stewart. Simplified Gravimetric Determination of Total Fat in Food Composites After Chloroform-methanol Extraction. *Journal of the American Oil Chemists' Society,* 74: 137-142, 1997.

186. M C. Pérez-Camino, W. Moreda, R. Mateos and A. Cert. Determination of Esters of Fatty Acids with Low Molecular Weight Alcohols in Olive Oils. *Journal of Agricultural and Food Chemistry*, 56: 4721-5, 2002.

187. N. Cicco, M. Lanorte, M. Paraggio, M. Viggiano and L. Lattanzio. A reproducible, rapid and inexpensive Folin-Ciocalteu Micro-Method in Determining Phenolics of Plant Methanol Extracts. *Microchemical Journal,* 91: 107-110, 2009.

188. H. C. Karantonis, N. Tsantila, G. Stamatakis, M. Samiotaki, G. Panayotou, S. Antonopoulou and C. A. Demopoulos. Bioactive Polar Lipids in Olive Oil, Pomace and Waste Byproducts. *Journal of Food Biochemistry*, 32: 443-459, 2008.

189. W. Stelte. Steam Explosion for Biomass Pre-treatment, Resultat Kontrakt Report, Energy and Climate Centre for Renewable Energy and Transport Section for Biomass in Danish Technological Institute,
http://www.teknologisk.dk/_/media/52681_RK%20report%20steam%20explosion.pdf
[retrieved 18 October 2016].

190. L. Gallardo-Guerrero, M. Roca and M. I. Mínguez-Mosquera. Distribution of Chlorophylls and Carotenoids in Ripening Olives and Between Oil and Alperujo When Processed Using a Two-Phase Extraction System, *Journal of the American Oil Chemists' Society*, 79: 105-109, 2002.

191. M. Servili and G. F. Montedoro. Contribution of Phenolic Compounds to Virgin Olive Oil Quality. *European Journal of Lipid Science Technology*, 104: 602-613, 2002.

192. A. El-Abbassi, H. Kiai and A. Hafidi. Phenolic Profile and Antioxidant Activities of Olive Mill Wastewater. *Food Chemistry*, 132: 406-412, 2012.

193. T. C. Wallace. Analysis of Procyanidins and Anthocyanins in Food Products Using Chromatographic and Spectroscopic Techniques, *Dissertation, The Ohio State University*, 2010.

194. X. Sui, X. Dong and W. Zhou. Combined Effect of Ph and High Temperature on the Stability and Antioxidant Capacity of Two Anthocyanins in Aqueous Solution. *Food Chemistry*, 163: 163-170, 2014.

195. E. Sadilova, F. C. Stintzing and R. Carle. Thermal Degradation of Acylated and Nonacylated Anthocyanins. *Journal of Food Science*, 71: 504-512, 2006.

196. T. Gomes and F. Caponio. Evaluation of the State of Oxidation of Olive−Pomace Oils. Influence of the Refining Process. *Journal of Agricultural and Food Chemistry*, 46: 1137-1142, 1998.

197. M. Brenes, A. Garcia, P. Garcia and A. Garrido. Acid Hydrolysis of Secoiridoid Aglycons During Storage of Virgin Olive Oil. *Journal of Agricultural and Food Chemistry*, 49: 5609-14.

198. G. Rodríguez-Gutiérrez, A. Lama, M. Trujillo, J. L. Espartero and J. Fernandez-Bolanos. Isolation of a Powerful Antioxidant from Olea Europaea Fruit-mill Waste: 3,4-Dihydroxyphenylglycol. *LWT - Food Science and Technology*. 42: 483-490, 2009.

199. M. Plaza, M. Amigo-Benavent, M. D. del Castillo, E. Ibáñez and M. Herrero. Facts about the Formation of New Antioxidants in Natural Samples after Subcritical Water Extraction. *Food Research International*, 43: 2341-2348, 2010.

200. J. Wiboonsirikul, Y. Kimura, Y. Kanaya, T. Tsuno and S. Adachi. Production and Characterization of Functional Substances from a By-Product of Rice Bran Oil and Protein Production by a Compressed Hot Water Treatment. *Bioscience, Biotechnology, and Biochemistry*, 72: 384–392, 2008.

201. A. Kazan, M. S., Çeliktaş, S. Sargın and Ö. Yeşil-Çeliktaş. Bio-based Fractions by Hydrothermal Treatment of Olive Pomace: Process Optimization and Evaluation. *Energy Conversion and Management*, 103: 366-373, 2015.

APPENDIX A: SUPPLEMANTARY FIGURES

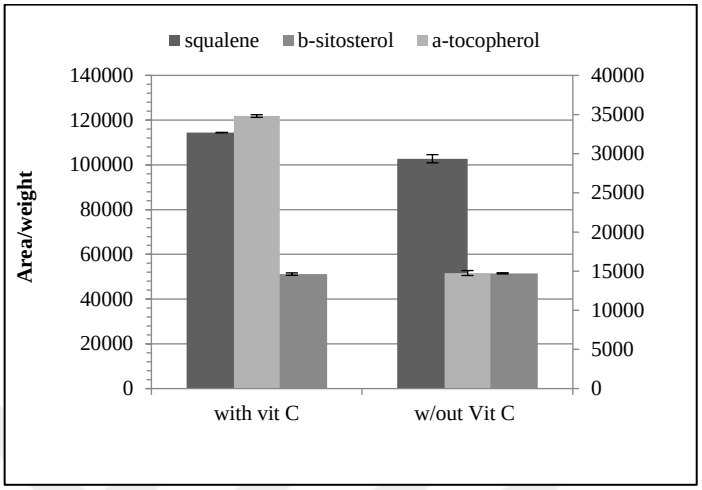


Figure A.1. The effect of antioxidant addition (vitamin C; 0.5 mL of 20 per cent (w/v)) on squalene (y1), β-sitosterol (y1) and α-tocopherol (y2) (n=2)

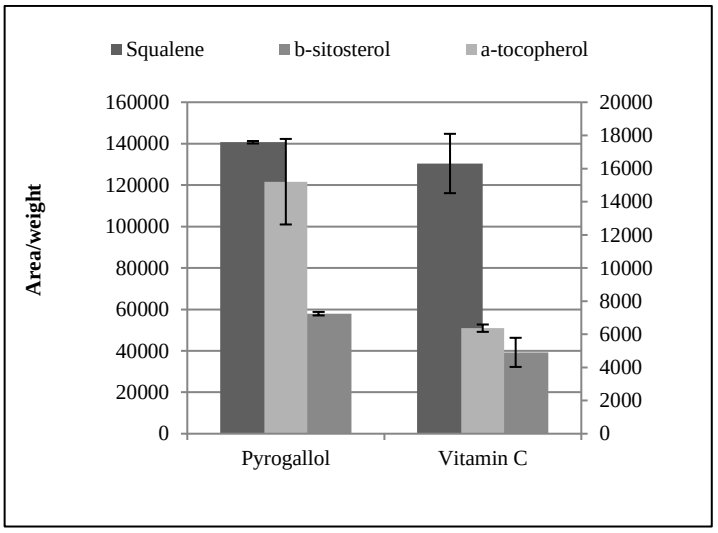


Figure A.2. The effect of different antioxidants (pyrogallol (3 per cent (w/v) in pure ethanol) and vitamin C (0.5 mL of 20 per cent (w/v) aqueous solution) addition on squalene (y1), β-sitosterol (y1) and α-tocopherol (y2) (n=2)

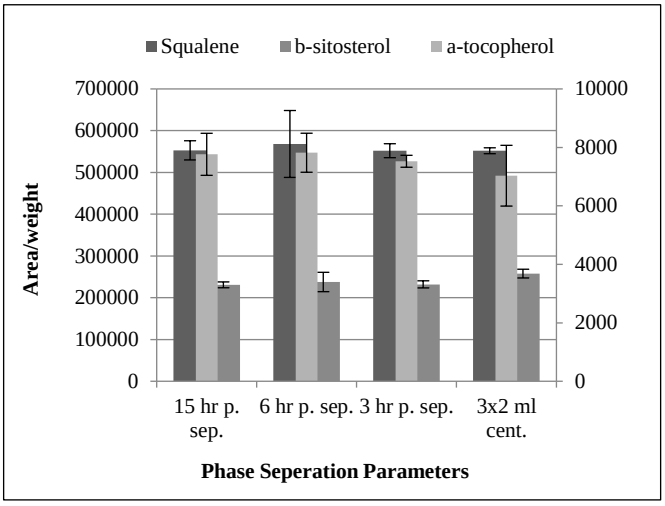


Figure A.3. The effect of phase separation parameters (3, 6 and 15 hr phase separation and centrifugation (3 x 2 ml) on squalene (y1), β-sitosterol (y1) and α-tocopherol (y2) (n=3)

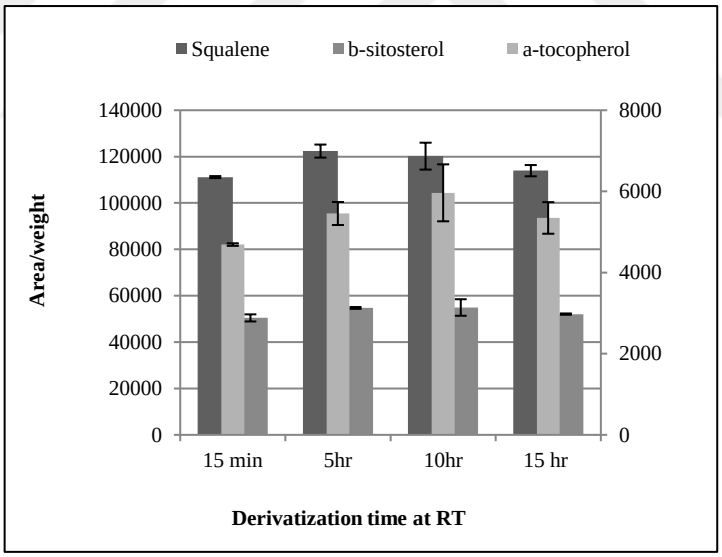


Figure A.4. The effect of derivatization time at RT on squalene (y1), β-sitosterol (y1) and α-tocopherol (y2) (n=2)

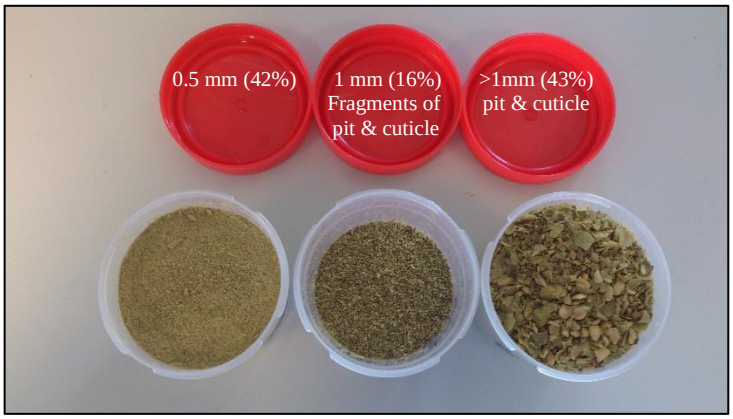


Figure A.5. Different mesh size fractions obtained by sieving dried olive pomace

Figure A.6. Flash hydrolysis laboratory pilot unit designed in the Instituto de la Grasa (Sevilla, Spain): 1) Steam generator; 2) Accumulator; 3) Reactor (2 L); 4) Steam expansion chamber (CSIC, Manual de usuario del sistema de control del reactor de autohidrolisis rapida, Rev.1, 1998)

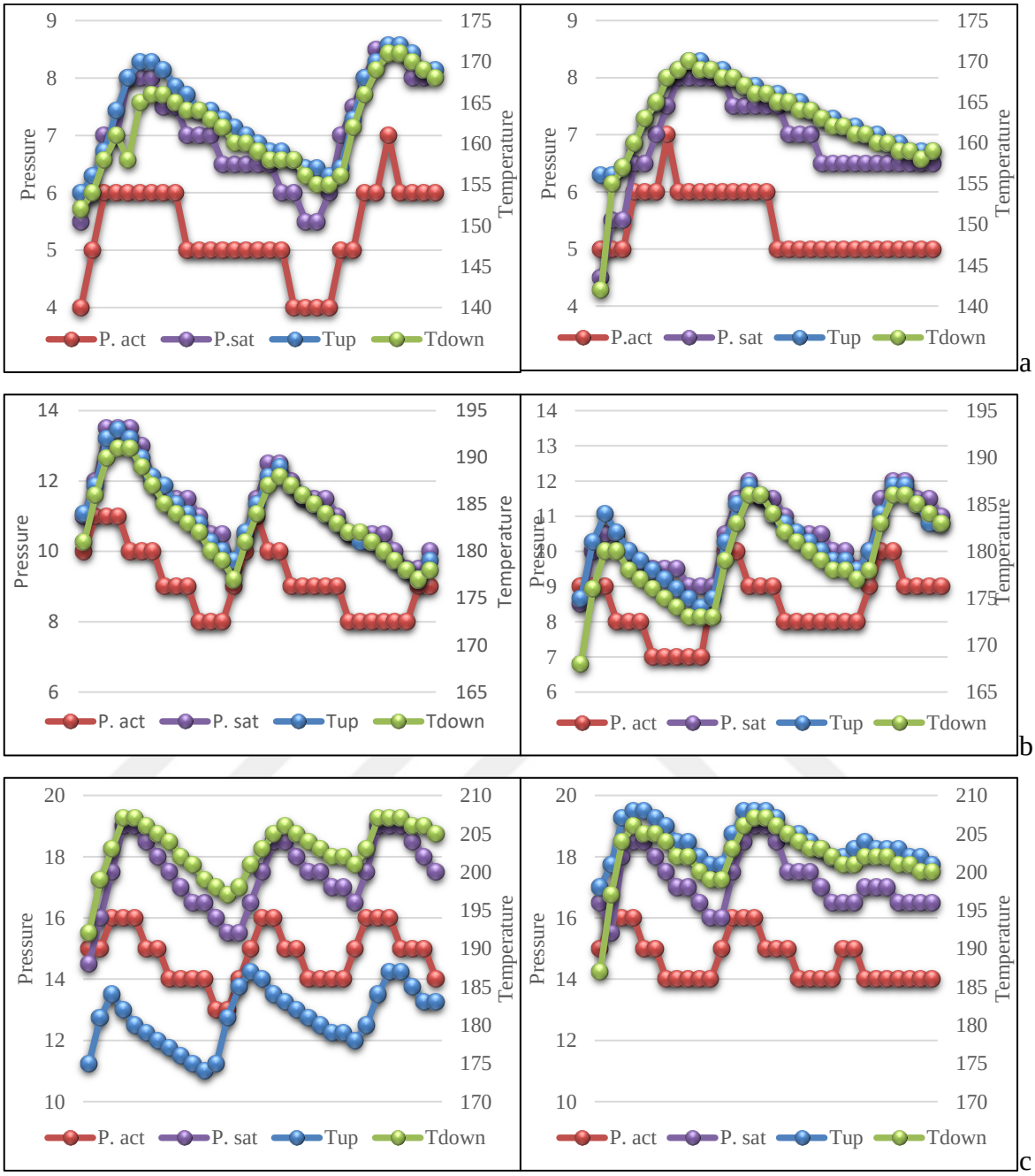


Figure A.7. Pressure (y1) and temperature (y2) (probes located up and down) values of six steam treatments of pomace for T_{target}; a) 160 °C , b) 180 °C, c) 200 °C with two replicate runs

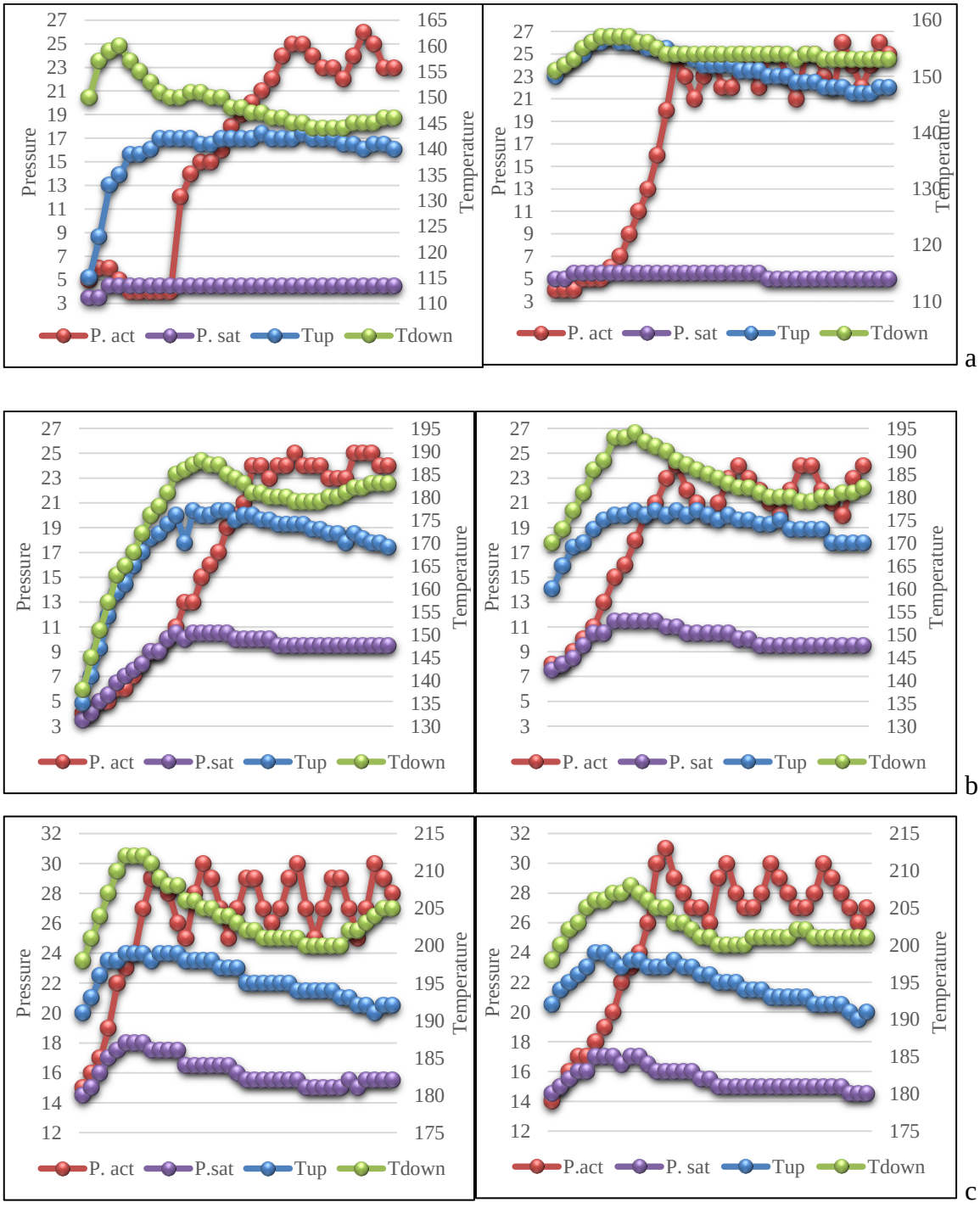


Figure A.8. Pressure (y1) and temperature (y2) (probes located up and down) values of six subcritical water treatments of pomace for T_{target}; a) 160 °C , b) 180 °C, c) 200 °C with two replicate runs

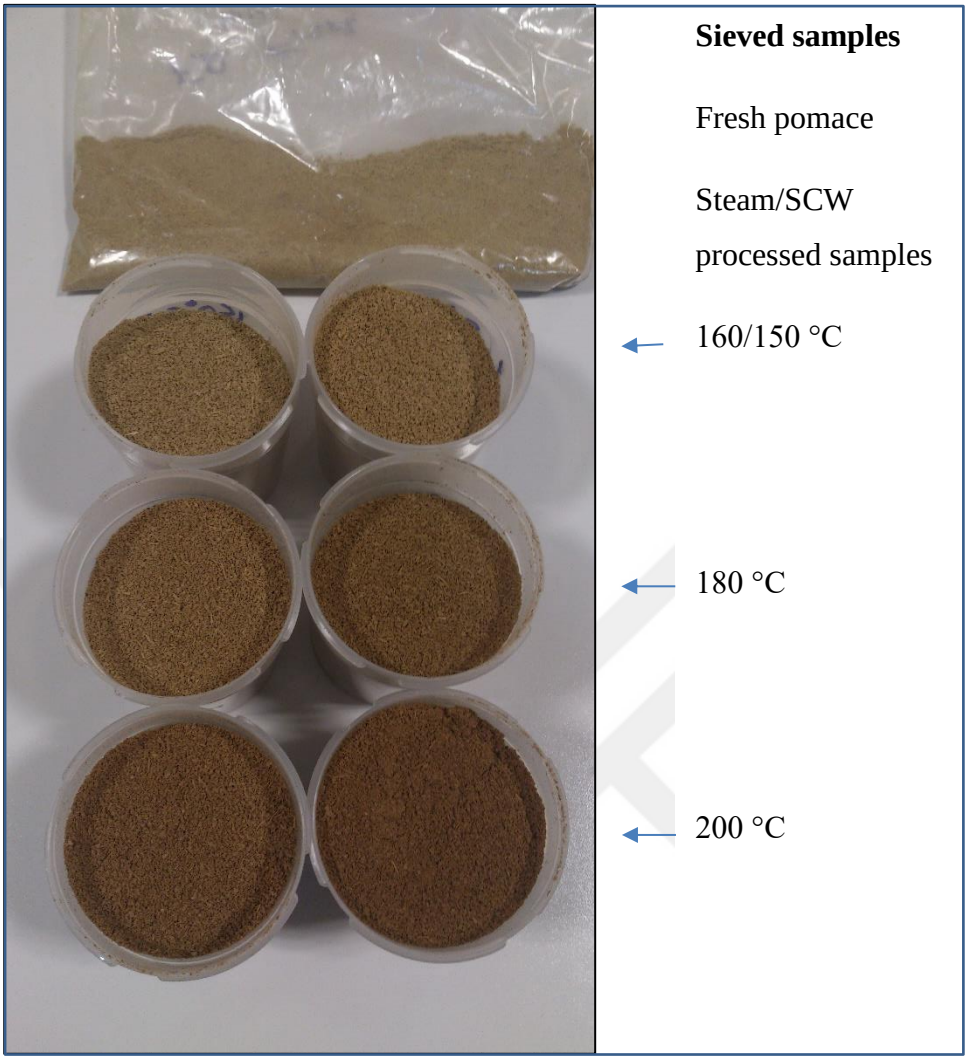


Figure A.9. Matrix differences after sieving fresh pomace and processed samples