UTILIZATION OF PISTACHIO BY-PRODUCTS FOR THE RECOVERY OF PHENOLIC ANTIOXIDANTS

by
Sevcan Erşan

Submitted to Graduate School of Natural and Applied Sciences
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in
Biotechnology

Yeditepe University
2017

UTILIZATION OF PISTACHIO BY-PRODUCTS FOR THE RECOVERY OF PHENOLIC ANTIOXIDANTS

APPROVED BY:

Assist. Prof. Dr. Özlem Güçlü Üstündağ (Thesis Supervisor) .....

Prof. Dr. Artemis Karaali .....

Prof. Dr. Beraat Özçelik .....

Prof. Dr. Mustafa Özilgen .....

Assoc. Prof. Dr. Esra Çapanoğlu Güven .....

DATE OF APPROVAL: ..../..../2017

Dedicated to

Prof. Dr. habil. Dr. h. c. Reinhold Carle…

ACKNOWLEDGEMENTS

Ph.D. is a long journey that is only realized with the support of many actors. But most importantly, I would like to thank TÜBİTAK (The Scientific and Technological Research Council of Turkey) 2211/C and 2214/A Programs for their financial support. This study could not be realized without their support.

Although my Ph.D. journey has officially started in March 2011, actual experimental work for this project started in February 2014 with the acceptance of my thesis proposal, and continued until September 2017. Studies conducted from October 2015 to September 2017 were placed in University of Hohenheim, Germany while sample collection and subcritical water extractions were performed in Yeditepe University, Turkey, beforehand.

I would like to express my greatest thanks to Prof. Dr. habil. Dr. h. c. Reinhold Carle, who welcomed me to his chair, Plant Foodstuff Technology and Analysis at University of Hohenheim for two years. Many thanks for his positive energy, great support, and always having solutions for me. I would like to express my gratitude to P. D. Dr. Ralf Schweiggert for his continuous guidance, encouragement, and patience. I also would like to thank Martin Leitenberger for all useful discussions and technical support on chromatographic analysis, and my colleagues from Plant Foodstuff Technology and Analysis at University of Hohenheim who were always there to support me in a foreign country.

I would like to thank my thesis steering committee members Prof. Dr. Artemis Karaali, and Prof. Dr. Beraat Özçelik for their time and valuable advices.

I also would like to thank Kenan Bozhöyük and Hazar Emir Food Industry and Trade. Ltd. Co. for providing pistachio samples whenever I need.

I would like to express my special thanks to my parents and my little brother for their support throughout of my life.

A very special thanks should go to my fiancé Dr. Osman Ceylan for all kind of supports he gave throughout my whole graduate life.

Finally, many thanks to whom be a part of my life during this period, and contribute somehow to my journey!

September, 2017

Sevcan Erşan
Food Engineer, M.Sc.

ABSTRACT

UTILIZATION OF PISTACHIO BY-PRODUCTS FOR THE RECOVERY OF PHENOLIC ANTIOXIDANTS

Pistachio processing accrues high amounts of by-products. Pistachio hulls, the main by-product of pistachio processing, has yet no commercial value. The main objective of this study was utilization of pistachio hull focusing on the recovery of the phenolic antioxidant compounds. First, detailed analysis of phenolic constituents of the pistachio hull was assessed using high-performance liquid chromatography coupled with diode array detector and electrospray ionization mass spectrometry (HPLC-DAD-ESI-MS^n) as well as by high-resolution mass spectrometry (HR-MS). Then, a rapid method was developed and validated for the simultaneous extraction and quantitation of phenolic compounds from pistachio hull after screening of various extraction parameters, and solvent systems with variable polarity and acidity. Moreover, a method using ultra-high-performance liquid chromatography coupled with diode array detector and evaporative light scattering detector (UHPLC-DAD-ELSD) was developed for faster separation of pistachio hull phenolics. Optimized method was then applied for the quantitation of pistachio hull phenolics from different varieties. Finally, an environmentally friendly extraction process using subcritical water (SCW) was developed for the recovery of pistachio hull phenolics. A total of 66 individual phenolic compounds were identified in the aqueous methanolic extracts in three different classes, namely gallotannins, flavonoids, and anacardic acids. The total amount of individual phenolics were ranged from 61.2 to 100.7 g/kg dry matter, DM for different varieties where anacardic acids predominated (64.6-80.4 per cent), followed by gallotannins (13.4-21.2 per cent), and flavonol glycosides (5.7-16.3 per cent). By using SCW extraction at 110-150 °C, extracts rich in gallic acid (22.2 g/kg DM), penta-*O*-galloyl-*β*-D-glucose (9.77 g/kg DM), and flavonols (4.37-5.65 g/kg DM) were obtained, while anacardic acids (up to 50.7 g/kg DM) were retained in the extraction residue. Moreover, SCW extracts showed superior antioxidant properties compared to those obtained for aqueous methanol extracts. In conclusion, pistachio hulls were shown to be a rich source of bioactive phenolic compounds.

ÖZET

ANTEPFISTIĞI ATIKLARININ FENOLİK ANTİOKSİDAN MADDE GERİ KAZANIMI AÇISINDAN DEĞERLENDİRİLMESİ

Antepfıstığı üretimi atıklarından antepfıstığı yumuşak kabuğu ticari değeri olmayan bir üründür. Bu çalışmanın amacı, antepfıstığı kabuğunu fenolik antioksidan maddelerin geri kazanımı açısından değerlendirilmesidir. Öncelikle, antepfıstığı kabuğunun fenolik madde içeriği yüksek performanslı sıvı kromatografisi-diode-array dedektör-elektrosprey iyonizasyon kütle spektrometresi (HPLC-DAD-ESI-MS^n) ve yüksek çözünürlüklü kütle spektrometresi (HR-MS) kullanılarak ayrıntılı olarak analiz edilmiştir. Çeşitli çözgen sistemleri ve ekstraksiyon parametreleri denendikten sonra antepfıstığı kabuğu fenoliklerinin eş zamanlı ayrımı ve kantitatif tayini için ultrasonik destekli ekstraksiyon metodu geliştirilmiştir. Ayrıca, antepfıstığı fenoliklerinin hızlı ayrımı için ultra-yüksek performanslı sıvı kromatografisi-diode-array dedektör-buharlaştırmalı ışık saçılması dedektörü (UHPLC-DAD-ESLD) kullanılarak yeni bir metot geliştirilmiş ve valide edilmiştir. Son olarak, antepfıstığı fenoliklerinin geri kazanımı için çevre dostu subkritik su ekstraksiyon yöntemi uygulanmıştır. Antepfıstığı kabuğu su bazlı metanol özütlerinde, başlıcaları gallotanin, flavonoit ve anakardik asit olmak üzere üzere toplam 66 adet fenolik madde belirlenmiştir. Farklı antepfıstığı varyetelerinden elde edilmiş kabukların toplam fenolik madde miktarı kuru madde bazında 61,2 ile 100,7 g/kg aralığında bulunmuştur. Antepfıstığı kabuğu fenolikleri çoğunluğu anakardik asit (64,6-80,4 yüzde) olarak üzere, gallotanin (13,4-21,2 yüzde), ve flavonol glikozitlerlerden (5,7-16,3 yüzde) oluşmaktadır. Subkritik su ekstraksiyonu ile 110 ve 150 °C aralığında gallik asit (22,2 g/kg kuru bazda), pentagalloglukozit (9,77 g/kg kuru bazda), ve flavonolce (4,37-5,65 g/kg kuru bazda) zengin özütler elde edilirken anakardik asitler ekstraksiyon kalıntısında tutulmuştur. Ayrıca, subkritik su özütlerinin antioksidan aktivitesi su bazlı metanol özütlerinden önemli miktarda fazla bulunmuştur. Sonuç olarak, antepfıstığı kabuğunun biyoaktif fenolik bileşenler açısından zengin olduğu tespit edilmiştir.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS/ABBREVIATIONS

ε	Dielectric constant
E_h	Redox potential
R_S	Peak resolution
S	Slope
σ	Standard deviation
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
AU	Absorbance unit
(11:0)-Anacardic acid	Undecylsalicylic acid
(13:0)-Anacardic acid	Tridecylsalicylic acid
(13:1)-Anacardic acid	Tridecenylsalicylic acid
(13:1)-Anacardic acid	Tridecadienylsalicylic acid
(15:0)-Anacardic acid	Pentadecylsalicylic acid
(15:1)-Anacardic acid	Pentadecenylsalicylic acid
(15:3)-Anacardic acid	Pentadecatrienylsalicylic acid
(17:0)-Anacardic acid	Heptadecylsalicylic acid
(17:1)-Anacardic acid	Heptadecenylsalicylic acid
(17:3)-Anacardic acid	Heptadecadienylsalicylic acid
APCI	Atmospheric-pressure chemical ionization
BEH	Ethylene-bridged-hybrid
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CatE	Catechin equivalents
CID	Collusion induced dissociation
cv.	Cultivar
CV	Coefficients of variation
Da	Dalton
DAD	Diode array detector

DM	Dry matter
ELS(D)	Evaporative light scattering (detector)
ESI	Electrospray ionization
ET	Electron transfer
FAO	Food and Agricultural Organisation of the United Nations
FAOstat	Food and Agricultural Organisation Corporate Statistical Database
FLD	Fluorescence detector
FRAP	Ferruc reducing antioxidant power
GAE	Gallic acid equivalents
GC	Gas chromatography
HAT	Hydrogen atom transfer
HHDP	Hexahydroxydiphenic acid
HPLC	High performance liquid chromatography
HR-MS	High resolution mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantitation
nd	not detected
NMR	Nuclear magnetic resonance
nq	not quantified
MALDI	Maser-assisted laser desorption and ionization
MS^n	Mass spectrometry at the n^{th} level
m/z	Mass-to-charge ratio
ORAC	Oxygen radical scavenging capacity
PDA	Photodiode array (detector)
QE	Quercetin equivalents
Res-SCW	Subcritical water extraction residue
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
SCW	Subcritical water
U(H)PLC	Ultra (high) performance liquid chromatography
UV/Vis	Ultraviole/visible

TAE	Tannic acid equivalents
TBHQ	Tert-buthylhydroquinone
TE	Trolox equivalents
TOF	Time-of-flight
tr	Traces

1. INTRODUCTION

Increased consumer awareness on nutrition and health has forced the industry to find new natural alternatives for synthetic food ingredients providing techno-functional and health-promoting properties [1, 2]. Agricultural and food processing by-products contain considerable amounts of bioactive compounds such as phenolic compounds possessing various potential biological activities such as antioxidant, and antimicrobial. They also offer a cheap source for these bioactive compounds, especially compared to the cost of their disposal and maintaining sustainability in the food industry [3]. However, they mostly have no or very low commercial value. Therefore, utilization of agricultural and food processing by-products for the recovery of bioactive compounds is an emerging research topic.

Pistachio (*Pistacia vera* L.) is an important commercial product with approximately one million tons of worldwide production each year by Iran as the top producer, followed by USA and Turkey [4]. It generates a significant amount of waste with no or low commercial values. However, pistachio belongs to the Anacardiaceae family [5], whose members such as mango [6–8], Brazilian pepper [9] and cashew [10] are characterized by the presence of diverse classes of phenolic compounds including flavonol glucosides, gallotannins and phenolic lipids. A limited number of previous studies also suggest that extracts derived from pistachio hull, the main pistachio processing by-product, exert antioxidant [11–14], antitumor [15], and anticancer [16] activities. However, the complete phenolic profile of pistachio hull remained incompletely investigated which also hamper its utilization. Therefore, the presented thesis aimed at providing a comprehensive and systematic overview of pistachio hull phenolics including their characterization and extraction. Additionally, sustainable utilization strategies should be investigated. Detailed aims and scope of this thesis were given in Section 2.9 after research topics involved are first described in the following sections (2.1-2.8).

2. THEORETICAL BACKGROUND

2.1. PISTACHIO

Pistachio (*P. vera* L.) is one of the oldest foods consumed. Its history dates back to as early as 7000 BC when it was cultivated for royal families and the court in the time of Babylon [17]. It is native to high desert regions of Iran, Afghanistan, and Central to Western Asia. It was later distributed to Mediterranean region of Middle East and Europa by trades. Pistachios were imported to America in the 1880s, and their cultivation was started after 1950s in California [18]. More recently, pistachio has also been cultivated in Australia [17]. Today, pistachio is produced in many different parts of world, i.e. Central Asia (China, Afghanistan, and Pakistan), Middle East (Syria, Iran), North Africa (Morocco, Tunisia, and Egypt), Europe (Italy, Spain, and Greece), America (USA, Mexico) and Australia [4, 19]. In 2014, 857 878 tons of pistachio kernel was produced in 826 523 ha field (Figure 2.1). Top producers are Iran (48 per cent), followed by USA (27 per cent), and Turkey (nine per cent) (Figure 2.2). Noteworthy, as seen in Figure 2.1, pistachio production has doubled during the last decade due to the increased consumer interest in pistachio as a healthy snack [20].

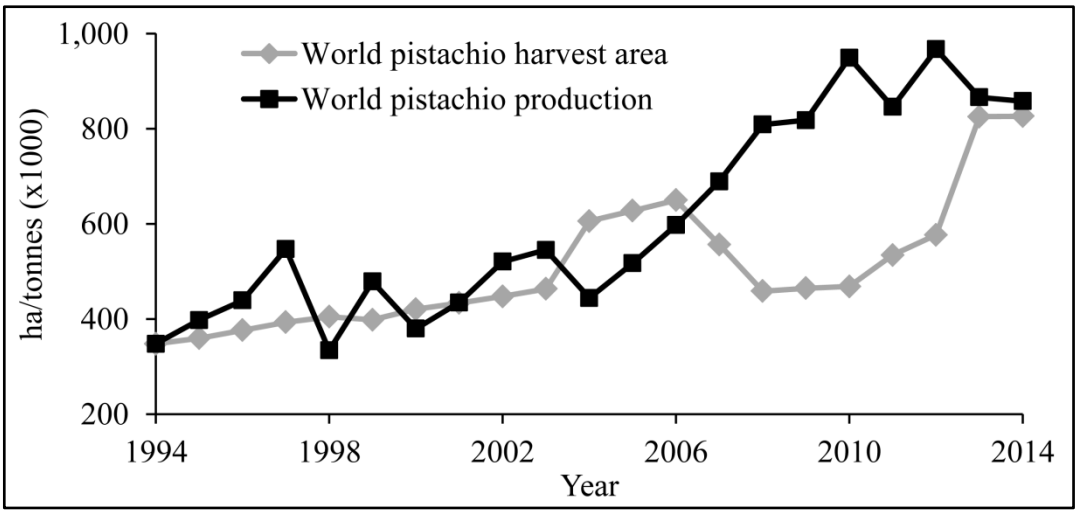

Figure 2.1. Amounts of world pistachio production and harvest area: Changes over years. Data were taken from FAOstat [4].

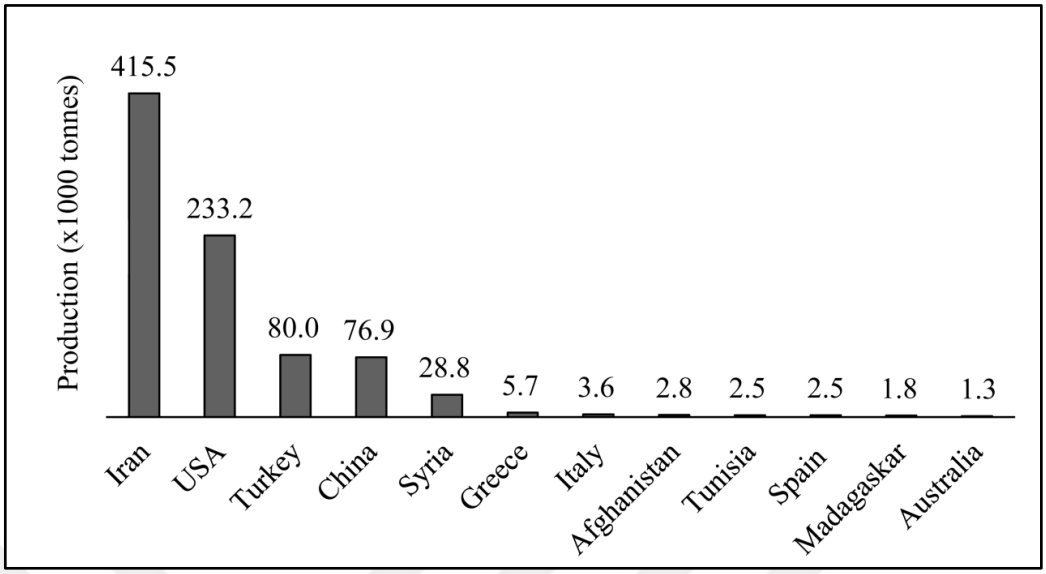

Figure 2.2. Pistachio production by country in 2014. Data were taken from FAOstat [4].

Pistachio belongs to the Anacardiaceae family, which also includes other commercially important plant products such as mango (*Magnifera indica* L.), cashew (*Anacardium occidentale* L.), Brazilian pepper (*Schinus terebinthifolius* Raddi), and sumac (*Rhus* L.). *P. vera* L. is the only specie that produces edible nuts, and thus, have commercial importance among 11 species of genus *Pistacia*. Other species of pistachio such as *P. atlantica* and *P. terebinthus* are grown as rootstock or for ornamental purposes [21].

Pistachio is a dicotyledonous plant grown on a tree reaching significant production in almost 10 years. Pistachio trees are dioecious with male and female flowers borne in different trees and pistachios are wind-pollinated [17]. Pistachio drupe is a fruit of pistachio tree consisting of a green kernel, a red colored skin, a lignocellulosic hard shell, and green to red hull (Figure 2.3) [22].

2.2. PISTACHIO PROCESSING

Pistachios are grown in the tree as grape-like bunches. They are harvested manually or mechanically when the fruit reaches the harvest maturity [23]. Based on the growth location and variety, pistachios reach their full maturity at different times, generally between June and October, when kernels start to grow, and shells harden and split-open [17].

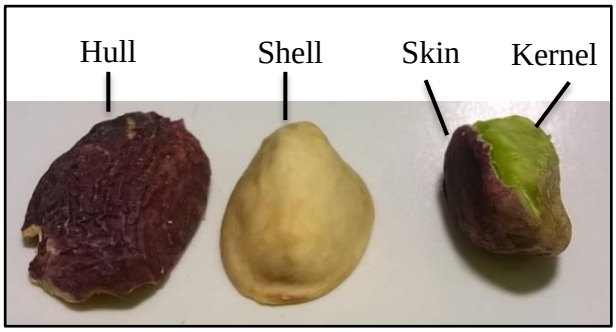

Figure 2.3. Different parts of dried pistachio drupe.

Harvested fruits are either processed directly or dried and stored until they are processed. Otherwise, when undried drupes are stored, shell staining might occur due to hull trapped moisture which is undesirable due to cosmetic reasons, and might indicate pathogenic infestation [18]. During drying, the moisture content of pistachio fruit is decreased from 40-50 per cent to three-five per cent, mostly by sun-drying in the field or in steam ovens with forced air [23].

Pistachios are mainly processed to obtain two different end-products; (i) in-shell pistachios, or (i) pistachio kernels. Ninety percent of pistachios are processed as in-shell pistachios, and marketed as snack food after salting and roasting. Remaining small amounts of pistachios are processed to obtained pistachio kernels, and used as nut meat in confectionery products such as pastries, and ice-cream to enhance their taste, color, flavor and nutritional value [23].

In-shell pistachio production briefly contains dehulling, shell splitting, and optionally roasting steps (Figure 2.4). Kernel production requires additional steps for the removal of shells and skins. In dehulling and skin removal steps, pistachios are optionally immersed in water to soften hulls or skins. Then, the outer layer of pistachios is crushed in the stone rollers, followed by separation of nuts from hull or skins using fanning mill [24]. After hull and/or skin removal, pistachios are dried to prevent microbial deterioration in the product. In the case of unshelling, pistachio shells are cracked manually using hammers or by machines to separate shells [23].

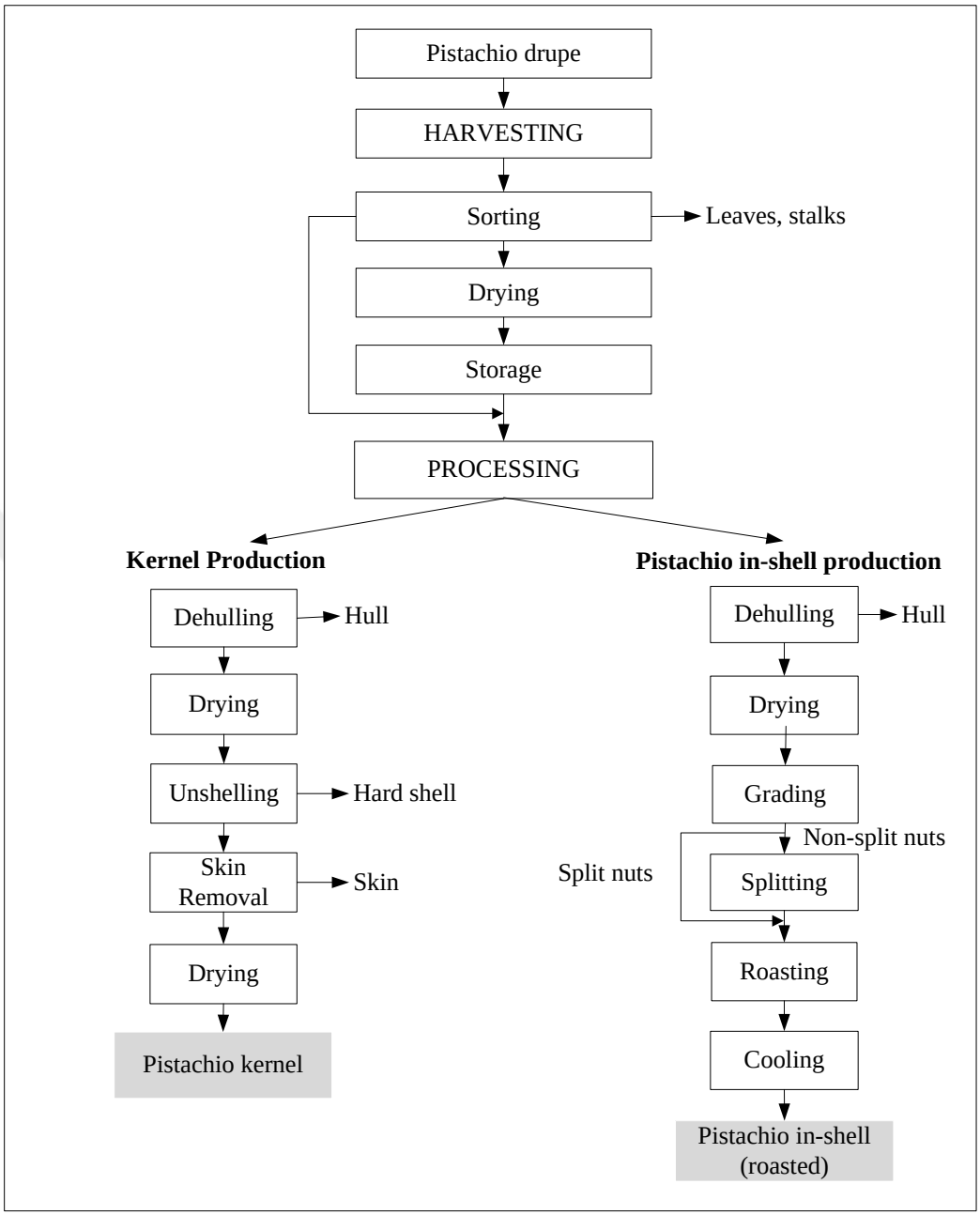

Figure 2.4. Overview of pistachio processing. Adapted from [17].

2.3. UTILIZATION POTENTIAL OF PISTACHIO BY-PRODUCTS

Pistachio processing by-products include hull, shell, and skin in variable portions depending on the final pistachio product. Pistachio hull is the main by-product of pistachio processing considering dehulling step is applied during the processing of all pistachios regardless of the

desired final product (Figure 2.4). Pistachio hull is a highly underutilized resource, however, with great potential. According to the study of Bartzas et al. on life cycle assessment of pistachio production in Greece, pistachio hulls valued as 3290 kg/ha are lost during post-harvest handling and 80 per cent of them are dumped on a farm or illegally disposed while only 20 per cent is used as animal feed [25]. Accordingly, using pistachio by-products (mainly hulls) as an animal feed supplement is the main activity for their utilization in Iran, the largest producer of pistachio [26–28]. Another possibility might be using pistachio hulls as compost material for pistachio seedlings or other plants [11, 12]. However, using pistachio hulls as an animal feed or compost material are limited to maximum 15 per cent of the diet of animal [26], or 10 per cent of compost/soil ratio [30], respectively, due to the presence of anti-nutritional phenolic compounds. Therefore, removal of the phenolic fraction is required to increase utilization potential of pistachio hull in these fields. Besides, a great research interest was recently given on pistachio hull phenolics aiming their use as a natural antioxidant and functional ingredients as further discussed in Section 2.5 [11, 14, 31–34].

Pistachio hull was also offered as an adsorption material for the removal of contaminants, i.e. cyanide, from industrial wastewater [35], or as a natural biomass resource for the production of bio-oil [36]. However, these studies remain only as scientific research.

2.4. PHENOLIC COMPOUNDS

2.4.1. Chemistry and Sources

Phenolics are at least one phenolic ring bearing molecules with many hydroxyl groups and various molecular attachments (Figure 2.5). They widely range from low molecular weight (<500 Da) simple molecules such as gallic acid and flavonoids to polymeric tannins (up to MW=300 000 Da) [37]. More than 8000 naturally occurring phenolic compounds were identified throughout the plant kingdom [38].

The main phenolic groups are phenolic acids, polyphenolics, stilbenes, and lignans [38]. Phenolic lipids are also considered as a subgroup of phenolics in the scope of this thesis due to their phenolic bearing structures. A more detailed classification leads separation of phenolic compounds into various subclasses (Figure 2.6).

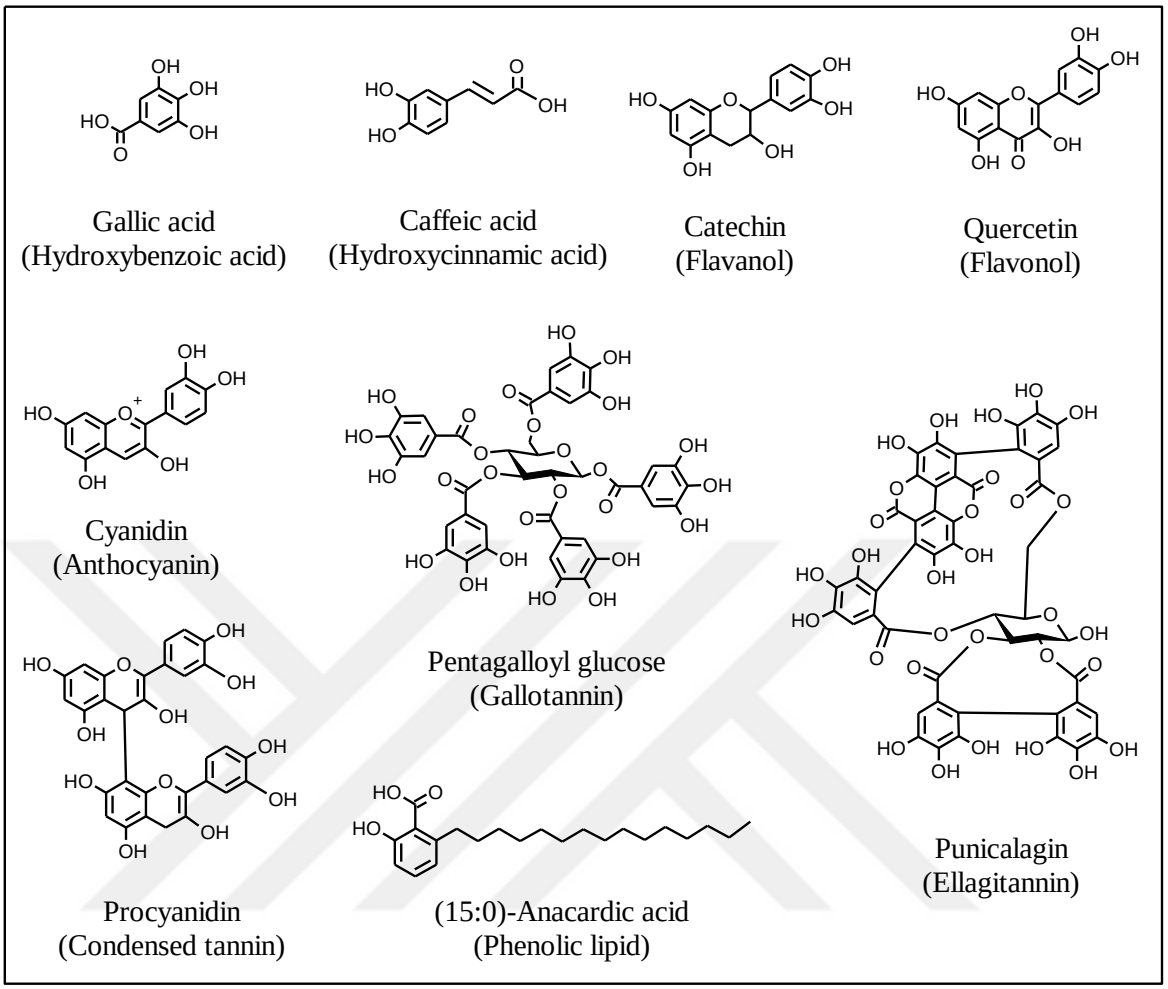

Figure 2.5. Chemical structures of some selected phenolics.

Phenolic acids consist of two subgroups of hydroxybenzoic and hydroxycinnamic acids. Hydroxybenzoic acids are molecules with C_6-C_1 structures such as gallic acid (Figure 2.5), ellagic acid, and protocatechuic acid. Hydroxybenzoic acids present in various fruits and vegetables, for instance, in tea, berries, and grapes. Hydroxycinnamic acids are C_6-C_3 structures with a three-carbon side chain (Figure 2.5). Coffee and blueberry are a rich sources of hydroxycinnamic acids such as ferulic, sinapic, *p*-coumaric and caffeic acid.

Polyphenolics are chemical compounds containing at least two aromatic rings. The most widely distributed polyphenolic class, flavonoids possess C_6-C_3-C_6 skeleton, two aromatic rings connected by a carbon bridge (Figure 2.5) [38]. Six sub-classes of flavonoids are flavones, flavanones, isoflavones, flavanols, flavonols, and anthocyanidins. Variations occur

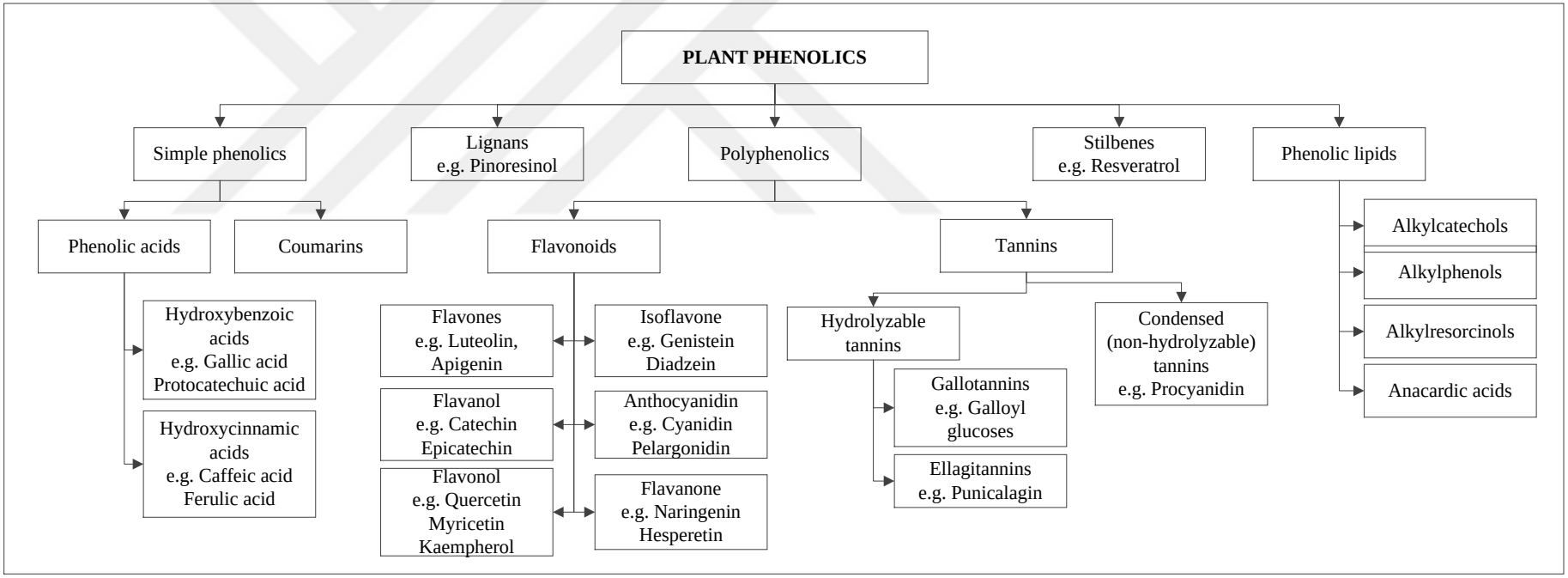

Figure 2.6. Classification of major plant phenolics. Adapted from [38–41]

in each group due to the differences in the number and arrangement of hydroxyl groups and with hydroxylation, methylation, glycosylation, acylation, and sulfonation of the structure [42]. Flavones such as luteolin, apigenin are abundantly found in parsley and celery. Green tea and grapes are rich in flavanols such as catechins while flavonols such as quercetin, myricetin, and kaempferol are ubiquitously found in many fruits and vegetables, such as onion, cranberry, chokeberry, and plums. Isoflavones such as daidzein and genistein are abundant in soy-based products. Anthocyanidins are coloring components giving blue, purple, and red color of many fruits such as black carrot, grapes, and radish. Naringenin and hesperitin are the main flavanones present in citrus fruits [38].

Tannins are polymers of phenolic compounds formed after various degrees of esterification and oxidative coupling of different phenolic moieties [43]. Based on the chemical structure and biosynthetic pathway (Figure 2.7), tannins are further divided into two subclasses: hydrolyzable tannins which are the polyesters of gallic acid with a core molecule, generally glucose, and condensed tannins (*syn*. proanthocyanidins) with flavonoid basis [44]. The most distinct property of tannins differing from other phenolic compounds is their ability to precipitate proteins and alkaloids [40]. Due to these properties, tannins are historically used as a tanning agent in the leather industry. Tannins were also used for the production of dyes and inks, or as medicine in traditional folk medicine, particularly in East Asia, as they are pharmacologically active components [45]. Tannins provide the taste of astringency, and a sense of dryness in many food and beverages such as red wine, tea and unripe fruits [43].

Hydrolyzable tannins are polyols of phenolic acids such as gallic acid or HHDP (hexahydroxydiphenic acid) with a core non-phenolic molecule. They can be fractionated to their components hydrolytically in the presence of hot water or tannase, and further separated into two classes as gallotannins and ellagitannins [40].

Gallotannins have galloyl units or their depsidic derivatives bound to a polyol sugar, mostly glucose, or less commonly organic acids such as quinic or shikimic acids [40]. Pentagalloyl glucose, one of the simplest gallotannin with five galloyl unit ester bonded to glucose, serves as a template for the formation of higher molecular weight tannins up to 12 galloyl groups [43]. Gallotannins are restricted to a small number of plant families including Anacardiaceae, and therefore, their presence is relatively rare in dietary sources [43].

However, they are the main source of commercial tannic acid used as a clarification agent in beer and fruit juice production [46].

Ellagitannins are formed by the oxidative linkage of two galloyl residues within the pentagalloyl glucose structure to form HHDP moiety. Characteristically, hydrolysis of HHDP moiety yields free hexahydroxydiphenolic acid, which is then transformed to ellagic acid spontaneously [43]. Furthermore, ellagitannins can form C-C and C-O linkages between different HHDP residues, and therefore, form highly polymerized structures [47]. Ellagitannins are more widespread in plant family such as pomegranates, walnut, and berries [48]. Punicalagin, present in pomegranate, is an example for ellagitannins (Figure 2.5).

Condensed tannins are oligomeric or polymeric proanthocyanidins with flavonol units linked with the inter-flavonoid bond. They are non-hydrolysable due to the C-C coupling of flavonol subunits [40]. However, in alcoholic solutions of strong minerals acids, condensed tannins can be broken down to their corresponding anthocyanins [43]. Condensed tannins are called procyanidin when catechin and epicatechin monomers are coupled, while prodelphinidin is a polymer of gallocatechin and epigallocatechin [37]. Condensed tannins are naturally present in cocoa beans, red wine, fruits, nuts, chocolate, and legumes [49].

Lignans are dimers of two phenylpropane units. The main dietary source is linseed containing secoisolariciresinol [50].

Stilbenes consist of two phenyl moieties connected by a two-carbon methylene bridge. Resveratrol, the main representative of this group, present in grape, wine, berries [38].

Phenolic lipids are amphiphilic molecules composed of a single phenolic ring attached with an alkyl side-chain [41]. They are hardly soluble in water [51]. They are subdivided into four classes, namely alkylphenols, alkyl catechols, alkylresorcinols, and anacardic acids where the latest two classes of compounds are a part of human diet due to their presence in some grains and fruits. Alkylresorcinols, in other terms resorcinol lipids, are an important component of various plants such as mango, rye, wheat [52]. They are present as a mixture of homologs possessing side chains of 13 to 27 carbons with varying degrees of saturation [41]. Anacardic acids are differentiated from alkylresorcinols by the addition of a carboxylic acid group to the phenol ring. Anacardic acids occur only in free form and present in few plants such as Anacardiaceae family and *Ginkgo biloba* [53]. Alkycatechols such as cardol

and cardanol are oxidation products of anacardic acids. Cashew nutshell is the main source of anacardic acids, alkyl catechols (cardol, cardanol), and alkylphenols [54].

2.4.2. Biosynthesis

Biosynthesis of phenolic compounds in plants relies on shikimate and polyketide (acetate) pathways with precursors originating from carbohydrate metabolism (Figure 2.7) [55]. Cinnamic acid derivatives are synthesized by shikimate pathway and derived from phenylalanine and/or tyrosine. Various different flavonoids are further synthesised from cinnamic acid after inclusion of malonic acid [38].

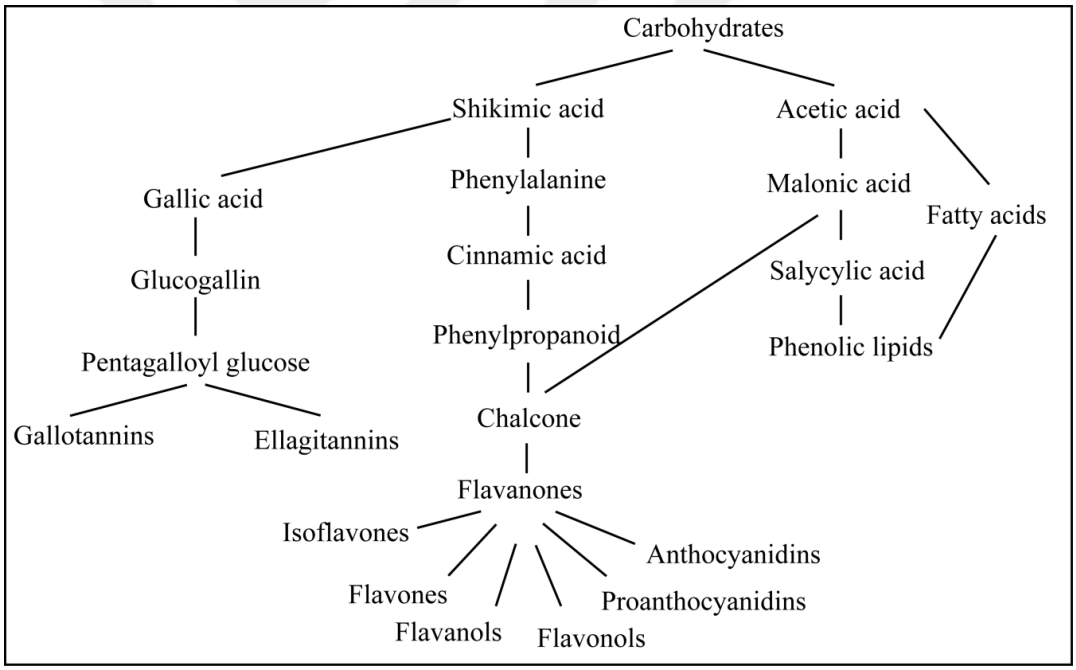

Figure 2.7. A brief overview of biosynthesis pathways for the gallotannins, flavonoids, and phenolic lipids [38, 47, 55, 56].

Gallic acid is directly synthesized by the desaturation of shikimic acid or oxidation of cinnamic acids. For the biosynthesis of gallotannins, gallic acid is first esterified to glucogallin, which is the first intermediate in the gallotannin synthesis, utilizing UDP-

glucose. Glucogallin (monogalloyl glucose) is further galloylated in a strictly position-specific manner up to pentagalloyl glucose [47]. Complex gallotannins with higher galloylation degree than pentagalloyl glucose are synthesized by the addition of galloyl units from glucogallin to pentagalloyl glucose with *meta*-depside bonds [44].

Distinctly, phenolic lipids are biosynthesized *via* the polyketide pathway. In anacardic acid biosynthesis, alkyl chains are derived from fatty acids while salicylic acid moiety is synthesized through polyketide pathway from malonic acid [56].

2.4.3. Antioxidant and Health Properties

Antioxidants are substances that significantly delay, or inhibit oxidation of a substrate [57]. Many plant-derived compounds such as phenolics exert antioxidant properties by scavenging reactive oxygen and nitrogen species (ROS/RNS) before they attach biological matrix, or by preventing the formation of ROS/RNS by, for instance, reducing hydrogen peroxide, capturing metal ions, or inhibiting specific enzymes responsible from free radical generation [59]. Therefore, phenolic antioxidants are important as they promote health by decreasing oxidative stress in living tissues, and prevent or decrease the incidence of oxidative stress-related diseases [58]. Numerous epidemiological studies suggested that diet containing fruits and vegetables rich in phenolic compounds are related to the reduced risk of many cancers [59]. Phenolic antioxidants are also important for food processing as they are suggested to be a natural alternative to the currently used synthetic antioxidants for food preservation, or for the production of functional foods exerting specific health benefits related to phenolic compounds [59, 60].

2.5. PISTACHIO HULL AS A SOURCE OF PHENOLIC ANTIOXIDANTS

2.5.1. Phenolic Composition of Pistachio Hull

Phenolic composition of pistachio hull was demonstrated to include various phenolic components including flavonoids, flavonols, and tannins as determined by spectrophotometric based assays [12–14, 31, 61, 62]. Total phenolic content of pistachio hull

was estimated between 0.01 and 43 mg GAE (Gallic acid equivalents) /g [54, 55] while total flavonoid, tannin and flavonol contents were reported as 0.688 mg QE (Quercetin equivalents) /g [11], 5-34 mg TAE (Tannic acid equivalents) /g [13], and 0.071 CatE (Catechin equivalents) /g [11], respectively. Individual phenolic compounds present in pistachio hull were shown to include various phenolic acids such as gallic, protocatechuic, hydroxybenzoic, *p*-coumaric, and vanillic acids, and flavonoids (quercetin glycosides, isorhamnetin glycosides, naringenin, eriodictyol, and catechin) [11, 12, 32]. However, recent studies suggested that pistachio hull contains a wider range of phenolic compounds including differently galloylated gallic acid derivatives, namely gallotannins, flavonols, and anacardic acids [22]. For instance, gallotannin content of pistachio hulls was recently investigated in detail in the scope of this thesis (Sections 3-4) [22, 63]. In these studies, various gallic acid esters of glucose, shikimic and quinic acids were shown to constitute 11-21 g/kg of pistachio hull. Although anacardic acid composition of pistachio hull was determined using GC and NMR more than three decades ago [64], this class of compounds did not get much attention until recently they were investigated again [22, 31, 63]. It was showed that anacardic acids are an important component of pistachio hull (39.5-73.8 mg/kg dry matter, DM), and constitute more than 65 per cent pistachio hull phenolics extracted with aqueous methanol [63].

2.5.2. Antioxidant and Health Effects of Pistachio Hull Extracts

A number of studies suggested that pistachio hull extracts are effective antioxidants that might be a natural alternative to commonly used synthetic ones such as BHA, BHT, and TBHQ [11, 14, 31–34]. For instance, aqueous pistachio hull extracts were shown to inhibit 76.5 per cent of DPPH radicals, while only 70.7 per cent and 55.9 per cent inhibition were observed when using TBHQ and BHT, respectively [14]. Moreover, methanol- and water-based pistachio hull extracts retarded lipid oxidation in soybean oil-based model system in a similar level to that achieved when BHA and BHT were used [13]. In another study, aqueous pistachio hull extracts were shown to be effective to inhibit linoleic acid oxidation and the formation of volatile organic compounds, and conjugated diene hydroperoxides in *β*-carotene-linoleic acid model system [14]. Recently, antioxidant activity of pistachio hull extracts was tested in real food systems [33, 34]. The quality of chicken burgers was

improved in terms of cooking properties and lipid peroxidation levels when they were treated with pistachio hull extracts [34]. In another study, oxidative stability was improved in fish fillets obtained from fishes fed by pistachio hull supplemented diet [33].

Pistachio hull derived products have already been used in Iranian traditional culture for medicinal purposes [65, 66]. The potential use of pistachio hull extracts for improving health status was also supported by a limited number of scientific literature where pistachio hull extracts showed radical scavenging activity against highly reactive superoxide anions [11], inhibit formation of reactive nitrogen and oxygen species in macrophage model system [31], and reduce lipid peroxidation levels in erythrocyte membranes and human peripheral blood lymphocytes [11]. Furthermore, aqueous pistachio hull extracts were shown to reduce liver cancer cell viability, and induce apoptosis by regulating apoptosis-related genes [67].

Pistachio hull was recently suggested to be a good therapeutic alternative for such diseases where available drug therapies are based on inhibition of specific enzymes; for instance, acetylcholinesterase, tyrosinase, and α-amylase in Alzheimer's disease, diabetes mellitus, and skin disorders, respectively [32].

In conclusion, pistachio hull extracts were shown to exert various biological activities, however mostly attributed to their phenolic composition.

2.5.3. Antioxidant and Health Effects of Pistachio Hull Phenolics

2.5.3.1. Gallic acid and gallotannins

Scientific research on the biological activity of gallic acid has a long history dates back to 90s when bioactivity of green tea was attributed to the galloylated catechin [68]. Since then, various biologically relevant activities were evidenced for gallic acid such as anti-inflammatory, antimutagenic, antiallergenic, antimicrobial, anticancer, antitumor, hepatoprotective, and neuroprotective activities [68–71]. The most pronounced biological activity of gallic acid is related to its radical scavenging activity corresponding to its molecular structure consisting of an aromatic ring and three available hydroxyl groups for hydrogen or electron donations [72]. Therefore, the presence of galloylated units in a

phenolic compound leads to the improved antioxidant activities compared to their nongalloylated parent molecules [68]. Accordingly, gallotannins with several galloyl groups are suggested to be 15-30 times more effective antioxidants than smaller phenolics, and Trolox, due to the increased number of hydroxyl groups in gallotannin molecule [73–76]. However, improvements on the level of antioxidant activity depend on the number and position of galloyl groups in a gallotannin molecule and restricted to a certain molecule size due to increase in hydrophobicity and steric hindrance in high molecular sizes [76]. Pentagalloyl glucose, a molecule with five galloyl groups attached to a glucose core, is one of the most studied gallotannin in the scientific literature for its health-promoting properties due to its less complex structure compared to higher gallotannins, and availability [77]. In these studies, pentagalloyl glucose was reported to reduce intracellular oxidative stress induced by carcinogens, and exhibited anticancer effect *in vitro* and *in vivo* against various cancer types such as prostate [78], lung [79], breast [80], and liver [81] by inhibiting the growth and/or invasion of cancer cells. Some of the other biological activities attributed to pentagalloyl glucose are anti-inflammatory [82], anti-allergy [83], anti-convulsion [84], anti-kidney stone formation, antivirus, antibacterial, radioprotective activities [76]. Researchers on the bioactivity of other gallotannins are rather limited due to their limited availability in pure form, and difficulties of tannin analysis [77, 85]. For instance, tannic acid, a commercially available gallotannin, is a mixture of differently polymerized gallotannins and its composition is poorly defined. However, numerous epidemiological studies suggested that consumption of fruits and vegetables containing hydrolyzable tannins is correlated with improved health status [37, 86]. Moreover, the interaction between gallotannins and proteins was claimed to be the reason of the therapeutical potential of certain herbal medicines [73]. Therefore, tannins are suggested for the treatment of diabetes [87] or Alzheimer's disease [88, 89] due to their ability to inhibit enzymes having the main function in respective disease, namely α-glucosidase and cholinesterase, respectively. Apart from their promising biological potential, gallotannins are also considered as anti-nutritional factors as they reduce protein bioavailability, and might exert toxic activities at high levels [90]. Therefore, their safety is under consideration for their use as a pharmaceutical product.

2.5.3.2. Flavonoids

Flavonoids are the main focus of polyphenolic research due to their prevalence in fruits and vegetables, and thus, being an integral part of human diet. As in the case of "French paradox" or Mediterranean diet, various health-related benefits were attributed to the diet rich in flavonoids containing fruits and vegetables [38, 39, 50].

Flavonoids are strong antioxidant molecules due to their capacity to donate hydrogen atom or electron, and their low redox potential ($0.23<E_h<0.75$ V) compared to that of highly active free radicals having high redox potential (1.0-2.13 V) [91]. In addition, flavonoids effectively chelate metal ions which are potential enhancers for the formation of reactive oxygen species, and inhibitors of enzymes responsible for the formation of superoxide anions [92].

Flavonol derivatives such as quercetin, myricetin and kaempferol, one of the most abundant sub-class of flavonoids, have attracted great attention due to the number of reported biological activities, including antioxidant, antimicrobial, anti-inflammatory, antiobesity, neuroprotective activities, and even the prevention of cardiovascular disease, diabetes, chronically high cholesterol levels, and some kinds of cancers [38, 93–96].

Quercetin is one of the most potent antioxidants due to its flavonoid based structure containing an *o*-dihydroxy structure in the B ring, two, three-double bond in the C ring, and hydroxyl groups at three and five positions in the A ring (Figure 2.8) [93]. With these structural features, quercetin can provide hydrogen or electron to reduce free radical, and later, stabilize itself by molecular resonance and electron delocalization within the aromatic structure [93]. Therefore, glucose addition to one of the hydroxyl groups was shown to reduce radical scavenging properties of quercetin [96]. However, galloyl group addition such as isoquercetin gallate was shown to increase radical scavenging activity compared to that of quercetin aglycone [96].

Among all flavonoids, anthocyanins are probably the most attractive group due to their coloring properties ranging from blue to red [97].

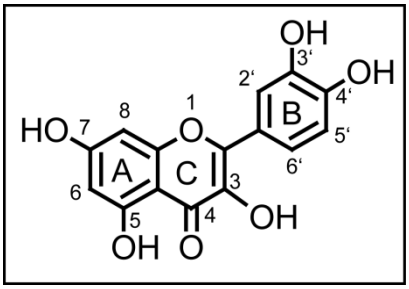

Figure 2.8. Quercetin structure.

2.5.3.3. Anacardic acids

Anacardic acids are biologically active amphiphilic molecules with alkyl side chain attached to a gallic acid moiety. Their main biological activities are related to their ability to inhibit prooxidant enzymes and chelate metal ions to prevent generation of reactive oxygen species, however, without showing radical scavenging activity [98]. Anacardic acids were reported to be efficient lipoxygenase inhibitors [99, 100], and therefore, prevent lipoxygenase associated low-density lipoprotein oxidation in the human body [100], and lipid oxidation in food systems such as egg yolk [101]. They are also suggested to be used as pharmaceutical agents due to their strong antioxidant, anticancer and anti-inflammatory activities [15, 41, 102, 103].

Due to their amphiphilic structure containing both hydrophilic phenolic acid moiety and hydrophobic alkyl side chain, anacardic acids are able to interact with biological membranes and can be incorporated into phospholipid bilayers in living cells to form stable monolayer membranes [41, 55]. The presence of alkyl side chain and ability to close enzyme active site by these hydrophobic tails is considered as the main reason for enzyme inhibitory activities of anacardic acids [99]. Therefore, alky side chain was suggested to have an important function for the biological activity of anacardic acids [98, 100]. However, apart from their biological activities, anacardic acids are suspected to induce allergenic and sensitizing reactions in human skin after contact [102], and they are believed to be the main cause of cashew allergy [104].

2.6. EXTRACTION OF PHENOLIC COMPOUNDS

2.6.1. Extraction Parameters

Complete extraction is a critical step for accurate analysis of phenolic compounds present in a plant material. Optimum conditions for the extraction of phenolic compounds may vary depending on the plant matrix and type of phenolic to be extracted and should be reconsidered for each unique plant material [105, 106]. Some of the several factors affecting extraction efficiency are the solvent type, extraction time, extraction temperate, sample-to-solvent ratio, and a number of repeated extractions [106].

The choice of extraction solvent is the first parameter to be considered. Several solvent systems with a range of polarity were proposed before for the extraction of different phenolic compounds, e.g. anacardic acids, gallotannins, and flavonoids, separately, from several sources such as mango [107–109], cashew nut [54], cashew nut-shell liquid [110], and apple [111]. In these studies, flavonoids, and gallotannins are generally extracted with polar solvents such as water or more commonly aqueous solutions of alcohols such as methanol and ethanol, or acetone [112, 113]. On the other hand, nonpolar solvents such as ethyl acetate [64], diethyl ether [114], or petroleum ether [115] were used for the extraction of anacardic acids due to their amphiphilic nature with a nonpolar lipophilic side chain, thus their higher solubility in nonpolar solvents. Other solvents such as dichloromethane [108, 116], acetone, methanol and ethyl acetate [117] were also purposed for the extraction of anacardic acid analogue molecules, alkylresorcinols. Diethyl ether and ethyl acetate are commonly used organic solvents for liquid-liquid separation and purification of phenolic acids, flavonoid aglycones, and glycosides from acidified aqueous crude extracts [118]. Therefore, selecting the appropriate combination of extraction solvent aiming exhaustive and quantitative recovery of phenolic compounds from plant matrices, i.e. pistachio hull containing a diverse type of phenolic compounds such as hydrophilic gallic acid derivatives to hydrophobic anacardic acids might be a challenge and should be investigated in detail.

Solvent acidity is another factor as generally small amounts of acids are added to increase extraction yields by disrupting plant matrix [106] as well as to diminish extraction of nonpolar compounds [119]. Solvent acidity also affects the stability of phenolic compounds

as they are relatively more stable at acidic conditions while alkaline conditions cause rapid degradation [106, 120]. Acidic conditions can also suppress some other physicochemical changes, for instance, methanolysis of gallotannins dominated in methanolic neutral conditions which cause the formation of their degradation products and methyl gallate/digallate [121, 122]. Mineral acids such as HCl in low concentrations (0.1-one per cent) or more preferably organic acids such as formic acid and acetic acid in the concentration range of one-to-five per cent are commonly used agents for solvent acidification for phenolic extraction [106].

Extraction temperature is one of the important factors largely affecting extraction efficiency. Increased extraction temperatures generally promote solubility of analytes and mass transfer rates while the rate of degradation and oxidation reactions might increase [57]. Therefore, optimum temperature range should be determined to obtain maximum extraction yields for the targeted analytes. When the target is free phenolic compounds, extraction temperatures between 20 and 50 °C are commonly used, because higher temperatures might cause degradation of phenolics, especially heat labile ones such as anthocyanins [106]. However, temperature related degradation of some phenolic compounds, such as gallotannins, might be desirable. For instance, hydrolysis of higher molecular weight gallotannins of witch hazel (*Hamamelis virginiana* L.), a tannin-rich medicinal plant, at 100 °C for 120 min was shown to produce extracts richer in gallic acid, and penta-*O*-galloyl-*β*-D-glucose compared to those obtained in non-heat-treated extracts [123]. Temperature-related degradation of gallotannins might also facilitate their analysis. Current methodologies for tannin analysis are mostly based on unspecific colorimetric assays as their structural diversity limits their separation by chromatographic methods [124]. Hydrolysis of higher gallotannins containing more than five galloyl units to yield penta-*O*-galloyl-*β*-D-glucose and methyl gallate during Soxhlet methanol extraction was proposed as an alternative for the quantification of mango kernels gallotannins to enable standardization of extracts [125, 126]. Temperature might also facilitate the release bound phenolics such as ferulic acid, and caffeic acid from matrix components, such as lignin [127]. Moreover, recent studies show that temperature related degradation of phenolic compounds might lead to the formation of phenolic/non-phenolic compounds having higher antioxidant activity [128]. Therefore, modification of phenolic composition by adjusting temperature might provide formation of novel extracts with higher biological activity.

Extraction efficiency is also influenced by the extraction time which affects solubilization of phenolics. However, longer times might lead to degradation of phenolics. Therefore extraction time and its combination with extraction temperature are critical and should be adjusted to give maximum extraction yields without leading oxidation or degradation of target analytes [57].

The sample-to-solvent ratio is directly related to the extraction yields. Yields increased with increasing sample-to-solvent ratio. Commonly, ratios of 10 to 50 are used. Higher solvent ratios may lead sample dilution and require longer evaporation times while a lower ratio may limit extraction due to saturation of solvent [106].

Extraction efficiency is also influenced by the repeated extraction procedure. Repeated extraction cycles are also preferred due to the minimization of oxidation and degradation of phenolics due to exposure to long extraction times and temperatures [106].

In conclusion, each parameter described above should be considered for the development of an extraction method giving optimum extraction yields.

2.6.2. Extraction Techniques

Conversion of by-products to high-value-added ingredients requires at first an extraction step of the target compounds from the plant matrix with a suitable technique [3]. Conventional solvent extraction technologies such as stirring, shaking, soaking, and maceration generally require long processing times using toxic organic solvents and suffer from low efficiency and lack of selectivity. Even sometimes harsh acidic or basic treatments may need to be applied to increase process efficiency and exhaustively recover both free and bound phenolic compounds [129]. At the end, expended toxic solvents, and toxic solvent-contaminated spent residues further contribute to the disposal problem. Similarly, recent studies regarding the pistachio hull phenolics focused on solvent based conventional extraction, *e.g.* aqueous methanol, due to its high yields [11, 31, 63]. Thus, there is still need for new and rapid extraction techniques to maximize phenolic yields and selectivity with little environmental effect. A number of novel extraction methods have been proposed for the extraction of target compounds from various matrices, including ultrasound-assisted extraction, sub/supercritical fluid extraction, microwave extraction etc.

2.6.2.1. Ultrasound-assisted extraction

Ultrasound-assisted extraction is a promising novel extraction technique that is considered as green and economically viable technology due to the requirements of less solvent and extraction time compared to those required for conventional methods [130]. Thereby, research for ultrasound application in food applications increased in terms of food processing, preservation or extraction [131].

Ultrasound-assisted extraction is a process where ultrasound waves having a frequency between audible (>20 kHz) and microwave (<10 MHz) range are used at either low (<one W/cm^2) or high (10-1000 W/cm^2) intensity range [130]. High-intensity sonication is employed for the ultrasound-assisted extraction of plants while low-intensity sonication finds application as a non-destructive analytical technique in, for instance, process quality control [130]. Acoustic cavitation, which refers to bubble formation, growth, and their rapid collapse during the propagation of ultrasound waves in a liquid medium is the main driving force in ultrasound-assisted extraction [131, 132]. Rapid formation and collapse of bubbles in a liquid medium by high-intensity ultrasound waves create local high temperature (up to 5000 K) and pressure (up to 1000 MPa) points, where intense matrix disruption occurs, and increased diffusion rates obtained by macro-turbulence and micro-mixing forces [130, 131]. At the end, enhanced extraction yields with minimal effect on the quality of end product were accomplished using ultrasound-assisted extraction, a relatively fast and easy to use a method which requires relatively low investment cost compared to other novel extraction methods [130].

Focusing on the extraction of phenolic compounds, ultrasound-assisted extraction has already been investigated for the extraction of phenolic and antioxidant compounds from various sources such as mango peel [8], black carrot [133], pomegranate peel [134], grape seeds [135], citrus peel [136], apple pomace [137], wheat bran [138], where enhanced analyte release, and thus, better extraction yields were obtained compared to conventional extraction methods. For instance, higher amount of alkylresorcinols (426 mg/kg) were obtained from rye bran after ultrasound-assisted extraction in 45 s while it was only 382 mg/kg after stirring for one h [8]. In another study, higher phenolic yields (14.8 per cent)

were obtained from pomegranate peels using ultrasound-assisted extraction in a shorter time (six min) compared to that obtained by stirring method (12 per cent) for 60 min [134].

Different ultrasound applications are distinguished, namely direct application of ultrasound using probe-type ultrasonic equipment, or indirect application by submerging in an ultrasound bath [131]. Bath type ultrasonic devices are commonly used for indirect ultrasound application since they are cheap and largely available. However, nowadays, probe-type ultrasonic equipments are preferred for extraction purposes as they are powerful, effective through the small surfaces, and provide better reproducibility compared to bath-type ultrasonic devices [133]. Combined processes such as ultrasounds assisted Soxhlet extraction, ultrasound-assisted Clevenger distillation, or coupling ultrasound with microwave and supercritical fluid extraction are also possible [135]. Although using ultrasound-assisted extraction for analytical purposes is very common as a sample preparation step prior to phenolic analysis, it is also of great interest to industry [131, 136].

2.6.2.2. Subcritical water extraction

Subcritical water (SCW), also called hot pressurized, superheated or pressurized low polarity water, extraction is an emerging environmentally-friendly separation technique that uses just water, a non-toxic and readily available solvent, in its subcritical state to extracts compounds with various polarities from mid-polar to nonpolar. Water is considered to be in its subcritical state above its boiling point (100 °C) and below its supercritical state (374 °C) under sufficient pressure to maintain it in a liquid state (Figure 2.9).

Although water is a highly polar solvent with an elevated dielectric constant (ε=80) at room temperature, its dielectric constant decreases dramatically under subcritical conditions (e.g. ε=30 at 220 °C) due to the weakening of hydrogen bonding, and reaches values similar to those of organic solvents, for instance, to ε=33 at room temperature for methanol [141]. Besides, the polarity and solvent power of SCW can be adjusted by changing temperature to extract selectively polar, mid-polar or nonpolar molecules due to the dependence of its dielectric constant to the temperature applied [142]. Moreover, decreased viscosity and surface tension of SCW enable better penetration to the plant matrix, and thus increased diffusion and mass transfer rates can be achieved [143].

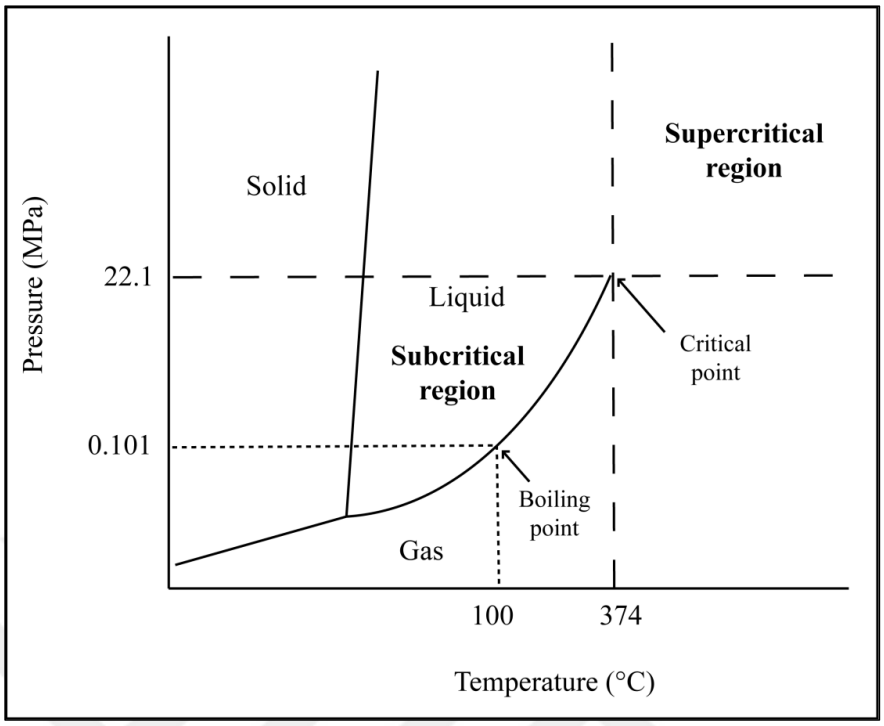

Figure 2.9. Phase diagram of water. Adapted from [139, 140].

Beyond its extraction properties, SCW also exhibits hydrolytic properties due to its increased ionic product, and support to release bound components, and break down bigger molecules to smaller ones [144, 145]. For instance, hydrolytic degradation of procyanidins under SCW conditions was shown from for red grape skin [146], and winery by-products extracted [147] to produce gallic acid. Owing to these properties and advantages, SCW extraction has already been used for the extraction of various bioactive compounds from natural materials [148, 149], such as flax shives [150, 151], rice bran [152, 153], and rice stem [154], canola meal [155] winery waste [156–158], grape seed [147], grape skin [146, 159], citrus pomace [160], apple pomace [161], pomegranate seed [162], mango peel [163], and potato peel [127]. Subcritical water extraction has been also applied to aromatic plants with high antioxidant activity such as oregano [164], rosemary [165], wild thyme, tarragon [166] and regional herbal plants that have medicinal properties such as Taiwan yams [167], kaffir lime [168], bitter melon [169] and *Terminalia chebula* Retz [170]. In those studies, variable results were obtained depending on the nature of plant matrix and extraction parameters applied, generally equivalent or higher extraction yields obtained compared to conventional

extraction methods. For instance, in a study where onion waste were extracted between 100 and 170 °C, 8-fold higher quercetin yields were obtained in 15 min SCW extraction compared to those obtained by conventional solvent-based extractions methods in two h [171]. In another study, SCW extraction of black tea, celery, and ginseng leaf at 170 °C gave 1.8- to 23.6-fold higher flavonol yields, namely quercetin, kaempferol, and myricetin, compared to stirring based solvent extraction [172]. However, SCW extraction of thermally liable compounds such as anthocyanins might only be possible at lower SCW temperatures, between 50 to 100 °C [157, 159]. In contrast to flavonoids, highly nonpolar components such as anacardic acids are hardly extracted using SCW [51]. Therefore, they can be separated from other mid-polar phenolic compounds using SCW extraction although anacardic acids and polyphenolic compounds are extracted together when solvents such as methanol and ethanol are used. Therefore, subcritical water extraction is a promising method for the selective recovery of phenolic compounds from plant materials. However, SCW extraction is still its early stages and large units are very rare [173].

2.7. ANALYSIS OF PHENOLIC COMPOUNDS

Analytical approaches for qualitative and quantitative determination of phenolic compounds can be broadly classified as spectrophotometric and chromatographic methods. Spectrophotometric methods are generally based on the measurement of color change upon reduction of a probe molecule. For instance, the most commonly used Folin-Ciocalteu reagent based total phenolic content assay is based on the blue color formation after reduction of a probe mixture of phosphomolybdate and phosphotungstate, giving specific absorption at 760 nm [174]. Some of the other spectrophotometric methods available for the analysis of different types of phenolic compounds are vanillin assay for flavonols, butanol-HCl assay for proanthocyanins, aluminium chloride based assay for total flavonoids, and rhodine assay for tannins [106]. In general, spectrophotometric assays provide simple and fast tool for screening of different types phenolic compounds. Their major limitation is being unspecific to phenolic compounds, but also reacting with other molecules such as ascorbic acid and aromatic amines [174, 175].

Chromatographic methods are more specific and sensitive tools providing information on individual phenolic compounds. High-performance liquid chromatography (HPLC) is the

most commonly used chromatographic method for the analysis of phenolic compound although gas chromatography (GC) analysis is also possible for some phenolic compounds after a derivatization step [176].

A typical HPLC system includes a pumping system, injector, column, and a detector. A mobile phase, a mixture of water, acetonitrile or methanol, is pressurized and pumped from a reservoir into the column, typically packed with a granular material based on silica having 2-50 micrometer pores, and then, to the detector. A sample dissolved in a liquid medium is injected into the flowing mobile phase, and its components are then separated in the column into peaks and produce a signal in the detector [176].

One of the most critical parts of HPLC analysis is the detection system. Absorbance-based detectors such as UV/Vis, PDA (photodiode array), and DAD (diode-array detector) are the most frequently used detector systems for the analysis of phenolic compounds due to their specific absorption properties at different wavelengths between 190 and 520 nm [177]. The principle of absorbance detectors is based on the measurement of the amount of absorbed light at a specific wavelength after the sample is irradiated with light in a flow cell. These types of detectors provide high sensitivity for the light absorbing analytes, broad linear range, ease of use, and compatibility with various solvents with isocratic or gradient elutions [106].

As an alternative detection system, evaporative light scattering (ELS) detectors are introduced for the analysis of a wider range of chemical compounds without a need for a chromophore base such that required for UV/Vis detection. Therefore, ELS detector systems have been increasingly used for the determination of sugars, fatty acids, steroids and saponins [178–180], especially in pharmaceutical and drug industry [181]. ELS detection consist of three stages: (i) nebulization where effluent coming from a liquid chromatography is atomized by the addition of a gas which is usually air or nitrogen, (ii) evaporation where solvent is evaporated in a so-called drift chamber, and (iii) detection with a light source where the amount of scattered light by the analyte molecule is measured [182]. Non-linear response behavior independent from compound concentration, no spectral data produced to identify certain peaks, its destructive nature that prevents coupling with mass detectors, and its lower precision compare to UV detectors are the disadvantages of ELS detectors [177].

An HPLC system equipped with UV/Vis and/or ELS detector only provides a tentative identification of an analyte based on the comparison of its retention time and UV/Vis properties with those of standard compounds. Since more than 8000 different phenolic compounds are available in plant kingdom [38], differentiation of similar phenolic structures might be challenging due to their similar/identical properties. For such cases, mass analysis using HPLC connected with a mass spectrometry (MS) provides more information about the structure, and thus, the identity of an analyte.

An MS detector consists of: (i) an ion source where solvent removal and analyte ionization occurs, (ii) an analyzer where charged molecules are separated and sorted based on their mass-to-charge ratio (*m/z*) using electromagnetic field, and (iii) a detector that measures *m/z* values and produces total ion chromatogram as an output [176]. Atmospheric-pressure electrospray ionization (ESI) is commonly used ion source for phenolic analysis. Other types of ionization sources such as atmospheric-pressure chemical ionization (APCI), and matrix-laser-assisted laser desorption and ionization (MALDI) are also available for the hardly ionize analytes. Different types of analyzers such as quadrupole, ion trap, time-of-flight (TOF), Fourier transform analyzer, and the magnetic sector can be used. In ion trap MS, analyte ions are collected for a period of time, and specific ions are selected based on their *m/z* values. Finally, *m/z* value of analyte is measured in detector part giving values in resolution range from one Da to 0.0004 Da depending on the sensitivity of equipment. High-resolution mass spectroscopy (HR-MS) is a highly sensitive mass analysis tool based on the determination of exact molecular masses up to 0.0004 Da resolutions. By using a precisely defined electromagnetic field in HR-MS, mono-isotopic masses with respective percentages of naturally occurring elemental isotopes are determined to provide a possible chemical formula of an analyte [183].

2.8. ANALYSIS OF ANTIOXIDANTS

Many methods are available for the analysis of antioxidant activity/capacity of a plant material, food or human plasma based on different antioxidant mechanisms and targeted molecules [184–186]. The most commonly used ones for the determination of antioxidant capacity of phenolic compounds are non-enzymatic radical scavenging antioxidant capacity

assays which further divided into hydrogen atom transfer (HAT) and electron transfer (ET) based assays [184–186].

HAT based assays measure the capability of an antioxidant molecule to donate a hydrogen molecule to reduce a free radical. HAT-based assays generally include competitive reaction between antioxidant molecules and a probe such as oxygen radical absorbance capacity (ORAC), total radical-trapping antioxidant parameter (TRAP), and crocin bleaching assays. On the other hand, ET-based assays are generally noncompetitive and measure the capacity of an antioxidant to reduce an oxidant probe by exchanging an electron. Ferric ion reducing antioxidant power (FRAP), ABTS, and DPPH are examples for ET-based assays. However, this classification is not absolute as DPPH and ABTS assays are also considered as mixed mode ET/HAT based assays [184, 185].

ET-based antioxidant capacity assays are mostly based on the color formation/reduction upon reaction of an antioxidant molecule and a synthetic free radical [184]. For instance, in DPPH assay, purple color of DPPH radical fades in the presence of an antioxidant molecule, and the color change is monitored spectrophotometrically at the absorbance of 517 nm [187].

2.9. AIMS AND SCOPE OF THE STUDY

The main objective of this thesis is the utilization of pistachio hull focusing on the recovery of the phenolic antioxidant compounds using sustainable technologies. The specific objectives of this Ph.D. thesis are below:

(i) To characterize of phenolic composition of pistachio hull.

(ii) To develop a methodology for the extraction, and quantitation of pistachio hull phenolics.

(iii) To recover phenolic and antioxidant compounds from pistachio hull using subcritical water technology.

The thesis is divided into six sections. Introduction (Section 1) is followed by a brief theoretical background as given in Section 2. Phenolic composition of pistachio hull investigated using HPLC-DAD-ESI (HR)-MS^n presented in Section 3. In Section 4, a quantitative methodology for extraction and analysis of pistachio hull phenolics were presented. Subcritical water extraction as a novel extraction technique is proposed to recover pistachio hull phenolics in Section 5. Each chapter has its own material and methods, results and discussion, and conclusion subsections. A conclusion resides after these five sections including general conclusions and suggestions for possible future work.

The presented Ph.D. thesis extended the current knowledge on the phenolic composition of pistachio (*P. vera* L.) hull (exo- and meso-carp) and relevant extraction techniques for the recovery of pistachio hull phenolics as each section was studied in depth for the first time.

3. IDENTIFICATION OF PHENOLIC COMPOUNDS IN RED AND GREEN PISTACHIO HULLS BY HPLC-DAD-ESI-(HR)-MS^n [1]

3.1. INTRODUCTION

Pistachio (*P. vera* L.) seeds are an important commercial crop, whose annual production has doubled during the past decade to reach a worldwide production of approximately one million tons in 2013. Top producing countries are Iran followed by the USA and Turkey [4]. Although the fruit of the pistachio tree is botanically considered a drupe, pistachio seeds are often regarded as "nuts", being most commonly consumed as roasted and salted snack food. In addition, a certain amount of pistachio seeds is used as a food ingredient, e.g., in pastry, ice-cream, chocolate, confectionery production, and mortadella. Pistachio drupes consist of an edible seed characterized by light-green cotyledons (kernels), a mauvish seed coat (testa) covered by a creamy lignified shell (endocarp), and a green to yellow-red colored outer hull (exo- and mesocarp) depending on the degree of ripeness [18]. According to their final use, they are processed to separate the non-lignified hull for snack pistachios or the entire hull, shell and seed coat to obtain the isolated kernels [24, 188]. In both cases, significant amounts of waste accrue having no or low commercial value, which need to be disposed at the processor's expense. Therefore, valorization of pistachio by-products is of great interest, so far being hampered by missing identification of valuable target compounds which merit utilization. Extracts of pistachio outer hull, the main by-product of pistachio processing, have been shown to exert antioxidant [11–14], antimicrobial [14, 115, 189], antimutagenic [14] and cytoprotective [12] as well as potential antitumor [15] and anticancer activities [16]. Despite such potent bioactivities, the phytochemicals in the pistachio hull remain to be comprehensively characterized.

---

[1]Reproduced with permission from Erşan, S., Güçlü Üstündağ, Ö., Carle, R., Schweiggert, R. M., Identification of phenolic compounds in red and green pistachio (*Pistacia vera* L.) hulls (exo- and mesocarp) by HPLC-DAD-ESI-(HR)-MS^n. Journal of Agricultural and Food Chemistry, 2016, *64*, 5334-5344. Copyright 2016, American Chemical Society.

Only a limited number of previous studies has aimed at elucidating the phenolic profiles of pistachio hulls (either ripe exocarp or green exo- and mesocarp), suggesting phenolic acids (gallic, protocatechuic, hydroxybenzoic, *p*-coumaric, and vanillic acids) and flavonoids (quercetin glycosides, isorhamnetin glycosides, naringenin, eriodictyol, and catechin) represent the main phenolic constituents, as only being identified by HPLC-DAD-FLD or HPLC-DAD-MS [11, 12], together with (13:0)-, (13:1)-, (15:0)-, (15:1)-, and (17:1)-anacardic acids [64]. Only a limited number of previous studies has aimed at elucidating the phenolic profiles of pistachio hulls (either ripe exocarp or green exo- and mesocarp), suggesting phenolic acids (gallic, protocatechuic, hydroxybenzoic, *p*-coumaric, and vanillic acids) and flavonoids (quercetin glycosides, isorhamnetin glycosides, naringenin, eriodictyol, and catechin) represent the main phenolic constituents, as only being identified by HPLC-DAD-FLD or HPLC-DAD-MS [11, 12], together with (13:0)-, (13:1)-, (15:0)-, (15:1)-, and (17:1)-anacardic acids [64]. Since pistachio belongs to the Anacardiaceae, a wide array of phenolic compounds is to be expected in pistachio hull [5]. For instance, flavonol glycosides, gallotannins, and specific phenolic lipids have been reported in other members of the Anacardiaceae such as mango (*Mangifera indica* L.) [6–8], Brazilian pepper (*Schinus terebinthifolius* Raddi) [9], and cashew (*Anacardium occidentale* L.) [10]. Thus, we sought to provide a comprehensive analysis of the phenolic constituents present in pistachio hull using highly sensitive tools such as HR-MS^n, particularly, aiming at the further utilization of pistachio hull as a source of potent phenolic bioactives.

Pistachio drupes are harvested at different maturity stages based on their desired properties and final use. A high portion of pistachio drupes are harvested red colored at full maturity, and preferentially used for the production of snack food, because their fully developed taste and high shell splitting ratio are important quality traits. However, green drupes, i.e. early-harvested drupes, are desired to produce intensely green colored kernels for pastry and confectionery industry of high market value. Thus, both green and red pistachio hulls, either in fresh or dried form depending on agricultural practices of the country, can be obtained in high amounts as by-products of pistachio processing. Particularly, dried pistachio hulls accrue in large amounts from numerous processors, because drying allows the off-season processing of the otherwise highly perishable pistachio drupes. Hitherto, no attention has been given to their compositional differences, and a lack of unambiguous sample descriptions in previous studies often hampers their comparative consideration [11–14].

Therefore, in this study, we additionally sought to compare the phenolic profiles of red and green pistachio hull as a further reference for their utilization as a phenolic source.

For the above-mentioned purposes, dried red and green hulls were obtained from a commercial pistachio processor to be extracted with aqueous methanol yielding phenolic-rich samples. Subsequently, these were screened for bioactive phenolic compounds by HPLC-DAD-ESI-MS^n and HPLC-HR-MS.

3.2. MATERIALS AND METHODS

3.2.1. Reagents

Gallic acid, protocatechuic acid, penta-*O*-galloyl-*β*-D-glucose, and (15:0)-anacardic acid were obtained from Sigma–Aldrich Chemie (Steinheim, Germany). *β*-Glucogallin (1-*O*-galloyl *β*-D-glucopyranose) was from PhytoLab (Vestenbergsgreuth, Germany), quercetin 3-*O*-glucuronide, quercetin 3-*O*-glucoside, quercetin 3-*O*-galactoside, myricetin 3-*O*-galactoside and cyanidin 3-*O*-*β*-D-galactopyranoside were from Extrasynthèse (Genay Cedex, France). HPLC grade methanol from VWR (Darmstadt, Germany), analytical grade formic acid from Merck (Darmstadt, Germany), and deionized water were used throughout the study.

3.2.2. Samples and Sample Preparation

Dried red and green pistachio drupes cv. ‘Uzun’ (Figure 3.1) were obtained from a pistachio processor (Gaziantep, Turkey). Both red and green pistachio drupes were harvested between August and September in 2013 in Gaziantep region of Turkey, traditionally sun-dried to decrease their moisture contents to 4.6 per cent ± 0.2 per cent as determined according to AOAC Official Method 934.01 [190] and stored for a year by the producer as usual in Turkey. Commonly, the moisture content of pistachio drupes during commercial storage was reported to range from 40-50 per cent in fresh form to three-five per cent in dried form [191]. Average air temperatures were between 3.4 °C and 28.6 °C for this region during the year the drupes were stored with a relative humidity of 29.0-78.9 per cent [192].

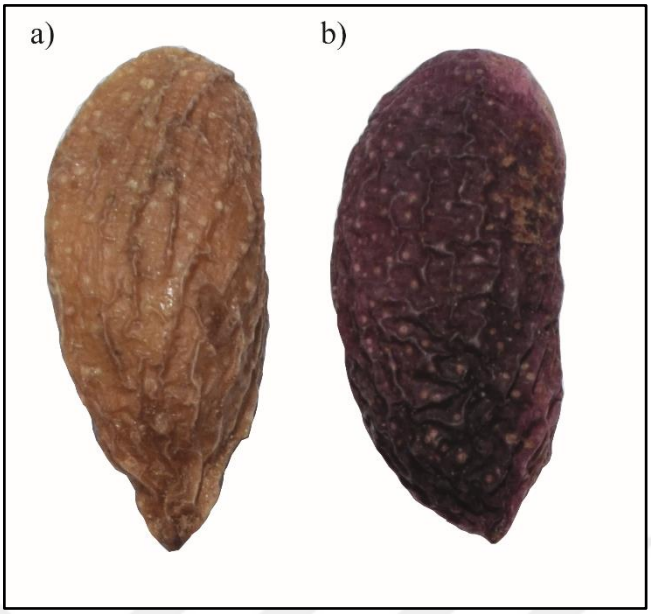

Figure 3.1. Photograph of dried (a) green and (b) red *P. vera* L. drupes. Background was cut off without further manipulation. Note that the green color has been lost upon drying and storage due to its instability.

Pistachio drupes were sampled in triplicate (each 500.0 g). Hulls and seeds were separated manually and weighted. Proportions of hulls and seeds of the whole pistachio drupe were approx. 20.5 per cent and 37.4 per cent on dry weight basis, respectively. Pistachio hulls were subsequently ground using an A11 laboratory mill (IKA, Staufen, Germany). Ground samples were stored at -20 °C until analyses.

3.2.3. Extraction of Phenolics

Ground red and green pistachio hulls (1.00 g) were combined with five mL of acidified (0.1 per cent HCl, v/v) aqueous methanol (80 per cent, v/v) and subjected to probe sonication at 70 per cent amplitude for 30 s. After centrifugation (1233 x *g*, three min), the supernatant was collected and the solid residues were re-extracted four times as described above. The combined methanolic extracts were evaporated to dryness in *vacuo* at 30 °C. Then, the dried extract was dissolved in one mL of 50 per cent aqueous methanol containing one per cent (v/v) formic acid and membrane-filtered (0.45 μm, regenerated cellulose) into amber vials prior to HPLC analyses.

3.2.4. HPLC-DAD-ESI-MS^n Analyses

An 1100 series HPLC system with G1322A degasser, a G1312A pump module, a G1313A autosampler, a G1316A column thermostat and a G1315A diode array detector (Agilent, Waldbronn, Germany) was equipped with a 250 mm × 4.6 mm i.d., five µm particle size, 100 Å pore size Kinetex C_{18} core-shell reversed-phase column fitted with 4.6 mm x two mm i.d. SecurityGuard Ultra C_{18} guard column both from Phenomenex (Aschaffenburg, Germany). Mobile phases were water for eluent A and methanol for eluent B both containing one per cent (v/v) formic acid. Chromatographic separation was achieved at 35 °C column temperature, one mL/min flow rate, and using the following gradient profile: isocratic at two per cent B for 10 min, from two-37 per cent B in 27 min, isocratic at 37 per cent B for five min, from 37-40 per cent B in 18 min, from 40-60 per cent B in 10 min, from 60-100 per cent B in 20 min, isocratic at 100 per cent B for 14 min, from 100-two per cent B in one min followed by isocratic conditioning at two per cent B for sevn min prior to the next run. Total run time was 112 min and injection volume was five µL.

For multi-stage mass spectrometry, the above described LC system was interfaced with an Esquire 3000+ ion-trap mass spectrometer (Bruker Daltonics, Bremen, Germany), which had been fitted with an ESI source operated in positive ion mode for anthocyanins and negative ion mode for all other analyses. Ion scan rate was in the range of *m/z* 100-2000 at a scan speed of *m/z* 13,000/s. Nitrogen was used both as drying and nebulizing gas at a flow rate of 11 mL/min and at a pressure of 60 psi, respectively. Nebulizer temperature was 365 °C. The potential on the capillary was set at ±2287 V for both negative and positive ion modes. Helium at a pressure of 4 x 10^{-6} mbar was used for collision induced dissociation (CID) at a fragmentation amplitude of 1.2 V. Ion chromatograms were analyzed using Esquire Control software.

3.2.5. HPLC-ESI-HR-MS Analyses

HPLC-HR-MS analyses were performed using micrOTOF-Q mass spectrometer (Bruker Daltonics, Bremen, Germany) coupled to an Agilent 1200 series HPLC system operated according to the above mentioned parameters. HR-MS was operated in negative ESI mode with +2200 V capillary voltages. Ion scan rate was in the range of *m/z* 250-3000 at a scan

speed of *m/z* 12,900/s (at *m/z* 996.8). Nitrogen was used both as nebulizing and drying gas at a pressure of 3.0 bar and a flow rate of eight L/min, respectively. Drying gas was heated to 300 °C. Instrument calibration was carried out according to the manufacturer's instructions using sodium formate. DataAnalysis 3.4 software was used to generate molecular formulas based on accurate mass measurements.

3.3. RESULTS AND DISCUSSION

3.3.1. Analysis of Phenolic Compounds by HPLC-DAD-ESI-MS^n and HPLC-ESI-HR-MS

A total of 66 phenolic compounds was detected in pistachio hull extracts, representative structures are presented in Figure 3.2. Monitoring was performed at different wavelengths as shown in Figure 3.3. All analytical data obtained for the examined compounds are listed in Table 3.1, including their retention times, UV absorption maxima, ESI-MS^n fragmentation pattern, and the respective high-resolution mass-to-charge (*m/z*) signals. The identification of the examined 66 compounds led to their allocation into three structurally related groups, i.e., gallotannins, flavonoids, and anacardic acids.

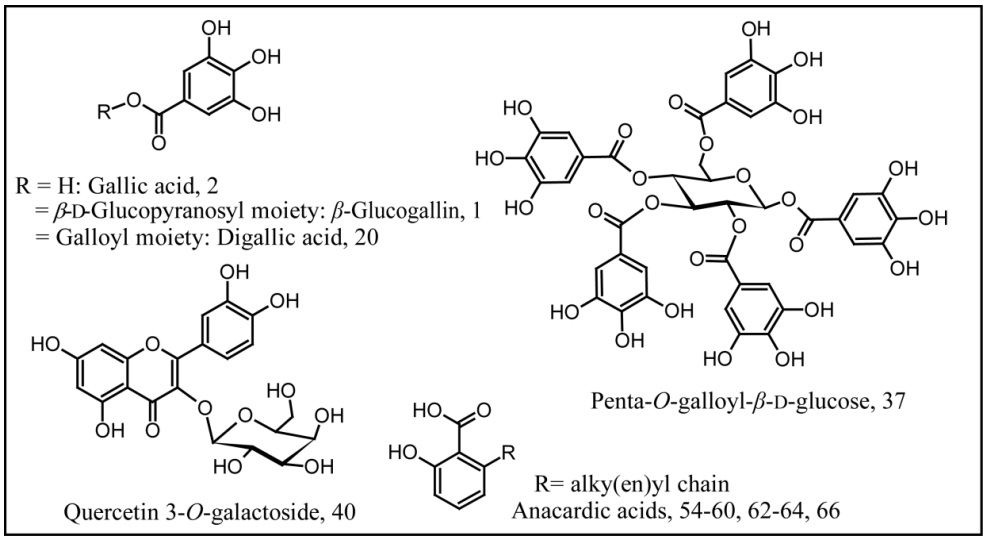

Figure 3.2. Representative structures of phenolic compounds detected at high signal intensity in aqueous methanolic extracts of *P. vera* L. hulls.

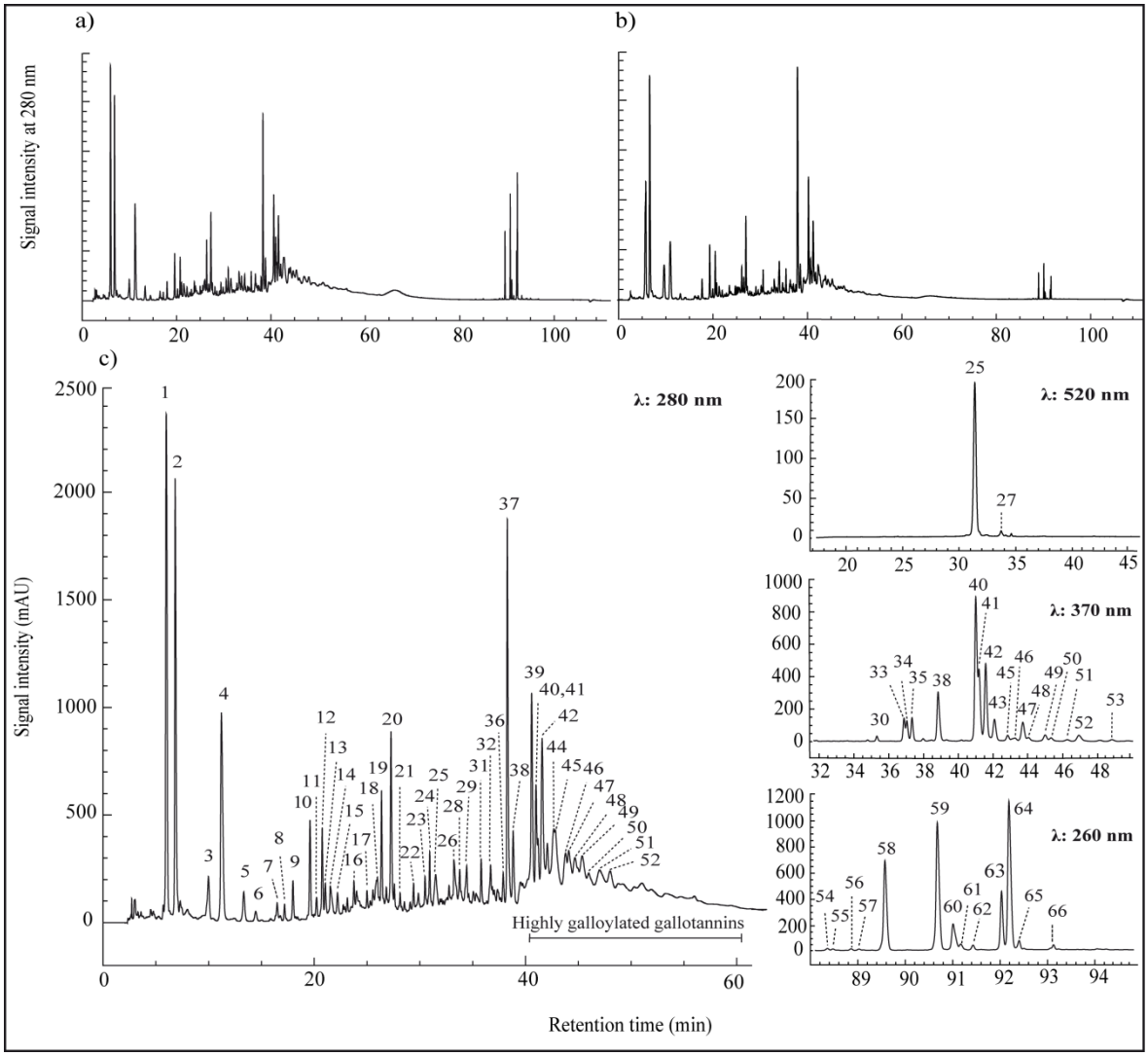

Figure 3.3. HPLC separation of phenolic compounds from (a) red and (b) green *P. vera* L. hulls at 280 nm. (c) Chromatogram of phenolic compounds from red hull monitored at different wavelengths. Peak assignments are shown in Table 3.1.

Table 3.1. HPLC retention times, UV/Vis spectra, and MS data of pistachio (*P. vera* L.) hull phenolics

Peak no.	**Ret. Time[1] (min)**	**Compound identity**	**HPLC-UV/Vis abs. max (nm)**	**HR-ESI(-)-MS $[M-H]^-$ *m/z* exp. (theo.)**	**Molecular formula**	**HPLC-ESI-MS^n experiment *m/z***
1	6.0	1-*O*-galloyl *β*-D-glucopyranose (*β*-Glucogallin) [2]	274	331.0684 (331.0671)	$C_{13}H_{16}O_{10}$	[331]: 169(100), 271(56), 193(31), 211(31), 183(25), 315(10) [331→169]:125(100)
2	6.8	Gallic acid [2]	272	na [3]	na	[169]: 125(100)
3	10.0	Gallic acid derivative (1)	276	296.0788 (296.0789)	na	[296]:169(100), 125(15), 195(5), 107(5), 171(2) [296→169]:125(100)
4	11.2	Galloyl quinic acid	275	343.0689 (343.0671)	$C_{14}H_{16}O_{10}$	[343]: 191(100), 169(17), 125(12)
5	13.4	Protocatechuic acid [2]	259	na	na	[153]: 109(100)
6	14.5	Galloyl dihexose (1)	274	493.1195 (493.1194)	$C_{19}H_{26}O_{15}$	[493]: 313(100), 169(39), 283(29), 331(10) [493→313]: 283(100), 169(44), 223(43), 135(39), 241(15)
7	16.5	Galloyl dihexose (2)	274	493.1209 (493.1194)	$C_{19}H_{26}O_{15}$	[493]: 271(100), 313(25), 211(20), 331(20), 169(7) [493→271]: 211(100), 125(4)
8	17.2	Galloyl dihexose (3)	274	493.1205 (493.1199)	$C_{19}H_{26}O_{15}$	[493]: 313(100) [493→313]: 169(100), 125(92), 189(30), 242(18)
9	18.0	Galloyl shikimic acid (1)	274	325.0571 (325.0565)	$C_{14}H_{14}O_9$	[325]: 169(100), 125(22), 139(9), 193(8) [325→169]: 125(100)
10	19.6	Galloyl shikimic acid (2)	274	325.0568 (325.0565)	$C_{14}H_{14}O_9$	[325]: 169(100), 125(15), 281(7), 111(4), 173(3) [325→169]: 124(100), 125(28)

Table 3.1. HPLC retention times, UV/Vis spectra, and MS data of pistachio (*P. vera* L.) hull phenolics (Continued)

Peak no.	**Ret. time (min)**	**Compound identity**	**HPLC-UV/Vis abs. max (nm)**	**HR-ESI(-)-MS $[M-H]^-$ *m/z* exp. (theo.)**	**Molecular formula**	**HPLC-ESI-MS^n experiment *m/z***
11	20.2	Gallic acid derivative (2)	276	na	na	[571]: 285(100), 169(7) [571→285]: 133(100), 169(49), 170(10)
12	20.8	Galloyl shikimic acid (3)	274	325.0576 (325.0565)	$C_{14}H_{14}O_9$	[325]: 169(100), 125(15), 137(3), 173(3) [325→169]: 125(100)
13	21.1	Digallic acid (1)	280	321.0261 (321.0252)	$C_{14}H_{10}O_9$	[321]: 169(100) [321→169]: 125(100)
14	21.5	Digalloyl hexose (1)	280	483.0805 (483.0780)	$C_{20}H_{20}O_{14}$	[483]: 331(100), 271[(16), 169(15) [483→331]: 169(100), 313(60), 271(51), 193(25), 123(15), 241(12)
15	22.3	Methyl gallate	272	na	na	[183]: 168(100), 124(10) [183→168]: 124(100)
16	23.8	Digalloyl hexose (2)	288	483.0794 (483.0780)	$C_{20}H_{20}O_{14}$	[483]: 331(100), 169(27), 332(17), 271(11) [483→331]: 169(100), 241(53), 125(15)
17	25.0	Gallic acid derivative (3)	274	509.0972 (509.0996)	$C_{15}H_{26}O_{19}$	[509]: 267(100), 429(11) [509→267]: 139(100)
18	26.0	Gallic acid derivative (4)	274	423.0931 (423.0933)	$C_{19}H_{20}O_{11}$	[423]: 313(100), 169(70), 125(36), 211(24) [423→313]: 169(100), 313(39), 295(17)
19	26.4	Digalloyl quinic acid	274	495.0776 (495.0780)	$C_{21}H_{20}O_{14}$	[495]: 343(100), 191(29), 344(16), 271(4) 169(3) [495→343]: 191(100)

Table 3.1. HPLC retention times, UV/Vis spectra, and MS data of pistachio (*P. vera* L.) hull phenolics (Continued)

Peak no.	**Ret. time (min)**	**Compound identity**	**HPLC-UV/Vis abs. max (nm)**	**HR-ESI(-)-MS $[M-H]^-$ *m/z* exp. (theo.)**	**Molecular formula**	**HPLC-ESI-MS^n experiment *m/z***
20	27.3	Digallic acid (2)	275	321.0246 (321.0252)	$C_{14}H_{10}O_9$	[321]: 169(100), 125(16)
21	28.1	Gallic acid derivative (5)	268	403.1254 (403.1246)	$C_{17}H_{24}O_{11}$	[403]: 169(100), 151(55), 313(37), 125(36), 271(21), 211(18), 179(15) [403→169]: 124(100), 125(34), 107(34)
22	29.5	Gallic acid derivative (6)	274	467.1197 (467.1195)	$C_{21}H_{24}O_{12}$	[467]: 313(100), 169(17), 295(7) [467→313]: 169(100), 153(10), 191(5)
23	30.6	Digalloyl shikimic acid (1)	275	477.0688 (477.0675)	$C_{21}H_{18}O_{13}$	[477]: 325(100), 169(47), 326(33) [477→325]: 169(100)
24	31.0	Digalloyl shikimic acid (2)	276	477.0703 (477.0675)	$C_{21}H_{18}O_{13}$	[477]: 325(100), 169(47) [477→325]: 169(100), 125(11), 281(10)
25	31.6	Cyanidin 3-O-*β*-D-galactopyranoside [2]	278, 517	na	na	[4][449]: 287(100) [449→287]: 137(100)
26	33.2	Luteic acid	278	319.0133 (319.0096)	$C_{14}H_8O_9$	[319]: 239(100), 139(15), 240(14) [319→239]: 139(100), 124(12)
27	33.7	Cyanidin pentoside	520	na	na	[4][419]: 287(100)
28	33.8	Trigalloyl quinic acid	273	647.0873 (647.0890)	$C_{28}H_{24}O_{18}$	[647]: 495(100), 343(22), 191(2) [647→495]: 343(100), 191(46)

Table 3.1. HPLC retention times, UV/Vis spectra, and MS data of pistachio (*P. vera* L.) hull phenolics (Continued)

Peak no.	**Ret. time (min)**	**Compound identity**	**HPLC-UV/Vis abs. max (nm)**	**HR-ESI(-)-MS $[M-H]^-$ *m/z* exp. (theo.)**	**Molecular formula**	**HPLC-ESI-MS^n experiment *m/z***
29	34.5	Quinic acid derivative (1)	276	523.1455 (523.1157)	$C_{24}H_{28}O_{13}$	[523]: 209(100), 371(45), 505(30), 313(19), 169(14), 191(14) [523→209]: 191(100), 165(21), 151(18), 123(2)
30	35.3	Myricetin galloyl hexoside	270, 354	631.0934 (631.0941)	$C_{28}H_{24}O_{17}$	[631]: 479(100), 317(10) [631→479]: 316(100), 317(69), 325(15), 271(11)
31	35.8	Tetragalloyl hexose	278	787.1010 (787.0999)	$C_{34}H_{28}O_{22}$	[787]: 617(100) [787→617]: 465(100), 589(55), 221(41), 277(34), 449(30), 600(26), 296(20), 235(18), 466(12)
32	36.7	Trigallic acid	274	473.0373 (473.0362)	$C_{21}H_{14}O_{13}$	[473]: 321(100), 169(25) [473→321]: 169(100), 125(6)
33	36.9	Myricetin hexuronide	357, 252	493.0640 (493.0624)	$C_{21}H_{18}O_{14}$	[493]: 317(100), 299(4), 151(3), 137(2) [493→317]: 227(100), 151(23), 137(18)
34	37.1	Myricetin 3-*O*-galactoside	359, 252	479.0848 (479.0831)	$C_{21}H_{20}O_{13}$	[479]: 317(100), 271(20), 179(19), 287(15) [479→317]: 215(100), 271(99), 164(24), 270(22), 287(22), 242(17)
35	37.4	Myricetin hexoside	357, 252	479.0884 (479.0831)	$C_{21}H_{20}O_{13}$	[479]: 316(100), 317(57), 271(12) [479→316]: 271(100), 179(40), 317(35), 180(29), 255(26), 137(23)
36	37.9	Methyl-digallate	274	335.0414 (335.0409)	$C_{15}H_{12}O_9$	[335]: 183(100), 253(5) [335→183]: 168(100)

Table 3.1. HPLC retention times, UV/Vis spectra, and MS data of pistachio (*P. vera* L.) hull phenolics (Continued)

Peak no.	**Ret. time (min)**	**Compound identity**	**HPLC-UV/Vis abs. max (nm)**	**HR-ESI(-)-MS $[M-H]^-$ *m/z* exp. (theo.)**	**Molecular formula**	**HPLC-ESI-MS^n experiment *m/z***
37	38.3	Penta-*O*-galloyl-*β*-D glucose [2]	280	939.1130 (939.1109)	$C_{41}H_{32}O_{26}$	[939]: 769(100), 617(18), 787(12) [939→769]: 617(100), 387(43), 601(40), 465(28), 323(21), 259(19), 403(19), 725(17), 245(12), 573(12), 386(11)
38	38.9	Quercetin galloyl hexoside (1)	257, 354	615.1031 (615.0992)	$C_{28}H_{24}O_{16}$	[615]: 463(100), 301(32) [615→463]: 301(100), 179(8), 229(7), 253(6), 272(6), 151(2)
39	40.6	Hexagalloyl hexose	263, 353	1091.1423 (1091.1213)	$C_{48}H_{36}O_{30}$	[1091]: 939(100), 769(16) [1091→939]: 769(100), 770(13), 617(10)
40	41.0	Quercetin 3-*O*-galactoside [2]	252, 352	463.0922 (463.0882)	$C_{21}H_{20}O_{12}$	[463]: 301(100), 179(8) [463→301]: 151(100), 229(58), 271(43), 343(30)
41	41.2	Quercetin 3-*O*-glucuronide [2]	255, 355	477.0715 (477.0675)	$C_{21}H_{18}O_{13}$	[477]: 301(100), 179(7), 151(2) [477→301]: 179(100), 152(20), 180(11), 121(8), 256(7), 273(6), 229(6)
42	41.6	Quercetin 3-*O*-glucoside [2]	260, 355	463.0915 (463.0877)	$C_{21}H_{20}O_{12}$	[463]: 301(100) [463→301]: 179(100), 271(52), 152(39), 272(32), 255(31), 151(27), 203(21)
43	42.1	Quercetin galloyl hexoside (2)	254, 358	615.1031 (615.0986)	$C_{28}H_{24}O_{16}$	[615]: 301(100), 313(15), 315(8), 273(6), 463(3) [615→301]: 179(100), 151(99)

Table 3.1. HPLC retention times, UV/Vis spectra, and MS data of pistachio (*P. vera* L.) hull phenolics (Continued)

Peak no.	**Ret. time (min)**	**Compound identity**	**HPLC-UV/Vis abs. max (nm)**	**HR-ESI(-)-MS $[M-H]^-$ *m/z* exp. (theo.)**	**Molecular formula**	**HPLC-ESI-MS^n experiment *m/z***
44	42.7	Gallic acid derivative (7)	279	347.0409 (347.0406)	$C_{16}H_{12}O_9$	[347]: 267(100), 139(5), 249(4), 269(4), 124(2) [347→267]: 139(100), 249(28), 83(16), 140(10), 205(8)
45	42.9	Quercetin galloyl hexoside (3)	276, 358	615.1098 (615.0986)	$C_{28}H_{24}O_{16}$	[615]: 301(100), 179(5), 463(3) [615→301]: 151(100), 170(32), 107(21), 179(20)
46	43.3	Quercetin galloyl deoxyhexose	274, 355	599.1075 (599.1037)	$C_{28}H_{24}O_{15}$	[599]: 463(100), 301(36) [599→463]: 301(100), 255(7)
47	43.7	Quercetin pentoside	254, 355	433.0810 (433.0771)	$C_{20}H_{18}O_{11}$	[433]: 301(100), 255(6), 242(4), 193(4) [433→301]: 271(100), 179(42), 151(41), 255(11), 243(9)
48	44.1	Unknown (1)	275, 357	na	na	[629]: 327(100), 459(15), 477(15), 328(11) [629→327]: 113(100), 169(49), 283(40), 175(38), 170(37), 177(35)
49	45.0	Kaempferol hexoside	272, 350	447.0963 (447.0927)	$C_{21}H_{20}O_{11}$	[447]: 285(100), 255(28), 327(19) [447→285]: 255(100), 223(6), 256(5)
50	45.4	Quercetin galloyl hexuronide	276, 352	629.0852 (629.0779)	$C_{28}H_{22}O_{17}$	[629]: 477(100), 301(26) [629→477]: 301(100), 323(5), 175(3)
51	46.3	Quercetin galloyl pentoside	277, 352	585.0910 (585.0881)	$C_{27}H_{22}O_{15}$	[585]: 301(100) [585→301]: 151(100), 179(45), 165(44), 121(9)
52	46.9	Kaempferol hexoside	270, 350	447.0952 (447.0927)	$C_{21}H_{20}O_{11}$	[447]: 285(100), 284(92), 255(63), 163(18), 151(17) [447→285]: 255(100), 197(13), 240(10), 198(8), 213(7), 227(6), 169(5)

Table 3.1. HPLC retention times, UV/Vis spectra, and MS data of pistachio (*P. vera* L.) hull phenolics (Continued)

Peak no.	**Ret. time (min)**	**Compound identity**	**HPLC-UV/Vis abs. max (nm)**	**HR-ESI(-)-MS $[M-H]^-$ *m/z* exp. (theo.)**	**Molecular formula**	**HPLC-ESI-MS^n experiment *m/z***
53	48.9	Kaempferol pentoside	270, 350	417.0907 (417.0822)	$C_{20}H_{18}O_{10}$	[417]: 284(100), 255(37) [417→284]: 255(100), 160(5), 165(4), 227(4), 195(3), 151(3)
54	88.4	(16:1)-Anacardic acid	238, 310	359.2521 (359.2586)	$C_{23}H_{36}O_3$	[359]: 315(100), 341(43), 161(21), 315(17), 107(13), 343(13), 317(11) [359→315]: 108(100)
55	88.5	(13:2)-Anacardic acid	238, 310	315.1968 (315.1966)	$C_{20}H_{28}O_3$	[315]: 271(100), 107(6) [315→271]: 107(100)
56	88.9	(11:0)-Anacardic acid	238, 308	291.1960 (291.1966)	$C_{18}H_{28}O_3$	[291]: 247(100) [291→247]: 106(100)
57	89.1	(15:3)-Anacardic acid	238, 310	341.2112 (341.2122)	$C_{22}H_{30}O_3$	[341]: 297(100) [341→297]:149(100)
58	89.6	(13:1)-Anacardic acid	246, 310	317.2115 (317.2117)	$C_{20}H_{30}O_3$	[317]: 273(100) [317→273]: 107(100)
59	90.7	(13:0)-Anacardic acid	248, 312	319.2289 (319.2279)	$C_{20}H_{32}O_3$	[319]: 275(100) [319→275]: 106(100)
60	91.0	(15:1)-Anacardic acid	240, 310	345.2429 (345.2434)	$C_{22}H_{34}O_3$	[345]: 301(100), 119(4) [345→301]: 119(100)

Table 3.1. HPLC retention times, UV/Vis spectra, and MS data of pistachio (*P. vera* L.) hull phenolics (Continued)

Peak no.	**Ret. time (min)**	**Compound identity**	**HPLC-UV/Vis abs. max (nm)**	**HR-ESI(-)-MS $[M-H]^-$ *m/z* exp. (theo.)**	**Molecular formula**	**HPLC-ESI-MS^n experiment *m/z***
61	91.2	Unknown (2)	260	455.3510 (455.3405)	$C_{30}H_{48}O_3$	[455]: 418(100) [455→418]: 434(100), 395(66), 375(60), 399(15)
62	91.4	(17:2)-Anacardic acid	238, 310	371.2599 (371.2592)	$C_{24}H_{36}O_3$	[371]: 327(100) [371→327]: 327(100), 119(18), 107(10)
63	92.0	(15:0)-Anacardic acid [2]	242, 311	347.2623 (347.2592)	$C_{22}H_{36}O_3$	[347]: 303(100) [373→303]: 106(100)
64	92.2	(17:1)-Anacardic acid	248, 312	373.2743 (373.2743)	$C_{24}H_{38}O_3$	[373]: 329(100) [373→329]: 106(100)
65	92.4	Unknown (3)	260	373.2748 (373.2748)	$C_{24}H_{38}O_3$	[373]: 329(100) [373→329]: 119(100)
66	93.1	(17:0)-Anacardic acid	238, 312	375.2951 (375.2977)	$C_{24}H_{40}O_3$	[375]: 331(100) [375→331]: 106(100)

[1]Ret. time: retention time
[2] Verified by reference standards
[3] na: not available
[4] Positive ionization mode was used for the identification of anthocyanins.
[5]All compounds were detected in both red and green pistachio hulls, except for the anthocyanins, 25 and 27, which were only found in red hulls.

3.3.1.1. Gallotannins

Gallotannins represent a subgroup of hydrolyzable tannins, more specifically being esters of at least one gallic acid molecule with polyols such as sugars, shikimic acids or quinic acids [40]. While gallic acid, 2, was tentatively identified by comparing the obtained retention time, UV absorption and mass spectra to those of an authentic standard, a total of 30 related gallic acid derivatives and gallotannins was identified in pistachio hull extracts, mainly based on the formation of characteristic product ions at *m/z* 169 ([gallic acid-H]$^-$) and 125 ([gallic acid-CO_2-H]$^-$) as well as due to the specific neutral loss of a dehydrated galloyl moiety (152 Da). The identification was corroborated by their UV absorption spectra and high-resolution MS data.

3.3.1.1.1. Galloyl hexoses

Compound one with a parent ion [M-H]$^-$ at *m/z* 331 revealed a daughter ion [M-H-162]$^-$ at *m/z* 169 upon CID fragmentation, indicating the loss of a hexose moiety. It was identified as 1-*O*-galloyl *β*-D-glucopyranose (*β*-glucogallin) after comparing its retention time, UV absorption and mass spectra with those of an authentic standard. Glucogallin has already been reported in many plants including other members of Anacardiaceae, such as Brazilian pepper [9] and mango [193], and is considered a primary metabolite and galloyl donor for gallotannin biosynthesis [194, 195].

The parent ion [M-H]$^-$ at *m/z* 493 of compound eight formed daughter ions [M-H-162]$^-$ at *m/z* 313 and [M-H-162-162]$^-$ at *m/z* 169. In agreement with its UV and high-resolution MS data, compound eight was tentatively identified as a galloyl dihexose. Similar galloyl dihexoses have been previously found in plant parts of other Anacardiaceae, namely in sumac (*Rhus coriaria* L.) [196]. Compounds six and seven exhibited both UV absorption spectra and high-resolution mass signals identical to those of compound eight (Table 3.1). However, their different fragmentation patterns indicated that they might represent distinct isomers of galloyl dihexoses as reported previously [197]. For instance, while predominant CID daughter ions [M-H-180]$^-$ at *m/z* 313 and [M-H-324]$^-$ at *m/z* 169 were observed for

compounds six and eight, those of compound seven were observed at *m/z* 271 ([M-H-162-60]$^-$), at *m/z* 211 ([M-H-162-120]$^-$) and at *m/z* 313 ([M-H-180]$^-$) (Table 3.1) [193].

Two further minor compounds, 14 and 16, were tentatively assigned as digalloyl hexoses (*m/z* 483) due to loss of a dehydrated galloyl (152 Da) and a hexose (162 Da) moiety, ultimately yielding gallic acid (*m/z* 169) as daughter ion. The loss of dehydrated galloyl units (152 Da) may indicate depsidically linked gallic acids due to previously reported a predominance of galloyl fission in these types of linkages [112].

The pseudo-molecular ions [M-H]$^-$ of three compounds, 31, 37 and 39, exhibited sequential losses of galloyl moieties (152 Da) from their parent ions at *m/z* 787, 939 and 1091, respectively. These compounds were tentatively identified as tetra- , penta- , and hexagalloyl hexose, respectively, based on their retention order, UV absorption maxima, high resolution MS data, and MS^n fragmentation pattern as compared with the literature [107,198]. The identity of compound 37 was further confirmed as penta-*O*-galloyl-*β*-D-glucose after comparing its analytical data with that of the corresponding authentic standard. Hexagalloyl hexose, 39, was the gallotannin having the highest degree of galloylation that was detected as a separate peak, although several unresolved peaks eluting after 40 min (Figure 3.3) may be attributed to the presence of higher degrees of galloylated tannins. The chromatographic separation of such highly galloylated gallotannins was previously shown to be most intricate due to the increased number of possible gallotannin isomers with the increase in the number of galloyl units [124]. In agreement, our extracted ion chromatograms containing the respective traces of penta-, hexa-, hepta-, octa-, and nonagalloyl tannins are shown in Figure 3.4 to illustrate the increasing complexity of these compounds and their related mass signals. In addition, characteristic doubly charged pseudo-molecular ions $[M\text{-}2H]^{2-}$ and corresponding fragment ions $[M\text{-}n \times 152\text{-}2H]^{2-}$ with (n = 1–4) were observed in the region of highly galloylated gallotannins [9,199].

3.3.1.1.2. Galloyl quinic acids

By analogy to differently galloylated hexoses, quinic acid was found to be galloylated to different degrees. Compounds 4, 19, and 28 were tentatively identified as mono-, di- and trigalloyl quinic acids due to sequential losses of galloyl moieties (152 Da) from their parent ions at *m/z* 343, 495 and 647, respectively, and the formation of a final product ion at *m/z*

191 (deprotonated quinic acid) [200, 201]. HR-MS measured exact molecular masses were also in good agreement with calculated masses of the respective galloyl quinic acids, corroborating their identification (Table 3.1).

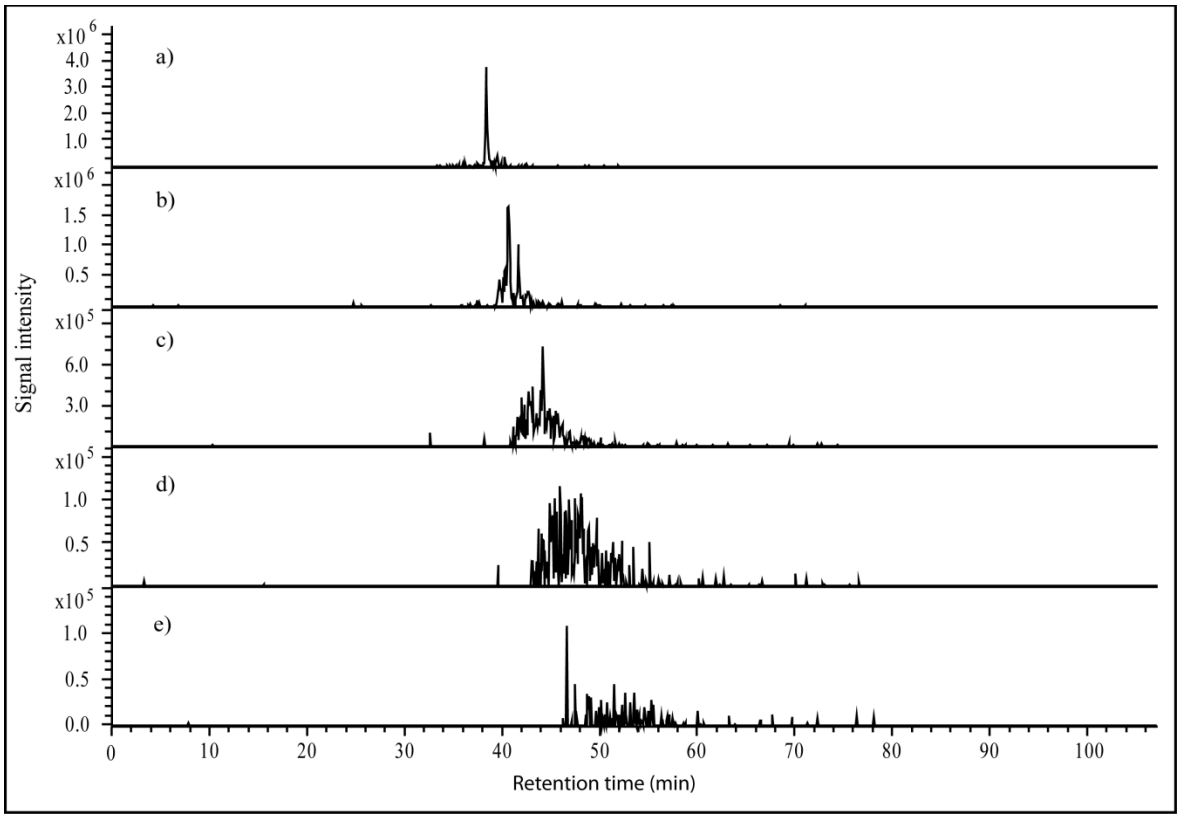

Figure 3.4. Extracted ion chromatograms indicating the putative presence of (a) penta- (*m/z* 939), (b) hexa- (*m/z* 1091), (c) hepta- (*m/z* 1243), (d) octa- (*m/z* 1395), and (e) nona- (*m/z* 1547) galloyl hexoses.

An additional quinic acid derivative (compound 29) was also tentatively identified due to the formation of a putative product ion at *m/z* 191 (deprotonated quinic acid) upon CID experiments. Compounds with different degrees of galloyl quinic acids were also reported in *Pistacia lentiscus* L. [200], *Myrtus communis* L. [202], green tea (*Camellia sinensis* L.), and tara (*Caesalpinia spinosa* (Molina) Kuntze) [201].

3.3.1.1.3. Galloyl shikimic acids

Three compounds, 9, 10 and 12, with identical UV absorption spectra, identical high-resolution MS data, and identical pseudo-molecular ions $[M-H]^-$ at *m/z* 325 were characterized by the loss of 156 Da, yielding a daughter ion at *m/z* 169. In agreement with our analytical data, these compounds were identified as monogalloyl shikimic acids. Two compounds, 23 and 24, with pseudo-molecular ions $[M-H]^-$ at *m/z* 477 produced daughter ions at *m/z* 325 due to the neutral loss $[M-H-152]^-$ of a putative galloyl moiety. Further fragmentation of *m/z* 325 yielded spectra similar to the above mentioned monogalloyl shikimic acids, 9, 10, and 12. In agreement with their chemical formula (Table 3.1), compounds 23 and 24 were tentatively identified as the isomers of digalloyl shikimic acids. Despite their uncommon presence in plants, differently galloylated shikimic acids were previously reported in other plants [203–206], including Brazilian pepper from the Anacardiaceae [9]. As shikimic acid has been reported as a precursor of gallate synthesis [207], galloylated shikimic acids may represent intermediates for the biosynthesis of higher molecular weight gallotannins.

The presence of gallotannins has previously been reported in other Anacardiaceae such as mango [107], and sumac [196]. However, this is the first detailed report on pistachio hull gallotannins. Behgar et al.[208] previously reported the total “tannin content” of pistachio hull as determined by an unspecific protein precipitation based radial diffusion assay.

Two peaks, 13 and 20, exhibited parent ions $[M-H]^-$ at *m/z* 321. Their CID fragmentations resulted in product ions at *m/z* 169 and 125 characteristic of gallic acid. Thus, these compounds were tentatively identified as digallic acids, although their differences on linkages between the two galloyl moieties remain unknown despite their slightly different UV absorption maxima (Table 3.1). The pseudo-molecular ion $[M-H]^-$ of compound 32 at *m/z* 473 tentatively indicated the presence of a trigallic acid due to sequential loss of two galloyl moieties, yielding product ions specific for gallic acid. We were unable to provide evidence that these three compounds, 13, 20, and 32, represented depsides, although depsidically linked gallic acids were previously found in tanoak acorns (*Notholithocarpus densiflorus* (Hook. & Arn.) Manos, Cannon & S. H. Oh) [197], and Anacardiaceae such as sumac [196], mango peel [198] as well as in *Rhus chinensis* Mill. leaves, a traditional Chinese herb [209].

Compound 15 exhibited a parent ion $[M-H]^-$ at *m/z* 183 with a corresponding demethylated product ion $[M-H-15]^-$ at *m/z* 168, thus being tentatively assigned as methyl gallate in agreement with previous reports [198]. Similarly, compound 36 with a parent ion at *m/z* 335 was identified as methyl digallate due to loss of a digalloyl moiety during CID. In addition, a product ion $[M-H-152]^-$ at *m/z* 183 (methyl gallate) was observed in the MS^n spectra. Noteworthy, methyl gallate, 15, and methyl digallate, 36, may represent potential artefacts, resulting from methanolysis of depsidically linked gallotannins in the course of extraction and analysis [112, 201]. In agreement, an increase in both peak area and height of compound 15 and 36 was observed upon re-analysis of methanolic extracts kept at room temperature for 24 h (data not shown).

Compound 26 produced a parent ion $[M-H]^-$ at *m/z* 319 and its HR-MS measured exact molecular mass revealed a good fit to luteic acid, a digallic acid with an additional C-C bond between its benzene rings (Table 3.1). Although compound 17, 26 and 44 had common MS^n product ions at *m/z* 139, which may indicate the formation methyl pyrogallol fragments upon CID, the identities of compounds 17 and 44 yet remain unknown. Luteic acid, a molecule present in the structure of myrobalanitannin, has been reported in the fruits of *Terminalia chebula* Renz. as an intermediate of ellagic acid biosynthesis [210].

Five compounds, 3, 11, 18, 21, and 22, were tentatively identified as gallic acid derivatives due to the formation of a deprotonated gallic acid at *m/z* 169 as MS^n daughter ion, although their further characterization remains pending. The yet unidentified gallic acid derivative, compound 3, may contain nitrogen due to its even-numbered mass-to-charge ratio at *m/z* 296 (Table 3.1).

3.3.1.2. Flavonoids

3.3.1.2.1. Flavonols

A total of 17 flavonols was tentatively identified in red pistachio hull extracts, including quercetin, myricetin, and kaempferol derivatives. Quercetin derivatives, 38, 40-43, 45-47, 50 and 51, were the major flavonol constituents of the hulls under investigation, according to their highly characteristic UV absorption maxima at ca. 350 nm, common fragment ions at *m/z* 301 (deprotonated quercetin), and a characteristic fragment of the quercetin aglycone

at *m/z* 179. The major quercetin derivatives, quercetin 3-*O*-galactoside, 40, quercetin 3-*O*-glucuronide, 41, and quercetin 3-*O*-glucoside, 42, were identified by comparing their retention times, UV absorption and MS data to those of authentic standards (Figure 3). In addition to quercetin hexosides, a quercetin pentoside, 47, was tentatively identified. Moreover, several types of galloylated quercetin glycosides were tentatively identified according to their analytical data shown in Table 3.1, such as quercetin galloyl hexosides, 38, 43, and 45, quercetin galloyl deoxyhexose, 46, quercetin galloyl hexuronide, 50, and quercetin galloyl pentoside, 51.

Four myricetin derivatives, 30, 33-35, were tentatively assigned based on their characteristic fragment ion at *m/z* 317 (myricetin aglycone) and their characteristic secondary fragments at *m/z* 299 and 271. Compound 33 was tentatively identified as myricetin hexuronide due to its parent ion $[M-H]^-$ at *m/z* 493 and the derived, previously reported [112] predominant daughter ion at *m/z* 317, indicating the loss of an uronic acid moiety in agreement with its high-resolution MS data. Compounds 34 and 35 were tentatively identified as myricetin hexosides based on the analytical data presented in Table 3.1. Compound 34 was further identified as myricetin 3-*O*-galactoside after comparison of its analytical data with that of an authentic standard. The parent ion $[M-H]^-$ of compound 30 at *m/z* 631 was characterized by the sequential loss of 162 Da (hexose) and 152 Da (galloyl moiety), thus being identified as myricetin galloyl hexoside in agreement with its high-resolution MS data. Moreover, three kaempferol derivatives, namely two hexosides and one pentoside, 49, 52 and 53, were identified in trace amounts. HR-ESI-MS accurate mass measurements were in agreement with all proposed flavonols (Table 3.1).

Flavonols of pistachio hull have been recently investigated using HPLC-DAD [11]. According to this study, quercetin rutinoside represented the major flavonol accompanied by lower amounts of quercetin, quercetin galactoside, quercetin glucoside, and kaempferol and isorhamnetin glycosides. The presence of these flavonol derivatives was confirmed by our study, except for quercetin rutinoside and isorhamnetin derivatives which were not detected in our samples. In further contrast, in our samples, quercetin galactoside, quercetin glucuronide and quercetin glucoside were the major flavonols accompanied by low amounts of myricetin and kaempferol derivatives (Table 3.1).

3.3.1.2.1. Anthocyanins

Compound 26 was the main peak observed in the chromatogram recorded at 520 nm (Figure 3), whose parent ion $[M]^+$ at *m/z* 449 exhibited a characteristic daughter ion $[M-162]^+$ at *m/z* 287, the cyanidin aglycone. After comparing its retention time, UV/Vis absorption and mass spectra with those of an authentic standard, compound 25 was tentatively identified as cyanidin 3-*O*-*β*-D-galactopyranoside. Minor amounts of a putative cyanidin pentoside, 27, were also tentatively identified based on the formation of a cyanidin aglycone at *m/z* 287 after the loss of a pentose (132 Da) upon CID fragmentation. The presence of cyanidin 3-*O*-galactoside in *P. vera* seed coat (named as "skin" in the study) [211, 212] and cyanidin 3-*O*-glucoside in leaves of *P. lentiscus* [200] has previously been reported. However, this is the first report on the occurrence and identification of cyanidin derivatives in red pistachio hull, although a previous study reported total anthocyanin contents of pistachio hulls as determined spectrophotometrically [61]. Interestingly, 7-*O*-methylated anthocyanins have not been observed in our study, although their presence in mango [213], cashew apple [214], Brazilian pepper [9], and sumac [196] has been proposed to be a chemotaxonomic marker of the Anacardiaceae. Noteworthy, our samples underwent drying and storage prior to analyses, and thus, further studies on freshly collected pistachio fruits should be done. On the other hand, it is worth mentioning that *P. vera* has occasionally been classified in its own family Pistaciaceae rather than in the Anacardiaceae, which may be supported by the aforementioned lack of 7-*O*-methylated anthocyanins.

3.3.1.3. Anacardic acids

A total of 11 anacardic acids 54-60, 62-64, 66, with different lengths of alkyl chains (C13, C15 and C17) and saturation degrees (fully saturated or mono-, di-, or tri-unsaturated) were identified in pistachio hull extracts (Table 3.1). They eluted late at 88-94 min (Figure 3.3) due to their lipophilic alk(en)yl side chain. In the following, the compounds are named according to the length of their side chain and the number of double bonds in the side chain (Figure 3.2). All of them produced similar UV absorption spectra with maximum absorbance at 250 and 311 nm. Their CID mass spectra had a product ion $[M-44]^-$ in common, indicating a CO_2 loss from the phenolic carboxyl group. Furthermore, characteristic product ions at *m/z*

106 or *m/z* 107 have been reported to occur due to the elimination of the phenol group, while product ions at *m/z* 119 and 149 were described to result from fragmentation at the allyl position of unsaturated anacardic acids [215, 216]. Compound 63 was assigned as (15:0)-anacardic acid after comparing its retention time, UV absorption and mass spectra including the characteristic loss of CO_2 (44 Da), and the product ion at *m/z* 106 with those of an authentic standard. The identification of further anacardic acids was based on UV and mass spectra including accurate mass measurements and the corresponding molecular formulas that were consistent with previously published data of Jerz et al. [215] As shown in Figure 3.3, the most abundant representatives were (13:0)-, 59, (13:1)-, 58, (15:0)-, 63, (15:1)-, 60, and (17:1)-anacardic acids, 64, being in agreement with an earlier study on phenolic acids of *P. vera* [64]. Their elution occurred later with increasing chain length and decreasing degree of saturation as previously shown [215]. Based on the oxidative degradation of these major compounds, Yalpani and Tyman [64] determined the localization of the double bond of the unsaturated (13:1)-, (15:1)-, and (17:1)-anacardic acids to be at the eight position of the alkyl chain for monounsaturated anacardic acids from green pistachio hull (named as "outer green shell" in their study). We assume that this allocation of the double bonds may also be valid for our results. Besides confirming these major compounds, our study is the first report of the occurrence of six minor anacardic acids, namely (16:1)-, (13:2)-, (11:0)-, (15:3)-, (17:2)-, and (17:0)-anacardic acids. In contrast to pistachio kernels [216], cardanols, decarboxylated derivatives of anacardic acids, were not detected in pistachio hulls. Interestingly, anacardic acids are currently considered to be chemotaxonomic markers of the Anacardiaceae [5], consistently occurring in cashew nuts and shells [215], as well as in mango [108]. These findings might be of interest for the above mentioned discussion on the assignment of the genus *Pistacia*.

3.3.1.4. Minor compound

Compound five with a parent ion $[M\text{-}H]^-$ at *m/z* 153 was tentatively identified as protocatechuic acid after comparison of its analytical data with those of an authentic standard. Protocatechuic acid has been reported in pistachio hull before [11, 12].

3.3.2. Comparison of Red and Green Hulls

When red and green type pistachio hulls were compared (Figure 3.3), virtually identical phenolic profiles were observed, except for the anthocyanins that only occurred in red hulls. These findings indicate that green and red pistachio hulls may be utilized without separation as a source of phenolic compounds. However, further research on the quantity and contribution of each class of phenolic compounds to the biological activity of red and green pistachio hull extracts should be performed to determine technologically optimal recovery strategies for phenolics from different types of pistachio hulls.

3.4. CONCLUSION

In conclusion, the complex phenolic profiles of dried red and green *P. vera* hulls were characterized in this study to provide basic knowledge for their future utilization. Apart from anthocyanins that are characteristic of red hulls, phenolic constituents of pistachio hulls may largely be grouped in three major phenolic classes: gallotannins, flavonoids and anacardic acids. The identity of the gallotannins of pistachio hull was elucidated for the first time, revealing the presence of galloyl hexoses with up to nine galloyl units, and galloyl quinic and shikimic acids with up to three galloyl units. Pistachio hulls also contained glycosides of flavonoids such as quercetin glycosides and a cyanidin 3-*O*-*β*-D-galactopyranoside. Furthermore, anacardic acids, a distinct class of polar phenolic lipids, were identified and may be useful as chemotaxonomic markers. In brief, a wide range of phenolic compounds was present in pistachio hulls, ranging from simple (gallic acid) to very complex ones (gallotannins) and from polar/mid-polar (gallotannins and flavonoids) to amphiphilic (anacardic acids) ones. Thus, pistachio hull represents an interesting source for the production of multifunctional phenolic extracts. Further research on the extraction and isolation of these compounds and the determination of their relation with attributed biological functions should be encouraged.

4. DETERMINATION OF PISTACHIO HULL PHENOLICS BY HPLC-DAD AND UHPLC-PDA-ELSD AFTER ULTRASOUND-ASSISTED EXTRACTION [2]

4.1. INTRODUCTION

Pistachio (*P. vera* L.) hull (exo- and mesocarp) is the main by-product of pistachio processing as it constitutes a high percentage (21 per cent) of dry pistachio drupe [22]. Several utilization strategies for pistachio hull have been previously explored, such feed supplement [217], raw material for biofuel or biogas production [55, 56], adsorbent for removal of toxic contaminants (e.g. cyanide) from wastewater [35], and as an ingredient of gelled food products [219]. However, to date, the hulls still accrue in enormous amounts and are considered as waste without any commercial value. Recently, pistachio hull has gained attention as a source of phenolic constituents with antioxidant and antimicrobial bioactivity [11, 13, 14]. Consequently, the hulls and particularly the phenolics derived thereof might be used in the food and nutraceutical industry as a natural alternative to synthetic antioxidants [14] or functional and nutraceutical ingredients with cytoprotective [11, 220] and anti-inflammatory activities [31]. Pistachio hulls were previously shown to contain a large diversity of phenolic compounds, including gallotannins, flavonoids, and anacardic acids. Among these, gallic acid, monogalloyl glucose, pentagalloyl glucose, quercetin galactoside, quercetin glucoside, and anacardic acids represent the most abundant constituents [22]. When using common technical extraction solvents such as methanol, ethanol, and acetone, most of these phenolic constituents are inevitably extracted together in variable yields depending on the solubility of compounds in the solvent system of choice [105].

---

[2] Reprinted from Journal of Food Composition and Analysis, 62, Erşan, S.; Güçlü Üstündağ, Ö.; Carle, R.; Schweiggert, R. M., Determination of pistachio (*Pistacia vera* L.) hull (exo- and mesocarp) phenolics by HPLC-DAD-ESI/MS^n and UHPLC-DAD-ELSD after ultrasound-assisted extraction, 103-114, Copyright 2017, with permission from Elsevier.

Thus, in past studies, an often poorly characterized mixture of phenolics has been used and might have been responsible for the widely differing and partly controversial reports on the biological activities attributed to so-called crude pistachio hull extracts.To date, most of the available analytical methods are based on the separate extraction and/or analysis of each of the individual phenolic groups, e.g. gallotannins [107], flavonoids [6], anacardic acids [31], or alkylresorcinols [108]. Analytical methods for quantitation of all different phenolic classes present in pistachio hulls, from the very hydrophilic gallic acids to the amphiphilic anacardic acids, are urgently needed to allow a better interpretation of the biological activity of pistachio hull extracts. The complex composition has been shown to require long HPLC gradient times (112 min) achieving acceptable separations [22]. Therefore, UHPLC with sub-two µm columns might be a suitable technique to enable high sample throughputs with short chromatographic run times at equal or even enhanced peak resolutions [221].

Therefore, in continuation of our previous comprehensive study on the identification of pistachio hull phenolics [22], the first aim of the present work was to develop a simultaneous and quantitative extraction procedure for the determination of gallotannins, flavonoids and anacardic acids from pistachio hull. First, exhaustive extraction conditions were targeted using different extraction solvents, sample/solvent ratios, and repetitive extraction cycles. Second, two alternative analytical methods, namely HPLC-DAD-MS^n and UHPLC-PDA-ELSD, were presented, and compared in terms of their separation and detection capabilities, and their detection and quantitation limits. Finally, the optimized extraction procedure was combined with the presented highly peak resolving HPLC-DAD method, validated, and applied to four different pistachio hull samples from three different varieties.

4.2. MATERIALS AND METHODS

4.2.1. Chemicals

Gallic acid monohydrate (≥98 per cent), protocatechuic acid (≥97 per cent), penta-*O*-galloyl-*β*-D-glucose (≥96 per cent), and (15:0)-anacardic acid (≥97 per cent) were from Sigma–Aldrich Chemie (Steinheim, Germany), *β*-glucogallin (1-*O*-galloyl-*β*-D-glucopyranose) (≥94 per cent) from PhytoLab (Vestenbergsgreuth, Germany), quercetin 3-*O*-glucuronide (≥95 per cent), quercetin 3-*O*-glucoside (≥99 per cent), quercetin 3-*O*-galactoside (≥98 per

cent), myricetin 3-*O*-galactoside (≥99 per cent) and cyanidin 3-*O*-*β*-D-galactopyranoside (≥97 per cent) from Extrasynthèse (Genay Cedex, France). HPLC grade methanol was purchased from VWR (Darmstadt, Germany). Analytical or higher grades of hexane, diethyl ether, dichloromethane, ethyl acetate, acetone, and ethanol, acetic acid, and hydrochloric acid (HCl, 37 per cent) were from Merck (Darmstadt, Germany). Deionized water was used throughout the study.

4.2.2. Sample and Sample Preparation

Four types of dried pistachio drupes were from the cultivars ‘Uzun’ (red and green), ‘Siirt’ and ‘Ohadi’, representing commercially used varieties for pistachio production in Turkey. All samples were harvested in 2013 and obtained from a local pistachio processor (Gaziantep, Turkey). Pistachios from cv. ‘Uzun’ are available for processing in the two different maturation stages (i) early harvested, i.e., green drupes, and (ii) fully mature, i.e., red drupes, depending on the purpose of use as described before [22]. In contrast, cultivars ‘Siirt’ and ‘Ohadi’ pistachios are only harvested and processed in fully mature form. Their growth, processing and storage conditions to yield drupes with a moisture content of three-to-five per cent have been reported previously [22]. Moisture contents of the drupes, as determined with an infrared moisture analyzer MA 40 (Sartorius, Göttingen, Germany), were 4.6 per cent, 4.3 per cent, 4.8 per cent and 5.0 per cent for cv. ‘Uzun’ red, Uzun’ green, ‘Ohadi’ and ‘Siirt’, respectively. The average drupe weight was determined by weighing three replicates of 100 drupes randomly sampled from 1000 g of pistachio drupes, being ca. 0.99, 0.98, 1.00, and 1.27 g/drupe containing 0.20, 0.15, 0.18, 0.19 g dry hull/drupe of cv. ‘Uzun’ red, Uzun’ green, ‘Ohadi’ and ‘Siirt’, respectively. Pistachio hulls were separated manually, finely ground using an A11 laboratory mill (IKA, Staufen, Germany), and stored at -20 °C until analyses. Hulls from cv. ‘Uzun’ red pistachio were used throughout method development and validation.

4.2.3. Phenolic Extraction

4.2.3.1. Ultrasound-assisted extraction of phenolic compounds

4.2.3.1.1. Optimization of extraction conditions

A detailed description of the extraction protocol is found below in the following section. Following this basic procedure, the effect of extraction solvent polarity and acidity, the number of repeated extractions, various sample-to-solvent ratios, and the inclusion of a pre-extractive water soaking step on the extraction yields of phenolic compounds was examined aiming at a simultaneous and exhaustive extraction of phenolic compounds from pistachio hull matrix.

The studied extraction solvents were hexane, diethyl ether, dichloromethane, ethyl acetate, acetone, methanol, and ethanol as well as aqueous solutions of methanol (methanol/water, 80/20 and 50/50, v/v), ethanol (ethanol/water, 80/20, v/v), acetone (acetone/water, 80/20, v/v), and pure water. The sample-to-solvent ratio was varied from one-to-five to one-to-20 (w/v). Furthermore, the methanol- and water-based solvents were acidified to yield the following extraction solvents methanol/water/formic acid (80/19/1, v/v/v), methanol/water/formic acid (80/15/5, v/v/v), methanol/water/acetic acid (80/19/1, v/v/v), methanol/water/HCl (80/19.9/0.1, v/v/v). After selecting the highest-yielding extraction solvent (methanol/water/formic acid (80/19/1, v/v/v) based on five-fold repeated extractions, the effect of repeated extractions was examined by collecting and analyzing each fraction separately. The yields (peak areas) obtained for each compound after each extraction cycle were summed up, set to 100 per cent, and then compared to the previous extraction cycles in a cumulative manner. The influence of sample soaking on the extraction yields was tested by adding one mL of water to the sample prior to extraction and soaking for five min. Results were evaluated based on the extraction yields of four selected representative major compounds present in high amounts, i.e., gallic acid, penta-*O*-galloyl-*β*-D-glucose, quercetin 3-*O*-galactoside/glucuronide, and (17:1)-anacardic acid, on HPLC-DAD peak area basis. Quercetin 3-*O*-galactoside and quercetin 3-*O*-glucuronide were determined together due to insufficient peak resolution.

4.2.3.1.2. Extraction procedure

Ground pistachio hulls (250 mg or 1000 mg) were combined with five mL of extraction solvent and probe sonicated (MS73 microtip, Sonopuls UW 3100, Bandelin Electronics, Berlin, Germany) for 30 s at 70 per cent amplitude while cooling the sample in an ice bath. After centrifugation (Labofuge 200; Heraeus, Hanau, Germany) at 3000 rpm (1233 x *g*) for three min, the supernatant was collected, and the solid residues were re-extracted up to four times as described above. Extracts were combined and evaporated to dryness in *vacuo* at 30 °C. Dried extracts were re-dissolved in pure methanol containing one per cent formic acid (v/v), using the same volume that has been used for the extraction in total, except for those samples that had been extracted with aqueous solvents, where dried extracts were made up with 80 per cent (v/v) aqueous methanol containing one per cent formic acid (v/v). The re-dissolved extracts were membrane-filtered through regenerated cellulose filters (0.45 µm pore size, Macherey-Nagel, Düren, Germany) into amber glass vials prior to HPLC analysis.

4.2.3.2. *Conventional stirring-based solvent extraction*

For comparison to ultrasound-assisted extraction, ground pistachio hull (250 mg) was combined with five mL of extraction solvent allowing the most exhaustive extraction, i.e. methanol/water/formic acid (80/19/1, v/v/v), at a sample-to-solvent ratio of 1/20 (w/v). Subsequently, the headspace of the extraction flask was flushed with nitrogen to prevent oxidation, and extraction was conducted under continuous magnetic stirring (ca. 600 rpm) for 30 min. After centrifugation at 3000 rpm (1233 x *g*) for three min, the supernatant was collected and the solid residue was re-extracted two more times as described above. The combined supernatants were membrane-filtered through regenerated cellulose filters (0.45 µm pore size, Macherey-Nagel, Düren, Germany) into amber glass vials prior to HPLC analysis.

4.2.4. Chromatographic Determinations

4.2.4.1. HPLC-DAD-ESI/MS^n analysis

The phenolic analysis was carried out using an HPLC-DAD-ESI/MS^n system as previously described [22] using a Kinetex C_{18} core-shell reversed-phase column (250 mm × 4.6 mm i.d., five µm particle size, Phenomenex, Aschaffenburg, Germany) with a SecurityGuard Ultra C_{18} guard column (4.6 mm × two mm i.d.) of the same material. In brief, water was used as eluent A and methanol as eluent B, both containing one per cent (v/v) formic acid. The gradient was as follows: isocratic at two per cent B for 10 min, from two-to-37 per cent B in 27 min, isocratic at 37 per cent B for five min, from 37 to 40 per cent B in 18 min, from 40 to 60 per cent B in 10 min, from 60 to 100 per cent B in 20 min, isocratic at 100 per cent for 14 min, from 100 to two per cent B in one min, and isocratic at two per cent B for seven min. Total run time was 112 min at a flow rate of one mL/min. The column temperature was 35 °C. Injection volume was three µL. UV/Vis absorption spectra were recorded in the range of 200-600 nm. Specific monitoring was performed at 280 nm (gallotannins), at 310 nm (anacardic acids), at 350 nm (flavonols), and 520 nm (anthocyanins).

4.2.4.2. UHPLC-DAD-ELSD analysis

An Acquity UPLC H-class system (Waters, Milford, MA, USA) including serially connected eλ DAD and ELS detector was used. The column used was an Acquity UPLC BEH (ethylene-bridged-hybrid) C_{18} (150 mm x 2.1 mm i.d., 1.7 µm particle size, Waters, Milford, MA, USA) with an Acquity UPLC BEH C_{18} VanGuard precolumn (five mm x 2.1 mm i.d., 1.7 µm particle size). The column was operated at 35 °C. The same binary solvent system as described for HPLC was used. UHPLC gradient program was as follows: isocratic at two per cent B for one min, from two-to-37 per cent B in 7 min, from 37 to 40 per cent B in 5.2 min, from 40 to 100 per cent B in 4.8 min, isocratic at 100 per cent for six min, from 100 to two per cent B in one min, and isocratic at two per cent B for five min. Total run time was 30 min at flow rate of 0.3 mL/min. Injection volume was 0.5 µL. UV/Vis spectra were collected in the range of 210-700 nm. For ELS detection, nebulizer gas (nitrogen) pressure

and drift temperature were set at 40 psi and 100 °C, respectively, while detector gain was set at 200.

4.2.4.3. Compound identification and quantitation

Compound identification was accomplished by comparing retention times, UV and mass spectra of the detected peaks with those of standard compounds. When authentic standards were unavailable, identification was based on the comparison of HPLC-DAD-MS^n data, peak elution orders and peak distribution with those of our previous study [22] as described in Section 3.3.

Quantitative analyses in UV/Vis based detection systems were accomplished using linear calibration curves generated with authentic standards of gallic acid, protocatechuic acid, penta-*O*-galloyl-*β*-D-glucose, quercetin 3-*O*-galactoside, cyanidin 3-*O*-*β*-D-galactopyranoside, and (15:0)-anacardic acid. Stock solutions of authentic standards (one mg/mL) were prepared in pure methanol, except cyanidin 3-*O*-*β*-D-galactopyranoside, which was prepared in methanol/HCl (99.9/0.1, v/v). Up to seven dilutions were made using methanol/water/formic acid (80/19/1, v/v/v) in the respective concentration range (Table 4.1). Three independent external calibration curves were prepared for each compound. When authentic standards were unavailable, structurally related substances, i.e., gallic acid for gallic acid derivatives, quercetin 3-*O*-galactoside for flavonols, and (15:0)-anacardic acid for other anacardic acids, were used for quantitation, including molecular-weight-correction factors obtained separately for each compound by dividing the molecular weight of the compound of interest by that of the respective authentic standard compound. Total phenolic contents represented the sum of gallotannins, flavonoids, anacardic acids and protocatechuic acid.

For quantitation by ELS detection, the same authentic standards as described for UV/Vis analyses were used. However, a linear relationship between analyte concentration and the signal response had to be established by logarithmic conversion of both parameters.

Peak resolution (R_s) was calculated using the following equation,

$$R_s = \frac{1.18 \times (t_1 - t_2)}{(w_{0.5,1} + w_{0.5,2})} \quad (4.1)$$

where t_1 and t_2 are retention times of respective peak maxima, and $w_{0.5,1}$ and $w_{0.5,2}$ are peak widths at half height [222].

4.2.5. Method Validation

The extraction procedure and the HPLC method were validated in terms of calibration linearity, limit of detection (LOD), limit of quantitation (LOQ), extraction recovery, and intra- and inter-day repeatability.

Calibration linearity was expressed based on the coefficient of determination (R^2) after linear regression analysis. Following ICH guidelines [223], LOD and LOQ were calculated using the standard deviation of the y-intercept (σ) and the slope of regression lines (S) based on three independent replicates of calibration curves for UV/Vis detection using the following formula.

$$\text{LOD} = \frac{3.3 \times \sigma}{S} \quad (4.2)$$

$$\text{LOQ} = \frac{10 \times \sigma}{S} \quad (4.3)$$

In the case of ELS detection, LOD and LOQ values were estimated using signal-to-noise ratios of three-to-one and ten-to-one, respectively.

Extraction recovery was determined by adding authentic standards at an expected high and low level (Table 4.2) to ground hull prior to extraction. Briefly, a methanolic solution containing the calculated amount of standard compound was transferred to an extraction tube and the solvent was evaporated under a gentle nitrogen stream. Subsequently, the pistachio hull sample was weighed into the extraction tube and the extractions were performed according to the procedure described above (Section 4.2.3.1).

Table 4.1. Linear concentration range, detection parameters, retention times, the slope of calibration curves, limit of detection (LOD) and limit of quantitation (LOQ) of reference standards as determined by HPLC and UHPLC.

Peak no.	**Analyte**	**Conc. range (mg/L)**	**Detection**	**Retention time (min)**		**Slope** [1]		**LOD (ng on column)**		**LOQ (ng on column)**	
				HPLC	**UHPLC**	**HPLC (mAU*min*L*mg^{-1})**	**UHPLC (mV*s*L*mg^{-1})**	**HPLC**	**UHPLC**	**HPLC**	**UHPLC**
2	Gallic acid	5.7-180.9	UV/Vis (280 nm)	6.7	3.2	9.3	4.1	3.0	17.6	9.0	53.4
5	Protocatechuic acid	1.6-50.0	UV/Vis (260 nm)	12.9	5.5	11.3	5.8	2.3	0.80	6.8	2.4
24	Cyanidin 3-*O*-*β*-D-galactopyranoside	0.7-23.2	UV/Vis (520 nm)	31.3	8.6	7.1	3.8	0.6	2.5	1.8	7.5
34	Penta-*O*-galloyl-*β*-D-glucose	5.8-185.8	UV/Vis (280 nm)	38.0	10.4	5.1	4.6	4.9	4.3	14.7	13.1
36	Quercetin 3-*O*-galactoside	3.1-100	UV/Vis (350 nm)	40.9	12.1	5.5	3.4	2.2	5.3	6.8	16.1
56	(15:0)-Anacardic acid	15.6-500	UV/Vis (310 nm)	92.0	22.1	1.4	0.7	11.4	10.8	34.4	32.6
		62.5-500	ELS [2]	-	22.1	-	0.8	-	15.6	-	62.5

[1] Means of three independent replicates of standard curve.
[2] ELS: evaporative light scattering.

Table 4.2. Recovery and repeatability of the developed ultrasound-assisted extraction and subsequent HPLC-DAD analysis.

Peak no.	**Analyte**	**Concentration spiked (mg/L)**	**Recovery (per cent)**	**Repeatability (CV per cent)[1]**	
				Intra-day (n=6)[2]	**Inter-day (n=4)[3]**
2	Gallic acid	24.1	98.4 ± 2.8	<5.4	5.4
		2.4	99.9 ± 5.6		
5	Protocatechuic acid	4.0	98.0 ± 2.5	<5.6	4.1
		-	-		
24	Cyanidin 3-*O*-*β*-D-galactopyranoside	3.1	99.5 ± 3.6	<3.8	3.6
		0.3	96.3 ± 5.1		
34	Penta-*O*-galloyl-*β*-D-glucose	24.8	95.0 ± 1.5	<10.1	12.4
		-	-		
36	Quercetin 3-*O*-galactoside	33.3	98.7 ± 1.2	<3.9	0.4
		3.3	98.8 ± 6.6		
56	(15:0)-Anacardic acid	106	101 ± 8.0	<3.7	2.9
		10.6	98.7 ± 4.6		

Results were expressed as mean ± standard deviation of three independent replicates.
[1] CV: coefficients of variation.
[2] Six determinations on one day.
[3] Six determinations each on four days within one month (n=four days)

Intra-day repeatability was assessed by six independent repeated determinations within one day, while inter-day repeatability was obtained from six determinations each on four different days within a month. Additionally, the stability of the phenolic compounds present in the extracts obtained after extraction was studied during storage at room temperature for 24 and 48 hours, and at low-temperature conditions (-20 °C and -80 °C) for 7 months with three independent replicates.

4.2.6. Statistical Analysis

All determinations were performed in triplicate and all data were expressed as a mean ± standard deviation. Significant differences of means ($P < 0.05$) were determined using analysis of variance (ANOVA) and Tukey's test for different independent samples using Minitab® 17.3.1 (State College, PA, USA).

4.3. RESULTS AND DISCUSSION

4.3.1. Simultaneous Extraction of Pistachio Hull Phenolics

4.3.1.1. Selection of extraction solvent

According to our previous study [22], the main phenolic compounds to be expected in pistachio hulls exhibit a wide range of polarity, ranging from highly water-soluble gallotannins and flavonoid glycosides to the less polar anacardic acids. In accordance with our earlier report, a selection of extraction solvents with widely different polarities was chosen for the screening as described above (Section 4.2.3.1) to compare the extractability of free phenolics from pistachio hulls.

As shown in Figure 4.1, apolar solvents including hexane, diethyl ether, ethyl acetate, and acetone were effective to selectively extract anacardic acids. Dichloromethane and ethanol allowed to additionally recover low amounts of gallic acid and quercetin 3-*O*-galactoside/glucuronide. Methanol, acetone/water (80/20, v/v), ethanol/water (80/20, v/v), and methanol/water (80/20, v/v) significantly increased the extracted amounts of gallic acid, quercetin 3-*O*-galactoside/glucuronide, and penta-*O*-galloyl-*β*-D-glucose ($P < 0.05$).

However, the yield of (17:1)-anacardic acid was similar to that of the aforementioned nonpolar solvents only when using methanol/water (80/20, v/v) or pure methanol ($P > 0.05$). With further increases in solvent polarity, e.g., by using methanol/water (50/50, v/v) or pure water, a substantial decrease in the yield of (17:1)-anacardic acid was observed ($P < 0.05$), presumably, due to the limited solubility of anacardic acids in aqueous solutions [224].

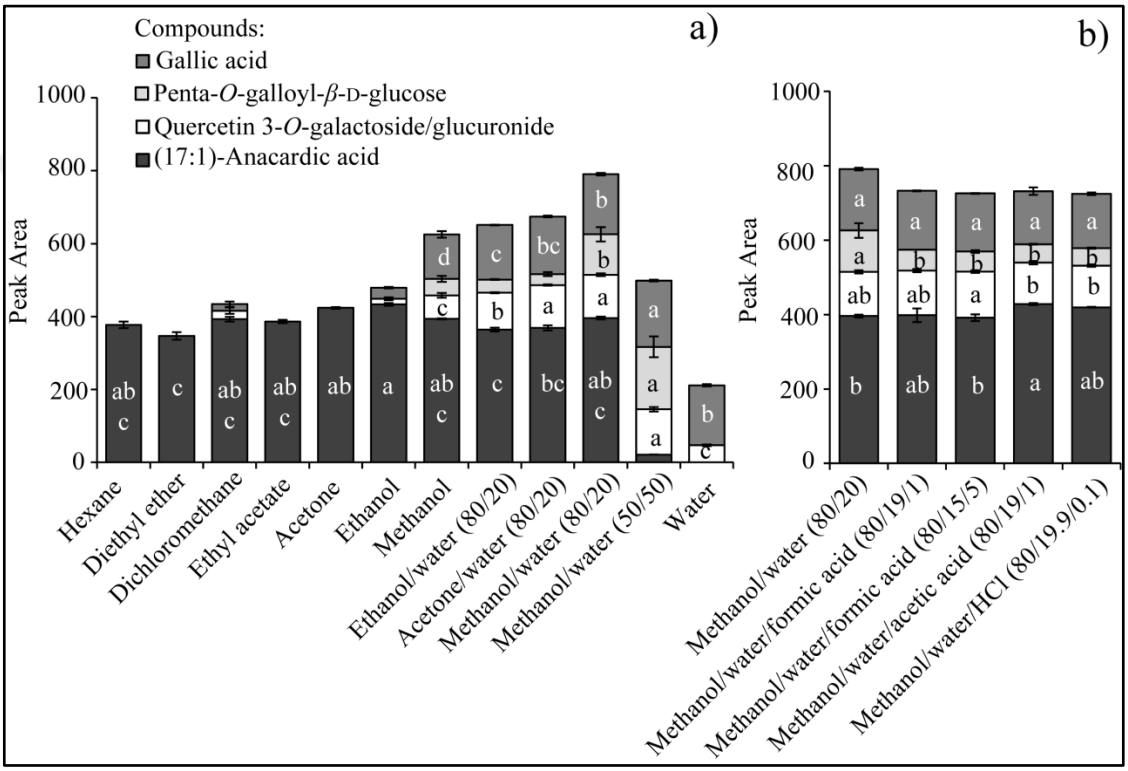

Figure 4.1. Extraction yields of major phenolic compounds from pistachio hulls of cv. 'Uzun' red (sample-to-solvent ratio of 1/20, w/v) using extraction solvents of different polarity (a) and acidity (b). Different letters indicate significant differences of means between the yielded peak areas of the corresponding compounds ($P < 0.05$). Values expressed as the mean ± standard deviation derived from triplicate (n = 3) analyses.

The highest extraction yields of quercetin 3-*O*-galactoside/glucuronide were observed when using aqueous solutions of acetone (80/20, v/v) and methanol (80/20 and 50/50, v/v), followed by ethanol/water (80/20, v/v), pure methanol and water ($P < 0.05$). Gallic acid and penta-*O*-galloyl-*β*-D-glucose were best extracted with methanol/water (50/50, v/v), followed

by methanol/water (80/20, v/v) ($P < 0.05$). Allowing high yields of all detected phenolic compounds, methanol/water (80/20, v/v) was selected for further optimization.

4.3.1.2. Effect of solvent acidification

Based on the findings of Section 4.3.1.1, a series of acidified methanol/water mixtures, namely methanol/water/formic acid (80/19/1, v/v/v), methanol/water/formic acid (80/15/5, v/v/v), methanol/water/acetic acid (80/19/1, v/v/v), and methanol/water/HCl (80/19.9/0.1, v/v/v), was compared to the non-acidified mixture (80/20, v/v) in terms of extraction yields (Figure 4.2). Extraction yields of gallic acid, quercetin 3-*O*-galactoside/glucuronide, and (17:1)-anacardic acid remained unchanged by acid addition as compared to non-acidified methanol/water (80/20, v/v) ($P < 0.05$). However, extraction yield of penta-*O*-galloyl-*β*-D-glucose was significantly higher in non-acidified methanol/water (80/20, v/v) extracts (pH 6.4), which might be attributed to the spontaneous hydrolysis of highly galloylated gallotannins in the aqueous methanolic environment around pH six to form methyl gallate/digallate and penta-*O*-galloyl-*β*-D-glucose [225, 226]. In agreement, the occurrence of trace amounts of artifacts (i.e., methyl gallate/digallate) was observed in sample chromatograms of non-acidified extracts. Therefore, although extraction yields in penta-*O*-galloyl-*β*-D-glucose were slightly lower, the slightly acidified mixture methanol/water/formic acid (80/19/1, v/v/v) was selected for the extraction of pistachio hull phenolics. Thereby, a further separation-enhancing acidification step of the injection solvent prior to HPLC analyses was redundant.

4.3.1.3. Number of extraction cycles and sample-to-solvent ratios

Using the selected solvent mixture methanol/water/formic acid (80/19/1, v/v/v), the necessity of up to five repetitive extraction cycles was evaluated at sample-to-solvent ratios of one-to-five and one-to-20 (w/v). As shown in Figure 4.2, extraction yields of ≥95 per cent were achieved after three extraction cycles for gallic acid, penta-*O*-galloyl-*β*-D-glucose, quercetin 3-*O*-galactoside/glucuronide and (17:1)-anacardic acid when the sample-to-solvent ratio of one-to-20 (w/v) was applied. For comparison, at a sample-to-solvent ratio of one-to-five (w/v), cumulative yields reached only to ≥76 per cent and ≥90 per cent after three

and four extraction cycles, respectively (data not shown). Thus, three extraction cycles at a sample-to-solvent ratio of one-to-20 (w/v) were found to be sufficient for an exhaustive and rapid extraction.

4.3.1.4. Effect of soaking step

Soaking the ground pistachio hull sample with water prior to the solvent extraction was tested because sample hydration and softening of plant material have been previously reported to be required for the analyses of dried samples of legumes and cereals [227, 228]. In our study, sample soaking resulted in insignificant differences in gallic acid, penta-*O*-galloyl-*β*-D-glucose, and quercetin 3-*O*-galactoside/glucuronide yields ($P > 0.05$) as compared to those obtained with a non-soaked sample. However, (17:1)-anacardic acid yield was significantly lower than without soaking (430 ± 20 versus 660 ± 8.0 AU * min, respectively, $P < 0.05$). Thus, sample soaking was omitted from the final extraction protocol.

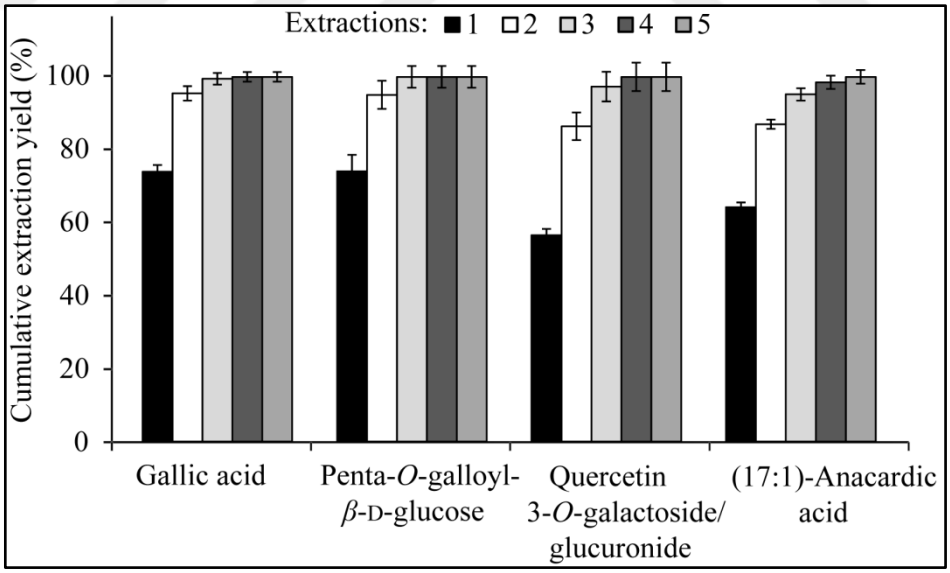

Figure 4.2. The influence of the number of repetitive extraction cycles on the extraction yields of major phenolic compounds from pistachio hulls of cv. 'Uzun' red (sample-to-solvent ratio of 1/20, w/v) using methanol/water/formic acid (80/19/1, v/v/v) as an extraction solvent. Values expressed as the mean ± standard deviation derived from triplicate (n = 3) analyses.

4.3.1.5. Optimized ultrasound-assisted extraction procedure

In brief summary, the optimized extraction method was based on the three-fold repeated extraction of ground pistachio hulls with methanol/water/formic acid (80/19/1, v/v/v) at a sample-to-solvent ratio of 1/20 (w/v) under probe-sonication. The developed procedure allows the simultaneous extraction of gallic acid, gallotannins, flavonoid glycosides and anacardic acids, making previously reported sequential extractions with solvents of different polarity unnecessary.

4.3.2. Comparison of Ultrasound-Assisted and Conventional Extraction Methods

Conventional extraction procedures are often based on stirring for relatively long times (from 30 min to several hours), thereby increasing the risk of analyte degradation [105]. However, stirring-based extractions are still in common use, since they are simple to apply, without requiring specialized equipment, and often easy to scale-up [57]. When comparing the ultrasound-assisted extraction method presented in this study with a conventional stirred extraction, the differences in extraction yields of gallic acid, penta-*O*-galloyl-*β*-D-glucose, quercetin 3-*O*-galactoside/glucuronide and (17:1)-anacardic acid were insignificant after three cycles of stirring extraction as compared to three cycles of ultrasound-assisted extraction (data not shown). Nevertheless, ultrasound-assisted extraction is still 20-fold faster (three x 30 s = 1.5 min total extraction time) than even one sole repetition of the stirring extraction (30 min).

4.3.3. Comparison of HPLC with UHPLC Separation and of UV- with ELSD Detection of Phenolic Compounds from Pistachio Hull

Chromatographic separations of pistachio hull phenolics on a core-shell C_{18} column (five µm particle size) under HPLC conditions and on a porous ethylene-bridged hybrid C_{18} column (1.7 µm particle size) under UHPLC conditions are shown in Figure 4.3.

The developed UHPLC method presented in the current study (30 min) was 3.7 times faster than the HPLC method (in 112 min), consuming 92 per cent less solvent. Nevertheless, separation efficiency in terms of the number of major peaks and peak profiles was highly

similar on both the core-shell particle HPLC column and the sub-2-µm porous particle UHPLC column with the following slight differences. Peak 34, one of the main peaks (for peak identification, see below and Table 4.3 and 4.4), was separated from minor peak 31 in the UHPLC system with R_S = 1.28, while it was co-eluted in the HPLC system with peak 35 (R_S = 0.62). However, UHPLC resolutions were observed to be inferior for peaks 53 and 54 (*Rs* = 0.97), and peak 56 and 57 (*Rs* = 1.2) as compared to HPLC conditions, where the resolution of the mentioned peaks was greater than 1.5.

Although smaller column particle sizes in UHPLC have often been associated with improved peak resolutions [229], core-shell columns with larger particle sizes have also been shown to allow comparable or sometimes even better peak resolutions [133, 230] without the high cost, the high maintenance requirements, and the often longer offline times of UHPLC systems. In conclusion, both HPLC and UHPLC methods appeared to be suitable for the separation of pistachio hull phenolics under consideration of their above-mentioned strengths and weaknesses.

The developed UHPLC method presented in the current study (30 min) was 3.7 times faster than the HPLC method (in 112 min), consuming 92 per cent less solvent. Nevertheless, separation efficiency in terms of the number of major peaks and peak profiles was highly similar on both the core-shell particle HPLC column and the sub-2-µm porous particle UHPLC column with the following slight differences. Peak 34, one of the main peaks (for peak identification, see below and Table 4.3 and 4.4), was separated from minor peak 31 in the UHPLC system with R_S = 1.28, while it was co-eluted in the HPLC system with peak 35 (R_S = 0.62). However, UHPLC resolutions were observed to be inferior for peaks 53 and 54 (*Rs* = 0.97), and peak 56 and 57 (*Rs* = 1.2) as compared to HPLC conditions, where the resolution of the mentioned peaks was greater than 1.5. Although smaller column particle sizes in UHPLC have often been associated with improved peak resolutions [229], core-shell columns with larger particle sizes have also been shown to allow comparable or sometimes even better peak resolutions [133, 230] without the high cost, the high maintenance requirements, and the often longer offline times of UHPLC systems. In conclusion, both HPLC and UHPLC methods appeared to be suitable for the separation of pistachio hull phenolics under consideration of their above-mentioned strengths and weaknesses.

Identification of 58 different phenolics as listed in Table 4.3 was achieved in HPLC chromatograms by comparing their retention times, UV and mass spectra with those of

standard compounds and our previous study [22] where a detailed description of peak identification was provided.

Peak identification in UHPLC chromatograms was carried out by the aid of authentic reference compounds which allowed the identification of *β*-glucogallin (1), gallic acid (2), protocatechuic acid (5), cyanidin 3-*O*-*β*-D-galactopyranoside (24), myricetin 3-*O*-galactoside (32), penta-*O*-galloyl-*β*-D-glucose (34), quercetin 3-*O*-galactoside (36), quercetin 3-*O*-glucuronide (37) and quercetin 3-*O*-glucoside (38), and (15:0)-anacardic acid (56).

Since UHPLC-MS signals were unavailable to us, a comparison of UV spectra, relative peak heights, and elution orders of peaks of the HPLC-DAD-MS^n analyses with those of the UHPLC-DAD system was used for tentative identification of other peaks when authentic standards were unavailable. Flavonols (peak 28, 31-33, 35-38, 42, 43, 46), and anacardic acids (peak 52-58) having characteristic UV/Vis absorption around 350 nm and 310 nm, respectively, were identified in the UHPLC chromatogram by following the above-mentioned approach. In total, based on the above-mentioned tentative peak identifications, our UHPLC system allowed sufficient peak separation and resolution to quantitate all major and most minor phenolics, accounting to more than 90 per cent (w/w) of the total HPLC-quantifiable pistachio hull phenolics based on the results given in Table 4.4.

Besides comparing separation efficiencies, the signals obtained by the UV/Vis detection systems used were compared with those of the ELS detection in UHPLC system. Interestingly, ELSD was limited to the detection of only five major anacardic acids (peaks 52-54, 56, 57) among all phenolics present in pistachio hull extracts (Figure 4.3), where (13:0)-anacardic acid produced the highest ELSD response. Thus, the use of ELSD for quantitation of anacardic acids appears to be inferior to that of UV detection, particularly, as the later one exhibited lower LOD and LOQ values than those obtained by ELS detection (Table 4.1 and Section 4.3.4).

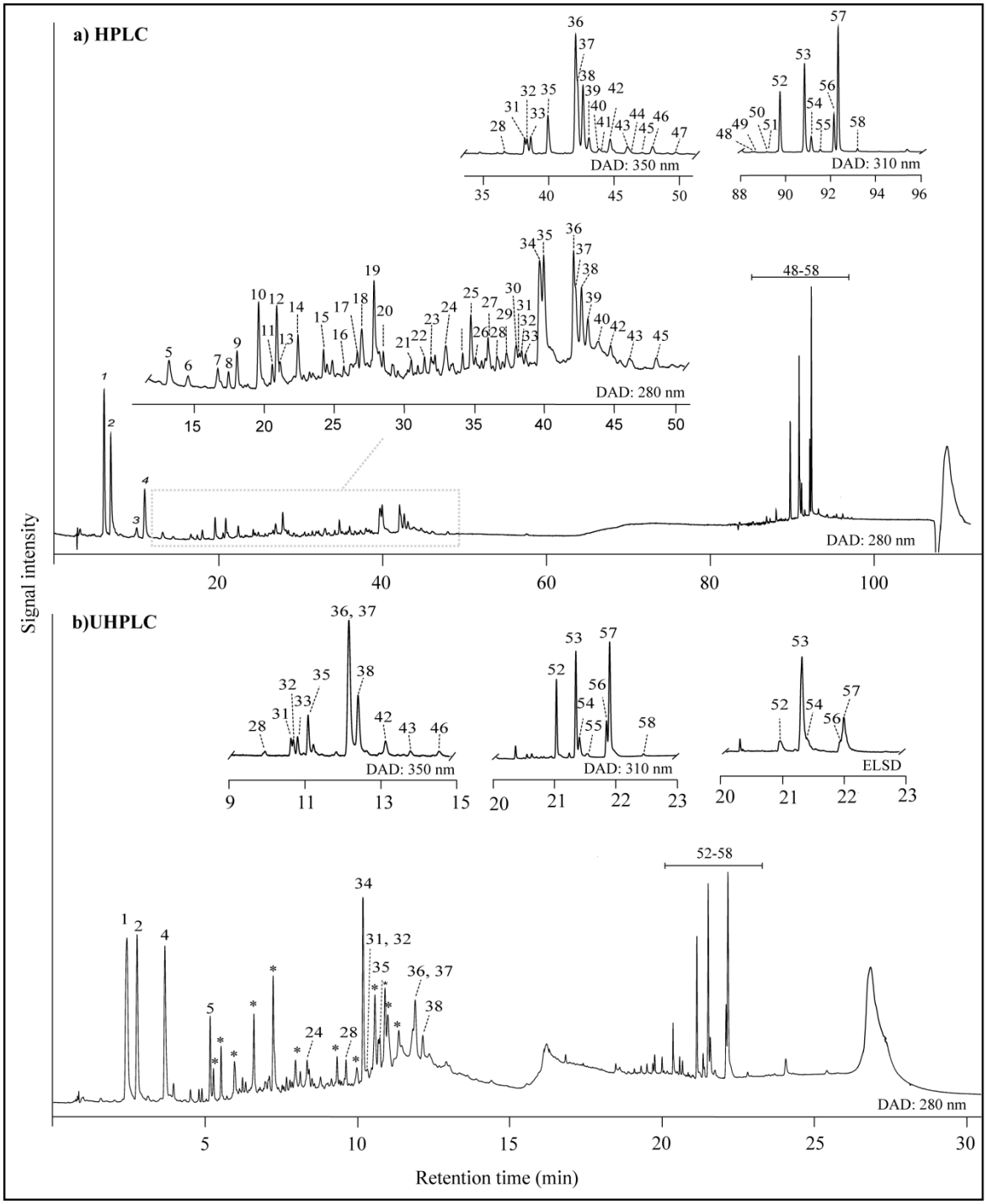

Figure 4.3. HPLC and UHPLC separation of methanol/water/formic acid (80/19/1, v/v/v) extract of cv. 'Uzun' red pistachio hull. Peak assignments are shown in Table 4.4. Peaks marked with an asterisk (*) displayed UV/Vis absorption maxima between 272 and 278 nm, supporting their cross-identification as gallic acid derivatives by comparison to HPLC-DAD-MS^n data (see Table 4.3).

Table 4.3. Retention times and HPLC-DAD-ESI-MS^n data of pistachio hull phenolics

Peak no.	Compound	Retention time (min)		UV/Vis abs max (nm)		HPLC-ESI(-)-MS^n *m/z* (per cent base peak)
		HPLC	UHPLC	HPLC	UHPLC	
1	*β*-Glucogallin [1]	6.0	2.9	274	278	[331]: 169(100), 271(27), 170(15), 125(10) [331→169]:125(100)
2	Gallic acid [1]	6.8	3.2	272	271	[169]: 125(100)
3	Gallic acid derivative (1)	10.0	na	276	na	[296]: 169(100)
4	Galloyl quinic acid	11.2	4.1	275	274	[343]: 191(100), 169(6)
5	Protocatechuic acid [1]	13.4	5.5	259	265	[153]: 109(100)
6	Galloyl dihexose (1)	14.5	na	274	na	[493]: 313(100), 283(49), 169(46), 331 (32), 433 (28) [493→313]: 283(100), 152(84), 223(80), 113(52)
7	Galloyl dihexose (2)	16.5	na	274	na	[493]: 271(100), 211(20), 313(14), 331(10), 169(7) [493→271]: 169(100), 211(12)
8	Galloyl dihexose (3)	17.2	na	274	na	[493]: 313(100), 364(38), 464(21) [493→313]: 169(100), 125(43), 177(37), 224(37)
9	Galloyl shikimic acid (1)	18.0	na	274	na	[325]: 169(100), 151(58), 125(51) [325→169]: 125(100)
10	Galloyl shikimic acid (2)	19.6	na	274	na	[325]: 169(100), 281(31), 301(17), 125(14), 155(10) [325→169]: 125(100)
11	Gallic acid derivative (2)	20.2	na	276	na	[571]: 285(100), 169 (10)
12	Galloyl shikimic acid (3)	20.8	na	274	na	[325]: 169(100), 233(39), 252(18), 125(13) [325→169]: 125(100)
13	Digallic acid (1)	21.1	na	280	na	[321]: 169(100)

Table 4.3. Retention times and HPLC-DAD-ESI-MS^n data of pistachio hull phenolics (Continued)

Peak no.	Compound	Retention time (min)		UV/Vis abs max (nm)		HPLC-ESI(-)-MS^n *m/z* (per cent base peak)
		HPLC	UHPLC	HPLC	UHPLC	
14	Digalloyl hexose (1)	21.5	na	280	na	[483]: 331(100), 271(16), 169(15)
	Methyl gallate [2]	22.3	na	272	na	[183]: 168(100), 124 (15)
15	Digalloyl hexose (2)	23.8	na	288	na	[483]: 331(100), 446(17), 313(13), 425(11), 241(11)
16	Gallic acid derivative (3)	25.0	na	274	na	[509]: 267(100), 430(15), 241(10), 357(10)
17	Gallic acid derivative (4)	26.0				[423]: 313(100), 169(73), 125 (26), 241(11), 272(10)
18	Digalloyl quinic acid	26.4	na	274	na	[495]: 343(100), 191(81), 344(41) [495→343]: 191(100)
19	Digallic acid (2)	27.3	na	275	na	[321]: 169(100), 125(16) [321→169]: 125(100)
20	Gallic acid derivative (5)	28.1	na	268	na	[403]: 169(100), 151 (40), 313 (15)
21	Gallic acid derivative (6)	29.1	na	268	na	[467]: 313(100), 169(59)
22	Digalloyl shikimic acid (1)	30.6	na	275	na	[477]: 325(100), 169 (22)
23	Digalloyl shikimic acid (2)	31.0	na	276	na	[477]: 325(100), 169 (7)
24	Cyanidin 3-O-β-D-galactopyranoside [1]	31.6	8.6	278, 517	276, 515 [3]	[449]: 287(100) [3] [449→287]: 137(100)
25	Luteic acid	33.2	na	278	na	[319]: 239 (100), 340 (15), 139 (15)
26	Trigalloyl quinic acid	33.8	na	273	na	[647]: 495 (100), 343 (24), 496 (12)
27	Quinic acid derivative (1)	34.5	na	276	na	[523]: 209 (100), 505 (64), 371 (54), 169 (21), 313 (18)
28	Myricetin galloyl hexoside	35.3	9.8	270, 354	270, 350	[631]: 479 (100), 316 (13) [631→479]: 316 (100)

Table 4.3. Retention times and HPLC-DAD-ESI-MS^n data of pistachio hull phenolics (Continued)

Peak no.	Compound	Retention time (min)		UV/Vis abs max (nm)		HPLC-ESI(-)-MS^n *m/z* (per cent base peak)
		HPLC	UHPLC	HPLC	UHPLC	
29	Tetragalloyl hexose	35.8	na	278	na	[787]: 617 (100) [787→617]: 465 (100)
30	Trigallic acid	36.7	na	274	na	[473]: 321 (100), 169 (8)
31	Myricetin hexuronide	36.9	10.5	252, 357	256, 367	[493]: 317 (100), 229 (13), 151 (10) [493→317]: 179 (100)
32	Myricetin 3-*O*-galactoside [1]	37.1	10.6	252, 359	269, 356	[479]: 317 (100), 214 (14), 287 (10) [479→317]: 271 (100), 287 (80), 242 (50)
33	Myricetin hexoside	37.4	10.7	252, 357	268, 355	[479]: 317 (100), 316 (76), 169 (43), 179 (36) [479→317]: 271 (100), 151 (38)
	Methyl digallate [2]	37.9	na	274	na	[335]: 183 (100)
34	Penta-*O*-galloyl-*β*-D glucose [1]	38.3	10.4	280	279	[939]: 769 (100), 617 (18), 787 (12) [939→769]: 618 (100), 602 (37)
35	Quercetin galloyl hexoside (1)	38.9	10.9	257, 354	266, 353	[615]: 463 (100), 301 (45), 362 (13) [615→463]: 300 (100), 301 (36)
36	Quercetin 3-*O*-galactoside [1]	41.0	12.1	252, 352	265, 355 [4]	[463]: 301 (100), 239 (27) [463→301]: 151 (100), 271 (50), 255 (25), 179 (24)
37	Quercetin 3-*O*-glucuronide [1]	41.2	12.1	255, 355		[477]: 301 (100), 273 (9) [477→301]: 179 (100)
38	Quercetin 3-*O*-glucoside [1]	41.6	12.3	260, 355	266, 356	[463]: 301 (100) [463→301]: 179 (100), 271 (67), 151 (64)

Table 4.3. Retention times and HPLC-DAD-ESI-MS^n data of pistachio hull phenolics (Continued)

Peak no.	Compound	Retention time (min)		UV/Vis abs max (nm)		HPLC-ESI(-)-MS^n *m/z* (per cent base peak)
		HPLC	UHPLC	HPLC	UHPLC	
39	Quercetin galloyl hexoside (2)	42.1	na	254, 358	na	[615]: 301 (100) [615→301]: 179 (100), 169 (85), 229 (78)
40	Quercetin galloyl hexoside (3)	42.9	na	276, 358	na	[615]: 301 (100), 463 (10)
41	Quercetin galloyl deoxyhexose		na	274, 355	na	[599]: 463 (100)
42	Quercetin pentoside	43.7	13.1	254, 355	268, 346	[433]: 301 (100), 271 (26) [433→301]: 271 (100), 255 (20)
43	Kaempferol hexoside (1)	45.0	13.7	272, 350	na	[447]: 285 (100), 255 (79), 284 (78), 151 (25) [447→285]: 255 (100)
44	Quercetin galloyl hexuronide	45.4		276, 352		[629]: 477 (100), 301 (26), 478 (25), 595 (13), 592 (12)
45	Quercetin galloyl pentoside	46.3	na	277, 352	na	[585]: 301 (100)
46	Kaempferol hexoside (2)	46.9	14.5	270, 350	na	[447]: 285 (100), 256 (29), 404 (16) [447→285]: 255 (100)
47	Kaempferol pentoside	48.9	na	270, 350	na	[417]: 284 (100)
48	(16:1)- Anacardic acid	88.4	na	310	na	[359]: 315 (100), 161 (32), 293 (11), 107 (10)
49	(13:2)- Anacardic acid	88.5	na	310	na	[315]: 271 (100), 107 (26), 272 (18), 269 (10)
50	(11:0)- Anacardic acid	88.9	na	308	na	[291]: 247 (100)
51	(15:3)-Anacardic acid	89.1	na	310	na	[341]: 297 (100)
52	(13:1)-Anacardic acid	89.6	21.0	310	310	[317]: 273 (100) [317→273]: 107 (100)

Table 4.3. Retention times and HPLC-DAD-ESI-MS^n data of pistachio hull phenolics (Continued)

Peak no.	Compound	Retention time (min)		UV/Vis abs max (nm)		HPLC-ESI(-)-MS^n *m/z* (per cent base peak)
		HPLC	UHPLC	HPLC	UHPLC	
53	(13:0)-Anacardic acid	90.7	21.4	312	310	[319]: 275 (100)
54	(15:1)-Anacardic acid	91.0	21.5	310	310	[345]: 301 (100)
55	(17:2)-Anacardic acid	91.4	21.6	310	na	[371]: 327 (100)
56	(15:0)-Anacardic acid[1]	92.0	22.0	311	310	[347]: 303 (100)
57	(17:1)-Anacardic acid	92.2	22.0	312	311	[373]: 329 (100)
58	(17:0)-Anacardic acid	93.1	22.7	312	na	[375]: 331 (100)

na: not available.
[1] Verified by reference standards.
[2] Not present in sample chromatogram.
[3] Positive ionization mode was used for the identification of anthocyanins.
[4] UV/Vis abs. max (nm) for peak 36 and 37

4.3.4. Method Validation

All calibration curves constructed showed good signal linearity (R^2 > 0.99) in the studied concentration ranges given in Table 4.1. Regarding the HPLC method, LOD and LOQ values ranged from 0.6 and 1.8 ng on column for cyanidin 3-*O*-*β*-D-galactopyranoside, respectively, to 11.4 and 34.4 ng on column for (15:0)-anacardic acid, respectively. These values are comparable to those of previous analytical reports of Gras et al. [133] (LOD: 0.35-1.20 and LOQ: 1.05-3.63 ng on column for cyanidin xyloside galactoside and cyanidin 3-*O*-glucoside, respectively), and Ziegler et al. [117] (LOD ≤ 20.41 and LOQ ≤ 61.85 ng on column for alkylphenols). Moreover, our developed method exhibited lower LOD (0.0008 mg/mL) and LOQ (0.003 mg/mL) values for quercetin 3-*O*-galactoside than those reported by Plazonić et al. [231] as 0.002 mg/mL and 0.005 mg/mL, respectively.

Regarding the above described UHPLC-DAD system, LOD and LOQ values were similar or higher than those obtained by HPLC, except for protocatechuic acid which presented lower LOD and LOQ under UHPLC conditions (Table 4.1). When comparing ELS with UV detection, LOD and LOQ values of (15:0)-anacardic acid for ELS detector were substantially higher than those obtained with both UV-based detectors.

Recovery rates ranged between 95.0 and 101 per cent for gallic acid, protocatechuic acid, penta-*O*-galloyl-*β*-D-glucose, quercetin 3-*O*-galactoside, cyanidin 3-*O*-*β*-D-galactopyranoside and (15:0)-anacardic acid, irrespective of the added amount of reference compounds (Table 4.2). Intra-day and inter-day repeatabilities of the method showed CV (coefficients of variation) values of 3.7-5.6 and 0.4-5.4 per cent, respectively, for all compounds, except for penta-*O*-galloyl-*β*-D-glucose (10.1-12.4 per cent). Stability of extracts during analysis (for 24 and 48 h) was further examined to exclude negative effects of long HPLC analysis time on the repeatabilities. The analyzed extracts were stable for up to 48 hours at room temperatures. The deviations from the initially measured values analyzed and expressed as CVs (per cent) ranged from 0.3 to 6.6 per cent for all compounds present in extracts stored at room temperature in HPLC vials, still remaining within the range of overall repeatabilities. Moreover, extracts (HPLC vials) can be stored at low temperatures (-20 °C and -80 °C) up to 7 months with CVs (per cent) ranging between 0.3 and 5.1 per cent for all compounds compared to freshly prepared extracts.

Table 4.4. Quantitation of individual phenolic compounds from different pistachio hull samples as determined by HPLC-DAD-MS^n.

Peak no.	Compound	Concentration (g/kg DM) [1]			
		'Uzun' green	**'Uzun' red**	**'Siirt'**	**'Ohadi'**
		Gallotannins			
1	*β*-Glucogallin	4.32 ± 0.53bc	4.76 ± 0.23b	3.91 ± 0.12c	5.89 ± 0.28a
2	Gallic acid	4.68 ± 0.07a	1.66 ± 0.08d	2.08 ± 0.06c	3.99 ± 0.06b
3	Gallic acid derivative (1)	1.53 ± 0.04c	0.20 ± 0.01a	tr	0.93 ± 0.01b
4	Galloyl quinic acid	1.87 ± 0.06a	2.09 ± 0.12b	0.46 ± 0.05d	1.33 ± 0.06c
9	Galloyl shikimic acid (1)	0.36 ± 0.01a	tr	tr	0.18 ± 0.01b
10	Galloyl shikimic acid (2)	0.97 ± 0.04d	0.46 ± 0.03a	0.57 ± 0.02c	0.83 ± 0.02b
11	Gallic acid derivative (2)	nd	nd	0.13 ± 0.01	nd
12	Galloyl shikimic acid (3)	0.75 ± 0.03b	0.42 ± 0.06a	0.34 ± 0.01b	0.81 ± 0.02a
14	Digalloyl hexose (1)	0.21 ± 0.08c	tr	0.44 ± 0.04a	0.33 ± 0.06ab
15	Digalloyl hexose (2)	nd	nd	nd	0.06 ± 0.02
17	Gallic acid derivative (4)	tr	tr	tr	0.25 ± 0.03
18	Digalloyl quinic acid	tr	0.56 ± 0.01a	tr	0.29 ± 0.03b
19	Digallic acid (2)	1.01 ± 0.06c	0.74 ± 0.01b	1.04 ± 0.05ab	1.14 ± 0.05a
20	Gallic acid derivative (5)	0.05 ± 0.02b	nd	nd	0.16 ± 0.05a
34	Penta-*O*-galloyl-*β*-D-glucose	5.01 ± 0.15a	1.30 ± 0.08d	2.38 ± 0.10c	4.30 ± 0.02b
		Flavonoids			
24	Cyanidin 3-*O*-*β*-D-galactopyranoside	-	0.31 ± 0.02b	nd	0.54 ± 0.01a
31	Myricetin hexuronide	0.27 ± 0.01b	0.19 ± 0.01c	0.35 ± 0.02a	0.33 ± 0.01a
32	Myricetin 3-*O*-galactoside	0.25 ± 0.01c	0.19 ± 0.02d	0.39 ± 0.01a	0.29 ± 0.01b

Table 4.4. Quantitation of individual phenolic compounds from different pistachio hull samples as determined by HPLC-DAD-MS^n (Continued)

Peak no.	Compound	Concentration (g/kg DM)[a]			
		'Uzun' green	**'Uzun' red**	**'Siirt'**	**'Ohadi'**
33	Myricetin hexoside	0.35 ± 0.01a	0.23 ± 0.02b	0.21 ± 0.01b	0.37 ± 0.01a
35	Quercetin galloyl hexoside (1)	1.15 ± 0.12b	0.69 ± 0.04c	2.20 ± 0.10a	2.41 ± 0.05a
36, 37	Quercetin 3-*O*-galactoside/ glucuronide	1.96 ± 0.10c	2.22 ± 0.14b	0.25 ± 0.02d	3.18 ± 0.06a
38	Quercetin 3-*O*-glucoside	1.00 ± 0.05c	0.95 ± 0.05c	4.20 ± 0.16a	1.45 ± 0.03b
39	Quercetin galloyl hexoside (2)	0.34 ± 0.02c	0.34 ± 0.02c	1.56 ± 0.06a	0.54 ± 0.02b
42	Quercetin pentoside	0.25 ± 0.01b	0.21 ± 0.05b	0.23 ± 0.01b	0.36 ± 0.00a
43	Kaempferol hexoside (1)	nd	nd	0.35 ± 0.01a	0.19 ± 0.00b
46	Kaempferol hexoside (2)	tr	tr	0.23 ± 0.01a	0.18 ± 0.00b
		Anacardic acids			
52	(13:1)-Anacardic acid	9.83 ± 0.38b	12.2 ± 0.34a	2.97 ± 0.08c	9.30 ± 0.12b
53	(13:0)-Anacardic acid	20.08 ± 0.30a	19.2 ± 0.74a	13.35 ± 0.12b	19.6 ± 0.29a
54	(15:1)-Anacardic acid	5.01 ± 0.13b	4.08 ± 0.19c	12.34 ± 0.24a	4.11 ± 0.14c
55	(17:2)-Anacardic acid	0.90 ± 0.10a	0.81 ± 0.08a	0.42 ± 0.03b	0.86 ± 0.14a
56	(15:0)-Anacardic acid	8.50 ± 0.03a	8.02 ± 0.35b	2.09 ± 0.03c	8.42 ± 0.06ab
57	(17:1)-Anacardic acid	26.1 ± 0.09b	28.7 ± 1.18a	8.11 ± 0.08c	26.7 ± 0.28b
58	(17:0)-Anacardic acid	0.59 ± 0.04b	0.76 ± 0.10a	0.26 ± 0.03c	0.57 ± 0.04b
5	Protocatechuic acid	0.36 ± 0.05b	0.34 ± 0.02b	0.36 ± 0.02b	0.87 ± 0.07a
	Total gallotannins	20.6 ± 0.63a (21.2 per cent) [2]	12.3 ± 0.53b (13.4 per cent) [2]	11.3 ± 0.26b (18.5 per cent) [2]	20.5 ± 0.40a (20.3 per cent) [2]

Table 4.4. Quantitation of individual phenolic compounds from different pistachio hull samples as determined by HPLC-DAD-MS^n (Continued)

Peak no.	Compound	Concentration (g/kg DM)[a]			
		'Uzun' green	**'Uzun' red**	**'Siirt'**	**'Ohadi'**
	Total flavonoids	5.56 ± 0.30b (5.7 per cent) [2]	5.33 ± 0.27b (5.8 per cent) [2]	9.97 ± 0.37a (16.3 per cent) [2]	9.83 ± 0.12a (9.8 per cent) [2]
	Total anacardic acids	71.0 ± 0.68ab (72.8 per cent) [2]	73.8 ± 2.95a (80.4 per cent) [2]	39.6 ± 0.34c (64.6 per cent) [2]	69.5 ± 0.53b (69.0 per cent) [2]
	Total of quantified phenolics [3]	97.5 ± 1.17a	91.8 ± 3.19b	61.2 ± 0.45c	100 ± 0.48a

Values expressed as the mean ± standard deviation derived from triplicate (n = 3) analyses.
Significant differences between the contents within cultivars are indicated by different letters ($P < 0.05$) within the row.
tr: traces, ≥ LOD (limit of detection), ≤ LOQ (limit of quantitation).
nd: not detected, < LOD: below the limit of detection for UV; MS analyses allowed the verification of compound presence.
LOD and LOQ values were given in Table 4.1.
[1] Moisture contents used for the calculation of dry matter (DM) content were 7.6 ± 0.3 per cent w/w (cv. 'Uzun' green), 6.5 ± 0.2 per cent (cv. 'Uzun' red), 7.1 ± 0.4 per cent (cv. 'Ohadi') and 8.2 ± 0.3 per cent (cv. 'Siirt').
[2] Percentages of total phenolics (per cent m/m).
[3] Total phenolic contents represented the sum of gallotannins, flavonoids, anacardic acids and protocatechuic acid.

4.3.5. Quantitation of Phenolic Constituents in Pistachio Hull Varieties

The above described, validated HPLC method was applied for the quantitation of phenolic compounds from pistachio hulls of three different varieties (cv. 'Uzun', 'Siirt', 'Ohadi'), of which one variety (cv. 'Uzun') was available in two different (green and red) maturity stages (total of four different samples).

The qualitative composition of phenolic compounds from all the studied varieties and maturation stages was widely similar, while their concentrations in the hulls showed substantial variation as outlined in Table 4.4. Total phenolic content ranged from 61.2 ± 0.45 in cv. 'Siirt' to 100 ± 0.48 g/kg DM in cv. 'Ohadi' green, mainly comprising compounds of the phenolic classes gallotannins, flavonoids and anacardic acids. For instance, the gallotannin content of hulls ranged from 11.3 ± 0.26 to 20.6 ± 0.63 g/kg DM. Gallotannin levels were higher in the hull of 'Uzun' green (20.6 ± 0.63 g/kg DM) than in those of cv. 'Uzun' red (12.3 ± 0.53 g/kg DM), whereas other phenolic classes were present at similar levels ($P > 0.05$). The higher levels of gallotannins present in green drupes may indicate that the accumulation of gallotannins, specifically gallic acid, and penta-*O*-galloyl-*β*-D-glucose, might represent a defense mechanism in the initial stage of fruit maturity to protect pistachio drupes from insects as explained by Haslam [232]. The major contributors of gallotannins were *β*-glucogallin, gallic acid, and penta-*O*-galloyl-*β*-D-glucose, accompanied by comparably lower amounts of other galloyl derivatives such as esters of gallic acid with hexoses, and shikimic or quinic acids. Penta-*O*-galloyl-*β*-D-glucose and other gallic acid derivatives such as galloyl quinic acid and shikimic acids were quantitated for the first time in the current study, although their presence was reported before [31] and confirmed by HR-MS [22]. Based on our results, pistachio hull represents a good source of gallic acid (1.66-4.68 g/kg DM) and penta-*O*-galloyl-*β*-D-glucose (1.30-5.01 g/kg DM) as compared to other known gallic acid sources such as grape seed (0.1- 1.0 g/kg DM) [233], green tea (av. 0.052 g/kg DM) [234], pomegranate peel (0.030 g/kg DM) [235], and penta-*O*-galloyl-*β*-D-glucose obtained by methanolysis of tannic acid (1.5 g/kg) [236].

Total flavonoid contents of pistachio hulls ranged between 5.56 ± 0.30 and 9.83 ± 0.12 g/kg DM (Table 4.4), constituting only a small portion (5.7-16.3 per cent) of the total phenolics. Flavonols such as quercetin 3-*O*-galactoside/glucuronide (0.25-3.18 g/kg DM), quercetin 3-

O-glucoside (0.95-4.20 g/kg DM), and quercetin galloyl hexoside (total of peak 35 and 39: 1.03-3.76 g/kg DM) were major contributors to pistachio hull flavonols. Flavonoid levels remained unaffected when comparing the two maturity levels of cv. 'Uzun' (red and green) ($P > 0.05$), except for the presence of cyanidin 3-*O*-*β*-D-galactopyranoside in the red one. Cyanidin 3-*O*-*β*-D-galactopyranoside was the only anthocyanin present and quantified in the hulls obtained from 'Uzun' red and 'Ohadi' drupes (0.31-0.54 g/kg DM). Similar flavonol profiles in fresh pistachio hull from an unknown variety of USA were reported in the study of Grace et al. [31]. Considering its flavonoid content (5.56-9.83 g/kg DM), pistachio hull might be considered as a good source, especially for quercetin glycosides, compared to other flavonol sources such as mango peel (0.27-3.80 g/kg DM) [6], onion (2.11-6.84 g/kg DM) [237], apple pomace (5.4-9.5 g/kg DM) [238], and different berry types (0.06-7.9 g/kg DM) [239].

Anacardic acids consistently constituted more than ca. 65 per cent of identified phenolics in all varieties and ranging from 39.6 to 73.8 g/kg DM (Table 4.4), where major anacardic acids were (13:1)-anacardic acid, (13:0)-anacardic acid, (15:1)-anacardic acid, (15:0)-anacardic acid, and (17:1)-anacardic acid. In agreement with Yalpani and Tyman [64], the relative homologue composition was characterized by 6-7 per cent of (15:1)-, 11-12 per cent of (15:0)-, 13-17 per cent of (13:1)-, 26-28 per cent of (13:0)-, and 37-39 per cent of (17:1)-anacardic acids, except for cv. 'Siirt', where its anacardic acid content was only half of the other pistachio varieties with (13:0)-, (15:1)- and (17:1)-anacardic acids being the main constituents. Concentrations of (13:0)-, and (17:1)-anacardic acids were highest, making pistachio hulls a source of both saturated and unsaturated anacardic acids with different chain lengths. Grace et al. [31] reported lower anacardic acid contents (32.0 ± 3.20 g/kg DM) than those found in our study. This might be attributed to the optimized procedure in our study ensuring their exhaustive recovery by means of the highly efficient ultrasound-assisted extraction method. However, pistachio hull may only be a secondary source for anacardic acid recovery (39.6-73.8 g/kg DM) compared to cashew, an exceptionally rich source of anacardic acids (215 and 354 g/kg in fruit and cashew nut shell liquid (CNSL), respectively) [54]. Nevertheless, its content is still considerably high compared to other sources of anacardic homologue alkylresorcinols such as mango peel (0.43-0.59 g/kg DM), rye grain (0.8-1.2 g/kg DM) [8], and different wheat species (0.65-0.74 g/kg DM) [52]. Moreover,

pistachio hull offers a unique mixture of anacardic acids with different chain lengths between C-13 to C-17 compared to cashew, which mainly contains C-15 chain-anacardic acids [54].

4.4. CONCLUSION

Simultaneous and quantitative extraction of gallotannins, flavonoids and anacardic acids from pistachio hull has been achieved using methanol/water/formic acid (80/19/1, v/v/v) for ultrasound-assisted extraction. For analyte quantitation, UHPLC-DAD-ELSD and HPLC-DAD separation and detection were comparatively validated. The validated HPLC method was applied to the determination of 58 different phenolics compounds from four different pistachio hull samples. Anacardic acids represented the most abundant phenolics in pistachio hulls, making them an alternative source to cashew products. Moreover, as compared to onion, grape seed, mango, apple, and several berries, pistachio hulls were shown to represent a rich source of potentially bioactive phenolics such as gallic acid, penta-*O*-galloyl-*β*-D-glucose, and quercetin glycosides. Thus, this by-product accruing in high amounts from pistachio processing, represents a yet underutilized source of phenolic compounds, being available at low cost in large quantities.

5. SUBCRITICAL WATER EXTRACTION OF PHENOLIC AND ANTIOXIDANT CONSTITUENTS FROM PISTACHIO HULL [3]

5.1. INTRODUCTION

The world market for phenolic compounds was estimated to exceed 700 million USD in 2015 [1]. Further growth is expected due to consumer preference of natural antioxidants being associated with various potential health benefits attributed to phenolic compounds such as a possible delay of the onset or even the prevention of cardiovascular disease, diabetes, chronically high cholesterol levels, and some kinds of cancers [2]. Residues arising from agricultural and food processing are of particular interest, as they are rich and cheap sources for the recovery of such valuable compounds. Otherwise, they need to be disposed at the expense of the food processors, who are increasingly aiming at improving sustainable production and complete exploitation of the raw materials of their processes. Pistachio (*P. vera* L.) hull (exo- and mesocarp) is the main by-product of pistachio processing. It has been shown to be a rich source of phenolic compounds, namely gallic acid, gallotannins such as galloyl glucose, and penta-*O*-galloyl-*β*-D-glucose, flavonoids such as quercetin and myricetin glycosides, and anacardic acids [22, 63]. Various biological activities including antioxidant, anticancer, anti-inflammatory, and antimicrobial have been assumed for gallic acid [240], penta-*O*-galloyl-*β*-D-glucose [77], various gallotannins [126], flavonols [95], and anacardic acids [241]. Pistachio hull extracts have previously been associated with similar biological activities, presumably being related to the contained phenolic compounds [14, 31]. Therefore, pistachio hulls represent a promising source to produce novel value-added phenolic extracts for their utilization in various fields such as food, pharmaceutical, cosmetic, and chemical industries. Commonly, the extraction of phenolic compounds is performed with organic solvents, and supercritical fluids [242]. The extraction with SCW is an environmentally friendly but much less explored alternative separation technique using

[3] Submitted for publication to a journal as "Erşan, S.; Güçlü Üstündağ, Ö.; Carle, R.; Schweiggert, R. M. Subcritical water extraction of phenolic and antioxidant constituents from pistachio (*Pistacia vera* L.) hull."

water at its subcritical state for the recovery of phenolic-rich extracts. Thereby, water is heated above its boiling point (100 °C) without reaching its supercritical state (374 °C) under pressure sufficient to maintain its liquid state. Under such extreme conditions, SCW possesses solvent properties highly suitable for the extraction of phenolic compounds, e.g., the dielectric constant is decreased and its polarity is similar to that of organic solvents [141]. Beyond that, SCW is also exhibiting hydrolytic properties due to its increased ionization constant leading to higher concentrations of OH^- and H^+ ions, thus often resulting in the liberation of bound components, the break-down of bigger molecules, and the *de novo*-formation of compounds having increased biological and antioxidant capacities [128]. Due to the aforementioned properties, research on SCW extraction has been intensified in recent years for the extraction of phenolic and antioxidant compounds from diverse agricultural and food by-products such as onion waste [171], rice bran [243], and grape [146]. However, SCW extraction has not been yet applied to the pistachio hull-like plant non-lignified matrixes containing large amounts and diverse class of phenolic compounds. Particularly, the extraction of gallotannins from pistachio hull, e.g., galloyl glycosides, galloyl quinic/shikimic acids, penta-*O*-galloyl-*β*-D-glucose, might be feasible by SCW extraction as previously exemplified by the hot water extraction of gallotannin-rich witch hazel (*H. virginiana* L.) at 100 °C [123].

Hence, the objective of the present work was to evaluate the potential of SCW extraction for the recovery pistachio hull phenolic and antioxidant compounds as a “green” alternative to solvent-based extraction methods. The effect of SCW temperature should be investigated between 110 and 190 °C aiming at optimization of polyphenol yields. Temperature-related qualitative and quantitative changes in phenolic composition and formation of degradation products should be investigated both in the extracts and extraction residues using a previously validated HPLC-DAD-ESI/MS^n method [22, 63]. In addition, antioxidant capacities of the extracts obtained were to be determined. Furthermore, the present study aimed at comparing the efficiency of SCW extraction to that of an ultrasound-assisted process using aqueous methanol as a solvent.

5.2. MATERIALS AND METHODS

5.2.1. Chemicals

Gallic acid monohydrate (purity ≥98 per cent), protocatechuic acid (purity ≥97 per cent), penta-*O*-galloyl-*β*-D-glucose (purity ≥96 per cent), (15:0)-anacardic acid (purity ≥97 per cent), HMF (5-hydroxymethyl furfural) (purity ≥98 per cent), Trolox ((±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) (purity 97 per cent), ABTS (2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) (purity ≥98 per cent), potassium persulfate (ACS grade, purity ≥99 per cent), DPPH (2,2-diphenyl-1-picrylhydrazyl), TPTZ (2,4,6-Tris(2-pyridyl)-*s*-triazine) (purity ≥99 per cent), acetic acid (analytical grade), HCl (37 per cent, analytical grade), $FeCl_3 \bullet 6H_2O$ (ACS reagent) were from Sigma-Aldrich (St. Louis, MO, USA). *β*-Glucogallin (1-*O*-galloyl-*β*-D-glucopyranose) (purity ≥94 per cent) was from PhytoLab (Vestenbergsgreuth, Germany), and quercetin 3-*O*-glucuronide (purity ≥95 per cent), quercetin 3-*O*-glucoside (purity ≥99 per cent), quercetin 3-*O*-galactoside (purity ≥98 per cent), myricetin 3-*O*-galactoside (purity ≥99 per cent), and cyanidin 3-*O*-*β*-D-galactopyranoside (purity ≥97 per cent) were from Extrasynthèse (Genay, France). HPLC grade methanol was from VWR (Darmstadt, Germany). Deionized water was used throughout the study.

5.2.2. Sample and Sample Preparation

Dried red pistachio drupes cv. ‘Uzun’ were obtained from a commercial pistachio processor (Gaziantep, Turkey) as detailed in our previous studies [22, 63]. Pistachio hulls were separated manually and ground using an A11 laboratory mill (IKA, Staufen, Germany). Ground pistachio hulls were sieved (Octagon D200, Endecotts, London, UK), and only the fraction having particle sizes between 0.5 μm and one mm was used for all experiments as described below, because usage of finer fractions rapidly led to clogging of the below-mentioned two μm frit.

5.2.3. Subcritical Water (SCW) Extraction

5.2.3.1. Extraction system

Subcritical water extraction (SCW) was performed using the extraction system shown in Figure 5.1. A high pressure pump (P, Dionex Ultimate ISO 3100SD, Thermo Scientific, Sunnyvale, CA, USA) was used to pump water from the water reservoir (R1) to the heating coil (HC) and, subsequently, to an extraction column made of 20 cm x 1.09 cm I. D. (1.27 cm O. D.) stainless steel tubing (Sandvik Materials Technology, Sandviken, Sweden). The length of the heating coil was three m to ensure the desired water temperature (110-190 °C) at its outlet. Thermocouples (T1-T2, TK 102S, Kimo Instruments, Bordeaux, France) were used to monitor water temperature entering and leaving the column. The extraction column containing the powdered sample was fitted with two μm pore size stainless steel frits (Chromotek Apple Valley, MN, USA) at both ends. Heating coil and the column were placed in a temperature controlled oven (Venticell 111, MMM Medcenter Einrichtungen, Munich, Germany). The eluate from the column was cooled to room temperature using the subsequent cooling coil (CC, three m). A back pressure regulator (BPR, IDEX Health and Science, Oak Harbor, WA, USA) allowed to precisely pressurize the system, while pressure gauge (PG, Ham-Let, Newburgh, IN, USA) enabled monitoring of the extraction pressure. Heating and cooling coils, as well as all the processing lines, were made of 0.26 cm I.D. stainless steel tubing (Sandvik Materials Technology, Sandviken, Sweden).

5.2.3.2. Extraction procedure

Ground pistachio hull (2.0) corresponding to a bed depth of five cm was placed into the extraction column. After connecting the column to the processing lines, water (ca. 50 mL) was pumped to the system until the pressure of 6.9 MPa (1000 psi) was reached. Subsequently, flow was stopped and oven temperature was set to the desired value (110-190 °C). After reaching the final temperature, water flow at four mL/min was turned on, and extracts (120 mL) were collected after discarding the first 11 mL of the eluate, representing the initial dead volume of the pipeline after the extraction column. All extracts and the solid extraction residues were freeze-dried and stored at -20 °C until analyses. Extraction yields

were determined gravimetrically after freeze-drying and expressed as per cent w/w on DM basis. In addition to physical cleaning of the column, the extraction system was flushed with 70 per cent (v/v) aqueous methanol and water between each run.

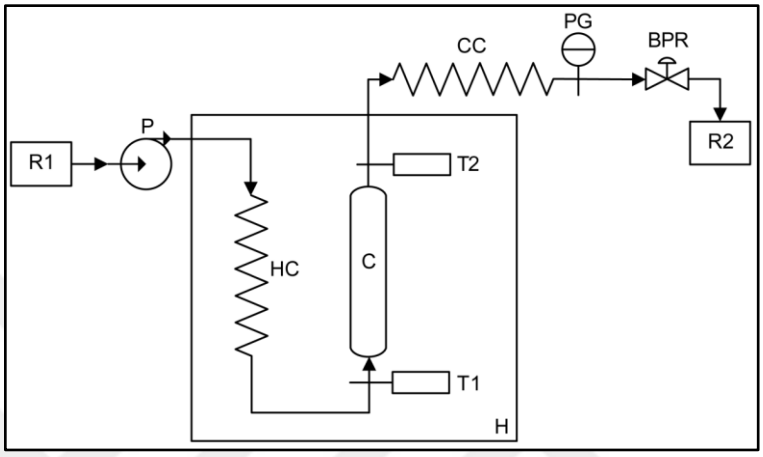

Figure 5.1. Schematic diagram of subcritical water extraction system. R1: Water reservoir, P: Pump, HC: Heating coil, T1-T2: Thermocouples, C: Extraction column, CC: Cooling coil, PG: Pressure gauge, BPR: Back pressure regulator, R2: Reservoir for the extract; H: Heating oven.

5.2.4. Ultrasound-Assisted Solvent Extraction

Ground pistachio hull (250) and freeze-dried SCW extraction residues (50), respectively, were extracted with a ternary mixture of methanol/water/formic acid (80/19/1, v/v/v) under ultrasonic probe-sonication as described previously [63].

5.2.5. Analytical Procedures

5.2.5.1. Spectrophotometric analyses

Antioxidant capacities were determined using three different *in vitro* assays, i.e., the DPPH radical scavenging [187], the ABTS radical scavenging [244], and the FRAP (Ferric

reducing antioxidant power) assays [245] using a microplate reader (Powerwave XS, Biotek Instruments, Winooski, VT, USA) as described previously in detail [246]. An aliquot of freeze-dried extract (10) was dissolved in two mL of methanol/water (80/20, v/v) and centrifuged (Labofuge 200; Heraeus, Hanau, Germany) at 3,000 rpm (1233 *g*) for three min. Supernatants were collected and used for the determination of antioxidant capacities. Absorbance readings were done immediately after 10 s of blending, and every five minutes over 120 min for ABTS assay, and over 300 min for both FRAP and DPPH assays to monitor reaction kinetics. Absorbance values obtained after the reaction had reached steady state were used for calculations. Up to seven different concentrations of each extract and Trolox standards were tested to probe the linear range of responses by plotting absorbance *versus* concentration. Results were expressed as mmol Trolox equivalent (TE) per g DM of sample after dividing slope of the absorbance-*versus*-concentration curve obtained with the sample by that of the respective curve obtained with the Trolox standard compound.

5.2.5.2. HPLC-DAD-ESI/MS^n analyses

HPLC-DAD-ESI/MS^n analyses of phenolic compounds were performed according to a previously described, validated procedure [63]. Prior to HPLC separation, an aliquot of freeze-dried extract (20 mg) was dissolved in one mL of a ternary mixture of methanol/water/formic acid (80/19/1, v/v/v) and filtered through regenerated cellulose membrane filters (0.45 µm pore size; Macherey-Nagel, Düren, Germany) into amber HPLC vials. Results were expressed as g per kg DM of sample.

5.2.6. Statistical Analysis

Data were expressed as a mean ± standard deviation of three technological repetitions. Analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons ($P < 0.05$) was used to determine significant differences between means, using Minitab© 17.3.1 (State College, PA, USA).

5.3. RESULTS AND DISCUSSION

5.3.1. Extraction Yields

Subcritical water extraction of dried ground pistachio hulls by the column extraction process (Figure 5.1) resulted in extraction yields ranging from 59.1 per cent (w/w, DM) to 70.9 per cent (w/w, DM) (Table 5.1).

Table 5.1. Extraction yields and the amount of remaining residue after extraction.

Extract	Extraction yield [1] (per cent w/w, DM [2])	Fraction of the extract soluble in aqueous methanol [3] (per cent w/w, DM)	Extraction residue (per cent w/w, DM)
SCW110	59.1 ± 0.8b	69.6 ± 4.5c	37.4 ± 1.5b
SCW130	59.3 ± 1.0b	66.2 ± 2.8c	31.7 ± 0.7c
SCW150	70.9 ± 3.2a	71.2 ± 2.4c	27.2 ± 1.3d
SCW170	67.1 ± 3.4a	74.7 ± 4.7bc	23.5 ± 0.7e
SCW190	65.3 ± 4.4ab	82.4 ± 2.4b	21.4 ± 0.5e
Aqueous methanol	59.5 ± 2.9b	94.5 ± 6.4a	45.0 ± 1.2a

Results were expressed as mean ± standard deviation of three independent technological replicates. Abbreviations: SCW110-190: Subcritical water extracts obtained by extracting at temperatures from 110 to 190°C. Significant differences between the contents of different samples are indicated by different letters ($P < 0.05$) within a column.
[1] Yields were calculated after freeze-drying of extracts.
[2] Moisture contents used for the calculation of dry matter (DM) content was 6.5 ± 0.2 per cent.
[3] Results were calculated after dissolving freeze-dried extracts in methanol/water/formic acid (80/19/1, v/v/v) at a concentration of 10 mg/mL.

The maximum yield (70.9 per cent) was observed following extraction at 150 °C, although differences to extraction at 170 °C (67.1 per cent) and 190 °C (65.3 per cent) were insignificant. Extraction yields at 110 and 130 °C were significantly lower (59.1-59.3 per cent). The amount of extraction residue consecutively decreased from 37.4 per cent when extracted at 110 °C to only 21.4 per cent at 190 °C (Table 5.1). Exceeding 150 °C resulted

in a significant drop in the amount of extraction residue from 27.2 per cent to 21.4-23.5 per cent (at 170-190 °C), while the extraction yield remained widely unchanged, thus suggesting the formation of volatile decomposition products. For comparison, pistachio hulls were extracted with acidified aqueous methanol under ultrasonic probe-sonication [63]. The extraction yield was 59.5 per cent w/w (DM), i.e., similar to SCW extraction at 110-130 °C, but lower than SCW extraction at 150-190 °C. Kilic et al. [32] previously reported yields of 36.1 per cent w/w methanolic pistachio hull extracts, while Grace et al. [31] obtained yields of ca. 31 per cent w/w DM of aqueous methanol extracts from defatted pistachio hulls.

The high extraction yields of aqueous methanol extracts obtained in our study might indicate a comparably high solubilization efficiency of the components of pistachio hull. Other plant sources previously extracted with SCW and their respective maximum yields are outer scale of onion, 17 per cent w/w at 165 °C [171], mango leaves, 24-36 per cent w/w at 100 °C [247], seabuckthorn (*Hippophae rhamnoides* L.) leaves, 49 per cent w/w at 200 °C [248], and ellagitannin-rich pomegranate peel, 43.3 per cent w/w at 40 °C [249]. Higher SCW extraction yields compared to that of aqueous methanol extracts might be due to the enhanced solubility of analytes and other matrix components [250]. For instance, water soluble components such as complex carbohydrates being insoluble in alcohols might be extracted under SCW conditions [251]. In agreement, the weight fraction of the SCW extracts soluble in aqueous methanol ranged between 66.2 per cent and 82.4 per cent, i.e., being less than total SCW extraction yields obtained after freeze-drying (Table 5.1). Moreover, thermal decomposition of yet unknown plant matrix constituents might have also contributed to the high SCW yields in our study.

5.3.2. Composition of Phenolic Compounds in the SCW Extracts

Qualitative and quantitative changes in the phenolic composition of SCW extracts depending on the extraction temperature between 110 and 190 °C were monitored by HPLC-DAD-ESI/MS^n (Figure 5.2). For comparison, an aqueous methanolic extract was produced by a previously reported ultrasound-assisted extraction procedure. In this extract, a total of 49 compounds was identified (Table 5.2), which was in agreement with our previous studies [22, 63].

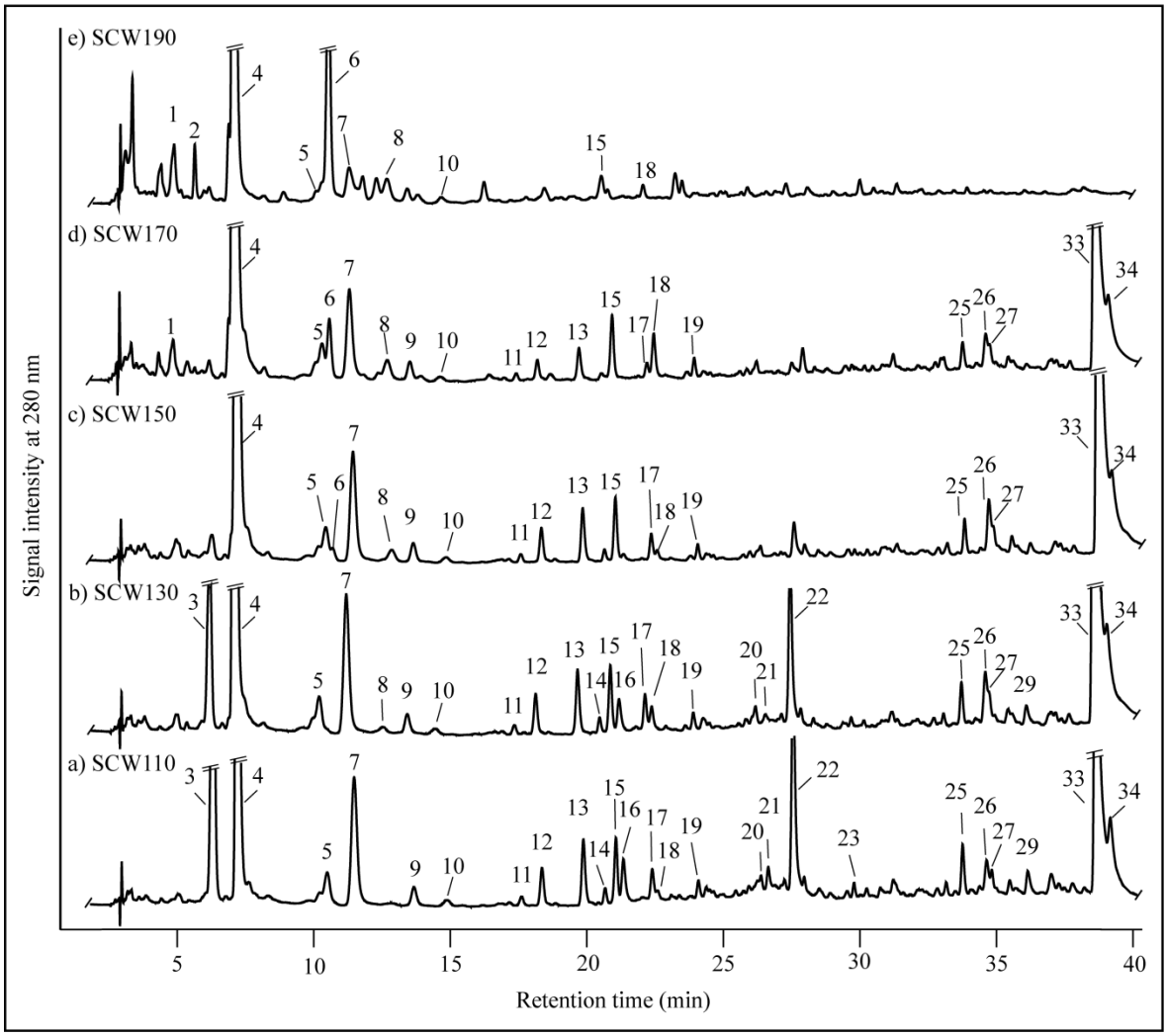

Figure 5.2. Chromatographic separation of SCW extracts at 280 nm. Peak assignments are shown in Table 4.2. Abbreviations: SCW110-190: Subcritical water extracts obtained between 110 and 190°C. Chemical structures of some representative phenolic compounds present in pistachio hull extracts are provided above.

Total yields of extracted phenolic compounds as determined by HPLC-DAD ranged from 22.2 to 39.5 g/kg DM. By trend, extraction yields in phenolic compounds increased when the extraction temperature was raised from 110 to 150 °C. However, when increasing from 170 to 190 °C, a significant decline from 39.3 to 22.2 g/kg DM was observed. Noteworthy, the total extraction yields of phenolic compounds obtained from SCW extracts (22.2-39.5 g/kg DM) were substantially lower than those from the aqueous methanol extract (81.8 g/kg DM), although being mostly attributed to a substantially lower yield in total anacardic acids

in the SCW extracts. Yields in total flavonol and total gallotannins were significantly higher in the SCW extracts than in the aqueous methanol extract as described in detail in the following sections.

5.3.2.1. Gallic acid and its derivatives

A total of 24 different gallic acid derivatives was determined in SCW extracts of pistachio hull (Table 5.2). Their total amounts ranged from 20.4 g/kg DM at 190 °C to 33.1 g/kg DM at 170 °C, constituting a high proportion (74-92 per cent) of total phenolics present in SCW extracts. SCW extraction temperature had a significant effect on gallotannin yields. For instance, a 1.4-fold increase of the total gallotannin level was observed when raising the extraction temperature from 110 to 170 °C ($P < 0.05$). However, a further temperature increase to 190 °C resulted in a significant decline of gallotannin levels to its lowest value (20.4 g/kg DM) in the studied temperature range.

In the extracts produced with repeated extractions using aqueous methanol, total gallotannin levels were 10.6 g/kg DM, being significantly lower than those of all SCW extracts. As aqueous methanol extraction is assumed to be widely exhaustive for the studied gallotannins [63], our findings suggest that a substantial amount of the gallotannins in the SCW extracts has been liberated from the matrix upon exposure to the high temperature, e.g., by hydrolysis of high molecular weight gallotannins or by hydrolytic liberation of matrix-bound gallotannins.

In agreement with our hypothesized hydrolytic gallotannin degradation, SCW extracts contained gallic acid as the main phenolic component (6.31-22.2 g/kg DM), while the aqueous methanol extract was characterized by specific gallotannins such as β-glucogallin (4.39 g/kg DM) and galloyl quinic acid (1.76 g/kg DM) occurring at higher levels than gallic acid (1.68 g/kg DM) in the aqueous methanol extract. Furthermore, an increase in SCW extraction temperature resulted in significantly increased yields of gallic acid, gradually increasing by four-to-six g/kg per 20 °C from 6.31 g/kg DM at 110 °C to 22.2 g/kg DM at 170 °C. At 190 °C, gallic acid content significantly decreased to 16.4 g/kg DM, but still remained at significantly higher levels compared to those obtained by aqueous methanol extraction (Table 5.2). Since gallic acid was exhaustively recovered from pistachio hull in our probe sonication-based aqueous methanol extraction [63], we assume that enhanced

liberation of gallotannins might have increased gallic acid levels observed after SCW extraction, while a heat-dependent degradation of gallotannins might have decreased their yield. In a previous study, heating of gallic acid was shown to produce pyrogallol by decarboxylation as a primary degradation product based on thermogravimetric analyses [243]. In agreement, pyrogallol was found at 190 °C in our study (Table 5.2).

Besides gallic acid, penta-*O*-galloyl-*β*-D-glucose, constituting the second most prevailed phenolic component of SCW extracts, was similarly found at higher levels when extraction temperature was raised from 110 to 130 °C, reaching a plateau at 9.70-9.77 g/kg DM when extracting at 130-150 °C (Table 5.2). When raising temperature from 150 to 170 °C, their content dropped from 9.77 to 5.24 g/kg DM. At 190 °C, penta-*O*-galloyl-*β*-D-glucose was undetectable in our study, indicating its complete thermal degradation.

In agreement with our observations about gallic acid and penta-O-galloyl-*β*-D-glucose, *β*-glucogallin, was best extracted at 110 °C, however, being completely absent above 150 °C. When compared to other gallotannins, *β*-glucogallin displayed a lower stability during SCW extraction than galloyl quinic acids, galloyl shikimic acids, and penta-*O*-galloyl-*β*-D-glucose. SCW extracts also contained various gallic acid esters such as galloyl quinic acids (1, 7-8, 21), galloyl shikimic acids (12, 13), or digallic acid (16, 22), and luteic acid (25) extracted at significantly higher concentrations (total 3.9-7.7 g/kg DM) than by aqueous methanol extraction (3.4 g/kg DM), remaining stable at variable extent between 110 and 170 °C without showing a clear-cut temperature-dependent degradation pattern. Small amounts of other galloyl hexoses such as tetragalloyl hexose (29), digalloyl hexoses (19) were also obtained (total 0.78 g/kg DM), however, being rapidly degraded at temperatures exceeding 110 °C. Tetragalloyl glucose (26) might have been formed by the cleavage of one galloyl unit from pentagalloyl glucose as a potential degradation product. Consequently, tetragalloyl glucose was only found in the SCW extracts, while methanol extracts were devoid of it (Table 5.2).

Table 5.2. Contents of individual compounds from different SCW extracts of pistachio hull samples as determined by HPLC-DAD-ESI/MS^n.

Peak no	Ret. time (min)	Compound identity	Concentration (g/kg DM[1])					
			SCW110	SCW130	SCW150	SCW170	SCW190	Aqueous methanol
1	4.8	Galloyl quinic acid (1)	nd	nd	nd	0.67 ± 0.15b	1.16 ± 0.17a	nd
2	5.5	Pyrogallol	nd	nd	nd	nd	0.20 ± 0,03	nd
3	6.0	*β*-Glucogallin	3.98 ± 0.36a	2.02 ± 0.25b	nd	nd	nd	4.39 ± 0.01a
4	6.8	Gallic acid	6.31 ± 0.61d	10.2 ± 0.70c	16.1 ± 1.26b	22.2 ± 1.79a	16.4 ± 1.27b	1.68 ± 0.04e
5	10.0	Gallic acid derivative (1)	0.39 ± 0.02a	0.44 ± 0.03a	nd	nd	nd	tr
6	10.7	Hydroxymethylfurfural	nd	nd	0.04 ± 0.02c	0.30 ± 0.09b	1.28 ± 0.21a	nd
7	11.2	Galloyl quinic acid (2)	2.22 ± 0.04a	2.14 ± 0.10a	2.18 ± 0.17a	1.63 ± 0.14b	1.49 ± 0.12b	1.76 ± 0.04b
8	12.8	Galloyl quinic acid (3)	nd	nd	0.22 ± 0.02c	0.58 ± 0.14b	0.86 ± 0.08a	nd
9	13.4	Protocatechuic acid	0.33 ± 0.02a	0.30 ± 0.00ab	0.37 ± 0.04a	0.30 ± 0.05ab	nd	0.24 ± 0.02b
10	14.5	Galloyl dihexose (1)	nd	nd	nd	nd	nd	nd
11	17.2	Galloyl dihexose (2)	nd	nd	nd	nd	nd	nd
12	18.0	Galloyl shikimic acid (1)	0.37 ± 0.03a	0.41 ± 0.04a	0.35 ± 0.02a	0.24 ± 0.02b	nd	0.05 ± 0.01c
13	19.6	Galloyl shikimic acid (2)	0.71 ± 0.02a	0.63 ± 0.03a	0.63 ± 0.05a	0.29 ± 0.06c	nd	0.45 ± 0.01b
14	20.2	Gallic acid derivative (2)	tr	nd	nd	nd	nd	nd
15	20.8	Galloyl shikimic acid (3)	0.62 ± 0.02ab	0.58 ± 0.03ab	0.79 ± 0.06a	0.78 ± 0.08a	0.43 ± 0.37ab	0.31 ± 0.01b
16	21.1	Digallic acid (1)	0.47 ± 0.07a	0.27 ± 0.05b	nd	nd	nd	nd
17	22.0	Procyanidin dimer	nq	nq	nq	nq	nq	nq

Table 5.2. Contents of individual compounds from different SCW extracts of pistachio hull samples as determined by HPLC-DAD-ESI/MS^n (Continued)

Peak no	Ret. time (min)	Compound identity	Concentration (g/kg DM[1])					
			SCW110	SCW130	SCW150	SCW170	SCW190	Aqueous methanol
18	22.3	Methyl gallate	nq	nq	nq	nq	nq	nq
19	23.8	Digalloyl hexose	0.28 ± 0.01a	tr	nd	nd	nd	nd
20	26.0	Gallic acid derivative (3)	0.21 ± 0.03a	nd	nd	nd	nd	tr
21	26.4	Digalloyl quinic acid	0.48 ± 0.12a	tr	nd	nd	nd	nd
22	27.3	Digallic acid (2)	2.41 ± 0.24a	1.56 ± 0.19b	nd	nd	nd	0.88 ± 0.01c
23	29.5	Gallic acid derivative (4)	tr	nd	nd	nd	nd	nd
24	31.6	Cyanidin 3-O-*β*-D-galactopyranoside	0.05 ± 0.04b	0.06 ± 0.01b	tr	nd	nd	0.21 ± 0.02a
25	33.4	Luteic acid	0.43 ± 0.06a	0.45 ± 0.01b	0.35 ± 0.05a	0.29 ± 0.07a	nd	nd
26	34.2	Tetragalloyl hexose	0.97 ± 0.11	1.53 ± 0.07	1.81 ± 0.26	0.59 ± 0.17	nd	nd
27	34.5	Quinic acid derivative	0.54 ± 0.15a	0.51 ± 0.08a	0.66 ± 0.05a	0.68 ± 0.14a	nd	0.20 ± 0.10b
28	35.3	Myricetin galloyl hexoside	tr	nd	nd	nd	nd	nd
29	35.8	Tetragalloyl hexose	0.50 ± 0.09	tr	nd	nd	nd	nd
30	36.9	Myricetin hexuronide	0.20 ± 0.02a	0.20 ± 0.01a	0.21 ± 0.02a	0.13 ± 0.01b	nd	tr
31	37.1	Myricetin 3-*O*-galactoside	0.17 ± 0.02a	0.16 ± 0.01ab	0.16 ± 0.02ab	0.13 ± 0.01b	nd	tr
32	37.4	Myricetin hexoside	0.22 ± 0.02a	0.21 ± 0.01a	0.22 ± 0.02a	0.18 ± 0.01ab	nd	0.17 ± 0.01b
33	38.3	Penta-*O*-galloyl-*β*-D glucose	5.34 ± 0.49b	9.70 ± 1.07a	9.77 ± 1.66a	5.24 ± 1.08b	nd	0.92 ± 0.07c

Table 5.2. Contents of individual compounds from different SCW extracts of pistachio hull samples as determined by HPLC-DAD-ESI/MS^n (Continued)

Peak no	Ret. time (min)	Compound identity	Concentration (g/kg DM[1])					
			SCW110	SCW130	SCW150	SCW170	SCW190	Aqueous methanol
34	38.9	Quercetin galloyl hexoside (1)	0.69 ± 0.07a	0.62 ± 0.05ab	0.62 ± 0.04ab	0.55 ± 0.04bc	0.27 ± 0.02c	0.50 ± 0.02d
35, 36	41.0	Quercetin 3-*O*-galactoside/glucuronide	2.05 ± 0.23a	1.79 ± 0.12ab	1.75 ± 0.17ab	1.26 ± 0.15c	0.22 ± 0.03d [2]	1.47 ± 0.09bc
37	41.6	Quercetin 3-*O*-glucoside	0.94 ± 0.11a	0.84 ± 0.05ab	0.84 ± 0.07ab	0.70 ± 0.06bc	0.22 ± 0.02d	0.64 ± 0.03c
38	42.1	Quercetin galloyl hexoside (2)	0.34 ± 0.04a	0.27 ± 0.02ab	0.24 ± 0.05b	0.12 ± 0.02c	nd	0.23 ± 0.01b
39	42.9	Quercetin galloyl hexoside (3)	0.12 ± 0.02a	0.10 ± 0.01a	tr	tr	nd	nd
40	43.7	Quercetin pentoside	0.19 ± 0.02a	0.17 ± 0.01a	0.15 ± 0.02a	0.08 ± 0.04b	nd	0.16 ± 0.02a
41	45.0	Kaempferol hexoside	0.11 ± 0.02a	0.10 ± 0.01a	0.08 ± 0.01a	tr	nd	nd
42	46.9	Kaempferol hexoside	0.12 ± 0.02a	0.10 ± 0.00a	0.09 ± 0.01a	tr	nd	nd
43	89.5	Unknown	nd	nd	nd	nd	nd	nd
44	89.6	(13:1)-Anacardic acid	0.91 ± 0.30cd	0.98 ± 0.10cd	1.42 ± 0.17bc	1.77 ± 0.21b	0.70 ± 0.04d	11.6 ± 0.39a
45	90.7	(13:0)-Anacardic acid	0.59 ± 0.37b	0.58 ± 0.06b	0.84 ± 0.10b	1.01 ± 0.15b	0.43 ± 0.01b	17.9 ± 0.59a
46	91.0	(15:1)-Anacardic acid	tr	tr	tr	tr	nd	3.84 ± 0.13
47	91.4	(17:2)-Anacardic acid	nd	nd	nd	nd	nd	0.70 ± 0.04
48	92.0	(15:0)-Anacardic acid	tr	tr	tr	tr	tr	7.16 ± 0.24
49	92.2	(17:1)-Anacardic acid	tr	tr	tr	tr	tr	25.5 ± 0.85

Table 5.2. Contents of individual compounds from different SCW extracts of pistachio hull samples as determined by HPLC-DAD-ESI/MS^n (Continued)

Peak no	Ret. time (min)	Compound identity	Concentration (g/kg DM[1])					
			SCW110	SCW130	SCW150	SCW170	SCW190	Aqueous methanol
50	93.1	(17:0)-Anacardic acid	nd	nd	nd	nd	nd	0.73 ± 0.04
		Total gallotannins	24.5 ± 1.59bc	30.0 ± 1.65ab	32.5 ± 3.47a	33.1 ± 1.91a	20.4 ± 1.07c	10.6 ± 0.00d
		Total flavonols	5.65 ± 0.50a	4.57 ± 0.31b	4.37 ± 0.43b	3.15 ± 0.33c	0.70 ± 0.07d	3.38 ± 0.18c
		Total anacardic acids	1.50 ± 0.66b	1.56 ± 0.15b	2.27 ± 0.27b	2.77 ± 0.36b	1.13 ± 0.04b	67.5 ± 2.20a
		Total phenolics	33.0 ± 1.22c	36.4 ± 1.93bc	39.5 ± 3.93b	39.3 ± 2.01b	22.2 ± 1.12d	81.8 ± 2.18a

Results were expressed as mean ± standard deviation of three independent of technological replicates. Abbreviations: SCW110-190: Subcritical water extracts obtained by extracting at temperatures from 110 to 190°C. Phenolic compounds which are not present in SCW extracts and detected below LOD in aqueous methanol extracts were excluded from the list. tr: traces, ≥LOD (limit of detection), ≤LOQ (limit of quantitation); nd: not detected, <LOD: below the limit of detection for UV; MS analyses allowed the verification of compound presence based on a previously published study [63]; nq: not quantified. Significant differences between the contents of different samples are indicated by different letters ($P < 0.05$) within a column.

[1] Moisture contents used for the calculation of dry matter (DM) content was 6.5 ± 0.2 per cent.

[2] Only quercetin galactoside was detected.

The above-mentioned interference of liberation and degradation of gallotannins might also partly explain the high variability of gallotannin yields described for SCW extractions in the literature. For instance, gallic acid yields were reported for the SCW extraction of black tea (0.67-5.5 g/kg DM at 100-200 °C) [252], defatted rice bran (0.25-1.7 g/kg DM at 125-175 °C), [243], and winery by-products (2.0 g/ kg DM at 50-150 °C) [147]. As compared to literature yields (0.25-5.5 g/kg), the SCW extracts of pistachio hull in our study still yielded by far the highest amounts of gallic acids (22.2 g/kg DM at 170 °C).

In agreement with the above-mentioned observations, release of gallic acid and penta-*O*-galloyl-*β*-D-glucose were observed upon heat extraction of witch hazel (*H. virginiana* L.), a tannin-rich medicinal plant, in an aqueous medium at 100 °C for 120 min [123]. Such a hydrolytic effect of SCW was also observed previously for red grape skin extracted between 110 and 160 °C [146], and winery by-products extracted at 150 °C [147], presumably liberating gallic acid upon hydrolysis of galloylated tannins (procyanidins) by cleavage of gallate esters.

5.3.2.2. Flavonoids

As shown in Table 5.2, flavonoids of the extracts mainly contained quercetin hexosides, pentosides, glucuronides, and galloylated hexosides. Minor amounts of other flavonols such as myricetin glycosides, hexuronides and galloyl hexosides as well as kaempferol hexosides were also found. In addition, as previously reported, one anthocyanin (cyanidin 3-*O*-*β*-D-galactopyranoside) was also detected [22, 63].

Highest total flavonol content of SCW extracts was obtained at 110-150 °C (4.37-5.65 g/kg DM), subsequently decreasing with an increase in temperature to 170 and 190 °C ($P < 0.05$). Presumably, this decline was due to their thermal degradation as reported earlier for SCW extraction performed in this temperature range [172]. For instance, the levels of quercetin 3-*O*-galactoside/glucuronide and quercetin 3-*O*-glucoside, representing the main flavonols of SCW extracts, were 1.75-2.05 and 0.84-0.94 g/kg DM, respectively, at 110-150 °C, decreasing to only ca. 0.2 g/kg DM when extracting at 190 °C. Similarly, quercetin galloyl hexoside contents (34, 38-39) were between 1.15 and 0.86 g/kg DM at 110-150 °C, declining to 0.27 g/kg DM at 190 °C. Based on our findings, quercetin glycosides might be considered

rather stable during SCW extraction until 130 and 150 °C, at least when using our column-based extraction system.

The aqueous methanolic extract contained significantly lower levels of total flavonols (3.38 g/kg DM) than that of SCW extracts obtained between 110 and 150 °C (4.4-5.7 g/kg DM), indicating SCW extraction to be more effective for the liberation of flavonols from pistachio hull matrix compared to aqueous methanol extraction. Compared to those of the aqueous methanol extract, a 1.3- to 1.6-fold increase in the yield of total flavonols and individual components such as quercetin 3-*O*-galactoside/glucuronide, quercetin 3-*O*-glucoside, quercetin galloyl hexosides (34, 38) was obtained. Moreover, an apparent liberation of small amounts of quercetin/myricetin galloyl hexoside (28, 39), myricetin and kaempferol hexosides (41-42) was observed in SCW extracts, while they were only present in at concentrations < LOD (limit of detection) in the aqueous methanol extracts.

In a previous study on onion waste, flavonol (quercetin and quercetin-4'-glucoside) yields were 4-fold increased when raising temperature from 100 °C to 170 °C in SCW extraction. Values comparable to those of methanolic extractions were only achieved at 170 °C in SCW extraction [171]. In contrast, in another study, flavonol yields obtained by SCW extraction at 170 °C were 1.8- to 23.6-fold higher than those obtained by ethanol- or methanol-based extraction for the recovery of specific flavonols, namely quercetin, kaempferol, and myricetin, from black tea, celery, and ginseng leaf [172]. These results might indicate that the nature of the sample matrix might exert a great influence on the achievable flavonol yields. In our study, pistachio hull flavonols, mainly quercetin 3-*O*-galactoside/glucuronide, quercetin 3-*O*-glucoside, myricetin galactosides, only remained stable up to 150 °C in our SCW extraction system. Nevertheless, flavonol yields obtained in our SCW system at 110-150 °C (4.37-5.65 g/kg DM) were higher than those of the aqueous methanol extracts (3.38 g/kg DM), showing the suitability of our system for flavonol extraction.

Cyanidin 3-*O*-*β*-D-galactopyranoside was also detected when extracting at 110 and 130 °C SCW (ca. 0.05 g/kg DM). However, the levels obtained by SCW extraction were significantly lower than those obtained by aqueous methanol extractions (0.21 g/kg DM), possibly due to the expectedly poor thermal stability of anthocyanins at elevated temperatures [146].

5.3.2.3. Anacardic acids

As previously described [63], pistachio hulls contain significant amounts of anacardic acids (Figure 5.2) which are biologically active [41] but also sensitizing and possible allergenic substances [102]. When using aqueous methanol for control extraction, large amounts (67.5 g/kg DM) of total anacardic acids were found in the extract (Table 5.2). Substantially lower amounts of anacardic acids, mainly (13:1)- and (13:0)-anacardic acids, were extracted with SCW (1.13 to 2.77 g/kg DM). Extraction yields were enhanced at higher temperatures, reaching a maximum for the unsaturated representative at 170 °C (1.8 g/kg DM, Table 5.2). Low anacardic acid yields in SCW extracts might be attributed to their limited solubility due to their hydrophobicity and degradation at high extraction temperatures due to their thermal lability [51]. In order to understand the fate of anacardic acids under SCW conditions, we further analyzed the extraction residues for their phenolic contents by the ultrasound-assisted aqueous methanol extraction. While all other phenolics were exhaustively diminished, the anacardic acids were retained at high levels in the extraction residues (Table 5.3). For instance, total anacardic acid content in the residues amounted to 50.7 g/kg DM at 110 °C, while it was only 1.50 g/kg DM in the SCW extract, but 81.8 g/kg DM in the aqueous methanol extract (Table 5.2). These findings demonstrate that 75.1 per cent of total anacardic acids were retained in the extraction residues, 2.2 per cent were extracted by SCW, and 22.7 per cent were degraded during SCW at 110 °C (15.3 g/kg DM, Table 5.2 and 5.3). When increasing SCW temperature to 130-190 °C, their levels in the extraction residues decreased, while their content in the SCW extract did not increase to the same extent. Thus, we assume that, at 130-190 °C, a temperature-dependent degradation of the anacardic acids did occur (Table 5.3).

5.3.2.4. Other components

Degradation at high temperatures was also evident from the overall reduction of the number of peaks in the HPLC chromatogram when temperature was increased (Figure 5.2). However, some compounds newly appeared upon SCW extraction (Table 5.2). For instance, a procyanidin dimer was tentatively identified, indicating the thermal degradation of matrix-components of larger molecular weights.

Table 5.3. Phenolic compounds in the aqueous methanolic extract of SCW extraction residues as determined by HPLC-DAD ESI/MS^n.

No	Ret. time (min)	Compound identity	Concentration (g/kg DM [1])				
			Res-SCW110	**Res-SCW130**	**Res-SCW150**	**Res-SCW170**	**Res-SCW190**
43	89.5	Unknown [2]	nd	0.6 ± 0.1	2.4 ± 0.2	nd	nd
44	89.6	(13:1)-Anacardic acid	12.6 ± 4.2a	8.8 ± 0.7ab	5.3 ± 0.4bc	1.8 ± 0.2cd	0.4 ± 0.1d
45	90.7	(13:0)-Anacardic acid	15.1 ± 0.6a	13.3 ± 0.5b	7.5 ± 0.5c	2.7 ± 0.2d	0.6 ± 0.1e
46	91.0	(15:1)-Anacardic acid	3.1 ± 0.3a	2.9 ± 0.1a	1.6 ± 0.1b	0.6 ± 0.1c	tr
47	91.4	(17:2)-Anacardic acid	tr	tr	tr	tr	nd
48	92.0	(15:0)-Anacardic acid	3.8 ± 1.9 ab	4.5 ± 0.1a	2.3 ± 0.2bc	0.8 ± 0.0 c	tr
49	92.2	(17:1)-Anacardic acid	16.1 ± 3.5a	16.3 ± 0.3a	8.4 ± 0.6b	3.0 ± 0.2c	0.8 ± 0.2c
50	93.1	(17:0)-Anacardic acid	tr	tr	nd	nd	nd
		Total anacardic acids	50.7 ± 0.9a	45.7 ± 1.2b	25.1 ± 1.8c	9.1 ± 0.5d	7.8 ± 0.4e

Results were expressed as mean ± standard deviation of three independent technological replicates.
Abbreviations: Res-SCW110-190: Subcritical water extraction residues obtained after extraction at temperatures from 110 to 190°C.
tr: traces, ≥LOD (limit of detection), ≤LOQ (limit of quantitation); nd: not detected, <LOD: below the limit of detection for UV; MS analyses allowed the verification of compound presence based on a previously published study [63].
All other compounds identified in the extracts (Table 5.2) were found <LOD.
Significant differences between the contents of different samples are indicated by different letters ($P < 0.05$) within a column.
[1] Moisture contents used for the calculation of dry matter (DM) content was 6.5 ± 0.2 per cent.

[2] Compound with λmax (nm): 273, 279(sh) did not provide useful MS signal.

Most importantly, the formation of 5-hydroxymethylfurfural (HMF) was observed when heating at least to 150 °C (0.04 g/kg DM), reaching its highest levels at 190 °C (1.28 g/kg DM), possibly due to thermo-oxidation or Maillard reactions occurring at high temperatures [128]. Considering maximum non-toxic HMF intake levels (80-100 mg/kg per day) previously recommended based on animal studies [253], SCW extracts obtained at high temperatures (150-190 °C) should be considered carefully for their contribution to the total HMF level of a final product when being intended to be used as a food additive.

5.3.3. Antioxidant Capacity of the Extracts

As the antioxidant capacity has been generally related to the biological activity of phenolic compounds, antioxidant capacities of SCW extracts were tested using three different commonly used *in vitro* antioxidant capacity assays, namely, the DPPH free radical scavenging assay, the ABTS radical scavenging assay, and the ferric reducing antioxidant power (FRAP) to reflect different potential antioxidant mechanisms of pistachio hull extracts.

Antioxidant capacities of SCW extracts ranged from 0.68 to 1.2 mmol TE/g DM and were largely consistent among all antioxidant capacity assays (Table 5.4). When extracts were obtained at 110 and 130 °C, antioxidant capacities of SCW extracts were in the same range, while a pronounced increase was observed when applying 170 °C or 190 °C for all assays ($P < 0.05$). Similarly, increased antioxidant capacities after applying high extraction temperatures were observed in previous studies on the SCW extraction of coffee silverskin [254], and pomegranate seed residues [249]. The authors of these studies have attributed the increased antioxidant capacities to the higher extraction efficiencies and heat-related formation of new antioxidant compounds *via* Maillard reaction, caramelization, and thermo-oxidation reactions at elevated temperatures, being related to the complex nature of plant extracts as previously discussed in the context of SCW extraction by Plaza et al. [128].

Aqueous methanol extracts exhibited the lowest antioxidant capacity (0.47-0.51 mmol TE/g DM) as compared to those of the obtained SCW extracts ($P < 0.05$). Based on our findings, pistachio hull might be considered as a promising antioxidant source (0.47-1.20 mmol TE/g DM) coming closed to other known antioxidant-rich sources such as green tea (FRAP: 0.386-1.14 mmol TE/ g DM) [255], grape (DPPH: 6.91 mmol TE/100 g DM) [256], and

pistachio nut and skin (ABTS: 0.015 and 2.19 mmol TE/ g FW, respectively) [211]. Our results further indicate that SCW extraction may be instrumental in obtaining pistachio hull extracts of high antioxidant capacity.

Table 5.4. Antioxidant capacity of pistachio hull extracts

Extract	Antioxidant capacity (mmol TE/g DM[1])		
	ABTS	DPPH	FRAP
SCW110	0.79 ± 0.03c	0.68 ± 0.03c	0.72 ± 0.03cd
SCW130	0.72 ± 0.08c	0.68 ± 0.00c	0.68 ± 0.03d
SCW150	0.89 ± 0.07bc	0.78 ± 0.02b	0.80 ± 0.06c
SCW170	0.98 ± 0.08b	0.77 ± 0.02b	1.04 ± 0.03b
SCW190	1.18 ± 0.08a	0.84 ± 0.00a	1.20 ± 0.04a
Aqueous methanol	0.47 ± 0.02d	0.51 ± 0.01d	0.49 ± 0.01e

Values were expressed as means ± standard deviation of three independent technological replicates. Abbreviations: SCW110-190: Subcritical water extracts obtained between 110 and 190°C.
Significant differences between the contents of different samples are indicated by different letters ($P < 0.05$) within a column.
[1] Moisture contents used for the calculation of dry matter (DM) content was 6.5 ± 0.2 per cent.

5.4. CONCLUSION

A semi-continuous, column-based extraction process using subcritical water (SCW) was developed and tested with regard to the extraction of phenolic compounds, namely, gallotannins, flavonols and anacardic acids, from pistachio hull. Using water as a "green solvent", our SCW extraction process allowed the recovery of extracts with gallotannin and flavonol levels being superior to those obtained by aqueous methanol-based extraction. Moreover, sensitizing and possible allergenic anacardic acids were excluded from SCW extracts while they were extracted together with gallotannin and flavonols when using aqueous methanol-based extraction. Furthermore, our findings show that the composition of phenolic compounds contained in SCW extracts of pistachio hull can be substantially modulated simply by varying the extraction temperature (110-190 °C). Higher temperatures enhanced the release of higher amounts of gallic acid and penta-*O*-galloyl-*β*-D-glucose from

the pistachio hull matrix, although being limited due to the thermal instability of the corresponding compounds at temperatures above 170 °C. The obtained SCW extracts exhibited a comparably high antioxidant activity. The high efficiency of SCW for the recovery of phenolic-rich extracts with high antioxidant capacities shows that this “green” and comparably environmentally friendly technology represents a promising alternative to the organic solvent-based extraction methods for the recovery of polyphenols from food processing by-products like pistachio hull.

6. CONCLUDING REMARKS

Pistachio hull accrues in large amounts as the main by-product of pistachio processing. Hitherto, it has to be disposed at the expense of the pistachio processor due to its current lack of commercial value. The lack of comprehensive reports on pistachio hull and the missing identification of its phenolic constituents hamper its valorization as a source of bioactive phenolics. Therefore, first, a comprehensive report about phenolic constituents in aqueous methanolic extracts of pistachio hull was presented in Section 3 to provide a basis for future studies on pistachio hull phenolics. Detailed HPLC-PDA-ESI-(HR)-MS^n data for a total of 66 phenolic compounds in red pistachio hull are presented. Several (a total of 31) phenolic compounds including differently galloylated hydrolyzable tannins, anthocyanins, and minor anacardic acids were identified for the first time. Furthermore, differences on red and green pistachio hulls were examined, since both are different by-products coming from different pistachio processing streams. For the first time, their composition in phenolic compounds was compared. Since it is currently debated if the genus *Pistacia* belongs to the Anacardiaceae or not, this results might be helpful for future chemotaxonomic considerations.

Based on the qualitative analysis done in Section 3, pistachio hull phenolics ranged from the very hydrophilic gallic acid derivatives to the amphiphilic anacardic acids, making their quantitative analytical determination a substantial challenge. Moreover, their partitioning between different solvent systems is of interest to allow a better interpretation of the biological activity of its extracts. Section 4 contains a comprehensive report about the simultaneous extraction and quantitation of phenolic constituents of pistachio hull. A rapid and simultaneous extraction method was presented for pistachio hull phenolics after screening of various extraction parameters and solvent systems with variable polarity and acidity to obtain in-depth insights into the extractability of free phenolics from pistachio hulls. Subsequently, two alternative post-extractive analytical methods, HPLC-DAD-ESI-MS^n and UHPLC-DAD-ELSD were presented and compared. Later, developed quantitation method was validated and its applicability was tested in four different pistachio hull samples from three different varieties. The phenolic composition of the hulls ranged between six and 10 per cent (w/w) and containing potentially bioactive phenolics such as gallic acid, penta-*O*-galloyl-*β*-D-glucose, quercetin glycosides, and anacardic acids. Extraction and

quantitation methodologies presented in this section are expected to be useful for future studies on pistachio hull phenolics such as comparison of different varieties, production years, or agricultural practices.

In Section 5, as an alternative to current poorly sustainable extraction processes requiring the use of organic solvents, subcritical water (SCW) extraction technology was evaluated, aiming at the production of value-added extracts rich in phenolic and antioxidant constituents from pistachio hull. SCW extracts were prepared at temperatures ranging from 110 to 190 °C. The extracts and the extraction residues were analyzed in detail using HPLC-DAD-ESI-MS^n for their composition of phenolic compounds as well as possible degradation products. Additionally, the antioxidant capacities of extracts were determined using three *in vitro* assays. Furthermore, the efficiency of SCW extraction was compared to that of aqueous methanol based ultrasound-assisted extraction developed in Section 4. SCW extracts were up to 3.0-, 13.2- and 10.6-fold richer in total gallotannins, gallic acid, and penta-*O*-galloyl-*β*-D-glucose contents, respectively, than those obtained by aqueous methanol extraction. In addition, the SCW extracts were depleted in anacardic acids, being widely retained in the SCW extraction residue, particularly, at lower temperatures. Thus, the proposed SCW process allows the selective extraction of gallotannin/flavonoids, and their separation from sensitizing and possible allergenic anacardic acids. However, SCW extraction should be further evaluated in terms of other process parameters such as flow rate and extraction time to have a better understanding of underlying extraction mechanisms. Moreover, evaluation of scaling-up options and feasibility of SCW extraction for the recovery of phenolic and antioxidant-rich extracts from pistachio hull should be evaluated before real-life application of this technology. Although SCW extraction units are currently very rare due to their high investment and operational costs in relation to the process requirements of high pressure and temperature, advancements in SCW is only limited by the advancements in technology that moves forward each day.

In summary, this thesis represents a detailed analytical report on the identification and quantitation of phenolic constituents of *P. vera* L. hull extracts, providing information for future studies on the utilization of pistachio hull phenolics. Moreover, the first assessment of SCW extraction of phenolics and antioxidants from pistachio hull were accomplished, creating a solid basis for future studies on not only the utilization of pistachio hull, but also of other plant matrices rich in gallotannins, flavonoids, and anacardic acids. Although the

initial objective of this thesis was exploring potential utilization of pistachio hull as a source of phenolic antioxidants (Section 2.9), the scope of this thesis remained limited with characterization and extraction studies due to the diversity and structural complexity of pistachio hull phenolics, and challenges on their analysis. Noteworthy, extraction is only one of the intermediate stages on the path of utilization of food and agricultural by-products as previously stated by Galanakis in detail [257]. As a next step, further studies are expected on purification, fractionation and/or isolation of pistachio hull phenolics together with investigations on their biological activities aiming final product formulations with intended biological activities. Based on the overview of the potential biological activity of pistachio hull phenolics given in the theoretical background part of this thesis (Section 2), they may find application, for instance, as a food preservative with antioxidant and antimicrobial activities, or can be used as pharmaceutical, or nutraceutical agent due to their health-promoting properties. However, despite the promising scientific results on pistachio hull phenolics, utilization potential of pistachio hull is still limited by the fact that they have no traditional use for human consumption. Therefore, current use of pistachio hull or extracts derived thereof for food applications is restricted to the assessment of their safety for human use.

Another limitation for the utilization of pistachio hull might be its seasonable production not being concentrated in one location and processing unit. For instance, for the specific case of Turkey, 80 000 tons of pistachio kernel production in 2014 can be estimated to produce 43850 tons of pistachio hull as a by-product [4, 22], however, distributed over a wide geographical region in southeast part of Turkey with many small processing units technologically differing. Therefore, characteristics, quantity and geographical distribution of pistachio by-products (mainly hull) should be investigated prior to establishing a processing unit for their utilization.

Finally, current trends in utilization of waste/by-products are directed towards to the next-generation biorefineries that are capable of producing multiple value-added products to increase feasibility and economic viability of this kind of processing units. Therefore, pistachio hull should be further evaluated in this concept to explore its full potential.

REFERENCES

1. Grand View Research. “Polyphenols Market Analysis By-Product (Grape Seed, Green Tea, Apple) by Application (Functional Food, Functional Beverages, Dietary Supplements) and Segment Forecasts to 2024”, http://www.grandviewresearch.com/industry-analysis [retrieved 1 June 2017].

2. P. M. Kris-Etherton, K. D. Hecker, A. Bonanome, S. M. Coval, A. E. Binkoski, K. F. Hilpert, A. E. Griel, and T. D. Etherton. Bioactive Compounds in Foods: Their Role in the Prevention of Cardiovascular Disease and Cancer. *American Journal of Medicine,* 113:71–88, 2002.

3. C. M. Galanakis. The Universal Recovery Strategy. In: C. M. Galanakis, editor, *Food Waste Recovery*, pages 59–81. Elsevier Academic Press Ltd., London, 2015.

4. FAOSTAT. “Database of the Food and Agriculture Organization of the United Nations”, http//faostat.fao.org/ [retrieved 5 September 2017].

5. N. Schulze-Kaysers, M. M. Feuereisen, and A. Schieber. Phenolic Compounds in Edible Species of the Anacardiaceae Family – A review. *Royal Society of Chemistry Advances,* 5:73301–14, 2015.

6. N. Berardini, R. Fezer, J. Conrad, U. Beifuss, R. Carle, and A. Schieber. Screening of Mango (*Mangifera indica* L.) Cultivars for Their Contents of Flavonol *O*- and Xanthone *C*-Glycosides, Anthocyanins, and Pectin. *Journal of Agricultural and Food Chemistry*, 53:1563–70, 2005.

7. C. Engels, M. G. Gänzle, and A. Schieber. Fractionation of Gallotannins from Mango (*Mangifera indica* L.) Kernels by High-Speed Counter-Current Chromatography and Determination of Their Antibacterial Activity. *Journal of Agricultural and Food Chemistry,* 58:775–80, 2010.

8. C. H. Geerkens, A. E. Matejka, R. Carle, and R. M. Schweiggert. Development and

Validation of an HPLC Method for the Determination of Alk(en)ylresorcinols Using Rapid Ultrasound-Assisted Extraction of Mango Peels and Rye Grains. *Food Chemistry*, 169:261–9, 2015.

9. M. M. Feuereisen, J. Hoppe, B. F. Zimmermann, F. Weber, N. Schulze-Kaysers, and A. Schieber. Characterization of Phenolic Compounds in Brazilian Pepper (*Schinus terebinthifolius* Raddi) Exocarp. *Journal of Agricultural and Food Chemistry*, 62:6219–26, 2014.

10. R. Paramashivappa, P. P. Kumar, P. J. Vithayathil, and A. S. Rao. Novel Method for Isolation of Major Phenolic Constituents from Cashew (*Anacardium occidentale* L.) Nut Shell Liquid. *Journal of Agricultural and Food Chemistry*, 49:2548–51, 2001.

11. D. Barreca, G. Laganà, U. Leuzzi, A. Smeriglio, D. Trombetta, and E. Bellocco. Evaluation of the Nutraceutical, Antioxidant and Cytoprotective Properties of Ripe Pistachio (*Pistacia vera* L., Variety Bronte) Hulls. *Food Chemistry*, 196:493–502, 2016.

12. F. Garavand, A. Madadlou, and S. Moini. Determination of Phenolic Profile and Antioxidant Activity of Pistachio Hull using HPLC-DAD-ESI-MS as Affected by Ultrasound and Microwave. *International Journal of Food Properties*, 20:19–29, 2017.

13. A. H. Goli, M. Barzegar, and M. A. Sahari. Antioxidant Activity and Total Phenolic Compounds of Pistachio (*Pistachia vera*) Hull Extracts. *Food Chemistry*, 92:521–5, 2005.

14. A. Rajaei, M. Barzegar, A. M. Mobarez, M. A. Sahari, and Z. H. Esfahani. Antioxidant, Antimicrobial and Antimutagenicity Activities of Pistachio (*Pistachia vera*) Green Hull Extract. *Food and Chemical Toxicology*, 48:107–12, 2010.

15. I. Kubo, M. Ochi, P. C. Vieira, and S. Komatsu. Antitumor Agents from the Cashew (*Anacardium occidentale*) Apple Juice. *Journal of Agricultural and Food Chemistry*, 41:1012–5, 1993.

16. D. Alam-Escamilla, E. Estrada-Muñiz, E. Solís-Villegas, G. Elizondo, and L. Vega.

Genotoxic and Cytostatic Effects of 6-Pentadecyl Salicylic Anacardic Acid in Transformed Cell Lines and Peripheral Blood Mononuclear Cells. *Mutation Research-Genetic Toxicology Environmental Mutagenesis*, 777:43–53, 2015.

17. E. M. Yahia. *Postharvest Biology and Technology of Tropical and Subtropical Fruits*, Woodhead Publishing Ltd., Philadelphia, 2011.

18. N. P. Seeram, Y. Zhang, and S. Bowerman. Phytochemicals and Health Aspects of Pistachio (*Pistacia vera* L.). In: C. Alasalvar, and F. Shahidi, editors, *Tree Nuts: Composition, Phytochemicals, and Health Effects*, pages 295–303. Taylor & Francis Groups CRC Press LLC, Boca Raton, 2008.

19. S. Padulosi, and A. Hadj-Hassan. Towards a Comprehensive Documentation and Use of *Pistacia* Genetic Diversity in Central and West Asia, North Africa and Europe. *Report of the IPGRI Workshop*, 14-17 December 1998, Irbid, Jordon.

20. M. L. Dreher. Pistachio Nuts: Composition and Potential Health Benefits. *Nutrition Reviews*, 70:234–40, 2012.

21. J. Wilkinson. *Nut Grower's Guide: The Complete Handbook for Producers and Hobbyists*. Landlinks Press, Collingswing, 2005.

22. S. Erşan, Ö. Güçlü Üstündağ, R. Carle, and R. M. Schweiggert. Identification of Phenolic Compounds in Red and Green Pistachio (*Pistacia vera* L.) Hulls (Exo- and Mesocarp) by HPLC-DAD-ESI-(HR)-MS^n. *Journal of Agricultural and Food Chemistry*, 64:5334–44, 2016.

23. M. L. Dewan, M. C. Nautiyal, and V. K. Sah. *Nut Fruits for the Himalayas*. Concept Publishing Co., New Delhi, 1992.

24. F. Bloch, and J. E. Brekke. Processing of Pistachio Nuts. *Economic Botany*, 14:129–44, 1960.

25. G. Bartzas, and K. Komnitsas. Life Cycle Analysis of Pistachio Production in Greece. *Science of the Total Environment*, 595:13–24, 2017.

26. A. Mokhtarpour, A. A. Naserian, A. M. Tahmasbi, and R. Valizadeh. Effect of Feeding Pistachio By-Products Silage Supplemented with Polyethylene Glycol and Urea on Holstein Dairy Cows Performance in Early Lactation. *Livestock Science*, 148:208–13, 2012.

27. S. Ghasemi, A. Naserian, R. Valizadeh, A. R. Vakili, M. Behgar, A. M. Tahmasebi, and S. Ghovvati. Partial and Total Substitution of Alfalfa Hay by Pistachio Byproduct Modulated the Counts of Selected Cellulolytic Ruminal Bacteria Attached to Alfalfa Hay in Sheep. *Livestock Science*, 150:342–8, 2012.

28. E. Bagheripour, Y. Rouzbehan, and D. Alipour. Effects of Ensiling, Air-Drying and Addition of Polyethylene Glycol on *In Vitro* Gas Production of Pistachio By-Products. *Animal Feed Science and Technology*, 146:327–36, 2008.

29. H. R. Karimi, A. Sajjadinia, V. Baghari, and H. Farahmand. Preliminary Evaluation of Composted Pistachio Hull and Rice Husk as Potting Medium on *Scindapsus aureus* Vegetative and Physiological Characteristics in Greenhouse Condition. *Journal of Plant Nutrition*, 36:2225–35, 2013.

30. A. Golchin, M. Nadi, and V. Mozaffari. The Effects of Vermicomposts Produced From Various Organic Solid Wastes on Growth of Pistachio Seedlings. *Acta Horticulture*, 726:301–6, 2006.

31. M. H. Grace, D. Esposito, M. A. Timmers, J. Xiong, G. Yousef, S. Komarnytsky, and M. A. Lila. Chemical Composition, Antioxidant and Anti-Inflammatory Properties of Pistachio Hull Extracts. *Food Chemistry*, 210:85–95, 2016.

32. I. H. Kilic, C. Sarikurkcu, I. D. Karagoz, M. C. Uren, M. S. Kocak, M. Cilkiz, and B. Tepe. A Significant By-Product of the Industrial Processing of Pistachios: Shell Skin -RP-HPLC Analysis, and Antioxidant and Enzyme Inhibitory Activities of the Methanol Extracts of *Pistacia vera* L. Shell Skins Cultivated in Gaziantep, Turkey. *Royal Society of Chemistry*

Advances, 6:1203–9, 2016.

33. J. Motamedi-Tehrani, E. Ebrahimi-Dorcheh, and S. A. H. Goli. Effect of Pistachio (*Pistacia vera*) Hull Extract on Growth Performance, Body Composition, Total Phenolic Compound and Fillets Peroxide Value of Common Carp, *Cyprinus carpio*. *Aquaculture Nutrition*, 22:479–84, 2016.

34. F. Al-Juhaimi, O. Q. Adiamo, O. N. Alsawmahi, K. Gahfoor, M .Z. Islam Sarker, I. A. M. Ahmed, and E. E. Babiker. Effect of Pistachio Seed Hull Extracts on Quality Attributes of Chicken Burger. *CYTA – Journal of Food*, 15:9–14, 2016.

35. G. Moussavi, and R. Khosravi. Removal of Cyanide from Wastewater by Adsorption onto Pistachio Hull Wastes: Parametric Experiments, Kinetics and Equilibrium Analysis, *Journal of Hazardous Materials*, 183:724–30, 2010.

36. İ. Demiral, N. G. Atilgan, and S. Şensöz. Production of Biofuel from Soft Shell of Pistachio (*Pistacia vera* L.). *Chemical Engineering Communications*, 196:104–15, 2008.

37. J. Serrano, R. Puupponen-Pimiä, A. Dauer, A.-M. Aura, and F. Saura-Calixto. Tannins: Current Knowledge of Food Sources, Intake, Bioavailability and Biological Effects. *Molecular Nutrition and Food Research*, 53:310-29, 2009.

38. A. Crozier, I. B. Jaganath, and M. N. Clifford. Dietary Phenolics: Chemistry, Bioavailability and Effects on Health. *Natural Product Reports*, 26:1001–43, 2009.

39. M. Naczk, and F. Shahidi. Biosynthesis, Classification and Nomenclature of Phenolics in Food and Nutraceuticals. In: *Phenolic in Food and Nutraceuticals*. Taylor & Francis Groups CRC Press LLC, New York, 2003.

40. K. Khanbabaee, and T. van Ree. Tannins: Classification and Definition. *Natural Product Reports*, 18:641–9, 2001.

41. M. Stasiuk, and A. Kozubek. Biological Activity of Phenolic Lipids. *Cellular and*

Molecular Life Sciences, 67:841–60, 2010.

42. K. B. Pandey, and S. I. Rizvi. Plant Polyphenols as Dietary Antioxidants in Human Health and Disease. *Oxidative Medicine and Cell Longevity*, 2:270–8, 2009.

43. A. E. Hagerman, Y. Zhao, and S. Johnson. Methods for Determination of Condensed and Hydrolyzable Tannins. In: F. Shahidi, editor, *Antinutrients and Phytochemicals in Food*, pages 209-222. American Chemical Society, Washington, 1997.

44. G. G. Gross. From Lignins to Tannins: Forty Years of Enzyme Studies on the Biosynthesis of Phenolic Compounds. *Phytochemistry*, 69:3018–31, 2008.

45. E. Haslam. Natural Polyphenols (Vegetable Tannins) as Drugs: Possible Modes of Action. *Journal of Natural Products*, 59:205–15, 1996.

46. J. Zhang, L. Li, S. H. Kim, A. E. Hagerman, and J. Lü. Anti-Cancer, Anti-Diabetic and Other Pharmacologic and Biological Activities of Penta-galloyl-glucose. *Pharmaceutical Research*, 26:2066–80, 2009.

47. R. Niemetz, and G. G. Gross. Enzymology of Gallotannin and Ellagitannin Biosynthesis. *Phytochemistry*, 66:2001–11, 2005.

48. E. Bakkalbaşi, O. Menteş, and N. Artik. Food Ellagitannins-Occurrence, Effects of Processing and Storage. *Critical Reviews in Food Science and Nutrition*, 49:283–98, 2009.

49. A. Smeriglio, D. Barreca, E. Bellocco, and D. Trombetta. Proanthocyanidins and Hydrolysable Tannins: Occurrence, Dietary Intake and Pharmacological Effects. *British Journal of Pharmacology*, 174:1244–62, 2017.

50. C. Manach, A. Scalbert, C. Morand, C. Rémésy, and L. Jiménez. Polyphenols: Food Sources and Bioavailability. *The American Journal of Clinical Nutrition*, 79:727–47, 2004.

51. M. Yuliana, N. Y. Tran-Thi, and Y.-H. H. Ju. Effect of Extraction Methods on

Characteristic and Composition of Indonesian Cashew Nut Shell Liquid. *Industrial Crops and Products*, 35:230–6, 2012.

52. J. U. Ziegler, C. B. Steingass, C. F. H. Longin, T. Würschum, R. Carle, and R. M. Schweiggert. Alkylresorcinol Composition Allows the Differentiation of *Triticum* spp. Having Different Degrees of Ploidy. *Journal of Cereal Science*, 65:244–51, 2015.

53. J. L. Gellerman, and H. Schlenk. Methods for Isolation and Determination of Anacardic Acids. *Analytical Chemistry*, 40:739–43, 1968.

54. M. T. S. Trevisan, B. Pfundstein, R. Haubner, G. Würtele, B. Spiegelhalder, H. Bartsch, and R. W. Owen. Characterization of Alkyl Phenols in Cashew (*Anacardium occidentale*) Products and Assay of Their Antioxidant Capacity. *Food and Chemical Toxicology*, 44:188–97, 2006.

55. A. Kozubek, and J. H. P. Tyman. Resorcinolic Lipids, the Natural Non-Isoprenoid Phenolic Amphiphiles and Their Biological Activity. *Chemical Reviews*, 99:2–25, 1999.

56. D. S. Walters, R. Craig, and R. O. Mumma. Fatty Acid Incorporation in the Biosynthesis of Anacardic Acids of Geraniums. *Phytochemistry*, 29:1815–22, 1990.

57. J. Dai, and R. J. Mumper. Plant Phenolics: Extraction, Analysis and Their Antioxidant and Anticancer Properties. *Molecules*, 15:7313–52, 2010.

58. M. S. Fernandez-Panchon, D. Villano, A. M. Troncoso, and M. C. Garcia-Parrilla. Antioxidant Activity of Phenolic Compounds: From *In Vitro* Results to *In Vivo* Evidence. *Critical Reviews in Food Science and Nutrition*, 48:649–71, 2008.

59. K. B. Pandey, and S. I. Rizvi. Plant Polyphenols as Dietary Antioxidants in Human Health and Disease. *Oxidative Medicine and Cell Longevity*, 2:270–8, 2009.

60. F. Shahidi, and Y. Zhong. Novel Antioxidants in Food Quality Preservation and Health Promotion. *European Journal of Lipid Science and Technology*, 112:930–40, 2010.

61. N. Nadernejad, A. Ahmadimoghadam, J. Hosseinifard, and S. Pourseyedi. Phenylalanin Ammonia-Lyase Activity, Total Phenolic and Flavonoid Content in Flowers, Leaves, Hulls and Kernels of Three Pistachio (*Pistacia vera* L.) Cultivars. *American-Eurasian Journal Agricultural and Environmental Sciences*, 12:807–14, 2012.

62. A. S. Sonmezdag, H. Kelebek, and S. Selli. Characterization and Comparative Evaluation of Volatile, Phenolic and Antioxidant Properties of Pistachio (*Pistacia vera* L.) Hull. *Journal of Essential Oil Research*, 29:262-270, 2016.

63. S. Erşan, Ö. Güçlü Üstündağ, R. Carle, and R. M. Schweiggert. Determination of Pistachio (*Pistacia vera* L.) Hull (Exo- and Mesocarp) Phenolics by HPLC-DAD-ESI/MS^n and UHPLC-DAD-ELSD after Ultrasound-Assisted Extraction. *Journal of Food Composition and Analysis*, 62:103–14, 2017.

64. M. Yalpani, and J. H. P. Tyman. The Phenolic Acids of *Pistachia vera*. *Phytochemistry*, 22:2263–6, 1983.

65. M. Bozorgi, Z. Memariani, M. Mobli, M. Hossein, S. Surmaghi, M. R. Shams-Ardekani, and R. Rahimi. Five *Pistacia* species (*P. vera, P. atlantica, P. terebinthus, P. khinjuk, and P. lentiscus*): A Review of Their Traditional Uses, Phytochemistry, and Pharmacology. *The Scientific World Journal*, 2013:1–33, 2013.

66. S. Jazayeri, A. Amanlou, N. Ghanadian, P. Pasalar, and M. A. Amanlou. Preliminary Investigation of Anticholinesterase Activity of Some Iranian Medicinal Plants Commonly Used in Traditional Medicine. *DARU Journal of Pharmaceutical Sciences*, 22:17, 2014.

67. J. Fathalizadeh, V. Bagheri, H. Khorramdelazad, M. K. Arababadi, A. Jafarzadeh, M. R. Mirzaei, A. Shamsizadeh, and M. R. Hajizadeh. Induction of Apoptosis by Pistachio (*Pistacia vera* L.) Hull Extract and Its Molecular Mechanisms of Action in Human Hepatoma Cell Line HepG2. *Cellular and Molecular Biology*, 61:128–34, 2015.

68. M. Daglia, A. Lorenzo, S. Nabavi, and Z. Talas. Polyphenols: Well Beyond the Antioxidant Capacity: Gallic Acid and Related Compounds as Neuroprotective Agents: You

are What You Eat! *Current Pharmaceutical Biotechnology*, 15:362–72, 2014.

69. B. Kroes, A. van den Berg, H. Q. van Ufford, H. van Dijk, and R. Labadie. Anti-Inflammatory Activity of Gallic Acid. *Planta Medica*, 58:499–504, 1992.

70. T. Gichner, F. Pospíšil, J. Velemínský, V. Volkeová, and J. Volke. Two Types of Antimutagenic Effects of Gallic and Tannic Acids towards *N*-Nitroso-Compounds-Induced Mutagenicity in the Ames *Salmonella* Assay. *Folia Microbiologica*, 32:55–62, 1987.

71. M. Inoue, R. Suzuki, N. Sakaguchi, Z. Li, T. Takeda, Y. Ogihara, B. Y. Jiang, and Y. Chen. Selective Induction of Cell Death in Cancer Cells by Gallic Acid. *Biological and Pharmaceutical Bulletin*, 18:1526–30, 1995.

72. C. A. Rice-Evans, N. J. Miller, and G. Paganga. Structure-Antioxidant Activity Relationships of Flavonoids and Phenolic Acids. *Free Radical Biology and Medicine*, 20:933–56, 1996.

73. A. E. Hagerman, K. M. Riedl, G. A. Jones, R. N. Sovik, N. T. Ritchard, P. W. Hartzfeld, and T. L. Rieche. High Molecular Weight Plant Polyphenolics (Tannins) as Biological Antioxidants. *Journal of Agricultural and Food Chemistry*, 4930:1–6, 1998.

74. M. C. Baratto, M. Tattini, C. Galardi, P. Pinelli, A. Romani, F. Visioli, R. Basosi, and R. Pogni. Antioxidant Activity of Galloyl Quinic Derivatives Isolated from *P. lentiscus* Leaves. *Free Radical Research*, 37:405–12, 2003.

75. C. Dufour, E. Da Silva, P. Potier, Y. Queneau, and O. Dangles. Gallic Esters of Sucrose as Efficient Radical Scavengers in Lipid Peroxidation. *Journal of Agricultural and Food Chemistry*, 50:3425–30, 2002.

76. D. Karas, J. Ulrichová, and K. Valentová. Galloylation of Polyphenols Alters Their Biological Activity. *Food and Chemical Toxicology*, 105:223–40, 2017.

77. J. Zhang, L. Li, S. H. Kim, A. E. Hagerman, and J. Lü. Anti-Cancer, Anti-Diabetic and

Other Pharmacologic and Biological Activities of Penta-galloyl-glucose. *Pharmaceutical Research*, 26:2066–80, 2009.

78. H. Hu, J. Zhang, H. J. Lee, S. H. Kim, and J. Lü. Penta-O-galloyl-beta-D-glucose Induces S- and G_1-Cell Cycle Arrests in Prostate Cancer Cells Targeting DNA Replication and Cyclin D1. *Carcinogenesis*, 30:818–23, 2009.

79. J.-E. Huh, E.-O. Lee, M.-S. Kim, K.-S. Kang, C.-H. Kim, B.-C. Cha Surh, and Y.-J. Kim S-H. Penta-O-galloyl-beta-D-glucose Suppresses Tumor Growth via Inhibition of Angiogenesis and Stimulation of Apoptosis: Roles of Cyclooxygenase-2 and Mitogen-Activated Protein Kinase Pathways. *Carcinogenesis*, 26:1436–45, 2005.

80. K.-T. Hua, T.-D. Way, and J.-K. Lin. Pentagalloylglucose Inhibits Estrogen Receptor A by Lysosome-Dependent Depletion and Modulates ErbB/PI3K/Akt Pathway in Human Breast Cancer MCF-7 Cells. *Molecular Carcinogenesis*, 45:551–60, 2006.

81. G.-S. Oh, H.-O. Pae, H. Oh, S.-G. Hong, I.-K. Kim, K.-Y. Chai, Y.-G.Yun, T.-O. Kwon, and H.-T. Chung. *In Vitro* Anti-proliferative Effect of 1, 2, 3, 4, 6-Penta-O-galloyl-beta-D-glucose on Human Hepatocellular Carcinoma Cell Line, SK-HEP-1 Cells. *Cancer Letters*, 174:17–24, 2001.

82. K. S. Feldman, K. Sahasrabudhe, M. D. Lawlor, S. L. Wilson, C. H. Lang, and W. J. Scheuchenzuber. *In Vitro* and *In Vivo* Inhibition of LPS-Stimulated Tumor Necrosis Factor-A Secretion by the Gallotannin Pentagalloylglucose. *Bioorganic and Medicinal Chemistry Letters*, 11:1813–5, 2001.

83. S. C. Cavalher-Machado, E. C. Rosas, F. de A. Brito, A. P. Heringe, R. R. de Oliveira, M. A. C. Kaplan, and M. D Henriques. The Anti-Allergic Activity of the Acetate Fraction of *Schinus Terebinthifolius* Leaves in Ige Induced Mice Paw Edema and Pleurisy. *International Immunopharmacology*, 8:1552–60, 2008.

84. A. Sugaya, T. Suzuki, E. Sugaya, N. Yuyama, K. Yasuda, and T. Tsuda. Inhibitory Effect of Peony Root Extract on Pentylenetetrazol-Induced EEG Power Spectrum Changes and

Extracellular Calcium Concentration Changes In Rat Cerebral Cortex. *Journal of Ethnopharmacology*, 33:159–67, 1991.

85. A. E. Hagerman, and L. G. Butler. Choosing Appropriate Methods and Standards for Assaying Tannin. *Journal of Chemical Ecology*, 15:1795–810, 1989.

86. P. Arapitsas. Hydrolyzable Tannin Analysis in Food. *Food Chemistry*, 135:1708–17, 2012.

87. L. Yue, J. Lee, L. Zheng, Y. Park, Z. Ye, and J. Yang. Computational Prediction Integrating the Inhibition Kinetics of Gallotannin on α-Glucosidase. *International Journal of Biological Macromolecules*, 103:829–38, 2017.

88. H. K. Bhakta, C. H. Park, T. Yokozawa, T. Tanaka, H. A. Jung, and J. S. Choi. Potential Anti-Cholinesterase and β-Site Amyloid Precursor Protein Cleaving Enzyme 1 Inhibitory Activities of Cornuside and Gallotannins from *Cornus Officinalis* Fruits. *Archieves of Pharmacal Research*, 40:1–18, 2017.

89. A. R. Afshari, H. R. Sadeghnia, and H. Mollazadeh. A Review on Potential Mechanisms of *Terminalia Chebula* in Alzheimer's Disease. *Advances in Pharmacological Sciences*, 2016:1-14, 2016.

90. K. Chung, C. Wei, and M. G. Johnson. Are Tannins A Sword In Biology and Health? *Trends in Food Science and Technology*, 9:168–75, 1998.

91. P.-G. Pietta. Flavonoids as Antioxidants. *Journal of Natural Products*, 63:1035–42, 2000.

92. F. Shahidi, and P. K. Wanasundara. Phenolic Antioxidants. *Critical Reviews in Food Science and Nutrition*, 32:67–103, 1992.

93. C. Rice-Evans, N. Miller, and G. Paganga. Antioxidant Properties of Phenolic Compounds. *Trends in Plant Science*, 2:152–9, 1997.

94. G. D'Andrea. Quercetin: A Flavonol with Multifaceted Therapeutic Applications? *Fitoterapia*, 106:256–71, 2015.

95. A. T. Jan, M. R. Kamli, I. Murtaza, J. B. Singh, A. Ali, and Q. M. R. Haq. Dietary Flavonoid Quercetin and Associated Health Benefits-An Overview. *Food Reviews International*, 26:302–17, 2010.

96. K. Kato, M. Ninomiya, K. Tanaka, and M. Koketsu. Effects of Functional Groups and Sugar Composition of Quercetin Derivatives on Their Radical Scavenging Properties. *Journal of Natural Products*, 79:1808–14, 2016.

97. J. He, and M. M. Giusti. Anthocyanins: Natural Colorants with Health-Promoting Properties. *Annual Review of Food Science and Technology*, 1:163–8, 2010.

98. I. Kubo, N. Masuoka, T. J. Ha, and K. Tsujimoto. Antioxidant Activity of Anacardic Acids. *Food Chemistry*, 99:555–62, 2006.

99. K. Tsujimoto, A. Hayashi, J. H. Tae, and I. Kubo. Anacardic Acids and Ferric Ion Chelation. *Zeitschrift für Naturforschung C, Journal of Biosciences*, 62:710–6, 2007.

100. K. Ubo, T. J. Ha, and I. Kubo. Lipoxygenase Inhibitory Activity of Anacardic Acids. *Journal of Agricultural and Food Chemistry*, 53:4350–4, 2005.

101. V. K. G. Abreu, A. L. F. Pereira, E. R. de Feritas, M. T. S. Trevisan, and J. M. da Costa. Effect of Anacardic Acid on Oxidative and Color Stability of Spray Dried Egg Yolk. *LWT - Food Science and Technology*, 55:466–71, 2014.

102. M. Hemshekhar, M. S. Santhosh, K. Kemparaju, and K. S. Girish. Emerging Roles of Anacardic Acid and Its Derivatives: A Pharmacological Overview. *Basic and Clinical Pharmacology and Toxicology*, 110:122–32, 2012.

103. F. B. Hamad, and E. B. Mubofu. Potential Biological Applications of Bio-Based Anacardic Acids and Their Derivatives. *International Journal of Molecular Sciences*,

16:8569–90, 2015.

104. J. G. Marks, T. DeMelfi, M. A. McCarthy, E. J. Witte, N. Castagnoli, W. L. Epstein, and R. C. Aber. Dermatitis from Cashew Nuts. *Journal of American Academy of Dermatology*, 10:627–31, 1984.

105. M. Naczk, and F. Shahidi. Extraction and Analysis of Phenolics in Food. *Journal of Chromatography A*, 1054:95–111, 2004.

106. A. Khoddami, M. A. Wilkes, and T. H. Roberts. Techniques for Analysis of Plant Phenolic Compounds. *Molecules*, 18:2328–75, 2013.

107. N. Berardini, R. Carle, and A. Schieber. Characterization of Gallotannins and Benzophenone Derivatives from Mango (*Mangifera indica* L. cv. "Tommy Atkins") Peels, Pulp and kernels by High-Performance Liquid Chromatography/Electrospray Ionization Mass Spectrometry. *Rapid Communications in Mass Spectrometry*, 18:2208–16, 2004.

108. M. Knödler, N. Berardini, D. R. Kammerer, R. Carle, and A. Schieber. Characterization of Major and Minor Alk(en)ylresorcinols from Mango (*Mangifera indica* L.) Peels by High-Performance Liquid Chromatography/Atmospheric Pressure Chemical Ionization Mass Spectrometry. *Rapid Communications in Mass Spectrometry*, 21:945–51, 2007.

109. A. Schieber, N. Berardini, and R. Carle. Identification of Flavonol and Xanthone Glycosides from Mango (*Mangifera indica* L. cv. "Tommy Atkins") Peels by High-Performance Liquid Chromatography-Electrospray. *Journal of Agricultural and Food Chemistry*, 51:5006–11, 2003.

110. M. Yuliana, B. T. Nguyen-Thi, S. Faika, L. H. Huynh, F. E. Soetaredjo, and Y. H. Ju. Separation and Purification of Cardol, Cardanol and Anacardic Acid from Cashew (*Anacardium occidentale* L.) Nut-Shell Liquid Using a Simple Two-Step Column Chromatography. *Journal of the Taiwan Institute of Chemical Engineers*, 45:2187–93, 2014.

111. A. Schieber, P. Keller, and R. Carle. Determination of Phenolic Acids and Flavonoids

of Apple and Pear by High-Performance Liquid Chromatography. *Journal of Chromatography A*, 910:265–73, 2001.

112. L. Regazzoni, E. Arlandini, D. Garzon, N. A. Santagati, G. Beretta, R. M. Facino, N. Alfredo, G. Beretta, and R. A. Maffei. Rapid Profiling of Gallotannins and Flavonoids of the Aqueous Extract of *Rhus coriaria* L. by Flow Injection Analysis with High-Resolution Mass Spectrometry Assisted with Database Searching. *Journal of Pharmaceutical and Biomedical Analysis*. 72:202–7, 2013.

113. J. P. Salminen, V. Ossipov, J. Loponen, E. Haukioja, and K. Pihlaja. Characterisation of Hydrolysable Tannins from Leaves of *Betula pubescens* by High-Performance Liquid Chromatography-Mass Spectrometry. *Journal of Chromatography A*. 864:283–91, 1999.

114. J. H. P. Tyman, and N. Jacobs. The Composition of the Unsaturated Phenolic Components of Anacardic Acid. *Journal of Chromatography A*, 54:83–90, 1974.

115. I. Castillo-Juárez, F. Rivero-Cruz, H. Celis, and I. Romero. Anti-*Helicobacter pylori* Activity of Anacardic Acids from *Amphipterygium adstringens*. *Journal of Ethnopharmacology*, 114:72–7, 2007.

116. C. H. Geerkens, P. Miller-Rostek, A. E. Matejka, S. Nene, D. R. Kammerer, R. Carle, and R. M. Schweiggert. Influence of Cultivar, Ripeness, Blanching, Drying, Irradiation, and Pectin Recovery on Alk(en)ylresorcinols In Mango Peels. *European Food Research and Technology*, 240:1235–45, 2015.

117. J. U. Ziegler, R. M. Schweiggert, and R. Carle. A Method for the Simultaneous Extraction and Quantitation of Lipophilic Antioxidants in *Triticum* sp. by HPLC-DAD/FLD-MS^n. *Journal of Food Composition and Analysis*, 39:94–102, 2015.

118. F. Ferreres, and F. A. Tomás-Barberán. Flavonoids: Flavonols, Flavones, and Flavanones. In: L. M. L. Nollet, F. Toldra, editors, *Handbook of Analysis of Active Compounds in Functional Foods*, pages 289-316. Taylor & Francis Groups CRC Press LLC, Boca Raton, 2012.

119. T. A. van Beek. Chemical Analysis of *Ginkgo biloba* Leaves and Extracts. *Journal of Chromatograpy A*, 967:21–55, 2002.

120. M. Friedman, and H. S. Jürgens. Effect of pH on the Stability of Plant Phenolic Compounds. *Journal of Agricultural and Food Chemistry*, 48:2101–10, 2000.

121. E. Haslam, R. D. Haworth, S. D. Mills, H. J. Rogers, R. Armitage, and T. Searle. Gallotannins. Part II. Some Esters and Depsides of Gallic Acid. *Journal of the Chemical Society*, 0:1836–42, 1961.

122. A. G. Newsome, Y. Li, and R. B. van Breemen. Improved Quantification of Free And Ester-Bound Gallic Acid in Foods and Beverages by UHPLC-MS/MS. *Journal of Agricultural and Food Chemistry*, 64:1326–34, 2016.

123. M. J. Gonzales, J. L. Torres, and I. Medina. Impact of Thermal Processing on the Activity of Gallotannins and Condensed Tannins from *Hamamelis virginiana* Used as Functional Ingredients in Seafood. *Journal of Agricultural and Food Chemistry*, 58:4274–83, 2010.

124. I. Mueller-Harvey. Analysis of Hydrolysable Tannins. *Animal Feed Science and Technology*, 91:3–20, 2001.

125. C. Engels, M. G. Gänzle, and A. Schieber. Fast LC-MS Analysis of Gallotannins from Mango (*Mangifera indica* L.) Kernels and Effects of Methanolysis on Their Antibacterial Activity and Iron Binding Capacity. *Food Research International*, 45:422–6, 2012.

126. C. Engels, A. Weiss, R. Carle, H. Schmidt, A. Schieber, and M. G. Gänzle. Effects of Gallotannin Treatment on Attachment, Growth, and Survival of *Escherichia coli* O157:H7 and *Listeria monocytogenes* on Spinach and Lettuce. *European Food Research and Technology*, 234:1081–90, 2012.

127. P. P. Singh, and M. D. A. Saldaña. Subcritical Water Extraction of Phenolic Compounds from Potato Peel. *Food Research International*, 44:2452–8, 2011.

128. M. Plaza, M. Amigo-Benavent, M. D. del Castillo, E. Ibanez, and M. Herrero. Facts about the Formation of New Antioxidants in Natural Samples Using Subcritical Water Extraction. *Food Research International*, 43:1123–9, 2010.

129. B. A. Acosta-Estrada, J. A. Gutiérrez-Uribe, and S. O. Serna-Saldívar. Bound Phenolics in Foods, A Review. *Food Chemistry*, 152:46–55, 2014.

130. B. K. Tiwari. Ultrasound: A Clean, Green Extraction Technology. *Trends in Analytical Chemistry*, 71:100–9, 2015.

131. F. Chemat, N. Rombaut, A. G. Sicaire, A. Meullemiestre, A. S. Fabiano-Tixier, and M. Abert-Vian. Ultrasound Assisted Extraction of Food and Natural Products. Mechanisms, Techniques, Combinations, Protocols and Applications. A Review. *Ultrasonics Sonochemistry*, 34:540–60, 2017.

132. S. Kentish, and M. Ashokkumar. *The Physical and Chemical Effects of Ultrasound*, Springer-Verlag New York Inc., New York, 2011.

133. C. C. Gras, R. Carle, and R. M. Schweiggert. Determination of Anthocyanins from Black Carrots by UHPLC-PDA after Ultrasound-Assisted Extraction. *Journal of Food Composition and Analysis*, 44:170–7, 2015.

134. Z. Pan, W. Qu, H. Ma, G. G. Atungulu, and T. H. McHugh. Continuous and Pulsed Ultrasound-Assisted Extractions of Antioxidants from Pomegranate Peel. *Ultrasonics Sonochemistry*, 19:365–72, 2012.

135. K. Ghafoor, Y. H. Choi, J. Y. Jeon, and I. H. Jo. Optimization of Ultrasound-Assisted Extraction of Phenolic Compounds, Antioxidants, and Anthocyanins from Grape (*Vitis vinifera*) Seeds. *Journal of Agricultural and Food Chemistry*, 57:4988–94, 2009.

136. J. Londoño-Londoño, V. R. de Lima, O. Lara, A. Gil, T. Beatriz, C. Pasa, G. J. Arango, and J. R. R. Pineda. Clean Recovery of Antioxidant Flavonoids from Citrus Peel: Optimizing an Aqueous Ultrasound-Assisted Extraction Method. *Food Chemistry*, 119:81–7, 2010.

137. M. Virot, V. Tomao, C. Le Bourvellec, C. M. C. G. Renard, and F. Chemat. Towards the Industrial Production of Antioxidants from Food Processing By-Products with Ultrasound-Assisted Extraction. *Ultrasonics Sonochemistry*, 17:1066–74, 2009.

138. J. Wang, B. Sun, Y. Cao, Y. Tian, and X. Li. Optimisation of Ultrasound-Assisted Extraction of Phenolic Compounds from Wheat Bran. *Food Chemistry*, 106:804–10, 2008.

139. J. W. King. Pressurized Water Extraction: Resources and Techniques for Optimizing Analytical Applications. In: C. Turner, editor, *Modern Extraction Techniques*, pages 79-95. American Chemical Society, Washington, 2006.

140. S. Gbashi, O. A. Adebo, L. Piater, N. E. Madala, and P. B. Njobeh. Subcritical Water Extraction of Biological Materials. *Separation and Purification Reviews*, 46:21–34, 2017.

141. 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*, 1217:2484–94, 2010.

142. M. Herrero, M. Castro-Puyana, J. A. Mendiola, and E. Ibañez. Compressed Fluids For the Extraction of Bioactive Compounds. *Trends in Analytical Chemistry*, 43:67–83, 2013.

143. M. Castro-Puyana, M. L. Marina, and M. Plaza. Water as Green Extraction Solvent: Principles and Reasons for Its Use. *Current Opinion in Green and Sustainable Chemistry*, 5:31–6, 2017.

144. N. Akiya, and P. E. Savage. Roles of Water for Chemical Reactions in High-Temperature Water. *Chem Reviews*, 102:2725–50, 2002.

145. 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*, 43:2341–8, 2010.

146. Z. Y. Ju, and L. R. Howard. Subcritical Water and Sulfured Water Extraction of Anthocyanins and Other Phenolics from Dried Red Grape Skin. *Journal of Food Science*,

70:270–6, 2015.

147. M. Garcia-Marino, J. Rivas-Gonzalo, E. Ibanez, and C. Garcia-Moreno. Recovery of Catechins and Proanthocyanidins from Winery By-Products Using Subcritical Water Extraction. *Analytica Chimica Acta*, 563:44–50, 2006.

148. J. E. Cacace, and G. Mazza. Pressurized Low Polarity Water Extraction of Biologically Active Compounds from Plant Products. In: J. Shi, editor, *Functional Food Ingredients and Nutraceuticals*, pages 177-198. Taylor & Francis Groups CRC Press LLC, Boca Raton, 2007.

149. M. Roberto, M. Junior, A. V. Leite, N. Romanelli, and V. Dragano. Supercritical Fluid Extraction and Stabilization of Phenolic Compounds From Natural Sources – Review. *Open Chemical Engineering Journal*, 4:51–60, 2010.

150. J.-W. Kim, and G. Mazza. Optimization of Extraction of Phenolic Compounds from Flax Shives by Pressurized Low-Polarity Water. *Journal of Agricultural and Food Chemistry*, 54:7575–84, 2006.

151. J.-W. Kim, and G. Mazza. Mass Transfer during Pressurized Low-Polarity Water Extraction of Phenolics and Carbohydrates from Flax Shives. *Industrial and Engineering Chemistry Research*, 46:7221–30, 2007.

152. O. Pourali, F. S. Asghari, and H. Yoshida. Production of Phenolic Compounds from Rice Bran Biomass under Subcritical Water Conditions. *Chemical Engineering Journal*, 160:259–66, 2010.

153. F. Salak, O. Pourali, and H. Yoshida. Application of Subcritical Water Treatment for Production of Phenolic Compounds from Rice Bran Biomass. *Proceedings of the World Congress on Engineering and Computer Science, 2009 WCECS*, San Francisco, 20–2, 2009.

154. B. Tangkhavanich, T. Kobayashi, and S. Adachi. Properties of Rice Stem Extracts Obtained by Using Subcritical Fluids. *Bioscience, Biotechnolology, and Biochemistry*,

77:2112–6, 2013.

155. M. Hassasroudsari, P. Chang, R. Pegg, and R. Tyler. Antioxidant Capacity of Bioactives Extracted from Canola Meal by Subcritical Water, Ethanolic and Hot Water Extraction. *Food Chemistry*, 114:717–26, 2009.

156. B. Aliakbarian, A. Fathi, P. Perego, and F. Dehghani. Extraction of Antioxidants from Winery Wastes Using Subcritical Water. *Journal of Supercritical Fluids*, 65:18–24, 2012.

157. J. K. Monrad, L. R. Howard, J. W. King, K. Srinivas, A. Mauromoustakos, and A. N. D. Y. M. Auromoustakos. Subcritical Solvent Extraction of Anthocyanins from Dried Red Grape Pomace. *Journal of Agricultural and Food Chemistry*, 58:2862–8, 2010.

158. J. K. Monrad, K. Srinivas, L. R. Howard, and J. W. King. Design and Optimization of a Semicontinuous Hot-Cold Extraction of Polyphenols from Grape Pomace. *Journal of Agricultural and Food Chemistry*, 60:5571–82, 2012.

159. Z. Y. Ju, and L. R. Howard. Subcritical Water and Sulfured Water Extraction of Anthocyanins and Other Phenolics from Dried Red Grape Skin. *Journal of Food Science*, 70:270–6, 2005.

160. J. Kim, T. Nagaoka, Y. Ishida, and S. Lee. Subcritical Water Extraction of Nutraceutical Compounds from Citrus Pomaces. *Separation Science and Technology*, 44:2598–608, 2009.

161. H. Wijngaard, and N. Brunton. The Optimization of Extraction of Antioxidants from Apple Pomace by Pressurized Liquids. *Journal of Agricultural and Food Chemistry*, 57:10625–31, 2009.

162. 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*, 90:1–9, 2011.

163. S. Tunchaiyaphum, M. N. Eshtiaghi, and N. Yoswathana. Extraction of Bioactive Compounds from Mango Peels Using Green Technology. *International Journal of Chemical Engineering and Applications*, 4:194–8, 2013.

164. I. Rodríguez-Meizoso, F. R. Marin, M. Herrero, F. J. Señorans, G. Reglero, A. Cifuentes, and E. Ibáñez. Subcritical Water Extraction of Nutraceuticals with Antioxidant Activity from Oregano. Chemical and Functional Characterization. *Journal of Pharmaceutical and Biomedical Analysis*, 41:1560–5, 2006.

165. E. Ibanez, A. Kubatova, F. J. Senorans, S. Cavero, G. Reglero, and S. B. Hawthorne. Subcritical Water Extraction of Antioxidant Compounds from Rosemary Plants. *Journal of Agricultural and Food Chemistry*, 51:375–82, 2003.

166. T. L. Miron, M. Plaza, G. Bahrim, E. Ibáñez, and M. Herrero. Chemical Composition of Bioactive Pressurized Extracts of Romanian Aromatic Plants. *Journal of Chromatography A*, 1218:4918–27, 2011.

167. P.-Y. Chen, Y.-X. Tu, C.-T. Wu, T.-T. Jong, and C.-M. J. Chang. Continuous Hot Pressurized Solvent Extraction of 1, 1-Diphenyl-2-Picrylhydrazyl Free Radical Scavenging Compounds From Taiwan Yams (*Dioscorea alata*). *Journal of Agricultural and Food Chemistry*, 52:1945–9, 2004.

168. P. K. Huwijitjaru, K. C. Halooddong, and S. A. Dachi. Note: Phenolic Content and Radical Scavenging Capacity of Kaffir Lime Fruit Peel Extracts Obtained by Pressurized Hot Water Extraction. *Food Science and Technology Research*, 14:1–4, 2008.

169. P. Budrat, and A. Shotipruk. Enhanced Recovery of Phenolic Compounds from Bitter Melon (*Momordica charantia*) by Subcritical Water Extraction. *Separation and Purification Technology*, 66:125–9, 2009.

170. P. Rangsriwong, N. Rangkadilok, J. Satayavivad, M. Goto, and A. Shotipruk. Subcritical Water Extraction of Polyphenolic Compounds from *Terminalia chebula* Retz. Fruits. *Separation and Purification Technology*, 66:51–6, 2009.

171. M.-J. Ko, C.-I. Cheigh, S.-W. Cho, and M.-S. Chung. Subcritical Water Extraction of Flavonol Quercetin from Onion Skin. *Journal of Food Engineering*, 102:327–33, 2011.

172. C.-I. Cheigh, S.-Y. Yoo, M.-J. Ko, P.-S. Chang, and M.-S. Chung. Extraction Characteristics of Subcritical Water Depending on the Number of Hydroxyl Group in Flavonols. *Food Chemistry*, 168:21–6, 2015.

173. C. Pronyk, and G. Mazza. Design and Scale-Up of Pressurized Fluid Extractors for Food and Bioproducts. *Journal of Food Engineering*, 95:215–26, 2009.

174. V. L. Singleton, R. Orthofer, and R. M. Lamuela-Raventos. Analysis of Total Phenols and Other Oxidation Substrates and Antioxidants by Means of Folin-Ciocalteu Reagent. *Methods in Enzymology*, 119:152–78, 1974.

175. B. Sun, J. M. Ricardo-da-Silva, and I. Spranger. Critical Factors of Vanillin Assay for Catechins and Proanthocyanidins. *Journal of Agricultural and Food Chemistry*, 46:4267–74, 1998.

176. M. C. McMaster. *LC/MS A practinal user's guide*. John Wiley & Sons Inc., Honoken, 2005.

177. N. Vervoort, D. Daemen, and G. Török. Performance Evaluation of Evaporative Light Scattering Detection and Charged Aerosol Detection in Reversed Phase Liquid Chromatography. *Journal of Chromatography A*, 1189:92–100, 2008.

178. S. Yan, G. Luo, Y. Wang, and Y. Cheng. Simultaneous Determination of Nine Components in *Qingkailing* Injection by HPLC/ELSD/DAD and Its Application to the Quality Control. *Journal of Pharmaceutical and Biomedical Analysis*, 40:889–95, 2006.

179. Q. T. Yu, L. W. Qi, P. Li, L. Yi, J. Zhao, and Z. Bi. Determination of Seventeen Main Flavonoids and Saponins in the Medicinal Plant Huang-qi (*Radix Astragali*) by HPLC-DAD-ELSD. *Journal of Separation Science*, 30:1292–9, 2007.

180. G. Yang, W. Fen, C. Lei, W. Xiao, and H. Sun. Study on Determination of Pentacyclic Triterpenoids in *Chaenomeles* by HPLC-ELSD. *Journal of Chromatography Science*, 47:718–22, 2009.

181. V. Douville, A. Lodi, J. Miller, A. Nicolas, I. Clarot, B. N. Prilleux. Megoulas, and M. Koupparis. Evaporative Light Scattering Detection (ELSD): A Tool for Improved Quality Control of Drug Substances. *Pharmeuropa Bio and Scientific Notes*, 2006:9–15, 2006.

182. M. J. Felton. Are ELSDs Coming Out of the Shadow? *Analytical Chemistry*, 1:631–4, 2002.

183. J. D. Pleil, and K. K. Isaacs. High-Resolution Mass Spectrometry: Basic Principles for Using Exact Mass and Mass Defect for Discovery Analysis of Organic Molecules in Blood, Breath, Urine and Environmental Media. *Journal of Breath Research*, 10:12001, 2016.

184. R. Apak, M. Özyürek, K. Güçlü, and E. Çapanoğlu. Antioxidant Activity/Capacity Measurement. 1. Classification, Physicochemical Principles, Mechanisms, and Electron Transfer (ET)-Based Assays. *Journal of Agricultural and Food Chemistry*, 64:997–1027, 2016.

185. R. Apak, M. Özyürek, K. Güçlü, and E. Çapanoğlu. Antioxidant Activity/Capacity Measuremen. 2. Hydrogen Atom Transfer (HAT)-Based, Mixed-Mode (Electron Transfer (ET)/HAT), and Lipid Peroxidation Assays. *Journal of Agricultural and Food Chemistry*, 64:1028–45, 2016.

186. R. Apak, M. Özyürek, K. Güçlü, and E. Çapanoğlu. Antioxidant Activity/Capacity Measurement. 3. Reactive Oxygen and Nitrogen Species (ROS/RNS) Scavenging Assays, Oxidative Stress Biomarkers, and Chromatographic/Chemometric Assays. *Journal of Agricultural and Food Chemistry*, 64:1046–70, 2016.

187. W. Brand-Williams, M. E. Cuvelier, and C. Berset. Use of a Free Radical Method to Evaluate Antioxidant Activity. *LWT - Food Science and Technology*, 28:25–30, 1995.

188. T. J. Payne. Nuts. In: D. M. Barrett, L. Somogyi, and H. Ramaswamy, editors. *Processing Fruits: Science and Technology*, pages 765–808. Taylor & Francis Groups CRC Press LLC, Boca Raton, 2004.

189. I. Kubo, H. Muroi, M. Himejima, Y. Yamagiwa, H. Mera, K. Tohushima, S. Ohta, and T. Kamikawa. Structure-Antibacterial Activity Relationships of Anacardic Acids. *Journal of Agricultural and Food Chemistry*, 14:1016–9, 1993.

190. AOAC Official Method 934.01. Moisture in Animal Feed. In: *Official Methods of Analysis of the Association of Official Analytical Chemists*. AOAC International, Gaithersburg, 2002.

191. MEB. *Antepfıstığı Yetiştiriciliği*. T.C. Milli Eğitim Bakanlığı, Ankara, 2010.

192. TUIK. *Turkey's Statistical Yearbook 2013*. Turkish Statistical Institute, Ankara, 2014.

193. K. A. Krenek, R. C. Barnes, and S. T. Talcott. Phytochemical Composition and Effects of Commercial Enzymes on the Hydrolysis of Gallic Acid Glycosides in Mango (*Mangifera indica* L. cv. "Keitt") Pulp. *Journal of Agricultural and Food Chemistry*, 62:9515–21, 2014.

194. G. G. Gross. Synthesis of Mono-, Di- and Trigalloyl-β-D-Glucose by β-Glucogallin-Dependent Galloyltransferases from Oak Leaves. *Zeitschrift für Naturforschung C, Journal of Biosciences*, 38:519–23, 1983.

195. R. Niemetz, J. U. Niehaus, and G. G. Gross. Biosynthesis and Biodegradation of Complex Gallotannins. In: G. G. Gross, R. W. Hemingway, and T. Yoshida, editors, *Plant Polyphenols 2: Chemistry, Biology, Pharmacology, Ecology*, pages 63-82. Springer-Verlag New York Inc., New York, 2012.

196. I. M. Abu-Reidah, M. S. Ali-Shtayeh, R. M. Jamous, D. Arráez-Román, and A. Segura-Carretero. HPLC-DAD-ESI-MS/MS Screening of Bioactive Components from *Rhus coriaria* L. (Sumac) Fruits. *Food Chemistry*, 166:179–91, 2015.

197. K. J. Meyers, T. J. Swiecki, and A. E. Mitchell. Understanding the Native Californian Diet: Identification of Condensed and Hydrolyzable Tannins in Tanoak Acorns (*Lithocarpus densiflorus*). *Journal of Agricultural and Food Chemistry*, 54:7686–91, 2006.

198. C. H. Geerkens, R. M. Schweiggert, H. Steingass, J. Boguhn, M. Rodehutscord, and R. Carle. Influence of Apple and Citrus Pectins, Processed Mango Peels, a Phenolic Mango Peel Extract, and Gallic Acid as Potential Feed Supplements on *In Vitro* Total Gas Production and Rumen Methanogenesis. *Journal of Agricultural and Food Chemistry*, 61:5727–37, 2013.

199. F. Luo, Y. Fu, Y. Xiang, S. Yan, G. Hu, X. Huang, G. Huang, C. Sun, X. Li, and K. Chen. Identification and Quantification of Gallotannins in Mango (*Mangifera indica* L.) Kernel and Peel and Their Antiproliferative Activities. *Journal of Functional Foods*, 8:282–91, 2014.

200. A. Romani, P. Pinelli, C. Galardi, N. Mulinacci, and M. Tattini. Identification and Quantification of Galloyl Derivatives, Flavonoid Glycosides and Anthocyanins in Leaves of *Pistacia lentiscus* L. *Phytochemical Analysis*, 13:79–86, 2002.

201. M. N. Clifford, S. Stoupi, and N. Kuhnert. Profiling and Characterization by LC-MS^n of the Galloylquinic Acids of Green Tea, Tara Tannin, and Tannic Acid. *Journal of Agricultural and Food Chemistry*, 55:2797–807, 2007.

202. A. Taamalli, I. Iswaldi, D. Arráez-Román, A. Segura-Carretero, A. Fernández-Gutiérrez, and M. Zarrouk. UPLC-QTOF/MS for a Rapid Characterisation of Phenolic Compounds from Leaves of *Myrtus communis* L. *Phytochemical Analysis*, 25:89–96, 2014.

203. L. Bravo, L. Goya, and E. Lecumberri. LC/MS Characterization of Phenolic Constituents of Mate (*Ilex paraguariensis*, St. Hil.) and Its Antioxidant Activity Compared to Commonly Consumed Beverages. *Food Research International*, 40:393–405, 2007.

204. M.-W. Lee, T. Tanaka, G.-I. Nonaka, and I. Nishioka. Dimeric Ellagitannins from *Alnus japonica*. *Phytochemistry*, 31:2835–9, 1992.

205. K. Ishimaru, G. I. Nonaka, and I. Nishioka. Gallic Acid Esters of *Proto*-Quercitol, Quinic Acid and (-)-Shikimic Acid from *Quercus mongolica* and *Q. myrsin aefolia*. *Phytochemistry*, 26:1501–4, 1987.

206. H. L. Cells, G. Nonaka, I. Nishioka, M. Nishizawa, T. Yamagishi, Y. Kashiwada, G. E. Dutschman, A. J. Bodner, R. E. Kilkuskie, Y. C. Cheng, and K. H. Lee. Anti-AIDS Agents, 2: Inhibitory Effects of Tannins on HIV Reverse Transcriptase and HIV Replication in H9 Lymphocyte Cells. *Journal of Natural Products*, 53:587–95, 1990.

207. M. E. Harbowy, D. A. Balentine, A. P. Davies, and Y. Cai. Tea Chemistry. *Critical Reviews in Plant Sciences*, 16:415–80, 1997.

208. M. Behgar, S. Ghasemi, A. Naserian, A. Borzoie, and H. Fatollahi. Gamma Radiation Effects on Phenolics, Antioxidants Activity and *In Vitro* Digestion of Pistachio (*Pistachia vera*) Hull. *Radiation Physics and Chemistry*, 80:963–7, 2011.

209. F. Tian, B. Li, B. Ji, G. Zhang, and Y. Luo. Identification and Structure-Activity Relationship of Gallotannins Separated from *Galla chinensis*. *LWT-Food Science and Technology*, 42:1289–95, 2009.

210. M. Nierenstein, and J. Potter. The Distribution of Myrobalanitannin. *Biochemical Journal*, 39:390–2, 1945.

211. A. Tomaino, M. Martorana, T. Arcoraci, D. Monteleone, C. Giovinazzo, and A. Saija. Antioxidant Activity and Phenolic Profile of Pistachio (*Pistacia vera* L., variety Bronte) Seeds and Skins. *Biochimie*, 92:1115–22, 2010.

212. M. G. Bellomo, and B. Fallico. Anthocyanins, Chlorophylls and Xanthophylls in Pistachio Nuts (*Pistacia vera*) of Different Geographic Origin. *Journal of Food Composition and Analysis*, 20:352–9, 2007.

213. N. Berardini, A. Schieber, I. Klaiber, U. Beifuss, R. Carle, and J. Conrad. 7-*O*-Methylcyanidin 3-O-*β*-D-Galactopyranoside, A Novel Anthocyanin from Mango

(*Mangifera indica* L. cv. “Tommy Atkins”) peels. *Zeitschrift für Naturforschung B*, 60:801–4, 2005.

214. R. M. Schweiggert, E. Vargas, J. Conrad, J. Hempel, C. C. Gras, J. U. Ziegler, A. Mayer, V. Jimenez, P. Esquivel, and R. Carle. Carotenoids, Carotenoid Esters, and Anthocyanins of Yellow-, Orange-, and Red-Peeled Cashew Apple (*Anacardium occidentale* L.). *Food Chemistry*, 200:274–82, 2016.

215. G. Jerz, J. A. Murillo-Velásquez, I. Skrjabin, R. Gök, and P. Winterhalter. Anacardic Acid Profiling in Cashew Nuts by Direct Coupling of Preparative High-Speed Countercurrent Chromatography and Mass Spectrometry (*prep*HSCCC-ESI-/APCI-MS/MS). In: S. Toth, C. Mussinan, editors, *Recent Advances in the Analysis of Food and Flavors*, pages 145–65. American Chemical Society, Washington, 2012.

216. M. Saitta, D. Giuffrida, G. L. La Torre, A. G. Potortì, G. Dugo, G. Loredana, L. Torre, A. G. Potortì, and G. Dugo. Characterisation of Alkylphenols in Pistachio (*Pistacia vera* L.) Kernels. *Food Chemistry*, 117:451–5, 2009.

217. M. Behgar, R. Valizadeh, M. Mirzaee, A. A. Naserian, and M. R. Nasiri. Correlation between the Physical and Chemical Properties of Some Forages and Non-Forage Fiber Sources. *Journal of Animal and Veterinary Advances*, 8:2280–5, 2009.

218. İ. Çelik, and G. N. Demirer. Biogas Production from Pistachio (*Pistacia vera* L.) Processing Waste. *Biocatalysis and Agricultural Biotechnology*, 4:767–72, 2015.

219. T. M. Moghaddam, S. M. A. Razavi, F. Malekzadegan, and A. S. Ardekani. Chemical Composition and Rheological Characterization of Pistachio Green Hull’s Marmalade. *Journal of Texture Studies*, 40:390–405, 2009.

220. E. Bellocco, D. Barreca, G. Laganà, A. Calderaro, Z. El Lekhlifi, S. Chebaibi, A. Smeriglio, and D. Trombetta. Cyanidin-3-*O*-Galactoside in Ripe Pistachio (*Pistachia vera* L. variety Bronte) Hulls: Identification and Evaluation of Its Antioxidant and Cytoprotective Activities. *Journal of Functional Foods*, 27:376–85, 2016.

221. M. E. Swartz. UPLC: An Introduction and Review. *Journal of Liquid Chromatography and Related Technologies*, 28:1253–63, 2005.

222. L. R. Snyder, J. J. Kirkland, and J. L. Glajch. *Practical HPLC Method Development*. John Wiley and Sons Inc., Hoboken, 1997.

223. ICH. "Validation of Analytical Procedures: Test and Methodology Q2 (R1)". http//www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Qual [retrieved 15 June 2016].

224. Cayman Chemical. "Product information: Anacardic acid". https//www.caymanchem.com/pdfs/13144.pdf [retrieved 15 December 2016].

225. P. Grundhöfer, R. Niemetz, G. Schilling, and G. G. Gross. Biosynthesis and Subcellular Distribution of Hydrolyzable Tannins. *Phytochemistry*, 57:915–27, 2001.

226. A. G. Newsome, Y. Li, and R. B. van Breemen. Improved Quantification of Free and Ester-Bound Gallic Acid in Foods and Beverages by UHPLC-MS/MS. *Journal of Agricultural and Food Chemistry*, 64:1326–34, 2016.

227. A. E. -M. M. R. Afify, H. S. El-Beltagi, S. M. A. El-Salam, and A. A. Omran. Biochemical Changes in Phenols, Flavonoids, Tannins, Vitamin E, β-Carotene and Antioxidant Activity during Soaking of Three White Sorghum Varieties. *Asian Pacific Journal of Tropical Biomedicine*, 2:203–9, 2012.

228. J. U. Ziegler, M. Flockerzie, C. F. H. Longin, T. Würschum, R. Carle, and R. M. Schweiggert. Development of Lipophilic Antioxidants and Chloroplasts during Sprouting of Diverse *Triticum* spp. *Journal of Agricultural and Food Chemistry*, 64:913–22, 2016.

229. K. M. Kalili, and A. De Villiers. Recent Developments in the HPLC Separation of Phenolic Compounds. *Journal of Separation Sciences*, 34:854–76, 2011.

230. P. Yang, T. McCabe, and M. Pursch. Practical Comparison of LC Columns Packed

With Different Superficially Porous Particles for the Separation of Small Molecules and Medium Size Natural Products. *Journal of Separation Sciences*, 34:2975–82, 2011.

231. A. Plazonić, F. Bucar, Z. Maleŝ, A. Mornar, B. Nigović, and N. Kujundžić. Identification and Quantification of Flavonoids and Phenolic Acids in Burr Parsley (*Caucalis platycarpos* L.), Using High-Performance Liquid Chromatography with Diode Array Detection and Electrospray Ionization Mass Spectrometry. *Molecules*, 14:2466–90, 2009.

232. E. Haslam. Plant Polyphenols (*syn.* Vegetable Tannins) and Chemical Defense-A Reappraisal. *Journal of Chemical Ecology*, 14:1789–805, 1988.

233. Y. Yilmaz, and R. T. Toledo. Major Flavonoids in Grape Seeds and Skins: Antioxidant Capacity of Catechin, Epicatechin, and Gallic acid. *Journal of Agricultural and Food Chemistry*, 52:255–60, 2004.

234. J.-K. Lin, C.-L. Lin, Y.-C. Liang, S.-Y. Lin-Shiau, and I.-M. Juan. Survey of Catechins, Gallic Acid, and Methylxanthines in Green, Oolong, Pu-Erh, and Black Teas. *Journal of Agricultural and Food Chemistry*, 46:3635–42, 1998.

235. C. B. Nasr, N. Ayed, and M. Metche. Quantitative Determination of the Polyphenolic Content of Pomegranate Peel. *Zeitschrift für Lebensmittel-Untersuchung und Forschung*, 203:374–8, 1996.

236. L. Li, A. A. Shaik, J. Zhang, K. Nhkata, L. Wang, Y. Zhang, C. Xing, S.-H. Kim, and J. Lü. Preparation of Penta-O-β-D Glucose from Tannic Acid and Plasma Pharmacokinetic Analyses by Liquid-Liquid Extraction and Reverse-Phase HPLC. *Journal of Pharmaceutical and Biomedical Analysis*, 54:545–50, 2011.

237. D. Caridi, V. C. Trenerry, S. Rochfort, S. Duong, D. Laugher, and R. Jones. Profiling and Quantifying Quercetin Glucosides in Onion (*Allium cepa* L.) varieties Using Capillary Zone Electrophoresis and High Performance Liquid Chromatography. *Food Chemistry* 105:691–9, 2007.

238. A. Schieber, P. Keller, and R. Carle. Determination of Phenolic Acids and Flavonoids of Apple and Pear by High-Performance Liquid Chromatography. *Journal of Chromatography A*, 910:265–73, 2001.

239. M. P. Khknen, A. I. Hopia, M. Heinonen, and M. P. Ka. Berry Phenolics and Their Antioxidant Activity. *Journal of Agricultural and Food Chemistry*, 49:4076–82, 2001.

240. C. Locatelli, F. B. Filippin-Monteiro, and T. B. Creczynski-Pasa. Alkyl Esters of Gallic Acid as Anticancer Agents: A Review. *European Journal of Medicinal Chemistry*, 60:233–9, 2013.

241. S. K. Mamidyala, S. Ramu, J. X. Huang, A. A. B. Robertson, and M. A. Cooper. Efficient Synthesis of Anacardic Acid Analogues and Their Antibacterial Activities. *Bioorganic and Medicinal Chemistry Lettters*, 23:1667–70, 2013.

242. C. D. Stalikas. Extraction, Separation, and Detection Methods for Phenolic Acids and Flavonoids. *Journal of Separation Sciences*, 30:3268–95, 2007.

243. C. Fabian, N. Y. Tran-Thi, N. S. Kasim, and Y-H. Ju. Release of Phenolic Acids From Defatted Rice Bran by Subcritical Water Treatment. *Journal of the Science of Food and Agriculture*, 90:2576–81, 2010.

244. N. Pellegrini, R. Roberta, M. Yang, and C. Rice-Evans. Screening of Dietary Carotenoids and Carotenoid-Rich Fruit Extracts for Antioxidant Activities Applying 2, 2'-Azinobis (3-Ethylenebenzothiazoline-6-Sulfonic Acid) Radical Cation Decolorization Assay. *Methods in Enzymology*, 299:379–89, 1999.

245. I. F. Benzie IF, and J. J. Strain. The Ferric Reducing Ability of Plasma (FRAP) as a Measure of "Antioxidant Power": FRAP assay. *Analytical Biochemistry*, 239:70–6, 1996.

246. Ö. Güçlü Üstündağ, S. Erşan, E. Özcan, G. Özan, N. Kayra, and F. Y. Ekinci. Black Tea Processing Waste as a Source of Antioxidant and Antimicrobial Phenolic Compounds. *European Food Research and Technology*, 242:1523–32, 2016.

247. M. T. Fernández-Ponce, L. Casas, C. Mantell, M. Rodríguez, and E. M. de la Ossa. Extraction of Antioxidant Compounds from Different Varieties of *Mangifera indica* Leaves Using Green Technologies. *Journal of Supercritical Fluids*, 72:168–75, 2012.

248. M. S. Y. Kumar, R. Dutta, D. Prasad, and K. Misra. Subcritical Water Extraction of Antioxidant Compounds from Seabuckthorn (*Hippophae rhamnoides*) Leaves for the Comparative Evaluation of Antioxidant Activity. *Food Chemistry*, 127:1309–16, 2011.

249. M. Çam, and Y. Hışıl. Pressurised Water Extraction of Polyphenols from Pomegranate Peels. *Food Chemistry*, 123:878–85, 2010.

250. A. Mustafa, and C. Turner. Pressurized Liquid Extraction as a Green Approach in Food and Herbal Plants Extraction: A Review. *Analytical Chimica Acta*, 703:8–18, 2011.

251. L. R. Adetunji, A. Adekunle, V. Orsat, and V. Raghavan. Advances in the Pectin Production Process Using Novel Extraction Techniques: A Review. *Food Hydrocolloids*, 62:239–50, 2017.

252. N. Yuda, M. Tanaka, M. Suzuki, Y. Asano, H. Ochi, and K. Iwatsuki. Polyphenols Extracted from Black Tea (*Camellia sinensis*) Residue by Hot-Compressed Water and Their Inhibitory Effect on Pancreatic Lipase *in Vitro. Journal of Food Science*, 77:254-61, 2012.

253. K. Abraham, R. Gürtler, K. Berg, G. Heinemeyer, A. Lampen, and K. E. Appel. Toxicology and Risk Assessment of 5-Hydroxymethylfurfural in Food. *Molecular Nutrition and Food Research*, 55:667–78, 2011.

254. Y. Narita, and K. Inouye. High Antioxidant Activity of Coffee Silverskin Extracts Obtained by the Treatment of Coffee Silverskin with Subcritical Water. *Food Chemistry*, 135:943–9, 2012.

255. I. F. Benzie, and Y. T. Szeto. Total Antioxidant Capacity of Teas by the Ferric Reducing/Antioxidant Power Assay. *Journal of Agricultural and Food Chemistry*, 47:633–6, 1999.

256. E. Capanoglu, R. C. H. de Vos, R. D. Hall, D. Boyacioglu, and J. Beekwilder. Changes in Polyphenol Content during Production of Grape Juice Concentrate. *Food Chemistry*, 139:521–6, 2013.

257. C. M. Galanakis. Recovery of High Added-Value Components from Food Wastes: Conventional, Emerging Technologies and Commercialized Applications. *Trends in Food Science and Technology*, 26:1–20, 2012.