

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE
ENGINEERING AND TECHNOLOGY

**HEALTH RELATED PROPERTIES OF DIFFERENT PARTS OF WALNUT
(*JUGLANS REGIA* L.) AND A WALNUT DRINK**

M.Sc. THESIS

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Department of Food Engineering

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JANUARY 2013

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

**CEVİZİN (*JUGLANS REGIA* L.) FARKLI KISIMLARI VE CEVİZ
İÇECEĞİNİN SAĞLIKLA İLGİLİ ÖZELLİKLERİ**

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To my family

FOREWORD

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ABBREVIATIONS

ABTS	: 2,2- azinobis 3-ethylbenzothiazoline-6-sulfonic acid diammonium salt
ANOVA	: Analysis of variance
APPH	: 2,2-azo-bis(2-amidinopropane) dihydrochloride
AOAC	: Association Official of Analytical Chemists
CE	: Catechin Equivalent
CUPRAC	: Copper Reducing Antioxidant Capacity
DF	: Dilution factor
DPPH	: 1,1-Diphenyl-2- picrylhydrazyl
DW	: Dry weight
FAO	: Food and Agriculture Organization of the United Nations
FL	: Fluorescein
FRAP	: Ferric Reducing Antioxidant Capacity
FW	: Fresh weight
GAE	: Gallic Acid Equivalent
GI	: Gastrointestinal
HPLC	: High Performance Liquid Chromatography
MW	: Molecular weight
ORAC	: Oxygen Radical Absorbance Capacity
PBS	: Phosphate buffer solution
PDA	: Photodiode array
SPSS	: Statistical Package for the Social Sciences
TAC	: Total antioxidant capacity
TEAC	: Trolox Equivalent Antioxidant Capacity
TF	: Total flavonoid content
TP	: Total phenolic content
TUIK	: Turkish Statistical Institute
WD	: Walnut Drink
WK	: Walnut Kernel
WMF	: Walnut Male Flower
WT	: Walnut Testa
WS	: Walnut Tree Sap

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LIST OF SYMBOLS

a^*	: red/green (color parameter)
b^*	: blue/yellow (color parameter)
L^*	: lightness/darkness (color parameter)
α	: alpha (in α -carotene)
β	: beta (in β -carotene or β -cryptoxanthin)
m	: milli (10^{-3})
μ	: micro (10^{-6})
p	: probability (statistical analysis)
R^2	: correlation coefficient (statistical analysis)
t	: tonnes

HEALTH RELATED PROPERTIES OF DIFFERENT PARTS OF WALNUT (*JUGLANS REGIA* L.) AND A WALNUT DRINK

SUMMARY

Walnut (*Juglans regia* L.), belongs to the Juglandaceae family, and is mostly cultivated in Northeast and East of Anatolia. Walnut has been well-documented to be an important source of polyunsaturated fatty acids, as well as antioxidant phenolics. Many epidemiological studies suggest that regular consumption of walnut can play an important role in preventing cancer and cardiovascular diseases. These nutritional characteristics of walnut have led to an increasing interest on this nut product, as well as on the effects of processing on its valuable compounds showing antioxidant activity.

In this thesis, different parts of the common walnut, including walnut kernel, walnut testa, male walnut flower, and walnut sap, as well as a walnut drink, prepared using testa, male flower, and sap, were evaluated for their contents of total phenolics, total flavonoids, and total antioxidant capacity, determined using five different *in vitro* assays (ABTS, DPPH, FRAP, CUPRAC, and ORAC) in parallel. The moisture and the fatty acid compositions, and the phenolic profiles were also determined in all samples. Additionally, the *in vitro* GI digestion model was applied to the walnut kernel and walnut drink in order to investigate and compare the *in vitro* bioavailability of walnut antioxidants in these samples.

The results obtained for moisture content analysis showed that walnut drink sample had the highest moisture content value (99.9%), which was followed by walnut sap (99.8%) walnut male flower (6.53%), walnut testa (6.30%), and walnut kernel (3.53%), respectively. Fifteen fatty acid components were identified in different walnut samples in total, having the linoleic, oleic, palmitic, and α -Linolenic acids as the major ones in common. The walnut samples, analyzed in the present work, were all found to be higher in contents of linoleic (5.2% in walnut drink to 54.2% in walnut kernel), oleic (5.2% in walnut male flower to 24.6% in walnut kernel), palmitic (6.3% in walnut kernel to 23.4% in walnut drink), and α -Linolenic acids (3.6% in walnut testa to 22.6% in walnut male flower). Total phenolic content values indicated that walnut kernel sample was significantly higher (44.2 mg GAE/g dry weight) compared to walnut testa (23.8 mg GAE/g dry weight), walnut male flower (2.7 mg GAE/g dry weight), walnut sap (3.0 mg GAE/g dry weight) and WD (2.9 mg GAE/g dry weight) ($p < 0.05$). Total flavonoid contents of different walnut samples were measured to range between 6.0-17.1 mg CE/g dry weight; giving the highest value in walnut testa (17.1 mg CE/g dry weight), followed by walnut kernel (12.9 mg CE/g dry weight), walnut male flower (6 mg CE/g dry weight), walnut sap (6 mg CE/g dry weight) and walnut drink (6 mg CE/g dry weight), respectively.

Among all five different antioxidant capacity methods performed in this study (ABTS, DPPH, FRAP, CUPRAC and ORAC), ORAC assay provided the highest values for all walnut samples; with the results determined for walnut kernel as 978 mg TE/g dry weight, for walnut testa as 612 mg TE/g dry weight, for walnut male flower as 411 mg TE/g dry weight, for walnut sap as 195 mg TE/g dry weight, and for walnut drink as 247 mg TE/g dry weight. The TAC of walnut drink sample was found to be significantly lower ($p<0.05$) than the TACs determined for walnut kernel and walnut testa samples, whereas walnut male flower and walnut sap did not much differ from walnut drink according to the results obtained with CUPRAC, FRAP, ORAC methods. In addition, CUPRAC method gave the highest linear correlations with TPC ($R^2=0.976$) and TFC ($R^2=0.705$) methods, as well as with the other TAC methods applied (except for DPPH) ($R^2=0.977$ for CUPRAC and ABTS, $R^2=0.961$ for CUPRAC and FRAP, and $R^2=0.955$ for CUPRAC and ORAC).

After the simulation of *in vitro* gastrointestinal digestion model, TPC, TFC and TAC of walnut kernel and walnut drink samples were evaluated for their PG (after gastric digestion, post gastric), IN (dialyzable fraction after intestinal digestion, the material that entered the serum), and OUT (the undialyzable fraction after intestinal digestion, the material that remained in the gastrointestinal tract) fractions. Results showed that 4.9%, 0.9%, and 0.4-6% (the range observed for four different tests, included DPPH, CUPRAC, FRAP, and ABTS) of TPC, TFC, and TAC were retained in the dialyzed fraction (IN) of walnut kernel compared to the initial values; whereas these values were 39.9%, 2.9%, and 6.5-27.1% for walnut drink sample.

CEVİZİN (*JUGLANS REGIA* L.) FARKLI KISIMLARI VE CEVİZ İÇECEĞİNİN SAĞLIKLA İLGİLİ ÖZELLİKLERİ

ÖZET

Ceviz (*Juglans regia* L.) Juglandaceae ailesine aittir ve ekonomik açıdan önemli bir ağaç türüdür. Dünyanın çoğu bölgesinde özellikle Amerika, Meksika, Asya'da yetiştirilmektedir. Türkiye'de ise çoğunlukla Kuzeydoğu ve Doğu Anadolu'da yetiştirilmektedir. Ceviz, ülkemiz için hem ekonomik hem de kültürel yönden önemli bir üründür. Türkiye, Dünya'da ceviz üretimi sırasında dördüncü ülke olarak yer almaktadır. Türkiye, bir çok ceviz çeşidinin bulunduğu önemli ülkeler arasında yer almasına rağmen günümüzde üretim ve tüketim istatistikleri incelendiğinde ceviz üretiminin istenilen seviyede olmadığını görülmektedir. Bu nedenle ceviz üretimini arttırmak için girişimler başlatılmıştır.

Cevizin fiziksel ve kimyasal özelliklerini belirleyebilmek için bir çok araştırma yapılmıştır. Bu analizler sonucunda cevizin besinsel olarak önemli bileşenlere sahip olduğu tespit edilmiştir. Bu bileşenler arasında esansiyel yağ asitleri ve proteinler önemli bir yer kaplamaktadır. Palmitik, stearik, oleik, linoleik ve linolenik yağ asitleri cevizdeki başlıca yağ asitlerindendir. Bu yağ asitlerinin insan sağlığına etkisini araştıran bir çok çalışma literatürde mevcuttur. Cevizde bulunan çoklu doymamış yağ asitleri kardiyovasküler hastalıklarının önlenmesinde ve damar tıkanıklarının önlenmesinde önemli rol oynamaktadır. Cevizin çoklu doymamış yağ asitleri yanı sıra antioksidan fenolikleri için iyi önemli bir kaynak olduğu detaylı belgelendirilmiştir. Bu çalışmalar sonucunda cevizin antioksidan içeriğinin oldukça yüksek olduğu tespit edilmiştir. Ceviz direk tüketiminin yanı sıra kurutulurak, reçel, pestil ve ezme olarak kullanıldığı bilinmektedir. Cevizin yeşil dış kabuğu, geleneksel bir ürün olan ceviz likörünün yapım aşamasında kullanılmaktadır. Böylece antioksidan içeriği ve fenolik bileşen olan ve bir çok hastalığı önlediği belirlenen juglon içeriği yüksek bir içecek elde edilmektedir.

Epidemiyolojik çalışmalar sonucunda elde edilen cevizde bulunan bileşenlerin sağlık üzerine etkileri oldukça fazladır. Cevizdeki yüksek orandaki omega-3 yağ asitleri kalp hastalıklarını, diyabeti, yüksek kan basıncını ve klinik depresyonu azaltır. Ceviz tüketimi kandaki kolesterol seviyesini düşürür, kalp atışlarında düzensizliği önler. Cevizdeki fitosteroller, kalın bağırsak, göğüs ve prostat kanseri gibi kanser türlerinden korunma sağlarken bağışıklık sistemini güçlendirir. Ceviz, damarlarda daha az pıhtılaşma özelliği olan kan tipinin üretimine ve iyi kolesterol oranının kötü kolesterol oranına göre artmasına yardım eder, kolesterolün damarları tıkama aşamasında önemli bir adım olan şişme ve kızarıklığı azaltır. Cevizdeki l-arginin kan damarlarının iç tarafının pürüzsüz ve düzgün olmasını sağlayarak kan-damar sisteminin rahatlamasını

sağlar. Cevizdeki yağ asitlerinin kalp hastalıklarını önleme etkileri vardır. Cevizdeki yağ profili, fitosteroller ve magnezyum, safra taşı oluşumunun önüne geçer. Cevizdeki melatonin, beyin bezesi tarafından salgılanan melatoninin insan vücudunun kullanıma hazır formunu içerir. Melatonin, gece çalışan ve zaman farkından dolayı uyku düzensizliği çeken kişilerde uyuma rahatsızlıklarını ortadan kaldırır. Ceviz, antioksidan savunmada önemli olan birtakım enzimlerde zorunlu kofaktörler olarak görev yapan manganez ve bakır içerir.

Fenolik bileşenlerce zengin olan cevizde gallik asit, elajik asit, kateşin, epikateşin gibi bir çok fenolik bileşen yapılan çalışmalarda tespit edilmiştir. Çoğu epidemiyolojik çalışmalar, cevizin düzenli olarak tüketildiğinde kanseri ve kardivasküler hastalıkları önlemekte önemli rol oynadığını belirtmektedir. Bu çalışmalarda cevizde özgü olan juglon bileşeni ayrıca incelenmiş ve bazı kanser çeşitlerini önlediği belirlenmiştir. Cevizin yağ asidi içeriği ve juglon bileşenini bulundurması cevizin besin değerine ayrı bir değer katmaktadır. Cevizin besin değeri, gıda işlemenin antioksidan aktiviteye sahip değerli bileşenlerine etkilerinin araştırılmasını önemli hale getirmiştir.

Ceviz ağacının meyvesi başlıca üç kısımdan oluşmaktadır; ceviz yeşil kabuğu, ceviz kabuğu ve ceviz içi. Diğer kısımları ise; erkek ve dişi çiçekler, yapraklardır. Literatürde yapılan araştırmalar; ceviz ağacının yaprağında antioksidan analizlerini kapsamaktadır. Bu çalışmalarda ceviz yaprağının antioksidan kapasitesi olarak yüksek bulunduğu tespit edilmiştir. Fakat, ceviz iç zarı ve ceviz çiçekleriyle ilgili literatürde herhangi bir araştırma bulunmamaktadır.

Bu çalışmada, ceviz içi, ceviz iç zarı, ceviz erkek çiçeği ve ceviz öz suyunu kapsayan yaygın olarak bulunan cevizin farklı kısımlarının yanı sıra iç zarı, erkek çiçeği ve öz suyundan hazırlanan ceviz içeceği toplam fenolik, toplam flavonoid içerikleri ve beş farklı *in vitro* analizi (ABTS, DPPH, FRAP, CUPRAC ve ORAC) kullanarak belirlenen toplam antioksidan kapasitesi değerlendirilmiştir. Nem miktarı ve yağ asidi kompozisyonu ve fenolik profilleri tüm örneklerde ayrıca belirlenmiştir. Ayrıca, bu örneklerde ceviz antioksidanlarının *in vitro* biyoyararlılığını araştırmak ve karşılaştırmak amacıyla ceviz içi ve ceviz içeceğine *in vitro* gastrointestinal sindirim simülasyon modeli uygulanmıştır.

Nem içeriği için elde edilen sonuçlar, ceviz içeceği örneğinin en yüksek nem içeriğini (%99.9) takiben sırasıyla ceviz öz suyu (%99.8), ceviz erkek çiçeği (%6.53), ceviz iç zarı (%6.30) ve ceviz içi (%3.53) olduğunu göstermiştir.

Ceviz örneklerinde; linoleik, oleik, palmitik ve α -linolenik asitler gibi yaygın olarak bulunan başlıca yağ asitleri gibi toplamda on beş yağ asidi tanımlanmıştır. Bu çalışmada analizlenen ceviz örneklerinin hepsinde içeceğinde daha yüksek oranda bulunan yağ asitleri; linoleik (5.2% in ceviz içeceğinde, %54.2 ceviz içinde), oleik (%5.2 ceviz erkek çiçeğinde, %24.6 ceviz içinde), palmitik (%6.3 ceviz içinde, %3.4 ceviz içeceğinde) ve α -linolenic acids (%3.6 ceviz iç zarı, %22.6 ceviz erkek çiçeğinde) bulunmuştur.

Ceviz içinin toplam fenolik içeriği (44.2 mg GAE/g kuru madde), ceviz iç zarı (23.8 mg GAE/g kuru madde), ceviz erkek çiçeği (2.7 mg GAE/g kuru madde), ceviz öz suyu (3.0 mg GAE/g kuru madde) ve ceviz içeceği (2.9 mg GAE/g dry kuru madde) ile karşılaştırıldığında önemli ölçüde yüksek olduğu belirlenmiştir ($p<0.05$). Farklı ceviz örneklerinin toplam flavanoid içeriği 6.0-17.1 mg CE/g kuru madde aralığında ölçülmüştür; en yüksek değeri ceviz iç zarı (17.1 mg CE/g kuru madde), ceviz iç zarını takiben ceviz içi (12.9 mg CE/g kuru madde), ceviz erkek çiçeği (6 mg CE/g kuru madde), ceviz öz suyu (6 mg CE/g kuru madde) ve ceviz içeceği (6 mg CE/g dry kuru madde) olarak bulunmuştur.

Bu çalışmada uygulanan beş farklı antioksidan kapasitesi belirleme yöntemi (ABTS, DPPH, FRAP, CUPRAC and ORAC) arasında, ORAC analizinde en yüksek değerler sağlanmıştır; sonuçlar, ceviz içi (978 mg TE/g kuru madde), ceviz iç zarı (612 mg TE/g kuru madde), ceviz erkek çiçeği (411 mg TE/g kuru madde), ceviz suyu (195 mg TE/g kuru madde) ve ceviz içeceği (247 mg TE/g kuru madde) için olarak belirlenmiştir. Ceviz içeceğinin toplam antioksidan kapasitesi ceviz içi ve ceviz iç zarının toplam antioksidan kapasitesine göre önemli ölçüde düşük ($p<0.05$) bulunurken ceviz erkek çiçeği ve ceviz öz suyunun toplam antioksidan kapasitesinden CUPRAC, FRAP, ORAC metodlarıyla elde edilen sonuçlara göre farklı bulunmamıştır. Ayrıca, CUPRAC metodu toplam fenolik ve toplam flavanoid metodlarıyla yüksek lineer korelasyon vermekle birlikte diğer uygulanan antioksidan metodlarıyla da (DPPH hariç), (CUPRAC ve ABTS için $R^2=0.977$, CUPRAC ve FRAP için $R^2=0.961$, ve CUPRAC ve ORAC için $R^2=0.955$) yüksek korelasyon sağlamıştır.

In vitro mide-bağırsak sistemi simülasyonunun ardından, ceviz içi ve ceviz içeceği için toplam fenolik madde, toplam flavonoid madde ve toplam antioksidan kapasitesi onların PG (mide sindiriminden sonra, post gastrik), IN (bağırsak sindirimden sonra zardan geçebilen fraksiyon, kana geçebilen materyal) ve OUT (bağırsak sindirimden sonra zardan geçemeyen fraksiyon, mide-bağırsak kanalında kalan materyal) fraksiyonlarını değerlendirilmiştir. Ceviz içinin başlangıç değerleri ile IN fraksiyonu karşılaştırıldığında; TPC, TFC ve TAC sonuçları için %4.9, %0.9, and %0.4-6 (DPPH, CUPRAC, FARP ve ABTS analizlerini içeren dört farklı testte aralıklar belirlenmiştir) bulunurken ceviz içeceği ele alındığında %39.9, %2.9, and %6.5-27.1 bulunmuştur.

1. INTRODUCTION

The walnut tree (*Juglans regia* L.) is cultivated in a wide range throughout the world, including the southern Europe, eastern Asia, and the USA as the primary commercial producers (Labuckas et al., 2008). Turkey had the fourth rank among the world's leading countries in walnut production, with a production rate of 183240 tons in 2011 (Anon., 2011). Walnut fruit is the second most produced hard-shell fruit after the almond fruit (Merikli and Akpinar, 2012).

Recent studies have indicated nuts, including walnut, hazelnut, etc., as the potential good sources of polyunsaturated fatty acids, as well as antioxidant phenolics (Arcan and Yemenicioglu, 2009; Li et al., 2007; McKay et al., 2010; Pellegrini et al., 2006). Among these products, walnut has been well-reported to exhibit greater antioxidant capacity in comparison to the other nuts (Arcan and Yemenicioğlu, 2009; Pellegrini et al., 2006). Epidemiological data, that have been conducted concerning the health promoting effects of antioxidants, have pointed out their protective effects in various diseases including cardiovascular disease, cancer, aging, and cataracts (Arcan and Yemenicioglu, 2009; Kaur and Kapoor, 2001). Walnut phenolics, that possess antiatherogenic and antioxidant properties, have been related to the favourable effects of walnut consumption on human health (Anderson et al., 2001; Fukuda et al., 2003).

In common walnut, the major phenolic compounds have been identified as flavonoids and naphthoquinones, among which juglone (5-hydroxyl-1,4-naphthoquinone) has gained an increasing interest due to its chemical reactivity (Duroux et al., 1998; Solar et al., 2006). Although several research studies have been performed on phenolic constituents and antioxidant activity of walnut leaves, walnut kernel, and walnut green husks (Oliveira et al., 2008; Pereira et al., 2007; Villarreal-Lozoya *et al.*, 2007), there is hardly any information on walnut testa and male flower of walnut.

Moreover, it is well-known that under *in vivo* conditions, polyphenols obtained from the diet are extracted following a gastrointestinal (GI) digestion. These bioactive compounds must be bioavailable, meaning that effectively absorbed from the gut into the circulation and transferred to the appropriate location within the body, in order to obtain any influence in a specific tissue or organ (McDougall *et al.*, 2005).

It has been proven that the measurement of bioavailability by *in vitro* models can be well correlated with the results obtained from human studies and animal models (Bouayed *et al.*, 2011). Based on our current knowledge, there is no previous study that has been carried out on determining the *in vitro* bioavailability of walnut using the simulation of *in vitro* gastrointestinal (GI) digestion.

This research thesis is presented as “literature”, “materials and methods”, “results and discussion”, and “conclusion” parts. In the “literature” section, parts of walnut, physical and chemical properties of walnut samples, as well as bioavailability issues were reviewed. “Materials and methods” section included the detailed protocols followed for the analyses (TPC, TFC, ABTS, FRAP, CUPRAC, ORAC, DPPH) performed. “Results and discussion” section included the results obtained for the spectrophotometric measurements and simulation of *in vitro* bioavailability of walnut kernel and walnut drink, as well as the evaluation and the comparison of the results with the current literature. In the “conclusion” part, the major conclusions have been provided for the specific results obtained for the analyzed walnut samples.

2. LITERATURE REVIEW

2.1. Walnut and Parts of Walnut

Walnut (*Juglans regia* L.), belongs to the Juglandaceae family, and is mostly cultivated in Northeast and East of Anatolia, as well as in Balkans, Lebanon, Caucasian, North Iraq, Persia, Afghanistan, Central Asia and China. Turkey is an important country in walnut production having a variety of specific walnut genes (Merikli and Akpinar, 2012). Walnuts are single-seeded stone fruits of the walnut tree which consist of three parts; green husk, walnut shell, and walnut kernel. The walnut kernel is covered with green husk and walnut shell. Other parts included in walnut tree are testa, leaves, and flowers (male and female). All parts of walnut tree are shown in Figure 2.1.



Figure 2.1 : Parts of walnut (1) green husk (2) kernel (3) shell (4) testa (5) flower (6) leaves

2.2. Rates of Production and Consumption

Walnut is cultivated in a wide range around the world today (Merikli and Akpinar, 2012). Although, the production rates of hard-shell fruits around the world are highly variable on year basis, the world's hard-shell fruit production has determined to be increased between 2008 and 2011 (Anon., 2011). According to the world's hard-shell fruit production statistics in 2011 (presented in Table 2.1), walnut was the second most produced hard-shell fruit with a production rate of 3418502 tonnes, followed by the cashew nuts with 4279738 tonnes.

Table 2.1: Worldwide hard-shell fruit production rates (in tonnes) (Anon., 2011).

Hard-shell fruits	2008	2009	2010	2011
Cashew nuts, with shell	3982640	3996393	3996622	4279738
Walnuts, with shell	2428949	2646663	2989437	3418502
Chestnut	1788997	1890391	1954824	2023019
Almonds, with shell	2442888	2407294	2537929	1942242
Pistachios	804284	816810	952452	942970
Nuts, nes	800191	815712	764867	805681
Hazelnuts, with shell	1069889	776166	855732	742993
Brazil nuts, with shell	85588	95735	102996	107508

Turkey had the fourth rank among the world's leading countries in walnut production, with an average production rate of 177394.3 t per year (in years between 2008-2011) (Table 2.2) (Anon., 2011). Walnut is an important hard-shell fruit depending not only on its nutritional value, but also on its economic value. Therefore, most countries have attempted to increase their walnut production.

Table 2.2: Worldwide walnut production rates (in tonnes) (Anon., 2011).

Countries	2008	2009	2010	2011	Average
China, mainland	828635	979366	1284351	1655508	1186965
Iran	433630	463000	475000	485000	464157.5
United States of America	395530	396440	457221	418212	416850.8
Turkey	170897	177298	178142	183240	177394.3
Mexico	79770	115350	76627	96476	92055.75
Ukraine	79170	83890	87400	112600	90765
India	37000	36000	38000	36000	36750
Romania	32259	38329	34359	35073	35005
France	36912	20417	31737	38314	31845
Chile	24000	26000	32500	35000	29375
Greece	15100	22000	22200	29800	22275
Serbia	24405	25172	21419	23938	23733.5
Egypt	25855	22445	20865	18389	21888.5
Italy	17000	15724	15087	17771	16395.5
Germany	18374	13097	12313	15083	14716.75

Turkey's hard-shell production rates are presented in Table 2.3. (TUIK, 2012). In 2012, the production rate of each hard-shell fruit increased (except for chestnut) in Turkey and walnut had the second rank, after hazelnut. Walnut production in Turkey was 183240 and 203212 tonnes in 2011 and 2012, respectively (TUIK, 2012). Turkey had the first rank in walnut production in the past, possessing various walnut genes. However, today, walnut production and exportation rates are lower than the expected rates. Therefore, Ministry of Forestry and Water Affairs in the Turkey was prepared 'Walnut Action Plan' and they planned to increase walnut production rates and planted five millions sapling end of the programme (2016) (Anon., 2012). More detailed research focusing on the nutritional quality and health-promoting components of walnuts will enhance our knowledge and encourage walnut consumption.

Table 2.3: Hard-shell fruit production (tonnes) in Turkey (TUIK, 2012)

YEARS	Almond (Tonnes)	Hazelnuts (Tonnes)	Walnuts (Tonnes)	Chestnuts (Tonnes)	Pistachios (Tonnes)
2000	47000	470000	116000	50000	75000
2001	42000	625000	116000	47000	30000
2002	41000	600000	120000	47000	35000
2003	41000	480000	130000	48000	90000
2004	37000	350000	126000	49000	30000
2005	45000	530000	150000	50000	60000
2006	43285	661000	129614	53814	110000
2007	50753	530000	172572	55100	73416
2008	52774	800791	170897	55395	120113
2009	54844	500000	177298	61697	81795
2010	55398	600000	178142	59171	128000
2011	69838	430000	183240	60270	112000
2012	80261	660000	203212	57881	150000
Average	50781	556676	151767	53410	84257

As shown in Table 2.4, Turkey's walnut production capacity reached up to 203212 t in 2012 while in 2008 only 170897 t has been produced, moreover it was increased total orchard area of 552019 da in 2012 while in 2008 only 328873 da.

Table 2.4: Walnut production in Turkey (TUIK, 2012)

YEAR	Production (tonnes)	Total orchard area (da)	Average yield per tree	Number of mature trees
2008	170897	328873	34	5094781
2009	177298	366736	34	5191724
2010	178142	413932	33	5441051
2011	183240	468378	33	5594576
2012	203212	552019	34	5977397

Although the production of walnut is being carried out in many regions of Turkey, high quality walnuts are especially grown in Black sea, Aegean and Southeast because of climatic and ecological conditions. In determining the appropriate climatic conditions for walnut, walnut and fruit development of the physiological and morphological structure is quite critical value considering are encountered. One of the most important factors limiting the walnut growers in the region at the beginning of late spring frosts and frost damage early autumn walnut growers also among the factors that limit counted. These all factor affect of walnut production rates.

2.3. Physical and Chemical Composition of Walnut

Walnuts contain nutrients providing positive effects on human health and therefore has an important place in the human diet. Several studies have been conducted concerning about chemical compositions and physical properties of walnut. For example, Ruggeri *et al.* (1998) is the earliest reference concerning the chemical composition of walnut cultivars such as Sorrento, Franquette, Hartley and Trento. In this study, in which the proximate analysis, soluble sugar content, and amino acid content of the walnut cultivars were analyzed, the two major groups were identified as oils and proteins in the walnut. Percentages of chemical composition have been reported in walnut 3.2-4.4% moisture, 12.0-19.6% protein, 61.3-73.8% oil, 1.8-2.3% ash, 2.2-4.5% sugar, respectively (Table 2.5) (Ruggeri *et al.*, 1998).

Table 2.5: Chemical compositions of four different kinds of walnut (Ruggeri *et al.*, 1998)

Ingredients (%)	Types of Walnut			
	Sorrento	Franquette	Hartley	Trento
Moisture	3.3	3.5	3.2	4.4
Protein	19.6	13.0	12.0	18.0
Oil	64.5	70.0	73.8	61.3
Ash	1.8	1.9	1.9	2.3
Sugar	2.7	2.2	2.3	4.5

The general proximate analysis of 6 different cultivars of walnuts grown in Portugal is reported by Amaral *et al.* (2003). Similar to the results observed in the previous study mentioned (Ruggeri *et al.*, 1998), fat fraction was the predominant fraction (ranging from 62.3 to 66.5%), followed by protein and carbohydrate fractions (ranging from 12.2-15.2% and 13.4-17.2%, respectively). The values obtained by Savage *et al.* (1999), who studied 12 cultivars of walnuts grown in New Zealand, were, on average, higher for moisture (6.1%) and crude protein (14.6%), but slightly lower for total fat (63.0%) than those obtained with walnuts grown in Portugal (4.2, 13.7, and 64.6%, respectively). Another study, performed to determine the physical properties and chemical composition of walnut is Bakkalbası *et al.* (2010), who studied on seven different cultivars of walnut in Turkey. Chemical properties of walnut, including titratable acidity, pH, protein, raw cellulose, and ash of shelled walnuts, were found to vary in the range of 0.32-0.65 mg/100g, 5.77-6.00, 12.48-16.90%, 5.72-8.93% and 1.57-1.90%, respectively (Table 2.6). This study also investigated physical parameters of walnuts such as fruit weight, kernel weight, yield and hunter color values, which varied in the range of, 9.48-18.79g, 4.37-8.58g, 44.90-59.54%, 39.41-47.63 (L), 0.84-1.77 (a) and 10.16-12.79 (b) respectively (Table 2.7).

Table 2.6: Chemical compositions of different kinds of walnuts cultivated in Turkey (Bakkalbasi *et al.*, 2010)

Types	Year	Titratable acidity(%)	pH	Protein (N×5.3)(%)	Raw Cellulose(%)	Ash(%)
Yalova 1	2004	0.45	5.77	15.32	5.99	1.68
	2005	0.65	5.85	16.90	6.10	1.79
Yalova 3	2004	0.39	5.98	14.57	6.01	1.74
	2005	0.57	5.79	14.69	6.27	1.57
Yalova 4	2004	-	-	-	-	-
	2005	0.34	5.90	13.0	7.92	1.76
Sebin	2004	0.32	6.00	13.09	9.22	1.94
	2005	-	-	-	-	-
Bilecik	2004	0.36	5.90	12.48	8.22	1.90
	2005	0.38	5.91	16.42	6.75	1.74
Sen 1	2004	-	-	-	-	-
	2005	-	-	16.00	8.93	1.88

Table 2.7: Physical compositions of different kinds of walnut cultivated in Turkey (Bakkalbasi *et al.*, 2010)

Types	Years	Fruit Weight (g)	Kernel Weight (g)	Yield (%)	Hunter Color Value		
					L	a	b
Yalova 1	2004	18.79	8.56	45.58	39.41	1.77	11.71
	2005	18.51	8.58	46.29	40.78	1.44	12.01
Yalova 3	2004	12.67	6.40	50.55	39.49	1.39	10.16
	2005	13.75	6.85	49.82	43.11	0.93	12.79
Yalova 4	2004	-	-	-	-	-	-
	2005	14.2	7.08	49.57	44.76	0.99	11.93
Sebin	2004	8.98	5.40	59.54	43.47	1.24	10.60
	2005	-	-	-	-	-	-
Bilecik	2004	13.30	6.22	46.64	44.62	1.16	11.59
	2005	9.48	4.37	45.87	44.96	1.37	11.45
Sen 1	2004	-	-	-	-	-	-
	2005	17.28	7.79	44.90	40.55	1.68	10.22
Kaman 5	2004	12.27	6.27	50.97	47.63	0.84	12.48
	2005	14.01	7.53	53.63	46.05	1.04	12.67

Although walnut has different kind of important nutritional ingredients, the most of important nutrional ingredient is oils. Walnut was contained approximately range of 52-70% oil and they are very important food because of their fatty acids compositions rather than their high amount of oil. Walnut oil was included essential fatty acids such as oleic, linoleic and linolenic acid (Bakkalbasi *et al.*, 2010).

Bada *et al.* (2010) was studied a total of 15 walnut samples from different geographical areas in Asturias, Spain. They found the higher percentages of fatty acids, as determined by capillary GC, corresponded to palmitic (6.11–7.49%), oleic (11.70–18.90%), linoleic (59.81–64.77%) and linolenic (11.11–15.65%) (Table 2.8).

There are normally five fatty acids such as palmitic, stearic, oleic, linoleic and linolenic in walnuts. Li *et al.* (2007) identified 30 different fatty acids in Combe Persian walnuts. The chemical composition, namely the oil content and the fatty acids compositions have been found to vary significantly.

This is dependent on the type of cultivar, growing location and other growing variables, such as irrigation (Amaral *et al.*, 2003; Bada *et al.*, 2010; Li *et al.*, 2007; Savage *et al.*, 1999; Villarreal-Lozoya *et al.*, 2007).

Table 2.8: Fatty acid compositions (%) of lipid fraction extracted from walnut samples (Bada *et al.*, 2010)

Cultivar	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
1	6.72	0.12	1.92	15.42	64.12	11.82
2	7.12	0.11	2.05	14.81	61.83	14.23
3	6.23	0.13	1.82	16.86	63.85	11.35
4	7.41	0.14	1.51	13.25	64.21	13.60
5	7.13	0.11	2.75	15.30	61.50	13.39
6	6.86	0.16	2.13	17.42	61.66	12.02
7	7.10	0.18	2.12	13.21	61.72	15.42
8	7.21	0.23	2.60	15.55	60.11	14.34
9	7.15	0.12	1.72	11.70	63.53	15.65
10	6.1	0.11	1.91	12.21	64.45	15.14
11	6.91	0.16	1.86	18.90	59.81	12.23
12	6.73	0.22	1.91	19.71	59.95	11.30
13	6.70	0.16	2.33	14.80	64.71	11.11
14	6.82	0.21	1.80	17.56	61.6	11.90
15	7.41	0.16	1.85	13.50	61.3	15.61

2.4. Antioxidant and Phenolic Compositions of Walnut and Walnut Parts

The consumption of walnut is increasing as consumers are showing an interest in their valuable fatty acid content and also their antioxidant capacity. Walnut is also consumed as, dried, walnut jam, walnut paste, walnut candy, walnut brine (Torun *et al.*, 1999). In the Slovenia, walnut green husk used for producing traditional walnut liqueur (Stampar *et al.*, 2006). Walnut is rich in an oil composed of unsaturated fatty acids, such as linoleic and oleic acid, which are susceptible to oxidation. Although the content of α -tocopherol, an antioxidant, in walnut is lower than in other nuts, such as almonds, hazelnuts, peanuts, etc. (Kagawa *et al.*, 2001), walnut is readily preserved.

This implies that the nut contains antioxidants inhibiting lipid auto-oxidation. Recently, a walnut extract containing ellagic acid, gallic acid, and flavonoids was reported to inhibit the oxidation of human plasma and low density lipoproteins (LDL) in vitro (Anderson *et al.*, 2001).

Phenolics can be found in all parts of a food, for example, the shell, leaf and kernel of a walnut. With varying amounts and types of different phenols from different parts of the walnut. The health protective effects of walnut fruit and its products have been characterized with their high contents of phenolics such as chlorogenic, caffeic, p-coumaric, ferulic, sinapic, ellagic, and syringic acid as well as syringaldehyde and juglone were identified in ripe fruits of 10 walnut cultivars (Colaric *et al.*, 2005). Phenolics were determined in the kernel and in the pellicle of the walnut. Also the pellicle was detected syringic acid, juglone, and ellagic acid predominate (average values of 33.83, 11.75, and 5.90 mg/100 g of kernel; 1003.24, 317.90, and 128.98 mg/100 g of pellicle, respectively), and the contents of ferulic and sinapic acid (average values of 0.06 and 0.05 mg/100 g of kernel and 2.93 and 2.17 mg/100 g of pellicle, respectively) were the lowest in all cultivars.

Table 2.9: Phenolic contents of walnut kernels from different cultivars (Colaric *et al.*, 2005)

Cultivar	Chlorogenic acid	p-coumaric acid	Syringic acid	Ellagic acid	Juglone	Syringaldehyde
Cisco	2.57	0.11	40.01	6.70	11.58	0.76
Fernette	1.39	0.05	16.57	3.26	7.27	1.03
Fernor	1.25	0.14	43.40	4.17	12.32	0.95
Rasna	2.44	0.13	57.45	6.59	17.02	1.16
A-117	2.41	0.18	26.29	9.77	19.16	1.04
Franquette	2.28	0.29	48.78	8.87	12.88	0.88
Adams	1.46	0.13	22.83	5.75	9.19	0.86
Lara	1.28	0.11	25.59	4.53	10.57	0.72
Chandler	1.06	0.15	39.77	4.30	10.55	0.84
Elit	1.96	0.07	17.60	5.09	6.93	0.49

Gallic acid, ellagic acid, catechin and epicatechin were identified by HPLC in six pecan cultivars and presence of gallic acid and ellagic acid in the range of 651 to 13001 $\mu\text{g/g}$ defatted kernel, 2505 to 47321 $\mu\text{g/g}$ defatted kernel, respectively. Moreover, the amounts of catechin and epicatechin in the HPLC phenolic profile were lower than gallic and ellagic acids. In addition to this phenolic acids, catechins and condensed tannins were identified in the shells of the different pecan cultivars (Villarreal-Lozoya *et al.*, 2007).

Flavonoids have been identified in most nuts with their aglycone profiles included in the USDA database. The highest total flavonoid concentrations are found in pecans at 34, almonds at 15, and pistachios and hazelnuts at 12 mg/100 g, respectively. Only among the flavonoids, cyanidin (2.71 mg/100 g) was identified in walnut. No flavonoids have detected in Brazil or macadamianuts (USDA, 2013).

Walnut has juglone as an important phenolic compound (Merikli and Akpinar, 2012). Extraction method is important for the detecting juglone in extracts of walnut. Periera *et al.* (2007) was not detected juglone in the walnut leaves extracts with HPLC-DAD. This study was mentioned that hot water extraction method is not available for the juglone however they was identified quantified: 3 and 5-caffeoylquinic acids, 3 and 4-pcoumaroylquinic acids, p-coumaric acid, quercetin 3-galactoside, quercetin 3-pentoside derivative, quercetin 3-arabinoside, quercetin 3-xyloside and quercetin 3-rhamnoside. Amaral *et al.* (2004) was identified juglone only in the chloroformic extract from a fresh walnut leaves with HPLC-DAD/MS and seven phenolic compounds were identified (3-caffeoylquinic, 3-p-coumaroylquinic and 4-p-coumaroylquinic acids, quercetin 3-galactoside, quercetin 3-arabinoside, quercetin 3-xyloside, quercetin 3-rhamnoside) and two other partially identified phenolics (quercetin 3-pentoside and kaempferol 3-pentoside derivatives) were also detected.

Stampar *et al.* (2006) was investigated four different walnut green husk. Thirteen phenolics was detected including chlorogenic acid, caffeic acid, gallic acid, protocatechuic acid, catechin with HPLC/DAD and also was identified the juglone that major phenolic in the husks with the highest content 1404 mg/100 g dry weight.

Jakopic *et al.* (2009) was studied with green walnut fruit and seven phenolics were identified gallic, chlorogenic, ellagic, sinapic and protocatechuic acid, catechin and juglone using HPLC method. Amount of juglone was compared other phenolic compound was highest content (~ 25 mg/g FW). An other study with green husk of walnut was Cosmulescu *et al.* (2010) and the juglone which is major phenolic 31308 mg/100 g sample was found.

Li *et al.* (2006) were studied on identifying polyphenolics in heartnut and Persian walnut by LC-ESI-MS method and major polyphenolics both in heartnut and Persian walnut were ellagic acid and valoneic acid dilactone. Persian walnuts contained an average of 0.29 and 1.31 mg of ellagic acid/g nut.

Researchers have reported the total phenolic and total antioxidant content of parts of walnuts. However studies are different from each others because of they were used different extraction method of parts of walnut and detected content of total phenolic and total antioxidant using different spectrophotometric methods and also not analyzed same walnut cultivars. Therefore, it is generally difficult to compare the results obtained in different studies. Table 2.10 shows some studies related to total phenolics and total antioxidant content of different parts of walnut, extraction method of walnut samples also present in this table.

2.5. Effects of Walnut on Human Health

Walnut has been well-reported to exhibit greater antioxidant capacity in comparison to the other nuts (Arcan and Yemenicioglu, 2009; Pellegrini *et al.*, 2006) and also walnuts are a good source of essential fatty acids (Li *et al.*, 2007). Therefore, epidemiological data, that have been conducted concerning the health promoting effects of both of antioxidants and fatty acids, have pointed out their protective effects in various diseases including cardiovascular disease, cancer, aging, and cataracts (Arcan and Yemenicioglu, 2009; Kaur and Kapoor, 2001; Lavedrine *et al.*, 1999).

Investigation in the France Lavedrine *et al.* (1999) was detected that polyunsaturated fatty acids contribute to the reduced risk of cardiovascular diseases and resources of polyunsaturated fatty acids was showed walnut oil and kernel.

Table 2.10: Total antioxidant capacity and the total phenolic content of different walnut parts reported in literature.

Walnut Part	Extraction Method	Analysis	Results	Unit	Refences
Kernel	Methanol/water	FRAP	114.92 ± 4.01	µmol TE/g dm	Arranz <i>et al.</i> ,(2008)
		ABTS	153.85 ± 16.23	µmol TE/g dm	
		ORAC	187.18 ± 9.13	µmol TE/g dm	
		DPPH	14.31 ± 0.07	g dm/g DPPH	
		TPC	1071 ± 35	mg GAE/100 g dm	
Kernel	Aqueous	TPC	414±18	mg GAE/100 g d.w.	Arcan <i>et al.</i> , (2009)
	Ethanol/water	ABTS	6498±77	µmol TE/100 g d.w.	
		TPC	175±17	mg GAE/100 g d.w.	
		ABTS	2555±196	µmol TE/100 g d.w.	
Leaf	Ethanol/water	ORAC	2.17 ± 0.22	µmoles TE/ ml extract	Ameida <i>et al.</i> , (2008)
		TPC	270 ± 3	mg of GAE/g lyophilised extract	
Green husk	Aqueous	TPC	(32.61- 74.08)±0.02	mg GAE/g	Oliveria <i>et al.</i> , (2008)
		DPPH	0.35-0.59	mg/ml	
Kernel	Methanolic/alkaline hydrolyzed	FRAP	412.29	mmol Fe2+/kg	Pellegrini <i>et al.</i> , (2006)
		TRAP	27.58	mmol Trolox/kg	
		TEAC	119.91	mmol Trolox/kg	
Kernel (Flour)	Metanol/water	TPC	490±27.3	mg GAE/g extract	Labuckas <i>et al.</i> , (2008)
Ethanol/water	479±42.1				
Kernel (Hull)	Methanol/water	TPC	17.3±0.4	mg GAE/g extract	
	Ethanol/water		14.9±3.25		
Kernel	Methanol/water	TPC	10.42	mg GAE/ g sample	Li <i>et al.</i> , (2006)
		FRAP	3127.32±0.07	µmol ascorbic acid/ g sample	

Abbey *et al.* (1994) reported that the significant reductions in total and LDL cholesterol was detected as 5% and 9%, respectively, after supplementation with walnut which is rich in polyunsaturated fatty acid (68 g/d), equivalent to 46 g fat.

Walnut phenolics, that possess antiatherogenic and antioxidant properties, have been related to the favourable effects of walnut consumption on human health (Anderson *et al.*, 2001; Fukuda *et al.*, 2003).

In common walnut, the major phenolic compounds have been identified as flavonoids and naphthoquinones, among which juglone (5-hydroxyl-1,4-naphthoquinone). Aithala *et al.* (2009) were investigated that potential of juglone and its effect on melanoma cells and a dose-dependent increase in the frequency of micronucleated binucleate cells indicated the potential of juglone to induce cytogenetic damage in melanoma tumor cells. There are more studies related to effects of juglone on human health such as Saling *et al.* (2011) were studied on the effect of juglone on several metabolic parameters in the isolated perfused rat liver and results of this study was showed that mild uncoupling increases longevity in mice a phenomenon that was associated with the improvement of several serological markers such as glucose, triglycerate and insulin levels. Ji *et al.* (2011) was designed to investigate the effect of juglone on the apoptosis of human gastric cancer SGC-7901 cells and they was found juglone can induce apoptosis in SGC-7901 cells through a mitochondrial pathway.

2.6. Bioavailability

The term ‘bioavailability’ has several working definitions depending on the research area it applies to. From the nutritional point of view, bioavailability refers to the fraction of the nutrient or bioactive compound ingested that is available for use in physiologic functions or to be stored (Fernández-García *et al.*, 2009). The bioavailability of a dietary compound depends on its digestive stability and its release from the food matrix that is referred as bioaccessibility (Tagliazucchi *et al.*, 2010).

In vitro digestion and dialysis methods enables the study of phenolic compound release from the food matrix and their transformation during digestion and has been applied to orange juice, strawberries and strawberry jam (Gil-Izquierdo *et al.*, 2002). *In vitro* digestion and dialysis methods for simulating the GI digestion are being extensively used since they are rapid, safe, and do not have the same ethical restrictions as *in vivo* methods (Liang *et al.*, 2012). In order to understand the bioactivity of polyphenols *in vivo* experiments in humans are required. However, *in vitro* methods have also been proven to be useful to determine their stability under GI conditions. Indeed, despite limitations such as constituting only a static model of digestion, the evaluation of bioavailability by *in vitro* models can be well correlated with results from human studies and animal models (Bouayed *et al.*, 2011).

All of the bioactive compounds such as tocopherols, phytosterols and phenols to be found and identified in walnuts has been well studied. However there is no previous study that has been carried out on determining the *in vitro* bioavailability of walnut using the simulation of *in vitro* gastrointestinal (GI) digestion and also *in vivo* bioactivity is not clear. Studies in humans are required to directly understand the bioaccessibility, bioavailability, metabolism, and elimination of nut flavonoids. Such clinical studies are required not only because of the differences in specific flavonoid profiles between nut varieties (and other foods) but the impact of the food matrix (and processing thereof) on these factors (Chen and Blumberg, 2008). Importantly, evidence is available from a limited number of studies that the antioxidant capacity of nuts can be demonstrated *in vivo* as well as *in vitro* (Anderson *et al.*, 2001).

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals

For extract preparation and determination of total phenolic, flavonoid and antioxidant contents, gallic acid ($\geq 98\%$), (+)-catechin ($\geq 98\%$), acetone ($\geq 99.8\%$), ethanol ($\geq 99.8\%$), hexane ($\geq 95\%$), Folin-Ciocalteu phenol reagent, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) and neocupraine (Nc) from Sigma-Aldrich Chemie GmbH (Steinheim, Germany); methanol ($\geq 99.9\%$), formic acid ($\geq 98\%$) hydrochloric acid (37%), *n*-butanol ($\geq 99.5\%$), sodium carbonate (Na_2CO_3), sodium nitrite (NaNO_2), sodium hydroxide (NaOH), sodium acetate trihydrate ($\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$), potassium persulfate ($\text{K}_2\text{S}_2\text{O}_8$), dipotassium hydrogen phosphate (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), copper (II) chloride (CuCl_2) and ammonium acetate (NH_4Ac) from Merck KGaA (Darmstadt, Germany); 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and aluminum chloride (AlCl_3) from Fluka Chemie (Buchs, Switzerland); ferrous sulfate (FeSO_4) and potassium chloride (KCl) from Riedel-de Haen Laborchemikalien GmbH (Hanover, Germany); ferric chloride (FeCl_3) from Lachema (Czech Republic) and 2,2'-azinobis(3-ethylbenzo-thiazoline-6-sulphonic acid) diammonium salt (ABTS) from Applichem GmbH (Darmstadt, Germany) were purchased.

The following standards and reagents were used for the quantification of phenolic compounds: (+)-catechin ($\geq 99\%$), (-)-epicatechin ($\geq 99\%$), gallic acid ($\geq 99\%$) and cyanidin 3-*O*-glucoside ($\geq 96\%$) from Extrasynthese (Genay, France); *p*-coumaric acid ($\geq 98\%$), chlorogenic acid ($\geq 98\%$), ellagic acid ($\geq 96\%$) and quercetin-3-*O*-glucoside ($\geq 98\%$) from Fluka (Buchs, Switzerland); rutin (95%), apigenin ($\geq 95\%$), kaempferol-rutinoside ($\geq 98\%$), cyanidin 3-*O*-rutinoside ($\geq 98\%$), trifluoroacetic acid (99%) and acetonitrile (99.8%) from Sigma-Aldrich (Steinheim, Germany).

For simulation of *in vitro* gastrointestinal digestion system, pepsin, pancreatin, bile salts, dialysis bags (Membra-Cel MD34) from Sigma-Aldrich and sodium bicarbonate (NaHCO_3) from BDH Chemicals Ltd. (Poole, UK) were purchased.

Water used for all analysis was distilled and purified with the water purification system (TKA GenPure, Germany).

3.1.2. Plant material

The samples investigated within this study included walnut kernel (WK), walnut testa (WT), and male flower of walnut (WMF). In addition, a special walnut drink (WD), prepared relying on a traditional recipe by using WT and WMF, was also supplied for the analyses.

WMF samples were collected in May-July, 2013 and stored after drying. The whole walnut fruits were collected in August, 2013 and October, 2013, and stored after drying. After one month of storage, the whole fruits were de-shelled and WT was separated from the WK. WT was stored in a special air-permeable package.

3.1.2.1. Preparation of walnut drink

Walnut tree sap (WS) was extracted from alburnum of walnut tree in November, 2013, and stored (in a stainless steel tank, at $< 4^\circ\text{C}$) until it was used.

A certain amount of WT was placed into 20 kg glass bell and added WS. This mixture WT/WS stored in air conditioning room after glass bell was closed. Same mixture was prepared with WMF according to same method with WT. All glass bells including mixture of WT/WS and WMF/WS were shaking periodically and waiting a certain time and also prepared clove/water (CW) mixture boiling with soft water and cooled.

The important point is first WMF/WS mixture was put into stainless steel tank including WS after that WT/WS mixture was added, followed by a certain amount of CW was involved into the stainless steel tank. Ascorbic acid and citric acid were also added to this mixture, which was subsequently bottled and stored at $< 4^\circ\text{C}$.



Figure 3.1: (1) Walnut drink (2) Walnut testa (3) Walnut kernel
(4) Walnut male flower

3.2. Methods

3.2.1. Determination of moisture content

Moisture contents of walnut samples were determined using freeze-drying process. Freeze drying is the removal of water by sublimation from the frozen state (ice). In this process, the food is first frozen and then subjected to a high vacuum, whereby the water ice evaporates without melting. The water vapor released is condensed on the surface of a condenser at very low temperature. A certain amount of walnut samples were placed on pre-weighed petri plate and weighed. Water content of all samples was removed by lyophilisation at -70°C and 0.010 mbar (Christ, Alpha 1-2 LD, Germany) (Figure 3.2) until the equilibrium moisture content was reached (48 h). After lyophilisation, petri plate including lyophilized walnut sample weighed again. All samples were analyzed in triplicate and average values were reported.



Figure 3.2: Christ freeze-dryer

3.2.2. Determination of FAMES

Fatty acid fraction of WK was extracted with soxhlet extraction method which is universal extraction using hexane. On the other hand fatty acids of WT, WMF and WD were extracted with chloroform and methanol mixture. The procedure used for the extraction of fatty acids was adapted from Folch *et al.* (1957), with some modifications. 1 g sample weight and chloroform/methanol (2:1 v/v) mixture waited for 24 hours. Mixture were filtered into seperated funnel and 6 mL 0.9% NaCl was added and allowed seperated into two phases. Under phase removed in vacuum and oil phase procured from upper phase. After fatty acids of all sample were extracted and fatty acids were methylated and fatty acids were identified using a GC system (Agilent Technologies 7820A, Santa Clara, CA, USA) equipped with a splitless injection port, a flame ionization detector (FID), an autosampler, a capillary column and a software system. The temperature of injector and detector was set at 250°C, and the column temperature at 230°C. H₂ served as the carrier gas at a flow rate of 1 (mL/min) across a period of 22.5 min. Quantification of the FAMES was based on the FID response, expressed as percent of total FAMES. The identification was based on the comparison of the relative retention times of eluting peaks with literature (AOAC, 1992).



Figure 3.3: Agilent Technologies 7820A, Gas Chromatography

3.2.3. Preparation of defatted samples

WK, WT and WMF were ground separately in a coffee mill (Sinbo SCM-2914, PRC). Water content of all samples was removed by lyophilisation for 48h at -70°C and 0.010 mbar (Christ, Alpha 1-2 LD, Germany) (Figure 3.2). 4 g dry weight sample was extracted with 20 ml hexane, which was followed by ultrasonification (USC900TH, VWR ultrasonic cleaner, US) (Figure 3.4) for 5 min and centrifugation (Universal 32R, Hettich Zentrifugen, Tuttlingen, Germany) (Figure 3.4) for 10 min at 4000 rpm at room temperature. The supernatants were removed and the defatted samples were air-dried to remove the residual hexane (Alasalvar *et al.*, 2006).



Figure 3.4 : (A) Hettich Zentrifugen Universal 32R centrifuge and (B) VWR Ultrasonic Cleaner

3.2.4. Preparation of extracts

The freeze-dried non-fat samples (WK, WT, and WMF) were subjected to an initial aqueous-acetone extraction process using the method described previously by Alasalvar *et al.* (2006). Four grams of each sample (except for WD and WS) was treated with 20 mL 80% aqueous-acetone, followed by ultrasonification at 50°C for 30 min, and centrifugation at 4000 rpm for 20 min. The supernatants were collected and a subsequent 10 mL 80% aqueous-acetone extraction was applied to the pellets twice, repeating the same conditions. The supernatants were combined and adjusted to a final volume of 40 mL. Then the solvent was removed from the combined supernatants under vacuum at 40°C, and the remaining water in the concentrated extract was removed by an additional freeze-drying step, under the same conditions as applied before. Finally, the obtained freeze-dried pellets were weighed as 1 g and extracted with 4 mL 100% methanol. The extracts were stored at -20°C until further analyses.

Walnut drink and walnut sap was extracted according to the same procedure, applied for WK, WT, and WMF, with some modifications. Approximately 0.3 g freeze-dried WD and WS samples were extracted with 10 mL 80% aqueous-acetone, followed by ultrasonification at 50°C for 30 min, and centrifugation at 4000 rpm for 20 min. The supernatants were collected and a subsequent 10 mL 80% aqueous-acetone extraction was applied to the pellets, repeating the same conditions. The supernatants were combined and adjusted to a final volume of 20 mL. After the removal of the solvent fraction under vacuum at 40°C and the additional freeze-drying step, the obtained WD and WS dry pellets were extracted with 100% methanol with a sample concentration of 0.01 g dry-weight sample/mL.

3.2.5. Determination of total phenolic content (TPC)

The total phenolic contents (TPC) of extracts were determined according to a modified version of the procedure described by Singleton and Rossi (1965) using Folin-Ciocalteu reagent. The results were expressed as mg Gallic acid equivalent (GAE) per g DW sample. 200 µL of extract was added to 1.5 mL of freshly prepared Folin-Ciocalteu reagent (1:10, v/v with distilled water). The mixture was allowed to stand for 5 min and then 1.2 mL of 7.5% sodium carbonate solution was added to the mixture.

After 90 min of incubation at room temperature, absorbance was read at 765 nm using a UV-Vis spectrophotometer (Shimadzu UV-1700, Tokyo, Japan) (Figure 3.5). Samples of each extraction were analyzed in triplicate. The calibration curve is shown in Appendix, Figure A.1.

3.2.6. Determination of total flavonoid content (TFC)

The TFC was measured colorimetrically as described by Kim *et al.* (2003) at 510 nm. 1 mL of sample was mixed with 0.3 mL of 5% NaNO₂ solution at time zero. After 5 min, 0.3 mL of 10% AlCl₃ was added. At the 6th min, 2 mL of 1 M NaOH was added to the mixture. After 2.4 mL of distilled water was added and vortexed. The TF of extracts was determined by a (+)-catechin standard curve and expressed as milligrams of (+)-catechin equivalent (CE) per g of DW of sample. Triplicate samples were analyzed for each extract. The calibration curve is shown in Appendix, Figure A.2.



Figure 3.5: Shimadzu UV-1700 spectrophotometer.

3.2.7. Determination of total antioxidant capacity (TAC)

The total antioxidant capacity (TAC) of extracts were determined by five different assays (ABTS, DPPH, FRAP CUPRAC and ORAC). A standard curve was prepared using trolox as reference reagent. TAC was expressed in mg of Trolox equivalent (TE) per g of DW of sample for all assays.

The measurements were performed in triplicate for each assay. The calibration curves obtained by each assay are shown in the Appendix, Figures A.3-A.6.

The ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) assay was performed according to Miller and Rice-Evans (1997) with some slight modifications. ABTS and potassium persulfate solutions were mixed and kept at room temperature in the dark for overnight. ABTS stock solution was diluted in 50 mM potassium phosphate buffer (pH 8.0) to an absorbance of 0.90 ± 0.05 at 734 nm to prepare the ABTS-working solution. Then, 100 μ L of sample extract was mixed with 1 mL of ABTS-working solution and the absorbance was measured at 734 nm exactly 1 min after initial mixing.

The DPPH (1,1-diphenyl-2-picrylhydrazyl) assay was determined by Kumaran and Karunakaran (2006). 100 μ L of each sample extract was mixed with 2 mL of 0.1 mM DPPH in methanol. After 30 min of incubation at room temperature, the absorbance of the mixture was recorded at 517 nm against methanol.

The FRAP (Ferric Reducing Antioxidant Power) assay was carried out according to the procedure of Benzie and Strain (1996). 100 μ L of extract was mixed with a 900 μ L aliquot of freshly prepared FRAP reagent (a mixture of acetate buffer (pH 3.6), 10 mM TPTZ solution and 20 mM ferric chloride in proportions of 10:1:1 (v/v/v), respectively) and the absorbance of the reaction mixture was measured at 593 nm after 4 min.

The CUPRAC (Copper Reducing Antioxidant Capacity) assay developed by Apak *et al.* (2004) was used in this study. 100 μ L of extract was mixed with 1 mL of 10 mM CuCl_2 , 7.5 mM neocuproine and 1 M NH_4Ac (pH:7). Immediately, 1 mL of distilled water was added and after 60 min of incubation at room temperature, absorbance was read at 450 nm.

The ORAC (Oxygen radical absorbance capacity) assay was described by de Prior *et al.* (2003). The automated ORAC assay was carried out on a Biotech Synergy HT. (Figure 3.6) multilabel counter for an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The reaction was performed at 37°C as the reaction was started by thermal decomposition of AAPH in 75 mM phosphate buffer (pH 7.0). A stock solution of FL was prepared by dissolving it in phosphate buffer (PBS) (75 mM, pH 7.0), and then storing it in complete darkness under refrigeration conditions.

The working solution (78 nM) was prepared daily by dilution of 0.167 ml of the stock solution in 25 ml of PBS. The AAPH radical was prepared daily by taking 0.2069 of AAPH and making it up to 5 ml with PBS. The reference standard used was a 20 μ M trolox solution that was prepared daily in PBS from a 1 mM stock standard solution kept in the freezer at -20°C . 150 μ l of FL (78 nM) and 25 μ l of sample, blank (PBS), or standard (trolox, 20 μ M) were placed, and then 25 μ l of AAPH were added. The plate was heated to 37°C for 15 min prior to the addition of AAPH. The fluorescence was measured immediately after the addition and measurements were then taken every 5 min until the relative fluorescence intensity (FI %) was less than 5% of the value of the initial reading. The measurements were taken in triplicate.



Figure 3.6 : Biotech Sinerji HT, Plate reader

3.2.8. Simulated *in vitro* gastrointestinal (GI) digestion

The digestion procedure adapted from McDougall et al. (2005) with some modifications. The method consists of two sequential steps: an initial stomach solution for 2 h at 37°C to simulate gastric conditions, followed by a digestion with bile salts/pancreatin for 2 h at 37°C to simulate small intestinal conditions.

3.2.8.1. Gastric phase

The simulated stomach solution was prepared as follows: 7.5 mL pepsin and 0.2 g NaCl was dissolved in distilled water, and then more water was added to obtain the volume of 100 mL, which was then adjusted to pH 2.0 using 5 M HCl. Subsequently, 1 g (WD and WS-lyophilized because of its high water content) or 5 g (WK-lyophilized) of samples were mixed with 20 mL stomach solution in a 250 mL beaker and pH of the mixture was adjusted to 2.0-3.0.

The mixture was incubated for 2 h in a Memmert shaking water bath (Nürnberg, Germany) (Figure 3.7) at 37°C and 100 rpm. After 2 h, the mixture was immediately cooled down in an ice bath and then 2 mL aliquots of the post-gastric (PG) digestion were collected. PG samples stored at -20°C until further analysis. A blank was prepared with identical chemicals but without food matrix, and underwent the same conditions as the samples.



Figure 3.7 : Memmert shaking water bath.

3.2.8.2. Intestinal phase

Pancreatin (4 mg/mL) and bile salt (25 mg/mL) mixture was added, with a volume of 4.5 mL, to the remainder in the glass beaker (from gastric digestion) and segment of cellulose dialysis tubing containing sufficient NaHCO_3 to neutralize the titratable acidity was added. Again, after 2 h of incubation in Memmert shaking water bath (Nürnberg, Germany) (Figure 3.7) at 37°C and 100 rpm, the solution in the dialysis tubing was taken as the IN sample representing the material that entered the serum and the solution outside the dialysis bags were taken as the OUT sample representing material that remained in the GI tract. A blank was prepared with identical chemicals but without food matrix, and underwent the same conditions as the samples. PG, IN and OUT samples were stored at -20°C until further analysis.

Prior to analysis, samples were thawed and centrifuged (Hettich Zentrifugen Universal 32R Tuttlingen, Germany) (Figure 3.4) at 18000 rpm and the supernatants were filtered through a 0.45- μ m-membrane filter and assayed for total phenolic content (TPC), total flavonoids content (TFC) and total antioxidant capacity (TAC) using a UV-Vis spectrophotometer as described above (UV-1700, Shimadzu, Japan) (Figure 3.5). The calibration curves obtained by TP, TFC and TAC are shown in the Appendix, Figures A.7-A.12.

3.2.9. Statistical Analysis

Data, collected from three independent experiments, were reported as mean values $\pm$ standard deviations. Data were subjected to statistical analysis using SPSS software (version 21.0) for the analysis of variance (ANOVA) and for the calculation of correlation coefficients (R^2) for spectrophotometric assays. Pairwise comparisons between the treatments were done using Tukey test with a 95% confidence level. Statistical analysis tables are given in the Appendix, Table B.1, B.2, B.3.

4. RESULTS AND DISCUSSION

4.1. Moisture Content

The moisture contents of the samples are represented in Table 4.1. The walnut drink was found to have the highest moisture content value (99.95%), which was followed by WS (99.81%), WMF (6.53%), WT (6.30%), and WK (3.53%), respectively.

Table 4.1: Moisture contents of walnut samples (WK, WT, WMF, WS, WD)¹

Sample	Moisture Content (%)
Walnut Kernel	3.53 ± 0.36
Walnut Testa	6.30 ± 1.69
Walnut Male Flower	6.53 ± 0.58
Walnut Sap	99.81 ± 0.03
Walnut Drink	99.95 ± 0.01

¹Data represent average quantities ± standard deviations of 3 independent samples. Different letters represent statistically significant differences ($p < 0.05$). Abbreviations: WK, walnut kernel; WT, walnut testa; WMF, walnut male flower; WS, walnut sap; WD, walnut drink.

The moisture content of the WK sample was in agreement with those reported by Ruggeri *et al.* (1998) (3.2-4.4%) and Amaral *et al.* (2003) (3.6-4.4%).

4.2. Fatty Acid Composition

The fatty acid compositions of the walnut samples are shown in Table 4.2. Totally, fifteen fatty acid components were identified in different walnut samples. Linoleic acid was the most abundant fatty acid in WK and WT samples (54.2% and 25.5%, respectively), whereas palmitic acid was found to be the major fatty acid in WMF and WD samples (22.7% and 23.4%, respectively). The samples were also determined to be higher in oleic (ranged in between 5.2 – 24.6%) and α -linolenic acids (ranged in between 3.6 – 22.6%).

On the other hand, WMF and WD samples differentiated from the other walnut samples with their relatively higher percentages of cis-11-Eicosenoic acid (10.6%) and stearic acid (9.9%), respectively (Table 4.2).

Table 4.2 : Fatty acid compositions (%) of different walnut samples¹

Fatty acid compositions (%)	WK	WT	WMF	WS	WD
Lauric acid [C 12:0]	-	0.2 ± 0.1 b	0.01 ± 0.00 b	-	5 ± 2 a
Myristic acid [C 14:0]	0.04 ± 0.01 b	0.30 ± 0.08 b	0.10 ± 0.01 b	-	8.5 ± 0.5 a
Pentadecanoic acid [C 15:0]	0.02 ± 0.00 bc	-	0.08 ± 0.07 b	-	4.50 ± 0.00 a
Palmitic acid [C 16:0]	6.3 ± 0.2 c	14 ± 2 b	23 ± 2 a	0.47± 0.00 d	23 ± 2 a
Palmitoleic acid [C 16:1]	0.09 ± 0.00 c	5 ± 2 b	10 ± 2 a	-	8 ± 4 ab
Heptadecanoic acid [C 17:0]	-	-	-	0.14± 0.00 a	-
Stearic acid [C 18:0]	2.60 ± 0.05 b	2.8 ± 0.4 b	1.8 ± 0.2 b	0.39± 0.00 c	10 ± 2 a
Oleic acid [C 18:1]	25 ± 2 a	11 ± 2 b	5.2 ± 0.3 c	0.46± 0.00 d	15 ± 5 b
Linoleic acid [C 18:2]	54 ± 1 a	26 ± 8 b	16 ± 2 c	0.07± 0.00 e	5 ± 1 d
γ-Linolenic acid [C 18:3n6]	-	9 ± 3 a	8 ± 2 a	-	3 ± 1 b
Arachidic acid [C 20:0]	0.15 ± 0.00 c	0.66 ± 0.00 b	0.42 ± 0.07 bc	-	2.0 ± 0.5 a
Cis-11-Eicosenoic acid [C 20:1]	0.22 ± 0.03 b	1.4 ± 0.3 b	11 ± 3 a	-	-
Cis-11,14-Eicosadienoic Acid [C 20:2]	0.03 ± 0.01 b	0.31 ± 0.00 b	0.07 ± 0.03 b	-	1.1 ± 0.4 a
α-Linolenic acid [C 18:3]	11.7 ± 0.2 b	4 ± 1 c	23 ± 3 a	-	10 ± 6 b
Behenic Acid [C 22:0]	0.04 ± 0.00 d	1.45 ± 0.00 b	0.43 ± 0.09 c	-	1.7 ± 0.2 a
Lignoceric Acid [C 24:0]	0.01 ± 0.00 b	0.57 ± 0.00 b	0.65 ± 0.08 b	-	2.8 ± 0.7 a
Non-identified	-	-	-	98.3± 0.00 a	-

¹Data represent average quantities ± standard deviations of 2 independent samples. Different letters represent statistically significant differences ($p < 0.05$). Abbreviations: WK, walnut kernel; WT, walnut testa; WMF, walnut male flower; WD, walnut drink.

The walnut samples, analyzed in the present work, were all found to be higher in contents of linoleic (5.2% in WD to 54.2% in WK), oleic (5.2% in WMF to 24.6% in WK), palmitic (6.3% in WK to 23.4% in WD), and α-Linolenic acids (3.6% in WT to 22.6% in WMF) (Table 4.2). This finding was in accordance with the literature studies that reported the major fatty acids in WK as palmitic, oleic, linoleic, and linolenic acids (Li *et al.*, 2007; Pereira *et al.*, 2007; Savage *et al.*, 1999).

The detected fatty acid components were determined to be more homogenously distributed in WD sample, which resulted in higher percent values in 9 out of 15 fatty acids in WD compared to the percent values in WK, WT, and WMF. Since WT and WMF were included in the process of WD, the most of the major fatty acids of WD were similar to those in WT and/or WMF (including palmitic, oleic, α -Linolenic, and palmitoleic acids). On the other hand, stearic and myristic acids were among the abundant fatty acids in WD, which differed from WT, WMF and also WS. This could arise from the production method of WD, that could contribute to the fatty acid profile of WD sample.

4.3. Total Phenolic Content (TPC)

The TPC values of the samples are given in Table 4.3 on dry weight (DW) basis. The results indicated that the WK sample was significantly higher in TPC (44.2 mg GAE/g DW) compared to WT (23.8 mg GAE/g DW), WMF (2.7 mg GAE/g DW), WS (3 mg GAE/g DW) and WD (2.9 mg GAE/g DW) ($p < 0.05$). On the other hand, WMF, WS and WD were determined to have significantly lower TPC ($p < 0.05$) (Table 4.3).

Table 4.3 : The contents of total phenolics in different walnut samples.¹

Sample	TPC (mg GAE/g DW)
Walnut Kernel	44.2 $\pm$ 2.0 a
Walnut Testa	23.8 $\pm$ 3.6 b
Walnut Male Flower	2.7 $\pm$ 1.3 c
Walnut Sap	3.0 $\pm$ 0.9 c
Walnut Drink	2.9 $\pm$ 0.7 c

¹Data represent average quantities $\pm$ standard deviations of 3 independent samples. Different letters represent statistically significant differences ($p < 0.05$). Abbreviations: WK, walnut kernel; WT, walnut testa; WMF, walnut male flower; WS, walnut sap; WD, walnut drink.

The TPC of the defatted extract of WK analyzed in this study was determined to be 44.2 mg GAE/g DW sample. This value was approximately 3- to 4-fold higher in comparison to the TPC values reported by Arranz *et al.* (2008) and Kornsteiner *et al.* (2006) as 1071 and 1686 mg GAE per 100 g DW in defatted WK samples, respectively. In another study, Labuckas *et al.* (2008) determined the TPCs of the defatted WK samples, from 3 different varieties, as 16.3-23.7 mg GAE/g of the extract.

In addition, walnuts were found to have the highest TPC values compared to the other nuts (including almonds, brazil nuts, cashew, hazelnuts, macadamias, peanuts, pecans, pine nuts and pistachios) analyzed in these studies (Chen and Blumberg, 2008; Kornsteiner *et al.*, 2006).

It is generally difficult to compare the results obtained in different studies since they employ different sample preparation and extraction methods, different assay procedures, etc. for the determination of the antioxidant properties (Arcan *et al.*, 2009). In this study, aqueous-acetone extraction was applied using the protocol described by Alasalvar *et al.* (2006) since this method was determined to give higher results in comparison to the various different extraction solvents such as ethanol/water, methanol/water, etc. (Arcan *et al.*, 2009; Arranz *et al.*, 2008; Pellegrini *et al.*, 2006). The relatively higher results obtained in the present work described here could be linked to the better extraction of walnut phenolics. On the other hand, the spectrophotometric assays were well-documented to be influenced by various factors. For example, Folin Ciocalteu assay is reported to be affected from several interfering substances, including sugars, aromatic amines, sulfur dioxide, ascorbic acid, organic acids, and Fe(II) (Box *et al.*, 1983), and it was strongly suggested that corrections for those interfering substances should be made in order to establish a uniformly acceptable method to determine the content of total phenolics and to compare the obtained results rationally (Prior *et al.*, 2005).

4.4. Total Flavonoid Content (TFC)

The TFCs of different walnut samples were measured colorimetrically (Table 4.4) and found to be higher in WT (17.1 mg CE/g DW) in comparison to the other walnut samples; included WK (12.9 mg CE/g DW), WMF (6 mg CE/g DW), WS (6 mg CE/g DW) and WD (6 mg CE/g DW). There were no significant differences between the TFCs of WK and WT ($p>0.05$), whereas these two samples were significantly higher in their total flavonoids than WMF, WS and WD samples ($p<0.05$) which had similar TFCs ($p>0.05$) (Table 4.4).

Table 4.4. The contents of total flavonoids in different walnut samples.¹

Sample	Total Flavonoids (mg CE/g DW)
Walnut Kernel	12.9 ± 2.4 a
Walnut Testa	17.1 ± 1.9 a
Walnut Male Flower	6 ± 0.6 b
Walnut Sap	6 ± 3 b
Walnut Drink	6 ± 2 b

¹Data represent average quantities ± standard deviations of 3 independent samples. Different letters represent statistically significant differences (p<0.05). Abbreviations: WK, walnut kernel; WT, walnut testa; WMF, walnut male flower; WS, walnut sap; WD, walnut drink.

In the literature, the highest TFCs have been reported in pecans, almonds, and pistachios and hazelnuts as 34 mg/100 g, 15 mg/100g, and 12 mg/100 g, respectively (USDA, 2013). Our result obtained for TFC of walnut kernel (1245 mg/100g FW) is approximately 36-fold higher than USDA datas (34 mg/100g). These differences might be due to the variations between the analyzed cultivars, as well as different extraction methods used in different studies.

4.5. Total Antioxidant Capacity (TAC)

Total antioxidant capacity was measured using five different methods (DPPH, CUPRAC, FRAP, ABTS, ORAC) in parallel, which showed similar trends (Table 4.8). The TAC values obtained for WK (ranged between 68.7 – 977.9 mg TE/g DW, measured using 5 different in vitro tests) were significantly higher (p<0.05) in comparison to the values obtained for the other samples. On the other hand, the TAC of WD and WS sample was found to be significantly lower (p<0.05) than the TACs determined for WK and WT samples, whereas WMF did not much differ from WD and WS according to the results obtained with CUPRAC, FRAP, ORAC methods.

Arranz *et al.* (2008) reported the TAC of WK as 114.9, 153.8, and 187.2 µmol TE/g DW (28.8, 38.5, and 46.8 mg TE/g DW, respectively) determined using FRAP, ABTS, and ORAC assays, which were lower than the results obtained in this study. Additionally, Villarreal-Lozoya *et al.* (2007) reported average TAC values for kernels of 7 different walnut varieties as 97 and 146 mg TE/g sample, measured using DPPH and ORAC methods, respectively. Our results for DPPH method were similar to the findings of the latter study, whereas the ORAC values were higher in our study. These

differences might be a result of the use of different species as well as different extraction methods in different studies.

It has been well reported that using a single method is not sufficient for the evaluation of the antioxidant activity. Even the methods based on the same principle, such as ABTS and DPPH, they can show several important differences in their response to antioxidants. Therefore, it is highly recommended to apply several test procedures to evaluate antioxidant activities properly (Contreras-Calderon *et al.*, 2011).

Table 4.5: The total antioxidant capacities of different walnut samples¹.

Sample	Total Antioxidant Capacity (mg TE/g dw)				
	DPPH	CUPRAC	FRAP	ABTS	ORAC
WK	101.9 ± 7.0 a	351.1 ± 46.9 a	68.7 ± 11.1 a	113.3 ± 2.6 a	977.9 ± 117.5 a
WT	45.3 ± 10.8 b	167.5 ± 31.5 b	25.0 ± 5.4 b	52.1 ± 1.2 b	611.7 ± 142.6 b
WMF	42.3 ± 5.4 b	54.8 ± 9.8 c	3.40 ± 0.04 c	22.7 ± 6.3 c	411.2 ± 24.6 c
WS	5 ± 2 c	18.4 ± 0.4 c	2.8 ± 0.5 c	9 ± 1 e	195 ± 10 d
WD	6 ± 1 c	44 ± 15 c	3.4 ± 0.2 c	14 ± 5 d	247 ± 47 cd

¹Data represent average quantities ± standard deviations of 3 independent samples. Different letters in columns represent statistically significant differences ($p < 0.05$). Abbreviations: WK, walnut kernel; WT, walnut testa; WMF, walnut male flower; WS, walnut sap; WD, walnut drink.

4.6. Correlation between Spectrophotometric Assays

The linear correlation coefficients (R^2), calculated between the results obtained for TPC, TFC, and TAC assays, were given in Table 4.6. The correlations between CUPRAC-TPC ($R^2=0.976$) and CUPRAC-TFC ($R^2=0.705$) were higher in comparison to the values obtained for the correlations of the other TAC assays with TPC/TFC. The highest correlation in between the 5 different TAC assay results was observed for CUPRAC and ABTS ($R^2=0.977$), while the lowest correlation was in between DPPH and FRAP ($R^2=0.827$). The correlation coefficients (R^2) calculated between the results of the applied spectrophotometric methods indicated the highest correlation between CUPRAC and ABTS assay results ($R^2=0.977$). In addition, all the other assays performed (except for DPPH) gave their highest correlations with CUPRAC assay. On the other hand, TFC results had the lowest correlations with the results of the other assays.

The good linear relationships between CUPRAC assay and the other spectrophotometric assays, especially TPC, could be linked to this method's ability to react with a variety of antioxidant compounds, regardless of chemical type or hydrophilicity, making it the most consistent method of total antioxidant measurement in relation to Folin reagent-responsive TPC (Apak *et al.*, 2006). On the other hand, the aluminium chloride (AlCl₃) colorimetric test, used to determine the TF, has been reported to be sensitive only for flavonoid groups that possess the characteristic chelating functional groups for Al binding (i.e. flavones and flavonols), which leads to the underestimation of the TFC by using this aluminium chloride method (Chang *et al.*, 2002). This could explain the relatively lower correlations observed between TFC assay and the other assays performed.

Table 4.6: Correlation coefficients (R^2 values) between TPC, TFC, and TAC assay results.

	TPC ¹	TFC ²	DPPH	CUPRAC	FRAP	ABTS	ORAC
TPC	1						
TFC	0.736**	1					
DPPH	0.893**	0.536**	1				
CUPRAC	0.976**	0.705**	0.914**	1			
FRAP	0.922**	0.671**	0.827**	0.961**	1		
ABTS	0.965**	0.614**	0.939**	0.977**	0.923**	1	
ORAC	0.919**	0.674**	0.929**	0.955**	0.915**	0.951**	1

** Significant difference between mean values of heartnuts and walnuts at $P < 0.01$. Abbreviations: TPC¹ (Total Phenolic Content); TFC² (Total Flavonoid Content)

4.7. *In vitro* Simulated Gastrointestinal (GI) Digestion

The changes in the TPC, TFC, and TAC of WK and WD samples during gastric and intestinal digestion were determined using spectrophotometric methods. In addition, the percent recoveries for TPC, TFC, and TAC were calculated by dividing the values obtained for the IN fraction to the initial values (Recovery (%) = (IN/Initial)x100) (see Table 4.7 for TPC and TFC, and see Table 4.8 for TAC). The percent recoveries obtained for WD were all found to be higher than those calculated for WK, in all assays (8-fold and 3-fold higher for TPC and TFC, respectively; and 5- to 23-fold higher for TAC, measured using 5 different tests) (Figure 4.1).

Table 4.7: Changes in TPC and TFC of walnut kernel and walnut drink samples during *in vitro* GI digestion

	TPC (mg GAE / g dw sample)		TF (mg CE / g dw sample)	
	WK	WD	WK	WD
Initial	44.2 ± 2.0a	2.9 ± 0.7c	12.9 ± 2.4a	5.8 ± 1.6b
PG	3.61 ± 0.08c	2.6 ± 0.89c	0.66 ± 0.02c	2.0 ± 1.4c
IN	2.15 ± 0.02c	1.2 ± 0.5c	0.12 ± 0.01c	0.17 ± 0.08c
OUT	8.5 ± 0.2b	2.6 ± 1.1c	2.5 ± 0.4c	0.5 ± 0.2c
Recovery (%)	4.9	39.9	0.9	2.9

* The data presented in this table consist of average quantities ± SD of three independent samples. Different letters in the columns within each group represent statistically significant differences ($p < 0.05$). The terms represent; Initial, as initially determined from sample matrix using 80% aqueous-acetone; PG, compounds remaining after gastric digestion; IN, dialyzed fraction after intestinal digestion; OUT, non-dialyzed fraction after intestinal digestion. Abbreviations used for samples are expressed in Table 4.2.

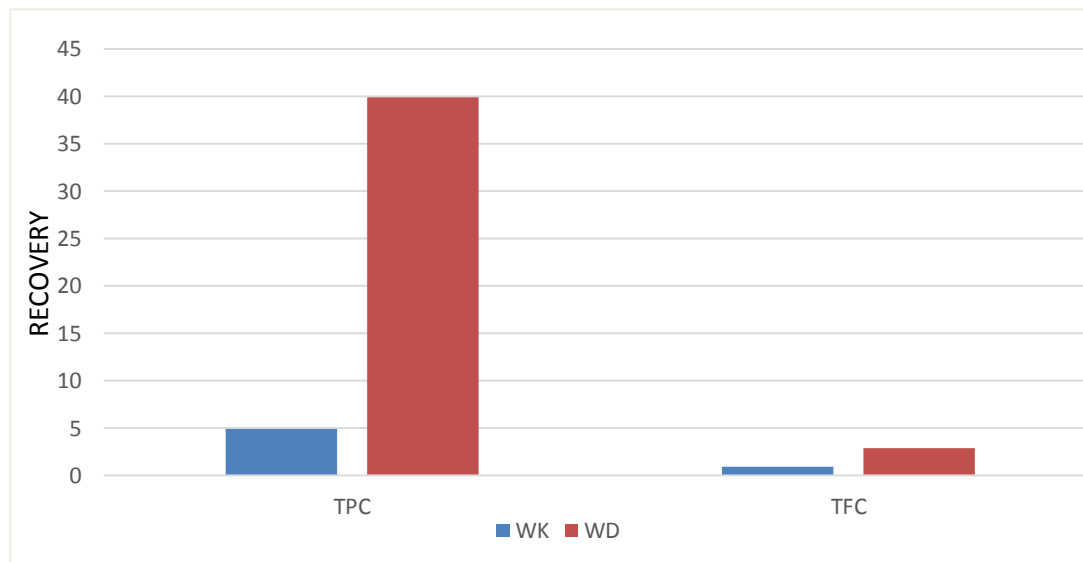


Figure 4.1: Recovery of total phenolics (TPC), total flavonoids (TFC) of walnut kernel and walnut drink after *in vitro* intestinal digestion, dialyzed fractions of the intestinal digestion (IN), expressed as percentage.

An *in vitro* simulation of GI digestion and absorption was applied to WK and WD samples. The calculated percent recoveries for TPC, TFC, and TAC values, in regarding to the IN fractions, were all found to be higher in WD sample (39.9% for TPC, 2.9% for TFC, and ranged between 6.5 – 27.1% for 4 different TAC tests applied) compared to those obtained for WK sample (4.9% for TPC, 0.9% for TFC, and ranged between 0.4 – 6.0% for 4 different TAC tests applied) (Table 4.7 and Table 4.8) (Figure 4.1 and 4.2). Although WD sample had significantly lower initial contents of total phenolics and total flavonoids, as well as TAC than those determined for WK ($p < 0.05$) (Table 4.3; Table 4.4; Table 4.5), the percent recoveries calculated for all these parameters were higher in WD than in WK. This indicates an enhanced serum availability of antioxidants in WD compared to WK. However, it should also be taken into consideration that although the percent recoveries were lower in WK, the contents observed in the serum available (IN) fractions of WK sample were not much lower or even higher (TPC, CUPRAC, ABTS, and FRAP) than those determined for WD sample, for the ingestion of the same amounts of samples.

In vitro GI digestion protocol applied to WK was also found to result in significantly lower TPC, TFC, and TAC values in the samples representing the gastric phase (PG) and the intestinal phase (IN and OUT), in comparison to those measured in initial acetone extractions of WK ($p < 0.05$) (Table 4.7 and Table 4.8) (Figure 4.1 and 4.2) which could indicate an incomplete release or degradation. On the other hand, there were no significant changes in these analyzed parameters of the same fractions of WD ($p > 0.05$).

In the applied *in vitro* GI digestion procedure, OUT fractions of WK sample were determined to have higher values than those obtained for PG fractions, in all assays. This might be due to the increase in the extractability of phenolics and flavonoids by the effect of their release as a result of enzyme application and incubation (2 hours) during the intestinal phase (Bouayed *et al.*, 2011). In addition, TAC of OUT fractions were also higher than initial and PG values. It is well known from the literature that the radical scavenger activity of polyphenols is strongly pH-dependent and higher pH values are reported to significantly increase this capacity. This increase in the radical scavenger activity has been attributed to the deprotonation of the hydroxyl moieties present on the aromatic rings of the phenolic compounds (Tagliazucchi *et al.*, 2010).

In order to understand the bioactivity of polyphenols, *in vivo* experiments in humans are required. However, *in vitro* methods have also been proven to be useful to determine their stability under GI conditions. Indeed, despite limitations, such as constituting only a static model of digestion, the evaluation of bioavailability using *in vitro* models can be well correlated with results from human studies and animal models (Bouayed *et al.*, 2011). *In vitro* digestion and dialysis methods for simulating the GI digestion are being extensively used since they are rapid, safe, and do not have the same ethical restrictions as *in vivo* methods (Liang *et al.*, 2012).

Table 4.8: Changes in TAC values of walnut kernel and walnut drink samples during *in vitro* GI digestion*

	DPPH (mg TE/g dw sample)		CUPRAC (mg TE/g dw sample)		FRAP (mg TE/g dw sample)		ABTS (mg TE/g dw sample)	
	WK	WD	WK	WD	WK	WD	WK	WD
Initial	101.9 ± 7.0a	6.0 ± 1.0bc	351.1 ± 46.9a	44.1 ± 15.1b	68.7 ± 11.1a	3.4 ± 0.2b	113.3 ± 2.6a	14.0 ± 5.1c
PG	8.08 ± 0.04bc	2.8 ± 1.6c	17.48 ± 0.4b	11.1 ± 5.3b	6.82 ± 0.14b	2.7 ± 1.4b	11.95±0.28cd	8.8 ± 3.7cd
IN	0.36 ± 0.01c	0.5 ± 0.3c	3.7 ± 0.2b	2.9 ± 1.2b	0.57 ± 0.04b	0.4 ± 0.1b	6.8 ± 0.6cd	3.8 ± 1.4d
OUT	12.0 ± 1.9b	0.7 ± 0.3c	17.2 ± 0.4b	7.7 ± 3.0b	7.4 ± 0.2b	0.7 ± 0.3b	31.0 ± 1.4b	8.8 ± 2.9cd
Recovery(%)	0.4	8.0	1.1	6.5	0.8	11.5	6.0	27.1

*The data presented in this table consist of average quantities ± SD of three independent samples. Different letters in the columns represent statistically significant differences ($p < 0.05$). The terms represent; Initial, as initially determined from sample matrix using 80% aqueous-acetone; PG, compounds remaining after gastric digestion; IN, dialyzed fraction after intestinal digestion; OUT, non-dialyzed fraction after intestinal digestion. Abbreviations used for samples are expressed in Table 4.2.

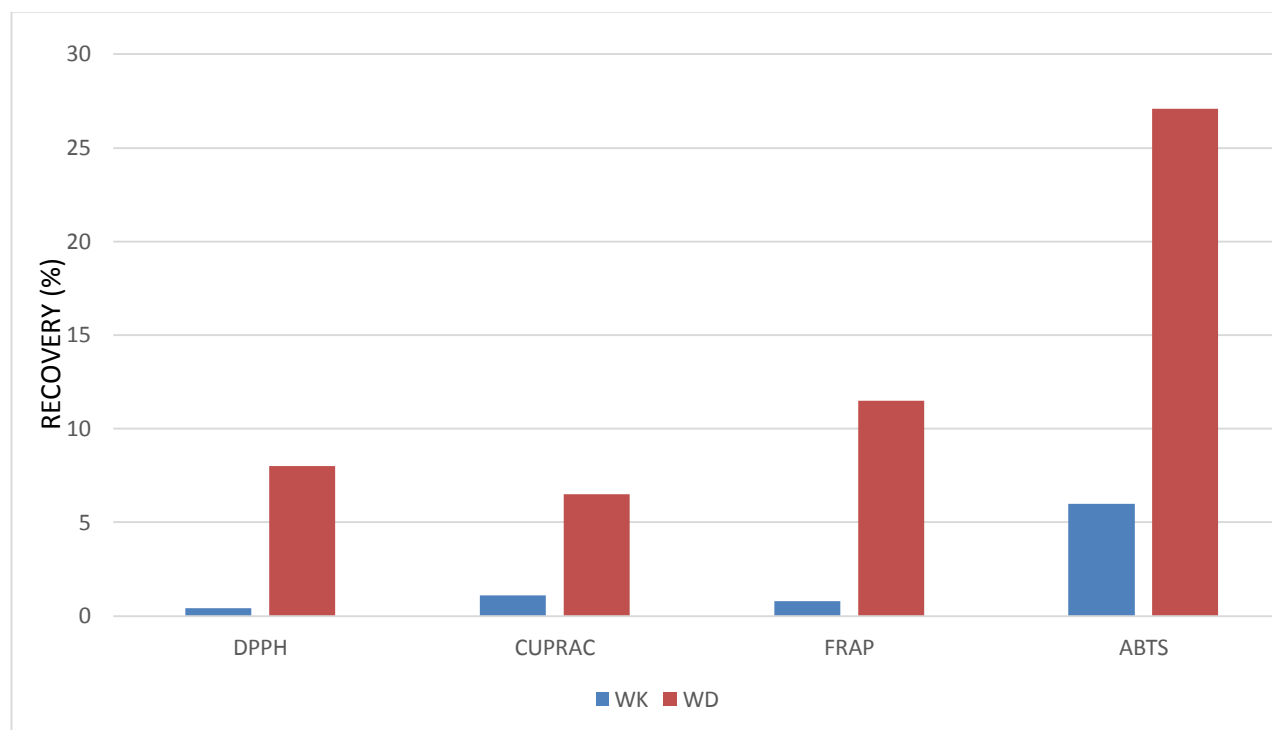


Figure 4.2: Recovery of total antioxidant capacities (TAC) of walnut kernel and walnut drink after *in vitro* intestinal digestion, dialyzed fractions of the intestinal digestion (IN), expressed as percent

5. CONCLUSIONS AND RECOMMENDATIONS

The different parts of walnut, including WK, WT, WMF, and WS, as well as the WD prepared using WT, WMF, and WS, were determined to contain linoleic, oleic, palmitic, and α -Linolenic acids as the major fatty acids. Differently, stearic and myristic acids were also found to be among the abundant fatty acids in WD. The antioxidant potential of WK was found to be the highest, followed by WT, WMF, WS, and WD, respectively. In this respect, traditional recipe of WD could be suggested to be modified in order to enhance the contents of phenolic constituents, and so the total antioxidant capacity of the final product. Since it was determined with the present work that WT had higher antioxidant capacity than WS and WMF, the amount of WT in WD could be increased, so as to observe an increased antioxidant capacity in WD sample.

In vitro simulation of GI digestion model applied to WK and WD indicated higher percent recoveries of total phenolics, total flavonoids and total antioxidant capacity in WD in comparison to the values obtained for WK, which could be linked to an improved serum availability of these compounds in WD.

Although the *in vitro* bioavailability studies have been well-documented to show good correlations with the *in vivo* human and animal studies, it is still necessary to perform and validate these results under the complex *in vivo* conditions of the human gut. Therefore, in addition to *in vitro* studies, clinical trials investigating the bioavailability of those compounds would provide valuable data for elucidating the effect of walnut kernel and walnut drink on human health.

This research study can contribute to the existing literature with its major findings on the antioxidant properties of a variety of walnut samples, including different walnut parts and an additional walnut drink. Although the results obtained with the model of simulated *in vitro* GI digestion cannot directly predict the human *in vivo* conditions, still this model is helpful for investigating the bioavailability of polyphenols. It would be interesting to perform further *in vivo* studies, in order to be able to verify the present results.

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APPENDIX

APPENDIX A. CALIBRATION CURVES

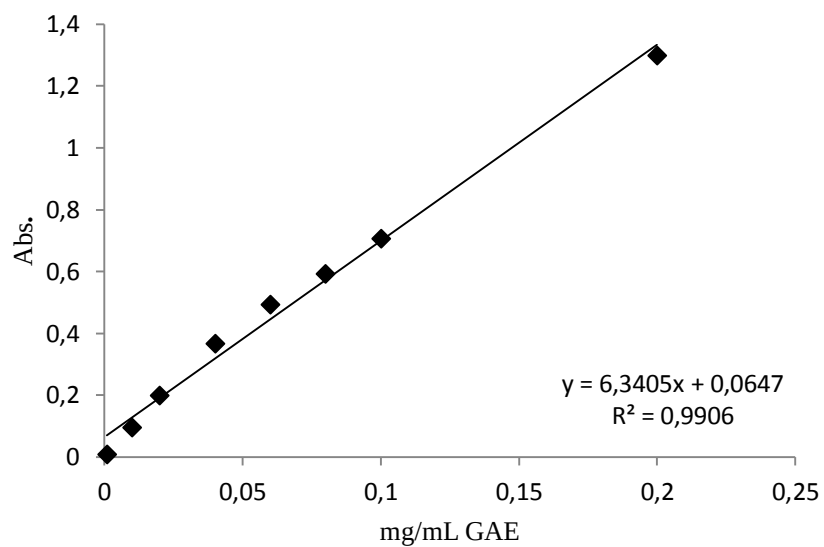


Figure A.1 : Calibration curve for total phenolics in 100% methanol

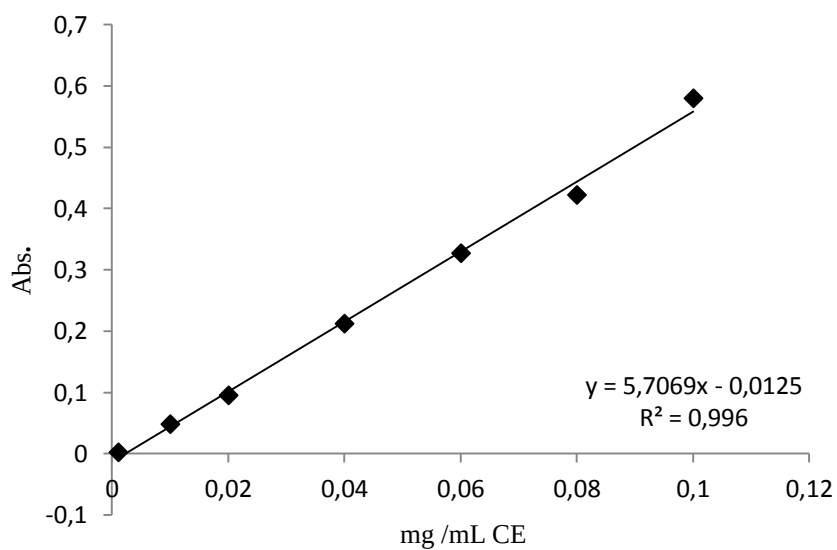


Figure A.2 : Calibration curve fot total flavonoids in 100% methanol

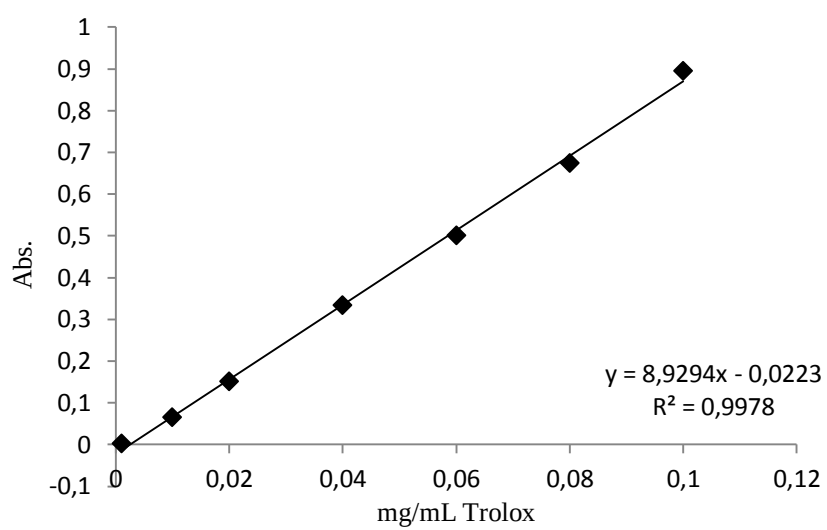


Figure A.3 : Calibration curve for ABTS assay in 100% methanol

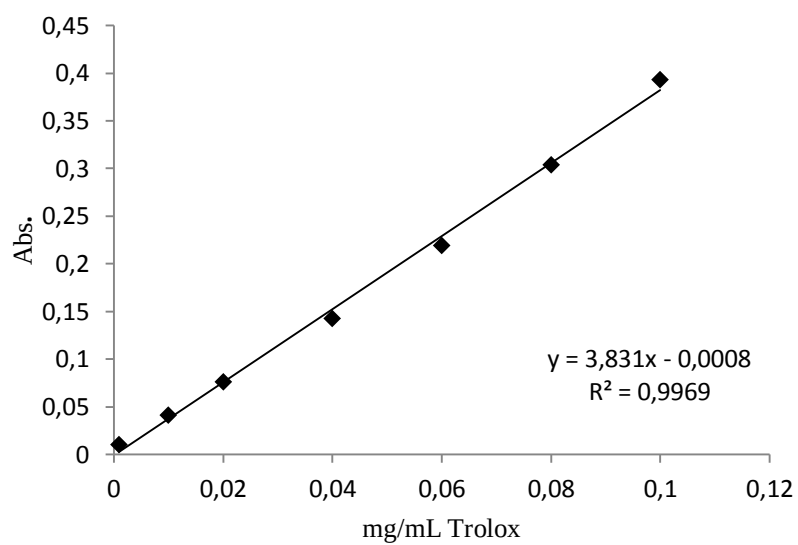


Figure A.4 : Calibration curve for DPPH assay in 100% methanol

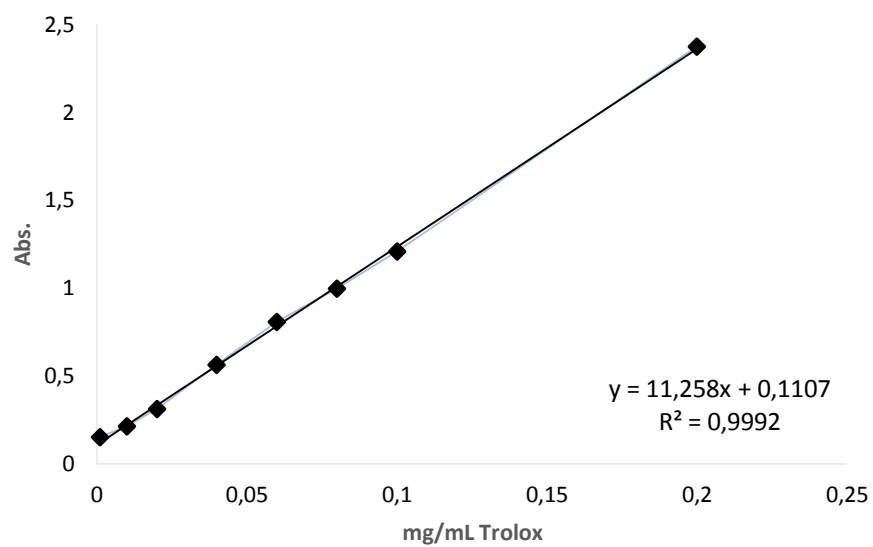


Figure A.5 : Calibration curve for FRAP assay in 100% methano

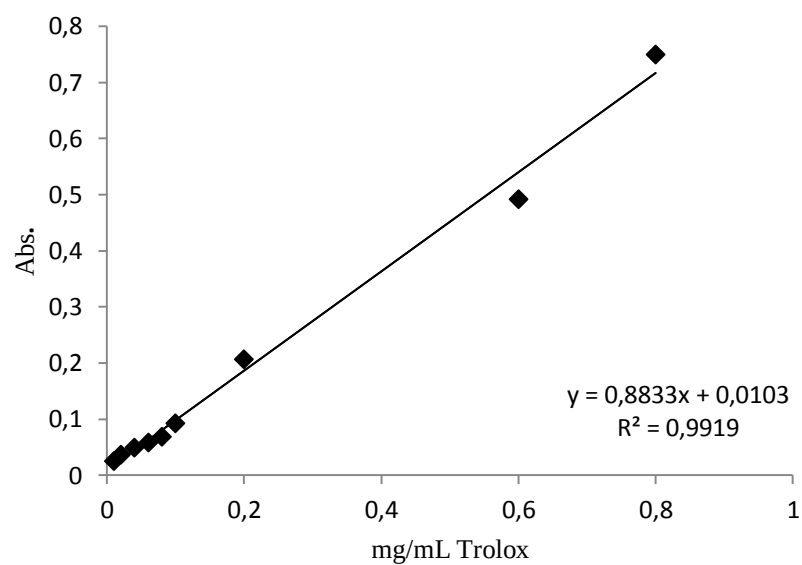


Figure A.6 : Calibration curve for CUPRAC assay in 100% methanol

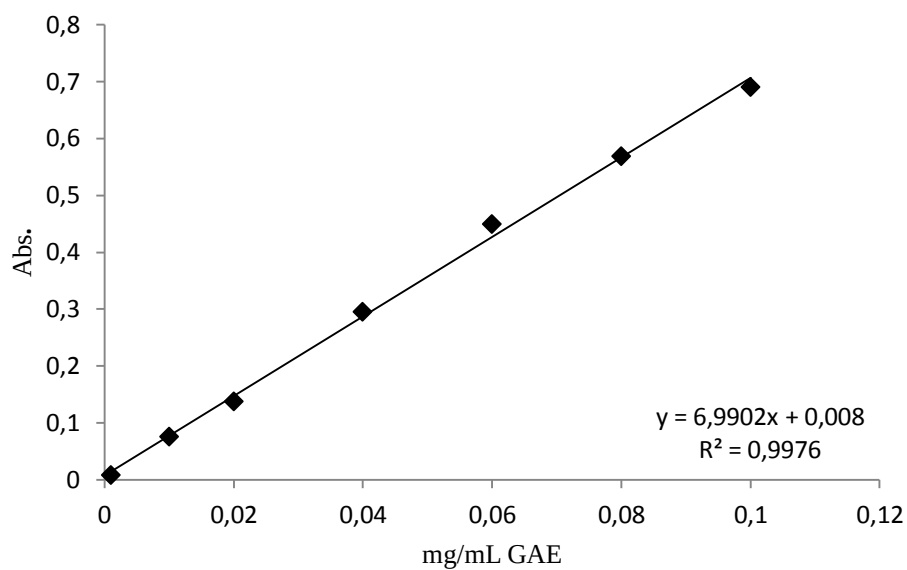


Figure A.7 : Calibration curve for total phenolic assay in 100% methanol (for IN, OUT, PG fraction)

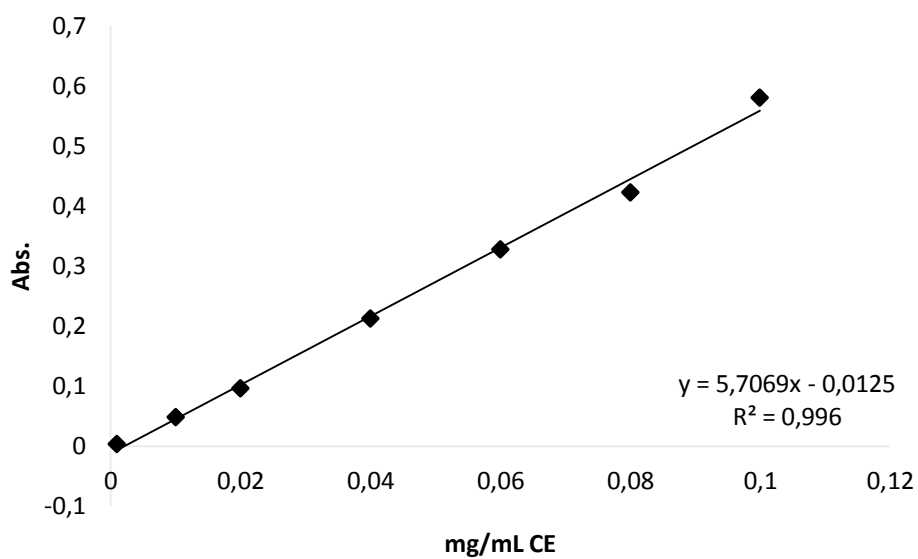


Figure A.8 : Calibration curve for total flavonoid assay in 100% methanol (for IN, OUT, PG fraction)

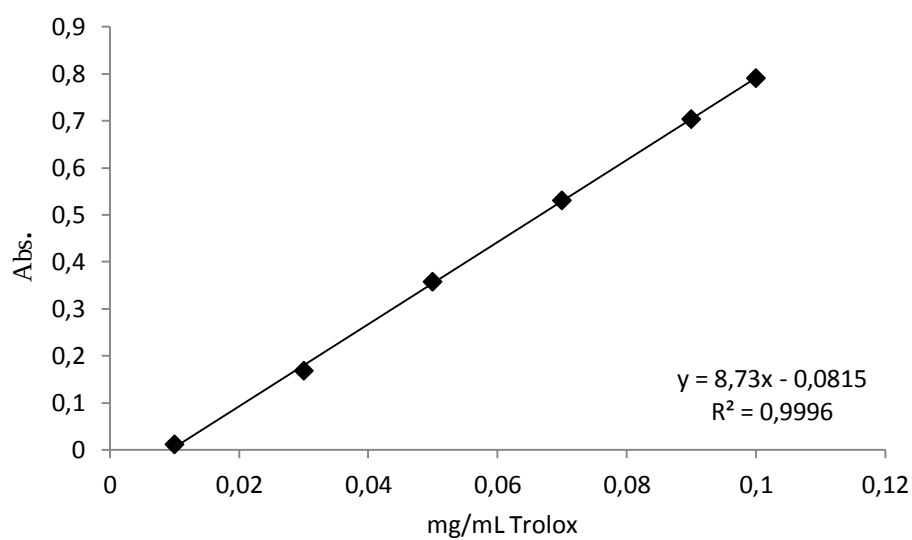


Figure A.9 : Calibration curve for ABTS assay in 100% methanol (for IN, OUT, PG fraction)

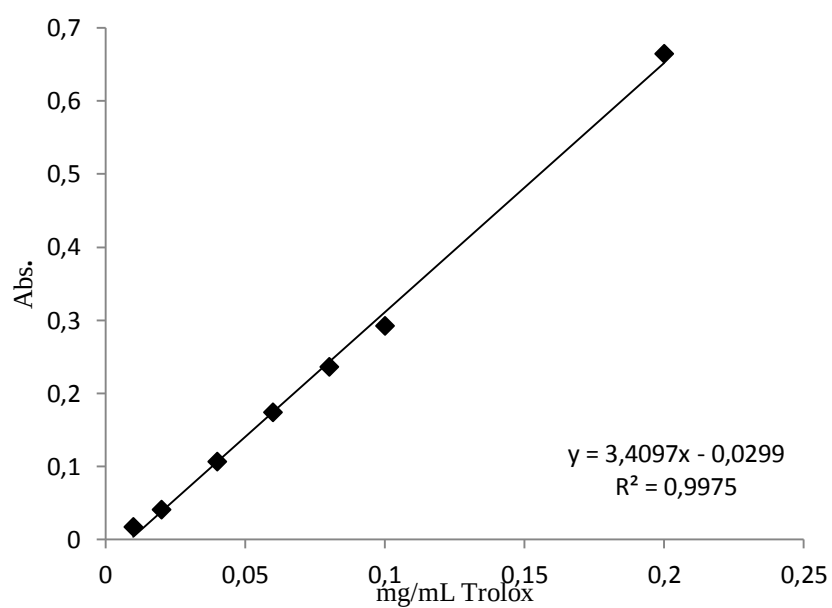


Figure A.10 : Calibration curve for DPPH assay in 100% methanol (for IN, OUT, PG fraction)

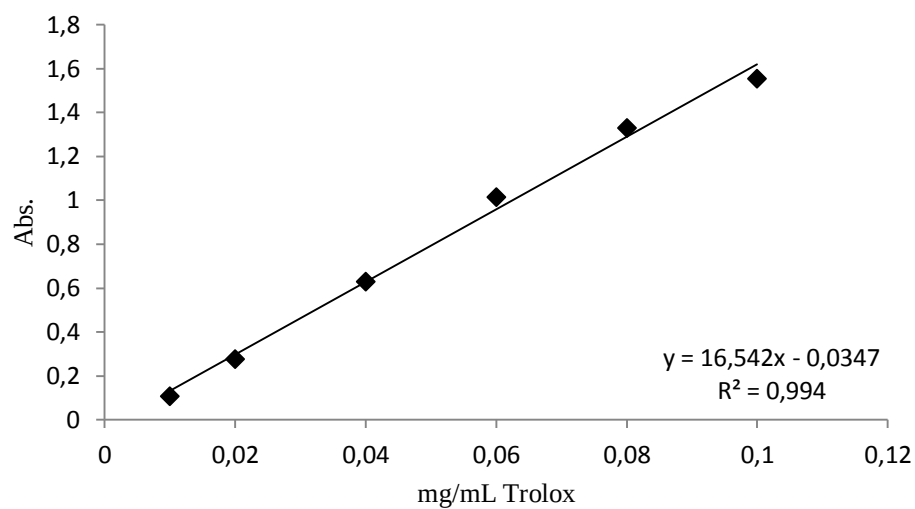


Figure A.11 : Calibration curve for FRAP assay in 100% methanol (for IN, OUT, PG fraction)

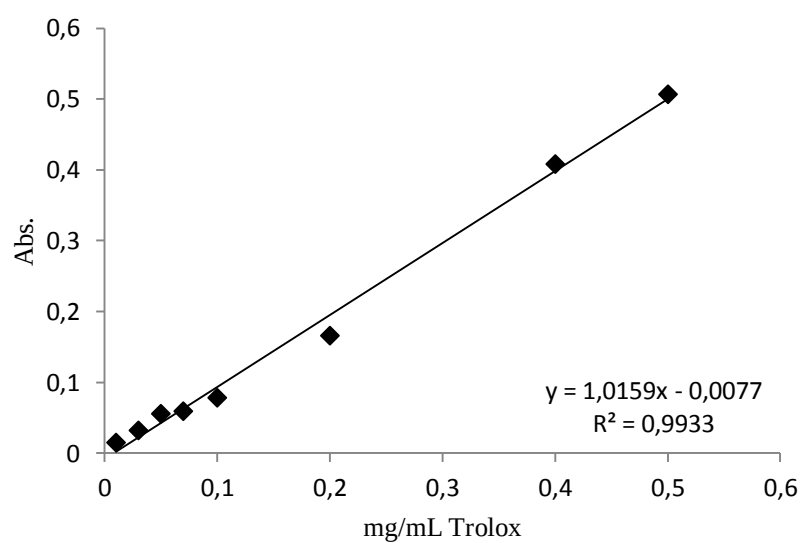


Figure A.12 : Calibration curve for CUPRAC assay in 100% methanol (for IN, OUT, PG fraction)

APPENDIX B. ANOVA TABLES

Table B.1: Statistical analysis fatty acid composition results of walnut samples.

		Sum of Squares	df	Mean Square	F	Sig.
C12	Between Groups	62,094	3	20,698	63,266	,000
	Within Groups	2,617	8	,327		
	Total	64,711	11			
C14	Between Groups	157,778	3	52,593	1821,917	,000
	Within Groups	,231	8	,029		
	Total	158,009	11			
C15	Between Groups	44,967	3	14,989	23667,066	,000
	Within Groups	,005	8	,001		
	Total	44,972	11			
C161	Between Groups	166,542	3	55,514	21,306	,000
	Within Groups	20,845	8	2,606		
	Total	187,387	11			
C16	Between Groups	589,244	3	196,415	110,086	,000
	Within Groups	14,274	8	1,784		
	Total	603,517	11			
C18	Between Groups	129,771	3	43,257	121,027	,000
	Within Groups	2,859	8	,357		
	Total	132,630	11			
C182	Between Groups	3951,084	3	1317,028	139,664	,000
	Within Groups	75,440	8	9,430		
	Total	4026,524	11			
C183n6	Between Groups	160,503	3	53,501	36,436	,000
	Within Groups	11,747	8	1,468		
	Total	172,250	11			
C181	Between Groups	600,382	3	200,127	56,742	,000
	Within Groups	28,216	8	3,527		
	Total	628,597	11			
C20	Between Groups	6,151	3	2,050	55,826	,000
	Within Groups	,294	8	,037		
	Total	6,444	11			
C20 1	Between Groups	229,203	3	76,401	96,051	,000
	Within Groups	6,363	8	,795		
	Total	235,567	11			

Table B.1: Statistical analysis fatty acid composition results of walnut samples.
(continuing).

C202	Between Groups	2,091	3	,697	28,879	,000
	Within Groups	,193	8	,024		
	Total	2,284	11			
C183	Between Groups	564,871	3	188,290	39,120	,000
	Within Groups	38,505	8	4,813		
	Total	603,375	11			
C22	Between Groups	5,574	3	1,858	374,078	,000
	Within Groups	,040	8	,005		
	Total	5,613	11			
C24	Between Groups	13,368	3	4,456	65,049	,000
	Within Groups	,548	8	,069		
	Total	13,916	11			

Table B.2: Statistical analysis spectrophotometric results of walnut samples samples

		Sum of Squares	df	Mean Square	F	Sig.
TP	Between Groups	8302,806	4	2075,702	157,082	,000
	Within Groups	330,352	25	13,214		
	Total	8633,159	29			
TF	Between Groups	653,727	4	163,432	25,394	,000
	Within Groups	160,894	25	6,436		
	Total	814,621	29			
DPPH	Between Groups	37162,232	4	9290,558	146,549	,000
	Within Groups	1584,886	25	63,395		
	Total	38747,118	29			
CUPRAC	Between Groups	467692,250	4	116923,062	121,445	,000
	Within Groups	24069,231	25	962,769		
	Total	491761,480	29			
FRAP	Between Groups	19208,255	4	4802,064	37,484	,000
	Within Groups	3202,765	25	128,111		
	Total	22411,021	29			
ABTS	Between Groups	43845,612	4	10961,403	1154,330	,000
	Within Groups	237,398	25	9,496		
	Total	44083,010	29			
ORAC	Between Groups	2360818,775	4	590204,694	71,455	,000
	Within Groups	206495,264	25	8259,811		
	Total	2567314,040	29			

Table B.3: Statistical analysis *in vitro* digestion analyses results of walnut samples

		Sum of Squares	df	Mean Square	F	Sig.
TP	Between Groups	4540,610	7	648,659	765,831	,000
	Within Groups	13,552	16	,847		
	Total	4554,162	23			
TF	Between Groups	406,183	7	58,026	51,184	,000
	Within Groups	18,139	16	1,134		
	Total	424,322	23			
DPPH	Between Groups	25515,085	7	3645,012	516,747	,000
	Within Groups	112,860	16	7,054		
	Total	25627,945	23			
CUPRAC	Between Groups	304059,784	7	43437,112	132,645	,000
	Within Groups	5239,485	16	327,468		
	Total	309299,269	23			
ABTS	Between Groups	28396,208	7	4056,601	540,536	,000
	Within Groups	120,076	16	7,505		
	Total	28516,284	23			
FRAP	Between Groups	11454,489	7	1636,356	105,283	,000
	Within Groups	248,679	16	15,542		
	Total	11703,168	23			

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