ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL

EFFECTS OF NOVEL FOOD PROCESSING TECHNIQUES ON BIOACCESSIBILITY AND TRANSEPITHELIAL TRANSPORT OF CRANBERRYBUSH POLYPHENOLS

Ph.D. THESIS

Gülay ÖZKAN

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

YENİ GIDA İŞLEME TEKNİKLERİNİN GİLABURUDA BULUNAN POLİFENOLLERİN BİYOERİŞİLEBİLİRLİĞİ VE BAĞIRSAK TAŞINIMLARI ÜZERİNDEKİ ETKİLERİ

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AĞUSTOS 2021

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To my family,

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Gülay ÖZKAN
(Food Engineer, M.Sc.)

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ABBREVIATIONS

5-DN : 5-demethylnobiletin

ABTS : 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt)

Al : Aluminum

ANOVA : Analysis of variance

Ba : Barium

Ca : Calcium

Caco-2 : Human colorectal adenocarcinoma cell line

CO_2 : Carbon dioxide

CUPRAC : Cupric ion reducing antioxidant capacity

CYP : Cytochrome P450 monooxygenases

DAD : Diode Array Detector

DE : Dextrose equivalent

DMEM : Dulbecco's Modified Eagle's Medium

DMPD : N, N-dimethyl-p-phenylenediamine dihydrochloride

DMSO : Dimethyl sulfoxide

DPPH : 2,2-diphenyl-1-picrylhydrazyl

DSC : Differential scanning calorimeter

DSMZ : German Collection of Microorganisms and Cell Cultures

EC : Ethyl cellulose

EDTA : Ethylenediaminetetraacetic acid

EE : Entrapment efficiency

EGCG : (−)-epigallocatechin-3-gallate

EPR : Electron Paramagnetic Resonance

ESI : Electrospray ionization

ET : Electron Transfer

EtOH : Ethanol

FA : Folic acid

FBS : Fetal Bovine Serum

FC : Flavonoid compound

FDA : Food and Drug Administration

FESEM : Field Emission Scanning Electron Microscope
FIR : Far infrared
FRAP : Ferric ion Reducing Antioxidant Power
GAE : Gallic Acid Equivalent
GI : Gastrointestinal digestion
HAT : Hydrogen atom transfer
HBSS : Hank's Balanced Salt Solution
HCA : (-)-hydroxycitric acid
HDPAF : High Degree of Polymerisation Agave Fructans
HepG2 : Human liver carcinoma cell line
HIPE : High Internal Phase Emulsions
HMF : Hydroxymethylfurfural
HPAF : High Performance Agave Fructans
HPLC : High Performance Liquid Chromatography
HPP : High Pressure Processing
HT-29 : Human colorectal adenocarcinoma cell line
HTST : High-Temperature Short Time
JA : Juice+almond milk blend
JM : Juice+bovine milk blend
JW : Juice+water blend
LC : Liquid Chromatography
LDH : Lactate Dehydrogenase Leakage
LDL : Low-density lipoprotein
LDPE : Low density polyethhylene pouches
LPIC : Lipid peroxidation
MCT : Medium chain triglycerides
MS : Mass Spectrometry
NEAA : Non-essential amino acids
O/W : Oil-in-water emulsion
ORAC : Oxygen Radical Absorbance Capacity
PAH : Polycyclic aromatic hydrocarbones
PBS : Phosphate Buffered Saline
Pc : Critical point of pressure
PEF : Pulsed Electric Field
PGSS : Particles from Gas Saturated Solutions

PID : Proportional–integral–derivative
PMF : Polymethoxylated flavonoids
PPO : Polyphenol oxidase activity
PSD : Particle size distribution
PVP : Polyvinylpyrrolidone
Q : Quercetin
QE : Quercetin Equivalent
Q-PVP : Quercetin-polyvinylpyrrolidone
QTOF : Quadrupole Time of Flight
R : Rutin
RA : Residual activity
RESS : Rapid Expansion of Supercritical Solutions
RH : Relative humidity
ROS : Reactive Oxygen Species
R-PVP : Rutin-polyvinylpyrrolidone
SAS : Supercritical Anti Solvent precipitation
SC- CO_2 : Supercritical carbon dioxide
SGF : Simulated gastric fluid
SIF : Simulated intestinal fluid
SRB : Sulforhodamine B
TAC : Total Anthocyanin Content
Tc : Critical point of temperature
TCA : Trichloroacatate
TE : Trolox® Equivalent
TEAC : Trolox® Equivalent Antioxidant Capacity
TEER : Transepithelial electrical resistance
TFC : Total Flavonoid Content
TIM-1 : *In vitro* dynamic digestion model
TPC : Total Phenolic Content
Trolox® : 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
TSSC : Total soluble solids content
UHT : Ultrahigh-Temperature
UPLC : Ultra High Performance Liquid Chromatography
VCEAC : Vitamin C equivalent antioxidant capacity
W/O/W : Water-in-oil-in-water emulsion

XRD **:** X-ray diffraction

α-TOC **:** α-tocopherol

β-CD **:** β-cyclodextrin

SYMBOLS

B	**:** magnetic flux density
C	**:** capacitance
Cp	**:** specific heat capacity
D	**:** electric flux density
E	**:** electric field
H	**:** magnetic field
Hz	**:** Hertz
J	**:** Joule
J	**:** total current density
k	**:** thermal conductivity
l	**:** pathlength
m	**:** milli (10^{-3})
n	**:** nano (10^{-9})
n	**:** number of pulses
p	**:** probability (statistical analysis)
Pa	**:** Pascal
Pr	**:** function of dielectric constant
R^2	**:** correlation coefficient (statistical analysis)
U	**:** voltage
V	**:** Volt
W	**:** Watt
Wspec	**:** specific energy intake
β	**:** beta (in β-carotene)
δ	**:** dielectric loss angle
ε	**:** molar extinction coefficient
ε′	**:** dielectric constant
ε″	**:** dielectric loss factor
θ	**:** angle of incidence
μ	**:** micro (10^{-6})
ρ	**:** density

ρ_e : total charge density

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EFFECTS OF NOVEL FOOD PROCESSING TECHNIQUES ON BIOACCESSIBILITY AND TRANSEPITHELIAL TRANSPORT OF CRANBERRYBUSH POLYPHENOLS

SUMMARY

Phenolic compounds, which are present in a wide variety of foods such as fruits, vegetables, flowers and leaf of plants, exhibit a variety of beneficial effects including antimicrobial, antioxidant, antidiabetic, diuretic, hypoglycemic, cough reliever, antiinflammatory and antiviral activities as well as prevention of cardiovascular, pancreas, liver and kidney diseases. However, most of the polyphenols have poor water solubility, chemical instability in gastrointestinal tract and, thus, a reduced bioavailability. Therefore, a wide variety of attempts have been investigated to improve the solubility, stability, bioaccessibility and bioavailability of phenolic compounds.

Considering the above, a research framework to study the effects of novel processing techniques on the antioxidant capacity, bioaccessibility and bioavailability of cranberrybush polyphenols has been developed. The objectives of this Ph.D. thesis were (i) to determine the effects of novel non-thermal food processing on cranberrybush polyphenols and vitamin C; (ii) to investigate the effects of non-thermal food processing and food matrix on bioaccessibility and transepithelial transportation of bioactive compounds, in particular chlorogenic acid, from cranberrybush (*Viburnum opulus*) using combined *in vitro* gastrointestinal digestion/Caco-2 cell culture model; (iii) to obtain an effective Supercritical Anti-Solvent (SAS) coprecipitation of quercetin or rutin with polyvinylpyrrolidone (PVP), enhancing the dissolution rate, and, therefore, improving the bioavailability of these natural antioxidant compounds; (iv) to determine the effects of SAS processing and food models on the antioxidant capacity, bioaccessibility and transport dynamics of flavonol-loaded microparticles by using combined *in vitro* gastrointestinal digestion/Caco-2 cell culture model.

To achieve these goals, four different experiments (*Chapters 3-6*) were conducted. Firstly, effects of high pressure processing (HPP) and pulsed electric field (PEF) treatments on physicochemical properties, bioactive compounds, antioxidant capacities and polyphenol oxidase activities of cranberrybush purée samples were evaluated (*Chapter 3*). Following that, non-thermal treated cranberrybush purée samples as well as cranberrybush juice/water, bovine or almond milk blends were subjected to combined *in vitro* gastrointestinal digestion/Caco-2 cell culture (*Chapter 4*). In line with the outcomes of previous chapter, in order to increase the bioavailability of some phenolic compounds that could not be absorbed across the gut epithelium after transport experiments with cranberrybush samples, the micronization of two flavonoids, quercetin and rutin, and their coprecipitation with PVP were studied by using SAS processing to increase their solubility and enhance their stability during gastrointestinal tract (*Chapter 5*). Finally, SAS-processed flavonoids in different

simulated food models were exposed to combined *in vitro* gastrointestinal digestion/Caco-2 cell culture in order to investigate their transport dynamics (*Chapter 6*).

In *Chapter 1*, research framework and objectives of this Ph.D. thesis are introduced. Following that, in *Chapter 2*, comprehensive reviews on the antioxidant properties, bioaccessibility and bioavailability of polyphenols are presented, with a specific focus on the application of novel processing techniques. Initially, a critical evaluation of the effects of novel non-thermal food processing technologies on the beverage antioxidants have been provided. Then, the studies about microencapsulation methods for food antioxidants regarding principles, advantages, drawbacks and applications have been reviewed. Afterwards, effects of encapsulation on the bioaccessibility and bioavailability of phenolic compounds were discussed. Lastly, *in vitro* and *in vivo* approaches on interactions of phenolics with food matrix were described.

In *Chapter 3*, the effects of high pressure processing (HPP; 200-600 MPa for 5 or 15 min) and pulsed electric field treatment (PEF; 3 kV/cm, 5-15 kJ/kg) on physicochemical properties (conductivity, pH and total soluble solids content), bioactive compounds (vitamin C, total phenolic, total flavonoid, total anthocyanin and chlorogenic acid contents), antioxidant capacities (DPPH and CUPRAC assays) and polyphenol oxidase activity of cranberrybush purée samples were evaluated. Results showed that conductivity increased significantly after PEF (15 kJ/kg) treatment. PEF and HPP treatments resulted with a better retention of bioactive compounds (increase in the total phenolic content in the range of ~4 – 11% and ~10 – 14% and total flavonoid content in the range of ~1 – 5% and ~6 – 8% after HPP and PEF, respectively) and antioxidant capacity compared to untreated sample. HPP reduced residual enzyme activity of PPO comparatively better than PEF. Besides, cranberrybush polyphenols were identified along with their detected accurate mass, molecular formula, error in ppm (between the mass found and the accurate mass < 10 ppm) of each phytochemical, as well as the MS/MS fragment ions. UPLC–QTOF–MS/MS analysis of cranberrybush led to the identification of flavan-3-ols (catechin, epicatechin, epi(catechin) hexoside), proanthocyanidins (procyanidin dimer, procyanidin trimer, procyanidin dimer monoglycoside), flavonols (quercetin, quercetin-deoxyhexose, quercetin-3-O-glucoside, quercetin pentoside hexoside, rutin, isorhamnetin-3-O-rutinoside), flavone (diosmetin-rhamnosylglucoside), phenolic acids (caffeic acid, chlorogenic acid, coumaric acid, *p*-coumaroyl-quinic acid) as well as anthocyanins (cyanidin-3-glucoside, cyanidin-3-rutinoside and cyanidin-3-xylosyl-rutinoside). In conclusion, high retention of bioactive compounds was achieved, with a potential extraction of vitamin C, phenolics, flavonoids and anthocyanins in cranberrybush purées after HPP and PEF treatments at selected processing intensities.

In *Chapter 4*, effects of food matrix and non-thermal food processing on bioaccessibility and transport dynamics of cranberrybush phenolics, in particular chlorogenic acid, in a combined *in vitro* gastrointestinal digestion/Caco-2 cell culture model were studied. Results showed that PEF treatment at 15 kJ/kg specific energy input resulted in a higher recovery of total flavonoid content (TFC; increase of 3.9% ± 1.1%, $p < 0.0001$), chlorogenic acid content (increase of 29.9% ± 5.9%, $p < 0.001$) and antioxidant capacity after gastrointestinal digestion. The present study also demonstrates that untreated and treated samples display comparable transport across the epithelial cell layer. Besides, addition of milk matrix have a positive effect on the stability and transportation of chlorogenic acid. JM increased the transport efficiency

of chlorogenic acid by 3.5% ± 0.8% ($p < 0.0001$), while JA increased the transport of chlorogenic acid by 3.3% ± 0.5% ($p < 0.001$) in comparison with JW blend. The *in vitro* gastrointestinal digestion/Caco-2 cell culture method applied in this chapter was used in the succeeding chapter (*Chapter 6*).

In *Chapter 5*, micronization of two flavonoids, quercetin and rutin, and their coprecipitation with polyvinylpyrrolidone were studied by using the SAS process. In particular, optimum conditions in terms of operating pressure, type of the solvent, total solute concentration and polymer/active ratio for the formation of spherical composite microparticles were determined. Morphology, mean size and size distribution of the particles were analyzed and discussed. The effectiveness of the process was also verified through entrapment efficiency and dissolution tests. Overall, amorphous microparticles were produced with total solute concentrations greater than 20 mg/mL. Furthermore, release studies confirmed the improvement of the flavonoids dissolution rates: 10 and 3.19 times faster dissolution rates were achieved with PVP/quercetin and PVP/rutin microparticles rather than those of unprocessed quercetin and rutin, respectively. Besides, the high entrapment efficiencies, up to 99.8%, were achieved for quercetin and rutin coprecipitates by using DMSO, which was the solvent chosen to coprecipitate the flavonoid compounds with PVP by the SAS process. Consequently, the characteristics of the powders could allow to use of these quercetin and rutin loaded microparticles in pharmaceutical and nutraceutical applications due to their high antioxidant and anticancer benefits for, in which the flavonoid compounds have high stability and bioavailability.

In *Chapter 6*, effects of SAS processing on bioaccessibility and transepithelial transportation of quercetin and rutin were investigated by using a recognized combined gastrointestinal digestion/cell-based assay. Moreover, aqueous hydrophilic and acidic conditions were simulated to analyze food-related factors that could have an impact on the transport of these compounds across the gut epithelium. SAS processing improved the recovery of the quercetin (94 and 13 times in hydrophilic and acidic conditions, respectively) and rutin (7 and 2 times in hydrophilic and acidic conditions, respectively) after *in vitro* digestion. Besides, transepithelial transportation of PVP/quercetin and PVP/rutin microparticles were found to be much higher rather than unprocessed quercetin and rutin.

Finally, in *Chapter 7*, based on the outcomes of the previous chapters, the general discussions and conclusions on the antioxidant properties, bioaccessibility and bioavailability of polyphenols were presented. The status and main outcomes of this thesis were discussed under the headings of fate of the polyphenols after application of novel non-thermal food processing techniques, effects of encapsulation on the food phenolics and interactions of phenolics and food matrix. During the discussion on the effects of encapsulation on the food phenolics, important factors to be considered during encapsulation, advantages and drawbacks of these techniques, their impacts on the antioxidant properties, bioaccessibility and bioavailability of phenolic substances were discussed. Besides, while referring to the interactions with food matrix, special attention has been paid to comparison of the different *in vitro* and *in vivo* digestion models.

YENİ GIDA İŞLEME TEKNİKLERİNİN GİLABURUDA BULUNAN POLİFENOLLERİN BİYOERİŞİLEBİLİRLİĞİ VE BAĞIRSAK TAŞINIMLARI ÜZERİNDEKİ ETKİLERİ

ÖZET

Bitkilerin meyve, sebze, çiçek ve yaprakları gibi çeşitli gıdalarda bulunan fenolik bileşikler, antimikrobiyal, antioksidan, antidiyabetik, idrar söktürücü, hipoglisemik, öksürük kesici, antiinflamatuar ve antiviral aktiviteler ile kardiyovasküler, pankreas, karaciğer ve böbrek hastalıklarının önlenmesi gibi gibi pek çok faydalı etkiye sahiptir. Bununla birlikte, polifenollerin çoğunun sudaki çözünürlüğünün zayıf olması ve gastrointestinal sistemde kimyasal olarak kararsız olmaları dolayısıyla oldukça düşük bir biyoyararlılığa sahiptir. Bu nedenle, fenolik bileşiklerin çözünürlüğünü, kararlılığını, biyo-erişilebilirliğini ve biyoyararlılığını iyileştirmek için çok çeşitli yöntemler geliştirilmiştir.

Yukarıdakiler dikkate alınarak, yeni gıda işleme tekniklerinin gilaburuda bulunan polifenollerin antioksidan kapasitesi, biyoerişilebilirliği ve biyoyararlılığı üzerindeki etkilerini çalışmak amacıyla bir araştırma çerçevesi oluşturulmuştur. Bu doktora tezinin hedefleri (i) yeni ısısal olmayan gıda işleme tekniklerinin gilaburuda bulunan polifenoller ve C vitamini üzerindeki etkilerini tespit etmek; (ii) ısısal olmayan gıda işleme yöntemleri ile gıda matrisinin gilaburuda bulunan biyoaktif bileşenlerin, özellikle klorojenik asidin, biyoerişilebilirliği ile bağırsak taşınımı üzerindeki etkilerini *in vitro* mide-bağırsak sindirim/Caco-2 hücre kültürü emilim modelini kullanarak araştırmak; (iii) polivinilpirolidon (PVP) ile kuersetin veya rutinin kopresipitasyonu amacıyla Süperkritik Anti-Solvent (SAS) yönteminin optimum işlem parametrelerini belirlemek; (iv) SAS işlemi ile farklı gıda koşullarının, flavonol (kuersetin ya da rutin) yüklü mikroparçacıkların antioksidan özellikleri, biyoerişilebilirliği ile bağırsak taşınımı üzerindeki etkilerini kombine *in vitro* mide-bağırsak sindirim/Caco-2 hücre kültürü emilim modelini kullanarak araştırmaktır.

Yukarıdaki hedeflere ulaşmak için dört farklı deneysel çalışma (*Bölüm 3–6*) yapılmıştır. Öncelikle farklı yüksek hidrostatik basınç (HPP) ve vurgulu elektrik alan (PEF) uygulamalarının gilaburu püresinin fizikokimyasal özellikleri, biyoaktif bileşikleri, antioksidan kapasitesi ve polifenol oksidaz aktivitesi üzerindeki etkileri değerlendirilmiştir (*Bölüm 3*). Bunu takiben, ısısal olmayan yöntemler ile işlenmiş gilaburu püresi örnekleri ile gilaburu suyu/su, süt veya badem sütü karışımları hazırlanarak *in vitro* mide-bağırsak sindirim ve emilim çalışmaları gerçekleştirilmiştir (*Bölüm 4*). Bir önceki bölümde gilaburu örnekleri ile yapılan emilim çalışmalarından elde edilen sonuçlar doğrultusunda, bağırsak epitelinden absorbe edilemeyen bazı fenolik bileşiklerin biyoyararlılıklarını arttırmak amacıyla, bir sonraki aşamada, iki flavonoidin, quercetin ve rutin, SAS yöntemi kullanılarak mikronizasyonu ve PVP ile birlikte kopresipitasyonu çalışılmıştır (*Bölüm 5*). Son olarak, SAS ile işlenmiş flavonoidler farklı gıda modellerinde *in vitro* mide-bağırsak sindirim/Caco-2 hücre kültürü emilimine tabi tutulmuştur (*Bölüm 6*).

İlk bölümde bu doktora tezinin araştırma çerçevesi ve hedefleri tanıtılmıştır. Bunu takiben *ikinci bölümde* yeni işleme tekniklerinin polifenollerin antioksidan özellikleri, biyoerişilebilirlikleri ve biyoyaralılıkları üzerindeki etkilerine ilişkin kapsamlı bir derleme sunulmuştur. Başlangıç olarak, yeni gıda işleme teknolojilerinden ve bu teknolojilerin içeceklerde bulunan biyoaktif bileşenlerin antioksidan özellikleri üzerindeki etkilerinden bahsedilmiştir. Sonrasında gıdalarda bulunan antioksidan maddelerin mikroenkapsülasyonu amacıyla kullanılan yöntemlerin prensipleri, avantajları, dezavantajları ve uygulamaları ile ilgili çalışmalar derlenmiştir. Daha sonra, enkapsülasyon işleminin fenolik bileşenlerin biyoerişelebilirliği ve biyoyararlılığı üzerindeki etkileri ele alınmıştır. Son olarak, fenolik madde – gıda matrisi etkileşimleri üzerine gerçekleştirilmiş *in vitro* ve *in vivo* yaklaşımlar anlatılmıştır.

Üçüncü bölümde HPP (200-600 MPa/5-15 dk) ve PEF (3 kV/cm, 5-15 kJ/kg) uygulamalarının gilaburu püresinin fizikokimyasal özellikleri (iletkenlik, pH ve toplam suda çözünür madde miktarı), biyoaktif madde miktarı (C vitamini, toplam fenolik, toplam flavonoid, toplam antosiyanin ve klorojenik asit içerikleri), antioksidan kapasitesi (DPPH ve CUPRAC) ve polifenol oksidaz aktivitesi üzerindeki etkileri değerlendirilmiştir. Sonuçlar PEF (15 kJ/kg) uygulamasından sonra iletkenliğin önemli ölçüde arttığını göstermiştir. Ayrıca, PEF ve HPP uygulamaları ile işlem görmemiş numuneye kıyasla sırasıyla toplam fenolik madde miktarında ~%4 - 11 ve ~%10 – 14 ve toplam flavonoid madde miktarında ise ~%1-5 ve ~ %6-8 aralığında artış tespit edilmiştir. PPO inaktivasyonu üzerinde HPP uygulamasının, PEF uygulamasına kıyasla daha etkin olduğu belirlenmiştir. Bunların yanı sıra, gilaburuda bulunan polifenoller, her bir fitokimyasalın kütlesi, molekül formülü, ppm düzeyindeki hataları (bulunan kütle ile doğru kütle arasında fark <10 ppm) ve MS/MS fragment iyonları ile birlikte tanımlanmıştır. UPLC – QTOF – MS/MS analiz sonuçlarına göre gilaburuda tespit edilen polifenoller şunlardır: flavan-3-oller (kateşin, epikateşin, epi(kateşin) heksosid), proantosiyanidinler (prosiyanidin dimer, prosiyanidin trimer, prosiyanidin dimer monoglikosit), flavonoller (kuersetin, kersetin-deoksiheksoz, kuersetin-*3*-*O*-glukozit, kuersetin pentosid heksosid, rutin, izorhamnetin-*3*-*O*-rutinosid), flavon (diosmetin-ramnosilglukosit), fenolik asitler (kafeik asit, klorojenik asit, kumarik asit, *p*-kumaroil-kuinik asit) ve antosiyaninler (siyanidin-3-glukozit, siyanidin-3-rutinosid ve siyanidin-3-ksilosil-rutinosid). Sonuç olarak, bu bölümde sunulan deneysel çalışma sonuçları seçilen işlem koşullarında HPP ve PEF uygulamalarının, gilaburuda bulunan biyoaktif bileşiklerin miktarında artış ya da yüksek oranda korunma sağlandığını göstermektedir.

Dördüncü bölümde gıda işleme yöntemleri ile gıda matrisinin gilaburu örneklerinde bulunan biyoaktif bileşenlerin, özellikle klorojenik asidin, biyoerişilebilirliği ile bağırsak taşınımı üzerindeki etkilerini incelemek amacıyla *in vitro* mide-bağırsak sindirim/Caco-2 hücre kültürü emilim modeli kullanılmıştır. Elde edilen sonuçlar, 15 kJ/kg spesifik enerji girdisi ile PEF işleminin, gastrointestinal sindirim sonrası toplam flavonoid içeriği (TFC; %3.9 ± 1.1% artış, $p < 0.0001$), klorojenik asit içeriği (%29.9 ± 5.9% artış, $p < 0.001$) ve antioksidan aktivitede daha yüksek bir kazanımla sonuçlandığını göstermiştir. Ayrıca, gilaburu suyuna sığır sütü ilavesi, *in vitro* mide-bağırsak sindirim sonrası toplam fenolik ve toplam flavonoid madde miktarları ile birlikte antioksidan kapasiteyi de olumlu yönde etkilemiştir. Sonuç olarak bu bölümde sunulan deneysel çalışma sonuçlarına göre, işlem görmüş gilaburu örneklerinde bulunan klorojenik asit ile herhangi bir işleme maruz bırakılmamış gilaburu

örneklerindeki klorojenik asidin bağırsaktan taşınımlarının benzer olduğu; ortama süt ilavesinin ise ilgili fenolik asidin mide-bağırsak sistemindeki kararlılığı ve bağırsaktan geçişi üzerinde olumlu etkileri olduğu tespit edilmiştir. Bu bölümde uygulanan *in vitro* mide-bağırsak sindirim/Caco-2 hücre kültürü sindirim modeli daha sonra *altıncı bölümde* de kullanılmıştır.

Beşinci bölümde SAS yöntemi kullanılarak kuersetin ve rutinin mikronizasyonu ve PVP ile birlikte kopresipitasyonu üzerine çalışılmıştır. Bu amaçla, mikro boyutta parçacık eldesi için sistem basıncı, çözücü tipi, toplam çözünür madde konsantrasyonu ve polimer/aktif oranı gibi işlem parametreleri için optimum koşullar belirlenmiştir. Ardından, elde edilen partiküllerin morfolojisi, ortalama partikül boyutu ve boyut dağılımları analiz edilmiştir. SAS yönteminin etkinliği ayrıca enkapsülasyon verimi ve suda çözünürlük testleri ile doğrulanmıştır. Genel olarak, 20 mg/mL'den daha yüksek toplam çözünen madde konsantrasyonlarında ve yüksek polimer/flavonoid oranlarında amorf yapıda mikroparçacıkların eldesi mümkün olmuştur. Ayrıca, ürünlerin sudaki çözünürlerini incelemek amacıyla salınım çalışması gerçekleştirilmiş ve SAS işlemine tabi tutulan kuersetin ve rutinin suda çözünme oranlarının işlem görmemiş kuersetin ve rutine göre sırasıyla 10 ve 3,19 kat arttığı tespit edilmiştir. Bunların yanı sıra, çözücü olarak DMSO kullanılarak elde edilen PVP/flavonoid mikroparçacıklar için %99,8'e varan enkapsülasyon verimi elde edilmiştir. Sonuç olarak, SAS uygulaması ile elde edilen flavonoid yüklü mikroparçacıkların karakteristik özellikleri, farmasötik ve gıda alanlarında, oldukça güçlü antioksidan potansiyele, yüksek kararlılığa ve biyoyararlılığa sahip takviyelerin üretilmesine olanak sağlamaktadır.

Altıncı bölümde SAS yönteminin flavonoidlerin (kuersetin ve rutin) biyoerişilebilirliği ile bağırsak taşınımı üzerindeki etkilerini incelemek amacıyla *in vitro* mide-bağırsak sindirim/Caco-2 hücre kültürü emilim modeli kullanılmıştır. Dahası, gıda ile ilgili faktörlerin bu bileşiklerin bağırsak epitelinden taşınması üzerindeki etkilerini değerlendirmek amacıyla hidrofilik ve asidik koşullar taklit edilmiştir. Elde edilen sonuçlara göre, SAS uygulamasının, kuersetin (sırasıyla hidrofilik ve asidik koşullarda 94 ve 13 kat) ve rutinin (sırasıyla hidrofilik ve asidik koşullarda 7 ve 2 kat) biyoerişilebilirliği oldukça yüksek oranda arttırdığı bulunmuştur. Ayrıca SAS uygulaması ile bu flavonoidlerin bağırsak taşınımları da olumlu etkilenmiştir.

Son olarak, y*edinci bölümde*, önceki bölümlerde elde edilen veriler dikkate alınarak polifenollerin antioksidan özellikleri, biyoerişilebilirliği ve biyoyararlılığı ile ilgili genel bir tartışma, sonuçlar ve gelecekteki araştırmalar için tavsiyeler verilmiştir. Bu tezden elde edilen sonuçlar yeni gıda işleme yöntemlerinin gıdalarda bulunan fenolik bileşenler üzerine etkisi, enkapsülasyon yöntemlerinin fenolik bileşenler üzerindeki etkileri ile fenolik bileşenlerin gıda matrisi ile etkilişimi başlıkları altında ele alınmıştır. Enkapsülasyon yöntemlerinin fenolik bileşenler üzerindeki etkileri tartışılırken enkapsülasyon işleminde dikkat edilmesi gereken faktörler, enkapsülasyon yöntemlerinin avantaj ve dezavantajları ile bu yöntemlerin fenolik bileşenlerin antioksidan özellikleri, biyoerişilebilirlikleri ve biyoyararlılıkları üzerindeki etkileri konularına değinilmiştir. Fenolik bileşenlerin sindirilebilirliği üzerinde gıda kaynaklı faktörlerin etkileri tartışılırken ise farklı *in vitro* ve *in vivo* sindirim modellerinden bahsedilmiştir.

1. INTRODUCTION

While the positive relation between nutrition and health become more and more pronounced, this forces the development of "superfoods" over the last years (Tichy et al., 2020). Epidemiological studies have shown that the potential beneficial effects of these kind of food products are attributed to the presence of bioactive compounds, in particular polyphenols. Phenolic substances are plant secondary metabolites which have several benefits to human health through antioxidant, anti-inflammatory, anticancer, anti-obesity, antiviral, antibacterial, antiaging and/or antiallergenic activities (Bao et al., 2019). However, the bioactive compounds in some fruits and vegetables may be lost during handling after harvest, processing, and storage. In this sense, novel nonthermal food processing technologies such as high pressure processing and pulsed electric field applications have been started to become widespread.

Furthermore, the biofunctional properties and possible beneficial effects of the polyphenols largely depends on their bioaccessibility and bioavailability (Parada and Aguilera, 2007). It is a fact that most of the phenolic substances possess low levels of solubility, stability, bioavailability and target tissue specificity in the body (Wang et al., 2014) depending on their molecular and physicochemical characteristics (Jafari and McClements, 2017). Overall, it is essential to develop a new approach to enhance the bioaccessibility and bioavailability of bioactive ingredients, including the incorporation them within micro-/nanoparticle delivery systems (Yang et al., 2008).

There are different methods used to examine the correlation between diet and health. However, *in vitro* models have been widely used to investigate human digestive tract rather than *in vivo* (human or animal) models owing to ethical issues. *In vitro* digestion methods are used to simulate the physiological conditions of the upper gastrointestinal tract (oral, gastric and small intestinal phases) (Brodkorb et al., 2019); thus provide some perception about the digestibility of controlled release systems and bioavailability of functional compounds (Alminger et al., 2014). Besides, in order to investigate the absorption efficiency and/or the transport through an epithelial

membrane of phenolic compounds, intestinal epithelial cells like the human colon adenocarcinoma cell line have been used (Sun et al., 2008; Gamboa and Leong, 2013).

Considering the above, a research framework to study the antioxidant capacity, bioaccessibility and bioavailability of cranberrybush polyphenols has been developed. Below are the major objectives of this Ph.D. thesis:

1. To evaluate the retention of polyphenols and vitamin C in cranberrybush purée (*Viburnum opulus*) by means of non-thermal treatments (*Chapter 3*)

2. To investigate the effects of food matrix and non-thermal processing on the bioaccessibility and transport dynamics of cranberrybush (*Viburnum opulus*) polyphenols in a combined *in vitro* digestion/Caco-2 cell culture model (*Chapter 4*)

3. To study micronization of two flavonoids, quercetin and rutin, and their coprecipitation with polyvinylpyrrolidone by using supercritical antisolvent process (*Chapter 5*)

4. To investigate the effects of supercritical antisolvent process and simulated hydrophilic and acidic food conditions on antioxidant capacity, bioaccessibility and transport dynamics of quercetin and rutin by combining *in vitro* digestion/Caco-2 cell culture model (*Chapter 6*)

2. LITERATURE REVIEW[1]

2.1 Effect of Novel Food Processing Technologies on Beverage Antioxidants

Over the past few decades, the role of dietary antioxidants such as polyphenols, carotenoids, and vitamins C and E has gained increased interest, especially associated with their numerous health beneficial effects including anticancer, antiinflammatory, antibacterial, antiviral, cardioprotective, and neuroprotective properties (Galano et al., 2016). Accordingly, the determination of antioxidant activity/capacity of food products is of great importance. However, the antioxidant compounds in some fruits and vegetables can be lost during handling after harvest, processing, and storage. It is a fact that plant-based foods are exposed to processing to increase shelf life and edibility while maintaining the sensory and nutritional properties as well as to ensure microbial safety. In this sense, novel nonthermal food processing technologies including high pressure processing (HPP), pulsed electric field (PEF), and ultrasound; novel thermal food processing technologies including microwave, ohmic, and infrared heating have been started to become widespread. HPP as a nonthermal processing is generally operated between 100 and 1000 MPa pressure and from −20°C to 60°C temperatures, providing cell wall and membrane disruption, enzyme inactivation, protein denaturation, and gel formation (Oey et al., 2008). HPP is an emerging food preservation method with satisfying the increasing consumer demand for fresh-like products with minimal alteration of nutritional and organoleptic properties (Rasanayagam et al., 2003). PEF, another nonthermal food processing technology, is

[1] This chapter is based on the papers;
Ozkan, G., Guldiken, B., and Capanoglu, E. (2019). Effect of Novel Food Processing Technologies on Beverage Antioxidants. Processing and Sustainability of Beverages: Volume:2, Woodhead Publishing, 413-449.
Ozkan, G., Franco, P., De Marco, I., Xiao, J., and Capanoglu, E. (2019). A review of microencapsulation methods for food antioxidants: Principles, advantages, drawbacks and applications. Food Chemistry, 272, 494-506.
Ozkan, G., Kostka, T., Esatbeyoglu, T., & Capanoglu, E. (2020). Effects of Lipid-Based Encapsulation on the Bioaccessibility and Bioavailability of Phenolic Compounds. Molecules, 25(23), 5545.
Pinarli, B., Simge Karliga, E., Ozkan, G., & Capanoglu, E. (2020). Interaction of phenolics with food matrix: In vitro and in vivo approaches. Mediterranean Journal of Nutrition and Metabolism, 13(1), 63-74.

utilized without significant adverse effects on the quality and nutrients in foods while extending shelf life (Dunn, 2001). The principle of this treatment is based on the dielectrical breakdown of the cell membrane (Tsong, 1991). Ultrasound as an attractive alternative to conventionaltreatments, related to collapse of cavitation bubbles which induces mechanical, chemical, and biochemical effects in the medium (Mason and Peters, 2002). Besides, it is a promising technology for food processing, particularly combination with other thermal or nonthermal technologies (Kim and Silva, 2016). Microwave drying is a method gaining popularity owing to its superior advantages such as high heating rates, significant reduction in cooking time, more uniform heating, safe handling, ease of operation, and low maintenance in a small extent of sensory and nutritional changes compared with conventional thermal applications (Salazar-González et al., 2012; Vadivambal and Jayas, 2010). Microwave heating is achieved by absorbing microwave energy and converting into heat. Microwave heating of food materials is mainly carried out due to dipolar and ionic mechanisms. The presence of moisture causes dielectric heating due to the dipolar nature of water (Datta and Davidson, 2000). A large number of potential applications exist for microwave heating including drying, pasteurization, sterilization, thawing, tempering, and baking of food materials (Gupta and Leong, 2008). Ohmic or electrical heating is generated by the superiority of the sample's electrical resistance against passing alternating current (FDA, 2000). A wide variety of applications of ohmic heating in food processing include blanching, evaporation, dehydration, fermentation (Sastry, 2008), and pasteurization (Castro et al., 2004). In addition, infrared radiation which is an indirect heating method can be applied to liquid foods such as milk (Krishnamurthy et al., 2008a), orange juice (Vikram et al., 2005), beer (Vasilenko, 2001), and honey (Hebbar et al., 2003).

The aim of this chapter is to provide a critical evaluation of the effects of nonthermal (HPP, PEF, and ultrasound) and novel thermal (microwave, ohmic, and infrared heating) food processing technologies on the beverage antioxidants. In order to achieve this purpose, this chapter will cover studies evaluating the impacts of technological treatments applied to various food beverages by comparing the effects of conventional thermal treatments. Furthermore, the principles, advantages, and disadvantages of these methods are highlighted.

2.1.1 Novel non-thermal technologies

Nonthermal food processing technologies has an ascending interest as alternative techniques due to sensory, quality, and nutritional modification of thermal food processing technologies. In addition to this, consumer demand has increased in accordance with the minimally processed, microbiologically safe, more natural flavors and colors, compromising the qualitative, sensory and nutritional attributes of the food products, and low-energy utilization (Barbosa-Cánovas et al., 2005; Varma et al., 2010; Dede et al., 2007).

HPP, PEF and ultrasound are nonthermal food processing techniques that gained popularity in the past decade in development of several food products. HPP is used for food pasteurization by the way of applying pressure between 100 and 1000 MPa at room or mild processing temperatures to the food products (Norton and Sun, 2008). On the other hand, PEF is utilized to pasteurize liquid foods at low temperature (<60°C), ambient, or refrigeration temperatures by means of exposing the target cell to strong electric field for sufficient time to obtain irreversible electroporation, accelerated mass transfer, and enhancement of tissue permeability (Mahnič-Kalamiza et al., 2014; Yang et al., 2016; Liu et al., 2011). The other promising nonthermal technology, ultrasound, is defined by propagation of sonic waves in the frequency range of 20–100 kHz for a few seconds to several minutes (Chemat et al., 2017a).

Therefore, the aim of this part of the study was to give detailed information including the principle, advantages, and disadvantages of the promising nonthermal technologies of HPP, PEF and ultrasound. Then, the effects of these technologies on beverage antioxidants were also discussed. Impacts of HPP and PEF and ultrasound processing on the bioactive compounds are covered in Table 2.1.

Table 2.1 : Effects of novel non-thermal food processing techniques on the antioxidant capacities of food products.

Applied Technology	Process Parameters	Optimum conditions	Product	Antioxidant Activity	References
HPP	200 – 600 MPa 30 – 70 °C 0 – 20 min	500 MPa 30 °C 15 min	Mixed fruit beverage (litchi juice, coconut water and lemon juice)	DPPH: 6.4 mg GAE/ 100 mL beverages	(Jayachandran et al., 2015)
HPP	400 – 600 MPa 30 – 60 °C 0 –15 min	400 MPa 30 °C 5 min	Aloe vera-litchi mixed fruit beverage	DPPH: 4.71 mg GAE/100 mL beverages	(Hulle et al., 2017)
HPP	300 – 500 MPa 5 – 15 min 0 – 2.5% *Stevia rebaudiana* Bertoni	300 MPa 14 min 2.5% *Stevia rebaudiana* Bertoni	Mixed fruit beverage (papaya, mango, orange)	ORAC: 38.8±1.7 mM TE TEAC: 26.5±0.8 mM TE	(Carbonell-Capella et al., 2013)
HPP PEF	For HHP: 600 MPa 42 °C 5 min For PEF: 36 kV/cm 100 µs	-	Blueberry juice	ABTS: 34.2 µmol/g for HPP ABTS: 33.5 µmol/g for PEF	(Barba et al., 2012)
HPP	200 – 600 MPa 10 – 20 min	600 MPa 20 min	Green asparagus juice	DPPH (IC_{50}): 115.23 mg/mL	(Chen et al., 2015b)
HPP	200–400 MPa 5–15 min	400 MPa 15 min	Bee-pollen added pineapple juice	FRAP: 126.5 µmol Trolox/g	(Zuluaga et al., 2016)
HPP	550 MPa 5 min	-	Papaya juice	DPPH: 7.07 mM FRAP: 6.69 mM	(Chen et al., 2015a)
HPP	550 MPa 6 min	-	Carrot juice	DPPH: 0.162% FRAP: 0.119%	(Zhang et al., 2016)
PEF	6.6 L/h juice flow rate 35 kV/cm 3 µs 45 Hz 45 ℃	-	*Opuntia dillenii* cactus juice	EPR: degradation of the free radical Fremy's salt ~80%	(Moussa-Ayoub et al., 2017)

Table 2.1 (continued) : Effects of *novel* non-thermal food processing techniques on the antioxidant capacities of food products.

PEF	56 exponential decay pulses 3 kV/cm	-	*Opuntia dillenii* cactus juice	EPR: degradation of the free radical Fremy's salt ~90%	(Moussa-Ayoub et al., 2016)
PEF	15 – 35 kV/cm Monopolar – bipolar pulse 500 – 2000 µs	35 kV/cm Bipolar pulse 1250 µs	Broccoli juice	Recovery: 95.9% by DPPH	(Sánchez-Vega et al., 2015)
PEF	0–35 kV/cm 0.2 – 2 µs	35 kV/cm 0.2 µs	Apple juice	DPPH: 1.02 mmol TE/L FRAP: 3.91 mmol TE/L ORAC: 63.35 mmol TE/L	(Bi et al., 2013)
PEF	Bipolar pulse 20 µs 50 Hz 1.5 kV/cm pulse numbers of 243 (PEF low) and 1033 (PEF high)	-	Grape juice	DPPH: 51.64% with PEF low DPPH: 64.61% with PEF high	(Leong et al., 2016)
Ultrasound	40 – 80% amplitudes 10 – 25 min	80% amplitude 15 min	Green cactus pear	ABTS: 102.60 mg VCEAC/100 mL % chelating activity: 54.2	(Cansino et al., 2013)
Ultrasound	40 – 80% amplitudes 3 – 25 min	60% -80% amplitude 15 min	Purple cactus pear	ABTS: 26.3 mg VCEAC/100 mL % chelating activity: ~65%	(Zafra-Rojas et al., 2013)
Ultrasound	25 kHz 0 – 60 min 20 °C	25 kHz 60 min 20 °C	Kasturi lime	DPPH: 777 mg Ascorbic acid equivalent/g	(Bhat et al., 2011)

DPPH: 2, 2-diphenyl-1-picrylhydrazyl; GAE: gallic acid equivalent antioxidant capacity; ORAC: oxygen radical absorbance capacity; TEAC: trolox equivalent antioxidant capacity; ABTS: 2,2'azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt; IC_{50}: the concentration of antioxidant required to cause of 50% reduction in the original concentration; FRAP: Ferric reducing antioxidant power; VCEAC: Vitamin C equivalent antioxidant capacity

2.1.1.1 Effect of HPP on beverage antioxidants

The potential application of high pressure processing (also called as high hydrostatic pressure (HHP) in some studies) has not been realized until the middle of the 1980s, although it was utilized in the chemical, ceramic, and plastic industries for decades. In 1914, Hite et al. could not show any solid arguments to investigate high pressure in order to preserve fruits and vegetables owing to the technological disadvantages at that period. Then, at the beginning of the 2000s, high-pressure technology was rediscovered and acquired to the food industry (Otero and Sanz, 2003).

HPP is a promising and novel nonthermal processing technique, achieving pasteurization in the balance of safety and quality characteristics rather than traditional thermal treatments, applied between 100 and 1000 MPa pressure and at room or mild processing temperatures (<60°C) to solid, liquid, packaged, or unpackaged foods (Clark, 2006; Balasubramaniam et al., 2008; Norton and Sun, 2008).

HPP, performing as a batch process, works according to the Pascal's principle that means pressure is transmitted instantly throughout the vessel which is independent of the size and geometry of the food product. This system consists of compression phase, pressure holding phase, and depressurization phase (Figure 2.1). In compression phase, the pressure is increased from ambient to the desired pressure; then it is kept constant for several minutes during pressure holding phase, followed by depressurization phase where the pressure is decreased to ambient pressure. During the stages of pressure holding phase and depressurization phase, an increase and a decrease of temperature take place because of compression and expansion of the food product as well as pressure-transmitting medium (usually water). Hereby, a heat exchange occurs between pressurizing chamber walls, pressure-transmitting medium, the packaging material of the food product, and the food product. When the water is used as a transmitting medium, temperature changes 3°C for every 100 MPa increase in the pressure, and is generally known as the compression heating value (Khurana and Karwe, 2009; Varma et al., 2010). The temperature increase of oil-rich products is three times higher than the water. Because, the fat molecules are considered to be bulky and nonpolar in nature while the water molecules are small and polar hence more compact. In addition to this information, it is known that oils show the highest compression heating compared to crude fats. Thus, a less compact structure would

have a tendency to be more compressed and it causes higher increase in the temperature during pressure application (Rasanayagam et al., 2003; Varma et al., 2010).

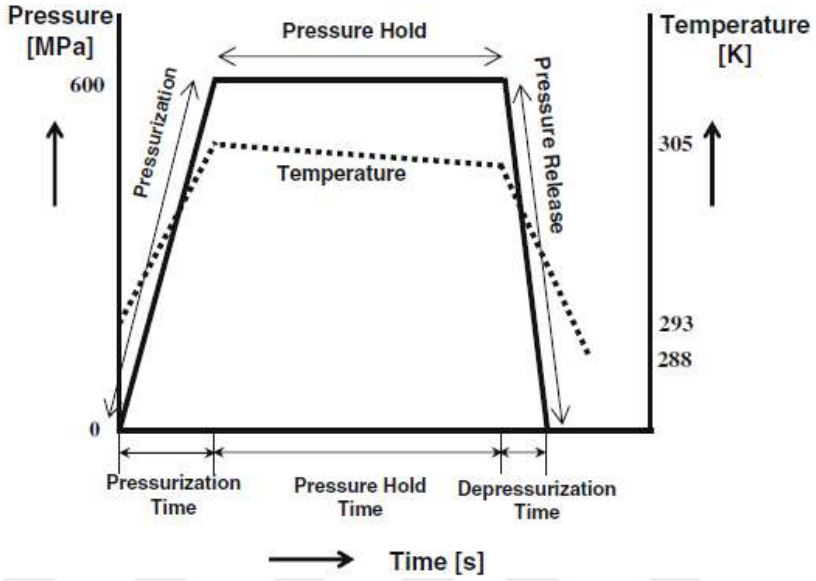


Figure 2.1 : Pressure and temperature variation during high pressure processing (Khurana et al., 2009).

HPP-treated foods generally show superior microbiological safety, improved quality including texture, flavor, and color, preferable nutritional value due to its limited effects on the covalent bonds, and low processing temperature which have an impact on the volatile compounds, pigments, vitamins, and antioxidant compounds compared to their thermally treated counterparts (Barba et al., 2015c; Ramirez et al., 2009; Zabetakis et al., 2000). HPP is a useful tool to inhibit microorganisms and inactivate enzymes such as peroxidase, polyphenol oxidase, and pectin methyl esterase (Saucedo-Reyes et al., 2009). The inhibition of microorganisms due to HPP is stemmed from the change in cell morphology, irreversible denaturation of enzymes and proteins, and inhibition of metabolic reactions (Mota et al., 2013). Moreover, this application is independent of size and shape of the food product that reduces the process duration (Sun, 2007), thus this situation facilitated the transition from scale-up laboratory to full-scale production (Torres and Velazquez, 2005); pressure is transmitted uniformly in fluid foods, hence, gradients do not occur as well (Thakur and Nelson, 1998; Toepfl et al., 2006).

On the other hand, it was reported that HPP has limitations on inhibition of Gram positive microorganisms, spores, and residual activity of endogenous enzymes which are responsible from long-term quality changes (Patterson, 2005; Chakraborty et al., 2014; Krebbers et al., 2003). Therefore, refrigeration conditions during storage (Scolari et al., 2015) and hurdle effect including HPP plus modified atmosphere packaging or carbonation are suggested (Sterr et al., 2015; Rode et al., 2015).

Up to date, a wide variety of food products have been developed by the most successful commercialized nonthermal food processing technique of HPP, which has already been used in fruits and vegetables, dairy products, meat, egg, juices, ready-to-eat meals, dips, and salads (Rastogi and Knorr, 2013; Wang et al., 2016; Jiménez-Sánchez et al., 2017).

The loss of sensory and nutritional quality of juices with the application of conventional thermal pasteurization is one of the main problem (Cardello et al., 2007). HPP is an efficient alternative technique that is applicable in juice industry with minimal impact on sensory and nutritional quality. In the study of Jayachandran et al. (2015), the effect of HPP (200–600 MPa/30–70°C/0–20 min) on the total antioxidant capacity of mixed fruit beverage including litchi juice, coconut water, and lemon juice was investigated. The total antioxidant capacity of juice samples was evaluated by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay and expressed as mg of gallic acid equivalents antioxidant capacity (GAE)/100 g of the fresh weight of sample. The increase of 1%–19% total antioxidant capacity at 500 and 600 MPa was obtained; owing to inducement of extractability of antioxidants from the modified tissue matrix into the extracellular environment (McInerney et al., 2007). Moreover, degradation of antioxidants initiated above 60°C. It was concluded that the combined effect of pressure and temperature on the antioxidant activity should be considered for a longer process time (Jayachandran et al., 2015). Similarly, the study on investigating HPP effect (400–600 MPa/30–60°C/0–15 min) on Aloe vera-litchi mixed fruit beverage was carried out by Hulle et al. (2017). It was found that extractability of phenolics and antioxidants for the samples treated at all pressures and temperature up to 50°C was increased, while the antioxidant capacity reduced up to 22% at 60°C (Hulle et al., 2017). In another study, Carbonell-Capella et al. (2013) studied the impact of HPP (300–500 MPa/5–15 min) on the total antioxidant capacity of a fruit juice mixture including papaya (32.5%, v/v), mango (10%, v/v), and orange (7.5%, v/v). It was

sweetened with different ratios of Stevia rebaudiana Bertoni (0%–2.5%). The greatest retention of beverage antioxidants was obtained with the HPP condition of 300 MPa pressure and 14 min treatment time by central composite face-centered design (Carbonell-Capella et al., 2013). Moreover, the antioxidant capacity of blueberry juice was better preserved by 600 MPa HPP treatment (Barba et al., 2012a). Chen et al. (2015b) observed that high pressures (200, 400, and 600 MPa/10–20 min) retained greater protective effect on the antioxidant capacity of green asparagus juice (Chen et al., 2015b). Zuluaga et al. (2016) studied about the HPP effect on antioxidant capacity [ferric reducing antioxidant power (FRAP)] of bee pollen added pineapple juice-based beverage. The results showed that the levels of FRAP increased with pressure and time in comparison with the control sample and a treatment of 395 MPa for 15 min was found as the optimal for the prevention of bioactive compounds (Zuluaga et al., 2016).

On the other hand, the effect of HPP (550 MPa/5 min) and high-temperature short time (HTST) (110°C/8.6 s) was compared in terms of total antioxidant capacity of papaya beverage after treatments and during storage of 40 days at 4°C. The results of total antioxidant capacity of juice samples were evaluated by DPPH and FRAP assays and expressed as mM of Trolox equivalents (mM). It was found that total antioxidant capacity of papaya beverage did not significantly change ($p > 0.05$) after HPP treatment when it was measured by DPPH and FRAP assays; it might be due to the insignificant changes of total carotenoid and total phenolic contents after HPP treatment. However, there was a significant ($p < 0.05$) decrease in total antioxidant capacities measured by DPPH and FRAP methods, which were attributed to the stable total carotenoids content and the significant decrease of total phenolic contents after HTST treatment. In addition, the HPP-treated sample showed higher total antioxidant capacity compared with the HTST-treated sample due to higher total carotenoid and total phenolic contents during 40 days of storage (Chen et al., 2015a). Zhang et al. (2016) also compared the impacts of HPP and HTST treatments in terms of total antioxidant capacity of carrot juice. Process conditions were selected as 550 MPa/6 min for HPP and 110°C for 8.6 s for HTST.

Higher antioxidant capacity retention measured by DPPH and FRAP was determined for HPP-treated juice rather than HTST-treated juice samples (Zhang et al., 2016). Similarly, Polydera et al. (2005) reported that high pressure led to higher antioxidant activity of orange juice compared to the thermal treatment (Polydera et al., 2005).

2.1.1.2 Effect of PEF on beverage antioxidants

PEF is an emerging and innovative nonthermal preservation technology which is considered to overcome the negative impacts of thermal treatments and enhance the shelf life of foods with improving nutritional and functional properties (Barba et al., 2012b, 2015b, Knorr et al., 2011). Moreover, PEF is one of the mild preservation techniques that can be applicable for liquid or semiliquid, viscous food products such as juices, milk, and yogurt (Torregrosa et al., 2006).

The short-duration electric field pulses, typically in the range of μs, of high intensity is applied on a food product located between the two electrodes (Asavasanti et al., 2011; Bouras et al., 2016) (Figure 2.2). The mechanism of the action includes electroporation and electropermeabilization (Teissie et al., 2005). Application of electric fields that is high enough to induce a transmembrane potential difference of approximately 0.2 V across the cell membrane leads to local instabilities, resulting with the formation of pores in the membrane (Wouters et al., 2001). As a detailed information, when the cells are exposed to an external electric field, the accumulation of oppositely charged ions on both sides of membrane causes membrane thickness reduction, then with the increase of electric field up to critical values gives rise to pore formation (Zimmermann, 1986). In the second step, as a result of expanded pores reversible or irreversible permeabilization is observed. For PEF processing, strong electric fields in the range of 5–50 kV/cm are applied to ensure complete, irreversible effects to ensure the inhibition of vegetative microbial cells (Toepfl et al., 2006; Knorr et al., 2011; Barba et al., 2015b). On the other hand, increasing permeability and cell disintegration in plant tissues by PEF treatment may also provide easier release of the intracellular bioactive substances (Puértolas et al., 2010). The degree of the electroporation depends on (i) the medium characteristics: temperature, electrical conductivity, ionic strength, and pH; (ii) process parameters: electric field strength, treatment time, number and type of pulse waveform, their width, and frequency; (iii) microorganism characteristics: type and growth stage of microorganisms, and (iv) treatment chamber characteristics: electrode configurations and mode of operation (Barba et al., 2015b; Jeyamkondan et al., 1999). Among these parameters, electric field strength and treatment time are the most important factors that have an influence on the electroporation which might be reversible or irreversible (Huang and Wang, 2009). On the other hand, it was found that square wave pulses were the most efficient,

whereas oscillatory pulses were the least efficient due to the prevention of the cells from continuously high-intensity electric field exposition for an extended period of time, hence prohibiting the cell membrane from irreversible breakdown. Moreover, bipolar pulses were obtained more efficient than monopolar pulses (Qin et al., 1994). Different goals such as arising mass transfer phenomena (Puértolas et al., 2012) or to inactivate microorganisms (González-Arenzana et al., 2015) can be achieved by adjusting the treatment conditions.

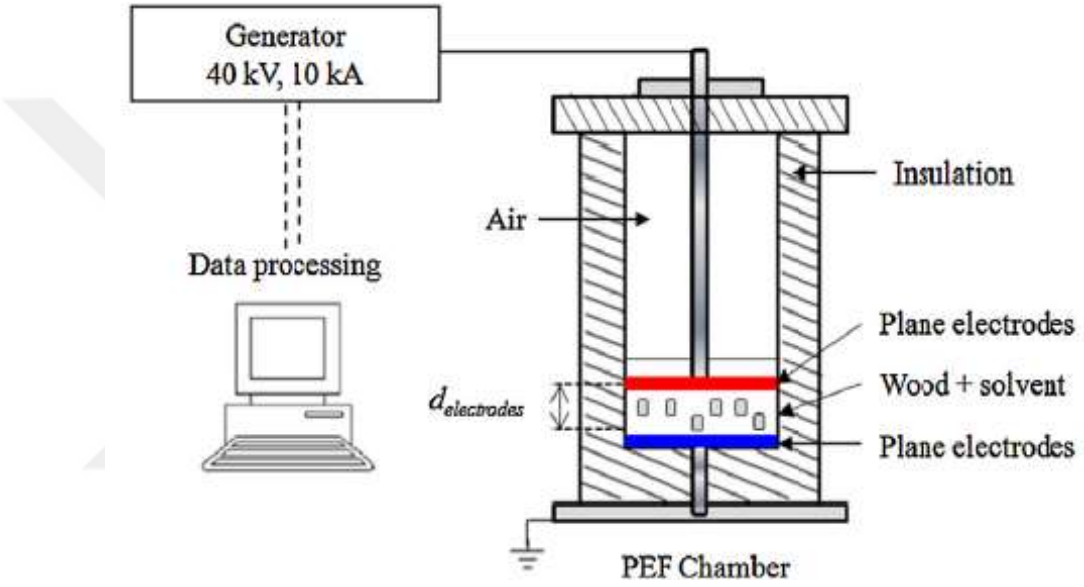


Figure 2.2 : Experimental set-up for PEF treatment (Bouras et al., 2016).

PEF is a preservation technique to obtain shelf-stable foods with high nutritional and sensory value (Odriozola-Serrano et al., 2013; Saldaña et al., 2014). This technology has also been extensively used to improve processes such as extraction by pressing or solvent diffusion, osmotic dehydration, drying, and freezing (Donsì et al., 2010) as well as the assessment of food wastes and by-products (Vorobiev and Lebovka, 2010). In addition, PEF draws on to use green extraction concept in terms of assuming alternative solvents [(water or agro-solvents (ethanol, methyl esters of fatty acids of vegetable oils]), reduction of energy consumption, production of high quality, and purity of extracts (Chemat et al., 2012). Moreover, PEF treatment allows to extract the bioactive compounds selectively without any detrimental effect to the treated sample (Barba et al., 2015b) which simplifies the downstream steps of separation and purification (Barba et al., 2015a; Vorobiev and Lebovka, 2010). Besides, pulsed

electric assistance empowers the yield and quality of the extracted compounds by descending the time and temperature of the extraction conditions (Donsì et al., 2010).

In spite of the fact that there has been a large quantity of information about the benefits of PEF processing, there are some limitations about its applications. It includes the effect of electric field on the cell activities which cause metabolic stress resulting with undesired effects on the quality of the final products, particularly fresh-cut products (Dellarosa et al., 2016). Another constraint is the high initial investment costs and elevated processing costs (Góngora-Nieto et al., 2002).

Due to the content of antioxidants, vitamins, and minerals which have important roles in human diet, consumer's demand tends to increase for fresh and nutritious food products such as unpasteurized fruit juices (Matthews, 2006). However, fresh fruit juices need to be heated immediately to prevent microbial, enzymatic, chemical, and physical deterioration and increase shelf life (Bates et al., 2001). In consequence of detrimental action of thermal treatments on the organoleptic, nutritional, and physicochemical properties of fluid foods, PEF application has been in the ascendant as a novel nonthermal application (Elez-Martínez et al., 2006; Mosqueda-Melgar et al., 2008). In the study of Moussa-Ayoub et al. (2017), the effect of PEF treatment (juice flow rate of 6.6 L h^{-1}, electric field strength of 35 V cm^{-1}, pulse width of 3 μs, frequency of 45 Hz, and preheating to 45°C) on the antioxidant activity of Opuntia dillenii cactus juice was compared with thermal treatment (95°C/3 min). The antioxidant activity of juice samples was evaluated by means of measuring the degradation of the stable synthetic radical Fremy's salt (potassium nitrosodisulfonate) in the presence of antioxidants in the samples by using electron paramagnetic resonance spectrometry (EPR) and expressed as mM Fremy's salt reduced by 100-μL diluted extract. It was found that antioxidant activity of PEF-treated samples was close to the antioxidant activity of the nonpasteurized juice, whereas the antioxidant activity of thermal treatment applied samples was decreased (Moussa-Ayoub et al., 2017). In another study, the same research group evaluated the antioxidant activities of O. dillenii cactus juice samples after PEF (56 exponential decay pulses at maximum electric field strength of 3 kV cm^{-1}), microwave heating (1800 W/3 min holding time) and enzymatic maceration (pectolytic enzymes) treatments. The antioxidant activity of juice samples was evaluated by using EPR method. The retention of antioxidant

activity of enzymatic maceration applied following PEF pretreated samples was found to be close to the nontreated samples. On the other hand, there was a decrease in the antioxidant activity of microwave-treated cactus juice samples due to the degradation of vitamin C or betacyanins. As a result, a combination of PEF and enzymatic pretreatments is recommended owing to the fact that this combination may protect the antioxidant activity in a better way (Moussa-Ayoub et al., 2016).

In another study, Sánchez-Vega et al. (2015) aimed to investigate the PEF processing effects including electric field strength, treatment time, and polarity on the broccoli juice antioxidants measured by the DPPH assay. Maximum relative antioxidant capacity (5.9%) was obtained with the process parameters of 25 and 35 kV cm^{-1} electric field strength, from 2000 to 500 μs treatment time and bipolar mode with the comparison of the results with the thermal treatment (90°C/60 s) (Sánchez-Vega et al., 2015). Similarly, Bi et al. (2013) also examined the effects of process parameters of electric field strength (0–35 kV cm^{-1}) and pulse rise time (2 and 0.2 μs) during PEF application on apple juice antioxidants measured using DPPH, FRAP, and oxygen radical absorbance capacity (ORAC) assays. While FRAP and ORAC values increased with increasing electric field and decreasing pulse rise time, the DPPH value was not changed after PEF implementation (Bi et al., 2013).

In a different study, health-promoting properties of grape juice after PEF treatment were investigated by means of measuring the antioxidant capacity of samples using DPPH and human intestinal Caco-2 cells assays. Oxidative stability of juice samples was assessed in terms of cell viability and lactate dehydrogenase leakage (LDH). Compared to untreated grape juice, PEF pretreatment (width of 20 μs, pulse frequency of 50 Hz, electric field strength of 1.5 kV cm^{-1}, and pulse numbers of 243 and 1033) on grape juices enhanced the DPPH scavenging activity by 31% and bioprotective capacity as 25% for cell viability, and 30% for LDH leakage (Leong et al., 2016a).

2.1.1.3 Effect of ultrasound on beverage antioxidants

Ultrasound is one of the emerging nonthermal technology to address the need to minimize processing and ensure safer and healthier food products with improved shelf life (Knorr et al., 2011). Ultrasound technology is characterized by the propagation of sonic waves in the frequency range of 20–100 kHz for a few seconds to several minutes

(Chemat et al., 2017a). These waves create alternate zones of compression and rarefaction which cause collapse of microscopic cavitation bubbles in the medium. Large surface area of these bubbles during the expansion cycle leads to enlargement of bubbles by increasing the gas diffusion. Then, these growing cavities become unstable owing to insufficient energy to retain the vapor phase in the bubbles, result with collapse releasing high temperatures and pressures that is responsible of mass transfer, highly efficient mixing and homogenization as well as enhanced bactericidal effect (Figure 2.3) (Piyasena et al., 2003; Paniwnyk, 2017).

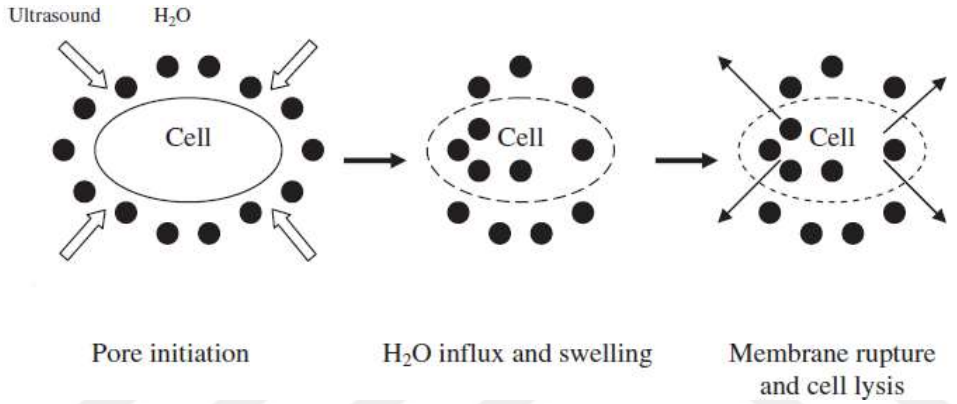


Figure 2.3 : Mechanism of ultrasound-induced cell damage (Chemat et al., 2011).

Ultrasound applications are based on three different methods including direct application to the product, coupling with the device, or submergence in an ultrasonic bath (Chemat et al., 2011). The application of ultrasound in food processing can be divided into low-intensity ultrasound and high-intensity ultrasound according to the frequency range. Low-intensity ultrasound with frequencies higher than 100 kHz at intensities below 1 W cm^2 could be employed for evaluating the composition of raw and fermented meat products, fish and poultry, quality control of fresh vegetables and fruits in both pre- and postharvest, cheese during processing, oil, bread, and cereal products, bulk and emulsified fat-based food products, food gels, aerated and frozen foods, detection of honey adulteration as well as for nondestructively supporting genetic improvement programs (Awad et al., 2012).

High-intensity ultrasound is characterized by the frequencies between 20 and 500 kHz at intensities higher than 1 W cm^{-2} that causes physical, mechanical, and chemical/biochemical alterations to the food being processed (Villamiel et al., 1999). This technology can be alternative to conventional treatments in terms of processing

and preservation. Besides, there are a large number of potential applications of high-intensity ultrasound in food processing including modifying textural characteristics of fat products, emulsification, defoaming, modifying the functional properties of different food proteins, inactivation or acceleration of enzymatic activity to enhance shelf life and quality of food products, microbial inactivation, freezing, thawing, freeze drying and concentration, drying and facilitating the extraction of various food and bioactive components (Gallego-Juárez et al., 2010; Awad et al., 2012).

The efficiency of ultrasound depends on the tissue structure, composition and volume of food product to be processed, amplitude of the ultrasonic waves, contact time with the microorganisms, the type of microorganism, and the temperature of treatment (FDA, 2000; Rodrigues et al., 2009).

Ultrasound as an innovative and environmental-friendly technology has superior advantages such as reduced water and solvent consumption during extraction, eliminating posttreatment of wastewater, efficiency in microbial decontamination, less energy consumption, and reduced processing time. Moreover, this technology regarded as cheap, simple, and reliable (Chemat et al., 2017a, b; Tiwari et al., 2008, 2009; Valero et al., 2007). Although there has been a large quantity of advantages of this technology, it is responsible from changes on flavor, color, viscosity, and chemical composition of food products due to free radicals and localized rise in temperature during ultrasound application (Garcia-Noguera et al., 2010).

There are several studies in which the influences of ultrasound treatment on the beverage antioxidants are investigated. In the study of Cansino et al. (2013), the effect of ultrasound (amplitudes 40%, 60%, and 80% / times 10, 15, and 25 min) on the antioxidant activity of green cactus pear juice was studied. The total antioxidant capacity of juice samples was evaluated by 2,2′ azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) and chelating activity of ferrous ions assays. The total antioxidant capacity measured by ABTS assay was expressed as μmol equivalents of Trolox per liter of juice (ET μmol L^{-1}), while total antioxidant capacity of juice samples by chelating activity of ferrous ions was obtained as % chelating activity. It was found that application time is an important parameter during sonication, because it is responsible from the release of antioxidants, particularly polyphenolic compounds. The antioxidant capacity measured by ABTS was the highest with the

treatment of 80% amplitude/15 min. On the other hand, ultrasound treatment did not have an influence on the chelating activity-related compounds (Cansino et al., 2013). Similarly, Zafra-Rojas et al. (2013) investigated the effect of different ultrasound conditions at 40% and 60% amplitude for 10, 15, and 25 min; 80% amplitude for 3, 5, 8, 10, 15, and 25 min on the antioxidant activity of purple cactus pear juice. The total antioxidant capacity of juice samples was determined by ABTS and chelating activity of ferrous ions as the previous study. Results showed that sonication at higher amplitudes and time released higher levels of antioxidants. However, the chelating activity in sonicated juices decreased except in juices treated at 80% amplitude for 25 min. Because of the fact that chelating activity is related with phenolic compounds and this activity depends on the type of the chelating activity of phenolic compounds released by ultrasound (Zafra-Rojas et al., 2013).

In another study, kasturi lime fruit juice was subjected to sonication for 25 kHz frequency/0, 30, and 60 min at 20°C to investigate the effect of process parameters on the antioxidant properties of the treated juices. DPPH free radical scavenging activity of the juices was increased from 14.59% to 50.93% with 60 min treatment time. This results might be due to the increase of extractability of antioxidant compounds by sonication (Bhat et al., 2011).

2.1.2 Novel thermal technologies

Since today, food processors have mostly used traditional thermal processes such as pasteurization, sterilization, and drying to ensure microbial safety and longer shelf life (Pereira and Vicente, 2010). However, concerns of customers on nutrition canalized food industry to seek new methods (Bingöl, 2015). Traditional heating methods generate heat outside the food by fuel combustion or electricity and transfer produced heat to the food via convection, conduction, or radiation mechanisms (Krishnamurthy et al., 2008b; Pereira and Vicente, 2010). On the other hand, dielectric energy is a form of electromagnetic energy which can be absorbed by the food material and transformed to heat whereas in ohmic heating electrical resistance of foods ensures converting of electricity to heat (Fellows, 2000). Unlike ohmic and microwave heating, infrared heating is an indirect heating method in which energy is transmitted to food surface by radiation then transformed to heat (Fellows, 2000).

The disadvantages of traditional heating methods are heat loss from the surfaces of the equipment, longer heating time, and lower heat transfer efficiency due to insufficient conduction of heat to the thermal center (Pereira and Vicente, 2010). Therefore, uniform, rapid heating technologies are required to ensure microbial safety and enhance food quality (Zhao et al., 2000). In addition, ohmic and dielectric heating provides rapid heating of food products without hot surfaces or surface browning or heat damage with small equipment (Fellows, 2000). In addition, ohmic heating may be more effective than microwave heating in some matters due to the use of all energy for heating without limitation of penetration depth (Li and Sun, 2002). Effects of ohmic, microwave, and infrared heating on the bioactive compounds are presented in Table 2.2.

2.1.2.1 Effect of ohmic heating on beverage antioxidants

Ohmic heating also called "resistance heating," "Joule heating," or "electro-conductive heating" is a novel food process method in which electrical resistance of food causes heating by passing electric current through the food product (Fellows, 2000). Nowadays, it is getting attention on heating of pumpable foods and provides important possibility for continuous processing of viscous and liquid food products (Icier and Ilicali, 2005). In food production process, unless the prior parameter for concerning product safety and quality is microbial inactivation, the presence of critical amounts of certain microorganisms may lead to product deterioration, quality loss, and/or health problems (Knirsch et al., 2010).

Basically, the heat transfer in an ohmic heating system (Figure 2.4), composed of AC supply, a variance, measurement units of current, voltage and temperature, an ohmic heater test unit, and digital system to record data, can be explained for liquid food by the following heat conduction equation (2.1) with an internal heat generation (Icier, 2012):

$$\nabla(k\nabla T) + \dot{u} = \rho C_p \frac{\partial T}{\partial t} \tag{2.1}$$

where k is the thermal conductivity (W/mK), ρ is the density, and Cp is the specific heat capacity (J/kgK) of liquid food.

Table 2.2 : Effects of novel thermal food processing techniques on the bioactive compounds.

Novel Thermal Method	Application	Applied Food	Treatment Effect	Ref
Ohmic Heating	Extraction (essential oils)	*Thymus vulgaris* L.	Reduced treatment time	(Gavahian et al., 2012)
	Extraction (essential oils)	*Zataria multiflora* Boiss (Shirazi thyme)	Shorter process, prevention of all essential oils	(Gavahian et al., 2011)
	Extraction (polyphenols)	Red grape pomace	Increase in polyphenol content	(El Darra et al., 2013)
	Heat treatment	Orange juice	Higher retention in Vitamin C content	(Vikram et al., 2005)
	Heat treatment	Acerola fruit	Higher ascorbic acid degradation and color change at 10Hz	(Mercali et al., 2014)
	Heat treatment	Blueberry pulp	Higher anthocyanin degradation with higher voltage levels	(Sarkis et al., 2013)
	Extraction (anthocyanin)	Black rice bran	Increased anthocyanin content as 50%	(Loypimai et al., 2015)
Microwave Heating	Heat treatment	Orange juice	Higher Vitamin C content	(Géczi et al., 2013)
	Concentration	Black mulberry juice	Less anthocyanin degradation and process time	(Fazaeli et al., 2013)
	Pasteurization	Grape fruit juice	Higher retention in total phenols and antioxidant capacity	(Igual et al., 2010)
	Pasteurization	Kava juice	Microbial inactivation without loss in juice quality	(Abdullah et al., 2013)
	Pasteurization	Tomato juice	Higher $ABTS^{+}$ values	(Stratakos et al., 2016)
	Pasteurization	Strawberry juice	Reduction in anthocyanin content as a function of time	(Mollov et al., 2007)
Infrared Heating	Heat treatment	Orange juice	Higher Vitamin C degradation with increasing temperature	(Vikram et al., 2005)
	Microbial inactivation	Honey	Increased HMF and reduced enzyme activity	(Hebbar et al., 2003)
	Microbial inactivation	Milk	Total microbial inactivation, no data for nutrient content	(Krishnamurthy et al., 2008)
	Heat treatment	Green tea	Increase in total polyphenols and flavanol content	(Kim et al., 2006)
	Heat treatment	Green tea	Increase in nitrite scavenging activity until 110°C	(Lee et al., 2006)
	Heat treatment	Green tea	Increase in total phenolic, flavanol content, and ascorbic acid content	(Park et al., 2009)

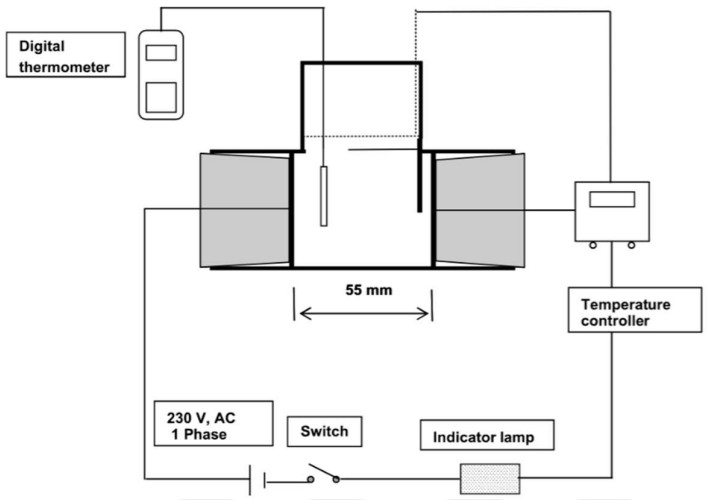


Figure 2.4 : Experimental setup for ohmic treatment (Vikram et al., 2005).

The electrical conductivity of food material depends on various factors such as temperature, voltage gradient, food composition (e.g., sugar content), etc. (Icier and Ilicali, 2005). For instance, electrical conductivity increases in the presence of ionic substances like acids and salts, however, decreases with nonpolar components (fats, lipids, etc.) (Lima et al., 2001). The process control parameters of ohmic heating include temperature, frequency, voltage gradient, the difference of electrical conductivity, food properties (acidity, composition, viscosity, etc.), electrode type, and flow properties (Icier, 2012). On the other hand, among all heating methods, thermal properties of food compounds have less effect on ohmic heating (Cokgezme et al., 2017). In addition, ohmic heating was reported as a faster heating method than microwave, and infrared heating methods due to internal heat generation (Vikram et al., 2005).

In the 19th century, ohmic heating was applied to pasteurize milk (Vicente and Castro, 2007). Ohmic treatment is also used for preheating, blanching, thawing, and extraction of foods (Icier, 2012; Li and Sun, 2002). In addition, in our day, ohmic heating is mainly used for ultrahigh-temperature (UHT) sterilization and pasteurization of liquid foods (Fellows, 2000).

Heating of liquid foods containing particles is a challenging task for the food industry that should be overcome (Bingöl, 2015). It was reported that fruit juices could be heated by ohmic heating due to their high electrical conductivity values (Cokgezme et al., 2017). In the case of ohmic heating, internal energy transformation within the food

material occurs (Knirsch et al., 2010). The advantages of ohmic heating are (1) there is no limit as penetration depth like microwave heating, (2) rapid heating, (3) there is no hot surfaces, surface fouling, or heat damage to sensitive foods, (4) heating possibility of viscous liquids, and (5) low cost (Fellows, 2000). In addition, ohmic heating does not generate waste (Cokgezme et al., 2017). The disadvantages of conventional heating methods are long processing time and low-energy efficiency (Darvishi et al., 2013). Furthermore, ohmic heating differs from other heating methods as requirements of electrodes contacting the foods, there is no restriction on used frequency and waveform (Vicente and Castro, 2007). In heterogeneous food material, it is important to arrange the heating rate of particles under ohmic heating and for a successful application, information on electric conductance is vital (Knirsch et al., 2010).

Many researchers investigated ohmic heat in extraction of different food compounds from various sources (Lakkakula et al., 2004). Electric field intensity, process time, temperature, and extracted food material affect the extracted juice yield (Praporscic et al., 2006). An important problem in extraction of essential oils from thyme via traditional methods was reported as long extraction time (1 h) and this problem was solved with ohmic-assisted hydrodistillation method (24.75 min) (Gavahian et al., 2012). In shorter processing time, all existing essential oils can be extracted via ohmic-assisted extraction method (Gavahian et al., 2011). In a similar manner, polyphenols were extracted from red grape pomace by pulsed ohmic heating (400 V cm^{-1}, 50°C, solvent: 30% ethanol/water) which resulted with an increase in polyphenol content from 440 to 620 mg GAE/100 g on dry basis after 60 min extraction time (El Darra et al., 2013). Rice bran is a rich source of various nutrients including tocopherols, vitamins, proteins, and dietary fibers (Loypimai et al., 2009). In a study performed by Lakkakula et al. (2004), extraction yield of rice bran oil increased from 53% to 92% by using ohmic heating. In a different study, beetroot pigment extraction was increased up to 3-fold at 42°C with 0.1% NaCl carrier fluid (Lima et al., 2001). On the other hand, in ohmic heating, frequency is another parameter for juice extraction in which lower frequencies resulted with higher apple juice yield at 40°C (Lima and Sastry, 1999). Potato and apple tissues became soft and available for juice treatment at moderate temperatures (<50°C) while electric field strength is lower than 100 V cm^{-1} (Praporscic et al., 2006). Furthermore, when considered all these studies, ohmic

heating may be a more convenient method for extraction compared to microwave due to higher yield and less input energy (Wang and Sastry, 2002).

In a previous study performed by Cokgezme et al. (2017), the effect of parameters was evaluated on ohmic heating of pomegranate juice. According to the results, voltage gradient increased as the total energy consumption and power increased. In addition, when the value of total soluble solids is 30%, the specific water removal ratio and energy efficiency were found to be at the highest values. Ohmic-assisted vacuum evaporation was utilized to concentrate sour cherry juice at three different voltage gradients (10, 12, and 14 V cm^{-1}) by Sabanci and Icier (2017) who reported that an increase in voltage gradient from 10 to 12 V cm^{-1} led to reduction in total evaporation time to half. Moreover, Darvishi et al. (2013) reported that voltage gradient affected heating rate, pH, and electrical conductivity in ohmic heating of pomegranate juice. According to this study, increase in voltage gradient resulted with a decrease in system performance, time, and pH. In the same manner, evaporation time of pomegranate juice reduced while voltage gradient increased and electrical conductivity increased to maximum values at critical dry matter contents which was different for different types of fruits (Icier et al., 2017). In addition, bubbling was reported at high voltage gradient while the temperature is higher than 81°C in the same study. Similarly, heating of orange juice with electromagnetic and conventional methods was evaluated for investigating the degradation rates of vitamin C (33.13 mg/100 g in fresh juice) and according to the results, heating and temperature were found to be responsible for the vitamin C degradation and retention of vitamin C was reported to be the highest in ohmic heating among all heat treatments (Vikram et al., 2005). In a similar manner, the negative effect of conventional thermal treatments on vitamin C content of acerola fruit (1000–4000 mg/100 g) wanted to be eliminated via ohmic heating in a study performed by Mercali et al. (2014). However, different from other studies, there was no positive effect of ohmic heating as electric field frequency at 10 Hz caused higher ascorbic acid degradation and color change, and above 100 Hz, the degradation reactions were found to be similar to that of conventional methods showing the requirements for further studies to understand the effect of electric field frequency on ascorbic acid degradation kinetics. In addition, Sarkis et al. (2013) reported similar results on anthocyanin degradation during the ohmic heating of blueberry pulp; in this

case, higher voltage levels caused higher degradation, but lower voltage levels reduced the degradation and similar results were obtained with conventional heating.

Another example of using ohmic heating is the prevention of degradation of rice bran oil (Loypimai et al., 2009; Lakkakula et al., 2004). During storage, lipid oxidation of rice bran was retarded with ohmic heating when electrical field strength was between 150 and 225V cm^{-1} and moisture content of the rice bran was 30%–40% (Loypimai et al., 2009). In addition, Loypimai et al. (2015) studied anthocyanin extraction from black rice bran (Oryza sativa L.) which is a rich source of anthocyanins (cyanidin-3-O-glucoside, cyanidin-3-O-rutinoside, delphinidin, cyanidin, perlargonidin, and malvidin), tocopherols, tocotrienols, and phenolic compounds. In this study, ohmic heating-assisted solvent extraction of anthocyanins increased the yield up to 50% with 30% moisture content and 150 V cm^{-1} electric field strength.

2.1.2.2 Effect of microwave heating on beverage antioxidants

After the World War II, dielectric heating was used for food components first between the 1 and 15 MHz frequency range. However, for microwave heating, a frequency range between 800 and 3000 MHz was used by the end of the 1950s (Bengtsson and Ohlsson, 1974). Although, Percy Spencer obtained the patent for popcorn production in 1949, widespread use of microwave energy occurred in the 1990s (Fito et al., 2005). Due to the dielectric properties of food materials, microwave (300 MHz–300 GHz) and radio frequency waves (0.003–300 MHz) generate heat via dipole rotation (Ramaswamy and Tang, 2008). Industrial, medical, and scientific use of microwaves were allocated to specific frequency bands to prevent interference between frequency rages of radio frequencies and microwaves (Bingöl, 2015). The Federal Communication Commission allocates some frequencies such as 915 ± 13, 2450 ± 50, 5800 ± 75, and 24,125 ± 125 MHz for heating purposes (Vicente and Castro, 2007).

In contrast to traditional heating methods, dielectric heating provides heat generation inside the food and achieving higher heat fluxes resulted rapid cooking (Bengtsson and Ohlsson, 1974). Among several applications in food processing, microwave energy can be used in thawing, tempering, heating of precooked products, cooking, baking, blanching, sterilization, pasteurization, and dehydration (Fito et al., 2005). Furthermore, microbial safety in microwave pasteurization and sterilization depends

on the function of microwave frequency, applicator design, composition, and properties of the food (Fu, 2004).

The major components of microwave equipment are magnetron, wave guide, and applicator (Figure 2.5) (Bingöl, 2015). The magnetron is a diode-type electron tube which is basically composed of the anode, the filament, the antenna, and the magnets (Vicente and Castro, 2007). Industrial (915 MHz) and household microwaves (2450 MHz) use different magnetrons. The reasons of 915 MHz magnetrons in industrial applications may provide higher electric power (up to 100 kW) and higher penetration depth of microwave energy at this frequency (Bingöl, 2015). Furthermore, the generated energy by the magnetron is directed to the waveguide, which is a hollow metal, propagates the waves to the heating chamber (Fellows, 2000; Vicente and Castro, 2007).

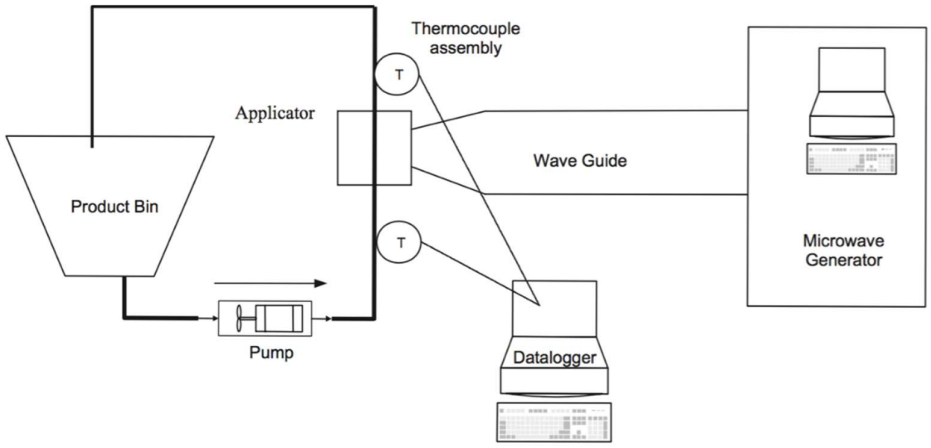


Figure 2.5 : Schematic diagram of microwave heating system (Sabliov et al., 2008).

Ionic polarization and dipole rotation are two ways of heat production by microwaves (Tewari, 2007). In ionic polarization, charged particles can move through the material, however, dipole rotation occurs when the charged particles are bound within the material and they move till the electric force is balanced by opposing forces (Hebbar and Rastogi, 2011). James Clerk Maxwell explained the distribution of electromagnetic energy by four fundamental equations (2.2, 2.3, 2.4 and 2.5) (Fu, 2004):

$$\nabla \times E = \frac{\partial B}{\partial t} \tag{2.2}$$

$$\nabla . D = \rho_e \tag{2.3}$$

$$\nabla \times H = J + \frac{\partial D}{\partial t} \tag{2.4}$$

$$\nabla . B = 0 \tag{2.5}$$

where E and H are defined as the electric and magnetic field, *J* is total current density, ρ_e is the total charge density, D is electric flux density, and B is the magnetic flux density.

The dielectric properties of a material are defined with permittivity which determines the conversion of electromagnetic energy to heat (Leonelli and Mason, 2010; Hebbar and Rastogi, 2011). The following equation (2.6) identifies the complex relative permittivity (Bingöl, 2015):

$$\varepsilon = \epsilon' - j\varepsilon'' = |\varepsilon| e^{-j\delta} \tag{2.6}$$

where ε' is the dielectric constant an expression of polarizability of the molecules and indicates their ability to store electrical energy, ε'' is the dielectric loss factor related to the energy absorption and dispersion of electromagnetic energy from the field (Bircan and Barringer, 2002), δ is the dielectric loss angle, and j is the imaginary number ($\sqrt{-1}$) (Hebbar and Rastogi, 2011).

Dielectric properties of foods indicate interaction of an electromagnetic field with non- or low-conducting material. In addition, foods, which can store and dissipate microwave energy, are poor electric insulators (Muthukumaran et al., 2011). The most common food products have $\varepsilon''<25$ (Venkatesh and Raghavan, 2005).

When microwave energy passes through a material, part of energy, which is called Pr, is reflected and other part is absorbed and another part is transmitted by the material. Pr is the function of dielectric constant and angle of incidence (θ) as shown in equation (2.6). In addition, the transmitted microwave power can be calculated as shown in equation (2.7 and 2.8) (Muthukumaran et al., 2011):

$$P_r(\theta) = \frac{\left(\sqrt{\varepsilon\prime}-1\right)^2}{\left(\sqrt{\varepsilon\prime}+1\right)^2} \tag{2.7}$$

$$P_{trans} = (1 - P_r) \tag{2.8}$$

The absorbed microwave energy is related with dielectric loss factor ε′ as expressed in equation (2.9):

$$P_{abs} = 5.56 \times 10^{-4} f \varepsilon'' E^2 \quad (2.9)$$

The dielectric properties of foods depends on temperature and composition of food material, frequency, and energy penetration (Bengtsson and Ohlsson, 1974). Dielectric constant of apples, which have 70% moisture content, reduces with increasing frequency. On the other hand, dielectric loss factor reduces to a minimum value and then increases with frequency (Feng et al., 2002). In addition, ionic conductivity is important at lower frequencies (<200 MHz), while not only ionic conductivity but also dipole rotation of free water are significant at microwave frequencies (Tang et al., 2005). The loss factor has bell-shape relationship with the frequency for edible oils. The ε″ reduces between 100 Hz and 13.2 kHz. After that point, ε″ increases while frequency rises (Muthukumaran et al., 2011).

For both salted and unsalted butter, temperature changes influenced dielectric properties. If process temperature increase, dielectric constant ε′ will reduce gradually. However, dielectric constant ε′ increases over 60°C. On the other hand, the loss factor ε″ reduces while temperature rises for unsalted butter. In addition, ε″ increases while temperature rises for salted butter (Muthukumaran et al., 2011). Free water behaves as liquid water; but bound water acts as ice on dielectric properties. While moisture decreases to critical moisture level, dielectric properties of food decrease. After that, decrease becomes less considerable owing to bound water (Hebbar and Rastogi, 2011). Furthermore, when foods are in drying process, water dipole mobility reduces that causes decreased loss factor. In addition, less available free water causes reduction at ionic conductivity (Feng et al., 2002). In a study with edible oils, unsaturation degree affects dielectric constant which increased by an increase in unsaturation (Muthukumaran et al., 2011).

In addition, volumetric heating of food material leads to less processing time and higher end-product quality and selective heating of food materials can be achieved due to their different dielectric properties in microwave processing (Thostenson and Chou, 1999). Whereas, microwave treatment to foodstuffs including high unbound water causes limited penetration depth and unequal electric field distribution which cause

nonuniform temperature distribution in foodstuff (Franco et al., 2015). In addition, only surface heating may occur while penetration depth of the microwave is remarkably less than the thickness of the material (Thostenson and Chou, 1999). The advantages of microwave heating in comparison to conventional heating systems can be summarized as rapid, uniform, and selective heating; small process equipment; high efficiency; clean environment; and rapid operation control (Leonelli and Mason, 2010).

Pasteurization temperature and process time affect inactivation of microorganisms and product quality. HTST heating is used for pasteurization of juices as a conventional method by the food industry, however, this continuous method leads to fouling and loss in nutrients due to the high surface temperatures (Math et al., 2014). On the other hand, continuous microwave process provides rapid heating due to the effect of high microwave power and protects product quality (Arjmandi et al., 2016). It is known that vitamins, pigments (carotenoids, anthocyanins, etc.), and phenolics are antioxidant compounds found in fruits and vegetables (Podsędek et al., 2003). In addition, chlorophyll stability was found to be higher in microwave-treated kiwifruit than conventional heat processing, however, the variation of carotenoid retention in microwave and conventional methods was reported as insignificant (Benlloch-Tinoco et al., 2015). Furthermore, vitamin C content and color properties of orange juice samples treated with continuous flow microwave were found to be similar to conventional heating method (water bath). However, color properties of milk samples were observed to be different from conventionally treated and untreated milk samples which may indicate changed product properties with microwave processing rather than thermal effects (Géczi et al., 2013).

Microwave heating, used as a concentration process for black mulberry juice, reduced required process time (approximately 30 min) and degradation of anthocyanins compared to rotary evaporator (Fazaeli et al., 2013). On the other hand, for the determination of vitamins A and E in beverage samples (milk, banana flavored milk energy drink, multivitamin multifruit juice, and orange juice), microwave-assisted saponification and extraction were performed (Höller et al., 2003).

In some previous studies, microwave heating was used to pasteurize various liquid food products such as orange juice (Tajchakavit and Ramaswamy, 1997), apple juice

(Cañumir et al., 2002), apple cider (Gentry and Roberts, 2005), grapefruit juice (Igual et al., 2010), milk (Valero et al., 2000; Clare et al., 2005), and tomato juice (Stratakos et al., 2016), to inactivate enzymes in green coconut water (Matsui et al., 2008), and to aseptically process sweet potato puree (Coronel et al., 2005) and salsa sauce (Kumar et al., 2007).

Igual et al. (2010) compared conventional and microwave pasteurization in grapefruit juice considering the total phenolic content and antioxidant capacity values. The results indicated that conventional pasteurization process led to the retention of total phenols and antioxidant capacity as 75% and 20%, respectively. In addition, microwave processing caused higher retention in total phenols and antioxidant capacity as 82% and 33%, respectively. Abdullah et al. (2013) studied the microwave pasteurization of fresh kava juice using continuous flow system. In this study, it is reported that microbial safety was achieved at 65.2°C without loss in juice quality. In addition, Stratakos et al. (2016) applied microwave pasteurization (6 × 3 kW, 2450 MHz) on tomato juice and reported changes in physicochemical properties, antioxidant capacity, and microbial characteristics. According to their results, initial $ABTS^{+}$ values were found to be higher in microwave-treated samples in comparison to conventionally pasteurized samples, but there were no significant differences during storage. Furthermore, the difference in the anthocyanin contents of fruit blends (pomegranate: carrot, beetroot: jamun, musk melon: grape, papaya: grape, papaya: banana: grape, mango: grape, watermelon: figs, and diluted jamun juice), which are processed through continuous microwave system (2 kW, 250 mL min^{-1}), were found to be statistically insignificant in comparison to the fresh sample (Math et al., 2014). Whereas, purified strawberry anthocyanins were determined in strawberry juice samples treated with microwave pasteurization, and anthocyanin content was observed to reduce from 40.4 to 34.1 mg L^{-1} (30 min), 38.7 mg L^{-1} (60 min), and 25.4 mg L^{-1} (90 min) depending on the treatment time (Mollov et al., 2007).

Feasibility of microwave sterilization (130°C, 5 kW) was studied on peanut-based liquids (peanut punch and Irish moss peanut drink) as an alternative to conventional UHT sterilization system and the results indicated that nutrient retention may be reachable due to temperature uniformity (Sabliov et al., 2008).

In addition, vitamin C content, chlorophyll content, and color properties of green tea leaves were quantified after seven different drying methods (sun, shade, microwave, freeze drying, oven 60°C, oven 80°C, and oven 100°C) among which the highest chlorophyll and vitamin C contents were determined with freeze drying and the worst drying method depending on the color properties were identified as sun drying (Roshanak et al., 2016). On the other hand, in another study, vitamin C content of orange juice samples remained similar after conventional and microwave processes (Villamiel et al., 1998).

2.1.2.3 Effect of infrared heating on beverage antioxidants

In the past, infrared radiation has been mainly applied in electronics, however, studies were also performed on food applications in recent times (Krishnamurthy et al., 2008b). Infrared radiation is placed between visible light and microwave in the electromagnetic spectrum within the wavelength range between 0.5 and 1000 mm (Rastogi, 2012). Infrared technology is used in the food industry for different purposes including heating process, chemical composition analyses, contactless temperature measurement from food surfaces (Bingöl, 2015). Different from ohmic and microwave heating, infrared heating is an indirect heating method in which energy is transmitted to food surface by radiation after that transformed to heat (Fellows, 2000).

The advantages of infrared heating are (1) instant heating due to direct heat penetration, (2) fast heat transfer, (3) higher heat transfer capacity, (4) uniform heating, (5) preservation of nutrients, (6) no heating of air, and (7) small equipment (Rastogi, 2012). Infrared radiation can be divided into three sections according to the wavelength as near (0.78–1.4 μm; ν = 12,500–4000 cm^{-1}), middle (1.4–3.0 μm; ν = 4000–200 cm^{-1}), and far (3.0–1000 μm; ν = 200–10 cm^{-1}) which is more convenient for food processing due to more effective absorption of energy in this range (Kümmerle et al., 1998; Rastogi, 2012). However, limited penetration depth is a negative side of infrared heating which requires additional energy source while drying or baking of larger volume of foodstuffs (Fellows, 2000). In addition, infrared heating is slower than microwave heat treatment (Hebbar et al., 2003). There are various processing options for solid food processing with infrared radiation including thawing, roasting, drying, baking, cooking, and blanching, but the advantages of infrared

heating are not that much while considering the heating of liquid foods (Rastogi, 2012).

The basic principle of heat generation from infrared radiation is molecular vibration, and therefore, infrared energy can be conducted throughout the food via conduction (Bingöl, 2015). There are several equipment designs for heat treatment of liquid foods (Figure 2.6) by infrared radiation. Providing information related with the equipments might be beneficial to better understand this process when considering challenges of infrared radiation in liquid food processing. For orange juice heating process, Vikram et al. (2005) developed a system composed of a heating chamber with infrared modules (250 W) which canalize infrared waves on to a platform by equipped reflectors. On the other hand, Krishnamurthy et al. (2008a) used a lab scale infrared heating system with a cone-shaped, aluminum waveguide due to its high emissivity (0.8) and six ceramic infrared lamps with a power of 500 W for heat treatment of milk. Hebbar et al. (2003) worked with a near infrared (NIR) batch oven with fitted infrared lamps (1.0 kW, peak wavelength 1.1–1.2 m) for heat treatment of honey. In this study, uniform power intensity was provided by fixing the distance between the sample and the heat source. In addition, uniform heating was achieved by mixing of honey samples between heating periods.

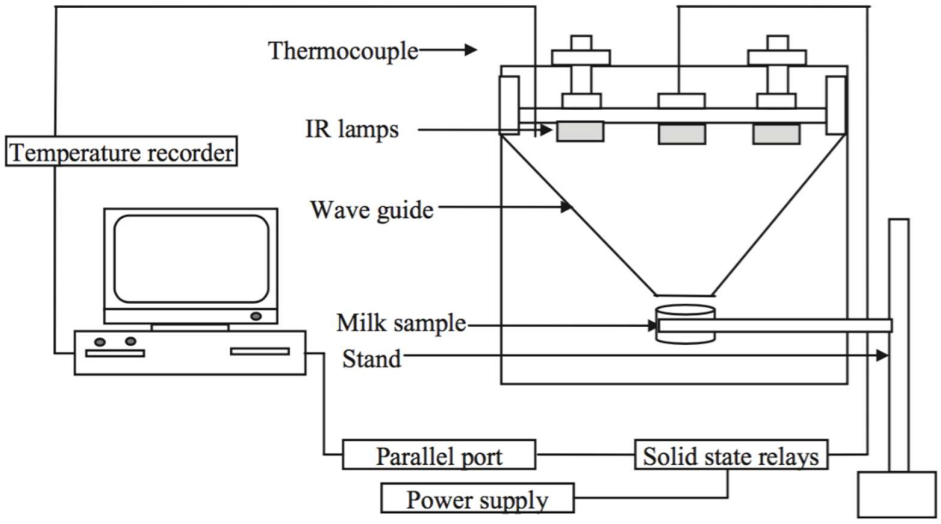


Figure 2.6 : Schematic diagram of infrared heating system (Krishnamurthy et al., 2008).

There are only few studies on infrared radiation of liquid foods and even fewer for beverages. Infrared radiation was applied as a heat treatment to milk (Krishnamurthy

et al., 2008a), orange juice (Vikram et al., 2005), honey (Hebbar et al., 2003), and beer (Vasilenko, 2001) in order to provide stabilization during storage period and the nutritional quality was evaluated after the treatment.

During infrared heating of orange juice samples, degradation of vitamin C [k-value (min^{-1}) was 0.0444 at 50°C, 0.0760 at 60°C, 0.0969 at 75°C, and 0.2284 at 90°C] was higher than the conventional method and the color [k-value (min^{-1}) was 0.0706 at 50°C, 0.1294 at 60°C, 0.3256 at 75°C, and 0.6108 at 90°C] increased with increasing processing temperature (Vikram et al., 2005).

The effect of infrared irradiation on yeast reduction and physicochemical characteristics of honey was investigated by Hebbar et al. (2003). In this study, heat treatment via infrared radiation could not reach the desired yeast inactivation level in short periods. Temperature of the honey samples increased to 85°C after 5 min heating which caused an increase in the hydroxymethylfurfural (HMF) content (220%) and a reduction in the enzyme activity (37%). In addition, yeast inactivation was achieved after 8 min of heat treatment.

Krishnamurthy et al. (2008a) used the infrared radiation for inactivation of Staphylococcus aureus in milk samples. This study revealed that infrared lamp temperatures, treatment time, and volume of treated sample are important parameters for the inactivation of microorganisms in milk samples. Total inactivation of S. aureus was achieved for 3 and 5 mL milk samples at a lamp temperature of 619 °C and treatment time of 4 min.

On the other hand, Kim et al. (2006) applied infrared radiation in the manufacturing process of green tea which is a rich source of polyphenols, especially catechins. This study indicated the positive effect of far infrared (FIR) radiation on polyphenol content of green tea. The total phenol content of green tea increased from 475.6 to 811.1 mg g^{-1} and the total flavanol content from 175.7 to 208.7 mg g^{-1} by FIR irradiation at 90 °C for 10 min which was applied instead of a roasting step. Furthermore, epigallocatechin and epigallocatechin gallate contents increased from 57.68 and 9.60 to 89.88 and 16.33 mg g^{-1}, respectively. In similar a manner, Lee et al. (2006) evaluated the catechin content and nitrite scavenging activity of green tea after FIR irradiation at eight temperatures (80 °C, 90 °C, 100 °C, 110 °C, 120 °C, 130 °C, 140

°C, and 150 °C). Total phenol and total flavanol contents of green tea increased from 244.7 to 368.5 mg g^{-1} and from 122.0 to 178.7 mg g^{-1}, respectively. In addition, increase in FIR radiation increased nitrite scavenging activity until the temperature reached 110°C. Park et al. (2009) reported increased total phenolic contents of green tea from 116.30 to 171.77 mg g^{-1}, total flavanol contents from 17.54 to 24.76 mg g^{-1}, ascorbic acid contents from 3.07 to 4.20 mg g^{-1}, and epicatechin gallate and epigallocatechin gallate from 2.41 to 4.59 mg mL^{-1} and 20.61 to 28.54 mg mL^{-1}, respectively. In addition, it was reported that nitrite scavenging ability was affected from pH. Nitrite scavenging ability was increased with FIR radiation at pH 3.0 and 4.2, however, there was no difference at pH 6.0. According to the results, it can be concluded that FIR radiation can be utilized to enhance green tea quality.

2.2 A Review of Microencapsulation Methods for Food Antioxidants: Principles, advantages, drawbacks and applications

Antioxidants as food preservatives have gained an increasing attention as they prevent foods from deterioration occured through oxidation, reduce loss of nutritional value and energy contents, allow freshness by ensuring flavours, odours, colour pigments, taste and texture. Consequently, numerous health benefits in reducing cancer, cardiovascular and neurological diseases as well as anti-inflammatory, antibacterial, anti-allergy, anti-hypertensive, antiviral and skin wound healing effects have been attributed to the role of dietary antioxidants (Alvarez-Suarez et al.2016; Ballesteros et al., 2017; Giampieri et al., 2013). Various food antioxidants have been classified into different categories based on their chemical structure and functions: water soluble bioactives including citrates, norbixin, betalains, most of the phenolics, flavanoids and anthocyanins, and lipid soluble components such as carotenoids, tocopherols, terpenoids and vitamin E (Carocho et al., 2017).

The relevant antioxidant activities of bioactive substances may be hampered due to their degradation triggered by light, oxygen, temperature, moisture and existence of unsaturated bonds in the molecular structures. Thus, microencapsulation with an appropriate carrier is an alternative technology for enhancing the storage and enviromental stability of bioactives as well as giving an advance to mask off-flavour, bitter taste and astringency of polyphenols (Ballesteros et al., 2017). Accordingly,

investigating the effects of microencapsulation techniques on food antioxidants is of great importance.

Microencapsulation is a process of packaging solids, liquids, or gaseous materials as active material with a continuous film as a coating to form capsules in micrometer to millimeter in size. Microencapsulation techniques are classified into three groups (Tyagi et al., 2011): (i) physical methods such as spray drying, lyophilization, supercritical fluid precipitation and solvent evaporation; (ii) physico-chemical methods including coacervation, liposomes and ionic gelation; (iii) chemical methods such as interfacial polymerization and molecular inclusion complexation (Table 2.3).

2.2.1 Physical methods

2.2.1.1 Spray drying

Spray-drying is an encapsulation technique related with the atomization of a liquid into a dry powder by means of an injector including a hot drying gas stream (Rattes and Oliveira, 2007). This technique comprises of three stages: (i) homogenization of feed liquid by an atomizer (ii) drying of feed solution by a hot gas carrier to achieve the evaporation of the solvent, (iii) collection of dry particles by cyclones or a filter (Schafroth, Arpagaus, Jadhav, Makne, & Douroumis, 2012). In detail, the feed liquid, which includes a core and wall material, may be a solution, an emulsion or a suspension (Gharsallaoui et al., 2007). Previously, this liquid is injected into the drying vessel through a nozzle or an atomizer in order to obtain small droplets followed by the evaporation of the solvent (Fatnassi et al., 2013), then, these dried particles are eliminated from the drying gas into a collector by a cyclone or filter (Schoubben et al., 2010) (Figure 2.7). The characteristics of spray-dried powders are related with the processing factors of spray drying including drying temperature, drying air flow rate, feed flow rate, speed of atomizer, sort of carrier agent and concentration of carrier agent (Schoubben et al., 2010).

Typical wall materials that have been used for spray drying are polysaccharides such as gum arabic, cyclodextrins and maltodextrin with different dextrose equivalent values, proteins such as whey proteins, sodium caseinate, soybean proteins and others including modified starch, gelatine, gellan gum, and chitosan (Lee and Wong, 2014).

Table 2.3 : Effects of microencapsulation techniques on the food antioxidants.

Applied Technique	Core material	Wall material	Results
Physical techniques			
Spray drying	Blueberry juice	Inulin and maltodextrin	Inulin based particles presents 5.1 and 1.5% of resver quercetin 3-d-galactoside, respectively, maltodextrin based particles include 21.1 and 28.5%
	Pitanga juice	High Performance Agave Fructans and High Degree of Polymerisation Agave Fructans and maltodextrin	The highest DPPH radical scavenging activity (59. ET/100 g solid) with maltodextrin-based micro (wall/core material ratio of 6/1)
	Blueberry polyphenols	Wheat flour, chickpea flour, coconut flour and soy protein isolate	90% retention of phenolic content by soy protein iso 16 weeks of storage at 4 and 20 °C
	Orange peel extract	Whey protein isolate	95% retention for both total phenolic content an radical scavenging activity
	Amla juice	Maltodextrin	Better retention of total phenolic content and DPP scavengin activity with 125 °C drying temperature maltodextrin concentration
	Pomegranate juice	Maltodextrin	Decrease in anthocyanin content with increasing maltodextrin concentration
	Beetroot juice	Maltodextrin	The highest betain retention (70.46%) with 15% maltodextrin Increasing DPPH scavenging activity (56.55 and 67.76%)
	Coffee phenolics	Maltodextrin, gum arabic and the mixture of both	Better retention with maltodextrin regarding flavono content (7.88 mg QE/100 ml) and total antioxidant a (380.25 mg α-TOC/100 ml)
	Guava	Maltodextrin and β-cyclodextrin	3% degradation in β-cyclodextrin based micropartic 2.5–3 times lower antioxidant potential of spray drie extract compared with concentrated extract
	Grape fruit	Gum Arabic and bamboo fiber	58% retention in phenolic content 92-94% retention in ascorbic acid

Table 2.3 (continued) : Effects of microencapsulation techniques on the food antioxidants.

Applied Technique	Core material	Wall material	Results	References
Lyophilization	Blueberry juice	Hydroxypropyl-β-cyclodextrin, β-cyclodextrin, maltodextrin	Higher retention values for HP-β-CD and β-CD, respectively of 105.21 and 104.55 μmol/L TE/g with ABTS, 1.83 and 1.80 mmol/L TE/g with DMPD, 79.70 and 67.28 mmol/L TE/g with FRAP	(Wilkowska et al., 2016)
	Green tea polyphenols	Maltodextrin, β-cyclodextrin, combination of both	Enhanced antioxidant activity (IC_{50} value 54.77 - 60.26 μg/ml)	(Pasrija et al., 2015)
	Gallic acid	Chitosan, β-cyclodextrin, xanthan	There were no significant effect	(da Rosa et al., 2013)
	Bayberry polyphenols	Ethyl cellulose	Increased free radical inhibition	(Zheng et al., 2011)
	Blackberry	Maltodextrin 10 and 20 DE	76% retention with maltodextrin 10DE 68% retention with maltodextrin 20DE	(Yamashita et al., 2017)
	Saffron petal	Cress seed gum, Gum Arabic	33% reduction in anthocyanin content of un-encapsulated extract No significant change after microencapsulation as well as storage of dried powders	(Jafari et al., 2017)
	Black glutinous rice bran anthocyanins	Black glutinous rice maltodextrin DE10, 20 and 30	~71.96% anthocyanin retention in the freeze dried powders Higher retention with black glutinous rice maltodextrin DE20 than commercial DE10	(Laokuldilok and Kanha, 2015)
	Garcinia fruit	Whey protein isolate, maltodextrin and the combination of both	Higher free (above 85%) and net (above 90%) HCA recovery	(Ezhilarasi et al., 2013a)
	Cherry juice	Maltodextrin and arabic gum	Enhanced monomeric anthocyanin content with freeze-dried encapsulated juice powder (67.5 mg/100 g) After 33 days of storage at 38 ºC, 90 % retention for freeze-dried powder	(Sanchez et al., 2015)

Table 2.3 (continued) : Effects of microencapsulation techniques on the food antioxidants.

Applied Technique	Core material	Wall material	Results	References
Supercritical fluids based techniques	Rutin	-	Completely dissolution of micronized rutin after 3 min in SGF and SIF	(Montes et al., 2016)
	Genistein	-	2 times higher dissolution rate comparing micronized and unprocessed genistein	(Xu and Luo, 2014)
	Yarrow extract	-	3 fold increase in the phenolic compound concentration of the precipitated particles	(Villanueva-Bermejo et al., 2017)
	Grape marc extract		250-350% increase of total phenolic content in SAS precipitates	(Natolino et al., 2016)
	Olive leave extract	-	1.35 antioxidant activity index for olive leave extract precipitates and 0.32 for unprocessed extract	(Chinnarasu et al., 2015)
	Quercetin	Ethyl cellulose	Enhanced antioxidant activity of quercetin/EC coprecipitates from 17.2 up to 25.1 μg DPPH/μg quercetin and from 13.7 up to 24.2 μg DPPH/μg quercetin after a year of storage at darkness and exposing to the visible light, respectively	(Fernández-Ponce et al., 2015)
	Grape pomace	Poly(-lactic-coglycolic acid)	Higher stability of extract released from the particles rather than crude extract	(Mezzomo et al., 2016)
	Folic acid	Polyvinylpyrrolidone	88% FA integrity comparing micronized folic acid (FA) with unprocessed FA, 95% folic acid integrity comparing coprecipitated particles including (PVP)–(FA) with the unprocessed physical mixture of PVP+FA	(Prosapio et al., 2015)
	Rosemary extract	Pluronic F88 and 127	High dissolution rate of coprecipitated particles	(Visentin et al., 2012)
	Lycopene	α-tocopherol	84,1; 90,2 and 97,1% lycope retention after 28 days storage at 25, 4 and −20 °C, Complete degradation of lycopene after storage at 25 and 4 °C, 27.2% retention after storage at -20 °C	(Cheng et al., 2017)
	Jabuticaba anthocyanins	Polyethyleneglycol	Enhanced stability against light and temperature with coprecipitated particles	(Santos et al., 2013)
	β-carotene	Poly-(ε-caprolactone)	Highest β-carotene concentrations with increasing pressure and temperature conditions	(de Paz et al., 2012)

Table 2.3 (continued) : Effects of microencapsulation techniques on the food antioxidants.

Applied Technique	Core material	Wall material	Results	References
Solvent evaporation	Quercetin	Polymethyl methacrylate	82% retention of quercetin after encapsulation	(Lee et al., 2007)
Coacervation	β – carotene	Casein/gum tragacanth	Functional contribution to scavenging activity of β – carotene	(Jain et al., 2016)
	β – carotene	Whey protein isolate/gum acacia	Increased fuctional stability and contribution to scavenging activity of β – carotene	(Jain et al., 2015)
	Broccoli particles	Gelatine/gum Arabic	Increasing chemical stability in terms of chlorophylls and polyphenol contents and antioxidant activity	(Sánchez et al., 2016)
	Black raspberry anthocyanins	Gelatin/gum Arabic	36% increase of anthocyanins after 2 months at 37 °C	(Shaddel et al., 2017a)
	Black raspberry anthocyanins	Gelatin/gum Arabic	48.57-70.10% retention after 60 days at 7 °C 12.92-23.66% retention after 60 days at 37 °C	(Shaddel et al., 2017b)
	Astaxanthin from shrimp waste	Gelatine/cashew gum	47% retention of the initial astaxanthin	(Gomez-Estaca et al., 2016)
	Capsanthin	Soybean Protein Isolate/chitosan	45.81-81.01% retention after 10 days at different RH 90.18-81.97% retention at different temperatures 85.84% and 62.91% retention after 10 days exposure to dark and outdoor light	(Xiao et al., 2014)
	Ascorbic acid	Gelatine/gum Arabic	57-80% retention after 30 days at 20 °C 32-44% retention after 30 days at 37 °C	(Comunian et al., 2013)
Liposomes	Carotenoids		Improving effect on the DPPH and FRAP activities of carotenoids Better protection with lutein and β-carotene by lipid peroxidation assay	(Tan et al., 2014)
	Green tea		No significant change on total phenolic content and antioxidant activity during one month storage of the processed compounds	(Dag and Oztop, 2017)
	Apigenin		High retention of the antioxidant properties of processed compounds	(Paini et al., 2015)
	Ascorbic acid		300-fold decrease in inhibition of ascorbic acid degradation	(Wechtersbach et al., 2012)

Table 2.3 (continued) : Effects of microencapsulation techniques on the food antioxidants.

Applied Technique	Core material - Wall material		Results	References
Liposomes	Eugenol α-lipoic acid	- -	A slight decrease (6-13%) in the inhibition capacity of the eugenol and α-lipoic acid loaded liposomes Potential applications for functional foods and nutraceuticals	(Trucillo et al., 2018) (Zhao et al., 2017)
Ionic gelation	Betalain	Sodium alginate sodium alginate-bovine serum albumin	The highest antiradical activity with calcium alginate beads is 88.5%	(Otálora et al., 2016)
	Yerba mate	Sodium alginate with/without corn starch	No significant effect of using starch filler in the antioxidant activities of beads	(López-Córdoba et al., 2014)
	Catechin	Pectin	1.8-fold higher FRAP value of entrapped catechin rather than free catechin	(Lee et al., 2009)
	Cocoa extract	Sodium alginate	Delayed release of polyphenols from internally produced beads	(Lupo et al., 2015)
	Dandelion extract	Sodium alginate, pectin and their whey protein blends	High retention of hydroxycinnamic acids (89.14%) with the combination of sodium alginate and whey protein	(Belščak-Cvitanović et al., 2016)
	Cocoa extract	Sodium alginate	60% retention of cocoa extract	(Lupo et al., 2014)
Chemical techniques				
	Chrysin	β-cyclodextrin	Enhanced antioxidant ability of chrysin in the inclusion with the increasing β-CD concentration	(Chakraborty et al., 2010)
	Vanillin	β-cyclodextrin	Protection of vanillin inside the cavity of β-CD from oxidation	(Karathanos et al., 2007)
	Quercetin	(2-hydroxypropyl)-β-cyclodextrin	129-fold increase in solubility of quercetin complexes; 85.6% retetion of quercetin in inclusion complex after 90 min radiation application, whereas 79.6% retention for uncomplexed quercetin	(Savic et al., 2015)
	Carvacrol	Hydroxypropyl-β-cyclodextrin	Lower antioxidant ability of carvacrol in the inclusion complexes	(Kamimura et al., 2014)

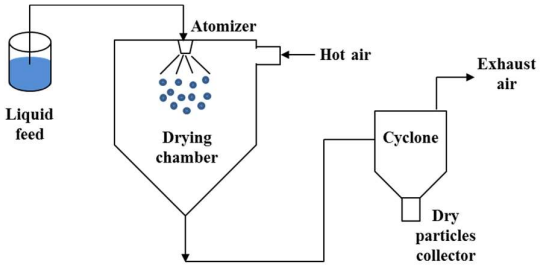


Figure 2.7 : Schematic diagram of spray drying.

This technology is applicable for an extensive range of food ingredients including flavors, colors, vitamins, minerals, fats and oils to prolong the shelf life stability against environmental conditions (Pillai et al., 2012).

A wide variety of food antioxidants have been encapsulated by spray drying method which were evaluated by means of total antioxidant capacity assays. Araujo-Díaz et al., (2017) studied the formation of microcapsulated blueberry juice by spray drying using inulin and maltodextrin as wall materials. The antioxidant activity of blueberry juice and blueberry juice loaded microcapsules were quantified by the content of resveratrol and quercetin 3-D-galactoside using HPLC. The results showed that there was a decrease in blueberry antioxidants after the spray drying process with both wall materials. In the case of using inulin, lower antioxidant concentration was obtained as 5.1 and 1.5%, for resveratrol and quercetin 3-D-galactoside, respectively; whereas, it was higher for maltodextrin as 21.1 and 28.5%. Results indicated that maltodextrin is a more effective wall material than inulin to coacerve the active materials in the microcapsule matrix due to the physicochemical properties of maltodextrin including amorphous microstructure. Similar trends were observed for microencapsulated pitanga juice in which the preservation efficiency of High Performance Agave Fructans (HPAF) and High Degree of Polymerisation Agave Fructans (HDPAF) were compared with the maltodextrin. The highest DPPH radical scavenging activity was found with maltodextrin based microcapsules as 59.53 mmol ET/100 g solid obtained under the conditions of 110 °C outlet air temperature and 1:6 core to wall material ratio, followed by HPAF as 53.38 mmol ET/100 g solid with 140 °C outlet air temperature and 1:6 core to wall material ratio (Ortiz-Basurto et al., 2017). It could be concluded that protection degree of the core material enhanced with the increasing proportion of the carrier agent. In addition to this, maltodextrin and fructants represent similar amorphous microstructures related with low water mobility which overcomes

the unfavorable biochemical reactions (Díaz et al., 2015). These outcomes are in agreement with the results reported by Ballesteros et al. (2017), who demonstrated that maltodextrin is superior against gum arabic and the mixture of both regarding phenolic and flavonoids, as well as their antioxidant activities.

In some studies, the impact of spray drying technique was discussed by means of antioxidant capacity as well as the total phenolic content. Correia, Grace, Esposito, and Lila et al. (2017) obtained particles of wild blueberry pomace extracts, which is rich in phenolic compounds, by spray drying, freeze drying, or vacuum oven drying using wheat flour, chickpea flour, coconut flour and soy protein isolate as encapsulating materials. It was clear that the soy protein isolate based microparticles obtained by spray drying showed the highest antioxidant capacity correlated with the anthocyanin content. Moreover, spray dried microparticles had higher storage stability in terms of total pheolic content (90% retention) during 16 weeks of storage period for both at 4 ℃ and 20 ℃ compared with other polyphenol-protein matrices (40% retention in chickpea flour+blueberry, vacuum oven dried). Sormoli and Langrish (2016) achieved 95% retention in total phenolic content and DPPH radical scavenging activity for orange peel extract encapsulated using whey protein isolate as encapsulants at 130 ℃ and 150 ℃ air inlet temperatures in comparison to the unprocessed core material. Mishra et al. (2014) reported the effect of spray drying conditions including drying temperature (125-200 ℃) and maltodextrin concentration (5–9%) on physicochemical properties of amla microparticles. Results indicated a significant decrease in total phenolic content and DPPH radical scavenging activity of amla powders by increasing the drying temperature and maltodextrin concentration because of the adverse effect of higher temperatures on the phenolic structure and concentration impact of maltodextrin. Similarly, Jafari et al., (2017) demonstrated that increasing maltodextrin concentration in pomegranate juice loaded microparticles led to a decrease in the anthocyanin content, which is highly correlated with the antioxidant activity. These results stated the utilization of maltodextrin just as a tool during spray drying process (Khazaei et al., 2014). In contrast, Bazaria and Kumar (2016) revealed the high retention of betalain and DPPH radical scavenging activity of encapsulated beetroot juice betalains with increasing amount of maltodextrin.

The influence of spray drying technique on flavonoid contents of dried microparticles was also studied. A glance at the results reveals the high stability of encapsulated guava extracts with maltodextrin based on the total flavonoid content; whereas there was a small degradation (3%) in β-cyclodextrin based microparticles. However, DPPH radical scavenging activities of microparticles found to be 2.5 – 3 times lower than the concentrated guava extracts for both maltodextrin and β-cyclodextrin wall materials (Fernandes et al., 2014).

Agudelo et al., (2017) assessed phenolic and ascorbic acid contents of microparticles containing grapefruit generated by gum arabic and bamboo fiber as encapsulating materials. In this sense, spray drying process caused a significant decrease in the content of phenolic compounds (42% decrease), nevertheless the retention levels were still high (92-94%) for the ascorbic acid.

When the advantages of this technique is evaluated, this technique is considered as rapid, continuous, simple, economic, reproducible, and easy to scale up in comparison with other drying processes such as freeze drying, hot air fluidized bed, and flash drying etc. which require high energy consumption (Gong et al., 2014). Besides, solid particles with low moisture content and water activity in addition to their high efficiency, quality and safety properties obtained after the process possess higher chemical, physical and microbial stability owing to the fact that final drying step is not necessary in spray drying, whereas it is required for other common techniques (Gula et al., 2013).

Regardless of the numerous advantages displayed by this technology, relatively high drying temperatures can damage sensitive compounds such as lycopene, β - carotene, anthocyanins, vitamin C, colors and flavors. Additionally, low product yield is reported due to loss of dry particles in the wall of drying vessel (Zhu et al., 2014). Moreover, its lack of controlling the droplet size and shape leads to a broad range of size distribution (Dalmoro et al., 2012). Another limitation of this technique is related with the wall materials. Low water solubility of polysaccharides (alginate, carboxymethylcellulose, guar gum) and proteins (whey proteins, soy proteins, sodium caseinate) result with limitations in practice of spray drying because of the higher water and lower dry matter contents which would need larger evaporation (Desai and Jin Park, 2005). Besides, it is not easy to process sugar rich materials without using

any carrier agents because of their low glass transition temperature and stickiness behavior (Bhandari et al., 1997). So, in conclusion the use of this technique should be decided considering the factors such as the solubility and heat sensitivity of bioactive compounds to be encapsulated, chemical structure and nature of the wall material, presence of other components such as sugars, proteins, etc., as well as economic and time aspects.

2.2.1.2 Lyophilization

Freeze drying, also known as lyophilization, is a multi-stage process that consists of freezing, sublimation (primary drying), desorption (secondary drying) and finally storage stages, resulting in a dry material (Laokuldilok and Kanha, 2015). The composition and structure of the wall material have a deep impact on the protection and controlled release of the core material (Young et al., 1993). Up to date, a plenty of food antioxidants have been encapsulated by freeze drying method. However, the effect of freeze drying process on food antioxidants in terms of antioxidant capacity were evaluated in few studies. Lowbush blueberry juice containing microcapsules using different wall materials such as hydroxypropyl-β-cyclodextrin, β-cyclodextrin and maltodextrin obtained by freeze drying method were examined in terms of antioxidant activity by 2,2'-Azinobis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging assay, N, N-dimethyl-p-phenylenediamine dihydrochloride (DMPD) radical scavenging assay and ferric reducing/antioxidant power (FRAP) assay. Results showed a significant retention of the antioxidant activity after the encapsulation process. The highest antiradical activity was determined by cyclodextrin preparations as 105.21 and 104.55 μmol/L TE/g with ABTS; 1.83 and 1.80 mmol/L TE/g with DMPD; 79.70 and 67.28 mmol/L TE/g with FRAP for hydroxypropyl-β-cyclodextrin and β-cyclodextrin, respectively (Wilkowska et al., 2016). In another study, green tea polyphenols were encapsulated by maltodextrin, β-cyclodextrin and combination of both to ensure the effectiveness of these phytochemicals in terms of antioxidative activity against the conditions of high temperature and alkaline pH. Freeze dried microcapsules (IC50 value 54.77 - 60.26 μg/mL) exhibited higher DPPH radical scavenging activity in comparison with spray dried microcapsules (IC_{50} value 58.13 – 72.86 μg/mL). Besides, wall material had also impact on the antioxidant activity and maltodextrin based microcapsules showed the highest rather than other wall materials used (Pasrija et al., 2015). On the other hand, wall materials had no

influence on the DPPH scavenging activity of gallic acid loaded microcapsules. Additionally, there was no significant difference of antioxidant activities between microencapsulated gallic acids (83.7 – 86.9%) and pure gallic acid (91.9%) (da Rosa et al., 2013). Similarly, Zheng, Ding, Zhang, and Sun (2011) carried out a study on free radical inhibition percentages of bayberry polyphenols in the presence of ethyl cellulose before and after microencapsulation process. According to the results, free radical inhibition percentages of the phenolic extract were found to be 75.35% for the pure extract and 83.13% for the encapsulated phenolic extract.

The influence of freeze drying technique on anthocyanin contents of dried microparticles was also studied. Yamashita et al., (2017) obtained microparticles of blackberry by-product extract, which is rich in anthocyanin content, by freeze drying using maltodextrin 10 and 20 dextrose equivalent (DE) as encapsulating adjuvants. Regarding anthocyanin content, a significant decline was obtained after microencapsulation process. After encapsulation of the extract, maltodextrin 10DE based microparticles (76%) showed better retention than maltodextrin 20DE based microparticles (68%). These results highlight the effect of DE on the retention of anthocyanins, in detail, larger mean diameter obtained by maltodextrin 10DE means smaller surface area for the possible degradation of the active material. Besides, Laokuldilok and Kanha (2015) investigated the influence of adjuvants with different DE values including enzymatically produced black glutinous rice maltodextrin 10, 20 and 30DE to encapsulate black glutinous rice bran anthocyanins. Results indicated that the average anthocyanin retention in the freeze dried powders was found to be 71.96 % and black glutinous rice maltodextrin 20DE showed the highest retention amoung other wall materials and also in comparison with commercial maltodextrin 10DE. On the other hand, Jafari et al., (2017) studied about the effect of wall materials in encapsulation process and revealed that there were no significant changes on the anthocyanin stability of saffron petal extract loaded dried powders including maltodextrin 20DE, maltodextrin 7-20DE mixture, gum Arabic-maltodextrin 20DE or gum Arabic-maltodextrin 20DE as wall materials. Besides, there was a 33% reduction in anthocyanin content of un-encapsulated extract after 10 weeks of storage, while there were no significant differences in microencapsulated powders after production as well as after 10 weeks of storage. In another study, enhanced monomeric anthocyanin content were found in freeze-dried encapsulated juice powder (67.5

mg/100 g) than in liquid cherry juices (23.5 mg/100 g). After 33 days of storage at 38 ºC, % monomeric anthocyanin retention was maintained at around 90% in the powder; whereas it decreased to 11% in the liquid juices (Sanchez et al., 2015).

Ezhilarasi et al. (2013a) encapsulated Garcinia cowa fruit rinds, which are rich sources of (-)-hydroxycitric acid (HCA), in whey protein isolate, maltodextrin and the combination of whey protein isolate and maltodextrin (1:1) by freeze drying technique. The effectiveness of wall materials was compared for their impact on HCA recovery. Microencapsulation of Garcinia extract using freeze drying resulted with higher free (above 85%) and net (above 90%) HCA recovery.

The most significant advantage of freeze drying is that it is a simple process carried out at low operating temperature with the absence of air resulting in prolonged and superior quality products by preventing deteriorations caused by oxidation or chemical modification (Anwar and Kunz, 2011). Freeze drying is the most suitable techique for dehydration of almost all heat sensitive substances such as natural oils, colors, aromas, drugs as well as water soluble components (Desai &and Jin Park, 2005).

However, freeze drying technique has some drawbacks such as long process time (more than 20 h), high capital and operating costs in comparison to others. Porous structure of freeze dried powders due to the ice sublimation during the process is also one of the major limitation (Anandharamakrishnan et al., 2010). Because of the fact that freeze dried powders must be crushed or gound into fine powders after drying, problems related with the lack of control over the particle size can be encountered. Besides, the active material within the capsule matrix are exposed to the atmosphere from the pores on the particle surface (Baldwin et al., 2011). As a result, the nature of the material to be encapsulated such as its porous structure seems to be the most critical factor to be determined for the use of this technique in addition to its cost.

2.2.1.3 Supercritical Fluids Based Techniques

A supercritical fluid is a solvent at a temperature and pressure above its critical point (Tc, Pc) at which it possesses properties between those of liquids, such as density and high solvating power, and gases, such as low viscosity, high diffusivities as well as high mass transfer rates. Numerous compounds could be stated at supercritical conditions including carbon dioxide, water, propane, nitrogen, etc. (Gouin, 2004).

Among these, carbon dioxide (CO_2) is the most commonly used supercritical fluid due to its moderate critical conditions (Tc = 31.1 °C, Pc = 7.38 MPa). Therefore, it is possible to avoid any alteration in properties of thermolabile substances in the case of several applications, such as micronization, encapsulation, extraction (Santos and Meireles, 2010), impregnation of drugs in aerogel (De Marco and Reverchon, 2017), membranes and scaffolds production (Baldino et al., 2015).

Various techniques depending on the use of supercritical fluids have been increasingly studied for several applications by means of coprecipitation and encapsulation. Coprecipitation is depicted as the simultaneous carrying down of an active compound with a carrier (Patnaik and Dean, 2004). In coprecipitates, the compounds can be entrapped, chemically bonded, absorbed in a polymer matrix or encapsulated by a polymeric coating (Ranjit and Baquee, 2013).

Supercritical fluid based processes are generally classified into three categories, regarding the role of the supercritical fluid (Munin and Edwards-Lévy, 2011):

- As a solvent: Rapid Expansion of Supercritical Solutions (RESS) and derived processes,

- As an anti-solvent: Supercritical Anti Solvent (SAS) precipitation and derived processes,

- As a solute: Particles from Gas Saturated Solutions (PGSS) and derived processes.

In RESS process, solutes including active compound and polymer are dissolved in a supercritical fluid followed by the expansion of solution using a small nozzle into a lower pressure region (Figure 2.8). This results with the precipitation of solutes due to dramatic decrease in solvent power of supercritical fluids (Debenedetti et al., 1993).

SAS process is based on bringing into contact a supercritical fluid, which acts as an antisolvent, with a solution including organic solvent and solutes of interest by injecting into a pressurized chamber through a nozzle (Sosa et al., 2011) (Figure 2.4). In contact with the solution, the supercritical fluid decreases the solubility of the solutes in the atomized particles, leading to their supersaturation, nucleation and formation of nano- or microparticles. Then, the organic solvent is eliminated from the particles under a continuous flow of supercritical fluid (Visentin et al., 2012). It is

possible to produce coprecipitates or microcapsules in a single step using a polymer and active compound soluble in the same solvent (Mattea et al., 2009).

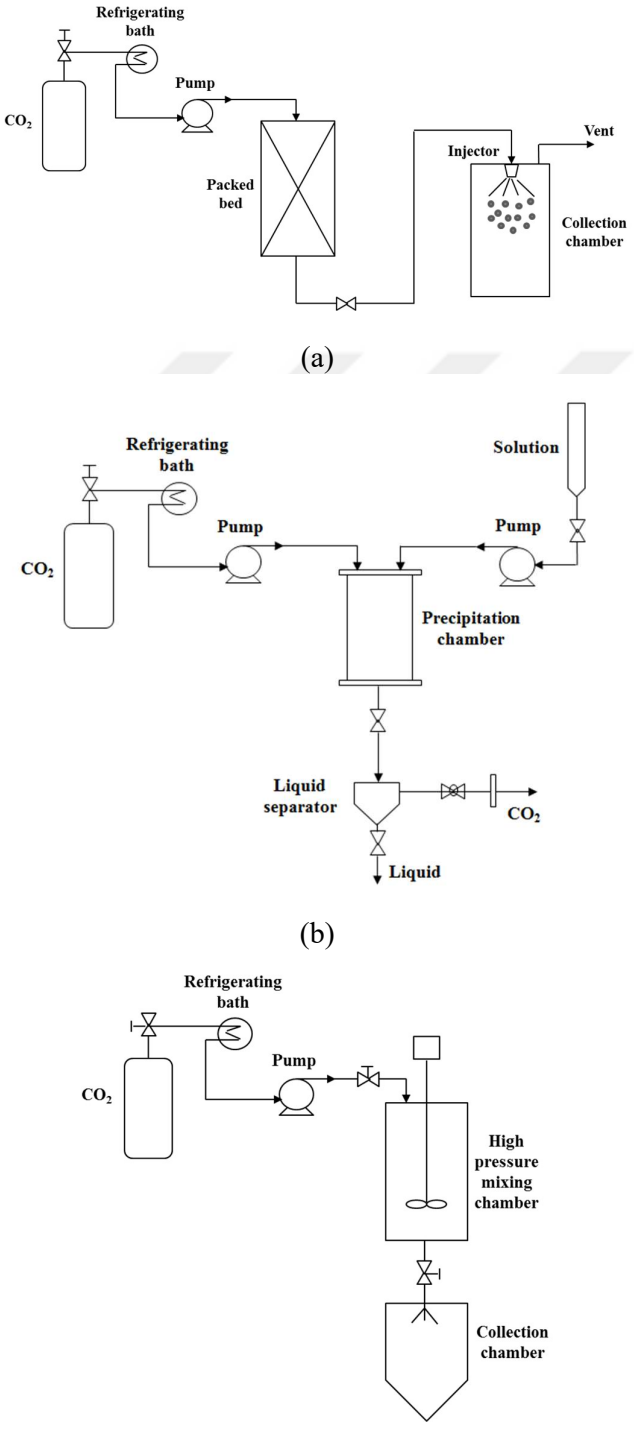


Figure 2.8 : Schematic diagram of the (a) RESS, (b) SAS and (c) PGSS apparatus.

PGSS is a process that includes the saturation of a solute with a supercritical fluid, followed by expansion through an atomization nozzle of this gas-saturated solution causing the formation of solid particles due to the cooling effect occurred by the release of the supercritical fluid (Mattea et al., 2009).

The encapsulation characteristics including morphology, particle size and encapsulation efficiency could be optimized by controlling the process parameters and formulations, such as pressure, temperature, supercritical fluid flow rate, supercritical fluid/solution ratio, overall concentration of solution and polymer/active compound ratio (Martín et al., 2007).

Coprecipitated microparticles can be successfully used in agricultural, biomedical, pharmaceutical, food and cosmetic fields in order to protect oxygen, heat or light sensible compounds against degradation and oxidation; to enhance solubility of poorly water solubles; to mask sensory properties such as color, taste and odor of the active substances as well as to achieve a controlled delivery of the active ingredients (De Marco et al., 2013).

Montes et al., (2016) carried out a study about the formation of spherical rutin sub-microparticles by SAS precipitation. The effect of SAS process on the solubility of rutin was quantified by following up the dissolution profiles of raw rutin and SAS processed particles in simulated gastric (SGF) and intestinal fluids (SIF). Results indicated that SAS processed particles completely dissolved after 3 min, whereas only 30 and 40% of the rutin dissolved in SGF and SIF. Furthermore, Xu and Luo (2014) acquired 2 times higher dissolution rates after 20 min comparing the micronized with unprocessed genistein. Additionaly, precipitates of ethanolic yarrow extract, which possesses high phenolic content related with the antioxidant activity, were produced using SAS technique. In the range of operating conditions studied, 3-fold increase in the phenolic compound concentration was covered with precipitated particles in comparison with the pure ethanolic yarrow extract (Villanueva-Bermejo et al., 2017). Natolino et al. (2016) also studied on micronization by SAS technology aiming to fabricate polyphenols containing precipitates from ethanolic grape marc extract. The results of this research highlighted the 250-350% increase of the total phenolic content in SAS precipitates in comparison with unprocessed extract. Chinnarasu et al. (2015) procured an antioxidant activity index equal to 1.35 for olive leave extract precipitated

using SAS technique, which is the value higher than those obtained as 0.32 for the unprocessed extract.

The micronization and coprecipitation of quercetin particles with ethyl cellulose (EC) was performed with SAS process. The antioxidant activity of unprocessed quercetin, the micronized quercetin particles and the quercetin/EC coprecipitates was analyzed by DPPH assay after one year of storage in darkness at room temperature (25 ℃) and after 20 days of exposure to visible light at room temperature (25 ℃). Results showed that unprocessed and micronized quercetin particles were prone to oxidation during storage in visible light at room temperature. However, the quercetin/EC coprecipitates did not show any reduction in the antioxidant activity in both during a year of storage at darkness and exposing to the visible light (Fernández-Ponce et al., 2015). Besides, Mezzomo et al. (2016) obtained coprecipitated particles of grape pomace extract, which is rich in antioxidants, by SAS process using poly (-lactic-coglycolic acid) to investigate the stability of SAS particles by comparing with crude extract. Results demonstrated higher stability of extract released from the particles rather than crude extract, unveiling the protective impact of the SAS process as well as to make use of a polymer as coprecipitate. Similarly, the enhanced stability of active material with the presence of a polymer in coprecipitates was proved by Prosapio et al. (2015). Folic acid integrity was found equal to 88%, comparing micronized folic acid (FA) with unprocessed FA; besides, 95% folic acid integrity was obtained comparing with coprecipitated particles including polyvinylpyrrolidone (PVP)–folic acid (FA) and the unprocessed physical mixture of PVP+FA. Moreover, pure precipitated and coprecipitated particles of rosemary extract using a polymer of Pluronic F88 and 127 by SAS process were examined in terms of dissolution rate. A glance at the results revealed the high dissolution rate, as 1 hour, of the phenolic content from the co-precipitated product, while only 15% of the phenolics of the pure precipitated dissolved after 8 hours (Visentin et al., 2012). Cheng et al. (2017) studied the stability of encapsulated particles of lycopene, obtained by SAS process using α-tocopherol, during 28 days of storage at 25, 4 and −20 ℃. According to the results, lycopene retention of encapsulated particles was found to be 84,1; 90,2 and 97,1% after storage at 25, 4 and −20 ℃, respectively. In contrast, non-encapsulated lycopene completely degraded after storage at 25 and 4 ℃, whereas 27.2% retention was determined after -20 ℃.

Regardless of the supercritical methods in which supercritical fluid is used as an antisolvent, there are also few studies based on RESS and PGSS technologies. Santos et al. (2013) obtained coprecipitated particles of jabuticaba extract, which is rich in anthocyanins, by RESS method using polyethyleneglycol, indicating enhanced stability against light and temperature comparing with the free extract. On the other hand, de Paz et al. (2012) presented a study related with the formation of coprecipitates with β-carotene and poly-(ε-caprolactone) using PGSS process. According to the results, diminished β-carotene content was obtained with coprecipitated particles; additionally, the highest β-carotene concentrations were ensured with increasing pressure and temperature conditions.

There is an increasing interest in using supercritical CO_2 (SC- CO_2) as a result of its nontoxicity, non-flammability, low price and easy removal from the final product by simple depressurization (Reverchon and Adami, 2006). Techniques assisted by supercritical fluids have become an efficient alternative to overcome some of the disadvantages of conventional processes: poor control of particle size and morphology, degradation and lost of biological activity of thermo sensitive compounds, low encapsulation efficiency and low precipitation yield (Santos and Meireles, 2010). Moreover, these technologies have potential advantages as avoiding complex purification steps during post-processing; indeed, in traditional precipitation the organic solvents have to be post precessed, whereas supercritical fluids enable the removal of solvent out of the droplets resulting in solvent-free particles (Martín et al., 2007). On the other hand, the only limiting factor of supercritical techniques is related with the selection of the supercritical process based on the solubility of the active material to be encapsulated and polymer matrix in the supercritical fluid (Bahrami and Ranjbarian, 2007).

2.2.1.4 Solvent Evaporation

Solvent evaporation is defined as solvent removal from an emulsion consisting of a polymer volatile organic solvent in water (Poncelet, 2006). This technique is based on four major steps: (i) dissolution of polymer as coating and active compound in an organic solvent to form a suspension, an emulsion or a solution; (ii) emulsification of the organic phase (dispersed phase) in an aqueous phase (continuous phase) by stirring, static mixing, extrusion or dripping; (iii) solvent removal by evaporation or liquid

extraction and (iv) recovery of particles by filtration or centrifugation and drying of the microspheres (Hwisa et al., 2013). Several process variables could influence the formation of microsphere such as the nature of the solvent, volume of the solvent, polymer concentration, emulsifier type and concentration, rate of solvent removal, addition of buffer or salts to the internal or external phase, phase volume ratio and temperature (Tiwari and Verma, 2011).

Solvent evaporation technique could be classified as solvent evaporation (emulsification-evaporation) including oil-in-water emulsions, multiple emulsions such as water-in-oil-in-water and nonaqueous emulsions, and solvent extraction (emulsification-extraction). The solvent evaporation method has attracted attention, due to its characteristics including use of mild conditions, ease of use and scale-up, lower residual solvent and no change on the activity of bioactive compounds (Hwisa et al., 2013). This method has been used to prepare polylactide, poly (lactic-co-glycolic) acid, polymethyl methacrylate, dimethylaminoborane, ethylcellulose, polyethylene glycol, polycaprolactone, eudragit, polyvinylic alcohol and kafirine based matrices (Munin and Edwards-Lévy, 2011).

Although this technique is well defined for its use in nanoparticle production and the investigations on drugs, as the methodology, there are only few studies about the impacts of the microencapsulation process on the food antioxidants. Lee et al. (2007) studied the formation of quercetin loaded particles by polyol-in-oil-in-polyol emulsion solvent evaporation method using polymethyl methacrylate as the wall material. Quercetin retention of free quercetin and quercetin loaded particles were quantified after 28 days of storage at 42 ℃. Results indicated a significant retention of quercetin after the encapsulation process (82%), whereas it was 18% for the free form. However, as there is lacking information on the effect of this technique, future studies are required.

2.2.2 Physico-chemical methods

2.2.2.1 Coacervation

Coacervation technique can be defined as a colloidal phenomenon that involves liquid – liquid phase separation of a single or a mixture of two oppositely charged polymers in aqueous solution triggered by electrostatic interactions, hydrogen bonding,

hydrophobic interactions, polarization-induced attractive interactions as well as chemical or enzymatic cross-linker agents including glutaraldehyde or transglutaminase (Xiao et al., 2014) (Figure 2.9). The power of the interaction between the biopolymers depends on various factors such as the biopolymer type (molar mass, flexibility, and charge), pH, ionic strength, concentration, and the ratio of the biopolymers (Turgeon et al., 2007).

The process of coacervation may be either simple or complex depending on the number of polymer used (Ezhilarasi et al., 2013b). While simple coacervation involves only one type of polymer with the addition of strong hydrophilic agents to the colloidal solution, complex coacervation is produced by mixing two or more polymers types for wall formation around an active core. In detail, complex coacervation method is carried out firstly preparation of an emulsion dispersing a core material into an aqueous polymer solution. Then, it is followed by wrapping of that phase as a uniform layer around the core material by adding the second aqueous solution promoted by the addition of salt, changing the pH, temperature or dilution of the medium. Finally, stabilization of the microcapsules by cross linking, desolvation or thermal treatment (Gaonkar et al., 2014). Soluble, aggregated or precipitated complexes are obtained after filtration or centrifugation applied to obtain these microcapsules, followed by washing with an appropriate solvent and drying (Livney, 2008).

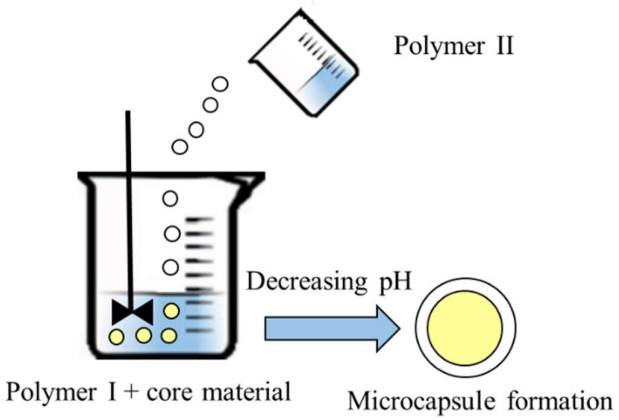


Figure 2.9 : Fabrication of microencapsules by complex coacervation.

A large numbers of coating materials have been evaluated for simple coacervation including gelatin, alginate, chitosan, glucan, and cellulose derivatives and for complex coacervation including gelatin/gum arabic, gelatin/caroboxymethyl cellulose, alginate/polylysine, alginate/chitosan, albumin/gum arabic, and glucan/cellulose

derivatives. The most studied coating system is gelatin/gum arabic in which gelatin is used as a a positive polyelectrolyte and gum arabic is used as a negative polyelectrolyte (Dubey, 2009).

Coacervation has been widely used for the encapsulation of lipophilic materials such as turmeric oleoresin (Zuanon et al., 2013), palm oil and β-carotene (Rutz et al., 2016), lycopene (Silva et al., 2012), lutein (Qv et al., 2011), vitamin E (Alencastre et al., 2006); however the process has also potential for encapsulation of hydrophilic substances (Comunian et al., 2013).

The effects of the coacervation process on food antioxidants have been investigated in some of the studies. Jain et al., (2016) studied the formation of microcapsulated β-carotene by complex coacervation using casein and gum tragacanth as wall materials. The antioxidant activity of free β-carotene and freeze dried carotene loaded microcapsules were measured by means of 2,2-diphenyl-1-picrylhydrazyl (DPPH) method up to 3 months to examine the impact of encapsulation of β-carotene. Results showed that there was a significant difference after 3 months in the percent scavenging activity of free β-carotene and freeze dried β-carotene loaded microcapsules, indicating that the prolonged stability of β-carotene was quite functionalized within the complex microcapsule matrix. Moreover, the same research group carried out a study to overcome poor water solubility and low stability properties of β-carotene by complex coacervation technique using whey protein isolate and gum acacia. Then, they evaluated the microcapsules in terms of percent scavenging activity using DPPH assay. According to the results, there was a significant difference after 3 months in the percent scavenging activity of free β-carotene and freeze dried β-carotene loaded microcapsules and it is clear that the antioxidant activity of β-carotene could be maintained by using micro-carriers for long time storage (Jain et al., 2015).

Sánchez et al., (2016) obtained microencapsulated broccoli particles, which are rich in chlorophyll content, by complex coacervation using gelatin/gum arabic as encapsulating materials to enhance its chemical stability. It was found that microencapsulation process enabled to preserve the chlorophyll content (10.00 ± 0.13 mg/kg), total phenolics content (4.33 mg 3,4-dihydroxybenzoic acid/g), and antioxidant activity (21.65±0.88 mg trolox/g) of broccoli.

Besides, black raspberry anthocyanins were investigated in terms of their storage stability for 60 days at different temperatures after applying double emulsion prior to complex coacervation using gelatin/gum arabic as wall materials. The coacervation process was studied as a function of wall and the core material ratios, the concentration of polymer solutions and pH values. Results demonstrated high retention of microencapsulated anthocyanins (up to 36%) after 2 months of storage at 37 ºC (Shaddel et al., 2017a), which have similar trends that were observed as 48.57-70.10% and 12.92-23.66% retention of black raspberry anthocyanins after 60 days of storage at 7 ℃ and 37 ℃, respectively (Shaddel et al., 2017b). It was also found that astaxanthin from shrimp waste was prevented (47% retention) from degradation when encapsulated by complex coacervation in a gelatine/cashew gum matrix (Gomez-Estaca et al., 2016).

Xiao et al. (2014) obtained 45.81-81.01% retention after 10 days at different relative humidity values (33, 58, 68 and 98%), 90.18-81.97% retention at different temperatures (60, 80 and 100 ℃) as well as 85.84% and 62.91% retention after 10 days exposure to dark and outdoor light for capsanthin encapsulated using complex coacervation with soybean protein isolate/chitosan as encapsulants.

On the other hand, ascorbic acid was evaluated as a core material within gelatine/gum arabic matrix which was acquired by complex coacervation technique. A glance at the results reveals the high stability with 57-80% and 32-44% retention of encapsulated materials after 30 days of storage at 20 and 37 ℃, respectively (Comunian et al., 2013).

Coacervation technique is superior to the other microencapsulation techniques because of its high loading capacity, low temperature, reduced evaporation losses or thermal degradation and compatibility to control the release of active materials (Taneja and Singh, 2012). Furthermore, a specific equipment is not required for its implementation (Gomez-Estaca et al., 2016) and it has simple preparation conditions such as non-toxic solvent and low agitation utilization (Jain et al., 2016). On the other hand, high cost of the particle isolation procedure and complexity of the technique should also be taken into account (Gouin, 2004).

2.2.2.2 Liposomes

Liposomes are vesicles consisting of single or multiple bilayers mainly composed of phospholipids which have both hydrophilic head and hydrophobic tail groups. Lamellar aggregates are carried out by the simple dispersion of phospholipids in water, while typical spherical shape of liposomes are obtained with the use of sufficient energy which is supplied by solvent evaporation, electroformation, thin film dehydratation/rehydratation, proliposome, membrane extrusion, dialysis, ultrasonication and high-pressure homogenization (Reza Mozafari et al., 2008) as well as superlip process (supercritical assisted liposome formation) (Trucillo et al., 2018). Size and structure of the liposomes depends on the composition, preparation method, and environmental conditions (Reza Mozafari et al., 2008).

Microencapsulation by liposomes has been investigated for drug delivery, cosmetics, pharmaceuticals and food industry (Reza Mozafari et al., 2008). With regard to food applications, it has a great inherent potential for encapsulating flavorings, essential oils, amino acids, vitamins, minerals, colorants, enzymes, microorganisms, antioxidants, antimicrobial agents, preservatives, and omega-3-fatty acid (Reza Mozafari et al., 2008).

Liposomes have been used for the evaluation of antioxidant properties of several lipophilic and hydrophilic antioxidants against oxidants. For example, in the study about carotenoids including lycopene, β-carotene, lutein, and canthaxanthin loaded liposomes delivery system, the process of encapsulation was examined by measuring the antioxidant activities using 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant powder (FRAP) and lipid peroxidation (LPIC) assays. Results demonstrated that encapsulation process improves the antioxidant activities of carotenoids measured with the DPPH and FRAP assays. Moreover, the highest antioxidant activity was observed with lutein, followed by β-carotene, lycopene, and canthaxanthin by incorporation into liposomes. According to the lipid peroxidation assay, lutein and β-carotene showed better protection against pro-oxidation, while it was weak for lycopene and canthaxanthin (Tan et al., 2014). Dag and Oztop (2017) carried out an experiment on green tea extract loaded liposomes to analyze the effect of encapsulation process on the stability of catechins. There was no significant change on the total phenolic content as well as antioxidant activity during one-month storage

of processed compounds. Similarly, the apigenin amount was preserved after the encapsulation process by liposome vesicles, which has been applied to overcome apigenin's low solubility and stability; the retention of the antioxidant properties of processed compounds was testified by calorimetric measurements (Paini et al., 2015). Furthermore, Wechtersbach et al. (2012) indicated up to 300-fold decrease in the inhibition of ascorbic acid degradation for co-encapsulation of citric and ascorbic acids into liposomes.

In another study, Trucillo et al. (2018) aimed to preserve beneficial effects of amphiphilic (eugenol) and a lipophilic (α-lipoic acid) antioxidants in liposome vesicles through a supercritical CO_2 assisted process. It was obtained that there was only a slight decrease (6-13%) in the inhibition capacity of the eugenol and α-lipoic acid loaded liposomes with respect to the unprocessed compounds. Hence, it could be concluded that the antioxidant power of eugenol and α-lipoic acid was protected after processing. Zhao et al. (2017) also utilized supercritical CO2 to generate liposomes for anthocyanin encapsulation and indicated the potential applications for functional foods and nutraceutical applications.

As an encapsulation system, the structural properties of liposomes arise from their ability of entrapping hydrophilic, lipophilic, and amphiphilic molecules (da Silva et al., 2010). Other unique properties of liposomes are their high bioavailability, biocompatibility, biodegradability, and high cell membrane permeability (Slingerland et al., 2012).

Regardless of the advantages displayed by this technology, the main limitations in liposome encapsulation are scaling up of the process at acceptable cost levels, poor physical and chemical stability, a wide range of particle size distributions, lipid oxidation (Tan and Misran, 2013) and the necessity of complex post-treatment steps (Trucillo et al., 2018). In summary, even though this method provides high bioavailability of the bioactive compound their low physical and chemical stability should be considered during its application.

2.2.2.3 Ionic Gelation

Ionic gelation is one of the microencapsulation techniques based on the ability of crosslinking polyelectrolytes in the presence of multivalent ions such as Ca^{2+}, Ba^{2+} and Al^{3+} (Yeo et al., 2001) and can be applied by either extrusion or emulsification/gelation (Lupo et al., 2015). Extrusion is the most common method to fabricate spherical gel particles by the way of dripping an aqueous polymer solution through a syringe needle or a nozzle into a $CaCl_2$ containing gelling bath (Paques et al., 2014). Emulsification/gelation method basically involves the production of an emulsion including hydrophobic active component in a polymer solution, then dripping into the calcium solution (Paques et al., 2014). Encapsulation by gelation technique could be carried out externally or internally. In external gelation, Ca^{2+} ions diffuse from an external source into the polymer solution (Davarcı et al., 2017). On the other hand, calcium salt including liquid solution is added dropwise into the polymer solution in internal gelation method, which results in the production of an aqueous-core calcium alginate capsules (Funami et al., 2009).

Alginate, chitosan, pectin, konjac, gellan gum, carboxymethyl cellulose are the polymers employed in cross linked gelling systems. In this context, alginate is the most commonly used polymer owing to its non-toxic, biodegradable and biocompatible properties as well as superior gelling properties under safe and mild conditions (Leong et al., 2016a).

Alginates have a wide range of applications including enzyme immobilization and controlled release of drugs. Otálora et al. (2016) studied the formation of betalain loaded beads by external ionic gelation using sodium alginate and the combination of sodium alginate-bovine serum albumin as wall materials. Antiradical activity of cactus betalain extract and cactus betalain extract containing beads were quantified after 25 days of storage at different conditions (25-50 °C/34.6-84.3% RH). The results showed that betalain retention was related with the type of matrix used in the beads and RH values. In detail, betalain retention diminished with the increase in moisture content. Antiradical activity in calcium alginate beads were the highest as 88.5%, whereas it was 80.6% for calcium alginate-bovine serum albumin beads at the best storage conditions of 34.6% RH at 25 °C, which are the values significantly greater than the cactus pulp extract as the control sample (75.1%). Similarly, yerba mate extracts were

encapsulated into alginate matrix, which contains corn starch as a filler material to enhance the structural properties, using external ionic gelation method to analyze the effect of gelation on antioxidant activity values of beads. It was reported that there was no significant effect of using starch filler in the antioxidant activities of beads. It could be concluded that there were no interactions between yerba mate polyphenols and the encapsulating matrix, thus the encapsulation method used did not alter the antioxidant activities (López-Córdoba et al., 2014).

In contrast, Lee et al. (2009) investigated the *in vitro* antioxidant activity of catechin loaded pectin matrix, produced by internal ionic gelation method, in a simulated intestinal fluid. The FRAP value of entrapped catechin increased continuously and finally reached a 1.8-fold higher value than that of free catechin, demonstrating the unstable property of catechin in an alkaline environment. On the other hand, Lupo et al. (2015) investigated the influence of two mechanisms including external and internal ionic gelation on the release of polyphenols from the alginate beads loaded with cocoa extract. Beads prepared by internal gelation displayed delayed release of polyphenols due to more homogeneous and compact structure.

Generally, emulsification/internal gelation has been suggested as an alternative due to its higher entrapment efficiency of bioactives than in case of extrusion/external gelation. In this context, hydrophilic polyphenols from dandelion extract and lipophilic β-carotene compounds were encapsulated in hydrogels based on sodium alginate, pectin and their whey protein blends by emulsification/internal gelation method. These results reveal that the combination of alginate with whey protein as the carrier matrix was superior due to its high retention of hydroxycinnamic acids (89.14%), accounting the descending diffusivity of polyphenols through the calcium alginate matrix as well as interactions between protein-polysaccharide and protein-polyphenol inside the bead structure (Belščak-Cvitanović et al., 2016). Lupo et al. (2014) also carried out an experiment regarding to emulsification/internal gelation method and found 60% retention of cocoa extract in sodium alginate based microspheres.

To conclude, emulsification/internal gelation method is proposed as an alternative to extrusion/external gelation for operating high quality microspheres with small diameters (Ahmed et al., 2013).

2.2.3 Chemical methods

2.2.3.1 Interfacial polymerization

Wall formation in this technique is characterized by polymerization, in which hydrophilic and lipophilic monomers interface at an oil-water emulsion and react to form a polymeric membrane on the surface of the droplet or particle (Yeo et al., 2001) (Figure 2.10). Due to the fact that this type of polymerization does not require catalysts and be empowered at low temperature, the interfacial polymerization technique can be applied for microcapsule preparation (Ichiura et al., 2005). The yield and quality of the polymeric membrane fabricated by this technique could be optimized by controlling the process parameters including the monomer concentrations, temperature, mixing rate, as well as the reaction time (Mathiowitz, 1999). Mainly four types of polymers have been developed to produce microcapsules by interfacial polymerization, consisting of polyamides, polyurethanes, polyureas and polyesters (Perignon et al., 2015).

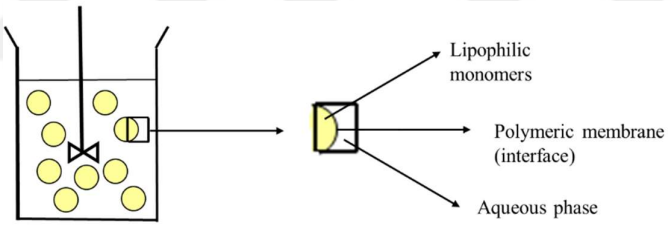


Figure 2.10 : Microcapsule formation by interfacial polymerisation.

Interfacial polymerization technique has potential advantages including possible control of capsule mean size and membrane thickness, high loading of active compound, versatile and stable mechanical and chemical properties of the membrane, low cost, easy to scale-up, simplicity and reliability of the process (Perignon et al., 2015). On the other hand, there are also some factors limiting the application of this technique. Indeed, it is difficult the production of large oil-water interface, where proteins or enzymes are prone to inactivation, altering biological activities of proteins in large amounts during polymerization reaction. This technique is lack of control on the polymerization characteristics including yield and quality of the polymer membrane. Additionally, requiring washing steps to remove monomers, by-products, organic solvents and surfactants, leading to loss of water soluble active substances as well as harm to acid labile actives by means of HCl by-product formation resulting

with a change in pH are other drawbacks of interfacial polymerization (Yeo et al., 2001). Relatively harsh initial conditions (high pH, toxic monomers, solvents and reaction products) for formulations delimitate the applications on the microencapsulation of active compounds (Perignon et al., 2015).

2.2.3.3 Molecular inclusion complexation

Molecular inclusion is an encapsulation technique which takes place at molecular level, consisting of entrapment of the guest (active) compound by a host (polymer) through physicochemical forces, such as hydrogen bonding, van der Waals forces or hydrophobic interactions (Marques, 2010). These complexes are formed through a reaction which takes place only in the presence of water (Desai and Jin Park, 2005). The most common "host" molecules are cyclodextrins (CDs), which are composed of a hydrophilic external part and an internal hydrophobic part. The guest molecule in apolar character could be entrapped into the apolar internal cavity by means of hydrophobic interactions (Pagington, 1986) (Figure 2.11).

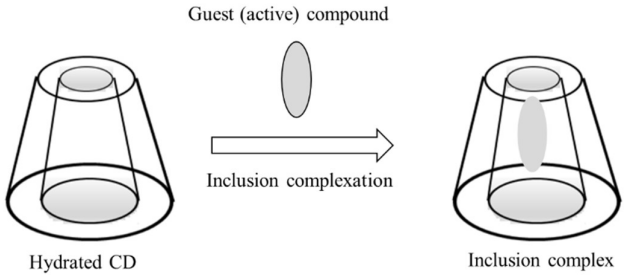


Figure 2.11 : A schematic drawing of molecular inclusion complexation.

Cyclodextrins, chemically and physically stable molecules, are produced by the enzymatic modification of starch and consisting of six (α-cyclodextrin), seven (β-cyclodextrin), eight (γ-cyclodextrin) or more glucopyranose units linked by α-(1,4) bonds (Karathanos et al., 2007). Only the first three cyclodextrins are generally recognized as safe by the United States Food and Drug Administration (López-Córdoba et al., 2014). There are three methods for fabricating the active- β -cyclodextrin complex. In the first method, β-cyclodextrin is dissolved in water and the actives are added to form an inclusion complex in crystalline form. In the second method, β -cyclodextrin is dissolved in a lower amount of water than in the first method to form a concentrated suspension and the actives are mixed to form an inclusion complex in crystalline form. In the last method, β-cyclodextrin is dissolved in a much

lower water content to form a paste and the actives are mixed during kneading to form an inclusion complex. The third method is superior due to the fact that it does not demand post-process applications, whereas the last step of former two methods is based on further separation and drying (Pagington, 1986).

A large number of poorly water-soluble molecules was entrapped in cyclodextrins in order to achieve high stability for food antioxidants. Chakraborty et al., (2010) studied the production of inclusion including entrapment of chrysin, which is a naturally occuring flavone, into β-CD as a drug delivery vehicle. The antioxidant activity of unprocessed active compound and inclusions were quantified by ABTS scavenging assay. The results indicated that inclusion complexes obtained in this study are based on van der Waal's interaction and hydrogen bonding. Additionally, the antioxidant ability of chrysin in the inclusion enhanced with increasing β-CD concentration. These results are in agreement with Karathanos et al., (2007) who reported that complexes of vanillin inside the cavity of β-CD not only allowed the active compound to be more soluble in water, but also protected from oxidation.

Savic et al. (2015) aimed to investigate the solubility and photostability of quercetin by forming an inclusion complex with (2-hydroxypropyl)- β-CD under the simpler operating conditions. A glance at the results revealed a 129-fold increase in the solubility of quercetin complexes. Moreover, while the quercetin content in the inclusion complex decreased to 85.6%, it was determined to be 79.6% for noncomplexed quercetin, after exposing to radiation for 90 min. In contrast, Kamimura et al. (2014) showed that carvacrol, which possesses high antioxidant activity, upon inclusion into hydroxypropyl- β-CD cavity comprised lower antioxidant activity, indicating inclusion complexes prevailed dimished amount of scavenging the free radicals.

The application of CD-assisted molecular encapsulation in foods has advantages not only for the protection of active ingredients against oxidation, heat and light-induced decompositions (Li and McGuffin, 2007) and to prolong the shelf-life of the products by control release of active substances, but also leading to enhance dissolution rate and bioavailability of guest compounds. Inclusion complexes have also been used for the encapsulation of volatile organic molecules, for masking of odors or flavors, or preservation of aromas (Ezhilarasi et al., 2013b). The efficiency of the molecular

inclusion could be controlled by the process parameters such as the geometric compatibility between compounds, the structure, charge and polarity of the guest and the host cavity as well as the solvent and the inclusion temperature (Astray et al., 2009).

2.3 Effects of Encapsulation on the Bioaccessibility and Bioavailability of Phenolic Compounds

The dynamic market of so called 'superfoods' grows steadily worldwide and offers new health-improving products regularly, although some of these foods evolved into established products or food additives e.g. Goji berries and Chia seeds, which are available in each supermarket (Wetters et al., 2018). While the demand for superfoods and healthier foods arose over the last years, the positive relation between nutrition and health become more and more pronounced and forces the development of these kind of products (Tichy et al., 2020). Moreover, the individual-related and specific nutrient supply, especially for the elderly, comes into focus. In 2050, nearly 16% of the world population will be aged over 65, whereby the demand for personalized functional foods will be increased in parallel as the population ages (Jafari and McClements, 2017).

Despite of the market growth in functional and healthier foods, their beneficial effects are controversially discussed e.g. as reviewed by Marian (2017). In this review, nutrition studies with healthy humans consuming dietary supplements were summarized. Conclusively, most of the studies showed health-improving effects induced by the supplements, but rather at high doses which are unusual for the dietary intake (Marian, 2017). For example, one of these food supplements is resveratrol, a naturally occuring phytoalexin, that is synthesized in plants e.g. grapes, as a response to injuries (Pannu and Bhatnagar, 2019). Resveratrol, as a food additive, possess various health promoting effects including high antioxidant and anti-inflammatory potential, anticarcinogenicity in breast and liver tissue, prevention of osteoporosis, improving ischemic diseases and muscle regeneration, etc. (Weiskirchen and Weiskirchen, 2016; De Vries et al., 2018). Unfortunately, these health-improving effects have been mainly analyzed in cell culture studies or preclinical models, which makes the application of effective concentrations and substances more difficult on humans (Weiskirchen and Weiskirchen, 2016). For example, the functionality of

resveratrol is limited owing to its low bioavailability (Pannu and Bhatnagar, 2019). While the solubility of resveratrol in aqueous solutions is 3 mg/L, the solubility is enhanced to 50 g/L in ethanol, which results in a higher uptake and plasma concentration of resveratrol with lipophilic-based food matrix. Besides, the bioavailability is too low to reach effective doses up to 1 g/day only by consumption of resveratrol-containing food. Theoretically, the consumption of about 3,500 L of rose wine, 2,600 kg of white grapes, up to 35,000 kg of peanuts or 2,500 kg of apples per day were found to be necessary to reach these daily intake doses (Weiskirchen and Weiskirchen, 2016).

These results illustrate the need of developing new delivery systems for bioactive compounds, which show low bioavailability values (Brglez Mojzer et al., 2016), by altering the molecular structure or the physiochemical characteristics of polyphenols (De Vries et al., 2018). The pharmaceutical industry developed technologies to improve drug delivery systems, which could be transferred to the food industry and may be also helpful for nanoscale delivery systems for food products (Jafari and McClements, 2017). The encapsulation of these compounds using nanoparticles, nanodelivery carrier or various emulsions could protect them against enzymatic degradation during digestion and increase the intestinal uptake, resulting in a higher gut concentration as well as increased plasma levels of encapsulated food additives (Kumari et al., 2010).

The purpose of the present review is to ensure a critical assessment based on the effects of different lipid-based encapsulation techniques on the retention of phenolic compounds. In order to achieve this purpose, studies investigating the effect of encapsulation on the bioaccessibility and bioavailability of bioactive compounds were covered.

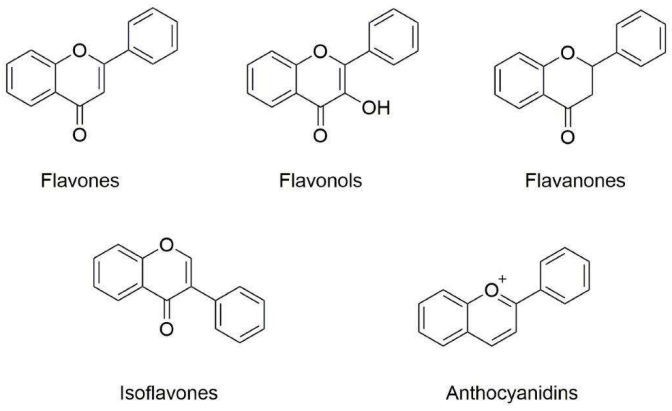
2.3.1 Overview of phenolic compounds bioaccessibility/bioavailability

Phenolic substances are secondary metabolites which are present in a wide variety of foods such as fruits, vegetables, cereals, horticultural crops, legumes, chocolate, etc. and in beverages, i.e. tea, coffee (Shahidi and Ambigaipalan, 2015). Polyphenols with at least one aromatic ring and one or more hydroxyl groups can be categorized primarily as flavonoids and non-flavonoids. Basic structure of the common classes of flavonoids and non-flavonoids are shown in Figure 2. 12. Flavonoids, as the most

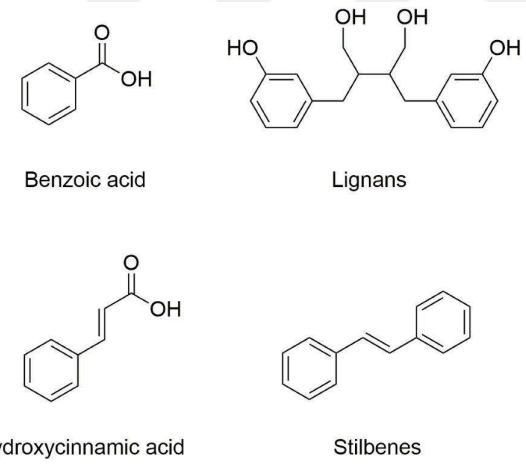
widespread and diverse group of polyphenols, can be further subdivided into flavonols (myricetin, quercetin, rutin, kaempferol etc.), flavones (aspigenin, luteolin, tangeretin etc.), flavanones (hesperetin, hesperidin, naringenin etc.), isoflavones (genistein, daidzein etc.) and anthocyanins (cyanidin, delphinidin, malvidin, pelargonidin etc.) depending on the degree of hydroxylation, methoxylation, prenylation and glycosylation (Gonzales et al., 2014). Non-flavonoids include diverse classes of polyphenols, such as stilbenes (resveratrol), lignans, hydrolyzable tannins and phenolic acids (hydroxybenzoic acids and hydroxycinnamic acids) (Esfanjani et al., 2018).

Phenolic compounds have been used for the production of functional foods due to their several benefits to human health through antioxidant, anti-inflammatory, anticancer, anti-obesity, antiviral, antibacterial, antiaging and/or antiallergenic activities (Bao et al., 2019). *In vitro* studies reported that flavonoids showed a high anticancer potential by inhibition of the proliferation, metastasis and angiogenesis of tumor cell lines, while the process of apoptotic cell death was activated. Such beneficial effects were also detected in mice fed with citrus peel extract, rich in phenolic compounds. The skin and colon carcinogenesis as well as the tumor size and volume of mice suffering from prostate cancer was significantly reduced in treated animals. In addition to the health-improving effects of a phenolic-enriched extract, specific effects of each individual phenolic compound can also be allocated; such as an anti-inflammatory potential of tangeretin and sinensetin or the suitability of hesperidin as an antioxidant (Koolaji et al., 2020).

Bioaccessibility and bioavailability of phenolic compounds are the main factors which effect the biofunctional properties and possible beneficial effects. Bioaccessibility as a clue of release and solubility of bioactive compounds during gastrointestinal digestion for further uptake, is a considerable factor for bioavailability (Parada andAguilera, 2007). Furthermore, various external and internal factors are also determinants on the bioavailability of phenolic compounds. The external factors comprise the nature of the bioactive agent including solubility, crystallinity etc. as well as the composition and structure of the food matrix, whereas the internal factors include gender, age, health, nutrient status, and life phase (Heaney, 2001).



(**a**)



(**b**)

Figure 2.12 : Basic structure of (**a**) common classes of flavonoids and (**b**) nonflavonoid-type phenolic compounds.

Several approaches have been used to enhance the bioaccessibility and bioavailability of bioactive ingredients, including chemical modifications of the molecules, dosing formulations, combination with other dietary components as well as incorporating them within micro-/nanoparticle delivery systems (Yang et al., 2008). A rapid dissolution of bioactive compounds within the gastrointestinal tract could be achieved by the relatively high surface area of these systems (Erfanian et al., 2014). Consequently, there is a great attempt to develop phenolic compound loaded micro/nano-scale delivery systems by pharmaceutical and food industries.

2.3.2 Improving the bioaccessibility of phenolic compounds by means of encapsulation

Bioaccessibility is the term used to define the amount of food compounds released from the food matrix in the gastrointestinal lumen, which is required to their intestinal absorption and bioavailability (Saura-Calixto et al., 2007). During digestion, food is exposed to three digestive phases including the oral, gastric and intestinal phase. In the oral phase, foods are submitted to mastication at the neutral environment with saliva, which contains amylase and mucin, to result an oral bolus. The factors effective on the mastication are food composition, food volume, number of chewing cycles, bite force, teeth condition, degree of hunger and habits (Fontijn et al., 2000; Engelen et al., 2005). In gastric phase, oral bolus is digested by gastric enzymes (e.g., pepsin and gastric lipase) and mechanical agitation (peristaltic movements) in the acidic environmental condition into a thick semifluid called chyme, which further be digested into macromolecules such as proteins, fats and polysaccharides prior to transfer to the small intestine (Bao et al., 2019). Subsequently, in intestinal phase, the digested food further breaks down into smaller constituents by bile salts and pancreatic enzymes (e.g., pancreatic lipase, trypsin, chymotrypsin) secreted from the intestinal mucosa at the environmental pH (Mao and Miao, 2015; Singh and Sarkar, 2011). The nutrients can be absorbed in the small intestine, whereas the non-absorbed digestion products pass to the large intestine for a fermentation process by the colonic microbiota. Finally, remaining metabolites are excreted from the human body (Guerra et al., 2012).

Bioaccessibility depends on various factors such as beverages consumed while eating, stomach contents, intestinal peristalsis, blood and lymph flow, physicochemical properties like pH, temperature and texture of the matrix and basic structure of phenolic compounds including the presence or absence of glycosylation, the type of conjugated sugar, the type of linkage to the aglycone, the site of glycosylation and the number of sugar moieties (Fernández-García et al., 2009; Poquet et al., 2010).

There are different methods used to examine the correlation between diet and health. However, *in vitro* models have been widely used to investigate human digestive tract rather than *in vivo* (human or animal) models owing to ethical issues. *In vitro* digestion methods that are commonly used for food can be divided into static and dynamic methods. These systems are used to simulate the physiological conditions of the upper gastrointestinal tract (oral, gastric and small intestinal phases) (Brodkorb et al., 2019);

thus provide some perception about the digestibility of controlled release systems and bioavailability of functional compounds (Alminger et al., 2014). Static models, which use a constant ratio of food to enzymes and electrolytes and a constant pH for each digestive phase, have been widely used for food and pharmaceutical purposes due to its simplicity, practicality and low cost (Alminger et al., 2014; Maldonado-Valderrama et al., 2010). Static digestion methods have some shortcomings including a lack of the gradual addition of simulated gastric fluids and gastric emptying, constant enzyme activity regardless the type of food as well as simulating the intestinal phase as one phase instead of the sequential duodenal, jejunal and ileal phases. Thus, the static *in vitro* digestion method should be used only to evaluate digestion endpoints rather than kinetic analysis of the different stages of the digestion process (Maldonado-Valderrama et al., 2010). On the other hand, dynamic digestion models, comprised by a multi-chamber apparatus, are multistage systems to mimic, as close as possible, the human digestion conditions. Moreover, it is possible to follow the simulation of the physicochemical changes such as pH transitions, enzyme secretion alteration and peristaltic movements that occur during *in vivo* digestion (Alminger et al., 2014; Li et al., 2013). Relatively complex structure, high expense to setup and maintenance are some of the limitations of this method (Brodkorb et al., 2019).

Health benefits of phenolic compounds in foods vary with the level of their bioaccessibility. Besides, the protection of phenolics in the gastrointestinal tract could be improved by encapsulating them by using carrier agents. Thus, up to date, bioaccessibility of a wide variety of food bioactives have been investigated. Impacts of encapsulation techniques on the bioaccessibility of phenolic compounds are covered in Table 2.4.

With regard to increasing water solubility of curcumin to improve its recovery, effective factors on the bioaccessibility of curcumin encapsulated within emulsion-based delivery systems have been analyzed by using the dynamic *in vitro* digestion model. Results highlighted that the bioaccessibility of curcumin depended on the type and amount of carrier lipids as well as droplet size of the nanoemulsion-based delivery systems, ranging from 1 to 58 % (Ahmed et al., 2012).

To understand the effect of coencapsulation of (−)-epigallocatechin-3-gallate (EGCG) and quercetin in a W/O/W emulsion gels, gastrointestinal stability tests have been

performed to analyze their bioaccessibility. According to the results, when simply suspended in water, the bioaccessibility of EGCG and quercetin were found to be 25.8 % and 12.9 %, respectively. In contrast, when coencapsulated in W/O/W emulsion gels, bioaccessibility of both EGCG and quercetin increased to around 48.4 % and 49 %, respectively (Chen et al., 2018).

Aditya et al. (2014) investigated the influence of the physical state and composition of the lipids on the achievement of quercetin loaded lipid nanocarriers by means of solid-lipid nanoparticles, nanostructured lipid carriers and lipid nanoemulsions, respectively. The results of this study provided a promising perspectives for the use of nanostructured lipid carriers and lipid nanoemulsions with the highest bioaccessibility value (~60 %) compared to solid lipid nanoparticles (~35 %) and free quercetin solution (~7 %). Similar trends were also ensured by Pool et al. (2013). Recovery of quercetin has been determined by a dynamic *in vitro* gastrointestinal model. Results highlighted an enhancement in the quercetin bioaccessibility from <5% in bulk water to 53% with 0.1 mg mL-1 and 29% with 0.5 mg mL-1 loading capacity when it was incorporated in the nanoemulsion. Ni et al. (2015) provided 33.6 % bioaccessibility value of quercetin in nanostructured lipid carrier, which is higher than that obtained in the bulk water (<2 %). Moreover, eudragit, an anionic copolymer based on methacrylic acid and ethyl acrylate, nanoparticles were also utilized to increase the stability and solubility of quercetin in the gastrointestinal tract. Pool et al. (2012) studied the formation of a delivery system for encapsulation of quercetin by using a solvent displacement method. After the simulated gastrointestinal digestion, quercetin release was around 7% for free quercetin dispersed in water, and around 22% for quercetin encapsulated within polymeric nanoparticles, indicating less amount of increase in the recovery of quercetin among other encapsulation techniques. In another study, Sessa et al. (2011) obtained chemically stable (no changes in the quantity and quality) resveratrol-loaded nanoemulsions during the gastric and intestinal digestions.

PMFs are one type of flavone compounds which possess methoxy groups on the flavonoid backbone and exist almost exclusively in the peel of numerous citrus fruits (Li et al., 2006). PMFs exhibit a wide spectrum of biological activity, including anti-inflammatory, anti-carcinogenic, anti-bacterial, anti-oxidant and neuroprotective effects (Lai et al., 2013; Gao et al., 2018); metabolic modulations (Sundaram et al., 2014); protection against cardiovascular diseases (Seo et al., 2011; Kurowska et al.,

2004); reducing serum triacylglycerol, very low-density lipoprotein (VLDL), and low-density lipoprotein (LDL) levels (Kurowska et al., 2004). Although PMFs have various health benefits, the potential applications of these compounds are limited due to their high hydrophobicity, low water solubility, high melting point and crystalline structure (Manthet et al., 2011; Kinoshita and Firman, 1996). Due to the fact that the oral efficacy of compounds is tightly dependent on the aqueous solubility, gut wall permeation, and metabolic stability, one of these strategies could be implemented to improve the bioaccessibility and bioavailability (Ting et al., 2015).

Tangeretin belongs to the class of PMFs (Xiao et al., 2009). Chen et al. (2015) investigated the effect of dietary lipids on the gastrointestinal fate of tangeretin-loaded zein nanoparticles. The recovery of tangeretin was found to be related with the concentration of co-ingested lipid phase. The bioaccessibility of the delivery systems was enhanced from 15% to 37% with the use of 4% initial oil concentration. In another study, different *in vitro* models were used to evaluate the effect of emulsification on bioaccessibility of tangeretin. *In vitro* lipolysis showed that bioaccessibility of emulsified tangeretin increased from 9.7 to 29.3% when compared with unprocessed tangeretin oil suspension. Besides, according to the dynamic *in vitro* gastrointestinal model (TIM-1) results, the bioaccessibility of tangeretin increased 2.6-fold when it was incorporated to the viscoelastic system rather than in the oil suspension (Ting et al., 2015). Similarly, a recent study (Wijaya et al., 2020) compared the effects of high internal phase emulsions (HIPE) stabilized by whey protein isolate – low methoxy pectin complexes and medium chain triglycerides (MCT) oil as a suspension on the bioaccessibility of tangeretin using *in vitro* lipolysis and dynamic *in vitro* intestinal digestion studies. The *in vitro* lipolysis results revealed that the bioaccessibility of tangeretin in HIPE-complexes was increased 2-fold than that of in the bulk oil. Additionally, the gastrointestinal model TIM-1 indicated 5-fold increase in the total bioaccessibility of tangeretin compared to PMFs in bulk oil. Nobiletin, another kind of PMFs, was also studied to improve its bioaccessibility. Ning et al. (2019) fabricated 5-demethylnobiletin (5-DN) loaded selenium-enriched peanut protein nanoparticles-stabilized Pickering emulsion. The bioaccessibility of 5-DN was found to be higher (18.3 %) with emulsion than in bulk oil (9.2%). Similar results were found by Wijaya et al. (2020). 1.5- and 2-fold increase in the bioaccessibility of nobiletin was obtained within HIPE-complexes compared to within bulk oil after *in vitro* lipolysis and the

gastrointestinal model TIM-1 digestion, respectively. Furthermore, with regard to increasing oral bioaccessibility of PMFs from aged citrus peel extracts, lipid-based delivery systems have been developed (Lu et al., 2019). Compared to the samples in bulk oil, the bioaccessibilities of PMFs in the nanoemulsion and Pickering emulsion were enhanced by 14-fold with the use of lipolysis model. On the other hand, results from the TIM-1 system demonstrated a 2- and 4-times increase in the bioaccessibilities of PMFs in the nanoemulsion and Pickering emulsion rather than that of bulk oil, respectively.

In conclusion, to generate a delivery system to enhance the solubility, stability, bioaccessibility and controlled release of a phenolic compound, factors including the solubility and thermal sensitivity of the phenolic compound, ratio and interaction between wall and core material as well as the phenolic loading ratio should be considered.

2.3.3 Intestinal transport mechanisms and effective factors on phenolic compounds bioavailability

An increase of bioaccessibility by encapsulation is the initial step for a higher exploitation of phenolic compounds. Nevertheless, the bioavailability is equally essential and represents the second step, which can be positively affected by encapsulation. With increasing intestinal absorption of phenolic compounds, their biological activities will be increased. The intestinal epithelial transport mechanisms can be divided into four different routes: the paracellular route, the transcellular route, the carrier-mediated transport and transcytosis (Figure 2.13) (Artursson et al., 2001; Laksitorini et al., 2014). While on the transcellular route substances diffuse through the membranes and the intracellular space of the epithelial cells, on the paracellular route ions and small molecules can passively diffuse through the tight junctions. More complex and hydrophilic molecules use vesicles along transcytosis or they bind to specific transporters, which are integrated in the membrane of the intestine, in case of a carrier-mediated transport (Laksitorini et al., 2014). Phenolic compounds were mainly absorbed by passive diffusion, whereby the lipophilicity and molecular weight of each molecule are crucial (Esfanjani et al., 2018). While such substance-specific features represent the first group of effective factors on polyphenolic bioavailability, the second group consists of all possibly consumed compounds of the dietary matrix,

which may influence the digestion processes and the composition of the person-related microbiome.

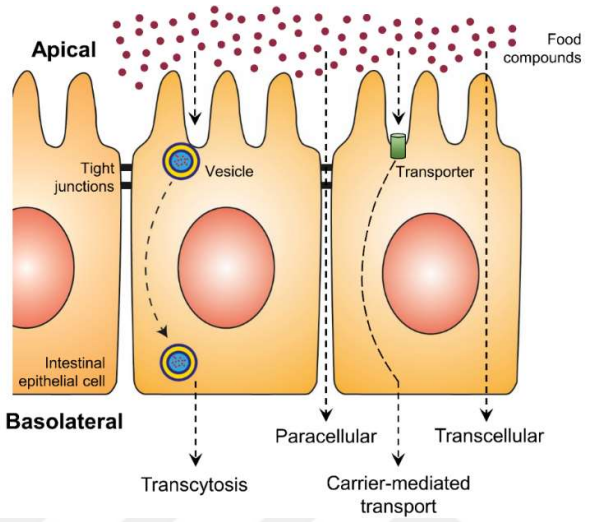


Figure 2.13 : Uptake of food compounds by intestinal epithelial transport mechanisms from the gut lumen (apical side) to the blood vessel (basolateral side).

One of the most important factors for a high bioavailability is the degree of polymerization as well as the methylation of the phenolic compound (Spencer et al., 2001; Wen and Walle, 2006; Appeldoorn et al., 2009; Monagas et al., 2010). (-)-Epicatechin, a flavan-3-ol, possess a moderate bioavailability in *in vivo* studies with an average absorption of 23% after 90 min (Spencer et al., 2001) or 46% after 2.5 h (Actis-Goretta et al., 2013). While 95.8% of transferred flavanol-related compounds were identified as (-)-epicatechin, the epicatechin dimers B2 and B5 showed a significantly lower content of < 1 % of the total transferred value (Spencer et al., 2001). Similar results were detected for further flavan-3-ols, whose monomers can directly be absorbed in the small intestine. More complex substances e.g. polymeric forms will be transferred to the colon, where gut bacteria metabolize the compounds by glucuronidation or sulfation prior to absorption (Monagas et al., 2010). Unfortunately, the health-improving potential of these microbial-derived metabolites are largely unknown. If a flavone will be directly absorbed or possibly metabolized, depends on the methylation state likewise. Wen and Walle (2006a) and Wen and Walle (2006b) analysed the stability of methylated and non-methylated flavones in addition to liver S9 fraction or in the presence of human hepatocytes. The methylated compounds showed a high resistance against metabolization in all assays compared to non-

methylated forms, suggesting that methylation of flavonoids eventually protect them from metabolization and excretion (Wen and Walle, 2006b). In further *in vitro* transport experiments, up to 8-times higher absorption rates were documented for methylated compounds, while the rate of the non-methylated forms was lower and correlated with their high potential of metabolic transformation (Wen and Walle, 2006a). Therefore, the replacement of hydroxyl groups by methylated groups may be another suitable method for increasing phenolic compounds bioavailability.

The metabolization of phenolic compounds by the microbiome and/or intestine epithelial cells plays an important role in the bioavailability. Nevertheless, elements of the dietary matrix can influence the bacterial growth and the composition of the microbiome, resulting in different digestion and metabolization pathways. Roowi et al. (2009) detected a high content of phenolic acids (3-hydroxyphenylacetic acid, 3-hydroxyphenylhydracrylic acid, dihydroferulic acid, 3-methoxy-4-hydroxyphenylhydracrylic acid and 3hydroxyhippuric acid) in the urine of participants after consumption of orange juice, which corresponded to 37% of total ingested flavanones. The excretion of these acids was significantly reduced by parallel consumption of orange juice with yoghurt, suggesting an increased metabolization by gut bacteria (Roowi et al., 2009). Similar to the effects of yoghurt, the naturally occurring dietary fiber pectin influenced the metabolic activity and/or composition of the intestinal flora and induced a higher quercetin plasma concentration after rutin digestion (Tamura et al., 2007). Moreover, glucose and insulin are effective factors on bioavailability. While the total anthocyanin content in red wine and red grape juice was comparable, the uptake of anthocyanins of red grape juice was significantly higher than that of red wine, which may due to the lower glucose content in red wine (Bitsch et al., 2004). Such synergistic effect of glucose and the phenolic compounds absorption may base on the stimulation of bacterial growth, whereby the bacteria use glucose as energy source (Tamura et al., 2007) or alternatively the high glucose content induce the release of insulin, which is able to influence the microbiome and the phenolic bioavailability (Piazza et al., 2007). Further, bacteria-independent effects may be induced by protein complexes and fat-enriched diets. Proteins e.g. the salivary protein histatine 5 can bind phenolic compounds and form insoluble complexes, which are related to a reduced absorption (Cai and Bennick, 2006). Otherwise, experiments with milk protein had no effect on the uptake of cocoa polyphenols (Keogh et al., 2007).

However, a high dietary fat content is associated with a greater absorption in a dose-dependent manner (Chen et al., 2013). Lesser et al. (2004) analysed the bioavailability of quercetin in pigs, whereby the dietary fat content was increased from 3 % to 17 %, resulting an enhanced absorption of 50 %. It is assumed that quercetin was incorporated in micelles, derived from the dietary fat, followed by absorption in the small intestine due to a higher solubility (Content, 2004). This principle of using lipid carrier is already used as an effective encapsulation method for higher phenolic absorption.

2.3.4 Improving the bioavailability of phenolic compounds by means of encapsulation

In order to compare the absorption efficiency of encapsulated vs. non-encapsulated compounds, several *in vitro* and/or *in vivo* assays were performed, followed by substance-specific quantification e.g. LC-MS/MS (Peng et al., 2018; Vitaglione et al., 2013; Mueller et al., 2018). For the *in vitro* assays, the absorption and/or the transport through an epithelial membrane were analysed, using intestinal epithelial cells like the human colon adenocarcinoma cell line Caco 2 (Sun et al., 2008; Gamboa et al., 2013). In the case of the absorption study e.g. described by Jain et al. (2013), Caco-2 cells were cultured and treated with encapsulated phenolic compounds. Afterwards the cells were washed for removing non-absorbed material, followed by cell lysis and a substance-specific quantification of the intracellular content. Finally, the intracellular concentration will be compared to the results of the non-encapsulated compound as well as the treatment concentration. Caco-2 absorption studies are fast and easy methods to analyse the bioavailability. Nevertheless, the experimental design contains undifferentiated cells without brush border formation, which rather mimic mature enterocytes in the human physiology (Kucki et al., 2017). Moreover, for health-improving effects induced by phenolic compounds, the transport of these compounds through the intestine to the blood flow is essential. Therefore, instead of uptake, the transport rate would give more insights into the bioavailability and efficiency of encapsulated substances. Caco-2 monolayer transport system has been established to investigate bioavailability with a much more complex in vitro model (Figure 2.14). Thereby, an insert, which represents a downsized version of a cell culture dish, is hanged in an e.g. 6-well of a plate, resulting in the separation of the well into an upper compartment (volume of the insert) and a lower compartment (volume of the 6-well).

The ground of the insert consists of a 10 µm thick membrane, made out of polyester or polycarbonate with µm-sized pores, which enable an exchange of molecules and media components but not cells between both compartments (Gamboa and Leong, 2013). On the membrane of the upper compartment, intestinal cells like Caco-2 can be cultured and differentiated to receive an intestinal epithelium, consisting of an enterocytes monolayer with tight junctions and brush border formation (Kucki et al., 2017). The differentiation of Caco-2 cells occurs spontaneously by reaching 100 % of confluence (Ferruzza et al., 2012) and is completed after 16-21 days of further cultivation, resulting in an intestinal membrane similar to the epithelium of the small intestine (Sun et al., 2008; Hidalgo et al., 1989). In this Caco-2 monolayer transport system, the upper compartment is comparable with the intestinal lumen or the apical side of the gut membrane, while the lower compartment is comparable with the blood vessels or basolateral side of the gut membrane (Sun et al., 2008). Thus, the in vivo processes can be simulated in more detail and in addition to the absorbed phenolic content in the cells, the concentration in the apical and basolateral compartment can be quantified (Gamboa and Leong, 2013). Yee (1997) verified the suitability of the Caco-2 monolayer transport system with a high correlation between the absorption results in humans and the permeability coefficient of the in vitro model. In spite of the good applicability of the Caco-2 monolayer transport system, this in vitro model can be extended with methotrexate-induced differentiated HT-29 cells, origins from a human colon adenocarcinoma, to get a mucus-secreting coculture (Gamboa and Leong, 2013; Lesuffleur et al., 1990; Behrens et al., 2001). The cultivation of colon epithelial cells in coculture with HT-29 goblet cells and mucus formation is a more sophisticated model for bioavailability, especially since the mucus represents a second physiological diffusion barrier, influencing the absorption time of digested compounds (Gamboa and Leong, 2013; Behrens et al., 2001).

An overview of in vitro studies analyzing the uptake of encapsulated phenolic compounds in Caco-2 absorption studies or the Caco-2 monolayer transport system are shown in Table 2.5.

The Caco-2 monolayer transport system is a useful tool for intestinal transport studies, nevertheless factors like the flow rate or gastrointestinal transit are not considered. For analyzing the digestion and uptake of food compounds, in vivo studies combine the influence of encapsulation on bioaccessibility and bioavailability, whereby for each

step and organ the phenolic concentration can be quantified e.g. as done by Augustin et al. (2011). Studies related with nutrition and health were mainly performed using humans, mice or rats as model organisms (Rubio-Aliaga, 2012), which run through several periods of consumption and/or fasting. The human nutrition studies of Vitaglione et al. (2013) and Mueller et al. (2018) started with a wash-out period over days, consisting of a phenol-free diet, followed by fasting for several hours (Vitaglione et al., 2013), and the consumption of the encapsulated/non-encapsulated phenolic compounds. Nallamuthu et al. (2015) studied the uptake of chlorogenic acid in rats after fasting for 14-15 h.

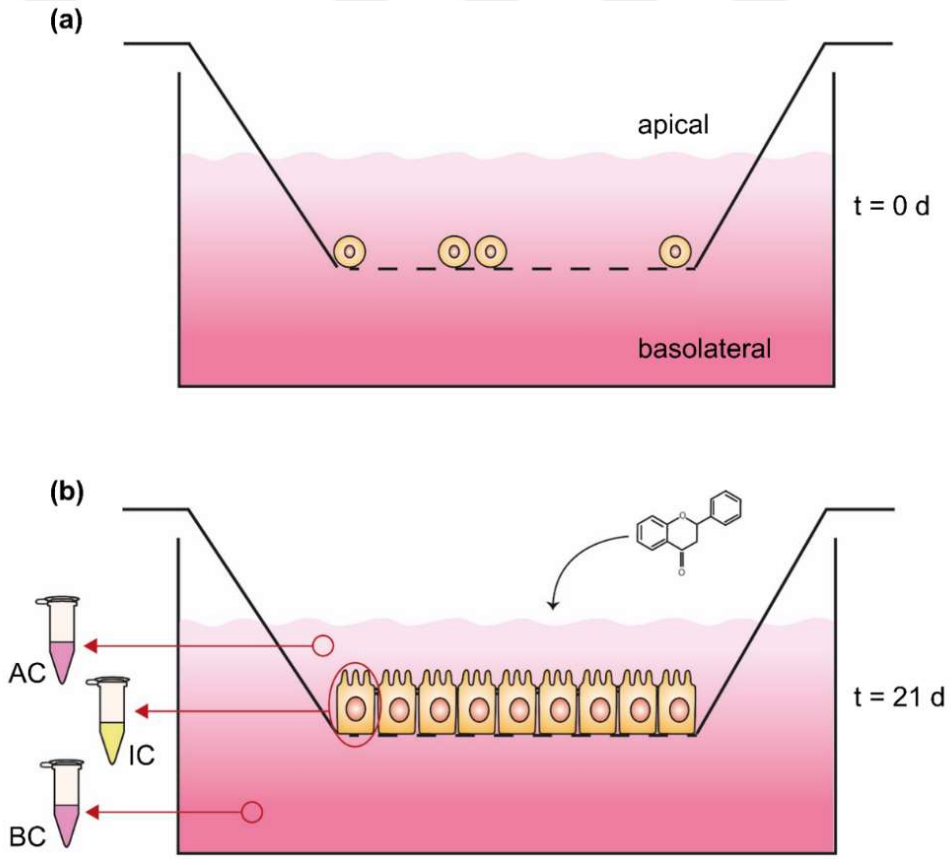


Figure 2.14 : Caco-2 monolayer transport system (a) at seeding time (t = 0 days) and (b) 21 days (t = 21 d) after seeding. Phenolic compounds will be applied in the apical compartment followed by quantification of the content in both compartments as well as the intracellular concentration; AC: medium sample of apical compartment, IC: washed and harvested cells for quantifying intracellular content, BC: medium sample of basolateral compartment.

Table 2.4 : Effects of different lipid-based encapsulation techniques on the bioaccessibility of selected phenolic compounds.

Applied Technique	Active Material	Carrier	Results	References
Nanoemulsion/ emulsion	Curcumin	Triacylglycerol	1 to 58% bioaccessibility of nanoemulsion-based delivery systems	(Ahmed et al., 2012)
W/O/W emulsion gels	EGCG and Quercetin	Gelatin	After coencapsulation in W/O/W emulsion gels, 48.4 and 49% bioaccessibility of EGCG and quercetin, respectively	(Chen et al., 2018)
Nanostructured lipid carriers, Lipid nanoemulsions, Solid lipid nanoparticles	Quercetin	Lecithin	~60% bioaccessibility with nanostructured lipid carriers and lipid nanoemulsions, ~35% with solid lipid nanoparticles and ~7% with free quercetin solution	(Aditya et al., 2014)
Nanoemulsion	Quercetin	Triacylglycerol	An enhancement in the quercetin bioaccessibility from < 5% in bulk water to 53% in nanoemulsions	(Pool et al., 2013)
Nanostructured lipid carrier	Quercetin	Glyceryl monostearate, glycerol monolaurate and caprylic capric triglyceride	33.6 and 2% bioaccessibility of quercetin in nanostructured lipid carrier and bulk water, respectively	(Ni et al., 2015)
Solvent displacement method	Quercetin	Eudragit	7 and 22% release of quercetin in water and polymeric nanoparticles, respectively	(Pool et al., 2012)
Nanoemulsion	Resveratrol	Peanut oil	No changes in the quantity and quality of the resveratrol-loaded nanoemulsions	(Sessa et al., 2011)
Antisolvent precipitation/emulsion	Tangeretin	Zein and β-lactoglobulin	15 to 37% bioaccessibility of tangeretin without and 4% initial oil concentration, respectively	(Chen et al., 2015c)
Viscoelastic emulsion	Tangeretin	MCT	According to *in vitro* lipolysis, 9.7 to 29.3% release of tangeretin within oil suspension and emulsion, respectively. According to TIM-1 model, 2.6-fold increase in tangeretin bioaccessibility within emulsion system	(Ting et al., 2015)

Table 2.4 (continued) : Effects of different lipid-based encapsulation techniques on the bioaccessibility of selected phenolic compounds.

Applied Technique	Active Material	Carrier	Results	References
High internal phase emulsions	Tangeretin	Whey protein isolate—low methoxy pectin	According to *in vitro* lipolysis, 2-fold increase in bioaccessibility within HIPE-complexes compared to that of the bulk oil According to TIM-1 model, 5-fold increase in bioaccessibility within HIPE-complexes compared to that of the bulk oil	(Wijaya et al., 2020)
Pickering emulsion	5-DN	Peanut protein	9.2 and 18.3% release of 5-DN in bulk oil and emulsion, respectively	(Ning et al., 2019)
High internal phase emulsions	Nobiletin	Whey protein isolate—low methoxy pectin	According to *in vitro* lipolysis, 1.5-fold increase in bioaccessibility within HIPE-complexes compared to that of the bulk oil According to TIM-1 model, 2-fold increase in bioaccessibility within HIPE-complexes compared to that of the bulk oil	(Wijaya et al., 2020)
Nanoemulsion/ Pickering emulsion	PMFs extract	MCT	According to *in vitro* lipolysis, 14-fold increase in bioaccessibility within nanoemulsion/emulsion compared to that of the bulk oil According to TIM-1 model, 2- and 4-fold increase in bioaccessibility within nanoemulsion and emulsion, respectively, compared to that of the bulk oil	(Lu et al., 2019)

TIM-1: *in vitro* dynamic digestion model; HIPE: high internal phase emulsions; 5-DN: 5-demethylnobiletin; EGCG: (−)-epigallocatechin-3-gallate, PMF: polymethoxylated flavonoids; W/O/W: water-in-oil-in-water emulsion.

Table 2.5 : Effects of lipid-based encapsulation techniques on the *in vitro* bioavailability of phenolic compounds.

Applied Technique	Active Material	Carrier	*In Vitro* Model: Analyzed Material and *Results*	References
Antisolvent precipitation	Quercetin	Shellac and almond gum	Caco-2 absorption study: analysis of intracellular quercetin level *Results of the cellular uptake could not be compared with nonencapsulated quercetin sample due to cytotoxic effects of nanoparticles*	(Doost et al., 2019)
Emulsion−diffusion solvent evaporation	Quercetin	Poly(lactic-co-glycolic acid)	Caco-2 absorption study: analysis of intracellular quercetin level *6-fold higher uptake efficiency by encapsulation*	(Jain et al., 2013)
Self-nanoemulsion	Quercetin	Castor oil	Caco-2 monolayer transport system: analysis of the supernatant from apical and basolateral compartment *Encapsulation enabled a 2-fold higher transportation of quercetin*	(Tran et al., 2014)
Pickering emulsion	Curcumin	Milled starch particles	Caco-2 absorption study: analysis of intracellular curcumin level *Encapsulation enabled a significantly higher uptake efficiency*	(Lu et al., 2019)
Antisolvent precipitation/emulsion	Tangeretin	Zein and β-lactoglobulin	Caco-2 monolayer transport system: analysis of the supernatant from apical and basolateral compartment *Oil dose-dependent increase in permeability*	(Chen et al., 2015c)
Pickering emulsion	5-DN	Peanut protein	Caco-2 absorption study: analysis of intracellular 5-DN level by HPLC *Higher uptake rate of 5-DN by encapsulation* Caco-2 monolayer transport system: analysis of the supernatant from apical and basolateral compartment *Higher permeability of 5-DN micelles than nonencapsulated 5-DN*	(Ning et al., 2019)
O/W nanoemulsion	Resveratrol	Lipophilic soy lecithin with defatted soy lecithin and peanut oil	Caco-2 monolayer transport system: analysis of the supernatant from apical and basolateral compartment *Significantly lower permeability of encapsulated resveratrol*	(Sessa et al., 2014)

Table 2.5 (continued) : Effects of lipid-based encapsulation techniques on the *in vitro* bioavailability of phenolic compounds.

Applied Technique	Active Material	Carrier	*In Vitro* Model: Analyzed Material and *Results*	References
O/W nanoemulsion	Resveratrol	Soy lecithin with peanut oil	Caco-2 absorption study: analysis of intracellular resveratrol level *Significantly higher uptake of resveratrol by encapsulation* Caco-2 monolayer transport system: analysis of the supernatant from apical and basolateral compartment *Significantly lower permeability of encapsulated resveratrol*	(Sessa et al., 2014)
O/W nanoemulsion	Resveratrol	Lipophilic soy lecithin with peanut oil	Caco-2 absorption study: analysis of intracellular resveratrol level *Significantly lower uptake of resveratrol by encapsulation*	(Sessa et al., 2014)
O/W nanoemulsion	Resveratrol	Tween 20 with glycerol monooleate and peanut oil	Caco-2 absorption study: analysis of intracellular resveratrol level *Significantly lower uptake of resveratrol by encapsulation* Caco-2 monolayer transport system: analysis of the supernatant from apical and basolateral compartment *Significantly lower permeability of encapsulated resveratrol*	(Sessa et al., 2014)

Ideally, all participants or test animals should run through all kinds of encapsulated samples, separated by a further wash-out period with normal consumption habits (Vitaglione et al., 2012), to directly compare and evaluate the effects of encapsulation methods. In order to quantify such increased or decreased effects on the phenolic absorption, blood, urine and/or fecal sample are collected regularly (Nallamuthu et al., 2015), which enable a time-dependent distribution and excretion analysis of the test substance within the digestion system and the blood flow. For a whole body distribution analysis including the separation of stomach, small intestine, cecum, colon and liver, Augustin et al. (2011) fed rats with radiolabelled phenolic compounds and measured the radioactivity in each organ 3, 6, 12 and 24 h after dosing. While in this study the rats need to be dissected, Rubio-Aliaga (2012) used a specific gamma camera to visualize the radiolabelled nanoparticles in the gastrointestinal tract of test animals similar to magnetic resonance imaging. Independent of the imaging method, the transport of radiolabelled, encapsulated compounds could be detected in more accuracy, especially if the encapsulation led to a slower but sustained absorption as described by Nallamuthu et al. (2015) and Liu et al. (2017). Detailed results of the above-mentioned studies as well as further bioavailability experiments for the encapsulated phenolic compounds are shown in Table 2.6.

Curcumin becomes more and more popular as food additive and nutritional supplement due to its antioxidant and anti-inflammatory effects (Anand et al., 2011). Nevertheless, it is weakly soluble in water, which restricts its bioavailability as well as the health-improving potential after consumption (Dei Cas and Ghidoni, 2019). Lu et al. (2019) increased significantly the uptake of curcumin by encapsulation using milled starch particles in form of a Pickering emulsion. Compared to a standard curcumin solution dissolved in DMSO, the emulsion led to a higher intake in Caco-2 absorption study (Lu et al., 2019). While the results of *in vitro* models are limited and cannot be directly transferred to the digestion system of animals and humans (Jain et al., 2013), further studies reported various encapsulation techniques which were tested *in vivo*, followed by the quantification of their bioavailability. Curcumin was encapsulated with cellulose derivatives in oil (Vitaglione et al., 2012), organogel (Yu and Huang, 2012), sophorolipid micelles (Peng et al., 2018) or a specific protease inhibitor from soybeans (Liu et al., 2017).

Table 2.6 : Effects of lipid-based encapsulation techniques on the *in vivo* bioavailability of phenolic compounds.

Applied Technique	Active Material	Carrier	*In Vivo* Model: Analyzed Material and *Results*	References
Emulsification/thermal gelation	Anthocyanins from bilberry extract	Whey protein	Humans: analysis of serum and urine samples *28% less anthocyanins in serum but 108% more anthocyanins in urine than nonencapsulated extract*	(Muller et al., 2018)
Emulsification/thermal gelation	Anthocyanins from bilberry extract	Citrus pectin	Humans: analysis of serum and urine samples *80% less anthocyanins in serum and 8% less anthocyanins in urine than nonencapsulated extract*	(Muller et al., 2018)
Ionic gelation	Chlorogenic acid	Chitosan nanoparticles	Rats: analysis of serum samples *Encapsulation enabled a slower and sustained absorption*	(Vitaglione et al., 2012)
Spray drying	Polyphenol extract from cocoa nibs	High-amylose maize starch	Humans: analysis of serum and urine samples *Significantly lower content of phenolic acids in serum and urine induced by encapsulation of polyphenols*	(Vitaglione et al., 2013)
O/W emulsion	Resveratrol	Sodium caseinate with high amylose maize starch and glucose	Rats: analysis of radiolabelled [^{3}H]-resveratrol along digestive system *Encapsulation led to a significantly higher content of resveratrol in the gut lumen of small intestine*	(Augustin et al., 2011)
Emulsion−diffusion solvent evaporation	Quercetin	Poly(lactic-co-glycolic acid)	Rats: analysis of serum samples *2.9-fold higher uptake efficiency by encapsulation*	(Jain et al., 2013)
Self-nanoemulsion	Quercetin	Castor oil	Rats: analysis of serum samples *2-fold significantly higher uptake efficiency by encapsulation*	(Tran et al., 2014)
Organogel-based nanoemulsion	Curcumin	Organogel	Mice: analysis of serum samples *Encapsulation led to a 9-fold higher bioavailability of curcumin*	(Anand et al., 2007)

Table 2.6 (continued) : Effects of lipid-based encapsulation techniques on the *in vivo* bioavailability of phenolic compounds.

Applied Technique	Active Material	Carrier	*In Vivo* Model: Analyzed Material and *Results*	References
Sophorolipid-coated nanoparticle	Curcumin	Sophorolipid micelles	Rats: analysis of serum samples *Significantly higher (3.6-fold) absorption of encapsulated curcumin*	(Peng et al., 2018)
Bowman−Birk inhibitor nanodelivery carrier	Curcumin	Soybean	Rats: analysis of serum samples *Encapsulation with Bowman−Birk inhibitor led to a slower but significantly 3.1-fold higher uptake compared to curcumin-loaded sodium caseinate nanoparticles*	(Liu et al., 2017)
Fluidized bed spray coating	Curcumin	Cellulose derivative with vegetable oil	Humans: analysis of serum samples *7.3-fold higher content of curcuminoids in serum, while the urinary concentration was not significantly affected by encapsulation*	(Vitaglione et al., 2012)
Casein nanoparticle	Resveratrol	Sodium casein	Rats: analysis of serum samples *10-fold higher bioavailability of encapsulated resveratrol than dissolved in polyethylenglykol*	(Rubio-Aliaga, 2012)

In all these experiments, the bioavailability of curcumin was analysed by measuring the serum concentration over several hours after consumption, in comparison to the non-encapsulated polyphenol- or curcumin-loaded sodium caseinate nanoparticles (Liu et al., 2017). After encapsulation, the uptake of curcumin was increased in the range of 3- to 9-fold higher serum concentrations, in detail, the cellulose-oil mixture (7-fold higher) and the organogel technique (9-fold higher) were found to be the most effective methods for encapsulation (Peng et al., 2018; Vitaglione et al., 2012; Liu et al., 2017; Yu and Huang, 2012). Although the experiments were partly done with mice and rats, similar uptake-improving effects were assumed for human bioavailability of encapsulated curcumin.

As mentioned above, *in vivo* studies combine the results of bioaccessibility and bioavailability. Therefore, the described higher serum concentrations of encapsulated curcumin give also insights into a constant or possibly higher bioaccessibility induced by nanoemulsions, nanoparticles or organogels. Such a combination of a higher occurrence and uptake of curcumin-loaded sophorolipid-coated nanoparticle was measured by Peng et al. (2018), who detected 2.7-fold higher bioaccessibility and 3.6-fold higher serum concentration than free curcumin. In this case, the higher bioavailability was mainly affected by the increased stability and occurrence of curcumin due to the encapsulation and less affected by uptake. In contrast, an O/W emulsion of resveratrol showed 2- to 4-fold increased uptake, resulting in significantly higher concentrations in blood and liver of rats, while no effects on the bioaccessibility of encapsulated resveratrol were detected (Augustin et al., 2011).

Another important factor of bioavailability is the particle size as well as the use of phospholipids for encapsulation. Sessa et al. (2014) analyzed the *in vitro* uptake and permeability of multiple resveratrol emulsions, which all based on peanut oil but differ in their composition of soy lecithin and droplet size. An increase in mean droplet size was negatively associated with the permeability but positively associated with cellular uptake. While smaller particles (128 or 137 nm in mean) were transported through the Caco-2 monolayer and accumulates in the basolateral compartment, it is assumed that larger particles (211 or 235 nm in mean) remain in the cells and could be responsible for the higher uptake contents. For permeability, the use of phospholipids in the encapsulation are beneficial compounds, resulting in a better interaction between nanoemulsion-based delivery system and the cell membrane. By generation of a

phospholipid layer from soy lecithin, the permeability of encapsulated resveratrol was significantly higher compared to particles consisting of Tween 20 and glycerol monooleate. Moreover, in the same study, the degradation of resveratrol in water could be reduced to 15-25 % by encapsulation with soy lecithin, while the non-encapsulated substance showed a degradation rate of 52 %. To sum up, small particles in combination with phospholipids enables the inhibition of degradation processes, as well as a high permeability of substances with low water solubility like resveratrol, curcumin and anthocyanins (Sessa et al., 2014).

2.3.5 Potential risk of a higher phenolic bioavailability

Although phenolic compounds are widely used for disease prevention due to their antioxidant properties, there are some evidences for a toxic potential of polyphenols and flavonoids as reviewed by Kyselova (2011). For instance, flavonoids showed a mutually influence with cytochrome P450 monooxygenases (CYPs), which are essential enzymes in metabolism and activation of ingested food compounds, medications or environmental toxins like polycyclic aromatic hydrocarbones (PAHs) (Peter et al., 2002). Flavonoids e.g. quercetin and diosmin are able to increase the biosynthesis and/or activity of CYPs, may promoting the formation of such carcinogens and increase their toxicity (Ciolino et al., 1998; Ciolino et al., 1999; Sergent et al., 2009). While CYPs are affected by flavonoids, their chemical structure can be affected by these enzymes as well. CYP-generated metabolites of flavonoids may bind to DNA and induce similar effects like mutagenic DNA alkylating agents (Peter et al., 2002). Walle et al. 2003 documented the covalently binding of ROS-activated quercetin to DNA and proteins in several cancer cell lines (Walle et al., 2003). These binding to the DNA induce the destabilization of the helix (Kanakis et al., 2005), may resulting in apoptosis, cell cycle arrests or mutations. The carcinogenic potential of quercetin was already shown in rats by the formation of kidney tumors, whereby the authors assumed the combination of genotoxic and non-genotoxic effects (Dunnick and Halley, 1992). In addition to the DNA alkylating potential, a clastogenic activity was reported for several flavonoids, although these two seems to be caused independently from each other (Snyder and Gillies, 2002). Similar to flavonoids, a wide range of polyphenols is able to induce toxic effects e.g. by an increase of the mutagenicity of *N*-Nitrosopyrrolidine, a nitrosamine mainly occurred in nitrite-rich food after cooking (Catterall et al., 2000). All these studies evaluation the toxic

potential of phenolic compounds were done *in vitro* using cancer cell lines or *in vivo* with rats. Therefore, the transfer to human beings is limited and need to be verified in further experiments (Mennen et al., 2005). Nevertheless, the above-mentioned toxic effects depend on the bioaccessibility and bioavailability of the phenolic compounds and will be strengthen by an encapsulation. Finally, benefits as well as risks of phenolic compounds should be reconsidered prior to application.

2.4 Interactions of Phenolics with Food Matrix: *In Vitro* and *In Vivo* Approaches

Phenolics are plant-derived, secondary metabolites produced by plants against stress conditions such as injury, infection, and UV radiation (Naczk and Shahidi, 2004). Numerous compounds such as phenolic acids, acetophenones, phenylacetic acid, hydroxycinnamic acids, coumarins, naphthoquinones, xanthons, stilbenes, and flavonoids belong to phenolics. Their chemical structure can range from very simple molecules to very complex ones (Jakobek, 2015).

In recent years, the antioxidant properties of phenolics and their preventive and supportive effects on several diseases including cancer, diabetes, bone, skin, and heart diseases have been discovered by researchers (Bohn, 2014). It has also been pointed out in the literature that some phenolic compounds promote the growth of useful bacteria and may inhibit the growth of pathogenic bacteria (Naczk and Shahidi, 2004).

The bioavailability is generally defined as the fraction of a given compound that reaches the systemic circulation after gastrointestinal digestion, absorption, metabolism, tissue distribution, and exerts bioactivity (Carbonell-Capella et al., 2014). Recently, there has been an increasing popularity about the studies on the bioavailability of phenolic compounds in various food matrixes (e.g. protein, fat, dietary fiber). These studies are important to determine the effects of food matrix and conditions on the recovery of the phenolic compounds which may play an influential role in the treatment of various diseases (Zhang et al., 2016). Furthermore, phenolic compounds need to be released during digestion and then absorbed in a certain amount in the intestine to be effective in the human organism. The bioavailability of phenolics depends on the nature of the phenolic compound, the complexity of the structure of the phenolic - food matrix, possibility of enzymes reaching the compound (Naczk and Shahidi, 2004) and the level of intake (Rodriguez-Mateos et al., 2016).

Macromolecules, such as lipids, proteins, and carbohydrates, can act as carriers of phenolics throughout the digestive tract, and these associations can also protect phenolics from oxidative degradation. Thus, it has been proposed that interactions with lipids, proteins and carbohydrates may affect the bioavailability of phenolics (Hollman et al., 2011).

The aim of the present review is to provide a critical assessment on the relationship between components in the food matrixes and different phenolics. In order to achieve this purpose, the interaction between carbohydrates, lipids, proteins, other food constituents including vitamins and phenolic compounds with phenolic compounds are highlighted. Additionally, the positive or negative effects of these components on the bioavailability of phenolics are emphasized.

2.4.1 Phenolic – Carbohydrate Interactions

Carbohydrates, one of our body's main sources of nutrients and energy, can interact with phenolic compounds and may alter the bioaccessibility/bioavailability of the phenolic compounds (Jakobek, 2015). For example, metabolism and bioavailability of procyanidins have been investigated by means of *in vitro* and *in vivo* models. Addition to these, matrix effect of a carbohydrate-rich food on the digestibility and bioavailability of procyanidins have also been evaluated. According to the results, a superior stability of procyanidins under gastric and duodenal digestion conditions has been obtained. However, the pharmacokinetic study presented a limited absorption (Serra et al., 2010).

On the other hand, the interaction of carbohydrates with polyphenol-protein complexes results with the prevention of some adverse effects of these complexes, such as inhibiting the activity of the enzymes, or may affect the shrinkage of certain food products (Jakobek, 2015).

In most of the studies, dietary fibers have been the main carbohydrate under investigation in terms of its interaction with phenolic substances. Interactions were observed to appear through non-covalent bonds as electrostatic forces and hydrogen bonds as Van der Waals forces between hydroxyl groups of phenolic compounds and various components of dietary fibers (Velderrain-Rodriguez et al., 2016). In Table 2.7,

several studies that investigated the effect of dietary fibers on bioavailability/bioaccessibility of phenolic compounds are summarized.

Ortega et al. (2011) stated that the dietary fibers from carob provide stability and increase potential bioaccessibility of phenolic substances during digestion (Ortega et al., 2011). Velderrain-Rodriguez et al. (2016) analyzed the possible interactions between dietary fibers and phenolic substances of mango, papaya and pineapple fruits. Results showed that the bioaccessibility of phenolic substances was not affected by dietary fibers in the fruits (Velderrain-Rodriguez et al., 2016). Moreover, Pérez-Jiménez et al. (2009) investigated the effect of dietary fibers on the bioavailability of phenolic substances in grapes (over short and long periods). When the blood samples of the volunteers were analyzed, it was demonstrated that total antioxidant capacity of grape dietary fibers and phenolic substances were increased compared to the control group. However, dietary fibers have no significant effect on the recovery of the phenolic substances in the long term (Pérez-Jiménez et al., 2009). M Tamura et al. (2007) investigated the effects of pectin and cellulose on the bioavailability of quercetin and isorhamnetin. According to the results, plasma concentrations of quercetin and isorhamnetin metabolites of mice was found to be higher (2.50 ± 1.30 and 6.66 ± 4.41, respectively) in the presence of pectin than that of the cellulose containing diet group (0.89 ± 0.25 and 0.64 ± 0.29, respectively). Moreover, in both groups, tamarixetin was not be detected (Tamura et al., 2007). On the other hand, there are numerous studies that showed a negative effect of dietary fibers on bioaccessibility or bioavailability of the polyphenols. Tomas et al. (2018) studied the effect of inulin addition in different ratios (5% and 10%) on the bioaccessibility of tomato sauce phenolic compounds. The results showed that there was a decrease in total phenolic content, antioxidant capacity and bioaccessibility in rutin, rutin apioside, naringenin chalcone and naringenin when the inulin content was increased. Addition of 5% and 10% of inulin caused to decrease of total phenolic content by 57% and 68%; and total flavonoid content by 48% and 60%, respectively (Tomas et al., 2018). Similar trends were also obtained by Cebeci and Şahin-Yeşilçubuk (2014) that the bioaccessibility of caffeic acid, ferulic acid, gallic acid, catechin, chlorogenic acid, p-coumaric acid, quercetin-3-galactoside, quercetin-3-β-D-glucoside and anthocyanidins in the blueberries reduced by the comsumption of oats together (Cebeci and Şahin-Yeşilçubuk, 2014).

Table 2.7 : Studies on dietary fiber – phenolic interactions.

Source of the Dietary Fiber	Phenolic Compounds	Methods	Results	References
Pectin and cellulose	Quercetin, rutin, isorhamnetin, tamarixetin	*in vivo* bioavailability in mice blood and feces samples	(+) Bioavailability	(Tamura et al., 2007)
Inulin	Daidzein, genistenin	*in vivo* bioavailability in human blood samples	(+) Bioavailability	(Piazza et al., 2007)
Mango, papaya and pineapple dietary fibers	Mango, papaya and pinapple phenolic compounds	*in vitro* gastrointestinal digestion method	(=) Bioaccessibility	(Velderrain-Rodriguez et al., 2016)
Grapes dietary fibers	Benzoic acid, catechins, hydroxycinnamic acid, other flavonoids and anthocyanidins	*in vivo* bioavailability in human blood samples	(=) Bioavailability	(Pérez-Jiménez et al., 2009)
Carob flour dietary fibers	Phenolic acids, flavonoids aglycones glucosides	*in vitro* gastrointestinal digestion method	(=) Bioaccessibility	(Ortega et al., 2011)
Inulin	Rutin, rutin-apioside, naringenin chalcone, naringenin, glycoalkaloid α-tomatin	*in vitro* gastrointestinal digestion method	(-) Bioaccessibility	(Tomas et al., 2018)
Oat dietary fiber	Caffeic acid, ferulic acid, gallic acid, catechin, chlorogenic acid, p-coumaric acid, quercetin-3-galactoside, quercetin-3-β-D-glucoside, anthocyanidins	*in vitro* gastrointestinal digestion method	(-) Bioaccessibility	(Cebeci and Şahin-Yeşilçubuk, 2014)
Rice Bran Hemi-cellulose	Daidzein	*in vivo* bioavailability in mice blood and feces samples	(-) Bioavailability	(Tamura et al., 2009)
Cellulose and Pectin	Gallic acid, punicalagin A, ferulic acid, chlorogenic acid, caffeic acid and anthocyanins	*in vitro* gastrointestinal digestion method	(-) Bioaccessibility	(Sengul et al., 2014)

(+: increase, -: decrease, =: not significant difference)

Saura-Calixto and Díaz-Rubio (2007) studied about the relationship between dietary fibers and phenolic substances naturally found in the wine. According to the results, nearly 35-60% of total polyphenols in red wine and about 10% of polyphenols in white wine were bound by dietary fibers. It was stated that the absorption of the phenolic compounds in small intestine could not be achieved due to the formation of a complex structure between polyphenols and dietary fibers, resulting with reduced bioaccessibility of the polyphenols (Saura-Calixto and Díaz-Rubio, 2007). Similarly, Motoi Tamura et al. (2009) were also gained descending polyphenol contents in the mice blood plasma samples which were fed by 5% rice bran hemicellulose and 5% cellulose - 0.1% daidzein diet (RBI diet) rather than 5% cellulose - 0.1% daidzein diet for 30 days. (CI diet). It was stated that the diabetic fibers were bound with isoflavones to form a bulk structure, resulting in low concentrations of daidzein in the blood plasma in the RBI diet group (Motoi Tamura et al., 2009). Furthermore, in another study, cellulose decreased the total phenolic content of pomegranate samples in all fractions (PG, IN and OUT). Addition to this, pectin caused to 2-fold decrease of total phenolic content in the serum fraction (IN) (Sengul et al., 2014).

In summary, carbohydrates have an impact on bioaccessibility/bioavailability of the phenolic compounds. Especially, phenolic compounds – dietary fibers interaction may alter the digestion of phenolic compounds in different ways. On the one hand, when phenolic compounds are incorporated by insoluble (non-extractable) dietary fibers with hydrogen or covalent bonds, the absorption of phenolic compounds in the small intestine becomes ineffective, then these compounds are fermented by bacteria in large intestine and their bioavailability can be altered. On the other hand, it was stated that some fermentable fibers such as inulin, resistant starches and fructooligosaccharides could cause to increase the microbiota fermentation of polyphenols (Bohn, 2014).

2.4.2 Phenolic – Lipid Interactions

Polyphenols can interact with lipids from food sources by generally hydrophobic bonding as well as hydrogen and covalent bonds. Lipid-polyphenol complexes may cause a reduction in lipid absorption. In addition, the access of free radicals to lipid molecules can be prevented by the presence of polyphenols in the lipid molecules, resulting in reduced lipid oxidation and consequently reduced formation of harmful lipid oxidation products (Jakobek, 2015).

Dietary lipids increase the gastrointestinal transit time and may alter the kinetics of polyphenol absorption (Bohn, 2014). When lipids interact with polyphenols, they can capture polyphenols and protect them during passage through the gastrointestinal tract (Gorelik et al., 2013).

Various studies have been carried out to understand the bioavailability of phenolic substances in the presence of the lipids in different foods (Table 2.8).

Table 2.8 : Studies on lipid – phenolic interactions.

Source	Phenolic Compounds	Methods	Results	References
Refined olive oil and tomato sauce	Naringenin, Routine, 5-CQA, Caffeic and Ferulic Acid	*in vivo* bioavailability in human blood and urine samples	(+) Bioavailability	(Martínez-Huélamo et al., 2016)
Breakfast meals in different amounts of fat	Quercetin	*in vivo* bioavailability in human blood plasma samples	(+) Bioavailability	(Guo et al., 2013)
Extra Virgin/Refined Olive Oil	Naringenin, Ferulic and Caffeic Acid	*in vivo* bioavailability in human blood plasma and urine samples	(+) Bioavailability	(Tulipani et al., 2012)
Olive oil	Olive Oil/Thyme Phenols	*in vivo* bioavailability in human blood and urine samples	(+) Bioaccessibility	(Rubió et al., 2014)
Fat-rich breakfast meals	Xanthone	*in vivo* bioavailability in human blood and urine samples	(+) Bioavailability	(Chitchumroonchokchai et al., 2012)
Oil (Triacylglycerol) & Mango	Mango Polyphenols	*in vitro* gastrointestinal digestion model	(+) Bioaccessibility	(Liu et al., 2016)
Fatty Yogurt (Milk Oil) & Curcumin	Curcumin	*in vitro* gastrointestinal digestion model	(+) Bioaccessibility	(Fu et al., 2016)
Oil (Dairy Oils) & Blueberry	Anthocyanins	*in vitro* gastrointestinal digestion model	(+) Bioaccessibility	(Ribnicky et al., 2014)
Hazelnut Oil & Goat Horn Flour	Phenolic acids Flavonoids	*in vitro* gastrointestinal digestion model	(+) Bioaccessibility	(Ortega et al., 2011)
Oil (Linoleic Acid) & Pomegranate	Anthocyanins	*in vitro* gastrointestinal digestion model	(+) Bioaccessibility	(Sengul et al., 2014)
Soybean oil, Olive oil & Corn oil	Galangin, Quercetin, Kaempferol, Myricetin	*in vitro* Caco-2/ HT29-MTX cell culture model	(+) Bioaccessibility	(Jailani and Williamson, 2014)

(+:increase, -:decrease, =:not significant difference)

Martínez-Huélamo et al. (2016) examined the effect of refined olive oil addition on the tomato polyphenols recovery and results showed that the bioavailability of the polyphenols in the blood and urine samples increased (Martínez-Huélamo et al., 2016). Moreover, Guo et al. (2013) conducted a study with overweight and adult volunteers whom were provided by non-fat (<0.5 g), low-fat (4 g) and high-fat (15.4 g) breakfasts, each containing equal amounts of quercetin. It was found that plasma quercetin concentration levels enhanced by 45% with the consumption of high fat containing breakfast than that of the fat free trial (Guo et al., 2013). Similarly, ascending bioavailability of tomato phenolic substances in blood plasma and urine samples were achieved with the addition of extra virgin and refined olive oil (Tulipani et al., 2012). Furthermore, Chitchumroonchokchai et al. (2012) investigated the recovery of mangosteen juice polyphenols when consumed with a high-fat breakfast. The absorption of the xanthines was found to be 2% higher in the urine and blood samples when the juice was consumed with oil (Chitchumroonchokchai et al., 2012). In another study, Liu et al. (2016) explored the effect of triacyl glycerols on the bioaccessibility of the phenolic compounds in mango phenolic compounds. This study showed that the bioaccessibility of carotenoids in mangoes increased in long-chain triglycerides containing emulsion than that of medium chain triglycerides one; resulting with the large enough hydrophobic regions in long-chain triglycerides to incorporate long nonpolar bioactive molecules (Liu et al., 2016). Fu et al. (2016) compared the impact of different food matrix including aqueous and fatty yogurt on the recovery of curcumin. A glance at the results reveals that curcumin in yoghurt was 15 times more bioaccessible than that of aqueous phase (Fu et al., 2016). The change in the bioaccessibility of pomegranate anthocyanins within the fat-rich food matrixes were also examined by *in vitro* gastrointestinal digestion model. According to the results, it was obtained that linoleic acid had a positive effect on total antioxidant capacity and bioaccessibility of the pomegranate phenolics (Sengul et al., 2014).

Moreover, absorption behavior of flavonoids – dietary lipids complexes was investigated by means of Caco-2 / HT29-MTX co-culture model. It was found that the hydrophobicity of the flavonoids was the main factor effecting the recovery. In the baser lateral chamber, the addition of oil increased the formation of quercetin and kaempferol conjugates 3 and 4 times, respectively, than that of control sample (without oil) (Jailani and Williamson, 2014).

In conclusion, when the interactions between lipid food matrix and phenolic substances were examined by the *in vivo* and *in vitro* studies, it was shown that bioaccessibility and/or bioavailability of the polyphenols generally found to be positively affected from this food matrix.

2.4.3 Phenolic – Protein Interactions

Phenolic compounds and proteins in food matrix could bind in different ways such as reversible - hydrogen bonds (non-covalent hydrophobic interactions) and irreversible - covalent bonds. Several parameters including molecular weight, structural flexibility and number of OH groups of polyphenols affect the strength of the bonds (Jakobek, 2015). The interactions between protein and phenolic substances can also be influenced temperature, pH, type and concentration of protein and phenolic compounds (Ozdal et al., 2013).

The influence of the protein – phenolic interactions on the bioaccessibility/bioavailability of the phenolic substances have been investigated by both *in vivo* and *in vitro* experiments (Table 2.9).

Green et al. (2007) studied about the effects of different protein sources (bovine, soy and rice milk) on green tea phenolic substances. Results indicated that total catechin recovery slightly increased to 52%, 55% and 69% with the addition of bovine, soy and rice milk, respectively (Green et al., 2007). Similar results were also obtained by Qiu et al. (2017) that bioaccessibility of resveratrol was increased from 54.9% to 70.1% with the formation of gliadin - resveratrol complex (Qiu et al., 2017). In another study, Keogh et al. (2007) evaluated the effect of milk proteins on the bioavailability of the cocoa phenolic compounds. According to the results, milk proteins did not influence the average concentration of polyphenols; however, they accelerated the absorption of polyphenols (Keogh et al., 2007). Neilson et al. (2009) were also demonstrated that milk proteins did not affect the bioaccessibility and bioavailability of the polyphenols in various confectionary products including cocoa beverages, dark, high sucrose and high milk chocolate (Neilson et al., 2009). Furthermore, the effects of proteins from various sources on the recovery of the phenolic substances were investigated. It was concluded that proteins had no effect on the bioavailability of phenolic compounds in the human blood and urine samples (Draijer et al., 2016).

Table 2.9 : Studies on protein – phenolic interactions.

Protein Source	Phenolic Compounds	Methods	Results	References
Bovine, soy and rice milk proteins	Green tea catechins (epicatechin, epigallo-catechin, epigallocatechin-gallate, epicatechin-gallate)	*in vitro* gastrointestinal digestion model	(+) Bioaccessibility	(Green et al., 2007)
Gliadin	Resveratrol	*in vitro* gastrointestinal digestion model	(+) Bioaccessibility	(Qiu et al., 2017)
Milk proteins	Cocoa phenolic compounds (catechins and epicatechins)	*in vivo* bioavailability in human blood samples	(=) Bioavailability	(Keogh et al., 2007)
Milk proteins	Cocoa phenolic compounds ((–)- epicatechins)	*in vivo* bioavailability in human blood samples	(=) Bioavailability	(Roura et al., 2007)
Milk proteins	Chocolate phenolic compounds (flavan-3-ols)	*in vivo* bioavailability in human blood samples *in vitro* Caco-2 cell culture method	(=) Bioavailability (=) Bioaccessibility	(Neilson et al., 2009)
Milk & soy proteins	Grapes and wine phenolic compounds (resveratrol, epicatechin, catechin, valerolactone, m-valerolactone, m-gallic acid, isorhamnetin)	*in vivo* bioavailability in human blood and urine samples	(=) Bioavailability	(Draijer et al., 2016)
Milk proteins	Black tea phenolic compounds (catechins, quercetins and kaempferol)	*in vivo* bioavailability in human blood samples	(=) Bioavailability	(Kyle et al., 2007)
Milk proteins	Instant coffee phenolic compounds (caffeic acid, ferulic acid, isoferulic acid)	*in vivo* bioavailability in human blood samples	(=) Bioavailability	(Renouf et al., 2009)

Table 2.9 (continued) : Studies on protein – phenolic interactions.

Protein Source	Phenolic Compounds	Methods	Results	References
Milk proteins	Coffee phenolic compounds (chlorogenic acid, caffeic acid, p-coumaric acid, ferulic acid)	*in vitro* Caco-2 cell culture method	(=) Bioaccessibility	(Dupas et al., 2006)
Probiotic yoghurt proteins	Soy isoflavone	*in vivo* bioavailability in human blood and urine samples	(=) Bioavailability	(Larkin et al., 2007)
Milk proteins	Jujube juice phenolic compounds (caffeic acid, gallic acid, 4-hydroxybenzoic acid, 3-(4-hydroxyphenyl) propionic acid, mandelic acid, protocatechuic acid, quercetin,quercetin galactoside)	*in vivo* bioavailability in rat blood samples	(=) Bioavailability	(Zhang et al., 2012)
Milk proteins	Coffee phenolic compounds (chlorogenic acid, caffeoyl-1,5-quinolactones)	*in vivo* bioavailability in human urine samples	(-) Bioavailability	(Duarte and Farah, 2011)
Milk proteins	Blueberry phenolic compounds (caffeic and ferulic acid)	*in vivo* bioavailability in human blood samples	(-) Bioavailability	(Serafini et al., 2009)
Milk proteins	Fruit juice phenolic compounds (Caffeic acid Chlorogenic acid Ferulic acid p-coumaric acid p-hydroxybenzoic acid Hesperidin Naringenin Quercetin Rutin)	*in vitro* gastrointestinal digestion method	(-) Bioaccessibility	(Rodríguez-Roque et al., 2015)

Table 2.9 (continued) : Studies on protein – phenolic interactions.

Protein Source	Phenolic Compounds	Methods	Results	References
Milk proteins	Black tea phenolic compounds (theobromine, theogallin, epicatechin, epigallocatechin, epicatechin gallate, epigallocatechin gallate)	Flow-mediated dilation (FMD) of human blood vessels	(-) Bioavailability	(Lorenz et al., 2007)
Milk Proteins (casein and bovine serum albumin)	Black and green tea phenolic compounds (quercetin, rutin)	*in vitro* colorimetric method by using electrogenated bromine	(-) Bioaccessibility	(Nizamova et al., 2011)
Casein and Whey Protein	Oat phenolic compounds (avenanthramide, gallic acid, protocatechuic acid, 4-hydroxybenzoic acid, vanillic acid, caffeic acid, ferulic acid)	*in vitro* gastrointestinal digestion model	(-) Bioaccessibility	(Chen et al., 2019)
Milk/yoghurt/soy milk proteins	Anthocyanins	*in vitro* gastrointestinal digestion model	(-) Bioaccessibility	(Sengul et al., 2014)
Mucin protein	Olive oil phenolic compounds (cinnamic, p-coumaric, caffeic, vanillic, protocatechuic acids, tyrosol, hydroxytyrosol oleuropein)	Polyphenol–mucin interaction assay	(-) Bioaccessibility	(Quintero-Flórez et al., 2015)

(+: increase, -: decrease, =: not significant difference)

Similarly, Kyle et al. (2007), Renouf et al. (2009), Dupas et al. (2006a), Larkin et al. (2007) and Zhang et al. (2012) also found that there was not any effect of proteins on the bioaccessibility/bioavailability of phenolic compounds. On the other hand, Duarte and Farah (2011) indicated that consumption of milk with coffee influenced adversely and decreased the bioavailability of chlorogenic acids. The recovery of the chlorogenic acid was found to be 40% ± 27% for coffee-milk, whereas 68% ± 20% for coffee alone (Duarte and Farah, 2011). In another study, milk proteins had a descending effect on the absorption of ferulic acid (-19.8%) and caffeic acid (-49.7%) in blueberries (Serafini et al., 2009). Additionally, Rodríguez-Roque et al. (2015) examine the potential efficacy of milk and phenolic compounds interactions from different kinds of fruit juices (orange, kiwi, pineapple and mango). The results indicated that the milk proteins could reduce the bioaccessibility of phenolic substances. While total phenolic compound content was found to be 16.8 ± 0.3 in water-fruit juice sample, it was 15.0 ± 0.3 in soy-milk fruit juice blend and 12.7 ± 0.3 in milk-fruit juice blend (Rodríguez-Roque et al., 2015). Similarly, Lorenz et al. (2007), Nizamova et al. (2011), Chen et al. (2019), Sengul et al. (2014) and Quintero-Flórez et al. (2015) were also found decreased bioaccessibility/ bioavailability of phenolic substances by addition of the proteins.

On the other hand, interactions between protein and phenolic substances can be influenced by many factors such as temperature, pH, type and concentration of protein and phenolic compounds Ozdal et al. (2013). Furthermore, Ozdal et al. (2013) stated that temperature have strengthening and debilitating effect on protein-phenolic bond structure. Also, it was noted that this bond structure will strengthen in the low pH range. Strengthening of bonds in low pH environment is interpreted as a factor that decreases bioavailability of both substances; this finding may be a valid reason for the decrease in bioavailability of phenolic substances in acidic fruits. In addition to these, it was reported that there is a linear relationship between the molecular size of phenolic substances and the binding capacity of proteins (Ozdal et al., 2013).

2.4.4 Others

In addition to major food matrix constituents such as proteins, lipids and carbohydrates, phenolic compounds as well as vitamins are also able to influence the bioaccessibility/ bioavailability of the polyhenols. The studies including phenolic-phenolic and vitamin-phenolic interactions are reported in Table 2.10.

Table 2.10 : Studies on phenolic - phenolic and phenolic - vitamin interactions.

Phenolic Compound	Phenolic/Vitamin Compound	Methods	Results	References
Oligomers-Heptamers	Procyanidins	*in vitro* Caco-2 cell culture method	(+) Bioaccessibility	(Kosińska and Andlauer, 2012)
Tetramer proanthocyanidins	Procyanidin B2	*in-situ* perfusion of the small intestine of rats	(+) Bioavailability	(Appeldoorn et al., 2009)
Apple procyanidin oligomers	Apple procyanidins with high molecular weight	*in vivo* bioavailability in rat blood samples	(+) Bioavailability	(Shoji et al., 2006)
Rosmarinic acid	Apigenin and luteolin	*in vitro* Caco-2 cell culture method	(+) Bioaccessibility	(Falé et al., 2013)
Hesperetin	Isoflavones and flavones	*in vitro* Caco-2 cell culture method	(+) Bioaccessibility	(Brand et al., 2010)
Catechin	Ascorbic acid	*in vitro* Caco-2 cell culture model	(+) Bioaccessibility	(Peters et al., 2010)
(-) Epigallocatechin-3-gallate	Ascorbic acid	*in vivo* bioavailability in human blood samples	(+) Bioavailability	(Gawande et al., 2008)

(+: increase, -: decrease, =: not significant difference)

Kosińska and Andlauer (2012) stated that some constituents (oligomers up to heptamers) in the purified cocoa powder extract could enhance the bioaccessibility of small procyanidins (Kosińska and Andlauer, 2012). In another study, Appeldoorn et al. (2009) pointed out a study that bioavailability of procyanidin B2 was increased by the addition of tetramer proanthocyanidins (Appeldoorn et al., 2009). Similar trends were also obtained by Shoji et al. (2006) that absorption of apple procyanidin oligomers was affected by procyanidins with high molecular weight (≥ 8-mer). Due to the fact that procyanidins with high molecular weight bound to mucosal proteins, procyanidin oligomers did not be affected from these proteins and were absorbed more, resulting with the increasing bioaccessibility of procyanidin oligomers (Shoji et al., 2006). Moreover, bioaccessibility of rosmarinic acid has been ascended by the addition of apigenin and luteolin (Falé et al., 2013). Brand et al. (2010) were also obtained that similar transporting the hesperetin through Caco-2 cells could be enhanced by the use of some phenolic compounds such as isoflavones and flavones (Brand et al., 2010).

Addition to these, the positive effects of phenolic-vitamin interactions on the recovery of the phenolic compounds have been stated. Peters et al. (2010) declared that catechin absorption rate was found to be 2.5 to 3 times higher with the addition of ascorbic acid (Peters et al., 2010). Similarly, the intake of (-) epigallocatechin-3-gallate enhanced by 14 to 27% with the use of ascorbic acid (Gawande et al., 2008).

Consequently, it has been noticed that bioavailability of the phenolic compounds are generally limited due to the some transporter constituents in the small intestine. These transporter compouns are blocked by the addition of phenolic or vitamins; therefore there is an enhanced effect of phenolic/vitamin- phenolic interactions on the bioaccessibility/bioavailability of the phenolic compounds.

3. RETENTION OF POLYPHENOLS AND VITAMIN C IN CRANBERRYBUSH PURÉE (*VIBURNUM OPULUS*) BY MEANS OF NON-THERMAL TREATMENTS[2]

3.1 Abstract

The effect of high pressure processing (HPP; 200-600 MPa for 5 or 15 min) and pulsed electric field (PEF; 3 kV/cm, 5-15 kJ/kg) treatment on physicochemical properties (conductivity, pH and total soluble solids content), bioactive compounds (vitamin C, total phenolic (TPC), total flavonoid (TFC), total anthocyanin (TAC) and chlorogenic acid contents), antioxidant capacities (DPPH and CUPRAC assays) and polyphenol oxidase (PPO) activity of cranberrybush purée were evaluated immediately after processing. The results were compared to an untreated purée. Results showed that conductivity increased significantly after PEF (15 kJ/kg) treatment. PEF and HPP treatments resulted with a better retention of bioactive compounds (increase in the TPC in the range of ~4 – 11% and ~10 – 14% and TFC in the range of ~1 – 5% and ~6 – 8% after HPP and PEF, respectively) and antioxidant capacity (as measured with CUPRAC method) compared to untreated sample. HPP reduced residual enzyme activity of PPO comparatively better than PEF.

Key words: Non-thermal technologies, high pressure processing (HPP), pulsed electric field (PEF), cranberrybush, bioactive compounds, polyphenols, antioxidant capacity, enzyme activity

3.2 Introduction

Viburnum species (Adoxaceae) exhibit a widespread natural distribution in the world. Among 230 species, *V. opulus* L., *V. orientale* Pallas, *V. lantana* L., and *V. tinus* L. are recorded in the flora of Turkey. *V. opulus* (European cranberrybush) fruits are called as Gilaburu in Anatolia (Barak et al., 2019). Red-coloured berries of European

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cranberrybush have a strong astringent taste and its own unique aroma (Velioglu et al., 2006). They are also used locally for making traditional drink, jam, jelly and marmalade, and considering their high pectin contents at consumption ripeness in early autumn, additional pectin is not required (Cam and Hisil, 2007). Since the berries have resistance to cold weather, it is also possible to harvest the fruits in winter months with decreased bioactive substances, but increased dry matter and sugar contents (Nikitina, 1998). In Turkish folk medicine, *V. opulus* have been used for its healing properties due to antimicrobial (Sagdic et al., 2006), antioxidant (Ersoy et al., 2017), antidiabetic (Altun et al., 2008), diuretic (Bae et al., 2010), hypoglycemic and cough reliever (Fujita et al., 1995) properties. Meanwhile, it is also reported that fruits are used against kidney problems, including to pass stone/sand (Ilhan et al., 2014).

Conventional thermal treatments have a deleterious impact on bioactive contents of berry purées, juices and extracts. For example; Klopotek et al. (2005) reported 65 and 85% loss for ascorbic acid and total phenolics, respectively, after thermal pasteurization of strawberry juice. Due to the increasing demand on minimally processed healthy foods with improved nutritional quality, sensory properties and long shelf-life, novel non-thermal food processing technologies such as high pressure prosessing (HPP), pulsed electric fields (PEF) or ultrasound are widely investigated and partly find industrial application (Ozkan et al., 2019). HPP is increasingly used to improve microbiological safety and extend the shelf-life of food products with minimal influence on the physical, nutritional and sensory properties (Jofré et al., 2010). Hydrostatic pressure is applied from 100 to 1000 MPa for 30 s to a few minutes at room or mild processing temperatures (<60 °C) to solid, liquid, packaged, or unpackaged foods (Norton and Sun, 2008). With regard to the use of PEF, short duration pulses of the applied electric field affect the permeability of biological membranes resulting in reversible or irreversible permeabilization. Strong electric fields in the range of 5–50 kV/cm are applied to ensure complete, irreversible inhibition of vegetative microbial cells (Toepfl et al., 2006). In addition to these, greater permeability and cell disintegration in the plant tissues may also enhance the release of the intracellular bioactive substances (Puértolas et al., 2010). Besides, it has been demonstrated that PEF treatment at electric field strengths between 0.5 and 1.5 kV/cm is sufficient in terms of release of anthocyanins from grape skin cells (Delsart et al., 2012). Both technologies have been tested in several fruits and vegetables,

including raspberry and blueberry purées (Medina-Meza et al., 2016), grape juice (Leong et al., 2016a), carrot purée (Leong et al., 2015), carrot juice (Stinco et al., 2019), apple and cranberry juice blend (Caminiti et al., 2011), aronia purée (Yuan et al., 2018), orange juice (Timmermans et al., 2011), exotic fruit juice blend (Buniowska et al., 2017), strawberry purée (Aaby et al., 2018; Marszałek et al., 2015; Patras et al., 2009) and tomato juice (Min et al., 2003), among others.

Therefore, the aim of this research was to understand the effect of different HPP (at 200 600 MPa for 5 or 15 min) and PEF (3 kV/cm, 5-15 kJ/kg) treatments on the release of bioactive compounds from cranberrybush purée. To achieve this objective, physicochemical properties, residual enzyme activities, bioactive contents as well as antioxidant capacities of HPP/PEF-treated and untreated cranberrybush purées were critically evaluated. Although there have been some reports on the bioactive characteristics of cranberrybush grown in different parts of the world, to the best of our knowledge, this is the first study that has been conducted to investigate the effects of novel approaches to obtain extracts from Turkish *V. Opulus* fruits.

3.3 Materials and Methods

3.3.1 Chemicals

All chemicals and standards used in the analyses were purchased from Sigma-Aldrich (Steinheim, Germany). The quality of all chemicals was of analytical or LC-MS grade.

3.3.2 Preparation of cranberrybush purée

Fresh cranberrybush (*Viburnum opulus* L.) at commercial maturity was obtained in December 2019 from a local grower in Kayseri (Turkey). After harvest, fruit samples were stored in water at room temperature in dark until further use. Before treatments, they were visually screened for any damage and manually destemmed. Cranberrybush purée was prepared on the day of the experiment by homogenising whole fruit samples (2 kg for each batch) using a blender (Blendtec Classic 575, Bad Homburg, Germany) for two cycles of 10 s at low speed (16 000 g), followed by a subsequent 10 s cycle at high speed (22 000 g). Three independent batches (n = 3) were prepared for each untreated control, HPP- and PEF-treated samples.

3.3.3 High pressure processing (HPP)

The cranberrybush purée was processed by using industrial-scale HPP equipment (Wave 6000/55, Hiperbaric S.A., Burgos Spain). Prior to the treatment, the cranberrybush purée was vacuum-packed and heat sealed in low density polyethhylene pouches (LDPE). The packed purée was then subjected to a pressure of 200, 400 and 600 MPa for 5 or 15 min at ambient temperature (20 ± 2°C). Water was used as medium for transmitting pressure. Finally, the purée was packed into sterilized falcons and stored at -80 °C until analysis.

3.3.4 Pulsed electric field (PEF) treatment

PEF treatment was conducted by using a pilot scale PEF batch system (PEF Pilot, Elea GmbH, Germany). The system provided a voltage up to 30 kV and monopolar, exponential decay pulses with pulse duration of 40 ms. The PEF operating settings applied were: constant pulse width of 20 µs, 3 kV/cm electric field strengths, 6 kV voltage, different specific energy inputs as 5, 10 and 15 kJ/kg and constant pulse frequency of 2 Hz (0.5 s). The specific energy intake *Wspec* (kJ/kg) and the electric field strength *E* (kV/cm) were calculated according to the following equations (3.1) and (3.2).

$$Wspec = (U^2C\,.n)/2m \tag{3.1}$$

$$E = U/d \tag{3.2}$$

where *n* (dimensionless) is the number of pulses; *m* is the total weight of cranberrybush purée (kg); U is the voltage (kV) and d is the distance between the electrodes (cm); C is the capacitance (1 µF).

The treatment chamber had a 2 cm electrode gap and a volume of 17 ml. Following the treatment, the samples were stored at -80 °C until analysis.

3.3.5 Conductivity, pH and total soluble solids measurements

Conductivity and pH were determined using an electrical conductivity meter (LabQuest 2-Vernier, Beaverton, USA) and pH meter (Inolab, Wuppertal, Germany), respectively.

Total soluble solids content (TSSC) of cranberrybush purée, expressed in °Brix, was measured at 20 °C using a hand-held refractometer (Atago, Master Refractometer, Japan).

3.3.6 Determination of vitamin C content by HPLC with fluorescence detection

Dehydro-/Ascorbic acid was determined according to the method of Kneifel & Sommer (1985). A sample of 5 g cranberrybush purée was homogenized with 100 mL acid composed of 0.77 M *m*-phosphoric acid and 3.5 M acetic acid solution in water. The homogenate was mixed with activated charcoal for the reduction of L-ascorbic acid to dehydro-L-ascorbic acid and followed by separation with a pleated filter. Then, 10 mL phenylenediamine solution was added to 4 mL of the diluted filtrate (x 100) and mixed for 90 min on a magnetic stirrer. After membrane filtration (0.45 µm) and incubation for 16 hours in the dark at room temperature, the samples were injected (20µL) and separated on an HPLC system (HPLC Alliance Waters Separations Modul 2695, Eschborn, Germany) equipped with a column LiChrospher 100 RP 18 (5 µm, 250 x 4 mm) and detected by a fluorescence detector (Fluorescence Detector 2475 Multi Lambda, Eschborn, Germany). The emission was set at 430 nm and the extinction at 350 nm. The mobile phase was consisted of 50% methanol with 92 mM sodiumacetate, 4.6 mM phosphoric acid and 21 mM acetic acid at isocratic conditions with a flow rate of 0.8 mL/min.

3.3.7 Determination of polyphenol oxidase (PPO) activity

The extraction of PPO was carried out according to the procedure described by Zhao et al. (2010) with some modifications. Cranberrybush purée samples (X g) were homogenised with (1.5 X mL) Na-phosphate buffer (50 mM, pH 6.0, containing 1 M NaCl). The homogenates were centrifuged at 11,000 g and 4 °C for 20 min (Megafuge 8R; Thermo Scientific, Darmstadt, Germany). The supernatant was collected and kept in an ice-water bath until performing the assay, but no longer than 3 h.

The measurement was performed at 25 °C at 420 nm using UV/visible spectrophotometer (Tecan Spark, Männedorf, Switzerland) (Siguemoto and Gut, 2017). The reaction mixture consisted of 100 µL of Na-phosphate buffer (pH 6.5) and 33 µL of the sample incubated for 1 min. A freshly prepared solution of pyrocatechol

(50 mM, 67 µL) was added and the change in absorbance was monitored every 10 s for 3 min. Determinations were performed in triplicate.

The reference value was determined using a blank solution containing Na-phosphate buffer and pyrocatechol. One unit of enzymatic activity (U) is defined as an absorbance increase of 0.001 per minute under the assay conditions.

The residual activity (RA) of enzymes is estimated with the following equation (3.3):

$$Residual\ PPO\ activity = A/A0 \quad (3.3)$$

where A is the PPO activity after treatment and A_0 is the PPO activity before treatment.

3.3.8 Extraction of polyphenols

The extraction of polyphenols was performed according to the procedure described by Leong et al. (2016a) with some modifications. Extraction of polyphenols in cranberrybush purée (5 g) was performed by adding 5 mL acidified methanol with 0.1% formic acid (v/v) to the sample. Afterwards, the mixture was vortexed for 10 s and left overnight (12 h) at 4 ℃. The following day, the mixture was centrifuged for 15 min at 4,000 rpm and 4 ℃ (Megafuge 8R; Thermo Scientific, Darmstadt, Germany). This extraction protocol was repeated three times and the supernatants were pooled to a final volume of 15 mL. Then, the supernatant was filtered through a 0.20 µm pore size polytetrafluoroethylene filter and stored at -80 ℃ until further analysis.

3.3.9 Identification of polyphenols by UPLC-QTOF-MS/MS

Identification of cranberrybush polyphenols was adapted from a previously published method with minor modifications (Kamiloglu, 2019). Before LC-MS analysis, extracts of different processing conditions were passed through 0.20 µm membrane filters. LC-MS analysis was performed on a Waters Acquity UPLC system (Waters Co., Milford, MA, USA) connected to a Waters Q-Tof Premier mass spectrometer equipped with an electrospray ionisation (ESI) source. The UPLC system was equipped with a binary pump with degasser, autosampler, tunable UV (TUV) detector and a column manager. For chromatographic separation, a Waters Acquity UPLC BEH Phenyl (2.1*100mm, 1.7µm) column was used. Mobile phase consisting of formic acid/MQ water (1/1000, v/v; eluent A) and formic acid/acetonitrile (1/1000, v/v; eluent B) was used. The linear

gradient was as follows: 0 min, 5% B; 0-6.48 min, 35% B; 6.48-6.77 min, 100% B; 6.77-8.00 min, 100% B; 8.00-8.10 min, 5% B. The injection volume was 10 µL, and the flow rate was set at 0.6 mL/min. The column temperature was kept at 45 °C, while the temperature of the autosampler was held at 10 °C. ESI-MS analysis was performed in negative and positive modes. Collision energies of 15 V (for low energy) and 30 V (for high energy) were used for full-scan LC-MS in the *m/z* range 100−1500. Acquisition and integration of chromatograms were performed using the Masslynx V4.1 software from Waters.

3.3.10 Quantification of chlorogenic acid using UPLC-UV

The method for determination of chlorogenic acid by UPLC was adapted from Kamiloglu et al. (2019) with minor modifications. Extracts obtained from different processing conditions were passed through 0.20 µm membrane filters and injected into a Waters Acquity UPLC system (Waters Co., Milford, MA, USA) coupled with a TUV detector. A Waters Acquity UPLC BEH Phenyl (2.1*100mm, 1.7µm) column was used as stationary phase. The following solvents with a flow rate of 0.6 mL/min and injection volume of 10 µL at 330 nm were used for spectral measurements: formic acid/MQ water (1/1000, v/v; eluent A) and formic acid/acetonitrile (1/1000, v/v; eluent B). The linear gradient was as follows: 0 min, 5% B; 0-6.48 min, 35% B; 6.48-6.77 min, 100% B; 6.77-8.00 min, 100% B; 8.00-8.10 min, 5% B. Chlorogenic acid was quantified by using its authentic standard. For calibration curves, chromatographic peak area of the standards versus nominal concentrations were plotted. All results were expressed as milligrams chlorogenic acid per 100 g fw (fresh weight) of sample. Each measurement was carried out in three replicates.

3.3.11 Spectrophotometric assays

Total phenolic content (TPC) was determined using Folin-Ciocalteu reagent as described previously by Singleton and Rossi (1965). The measurement was performed at 765 nm and the results were expressed as mg gallic acid equivalents (GAE) per 100 g fw sample.

Total flavonoid (TFC) content assay was performed according to Dewanto et al. (2002). The samples were analyzed at 510 nm and the results were expressed as mg rutin equivalents (RE) per 100 g fw sample.

The total monomeric anthocyanin content (TAC) was determined by pH differential method (Giusti and Wrolstad, 2001). Absorbances of samples diluted with pH 1.0 and pH 4.5 buffers were measured at 520 and 700 nm. The results are expressed as cyanidin-3-*O*-glucoside equivalents per 100 g fw by using the following formula:

$$TAC\ (mg/L) = (A\ x\ MW\ x\ DF\ x\ 1000)/(\varepsilon\ x\ 1) \quad (3.4)$$

where A = $(A520nm - A700nm)_{pH1.0} - (A520nm - A700nm)_{pH4.5}$, MW is the molecular weight of cyanidin-3-*O*-glucoside (449.2 g/mol), DF is the dilution factor, 1000 is the conversion factor from g to mg, ε is the molar extinction coefficient of cyanidin-3-*O*-glucoside (26900 L/(mol.cm)), and l is the path length (cm).

The antioxidant activities were estimated by using the cupric ion reducing antioxidant capacity (CUPRAC) (Apak et al., 2004) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Molyneux, 2004) assays. In the assays, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was used as a standard. Results were expressed as mg Trolox equivalents (TE) per 100 g of fw sample.

3.3.12 Statistical analysis

All experiments were conducted at least in triplicate. Results were reported as mean ± standard deviation. Error bars on figures represented standard deviations. Statistical analysis was applied using SPSS software (version 20.0, SPSS Inc. Chicago, IL, USA). Treatments were compared using one-way analysis of variance (ANOVA) followed by a Tukey post hoc test ($p < 0.05$). The correlation coefficients (R^2) were calculated using Microsoft Office Excel 2016 software (Microsoft Co. Redmond, WA, USA).

3.4 Results and Discussion

3.4.1 Physicochemical properties

The physicochemical characteristics measured in this study for all cranberrybush purée samples (control, HPP- and PEF-treated) included pH, total soluble solids and conductivity (Table 3.1). The initial pH value of cranberrybush purée was 3.09 ±0.02. No significant differences were observed in all treated and untreated samples ($p>0.05$). Results indicated that HPP did not accelerate further liberation of organic acids from the intracellular matrix of cells, as already reported by Varela-Santos et al. (2012) in

pomegranate juice, Huang et al. (2016) in strawberry and raspberry purées, Yi et al. (2016) in kiwifruit purée, and Aaby et al. (2018) in strawberry purée. Furthermore, the results of this study are in line with earlier reports wherein pH of the raspberry and blueberry purées (Medina-Meza et al., 2016) and orange juice (Sánchez-Moreno et al., 2005) did not have significant variations after PEF treatment.

Table 3.1 : Physicochemical properties of cranberrybush purée samples*.

	Treatment	TSSC (°Brix)	Conductivity (µS/cm)
HPP	Control	9.00 ± 0.001^{bcA}	317.05 ± 0.24^{ab}
	200 MPa/5 min	8.90 ± 0.14^{bcA}	n.d.
	400 MPa/5 min	9.00 ± 0.08^{bcA}	n.d.
	600 MPa/5 min	8.90 ± 0.08^{bcA}	n.d.
	200 MPa/15 min	9.07 ± 0.05^{bcA}	n.d.
	400 MPa/15 min	9.03 ± 0.05^{bcA}	n.d.
	600 MPa/15 min	8.97 ± 0.12^{bcA}	n.d.
PEF	5 kJ/kg	9.20 ± 0.16^{abcA}	317.10 ± 0.25^{ab}
	10 kJ/kg	9.47 ± 0.41^{abA}	317.30 ± 0.37^{ab}
	15 kJ/kg	9.67 ± 0.09^{aA}	317.97 ± 0.17^{a}

*Data is given as average values ± standard deviation of three independent batches. Different small letters in the same column represent statistically significant differences ($p < 0.05$) between treatments. Different capital letters in the same column indicate significant differences ($p < 0.05$) within HPP or PEF treatments, individually, in comparison with the control. TSSC; total soluble solids content; n.d. : not determined.

The TSSC value of the control sample was found to be 9.00±0.001 % and HPP treatment did not have a significant effect on TSSC ($p > 0.05$). Similar results were also found in the studies on papaya beverage, apple juice, strawberry purée and multi-vegetables smoothies (Chen et al., 2015c; Abid et al., 2014; Aaby et al., 2018; Hurtado et al., 2019). On the other hand, the highest TSSC values were obtained after PEF, in particular with 10 and 15 kJ/kg specific energy inputs. The intracellular substances including solutes, sugars, bioactive compounds and juices are held by the cell membrane of any plants which acts as a physical barrier (Lamanauskas et al., 2015). The use of mild or moderate PEF can induce a reversible pore formation or the local rupture of the cell membrane in the tissues of plants, which encourage and accelerate the free flow of these intracellular substances (Pataro et al., 2011; Lamanauskas et al., 2015). Note that the previous studies evidence improvement of the extraction of phenolic compounds by PEF from red cherries (Sotelo et al., 2018), pomegranate peels (Rajha et al., 2019), apple juice (Praporscicet al., 2007), apple peels (Wang et al., 2020) as well as extraction of sugar from sugar beet (Vorobiev and Lebovka, 2012).

Electrical conductivity is a characteristic of a biological tissue which is used to evaluate the effectiveness of PEF treatment in terms of electric field strength output (Aronsson and Rönner, 2001). Following electroporation and intracellular content leakage, electrical conductivity of plant materials increases, resulting in cell membrane rupture (Tylewicz et al., 2016). Thus, it is necessary to monitor conductivity of the product to achieve a consistent output of electric field strengths. There was a little change in conductivity of the samples, especially PEF treatment at 15 kJ/kg specific energy input indicating that cell disruption with higher specific energy input caused release of minerals.

3.4.2 PPO activity

The activity of some enzymes could be critical during purée processing due to their detrimental effects both on bioactives and the organoleptic characteristics of the final product (Martinez and Whitaker, 1995). The residual activity (RA; %) of PPO in cranberrybush purées after HPP and PEF treatments was measured (Figure 3.1). As shown in Figure 3.1, at 200 or 600 MPa, PPO activity gradually decreased by increasing pressure levels and extending the treatment times for HPP; the highest inactivation of PPO activity was 72.2 % at 600 MPa for 15 min. Cao et al. (2011) reported that the highest reduction of PPO activity in strawberry pulps was 51.5% at 600 MPa for 25 min. Aaby et al. (2018) reported that pressure treatment at 400, 500 and 600 MPa for 3 min reduced PPO activity with 11, 12 and 30%, respectively. Other studies indicated a complete inactivation of PPO. For example; Garcia-Palazon et al. (2004) showed a complete inactivation of PPO in strawberry fruits at 600 MPa for 15 min at room temperature. Dalmadi et al. (2006) indicated that there was a decrease in PPO activity in strawberries after HPP treatment at 500 MPa for 15 min; only 5% RA was detectable at 800 MPa for 15 min. Furthermore, PPO was completely inactivated in mango nectars after HPP treatment at 600 MPa for 1 min (Liu et al. 2014). The enzyme inactivation may be due to the dissociation or modification of the prosthetic group and conformational change in the enzyme (Akyol et al., 2006). The difference in pressure resistance of various PPOs to HPP may be affected by the cultivars, form of the products, pH, processing conditions, etc. (Cao et al. 2011). According to the results of this research, the reduction of PPO activity was different at 400 MPa compared with 200 or 600 MPa; RA of PPO was increased when the application time was increased from 5 min to 15 min. These results are in close agreement with other

studies on the application of HPP for PPO inactivation in carrot and feijoa juices, where the authors also noted an increase in RA of PPO (Stingo et al., 2019; Ortuno et al., 2013). Higher residual PPO activity at higher pressure levels or treatment times may be as a result of mechanical disruption of plant cells and releasing of bound enzymes from the plant tissue (Stinco et al., 2019). Moreover, it could be also related to the pressure-activated latent form due to conformational changes of the enzyme (Akyol et al., 2006).

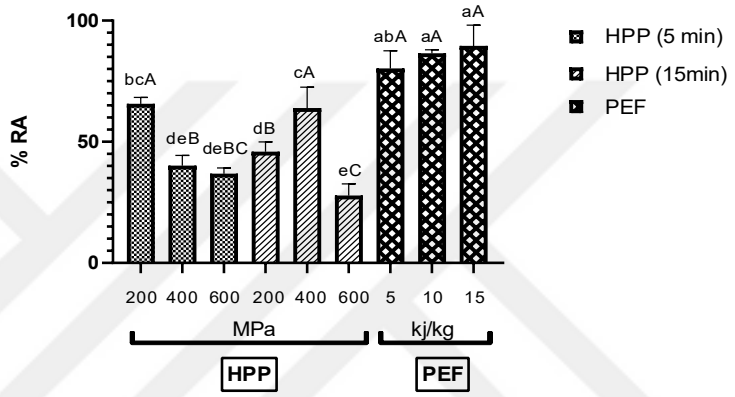


Figure 3.1 : Residual PPO activity of HPP- and PEF-treated samples.

In addition, PEF treatment showed a significant effect ($p < 0.05$) on the activity of PPO, with about 80.2 – 89.6 % of RA, depending on the specific energy input. This data indicates that PPO in cranberrybush purée showed higher resistance to PEF at selected intensities. The decrease of residual PPO activity with PEF treatment may stem from the conformational changes in enzyme structure (Huang et al., 2012). Medina-Meza et al. (2016) reported about 98 % RA of PPO in raspberry and about 80 % in blueberry purées after PEF treatment (388.1 and 244.4 W total specific energy for raspberry and blueberry purées, respectively and 66 μs treatment time). A remarkable inactivation of PPO after PEF treatment was also reported in several studies. Giner et al. (2001) reported a pronounced decrease in residual PPO activity up to 3.15% and 38.0% in apple extract and pear extract, respectively, after PEF treatment (22.3 kV/cm, 6 ms). These results are in agreement with Noci et al. (2008), who reported that there was about 45% RA in fresh apple juice after PEF treatment (6.67 s residence time and 100 pulses per transit), by using a lab-scale equipment. Furthermore, the RA of PPO in apple juice was reduced by 7.1–98.5%. PPO was almost completely inactivated after PEF treatment at 35 kV/cm and 2 μs pulse rise

time (Bi et al., 2013). On the other hand, Huang et al. (2019) presented that there was no significant effect ($p > 0.05$) on the activity of PPO in apricot with 0.625 – 1.25 kV/cm electric field strength and 30 – 60 s treatment time application. Similar results were also observed by Meneses et al. (2013). It was indicated that there was no inactivation of PPO in mushrooms after PEF treatment (13–32 kV/cm, 6–48 μs) at temperatures below 55 °C. According to the results of this study, RA was found to be ascended with increasing specific energy input, which was attributed to the release of PPO from cells as a result of electroporation. Indeed, similar results were previously obtained by Van Loey et al. (2001), who reported an enhanced PPO activity in squeezed apple juice after PEF treatment (31 kV/cm, 1000 pulses, 1 μs). These studies showed that the crucial factors affecting the PPO activity depend on the PEF processing conditions including electric field strength, number of pulses, pulse width, electrical energy density, pulse frequency, pulse shape, and pulse polarity (Poojary et al., 2017); characteristics of the treatment chamber and the liquid nature (conductivity) of the treated materials (Huang et al., 2012).

3.4.3 Vitamin C content

Vitamin C is prone to degradation under the influence of pH, temperature and the presence of metal ions, oxygen, light and oxidative enzymes due to its less stable nature than other vitamins (Davey et al., 2000). The results regarding the effects of treatments of cranberrybush purée on bioactive compounds are shown in Figure 3.2. The vitamin C content of the control cranberrybush purée was 37.9±0.01 mg/100 g fw. This value is in the range of those previously reported by other authors (Ersoy et al., 2017). They reported from 27±2.8 to 35±3.1 mg vitamin C/100 g fw in ten cranberrybush genotypes. While vitamin C content in the control samples showed significant reduction after HPP application, there was no significant change in vitamin C content for the PEF-treated purées. This indicated that vitamin C was better preserved through PEF processing. Different studies have proven the effectiveness of PEF in achieving higher vitamin C retention. For instance, Leong et al. (2015) declared that the content of vitamin C in carrot juice was not significantly affected by PEF, which demonstrated that low intensity PEF did not have a promoting impact for the release of vitamin C from purée into the juice at the electric field strength from 0.1 to 1.1 kV cm^{-1}. Additionaly, vitamin C content remained at initial levels after PEF

processing (1.5 kV/cm at 15 or 70 kJ/kg) of grape juice (Leong et al., 2016b); high-intensity pulsed electric filed (HIPEF) processing (35 kV/cm for 1,700 μs in bipolar 4 μs pulses at 100 Hz) of strawberry juice (Odriozola-Serrano et al., 2008) and PEF treatment (40 kV/cm, a pulse duration time of 2 μs, and a total PEF treatment time of 57 μs) of tomato juice (Min et al., 2003).

Furthermore, significant differences were determined in the HPP-treated samples depending on the treatment pressure and exposure time. Levels of vitamin C in the samples treated at 200 and 400 MPa were significantly lower (~23% loss) than the fresh purée (p<0.05). On the other hand, pressurisation at 600 MPa/5 min preserved the majority of vitamin C in the samples (~90%). These results are in line with Patras et al. (2009), who indicated a significant reduction in ascorbic acid content of HPP-treated strawberry purées at 400 and 500 MPa/15 min, while retention (94%) of ascorbic acid content was found to be the highest by processing at 600 MPa/15 min. On the other hand, Marszałek et al. (2015) showed a decrease in the content of vitamin C in HPP-preserved strawberry purée at at 300 or 500 MPa for 1, 5 or 15 min.

3.4.4 Bioactive compounds

Spectrophotometric quantitation of phenolics, flavonoids, and anthocyanins are summarized in Figure 3.2. TPC and TFC were increased at elevated pressure levels and extended treatment times for HPP; and increasing the specific energy input for PEF. HPP and PEF caused an increase in the TPC in the range of ~4 – 11% and ~10 – 14%, respectively under different treatment conditions. Similar findings were reported by Yuan et al. (2018), indicating a 3–13% increase in TPC of HPP – treated (200 – 600 MPa/2.5-5 min) aronia purée. In addition, the increase in TFC was found to be ~1 – 5% for HPP- and ~6 – 8% for PEF- treated samples. However, mostly this increase was not statistically significant (p > 0.05). Only, the cranberrybush purée sample processed at 15 kJ/kg specific energy input resulted with significantly higher values (p < 0.05) for TPC and TFC compared to all other samples. Moussa-Ayoub et al. (2017) also demonstrated that PEF (specific energy input of 85 kJ/kg) and HPP (600 MPa/10 min) led to a significant increase in the content of cactus juice flavonols in comparison to the untreated juice.

The present study showed that the application of HPP and PEF on cranberrybush purée preserved TAC in all samples regardless of the processing intensity. There were no

significant changes in TAC; however a slightly increasing trend was obtained. These results were in agreement with the results from Yuan et al. (2018), which showed that high pressure processing at 400–600 MPa for 5 min did not influence the content of anthocyanins in aronia purées. Patras et al. (2009) reported that there was no significant change in TAC after HPP processing (400, 500, 600 MPa/15 min) of the strawberry and blackberry purées. Regarding the PEF application, it was reported that TAC content was increased by the high energy input PEF processing as compared to the control, and the low energy input PEF processing (Leong et al., 2016b). On the other hand, Howard et al. (2012) reviewed the processing (thermal pasteurization) effect on berry polyphenols and emphasized a degradation (~10%) in TAC of blueberry juices.

The slight increase in TPC, TFC and TAC may be triggered by the enhanced extractability of phenolic compounds in the HPP/PEF treated cranberrybush purée. High hydrostatic pressure applied to the food material could bring along a large pressure difference on either side of the cell membrane, resulting with improved cell permeability, mass transfer, and liberation of matrix bound phenolic compounds (Grunovaitė et al., 2016). Besides, the higher level of TPC, TFC and TAC in the HPP- and PEF-treated cranberrybush purée may also arise from the inactivation of the endogenous deteriorative enzymes (Błaszczak et al., 2017). While PPO and peroxidase (POD) activity degrading phenolic compounds, β-glucosidase is responsible for the degradation of anthocyanins (Suthanthangjai et al., 2005).

In this study, phenolic compounds in samples were analyzed both by spectrophotometric and chromatographic methods. It has been reported that analysis of TPC using Folin-Ciocalteu reagent may not be specific to phenolic compounds, because reducing compounds present in the sample including reducing sugars, certain amino acids, citric acid, dehydroascorbic acid may also interfere and the values can be overestimated (Capanoglu et al., 2018). Similarly, in the aluminium chloride assay used for the determination of TFC, phenolic acids may also interfere and give a quite strong reaction in the assay (Ho et al., 2012). Considering the lack of specificity in these spectrophotometric assays, chromatographic analysis of individual main polyphenols was also performed to obtain more accurate results.

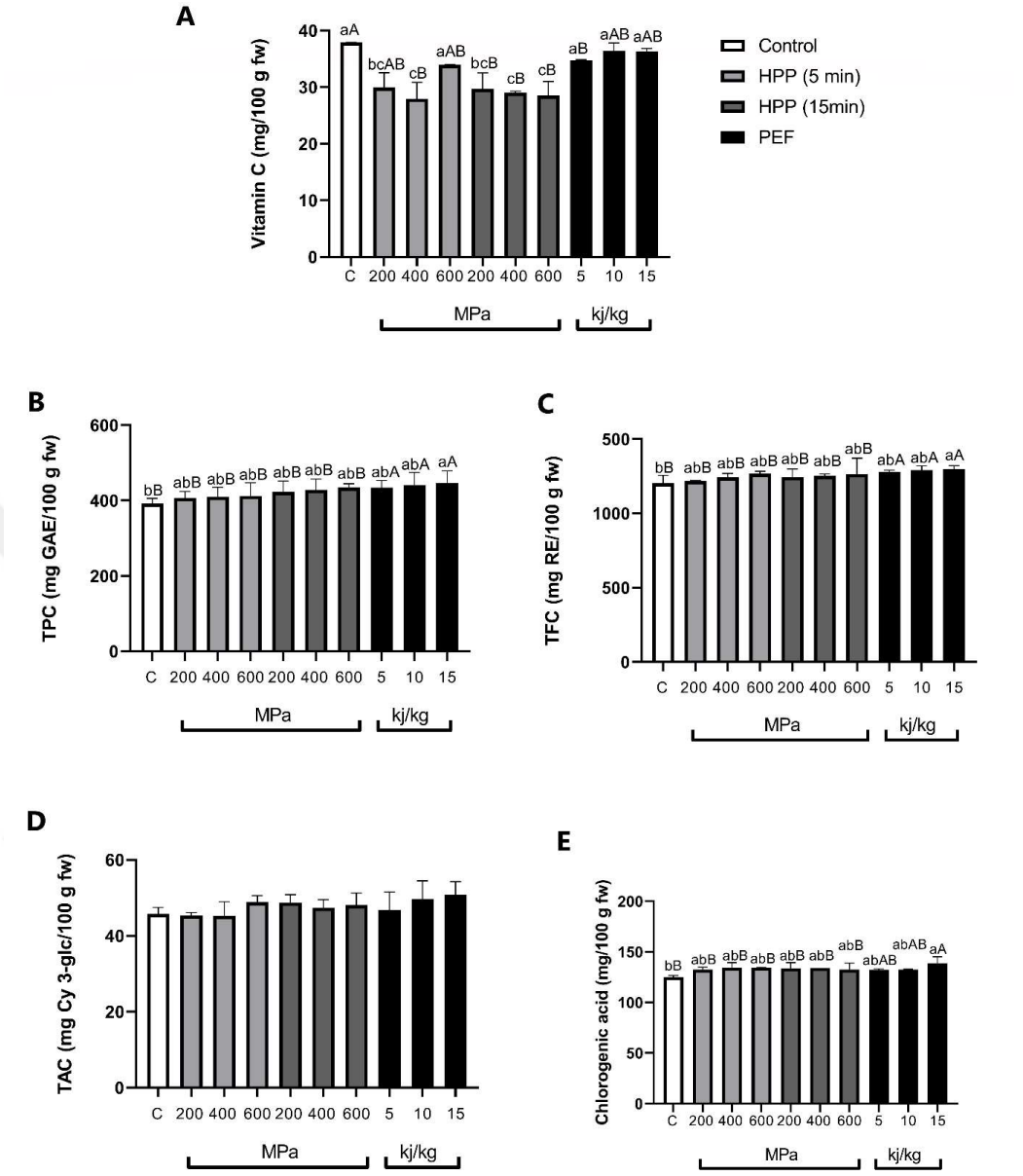


Figure 3.2 : Changes in bioactive compounds (A) vitamin C, (B) TPC, (C) TFC, (D) TAC and (E) chlorogenic acid contents of the untreated, HPP- and PEF-treated cranberrybush samples.

Table 3.2 presents the list of identified compounds along with their detected accurate mass, molecular formula, error in ppm (between the mass found and the accurate mass < 10 ppm) of each phytochemical, as well as the MS/MS fragment ions. UPLC–QTOF–MS/MS analysis of cranberrybush led to the identification of flavan-3-ols (catechin, epicatechin, epi(catechin) hexoside), proanthocyanidins (procyanidin dimer, procyanidin trimer, procyanidin dimer monoglycoside), flavonols (quercetin, quercetin-deoxyhexose, quercetin-3-*O*-glucoside, quercetin pentoside hexoside, rutin, isorhamnetin-3-*O*-rutinoside), flavone (diosmetin-rhamnosylglucoside), phenolic

acids (caffeic acid, chlorogenic acid, coumaric acid, *p*-coumaroyl-quinic acid) as well as anthocyanins (cyanidin-3-glucoside, cyanidin-3-rutinoside and cyanidin-3-xylosyl-rutinoside). The identification of each polyphenol was carried out in negative and positive modes, based on the MS and fragmentation pattern, as well as by comparison with the data already reported in the literature. The polyphenol profile of cranberrybush was in agreement with those reported previously (Dienaitė et al., 2020; Barak et al., 2019; Yurkiv and Grytsyk, 2017). It has been reported that chlorogenic acid is the major compound of *V. opulus* (Barak et al., 2019, Karaçelik et al., 2015; Velioglu et al., 2006). Therefore, in this study, in order to screen the effects of HPP and PEF treatments on the individual phenolic compounds, chlorogenic acid content was investigated. Chlorogenic acid content of the untreated sample was found to be 125 mg/100 g fw of sample (Figure 3.2) which is much higher than those of other berry fruits (Mattson et al., 2021). Different cranberrybush samples were collected from Russia and investigated in terms of their chlorogenic acid content. Results varied from 250 to 580 mg chlorogenic acid per 100 g fresh fruits (Perova et al., 2014). Besides, it was determined as 34.42 ± 1.22 mg/g dry extract by Barak et al. (2019) and 2037 mg/kg in juice by Velioglu et al. (2006) in cranberrybush samples collected from Turkey. The difference between the results may arise from the origin of the sample and/or storage conditions. Chlorogenic acid content was increased at elevated pressure levels and extended treatment times for HPP; and increasing the specific energy input for PEF. HPP and PEF caused an increase in the chlorogenic acid content in the range of ~6 – 7% and ~5.6 – 11%, respectively under different treatment conditions.

3.4.5 Antioxidant capacity

Antioxidant capacity measurement methods may be classified as hydrogen atom transfer (HAT)-based, electron transfer (ET)-based and mixed mode (ET- and HAT-based) assays (Capanoglu et al., 2018). The antioxidant quenching/scavenging activities of berries and fruits were found to be diverse by using different radical/oxidant sources (Prior et al., 2016). Thereby, in order to determine the influence of non-thermal food processing on the antioxidant capacity from a general perspective, the antioxidant capacity was determined by the CUPRAC and DPPH methods (Figure 3.3). CUPRAC is based on ET and DPPH is based on HAT. When CUPRAC method was used to measure antioxidant capacity, non-significant

differences were obtained after the HPP treatments, whereas PEF treatment at 15 kJ/kg resulted in a significant increase compared to the untreated sample due to greater amounts of phenolics and flavonoids being extracted. Moreover, positive significant correlations were found between TPC and CUPRAC (R^2 = 0.68) and TFC and CUPRAC (R^2 = 0.73); but a lower correlation was found as R^2 = 0.32 between TAC and CUPRAC. Additionally, there was no significant variation in DPPH scavenging activity of all the corresponding extracts. A moderate correlation coefficient was found as R^2 = 0.50 for vitamin C content. These results are in accordance to those found by other previous studies. Yuan et al. (2018) presented that HPP treatment (200 – 600 MPa/2.5-5 min) led to an insignificant increase in DPPH radical scavenging activity of aronia purée. Patras et al. (2009) reported that blackberry purée treated at 600 MPa for 15 min exhibited a significant increase by DPPH assay, while, strawberry purée treated at 400 MPa for 15 min had significantly lower antioxidant capacity as compared to the control. Medina-Meza et al. (2016) reported that while raspberry purée subjected to PEF at 388.1 W total specific energy input showed a significant increase in DPPH radical scavenging activity, there was no variation in the blueberry purée samples after 244.4 W specific energy input PEF application. Apple juice treated with PEF (1, 3 and 5 kV/cm electric field strength and 30 pulses total) showed no variation in the antioxidant capacities as measured with different methods (Soliva-Fortuny et al., 2009). Leong et al. (2016a) stated that all the PEF-treated grape juices showed an equivalent inhibitory effect against DPPH radicals (in the range of 62% and 66%). Similar results were also obtained by Buniowska et al. (2017), who reported an insignificant difference in DPPH results of exotic fruit juice blend obtained after PEF treatments (32 and 256 kJ/kg specific energy input).

Evaluating the effects of HPP and PEF techniques on the antioxidant capacity of the food products, it should be noticed that non-phenolic antioxidants such as ascorbic acid may be partially responsible for the overall antioxidant capacity of the samples. Besides, flavanones are anticipated to make a minor contribution in the DPPH assay (Khan et al., 2010). Furthermore, this was similar to the changes observed in TPC, TFC and TAC values which may be attributed to the combined influence of enhanced permeability and endogenous enzyme inactivation is discussed previously about the effect of HPP/PEF on vitamin C, enzyme activity and bioactive compounds.

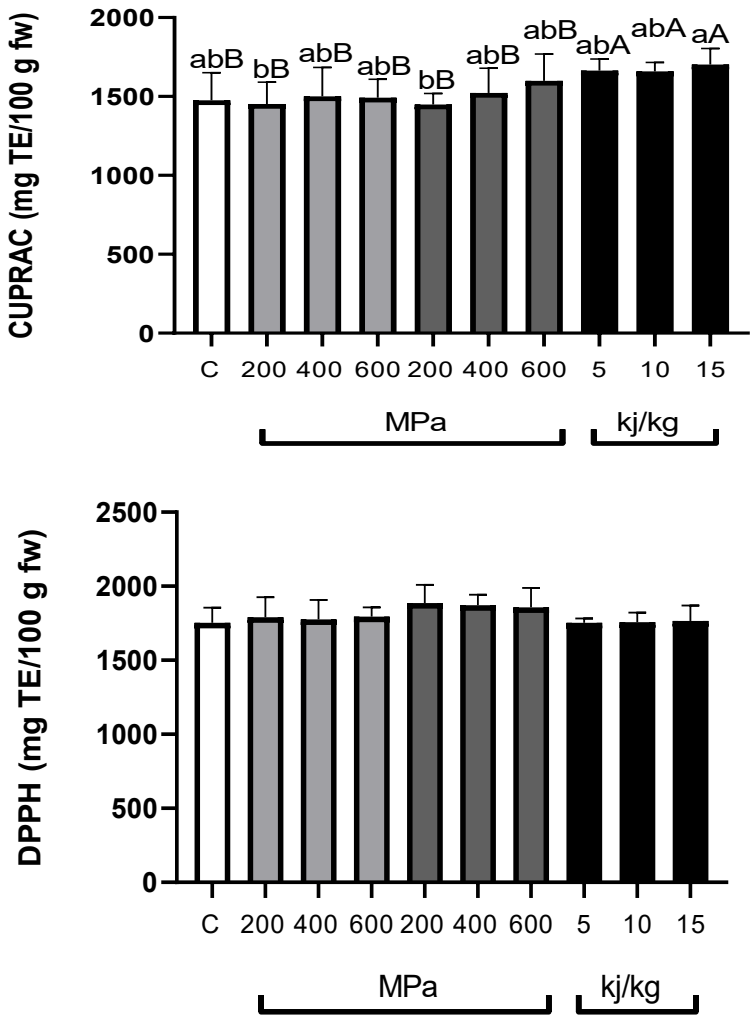


Figure 3.3 : Changes in total antioxidant capacity (CUPRAC, DPPH) of the untreated, HPP- and PEF-treated cranberrybush samples.

3.5 Conclusion

In this study, increasing trend or high retention of bioactive compounds was achieved, with a potential extraction of vitamin C, phenolics, flavonoids and anthocyanins in cranberrybush purées after HPP and PEF treatments at selected processing intensities. The antioxidant capacity measured with CUPRAC assay evidenced a noticeable improvement after PEF processing at high specific energy input (15 kJ/kg). The results of this study provided promising perspectives for the use of HPP and PEF as novel non-thermal treatments for the release of cranberrybush polyphenols. Considering the different processing conditions for HPP and PEF as well as different matrices in every study, it is essential to investigate the endogenous enzyme activities, bioactive

compounds and antioxidant capacities together to verify the interactions between these parameters in each particular food.

Furthermore, physicochemical, nutritional, microbiological, and organoleptic properties of raw and treated samples should be evaluated during storage to analyze the long-term quality changes for future studies. Besides, some of the bioactive compounds may need time to be released after pressurization or electroporation. As a future aspect, it can be suggested that conducting *in vitro* or *in vivo* bioavailability studies to assess the final absorption and metabolism of cranberrybush antioxidants may provide beneficial data to obtain the full picture on the effects of non-thermal treatments. To conclude, a glance at the results reveals that HPP and PEF technologies could be used to enhance nutritional quality of berry purées.

Table 3.2 : Identification of the major polyphenols detected in the cranberrybush using UPLC–QTOF–MS/MS.

RT (min)	Compound	Molecular Formula	Mode of ionization (ESI^-/ESI^+)	Theoretical m/z	Observed m/z	Mass Error (ppm)	*m/z* MS^n	References
1.17	Coumaroyl-quinic acid	$C_{16}H_{18}O_8$	$[M-H]^-$	337.0929	337.094	3.26	174	(Tang et al., 2020)
1.29	Cyanidin-3-glucoside	$C_{21}H_{20}O_{11}$	$[M+H]^+$	449.1078	449.1078	0	287	(Perova et al. 2014)
1.39	Cyanidin-3-rutinoside	$C_{27}H_{31}O_{15}$	$[M+H]^+$	595.1663	595.1660	-0.50	449, 287	(Perova et al. 2014)
1.42	Coumaroyl-quinic acid	$C_{16}H_{18}O_8$	$[M-H]^-$	337.0929	337.094	3.26	174	(Tang et al., 2020)
1.71	Caffeic acid	$C_9H_8O_4$	$[M-H]^-$	179.0350	179.0341	-5.03	135	(Voynikov et al., 2019)
2.09	Cyanidin-3-xylosyl-rutinoside	$C_{30}H_{44}O_8$	$[M+H]^+$	727.2185	727.2159	-3.58	581, 287	(Perova et al. 2014)
2.86	Procyanidin dimer	$C_{30}H_{26}O_{12}$	$[M-H]^-$	577.1351	577.1387	6.24	289, 407, 425, 451	(Dienaitė et al., 2020)
3.08	Chlorogenic acid	$C_{16}H_{18}O_9$	$[M-H]^-$	353.0879	353.0849	-8.50	135, 161, 179, 191	(Dienaitė et al., 2020)
3.19	Catechin	$C_{15}H_{14}O_6$	$[M-H]^-$	289.0716	289.0729	4.50	109, 125, 203, 245	(Dienaitė et al., 2020)
3.30	Procyanidin trimer	$C_{45}H_{38}O_{18}$	$[M-H]^-$	865.1985	865.1987	0.23	289, 577	(Tang et al., 2020)
3.52	Procyanidin dimer	$C_{30}H_{26}O_{12}$	$[M-H]^-$	577.1351	577.1387	6.24	289, 407, 425, 451	(Dienaitė et al., 2020)
3.63	Chlorogenic acid	$C_{16}H_{18}O_9$	$[M-H]^-$	353.0879	353.0849	-8.50	135, 161, 179, 191	(Dienaitė et al., 2020)
3.74	Epicatechin	$C_{15}H_{14}O_6$	$[M-H]^-$	289.0714	289.0729	5.19	109, 125, 203, 245	(Dienaitė et al., 2020)
3.80	Coumaric acid	$C_9H_8O_3$	$[M-H]^-$	163.0403	163.0416	7.97	119	(Kim et al., 2009)
4.07	Procyanidin trimer	$C_{45}H_{38}O_{18}$	$[M-H]^-$	865.1985	865.1987	0.23	289, 577	(Tang et al., 2020)
4.18	Procyanidin dimer	$C_{30}H_{26}O_{12}$	$[M-H]^-$	577.1351	577.1387	6.24	289, 407, 425, 451	(Dienaitė et al., 2020)
4.18	Quercetin pentoside hexoside	$C_{26}H_{28}O_{16}$	$[M-H]^-$	595.1377	595.1362	-2.52	301	(Dienaitė et al., 2020)
4.51	Rutin	$C_{27}H_{30}O_{16}$	$[M-H]^-$	609.1453	609.1411	-6.89	301	(Dienaitė et al., 2020)
4.62	Procyanidin dimer monoglycoside	$C_{31}H_{48}O_{20}$	$[M-H]^-$	739.2665	739.2693	3.79	289, 407, 425, 449	(Dienaitė et al., 2020)
4.68	Coumaric acid	$C_9H_8O_3$	$[M-H]^-$	163.0403	163.0416	7.97	119	(Kim et al., 2009)
4.73	Quercetin-3-*O*-glucoside	$C_{21}H_{20}O_{12}$	$[M-H]^-$	463.0879	463.0894	3.24	301	(Dienaitė et al., 2020)
5.1	Isorhamnetin-3-*O*-rutinoside	$C_{28}H_{32}O_{16}$	$[M-H]^-$	623.1616	623.1616	0.00	315	(Tsamo et al., 2015)
5.29	Quercetin-deoxyhexose	$C_{21}H_{20}O_{11}$	$[M-H]^-$	447.0933	447.0916	-3.80	301	(Barbosa et al., 2019)
5.62	(Epi)catechin hexoside	$C_{21}H_{24}O_{11}$	$[M-H]^-$	451.1245	451.1259	3.10	289, 341	(Dienaitė et al., 2020)
6.06	Diosmetin-rhamnosylglucoside (diosmin)	$C_{28}H_{32}O_{15}$	$[M-H]^-$	607.1668	607.1668	0.00	299	(García-Salas et al., 2013)
6.72	Quercetin	$C_{15}H_{10}O_7$	$[M-H]^-$	301.0346	301.0328	-5.98	151	(Shabana et al., 2011)

4. EFFECTS OF FOOD MATRIX AND NON-THERMAL PROCESSING ON BIOACCESSIBILITY AND TRANSPORT DYNAMICS OF CRANBERRYBUSH (*VIBURNUM OPULUS*) POLYPHENOLS IN A COMBINED *IN VITRO* DIGESTION/CACO-2 CELL CULTURE MODEL[3]

4.1 Abstract

The present study was developed to investigate the effects of food processing and food matrix on bioaccessibility and transepithelial transportation of antioxidant bioactive compounds, in particular chlorogenic acid, from cranberrybush (*Viburnum opulus*) after by using a combined *in vitro* gastrointestinal digestion/Caco-2 cell culture model. Results showed that PEF treatment with 15 kJ/kg specific energy input resulted in a higher recovery of total flavonoid content (TFC; increase of 3.9% ± 1.1%, $p < 0.0001$), chlorogenic acid content (increase of 29.9% ± 5.9%, $p < 0.001$) and antioxidant capacity after gastrointestinal digestion. Moreover, cranberrybush juice products were supplied in three diverse matrices: water (JW), bovine (JM, 25%) and almond (JA, 25%) milk. The addition of bovine milk affect posivitely the bioaccessibility of total phenolic content (TPC), TFC as well as antioxidant capacity. The present study also demonstrates that untreated and treated samples display comparable transport across the epithelial cell layer. Addition of milk matrix have a positive effect on the stability and transport of chlorogenic acid.

Key words: cranberrybush, chlorogenic acid, high pressure processing (HPP), pulsed electric field (PEF), matrix effect, bioaccessibility, Caco-2 cell model, bioavailability, transepithelial transport

[3] This chapter is based on the paper "Ozkan, G., Kostka, T., Drraeger, G., Capanoglu, E., and Esatbeyoglu, T. (2021). Effects of food matrix and non-thermal processing on bioaccessibility and transport dynamics of cranberrybush (Viburnum opulus) polyphenols in a combined in vitro digestion/Caco-2 cell culture model, Submitted."

4.2 Introduction

Viburnum opulus, also known as Gilaburu in Turkey, belongs to the Adoxaceae family. In Turkish folk medicine, consumption of *V. opulus* have linked to a variety of beneficial effects, such as antimicrobial (Sagdic et al., 2006), antioxidant (Ersoy et al., 2017), antidiabetic (Altun et al., 2008), diuretic (Bae et al., 2010), hypoglycemic, cough reliever (Fujita et al., 1995) as well as prevention of kidney problems (Ilhan et al., 2014). These health benefits were attributed to its high phenolic content which include mostly procyanidins, flavonoids and phenolic acids (Barak et al., 2019). According to Velioglu et al. (2006), the total amount of cranberrybush phenolic acids, in which chlorogenic acid is the key component, was found to be about 2000 mg/kg. This content is quite high when compared with other berries. Cranberrybush procyanidins consisting of catechin, epicatechin and their oligomers, were determined as 290.4, 26.9 and 82.8 mg/kg, respectively. Flavonoids (quercetin derivatives) were also present in cranberrybush as around 170 mg/kg (Sedat Velioglu et al., 2006).

To exert any bioactivity in a specific tissue, polyphenols must be effectively absorbed from the gut into the blood circulation and transferred to the appropriate location within the body. However, there are many parameters that have an impact on the release from the food matrix, stability through the gastrointestinal tract, bioaccessibility, bioavailability and thus bioactivity of the phenolic compounds.

Food processing methods and matrices may have potent effects on the retention of polyphenols in the final product. Even though thermal processes are widely applied for preserving food products to extend shelf life, they can have a negative effect on nutritional value of food products (Bevilacqua et al., 2018). On the other hand, non-thermal treatments such as high pressure processing (HPP) and pulsed electric field (PEF) applications have been identified as a useful tool for not only extending the shelf-life, but also preserving the nutritional and functional characteristics of food products (Ozkan et al., 2019). In the current context of functional foods, fruit beverages are generally supplemented with milk, due to its plenty content of protein, fat, and vitamin, to improve the nutritional value. In addition, the use of almond milk in the beverage blends of this type can be an alternative to milk for people who are vegetarian or have an allergy to protein as well as intolerant to lactose in cow's milk (Kundu et al., 2018). Therefore, comparing the fate of polyphenols from cranberrybush

juice between water, milk and milk blends represent a valuable opportunity to study the effect of milk matrix effect on bioavailability. A recent study showed that addition of milk matrix to pomelo juice effectively increased phenolic (TPC) bioaccessibility 60.1% to 63.3%, whereas soy milk reduced TPC bioaccessibility by 14.9% (Quan et al., 2020). However, Helal et al. (2014) demonstrated that the addition of bovine milk to cinnamon beverage had no any effect on polyphenols bioaccessibility. A variety of evidences from the studies investigating the milk matrix effect are probably caused by different protein-polyphenol interactions, which may protect phenolics from degradation or hinder the release and solubilization of phenolic compounds during digestion (Rodríguez-Roque et al., 2014b).

One of the key factors affecting the bioavailability of polyphenols are encompassing their bioaccessibility and the ability to transport through the gut epithelium into the blood. The bioaccessibility comprises the release of compounds from food matrices, their stability in gastrointestinal tract and, finally, availability for the subsequent absorption in the small intestine (Tagliazucchi et al., 2010). *In vitro* methods for assessing bioavailability are good alternatives to *in vivo* procedures. In a first step, these methods are generally based on the simulation of gastrointestinal digestion followed by the determination of polyphenol bioaccessibility (soluble fraction) (Senem Kamiloglu et al., 2017). In more detail, these methods have been improved by the incorporation of a human intestinal epithelial cell model derived from a colon carcinoma cell line (Caco-2) to predict intestinal absorption of polyphenols due to many of the functional and morphological properties of mature human enterocytes (Wu et al., 2017). The small intestine is primarily responsible for the uptake of food components. Researches have indicated good correlations between *in vitro* cell permeability using differentiated Caco-2 cells and *in vivo* absorption rates in rats (Conradi et al., 1993) and humans (Yee, 1997).

Considering the above, the aim of this current study was to assess the stability, bioaccessibility and transport efficiency of the cranberrybush phenolics using *in vitro* simulated gastrointestinal digestion, coupled with the human intestinal Caco-2 epithelial cell system as a model. In the work described here, we have investigated (i) the bioaccessibility and the antioxidant capacity of cranberrybush polyphenols after application of non-thermal food processing treatments including HPP and PEF at

selected intensities, (ii) the bioaccessibility and the antioxidant capacity of polyphenols from cranberrybush juice beverages formulated with or without bovine or almond milk, (iii) influence of non-thermal processing on the apical and basolateral recovery as well as transport efficiency of cranberrybush phenolics - chlorogenic acid and (iv) bioavailability of chlorogenic acid from cranberrybush juice beverages as affected by different milk-based matrices. To the best of our knowledge, this is the first *in vitro* study that introduced the transported polyphenols from cranberrybush as a responce to non-thermal food processing as well as the interaction with food matrix constituents by using stimulated epithelial cells.

4.3 Materials and Methods

4.3.1 Chemicals

Pepsin (≥ 250 U/mg, EC 3.4.23.1, from porcine gastric mucosa), pancreatin (8x USP, EC 232.468.9, from porcine pancreas, contains trypsin, amylase and lipase) and bile were purchased from Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany). Caco-2 human colon adenocarcinoma cell line was obtained from German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin, non-essential amino acids (MEM NEAA 100x), trypsin/EDTA (10x) and Hanks' balanced salt solution (HBSS, 0.35 g/L $NaHCO_3$) were purchased from Pan Biotech (Aidenbach, Germany) and trypan blue was obtained from Carl Roth (Karlsruhe, Germany). All other reagents were of analytical grade.

4.3.2 Preparation of cranberrybush purée and cranberrybush juice-based beverages

Fresh cranberrybush (*Viburnum opulus* L.) at commercial maturity was obtained in October 2019 and 2020 from a local grower in Kayseri (Turkey). After harvest, fruit samples were stored in water at room temperature in dark until further use. Before treatments, they were visually screened for any damage and manually destemmed. Cranberrybush purée was prepared on the day of the experiment by homogenizing whole fruit samples (2 kg for each batch) using a blender (Blendtec Classic 575, Bad Homburg, Germany) for two cycles of 10 s at low speed (16,000 g), followed by a

subsequent 10 s cycle at high speed (22,000 g). Three independent batches (n = 3) were prepared for each untreated control, HPP- and PEF-treated samples.

Cranberrybush purée was centrifuged at 4500 rpm for 15 min at 4 °C to separate the pulp from the juice. The extracted juice was used immediately for making fruit juice blends. The bovine whole milk (M) and almond milk (A) products were purchased at a local supermarket (Hannover, Germany). The nutritional composition of bovine milk consisted of 3.6% (w/v) of fat, 3.3% (w/v) of protein and 4.8% (w/v) of carbohydrates, while 1.1% (w/v) of fat, 0.5% (w/v) of protein and 0.2% (w/v) of fibre were contained in almond milk (data provided by manufacturers).

Afterwards, three different fruit juice-based beverages were prepared by vortexing 75 % of the cranberrybush juice and 25 % of water (fruit juice-water beverage, JW), milk (fruit juice- milk beverage, JM) or almond (fruit juice-almond beverage, JA) for 15 s. The formulation of the beverage was selected based on a previous study (Rodríguez-Roque et al., 2014a). All treatments were performed in triplicate and used immediately after mixing for *in vitro* simulated gastrointestinal digestion.

4.3.3 Processing technologies

The processing parameters for HPP and PEF were selected based on a previous study (Ozkan et al., 2021).

Briefly, the cranberrybush purée was processed by using industrial-scale HPP equipment (Wave 6000/55, Hiperbaric S.A., Burgos Spain). Prior to the treatment, the cranberrybush purée was vacuum-packed and heat sealed in low density polyethhylene pouches (LDPE). The packed purée was then subjected to a pressure of 600 MPa for 5 min at ambient temperature (20 ± 2°C). Water was used as medium for transmitting pressure. Finally, the purée was packed into sterilized falcons and stored at -80 °C until analysis.

PEF treatment was conducted by using a pilot scale PEF batch system (PEF Pilot, Elea GmbH, Germany). The system provided a voltage up to 30 kV and monopolar, exponential decay pulses with pulse duration of 40 ms. The PEF operating settings applied were: constant pulse width of 20 μs, 3 kV/cm electric field strengths, 6 kV voltage, different specific energy inputs as 5 (PEF5) and 15 kJ/kg (PEF15) and constant pulse frequency of 2 Hz (0.5 s). The specific energy intake *Wspec* (kJ/kg) and the electric field strength *E* (kV/cm) were calculated according to the following equations (4.1) and (4.2).

$$Wspec = (U^2C\,.n)/2m \quad (4.1)$$

$$E = U/d \quad (4.2)$$

where *n* (dimensionless) is the number of pulses; *m* is the total weight of cranberrybush purée (kg); U is the voltage (kV) and d is the distance between the electrodes (cm); C is the capacitance (1 μF).

The treatment chamber had a 2 cm electrode gap and a volume of 17 ml. Following the treatment, the samples were stored at -80 °C until analysis.

4.3.4 *In vitro* simulated gastrointestinal digestion

The *in vitro* digestion procedure was performed according to the method described by (Sessa et al., 2011) with some modifications. The method consists of two sequential steps; an initial pepsin/HCl digestion to simulate gastric conditions, followed by a digestion with bile salts/pancreatin to simulate intestinal conditions. Briefly, 5 g cranberrybush purée or 5 mL cranberry juice samples were mixed with 5 mL of phosphate buffer saline (PBS) and preincubated in a water bath at 37 °C for 15 min. Before starting gastric digestion, the pH was adjusted to 2 using 1 M HCl and porcine pepsin was then added to a final concentration of 1.3 mg/mL. The samples were incubated at 37 °C in a shaking water bath (GFL 1092, Burgwedel, Germany) at 100 rpm for 2 h. For the intestinal digestion, the pH of the digest was raised to pH 5.8 with 1 M $NaHCO_3$ dropwise, and 2.5 mL of pancreatin and bile salts mixture were added to final concentrations of 0.175 and 1.1 mg/mL, respectively. The pH was then adjusted to pH 6.5 with 1 M $NaHCO_3$, and samples were incubated at 37 °C in a shaking water bath at 100 rpm for 2 h. After gastrointestinal digestion, the samples were cooled by immersing in an ice bath and then centrifuged at 10,000 rpm for 30 min at 4 °C (Megafuge 8R; Thermo Scientific, Darmstadt, Germany) to separate the soluble or bioaccessible fraction and the residual fraction.

A blank (without the added sample) was incubated under the same conditions to discard interferences due to the digestive enzymes and buffers used in the digestion process. All experiments were done in triplicates.

Bioaccessible fractions of the digests were preserved at -80 ℃ and used for spectrophotometric (total phenolic, flavonoid and anthocyanin contents, and antioxidant capacity) and Caco-2 cell culture experiments.

4.3.5 Determination of total phenolics and antioxidant capacity

All spectrophotometric assays were performed using a Infinite M200 UV–visible spectrophotometer (Tecan, Crailsheim, Germany).

Total phenolic content (TPC) was determined using Folin-Ciocalteu reagent as described previously by (Singleton & Rossi, 1965). The results were expressed as mg gallic acid equivalents (GAE) per 100 g fresh weight (fw) sample.

Total flavonoid content (TFC) assay was performed according to (Dewanto et al., 2002). The results were expressed as mg rutin equivalents (RE) per 100 g fw sample.

The total monomeric anthocyanin content (TAC) was determined by pH differential method (Mónica Giusti & Wrolstad, 2005). Absorbances of samples diluted with pH 1.0 and pH 4.5 buffers were measured at 520 and 700 nm. The results are expressed as cyanidin-3-*O*-glucoside equivalents per 100 g fw by using the following equation:

$$TAC\ (mg/L) = (A\ x\ MW\ x\ DF\ x\ 1000)/(\varepsilon \text{ x } 1) \quad (4.3)$$

where A = $(A520nm - A700nm)_{pH1.0} - (A520nm - A700nm)_{pH4.5}$, MW is the molecular weight of cyanidin-3-*O*-glucoside (449.2 g/mol), DF is the dilution factor, 1000 is the conversion factor from g to mg, ε is the molar absorptivity of cyanidin-3-*O*-glucoside (26900 L/(mol.cm)), and l is the path length (cm).

The antioxidant capacities were estimated by using the cupric ion reducing antioxidant capacity (CUPRAC) (Apak et al., 2004) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Molyneux, 2004) assays. 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was used as a standard in both of the assays. Results were expressed as mg Trolox equivalents (TE) per 100 g of fw sample.

4.3.6 Bioaccessibility calculations

The bioaccessibility was calculated using equation (4.4) and expressed as percentage.

$$\text{Bioaccessibility (\%)} = (\text{BCdigested}/\text{BCnon} - \text{digested}) * 100 \quad (4.4)$$

where $BC_{digested}$ was the amount of bioactive compounds (TPC, TFC, TAC or chlorogenic acid) recovered in the supernatants of the centrifuged final digesta (BF; bioaccessible fraction) and $BC_{non\text{-}digested}$ was the amount of bioactive compounds in non-digested purée or juice.

4.3.7 Cell culture

Caco-2 cells were cultured in growth medium (DMEM including 4.5 g/L glucose and stable glutamine supplemented with 20% FBS, 1% NEAAs, 100 IU/mL penicillin and 100 µg/mL streptomycin). Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. Cells were subcultured at 70–80% confluence using trypsinization. For the transport experiment, Caco-2 cells were seeded in inserts of 6-well transwell plates (0.4 µm pore diameter, 24mm insert, Sarstedt, Nümbrecht, Germany) at a density of $4*10^4$ cell/well, with 2 mL of medium in the apical side and 2.5 mL of medium in the basolateral side. Cells were allowed to grow and differentiate to confluent monolayers for 21 days post seeding. Cell monolayer integrity during differentiation of Caco-2 cells was monitored by measuring the transepithelial electrical resistance, TEER value, using a Millicell© ERS-2 Volt-Ohm Meter (Millipore, Bedford, MA, USA). Only differentiated enterocytes displaying unchanged transepithelial electrical resistance values were used in the experiment. Medium was changed every two days. Passage numbers of the cells used in this study were 7-10.

4.3.8 Cytotoxicity test

Caco-2 cells were seeded in 96 well plates, allowed to differentiate for 14 days after confluency and treated with digests at a dilution of 1/2, 1/5 and 1/10 (v/v) in HBSS (Pan, Biotech). After 4 h of treatment, the cytotoxic effect of samples on Caco-2 cells was assessed using the sulforhodamine B (SRB) assay as described by (Empl et al., 2018; Longo-Sorbello et al., 2006). For the SRB assay, differentiated cells were first fixed by addition of 50 µL of 50% TCA (trichloroacatate in Milli-Q water) and kept at 4 °C for 45 min. The plates were rinsed with tap water and air-dried. Next, the cells were stained with 70 µL SRB solution (0.4% in 1% glacial acetic acid) and left for 15 min. The plates were then rinsed with 1% glacial acetic acid in Milli-Q water, air-dried and the stain was re-suspended in 200 µL of 10 mM Tris-buffer. Thereafter, the

absorbance was determined at 490 nm, substracting the background measurement at 620 nm.

4.3.9 Transport experiment

Transport experiment was carried out according to previously described procedure (Wu et al., 2017). Briefly, the medium in the transwells was changed to HBSS and preincubated for 1 h and then removed. BF used for the Caco-2 experiments was diluted 1:5 (v:v) with HBSS, since cell viability after 4 h of exposure to the samples in such conditions, assessed by SRB assay, was higher than 85–90%. Incubation was performed at 37 ℃ with 5% CO_2 for 4 h. During incubation, 200 µL samples were collected from both the apical and basolateral compartments every 2 h, after which 200 µL of HBSS was added to adjust the volume of the media. TEER value of the cells was detected before treatment and immediately after the last sampling to ensure integrity of the monolayer. Afterwards, HBSS was replaced by growth medium, and the monolayers were incubated for another 20 h to measure irreversible damage to the cell monolayer. Therefore, after 24 h, monolayer integrity was tested using TEER measurement (should be > 200 Ωcm^2). Samples were stored at -80 ℃ until further analysis.

4.3.10 Identification and quantification of chlorogenic acid by UPLC-QTOF-MS/MS

Identification of chlorogenic acid in cranberrybush samples was performed according to a previously published method (Ozkan et al., 2021). Before LC-MS analysis, extracts of different processing conditions were passed through 0.20 µm membrane filters. LC-MS analysis was performed on a Waters Acquity UPLC system (Waters Co., Milford, MA, USA) connected to a Waters Q-Tof Premier mass spectrometer equipped with an electrospray ionisation (ESI) source. The UPLC system was equipped with a binary pump with degasser, autosampler, tunable UV detector and a column manager. For chromatographic separation, a Waters Acquity UPLC BEH Phenyl (2.1*100mm, 1.7µm) column was used. Mobile phase consisting of formic acid/MQ water (1/1000, v/v; eluent A) and formic acid/acetonitrile (1/1000, v/v; eluent B) was used. The linear gradient was as follows: 0 min, 5% B; 0-6.48 min, 35% B; 6.48-6.77 min, 100% B; 6.77-8.00 min, 100% B; 8.00-8.10 min, 5% B. The injection

volume was 10 µL, and the flow rate was 0.6 mL/min. The column temperature was kept at 45 °C, while the temperature of the autosampler was held at 10 °C. ESI-MS analysis was performed in both positive and negative modes. Collision energies of 15 V (for low energy) and 30 V (for high energy) were used for full-scan LC-MS in the *m/z* range 100−1500. Acquisition and integration of chromatograms were performed using the Masslynx V4.1 software from Waters.

The method for determination of chlorogenic acid by UPLC was adapted from Wu et al. (2019) (Wu et al., 2017). Extracts obtained from different processing conditions were passed through 0.20 µm membrane filters and injected into a Waters Acquity UPLC system (Waters Co., Milford, MA, USA) coupled with a TUV detector (DAD). An Acquity UPLC BEH Phenyl (2.1*100mm, 1.7µm) column was used as stationary phase. The following solvents with a flow rate of 0.6 mL/min and injection volume of 10 µL were used for spectral measurements: formic acid/MQ water (1/1000, v/v; eluent A) and formic acid/acetonitrile (1/1000, v/v; eluent B). The linear gradient was as follows: 0 min, 5% B; 0-6.48 min, 35% B; 6.48-6.77 min, 100% B; 6.77-8.00 min, 100% B; 8.00-8.10 min, 5% B. Chlorogenic acid was quantified by using its authentic standard. For calibration curves, chromatographic peak area of the standards versus nominal concentrations were plotted. All results were expressed as milligrams per 100 g fw of sample.

4.3.11 Statistical analysis

All experiments were conducted at least in triplicate. Results were reported as mean ± standard deviation. Error bars on figures represented standard deviations. Statistical analysis was applied using Prism (version 8.4.1; GraphPad, La Jolla, USA). Treatments were compared using one-way analysis of variance (ANOVA) followed by a Tukey post hoc test ($\alpha = 0.05$).

4.4 Results and Discussion

4.4.1 Retention of the bioactives during *in vitro* gastrointestinal digestion

4.4.1.1 Effects of food processing

TPC, TFC, TAC, chlorogenic acid content and antioxidant capacity were previously reported for untreated and HPP/PEF-treated cranberrybush purée samples (Ozkan et

al., 2021). The effect of the *in vitro* digestion and food processing on the bioaccessibility of cranberrybush bioactives is shown in Figure 4.1. The bioaccessibility of TPC and TFC in the untreated cranberrybush purée were found to be 43.5 and 15.1 %, respectively. Similar to the results obtained in the present work, Buniowska et al. (2017) obtained TPC bioaccessibility value of 30.5% in a fruit juice mixture of mango and papaya. Furthermore, the bioaccessibility of TPC and TFC in blended fruit juice (orange, kiwi, pineapple and mango) was found to be 25.9 and 15.1%, respectively (Rodríguez-Roque et al., 2015). Previous studies have noticed a loss of fruit phenolics during gastrointestinal digestion, due to polymerization, epimerization and auto-oxidation under intestinal digestion conditions (Rodríguez-Roque et al., 2014a). Indeed, phenolics are highly sensitive to alkaline conditions and may degrade during gastrointestinal digestion (Bermúdez-Soto et al., 2007). Processing had a variable influence on the bioaccessibility of phenolic compounds from cranberrybush purée samples, depending on the type of the treatment and the treatment intensity. A statistically significant ($p < 0.001$) improvement up to 4% in the bioaccessibility of TFC and 30% of chlorogenic acid content was obtained after PEF15 treatment in comparison with the untreated sample. Moreover, an increasing trend was also obtained after PEF15 treatment in TPC content, PEF5 and HPP treatments in TPC, TFC and chlorogenic acid bioaccessibility, in comparison to the untreated sample, as these techniques may promote the liberation of phytochemicals from the food matrix under the effect of the digestive enzymes action, temperature and pH conditions. These results were in agreement with the results from Rodríguez-Roque et al. (2015), which showed that HPP (400 MPa for 5 min) and High Intensity-PEF (35 kV/cm electric field strength) had a significant increase on the bioaccessibility of TFC in fruit juice samples. Furthermore, it was found that apple samples had 26% higher bioaccessible phenolic compounds after 0.01 kJ kg^{-1} PEF treatment (t= 24h) than untreated samples (Ribas-Agustí et al., 2019). On the contrary, in this study, bioaccessibility of TAC ranged from 7.88% to 9.94% and non-thermal processing (HPP and PEF) did not change the bioaccessibility of TAC content. Similar findings were reported by Buniowska et al. (2017), indicating a 12.71, 10.90 and 15.63% bioaccessibility in TAC of untreated and PEF-treated (32 and 256 kJ/kg specific energy input) exotic fruit juice blends, respectively. In fact, the bioaccessibility of anthocyanins has been demonstrated to be lower than that of other flavonoids (Yang et al., 2011). The high loss of anthocyanins could be related to structural rearrangements in response to

changes in pH in four molecular structures: quinoidal base (blue), flavylium cation (red), carbinol (colorless) and chalcone (yellowish) forms. Anthocyanins are stable in acidic solutions (pH 1-3) where they exist primarily as flavylium cations; whereas anthocyanins adopt the forms of the carbinol and chalcone at pH > 4. Then, chalcone may undergo chemical degradations to produce phenolic acids (Kamiloglu et al., 2017). Due to the fact that processing may induce modifications (hydroxylation, methylation, isoprenylation, dimerization, glycosylation, formation of phenolic derivatives) in the physicochemical features of phenolic compounds, the bioaccessibility of these compounds may be altered as increase or decrease (Dugo et al., 2005).

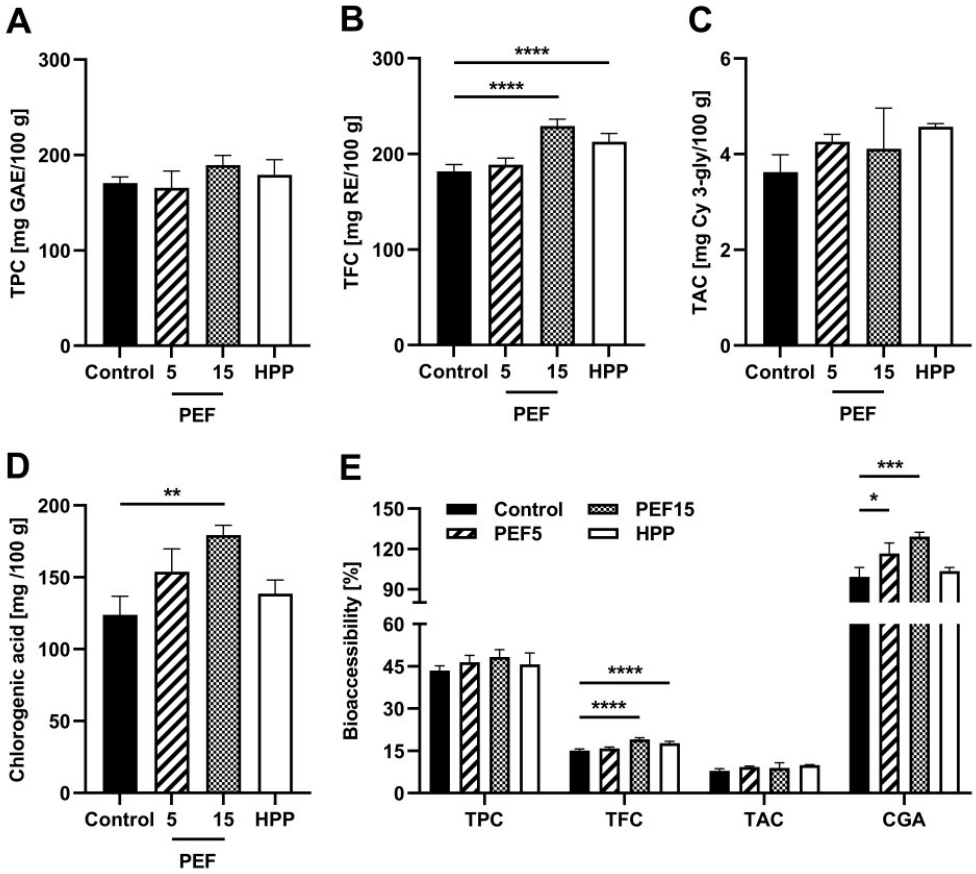


Figure 4.1 : Effects of PEF- or HPP-mediated food processing on the content and bioaccessibility of bioactive compounds after *in vitro* digestion of 100 g fresh weight cranberrybush purée samples.

In line with the antioxidant capacity measurements obtained in the untreated and HPP/PEF-treated samples after *in vitro* digestion, values of CUPRAC antioxidant capacity were found to be significantly ($p < 0.001$) higher for PEF15, suggesting

greater amounts of antioxidant compounds being extracted and becoming available for intestinal absorption (Figure 4.2). On the other hand, there was no significant change in the DPPH antiradical scavenging abilities of the samples. The differences in principles and mechanisms of the antioxidant capacity measurement assays could explain the different results (Ben Hlel et al., 2019). Overall, the bioaccessibility of the antioxidant capacity (CUPRAC and DPPH) in untreated and treated samples was found to be between 21.1 and 34.5%. In particular, a tendential improvement was obtained in the bioaccessibility of CUPRAC activities of bioactive compounds after PEF15 treatment in comparison with the untreated sample. Our results are in agreement with other researches performed on different food samples that demonstrated a ~16 – 36% bioaccessibility in total antioxidant capacity of untreated and non-thermal (PEF, high voltage electrical discharges and ultrasound) treated exotic fruit juice blends measured by Trolox equivalents antioxidant capacity (TEAC), oxygen radical antioxidant capacity (ORAC) and DPPH assays (Buniowska et al., 2017). In another study, Rodríguez-Roque et al. (2015) reported a decrease in the DPPH radical scavenging activity of digested fruit juice based beverages after High Intensity-PEF and HPP treatments by 19 and 14%, respectively, as compared with untreated products. Results imply that the impact of non-thermal processing techniques differs according to the nature of the food, its bioactive composition and their concentrations (Ben Hlel et al., 2019). Moreover, it can be concluded that the specific energy input of PEF treatment is an important factor that influences the bioactivity. Therefore, further investigations are necessary to find out the possible effects of the energy input of this technology on total antioxidant bioaccessibility.

4.4.1.2 Effects of food matrix

The effects of the *in vitro* digestion and food matrix on the bioaccessibility of cranberrybush bioactives are shown in Figure 4.3. With regard to the matrix effect, a significant decrease in the apparent concentrations of TPC and TFC contents was obtained in JM and TFC content in JA blend before digestion in comparison with JW blend. These decreases are due to the complexation between cranberrybush polyphenols and milk proteins. Procyanidins can bind tightly proline-rich proteins as milk caseins with maximum at pH values close or below the isoelectric point of the protein. Moreover, this affinity depends on the molecular weight, three-dimensional

structure and proline content of the protein as well as the degree of polymerization of the procyanidins (Hagerman & Butler, 1980). The ratio of procyanidin/protein is an important factor that determines the solubility of the complexes. While a higher ratio promotes the formation of insoluble complexes, a lower procyanidin/protein ratio leads to the formation of soluble complexes (Adamczyk et al., 2012). Indeed, the difference in the bioactive contents of JM and JA beverages would comprise the variety in the protein content of the food matrix.

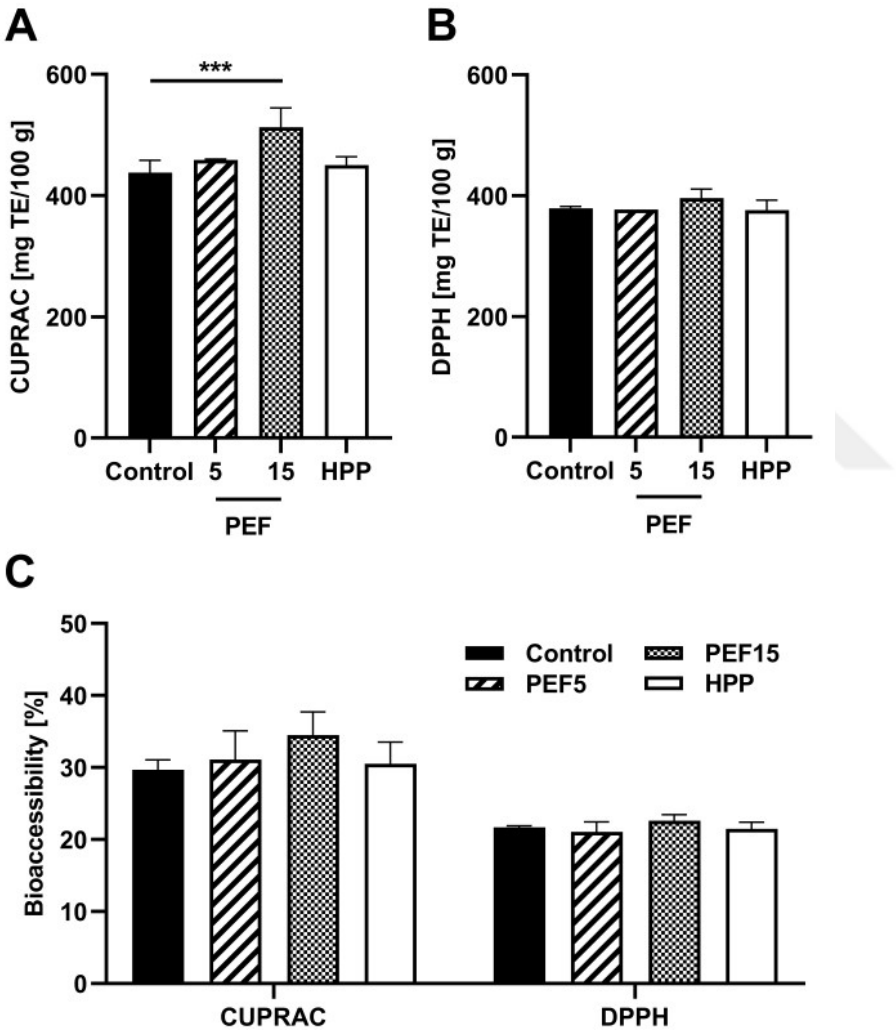


Figure 4.2 : Total antioxidant capacity after *in vitro* digestion of 100 g fresh weight cranberrybush purée samples.

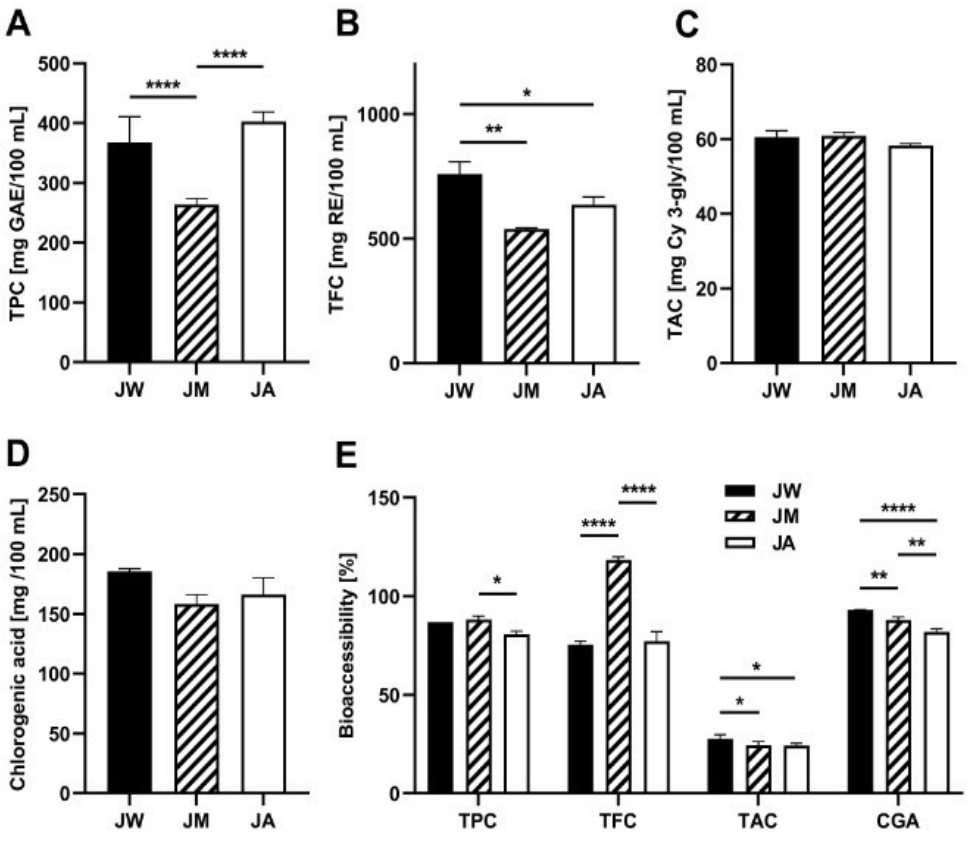


Figure 4.3 : Content and bioaccessibility of bioactive compounds after *in vitro* digestion of 100 mL cranberrybush juice samples mixed with water (JW), bovine milk (JM) or almond milk (JA).

In line with the results of the bioaccessibility, recovery of TPC, TFC, TAC and chlorogenic acid content were obtained as 86.9, 75.4, 27.6 and 93%, respectively, in JW blend after *in vitro* digestion. It can be deduced from the results that the enhancement in the bioaccessibility of bioactive compounds, in comparison with the results of cranberrybush purée, may arise from the difference in dietary fiber contents between purée and juice samples. It has been reported that a large number of soluble fibers, (e.g. pectin) that exist in raw fruits, have been widely used because of their crucial physiological functions and prebiotic effects. However, molecular interactions between the phenolics and pectin can affect the release of these phenolics from food matrix. Numerous studies reported an adverse impact of dietary fibers on the bioaccessibility of the polyphenols. Al-Yafeai & Böhm (2018) investigated the interactions between pectin contents and carotenoid bioaccessibility in tomato paste. It was stated that adding pectin led to significantly diminished bioaccessibility of total lycopene content to 16% compared with untreated samples (46%). Additionally, co-

digestion of pectin and pomegranate caused a 2-fold decrease in TPC of the dialyzed fraction (Sengul et al., 2014). In another study, the effect of inulin addition (5 and 10%) to a tomato sauce has been investigated in terms of the bioaccessibility of TPC and TFC and individual polyphenols. Results showed that the bioaccessibility of the polyphenols diminished proportionally with the increase in the inulin content in tomato sauces. In detail, TPC and TFC values for the sauce containing 10% inulin decreased by 31% and 36%, respectively, compared to tomato sauce ($p < 0.05$) (Tomas et al., 2018). Fiber – polyphenol complex possibly hinders the release and thus absorption of polyphenols in the gut; thereby this complexing has a negative effect during *in vitro* bioaccessibility (Sun-Waterhouse et al., 2008). Furthermore, increasing viscosity of the intestinal content with pectin lead to lower contact with intestinal enterocyte (Xu et al., 2015). It is also necessary to take into consideration the physiological properties of the food matrix as another aspect of the results. It is known that liquids are digested faster than semisolids or cellular structures (Norton et al., 2006).

In addition to these, a glance at the results revealed a statistically significant increase in the bioaccessibility of TPC ($p < 0.0001$) in JM when compared with LA as well as in the bioaccessibility of TFC ($p < 0.0001$) with respect to JW and JA. On the other hand, the almond milk matrix did not significantly affect the bioaccessibility of TPC and TFC, in comparison with JW samples. In JM samples, some of the phenolic substances could be bound to milk protein or fat and they can be released from these complexes through the action of digestive enzymes. Besides, a certain quantity of fat in milk favors the incorporation of bioactive compounds (especially lipophilic ones) into micelles, resulting in a higher bioactive content than that of JW (Quan et al., 2020; Rodríguez-Roque et al., 2015). The results obtained in this research agree with those of other studies. For instance, Quan et al. (2020) presented that there was a significant enhancement ($p < 0.05$) on the total phenolic bioaccessibility in kiwi or pomelo juice from 21.6 to 37.8% and 60.1 to 63.3%, respectively, with the addition of milk matrix. Besides, while soy milk matrix diminished the total phenolic bioaccessibility by 14.9% in pomelo juice, there was a 7.9% increase in kiwi juice-soy milk blend, compared with control juice contents (Quan et al., 2020). It maybe asumed from the results that findings of the present study may promote the protective effect of bovine milk on the cranberrybush phenolics during gastrointestinal digestion via the protein – phenolics interactions, resulted in a decreased digestive loss of the phenolics, improved

bioaccessibility and thus enhanced bioactivity. On the other hand, it was also claimed that bioaccessibility of phenolic compounds could decrease due to the phenolic compounds – protein interactions, resulting in precipitation of proteins and masking the phenolic compounds (Ozdal et al., 2013). It was noted that phenolic compound – protein interaction is stronger in the low pH range below the isoelectric point of the proteins, which results in a reduced bioaccessibility of phenolic substances in acidic fruits (Ozdal et al., 2013). In one study, the bioaccessibility of TPC and TFC in milk or soymilk-fruit juice (mixture of orange, kiwi, pineapple and mango) blends was found to be lower than that of water-fruit juice blend contents (Rodríguez-Roque et al., 2015). Furthermore, the results of the present study showed that the addition of milk or almond milk significantly ($p < 0.05$) lowered the TAC after gastrointestinal digestion. Similar findings were reported by Oksuz et al. (2019), indicating an inhibitory effect in TAC after gastrointestinal co-digestion of sour cherry-soymilk and sour cherry-milk, respectively (Oksuz et al., 2019).

The bioaccessibility of chlorogenic acid was found to be reduced in JM and JA with respect to JW. Similar results were reported by Rodríguez-Roque et al. (2014b, 2014a), who obtained a decrease in the bioaccessibility of some individual phenolic compounds (as ferulic acid, sinapic acid, quercetin, hesperidin and rutin) in fruit juice blends with the addition of milk or soymilk. Although hydrophilic bioactive compounds were found to be 3.4 times more bioaccessible in these juice samples rather than fruit juice-milk blends, the bioaccessibility of lipophilic (carotenoids) constituents was improved with milk addition (Rodríguez-Roque et al., 2014a; 2014b).

To evaluate the impact of the food matrix on the total antioxidant capacity, data from CUPRAC and DPPH assays were obtained from the undigested samples and bioaccessible fractions (Figure 4.4). When milk or almond milk was added to the juice, an immediate relevant loss of antioxidant capacity was observed. Likely, the decrease in the antioxidant capacity of undigested milk or almond milk blends is due to the precipitation of proanthocyanidins by milk proteins. The amount of precipitated polyphenols increased in bovine milk matrix rather than almond milk blends due to raising protein concentration. On the other hand, in line with the *in vitro* bioaccessibility of total antioxidant capacity, significant differences ($p < 0.05$) were obtained depending on the type of the matrix in fruit juice blends. The CUPRAC and

DPPH antioxidant capacity values of the juice samples changed in a manner similar to that of their bioactive contents during gastrointestinal digestion. While the greatest total antioxidant capacity was observed with the addition of milk matrix, almond milk matrix did not alter the results ($p > 0.05$). Similarly, Helal et al. (2014) stated that the addition of milk to cinnamon beverages enhanced the antioxidant capacity during gastrointestinal digestion probably linked to proteolysis, which releases free amino acids (tyrosine and tryptophan) and peptides with antioxidant properties. Besides, Quan et al. (2020) demonstrated that there was a significant increment ($p < 0.05$) on the bioaccessibility of ABTS scavenging ability in kiwi or pomelo juice with the addition of milk as well as soymilk matrix. Additionally, while the addition of milk to apple, grape and orange juices caused an increase in the antioxidant capacity values measured by ABTS assay after *in vitro* digestion, soymilk addition did not make any alteration in orange and grape juice samples, as compared with the control sample (He et al., 2016). In contrast, Rodríguez-Roque et al. (2015) reported that digested fruit juice-milk blend showed the lowest (12 – 15%) DPPH inhibition activity, whereas water and soymilk – fruit juice blends displayed the highest value (14 – 22%) with no significant differences among them (Rodríguez-Roque et al., 2015). As a consequence, there are different arguments related to the matrix effect on the bioaccessibility. It is clear from the results that matrix constituents like proteins, fat and minerals, most importantly their contents have a critical importance on the digestibility.

To conclude, findings indicate that the bioaccessibility of bioactive compounds depends on the processing technique to be applied, intensity and duration of the treatment, the type of the food matrix, the physicochemical properties of the bioactive compounds (i.e. nature and location in the food sample), interaction with dietary ingredients or gastrointestinal constituents (Rodríguez-Roque et al., 2015).

4.4.2 Caco-2 cytotoxicity

Before transport experiments, it is necessary to determine the maximum non-toxic sample concentration by a toxicity test. Thus, SRB assay, measuring the cellular viability based on cellular protein content, has been carried out. Cells were allowed to differentiate for 14 days after confluency. Then, Caco-2 cells treated with cranberrybush free and cranberrybush digests at different dilution ratios. 1/5 and 1/10 dilution ratios of the digests did not result in a major (i.e. biologically relevant)

cytotoxic effect (cell viability was never < 85%), indicating no cell release from the monolayer. Therefore, dilution of 1/5 was used in further Caco-2 transport experiments.

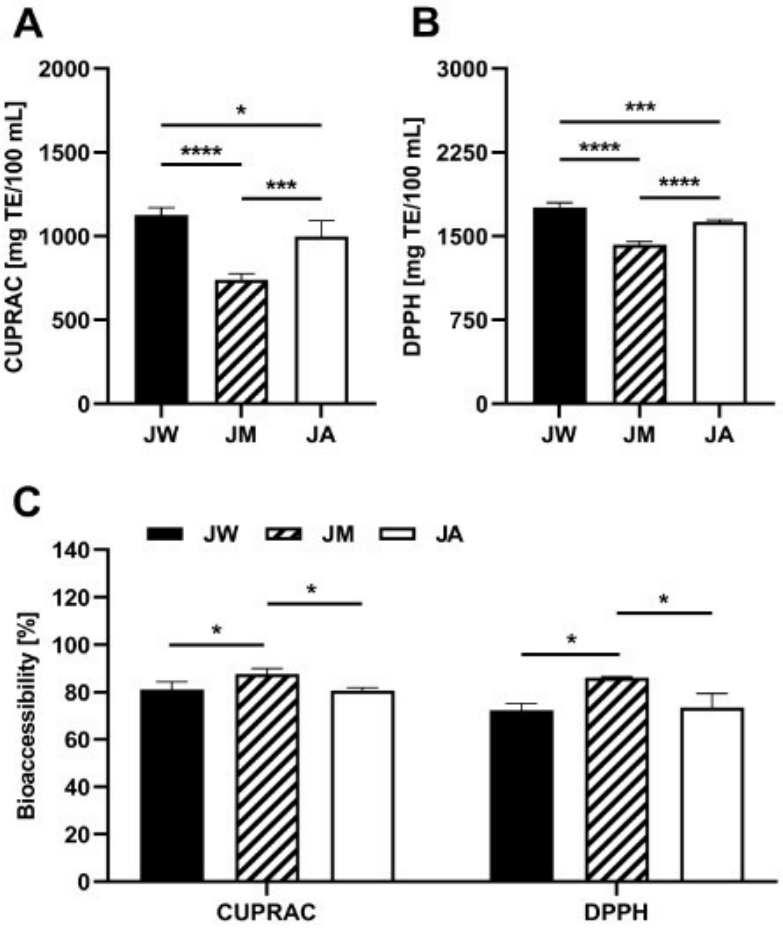


Figure 4.4 : Total antioxidant capacity after *in vitro* digestion of 100 mL cranberrybush juice mixed with water (JW), bovine milk (JM) or almond milk (JA).

4.4.3 Transport experiments

The TEER values before and after 4 and 24 h of treatment for all tested digests have been monitored. Before treatment, TEER values of all conditions were found to be higher than 300 Ωcm^2. TEER values did not change more than 10% of the original values (not shown) after 4 and 24 h, which are considerably above the recommended minimum level (200 Ωcm^2) needed for monolayer integrity (Palm et al., 1996). Therefore, we conclude that the monolayer integrity was not irreversibly damaged during transport experiments in the presence of a 1/5 diluted digestive matrix and

paracellular transport was not affected, which could influence bioavailability calculations.

4.4.3.1 Effects of food processing

One of the purposes of this study was to investigate the effect of novel non-thermal food processing on the absorption of phenolic compounds in the human digestive system using a recognized cell-based assay. Although there have been a series of studies conducted related to the effect of food processing on the bioaccessibility of micronutrients, the effects of non-thermal processing on the uptake of phenolic compounds from food matrices are largely unknown. Here we have used the *in vitro* digestion/Caco-2 cell system as a model to study the effect of HPP and PEF treatments on transport of cranberrybush polyphenols across the gut epithelium.

Identification of polyphenols in cranberrybush purée samples by using UPLC–QTOF–MS/MS analysis has been carried out previously (Ozkan et al., 2021). Three flavan-3-ols; catechin, epicatechin, epi(catechin) hexoside; three proanthocyanidins; procyanidin dimer, procyanidin trimer, procyanidin dimer monoglycoside, six flavonols; quercetin, quercetin-deoxyhexose, quercetin-*3*-*O*-glucoside, quercetin pentoside hexoside, rutin, isorhamnetin-*3*-*O*-rutinoside, a flavone; diosmetin-rhamnosylglucoside, four phenolic acids; caffeic acid, chlorogenic acid, coumaric acid and coumaroyl-quinic acid as well as three anthocyanins (cyanidin-3-glucoside, cyanidin-3-rutinoside and cyanidin-3-xylosyl-rutinoside) were detected in cranberrybush samples.

Catechin and epicatechin (*m/z* 289.0716, $C_{15}H_{14}O_6$) were detected at the basolateral compartment of all samples, whereas none of the oligomeric procyanidins could be detected, which is in line with previous *in vitro* and *in vivo* studies (Serra et al., 2010; Wu et al., 2017). It was reported that procyanidins were unstable at physiologic pH and were degraded into procyanidin monomers during digestion (Kahle et al., 2011). As for flavonols, while quercetin-deoxyhexose (*m/z* 447.0933, $C_{21}H_{20}O_{11}$) and quercetin-3-*O*-glucoside (*m/z* 463.0879, $C_{21}H_{20}O_{12}$) were found in the basolateral compartment of all the samples, other flavonoids present in the cranberrybush purée could not be detected. Similar results were also found in the studies on cocoa (Juarez-Enriquez et al., 2015) and buckwheat (Yao et al., 2020) polyphenols, the digestibility

of flavonoids such as quercetin and rutin were determined lower than that for the other phenol groups.

On the other hand, none of the anthocyanins could be detected in the basolateral compartment after 4 h exposure. This finding may be related to the low anthocyanin amount of the samples and chemical instability in the alkali conditions (as discussed in section 3.1).

The majority of the phenolic acids, namely, caffeic acid (*m/z* 179.0350, $C_9H_8O_4$), chlorogenic acid (*m/z* 353.0879, $C_{16}H_{18}O_9$) and coumaric acid (*m/z* 163.0403, $C_9H_8O_3$) were detected at the basolateral compartment of all the samples. In addition to these, quinic acid (*m/z* 191.0516, $C_7H_{12}O_6$) was also detected due to possible degradation of chlorogenic acid, present in the cranberrybush, by the cleavage of the chlorogenic acid moiety into quinic acid and caffeic acid by small intestine mucosal esterases (Dupas et al., 2006a). These results were in line with *in vitro* digestion/Caco-2 cell model study conducted by Wu et al. (2017), showing that the chlorogenic acid, present in the Aronia juice, was degraded throughout the different stages of digestion, while quinic acid significantly increased (Wu et al., 2017). Overall, we conclude that the cranberrybush derived phenolic acids are relatively stable in the small intestine. As chlorogenic acid is the major active metabolite of *V. opulus* (Barak et al., 2019), in the work described here, we have focused on the predominant phenolic acid, namely chlorogenic acid, to screen the transport efficiency through the Caco-2 cells (Table 4.1).

The digests of untreated and HPP/PEF-treated cranberrybush purée samples were added to the apical (top) side of the Caco-2 cells growing in transwells. Then, samples were collected from the apical compartment after 0, 2 and 4 h of incubation and from the basolateral (bottom) compartment after 2 and 4 h. At the end of 2 and 4 h of exposure, recoveries on the apical and basolateral sides were quantified for chlorogenic acid, as a percentage of the amount of chlorogenic acid loaded to the apical side at 0 h. Chlorogenic acid recoveries in the apical compartment after 2 h of exposure to samples ranged from 96 to 98%, which was in general not significantly different among samples. Furthermore, after 4 h of treatment with the samples, apical recoveries of chlorogenic acid obtained from HPP/PEF treated samples (71−73%) were not significantly different compared to untreated samples (72%). While the basolateral side recovery of intact chlorogenic acid was found to be in the range of 2.54 – 2.64%

after 2 h of treatment, after 4 h, the amount of compounds transported to the basolateral compartment increased to 4.24 – 4.51%. The basolateral side recovery (indicating transport) was corrected for the apical side recovery (indicating stability), defining as transport efficiency (Toydemir et al., 2013). The transport efficiency of chlorogenic acid in the untreated sample was still similar to those of HPP and PEF-treated samples after both 2 and 4 h exposure, indicating similar retention of chlorogenic acid even after HPP and PEF treatments at selected processing intensities in comparison with untreated samples. The major outcome of this work was that transport of the predominant cranberrybush phenolic acid was not negatively affected by processing.

The transepithelial transport of chlorogenic acid has been studied before using Caco-2 cells. It was presented that the transepithelial flux was found to be inversely correlated with the paracellular permeability of Caco-2 cells. Namely, the permeation of chlorogenic acid from apical to basolateral direction is many due to paracellular diffusion and the intestinal absorption could be restricted when the epithelial tight junction is tight enough. In addition to this, the permeation rate of chlorogenic was found to be concentration-dependent and was not saturable, indicating that passive diffusion might be involved (Konishi & Kobayashi, 2004). Similar to the results obtained here, Kamiloglu et al. (2017) also reported 4 – 6.1 % basolateral recovery of chlorogenic acid from black carrot, which is in the same range as observed here for chlorogenic acid present in untreated or HPP/PEF treated samples. On the other hand, it was indicated that a considerably low amount of chlorogenic acid standard (0.06%) was transported into the basolateral side (after 40 min), suggesting a restriction by the tight junction (Konishi & Kobayashi, 2004). Moreover, Monente et al. (2015) indicated only 0.55 and 1% of the chlorogenic acid and its metabolites from spent coffee grounds were transported from the apical to the basolateral side of cell after 0.5 h and 1 h, respectively. When compared the basolateral recovery values in this study and the literature, higher values seems to be likely to the extended exposure duration of the transport experiment (Monente et al., 2015). The results of this study were in line with *in vivo* studies conducted with volunteers, who had an ileostomy, consuming instant coffee, thereafter, ileal fluids were collected over the period of 24 h. Findings showed that 71% of chlorogenic acid and its metabolites were recovered in the ileal fluid after 24 h of the consumption, indicating 29% absorption of the intake in the small intestine and ~71% passage of the ingested chlorogenic acids from the small to

the large intestine (Stalmach et al., 2010). To conclude, polyphenols are generally poorly absorbed in the small intestine due to their glycosidic linkages, thereby the majority of these substances reach the colon to generate the aglycon form by colonic microbiota metabolism (Jaganath et al., 2006).

4.4.3.2 Effects of food matrix

The last objective in the present study was to evaluate the effect of milk food matrix on the bioavailability of the cranberybush juice polyphenols. Among other flavonols, quercetin-deoxyhexose and quercetin-3-*O*-glucoside were transported to the the basolateral compartment for all the samples.

On the other hand, catechin/epicatechin were transported through the Caco-2 cells in only JW samples. For procyanidin monomers, an interesting difference between cranberrybush juice and its milk blends (JM and JA) was observed owing to the complexation between cranberrybush procyanidins and proline-rich proteins, resulting in a reduction (as discussed in section 3.1.2). Besides, none of the oligomeric procyanidins could be detected and this observation was in the line of expectation. Most of the phenolic acids including caffeic acid, chlorogenic acid and coumaric acid were detected at the basolateral compartment of all the samples. Similar to the results of the processing effect on the recovery of phenolic acids, quinic acid was also detected in all the juice samples. Unlike other flavonoids, none of the anthocyanins could be detected in the basolateral compartment after 4 h exposure (as discussed in section 3.1).

As for transportation of chlorogenic acid from cranberrybush juice+water (JW) and juice+milk blends (JM and JA), apical recovery after 4 h, basolateral recovery as well as transport efficiencies after both 2 and 4 h exposure were found to be higher in juice samples than that of purée samples. For example, chlorogenic acid recovery at the basolateral side (t= 4 h) was 1.4, 2.2 and 2.1 times higher for the JW, JM and JA samples compared to the untreated purée sample. Reasons for greater retention of bioactive compounds in juice samples were explained as the type (especially pectin content) and physiological properties (liquid state) of the food matrix (see section 3.3.2).

What has become evident from this research is that the basolateral recovery and transportation of chlorogenic acid were altered with the addition of milk (bovine or almond milk). The recoveries of chlorogenic acid from JM and JA in the basolateral compartment after 4 h of exposures (9.61 and 9.46%, respectively) were significantly higher than that of JW samples (6.14%) ($p < 0.001$, $p < 0.0001$). Moreover, the transport efficiency after 4 h was also higher ($p < 0.001$) for the JM and ($p < 0.01$) JA samples compared to the JW. Similarly, Dupas et al. (2006b) investigated the effect of interactions between chlorogenic acid from coffee and semi-skimmed milk proteins (25%, g/L) on the absorption and bioavailability of the chlorogenic acid via Caco-2 cell model coupled with an *in vitro* digestion (t= 1 h). Results demonstrated that there is an increasing trend (not significant) in the absorption kinetics of chlorogenic acid in presence of milk proteins (casein) (0.25%) in comparison when chlorogenic acid is consumed out of a food matrix (0.14%) (Dupas et al., 2006b). These results suggest that the presence of milk proteins does not hinder the absorption of chlorogenic acid. However, particularly important from a milk food matrix perspective is that the natural food matrix including protein as well as lipid contents, phenolic profile and their interactions have an influence on transepithelial transportation of polyphenols, besides, should also be evaluated specifically for each individual polyphenols by taking into consideration the ratio between milk and polyphenols, as well.

4.5 Conclusion

In this study we aimed to investigate the effect of non-thermal food processing on the absorption of phenolic compounds in the human digestive system using a recognized combined gastrointestinal digestion/cell-based assay. Moreover, since the transport of polyphenols generally appears to be relatively poor (5 – 10%), it may be a significant attention to study other food-related factors like the matrix effect that could enhance the transport of these compounds across the gut epithelium. Processing method (HPP, PEF5 and PEF15) and milk matrix (bovine milk and almond milk) had different effects on the contents of phenolic compounds, bioaccessibility and transport efficiency in cranberrybush products. Both HPP and PEF enhanced or did not have a negative effect on the recovery of the bioactive. Particularly important from a food matrix perspective is that the addition of milk appears to have a positive effect on transepithelial transportation of chlorogenic acid. Therefore, from the results obtained here, it can be

concluded that high pressure and pulsed electric field treatments can be used as alternative processing methods in the production of functional foods with enhanced nutritional value. Furthermore, it would be interesting to conduct advanced investigations on the transport dynamics of polyphenols from different food matrices that will be treated by non-thermal food processing methods. In addition to these, due to the fact that majority of the polyphenols reach the colon and are consecutively metabolized by colonic microbiota, future studies involving the combination of *in vitro* digestion models and Caco-2 cell culture studies could also be included with the colonic fermentation. In this regard, sulfated, glucuronidated, or methylated metabolites of polyphenols could also be evaluated to present overall consideration of the fate of the polyphenols to be released from the food matrix.

Table 4.1 : Apical and basolateral side recovery and transport efficiency of chlorogenic acid from cranberrybush purée and juice samples.

Sample[A]	t= 2h			t= 4h		
	Apical side recovery (%)[B]	Basolateral side recovery (%)[C]	Transport efficiency[D]	Apical side recovery (%)[B]	Basolateral side recovery (%)[C]	Transport efficiency[D]
			Process effect			
Untreated	96±3	2.59±0.21	0.027±0.003	72±6	4.38±0.26	0.061±0.002
HPP	98±4	2.64±0.06	0.027±0.000	71±1	4.51±0.04	0.063±0.000
PEF5	98±7	2.54±0.09	0.026±0.003	72±9	4.24±0.30	0.060±0.006
PEF15	98±3	2.54±0.16	0.026±0.002	73±3	4.31±0.07	0.059±0.002
			Matrix effect			
JW	96±9	4.01±0.25	0.042±0.007	79±7	6.14±0.60	0.078±0.006
JM	94±4	4.69±0.99	0.050±0.011	73±6	9.61±0.25****	0.133±0.007***
JA	91±11	5.97±0.11*	0.066±0.008*	77±7	9.46±0.18***	0.123±0.011**

[A]The data presented in this table consist of average values ± standard deviation of three independent batches. Statistically significant differences are shown for each sample compared to each other. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. [B]Apical side recovery percentages were calculated as (chlorogenic acid concentration at the apical side after transport)/(chlorogenic acid concentration at the apical side at 0 h of incubation) × 100. [C]Basolateral side recovery percentages were calculated as (chlorogenic acid concentration at the basolateral side after transport)/(chlorogenic acid concentration at the apical side at 0 h of incubation) × 100. [D]Transport efficiency was calculated as (basolateral side recovery, %)/(apical side recovery, %).

5. PVP/FLAVONOID COPRECIPITATION BY SUPERCRITICAL ANTISOLVENT PROCESS[4]

5.1 Abstract

To date, various delivery systems have been developed to improve chemical stability and increase the bioavailability of polyphenolic compounds. In the present study, the micronization of two flavonoids, quercetin and rutin, and their coprecipitation with polyvinylpyrrolidone (PVP) were studied using the supercritical antisolvent process (SAS). SAS process parameters were optimized with the aim of obtaining composite microspheres with controlled mean size and particle size distribution. Spherical microparticles (with mean diameters in the range between 0.47 and 9.52 μm for PVP/quercetin and in the range 0.84–8.17 μm for PVP/rutin) were precipitated, depending on the operating conditions. In correspondence of the best operating conditions, the entrapment efficiency in PVP, for both flavonoids, was 99.8% and the dissolution rate from the coprecipitated powders was 10 and 3.19 times faster compared to the dissolution rates of unprocessed flavonoids for quercetin and rutin, respectively.

Key words: quercetin, rutin, polyphenols, polyvinylpyrrolidone, coprecipitated microparticles, (SAS) process

5.2 Introduction

Quercetin is a flavonoid compound, belonging to the class of flavonols (Fischer et al., 1997). A variety of foods and vegetables (Alinezhad et al., 2013), particularly onions, peppers, cranberries, blueberries, apples, cherries and grapes are considered to be rich sources of quercetin (Harnly et al., 2006). Dietary sources rich of quercetin are also flowers, tea, nuts, tomatoes, many seeds, barks, leaves as well as medicinal botanicals,

[4] This chapter is based on the paper "Ozkan, G., Franco, P., Capanoglu, E., and De Marco, I. (2019). PVP/flavonoid coprecipitation by supercritical antisolvent process. Chemical Engineering and Processing-Process Intensification, 146, 107689."

including *Ginkgo biloba*, *Hypericum perforatum*, and *Sambucus Canadensis* (Williamson et al., 2005; Wiczkowski et al., 2008). In plants, quercetin (structural formula shown in Figure 5.1a) is commonly found in the form of glycosides (Rice-Evans, 2001); among them, rutin (Figure 5.1b) is the most widespread glycoside form of quercetin (Manach et al., 1997). The use of these compounds has been associated with a wide range of biological activities, including antioxidant, anti-inflammatory, anticancer, antiviral as well as to prevent cardiovascular, pancreas and liver diseases (Wang et al., 2016; Cazarolli et al., 2008; Huang et al., 2017). On the other hand, quercetin and rutin undergo many chemical changes during food processing and storage, due to the effects of oxygen, temperature, pH, etc. Besides, these flavonoids show a poor water solubility and, thus, a reduced bioavailability (Wang et al., 2016).

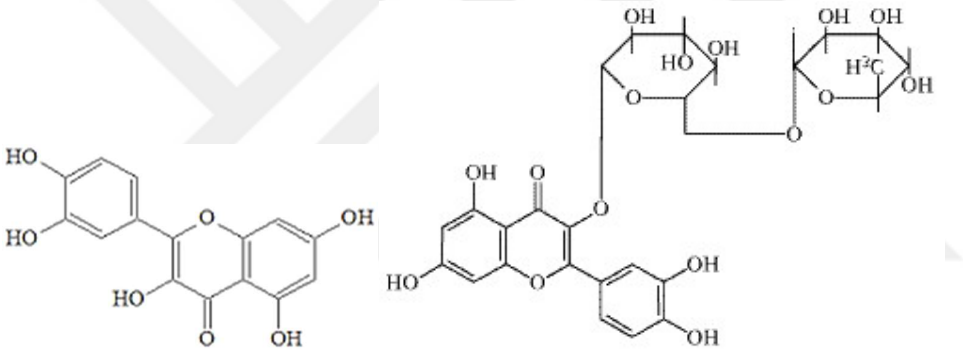


Figure 5.1 : Structural formulas of the flavonoids: (a) quercetin; (b) rutin.

Considering that they are very interesting in the pharmaceutical and nutraceutical fields because of their numerous benefits to human health, flavonoids are frequently taken as supplements, and functional foods. Therefore, in order to preserve their properties and improve their bioavailability, different delivery systems, based on the coprecipitation of flavonoids with a suitable polymer, have been developed (McClements et al., 2009).

Indeed, the active compound can be entrapped, impregnated in a polymer matrix, or encapsulated through a polymeric coating (Baldino et al.,2016; Concilio et al., 2015; Ranjit and Baquee, 2013).

Some conventional micronization techniques, such as, for example, spray-drying, emulsification/solvent evaporation, centrifugal extrusion, freeze-drying and coacervation have been used to obtain coprecipitated particles (Gonnet et al., 2010); however, using these processes, several limitations have been identified. Indeed, it is

not easy to control the particle size distribution, the product can be degraded because high temperatures and elevated quantities of residual solvent can cause the loss of its biological activity (Ozkan et al., 2019). Techniques assisted by supercritical fluids, in particular supercritical carbon dioxide ($scCO_2$), can overcome these limitations (Reverchon et al., 2008; Franco et al., 2018). Different processes have been proposed as an efficient alternative to conventional ones for the micronization and coprecipitation of compounds belonging to different categories, such as products in agricultural, biomedical, pharmaceutical, food and cosmetic fields (Fahim et al., 2014; Campardelli and Reverchon, 2015; Zahran et al., 2014). Supercritical carbon dioxide based techniques may be classified according to the role played by the $scCO_2$: indeed, it can play the role of solvent (like in the RESS, rapid expansion from supercritical solutions) (Chen et al., 2018), of antisolvent (like in the SAS, supercritical antisolvent process) (Rossmann et al., 2014), or of co-solvent (like in the SAA, supercritical assisted atomization process) (Peng et al., 2019). In the SAS process, carbon dioxide is used as the antisolvent for the product to be micronized or coprecipitated (Franco et al., 2018; Tabernero et al., 2012). Using the SAS technique, a wide variety of morphologies has been obtained, such as nanostructured filaments, nanoparticles with mean diameters in the range of 30–200 nm, spherical microparticles in the range of 0.25–20 μm, hollow expanded microparticles with diameters between 10 and 200 μm, and crystals of different dimensions (De Marco et al., 2013; De Marco and Reverchon, 2011; Montes et al., 2016). Because of its solubility in water, polyvinylpyrrolidone (PVP) is widely used as a carrier in pharmaceutical, biomedical and food applications; moreover, it has the GRAS (Generally Regarded as Safe) status and is included in the FDA (Food and Drug Administration) list (Prosapio et al., 2015; D'Souza et al., 2004). Besides, it has the capability of retarding the crystal growth (Ledet et al., 2015) and enhancing the dissolution rate of poorly water-soluble active compounds (Oth and Moes, 1985).

The literature regarding flavonoids coprecipitation using conventional techniques is scarce and is related to the formation of cyclodextrin inclusion complexes (Koontz et al., 2009; Praveena et al., 2017). In those articles, no information on the morphologies and on the dissolution rate of the active principles were given.

The literature concerning the production of quercetin and rutin coprecipitated particles by SAS process is limited. For example, Montes et al. proposed the use of SAS

technique to micronize rutin (Montes et al., 2016); however, the authors showed the difficulty in obtaining distinct and defined particles, and they highlighted the importance of choosing a proper solvent. Indeed, sub-microparticles were obtained only using a solvent mixture of acetone/dimethylsulfoxide (DMSO) at a ratio of 9/1, whereas, by using other solvent mixture proportions, the authors indicated that only a liquid phase was found in the precipitation chamber at the end of the experiments. Investigating the dissolution profiles of unprocessed and SAS processed rutin in simulated gastric (SGF) and intestinal fluids (SIF), an improvement of the dissolution rate was observed by the authors (Montes et al., 2016). Fernández-Ponce et al. (2015) attempted the micronization of quercetin and its coprecipitation using ethyl cellulose (EC) as the polymeric carrier and ethyl acetate as the solvent. The FESEM images revealed that, when quercetin was processed alone, smaller sized crystals were obtained compared to the ones of unprocessed quercetin; whereas, irregular and coalescent amorphous particles were obtained by coprecipitation. No dissolution tests were performed to evaluate whether the coprecipitation modified the dissolution rate of the active ingredient. In another paper, Fraile et al. (2014) coprecipitated quercetin with Pluronic F127 producing aggregated particles, as reported in FESEM micrographs (Fraile et al., 2014). For particulate solids, the product properties strongly depend on the dispersity of the material; for this reason, the aggregation and agglomeration have to be avoided when possible, for example, by using surfactants (Peukert et al., 2005).

Summarizing the literature results, to the best of our knowledge, the SAS micronization of quercetin and rutin and their coprecipitation with polymeric carriers have been attempted only in a limited number of articles without obtaining satisfying results.

In particular, SAS coprecipitation can be considered efficient when well-separated composite particles are produced (Prosapio et al., 2018); i.e., when the drug is homogeneously dispersed in the polymeric particle. However, until now, coalescent or irregular in shape and dimension particles have been obtained, as reported in the literature.

The objective of this study is to obtain an effective SAS coprecipitation of quercetin or rutin with PVP, enhancing the dissolution rate, and, therefore, improving the bioavailability of these natural antioxidant compounds. In particular, optimal

conditions in terms of the type of solvent used, operating pressure, overall concentration and polymer/flavonoid ratio for the formation of spherical composite microparticles were determined. Morphology, mean size and size distribution of the particles were analyzed and discussed. The effectiveness of the process was also verified through entrapment efficiency and dissolution tests.

5.3 Materials, Methods and Procedure

5.3.1 Materials

Polyvinylpyrrolidone (PVP, average molecular weight 10,000 g/mol), quercetin dihydrate (purity 98%), rutin hydrate (purity 95%) and dimethylsulfoxide (DMSO, purity 99.5%) were supplied by Sigma-Aldrich (Italy). Ethanol (EtOH, purity 99.9%) was purchased from Carlo Erba (Italy). Morlando group (Italy) provided carbon dioxide (purity 99%).

Solubility tests performed at room temperature showed that the solubilities of the materials in EtOH are about: 200 mg/mL in the case of PVP, 10 mg/mL in the case of quercetin and 20 mg/mL in the case of rutin; whereas, the solubilities in DMSO are: 250 mg/mL in the case of PVP, 90 mg/mL in the case of quercetin and 240 mg/mL in the case of rutin.

5.3.2 SAS apparatus and procedure

A schematic representation of the SAS apparatus used for micronization and coprecipitation is shown in Figure 5.2. Two high-pressure pumps are used to feed the supercritical CO2 ($scCO_2$) (P1) and the liquid solution (P2). A cylindrical vessel with an internal volume of 0.5 L is used as the precipitation vessel (P). The liquid solution, formed by a solute or two solutes (PVP and quercetin or rutin) dissolving in the selected solvent (EtOH or DMSO), is sprayed into the precipitator through a thin wall, 100 μm internal diameter stainless steel nozzle. Supercritical carbon dioxide ($scCO_2$) is preheated and, then, delivered to the vessel by another inlet located on the top of the precipitator. A micrometric valve enables to regulate the operating pressure in the vessel, whose value is assured by a test gauge manometer. Two electrically thin bands and a thermocouple are connected to a PID controller for the temperature measurement and control. The precipitated powders are collected on a stainless steel filter (pore

diameter equal to 0.1 μm), which is placed on the bottom of the precipitator. This filter allows the CO_2–solvent solution to reach the collection vessel at a lowerpressure (1.8–2.0 MPa) located at the exit of the precipitator. A rotameter, situated downstream of this second vessel, measures the CO_2 flow rate.

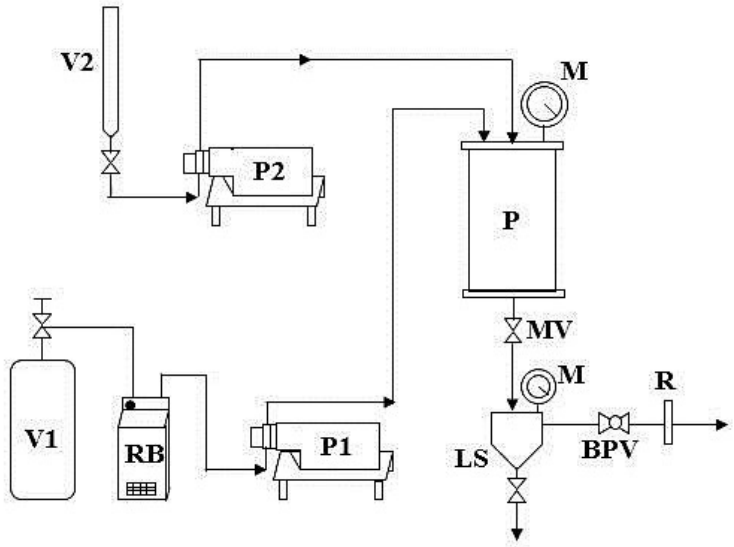


Figure 5.2 : Schematic representation of the SAS plant. V1: CO_2 supply; V2: liquid solution supply; RB: refrigerating bath; P1, P2: pumps; P: precipitation vessel; MV: micrometric valve; M: manometer; LS: liquid separator; BPV: back-pressure valve; R: rotameter.

A SAS experiment starts with the pressurization of the precipitation vessel with preheated scCO2 to assure the steady-state conditions. When temperature, pressure and antisolvent flow rate are stable, the liquid solution, with the active principle dissolved in it, is delivered through the nozzle, producing the precipitation of solute.

At the end of the solution injection, $scCO_2$ continues to flow to eliminate the residual solvent, avoiding to solubilize or modify the precipitates during the depressurization step. When the washing step is completed, CO_2 flow is interrupted, the precipitation vessel is slowly depressurized down to atmospheric pressure, and the precipitated powder can be collected and sampled for the subsequent analyses.

5.3.3 Analytical methods

The morphology of the precipitated powder was observed through a Field Emission Scanning Electron Microscope (FESEM, mod. LEO 1525, Carl Zeiss SMT AG, Oberkochen, Germany); considering that the powder has to be conductive to be

analyzed, it was placed on a stub with a sticky carbon cover and then coated with gold-palladium with the aid of a sputter coater (mod. 108 A, Agar Scientific, Stansted, United Kingdom).

Mean diameters, standard deviations and particle size distributions (PSDs) of the powders were determined using an image analysis software (Sigma Scan Pro 5.0, Aspire Software International Ashburn, VA). The samples were taken from various locations inside the precipitator and the corresponding PSD was elaborated considering approximately 1000 particles, whose diameters were measured using FESEM images taken at high enlargements. The elaboration of the PSDs was carried out using Microcal Origin Software (release 8.0, Microcal Software, Inc., Northampton, MA).

Calorimetric properties were studied using a differential scanning calorimeter (DSC, TC11 from Mettler-Toledo) under a nitrogen atmosphere (flow rate 50 mL/min); the sample (about 5 mg) was heated from 25 to 400 °C at 10 °C/min.

X-ray diffraction (XRD) analyses were performed by an X-ray powder diffractometer (model D8 Discover; Bruker, USA) with a Cu sealed tube source. Powders were placed on a holder and flattened with a glass slide to minimize preferred orientation of the particles. XRD patterns were recorded using the following conditions: Ni-filtered CuKα radiation, λ=1.54 Å, 2θ angle ranging from 5° to 55° with a scan rate of 0.2 s/step.

An UV/vis spectrophotometer (model Cary 50, Varian, Palo Alto, CA) was used to perform dissolution studies for each flavonoid compound (FC) at a wavelength of 369 nm and 257 nm for quercetin and rutin, respectively. A phosphate buffered saline solution (PBS) at pH 7.4 was used as release medium. A dialysis sack, containing the powder samples with an equivalents amount of FC (5 mg) suspended in 3 mL of PBS, was then incubated in 300 mL of PBS at pH 7.4 and 37 °C, continuously stirred at 150 rpm. The mean profiles were proposed in the present paper, since each analysis was performed in triplicate.

The entrapment efficiency of PVP/flavonoid coprecipitated particles (EE%) was also measured by UV–vis analysis, measuring the absorbance in PBS medium at the end of

the release, when all FC was released from the particles to the outer phase. The absorbance was converted into FC concentration using a calibration curve.

EE% was determined as follows:

$$EE\% = \frac{mg\ polyphenols\ in\ the\ processed\ sample}{mg\ polyphenols\ in\ the\ initial\ solution} * 100 \quad (5.1)$$

Considering that quercetin and rutin are not particularly soluble in PBS, the EE% measurements were repeated using ethanol as medium. Total antioxidant capacity was performed by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay according to Kumaran (Kumaran, 2006). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as a standard and the results were expressed in terms of mg Trolox equivalents (TE) per g dry weight of sample.

5.4 Results

SAS experiments were carried out using a CO2 flow rate of 30 g/min, a solution flow rate of 1 mL/min, and an operating temperature of 40 °C. The solvent and antisolvent flow rates were chosen to work with molar fractions to the right of the mixture critical point (MCP) of the binary system solvent/antisolvent at 40 °C (Reverchon and De Marco, 2011): in particular at CO_2 molar fractions approximately equal to 0.98. Moreover, the chosen operating pressures were higher than the critical pressure of the binary system solvent/CO_2, which is approximately 8.5 MPa for DMSO/CO_2 (Andreatta et al., 2007) and 8.0 MPa for EtOH/CO_2 (Lim et al., 1994) at 40 °C.

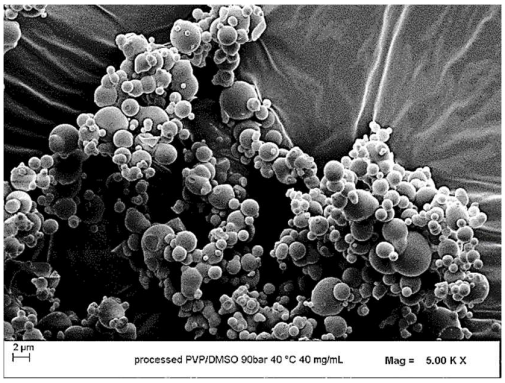
Table 5.1 shows a summary of the performed experiments. The effects of the type of solvent (EtOH or DMSO), the operating pressure, the total initial concentration of solutes in the organic solvent (C_{tot}, mg/mL) and the polymer/flavonoid ratio (PVP/FC, w/w) on the obtained morphology, mean diameter (m.d.), standard deviation (s.d.), and entrapment efficiency (EE%) on a volumetric basis was investigated for each compound. Each experiment was repeated twice; the values inserted in the last two columns of Table 5.1 are mean diameters, mean standard deviations and mean EE% of the duplicates.

Table 5.1 : Summary of SAS experiments (IP: irregular particles; C: crystals; MP: microparticles; cMP: coalescing microparticles; cSMP: coalescing sub-microparticles).

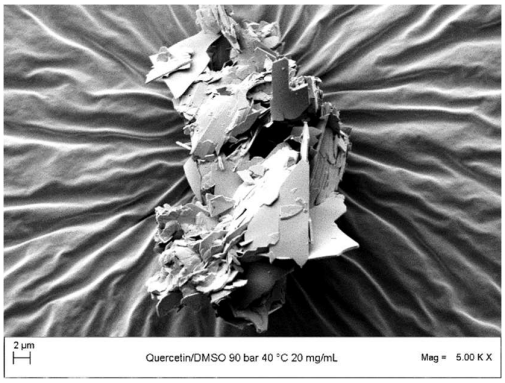
#	FC	Solvent	P [MPa]	C_{tot} [mg/mL]	PVP/FC [w/w]	Morphology	m.d. ± s.d. [µm]	EE%
1	-	DMSO	9.0	40	1/0	MP	2.61±0.82	-
2	quercetin	EtOH	9.0	20	20/1	MP+IP	3.51±1.09	31.7
3			9.0	40		MP+IP	5.56±1.43	34.0
4			9.0	20	0/1	C	-	-
5			13.0	20		-	-	-
6		DMSO	9.0	20	20/1	cMP	1.09±0.32	39.3
7			9.0	30		MP	8.33±2.64	92.2
8			9.0	40		MP	9.52±2.74	99.8
9			9.0	30	10/1	cSMP	-	54.4
10			9.0	40		cSMP	0.47±0.36	43.4
11		EtOH	9.0	20	20/1	MP+IP	2.24±0.67	33.3
12		DMSO	9.0	20	0/1	C	-	-
13	rutin	DMSO	13.0	20	20/1	-	0.84±0.26	-
14			9.0	20		cMP	1.15±0.34	34.7
15			9.0	30		MP	8.17±2.29	99.6
16			9.0	40	10/1	MP	0.94±0.25	99.8
17			9.0	40		cMP		90.1

5.4.1 Preliminary experiments

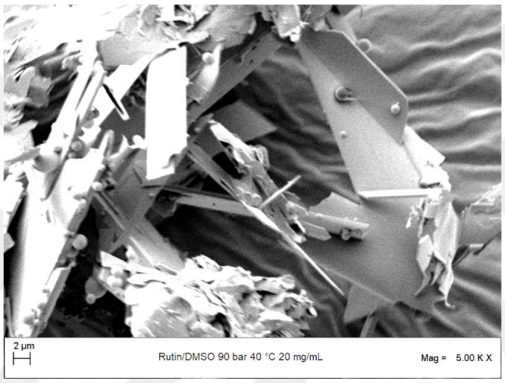
Some preliminary tests were carried out processing the polymer and each flavonoid separately, to investigate their behavior when processed by SAS. In particular, micronizing PVP at 40 °C, 9.0 MPa and a solute concentration in DMSO equal to 40 mg/mL (run #1 in Table 5.1), microparticles were produced (Figure 5.3a); PVP already showed the tendency to precipitate in the form of microparticles, at various operating pressures and various concentrations in the liquid solution, both using DMSO (De Marco et al., 2015) and EtOH (Rossmann et al., 2014) as the organic solvent.



(a)



(b)



(c)

Figure 5.3 : FESEM image of single compounds precipitated at 9.0 MPa, 40 °C: (a) PVP processed at 40 mg/mL$_{DMSO}$; (b) quercetin processed at 20 mg/mL$_{DMSO}$; (c) rutin processed at 20 mg/mL$_{DMSO}$.

Each FC was processed at 40 °C, 9.0 MPa, fixing the solute concentration in DMSO at 20 mg/mL (runs #4 and #12 in Table 5.1). Operating at these conditions, most of the solutes was extracted, whereas the remaining part, recovered from the filter and analyzed by FESEM, was constituted by large and irregular crystals, both in the case of quercetin (Figure 5.3b) and rutin (Figure 5.3c). These preliminary experiments on quercetin and rutin indicated that they are partially soluble in the mixture formed by $scCO_2$ and DMSO at the process conditions.

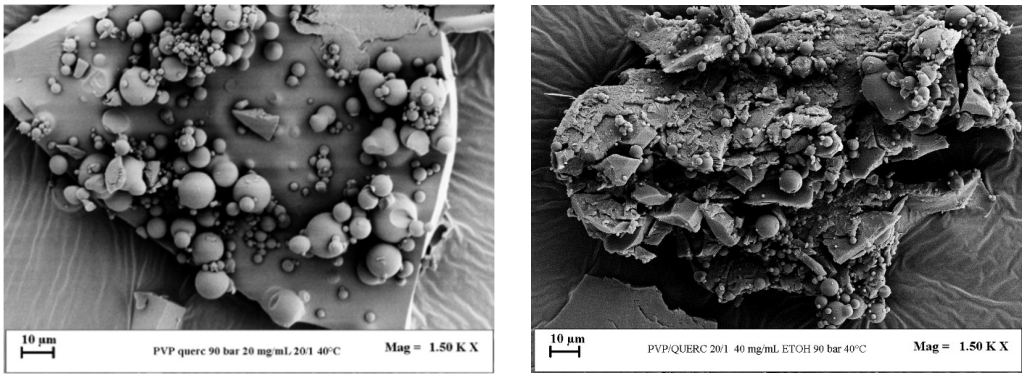
The flavonoids alone were also processed at the same operating conditions in terms of temperature and solute concentration but at higher pressure (13.0 MPa, runs #5 and #13 in Table 5.1). In these cases, no powder was recovered in the vessel at the end of the experiments, because the solute was completely dragged in the separator located downstream the precipitation vessel.

Based on these preliminary experiments, it is possible to assert that both active compounds were not good candidates for SAS micronization because of their solubility in the mixture formed by the organic solvent and the antisolvent; whereas PVP was a compound that can be effectively processed by SAS. Therefore, the following experiments were performed attempting the coprecipitation for the systems PVP/quercetin and PVP/rutin. Considering the results of the preliminary experiments, the coprecipitation at pressures higher than 9.0 MPa was not attempted.

5.4.2 Effect of the solvent

The first set of coprecipitation experiments was carried out to investigate the effect of the solvent on particle morphology, mean size and particle size distribution. Firstly, fixing PVP/quercetin ratio at 20/1 w/w, a test was performed with a total solute concentration in ethanol equal to 20 mg/mL (run #2). Then, another experiment was conducted by using a higher overall solute concentration (40 mg/mL) (run #3).

The FESEM analyses of the powders showed that some microparticles were precipitated, but also the presence of material with an irregular morphology was detected, both using a total concentration of PVP/quercetin in EtOH equal to 20 mg/mL (Figure 5.4a) as well as increasing the concentration at 40 mg/mL (Figure 5.4b).



(a) (b)

Figure 5.4 : FESEM images of PVP/quercetin 20/1 w/w particles precipitated at 9.0 MPa and 40 °C using ethanol as the organic solvent. Effect of overall concentration: (a) 20 mg/mL$_{EtOH}$; (b) 40 mg/mL$_{EtOH}$.

Considering that in both experiments not well defined particles were recovered, for the system PVP/quercetin, in the following experiments, DMSO was used as the organic solvent.

Regarding the system PVP/rutin, the effect of the solvent was investigated fixing a total concentration of the solute equal to 20 mg/mL and a PVP/rutin ratio to 20/1 w/w. Using ethanol as the solvent (Figure 5.5a, run #11), irregular material together with microparticles precipitated, whereas, using DMSO as the organic solvent (Figure 5.5b, run #14), coalescing spherical particles were obtained. As in the case of quercetin, DMSO seemed to be the preferable solvent to conduct the coprecipitation of PVP and rutin; indeed, with this solvent, spherical particles were formed. Therefore, also for the PVP/rutin system, the subsequent experiments were carried out using DMSO as the organic solvent.

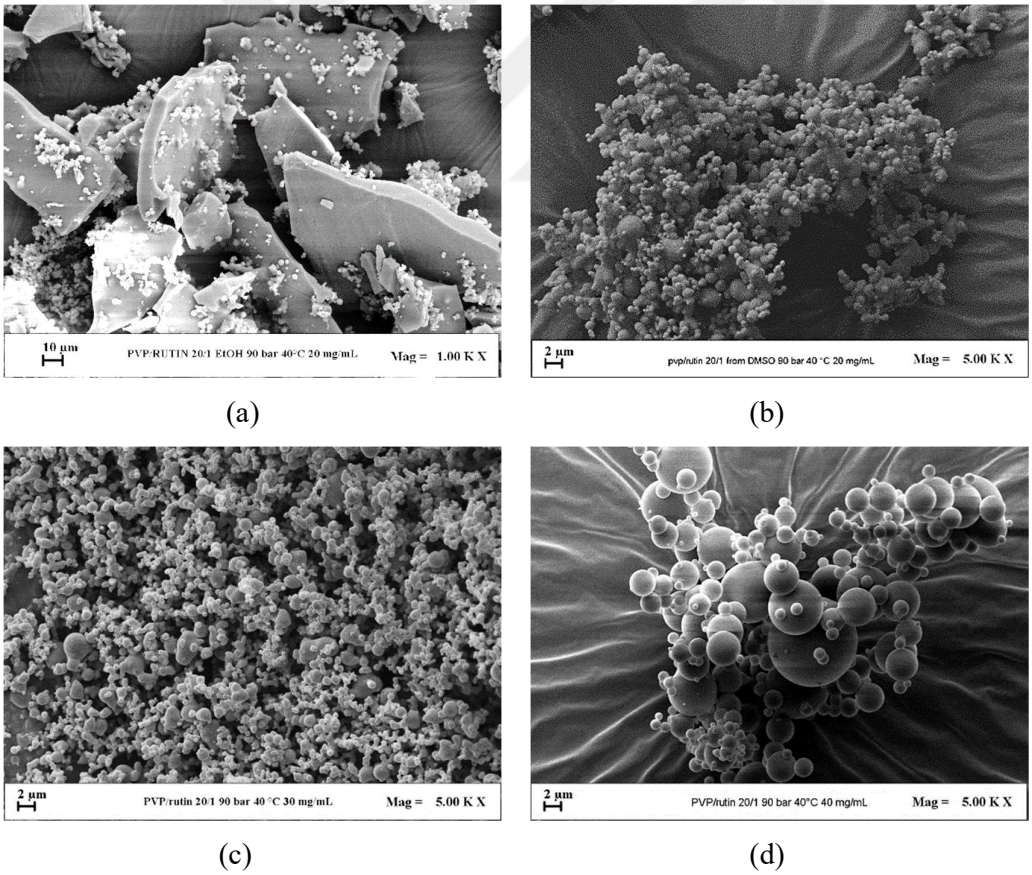
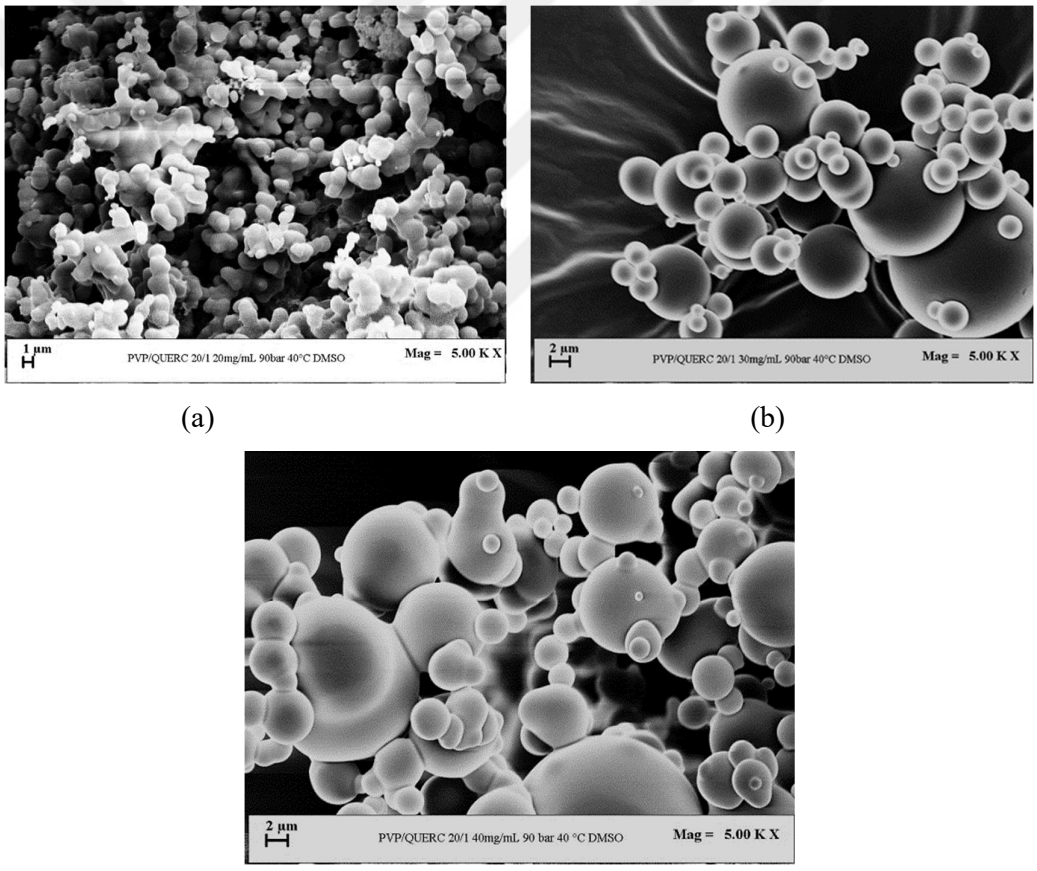


Figure 5.5 : FESEM images of PVP/rutin 20/1 w/w particles precipitated at 9.0 MPa and 40 °C. Effect of organic solvent and of overall concentration: (a) 20 mg/mL$_{EtOH}$; (b) 20 mg/mL$_{DMSO}$; (c) 30 mg/mL$_{DMSO}$; (d) 40 mg/mL$_{DMSO}$.

5.4.3 Effect of overall concentration of solutes

The effect of the total concentration in the liquid solution for the systems PVP/FC 20/1 w/w at 9.0 MPa and 40 °C was investigated, varying this parameter from 20 mg/mL$_{DMSO}$ to 40 mg/mL$_{DMSO}$ (runs # 6–8 and # 14–16).

In the case of PVP/quercetin, coalescing microparticles were precipitated when the total concentration of PVP/quercetin was equal to 20 mg/mL$_{DMSO}$ (Figure 5.6a, run #6), whereas spherical microparticles were obtained increasing the total concentration at 30 (Figure 5.6b, run #7) and 40 (Figure 5.6c, run #8) mg/mL$_{DMSO}$.



(a) (b)

(c)

Figure 5.6: FESEM images of PVP/quercetin 20/1 w/w particles precipitated at 9.0 MPa and 40 °C. Effect of overall concentration: (a) 20 mg/mL$_{DMSO}$; (b) 30 mg/mL$_{DMSO}$; (c) 40 mg/ mL$_{DMSO}$.

Comparing the volumetric cumulative distributions in Figure 5.7a, it is possible to observe that, increasing the total concentration of PVP/quercetin in DMSO, the mean particle dimension increased and the PSD enlarged.

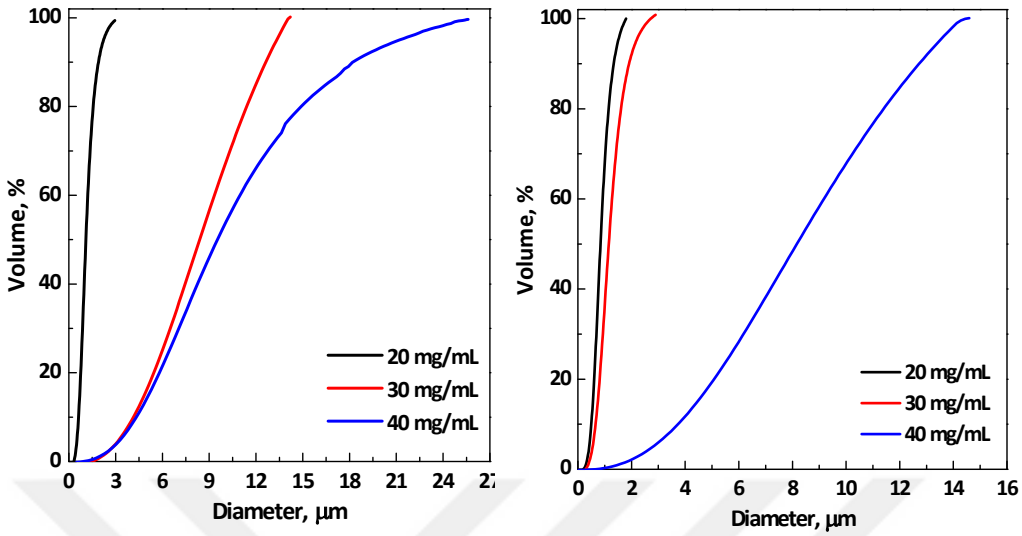


Figure 5.7 : Volumetric cumulative PSDs of PVP/flavonoid particles 20/1 precipitated from DMSO at 90 bar and 40 °C at different overall concentrations; (a) quercetin; (b) rutin.

In the case of the system PVP/rutin, coalescing microparticles (Figure 5.5b) were obtained operating with a total concentration of 20 mg/mL (run #14), whereas slightly coalescing microparticles were formed at 30 mg/mL (run #15), as shown in Figure 5.5c. On the other hand, increasing the total concentration of PVP/rutin at 40 mg/mL (run #16), well-defined spherical microparticles were produced, as shown in Figure 5.5d.

A comparison between the volumetric cumulative PSDs for PVP/rutin powders was shown in Figure 5.7b. Also in this case, increasing the overall concentration of PVP/rutin in DMSO, the particle size increased and the PSD enlarged.

5.4.4 Effect of PVP/flavonoid ratio

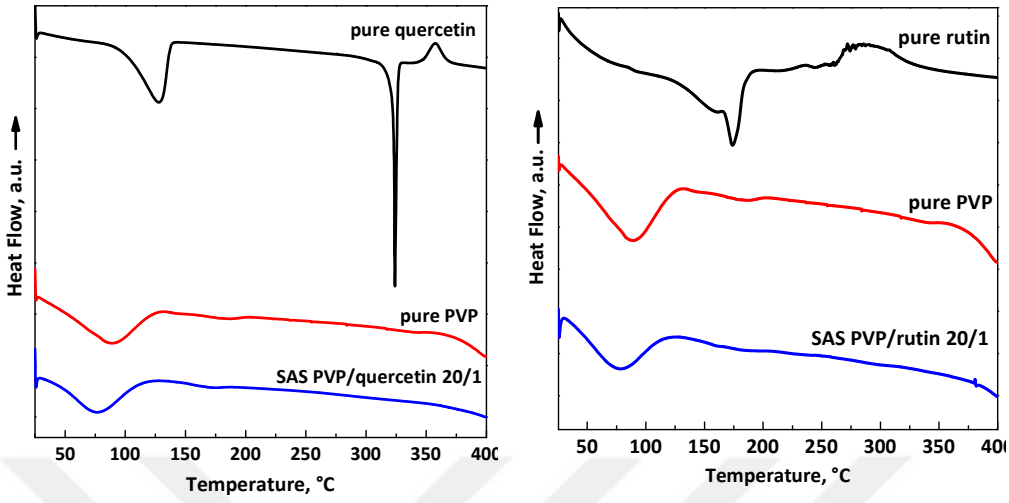
In order to complete the screening on the effect of the operating parameters on morphology, mean particle size and particle size distribution, the PVP/FC ratio was decreased from 20/1 w/w to 10/1 w/w. In the case of the system PVP/quercetin, the total concentration in DMSO was fixed first at 30 mg/mL (run #9) and, later, at 40 mg/mL (run #10). When the total process concentration was fixed at 30 mg/mL, sub-microparticles characterized by a high degree of coalescence were obtained. This prevented the determination of the mean particle size and PSD. At 40 mg/mL,

coalescing sub-microparticles with a decrease in particle dimensions with respect to the powders obtained at PVP/FC ratio equal to 20/1 (run #8) were precipitated. Therefore, in the case of the system PVP/quercetin, the 10/1 w/w ratio is not sufficient for the attainment of well separated coprecipitated particles.

The decrease of the PVP/flavonoid ratio from 20/1 to 10/1 w/w was also investigated in the case of the system PVP/rutin, fixing the total concentration of the solutes in DMSO at 40 mg/mL. Slightly coalescing microparticles were produced with a PVP/rutin ratio equal to 10/1 w/w and a decrease of particle size was observed, by reducing the polymer/flavonoid ratio. Indeed, the mean diameter ± standard deviation varied from 8.17 ± 2.29 μm (in the case of the ratio 20/1 w/w, run #16) to 0.94 ± 0.25 μm (for the test in correspondence of the ratio 10/1 w/w, run #17).

5.5 Characterization

DSC thermograms of unprocessed flavonoids, unprocessed PVP and SAS processed PVP/FC 20/1 w/w were reported in Figure 8a and b for quercetin and rutin, respectively. The DSC trace of unprocessed PVP was characterized by a wide endothermic peak corresponding to dehydration. DSC thermogram of pure quercetin showed two endothermic peaks, related to the loss of water and to the melting point (at 324 °C), respectively. Furthermore, an exothermic peak can be observed for quercetin at 358 °C due to the initial decomposition process (Muthurajan et al., 2015; da Costa et al., 2002). The DSC curve of unprocessed rutin exhibited a peak in correspondence of its phase transition at 174 °C, related to the molecular rearrangement of the rutin (Muthurajan et al., 2015). This first peak clearly represents its melting point (Asfour and Mohsen, 2018); furthermore, the subsequent peaks provide evidence of the phase transition of the flavonoid. SAS composites of PVP/quercetin and PVP/rutin showed an amorphous structure similar to the one of the polymer.



(a) (b)

Figure 5.8 : DSC thermograms of unprocessed PVP and flavonoids, SAS processed PVP/FC composites: (a) quercetin; (b) rutin.

XRD patterns of unprocessed and SAS processed PVP (run #1), unprocessed and SAS processed FC (runs #4, 12), SAS powders PVP/FC precipitated using EtOH (runs #2, 11) and DMSO (runs #7, 15) as the organic solvents were reported in Figure 9a and b for quercetin and rutin, respectively. Both the flavonoids showed a crystalline structure after the supercritical precipitation, confirming the considerations made observing the FESEM images reported in Figure 3b and c, whereas the PVP pattern clearly indicated its amorphous nature. The patterns related to the SAS coprecipitated PVP/FC powders confirmed the achievement of the amorphization both using ethanol and DMSO as the organic solvent.

Dissolution tests were performed using UV–vis spectroscopy on unprocessed FC and SAS processed PVP/FC composites. Release kinetics of each sample in PBS at pH 7.4 was monitored plotting the percentage of dissolved FC as a function of time (Figure 5.10). It should be noted that the working time of the UV–vis spectroscopy used in this work can perform the measurements up to 7999 min (corresponding to about 133.33 h). Dissolution profiles of unprocessed quercetin and SAS processed PVP/quercetin 20/1 (run #8) precipitated from DMSO were reported in Fig. 10a. Unprocessed quercetin achieved 48.58% of release in correspondence of the maximum limit of the instrument (about 133.33 h), whereas the sample PVP/quercetin 20/1 had a very fast dissolution rate and reached 48.58% release in only 13.5 h, which is nearly ten times faster than pure quercetin. From Figure 10a it is clear that quercetin from SAS

coprecipitated powders completely dissolved in about 125 h and its release profile was characterized by an initial burstlike effect (about 20%), due to an amount of quercetin precipitated near/on the microparticles surface.

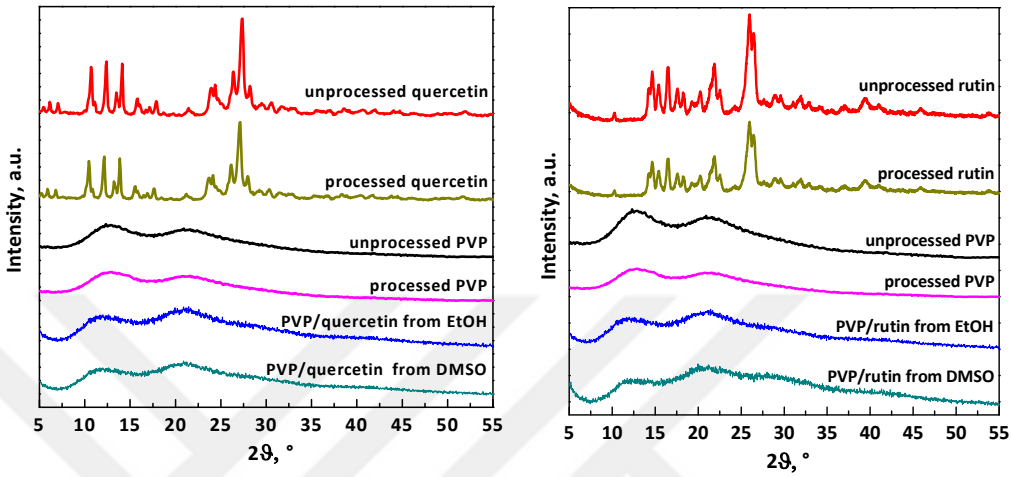


Figure 5.9 : XRD patterns for unprocessed FC and PVP, SAS processed FC and PVP, SAS processed PVP/FC precipitated using EtOH and DMSO as the organic solvents: (a) quercetin; (b) rutin.

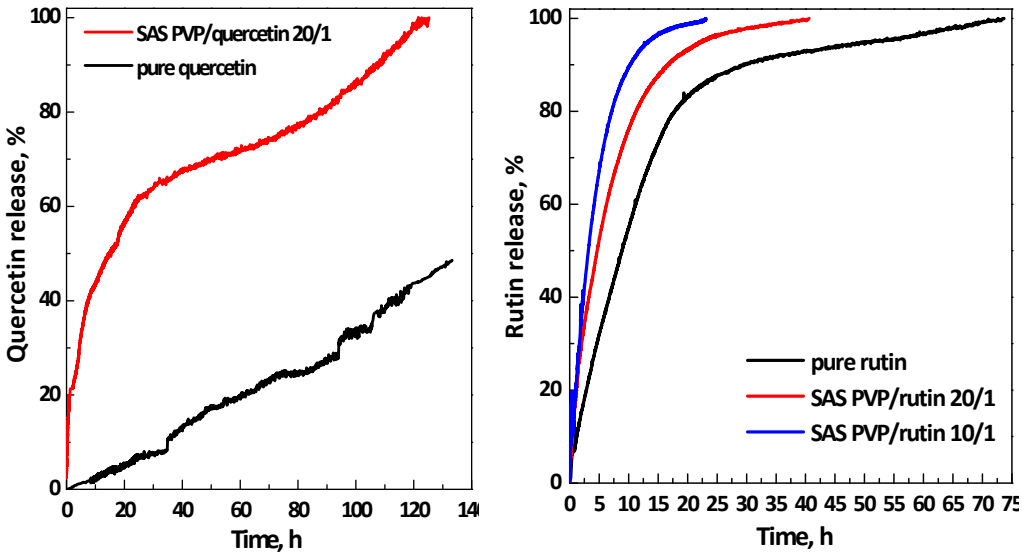


Figure 5.10 : Dissolution profiles in PBS at 37 °C and pH 7.4: (a) quercetin; (b) rutin.

Dissolution profiles of unprocessed rutin and SAS coprecipitated powders PVP/rutin 20/1 and 10/1 precipitated from DMSO were reported in Figure 10b. It can be concluded that unprocessed rutin completely dissolved in about 73.7 h; whereas,

PVP/rutin 20/1 w/w (run #16) and 10/1 w/w (run #17) samples took about 40.6 and 23.1 h, respectively; which means 1.82 and 3.19 times faster dissolution rate in comparison with unprocessed rutin.

Entrapment efficiency (EE%) was determined for all SAS samples using UV/vis analysis at the end of each release test; the results were reported in Table 5.1. For the samples PVP/quercetin 20/1 w/w precipitated from ethanol (run #2 and 3), EE% was in the range of 31.7–34.0 %, which means that a part of the quercetin was extracted during SAS process. On the other hand, for the samples PVP/quercetin 20/1 w/w precipitated from DMSO, the EE% ranged from 92.2 to 99.8 % using an overall concentration of 30 mg/mL (run #7) and 40 mg/mL (run #8), demonstrating a successful coprecipitation of the powders recovered at the end of the experiments. Moreover, it can be possible to observe that EE% decreased with a lower overall concentration (run #6) with coalescing microparticles or a PVP/quercetin ratio of 10/1 w/w (run #9 and #10) due to the partial extraction of the quercetin during SAS experiments and to the fact that, at lower concentration or at lower polymer/flavonoid ratios, the polymer cannot entrap more active compound.

Regarding PVP/rutin samples, the EE% was found to be 33.3 and 34.7 %, respectively in the case of using ethanol (run #11) and DMSO (run #14) as the organic solvent with 20 mg/mL overall concentration. The maximum EE% (from 90.1 to 99.8 %) was obtained at PVP/rutin 20/1 w/w samples precipitated from DMSO using an overall concentration of 30 mg/mL (run #15) and 40 mg/mL (run #16) as well as PVP/rutin 10/1 w/w with an overall concentration of 40 mg/mL (run #17), indicating a successful coprecipitation.

DPPH assay was used to evaluate the antioxidant stability of the original quercetin/rutin, SAS processed PVP/quercetin and PVP/rutin coprecipitated particles and SAS processed PVP particles. The antioxidant capacity results are shown in Table 5.2. As can be seen from the Table, the total antioxidant capacity of quercetin (1613 ± 60.4 mg TE/g DW) is much higher than that of rutin (726.2 ± 3 mg TE/g DW) ($p < 0.05$). Moreover, results indicated the preservation effect of SAS process on the antioxidant capacities of quercetin and rutin ($p > 0.05$). Fraile et al. obtained significantly higher antioxidant capacity of SAS- processed quercetin than unprocessed quercetin and explained this aspect by the dehydration of the compound

during SAS processing (Fraile et al., 2014). However, our results demonstrated that SAS process may not provide improved antioxidant levels but rather a protecting effect. On the other hand, it should be noted that the antioxidant power of the carrier in PVP/quercetin and PVP/rutin coprecipitates may also affect the results and should also be considered during the analysis.

Table 5.2 : Summary of SAS experiments (IP: Changes in the total antioxidant capacity of quercetin/rutin and SAS-processed samples*.

Sample	mg TE/g dry weight
Quercetin	1613 ± 60.4^{a}
SAS processed quercetin (run #7)	1633 ± 50.4^{a}
Rutin	726.2 ± 3^{b}
SAS processed rutin (run #15)	752.6 ± 18.6^{b}
SAS processed PVP	9.9 ± 0.5^{c}

*Different small letters in the columns represent statistically significant differences ($p < 0.05$).

5.6 Discussion

Summarizing the experimental results, different morphologies, such as coalescing particles, microparticles, nanoparticles, sub-microparticles and crystals, were obtained varying the operating conditions.

It is well known in the literature on SAS process that particle formation is explainable considering the interplay among different aspects; i.e., fluid dynamics of the injected solution in contact with $scCO_2$, high-pressure equilibria of the solute/solvent/antisolvent system and mass transfer to and from the injected solution (Reverchon and De Marco, 2011; Lengsfeld et al., 2000; Sarkari et al., 2000).

When the jet break-up occurs and the operating pressure is slightly higher than that of the MCP, spherical microparticles are generally precipitated. Micro-drops are formed during the liquid jet break-up, then the liquid is removed by drying with $scCO_2$. In the case of a quaternary system formed by $scCO_2$, an organic solvent, a polymer and an active compound, the attainment of microparticles makes the coprecipitation of the polymer and the active compound possible; indeed, both the compounds are confined by the micro-drop that behaves like an isolated reactor (Prosapio et al., 2018).

When the process conditions are located far above the MCP of the solvent-antisolvent system (at pressures much higher than MCP pressure), nanoparticles are commonly

obtained. Indeed, the surface tension of the injected liquid disappears before the liquid jet break-up and a gas plume formed (Lengsfeld et al., 2000; Sarkari et al., 2000); particles nucleate and grow from this gas plume. The polymeric carrier and the active compound tend to precipitate separately, considering that the heterogeneous nucleation is obtained very difficultly due to the different nucleation and growth times; as a result, the two compounds precipitate forming a sort of intimate physical mixture (Reverchon and De Marco, 2011).

The study of the effect of process parameters on the obtained morphology and on the average particle size allowed the ideal conditions for the PVP/flavonoid coprecipitation to be identified.

When the starting solutions were prepared by using ethanol as the organic solvent (runs # 2, 3 and 11), some microparticles together with material not well defined in morphology (Figure 4a, b and 5a) were recovered from the precipitator; on the contrary, the precipitation from DMSO always gives rise to spherical particles. This experimental evidence can be ascribed to the different properties of the two organic solvent, in terms of density and surface tension.

The effect of the total concentration (at fixed polymer/FC ratio) and of polymer/FC ratio (at fixed total concentration) can be discussed together. Indeed, at 20/1 w/w ratio, in correspondence of the lower total concentration (20 mg/mL) (runs # 6 and 14), and at 10/1 w/w ratio for all the tested total concentrations (runs # 9, 10 and 17), coalescent particles were obtained. It is also possible to note that the EE% in correspondence of these experiments is, in general, low. It is possible to hypothesize that, in these cases, the amount of PVP is insufficient to incorporate all the flavonoid, which partly precipitated on its own and was extracted by the mixture consisting of DMSO and $scCO_2$.

When, instead, higher total concentrations (30 and 40 mg/mL) in correspondence of a sufficient PVP/FC ratio (20/1 w/w) were chosen, the coprecipitation was successful: microparticles, with a mean diameter that increased and a PSD that enlarged at increased concentrations, and with high entrapment efficiency, were obtained (runs # 7, 8, 15 and 16).

Ultimately, PVP is confirmed as an excelled polymeric carrier for the SAS coprecipitation. Indeed, PVP can hinder the tendency of quercetin and rutin to precipitate in an irregular morphology, because it has the capability of blocking and controlling the morphology of the compound that precipitates in its presence. Similar results were previously observed, for example, by coprecipitating PVP with other vitamins or drugs (Franco et al.,2018; Prosapio et al., 2017).

5.7 Conclusion

In this work, it was demonstrated that it is possible to coprecipitate some poorly water-soluble flavonoid compounds (quercetin and rutin) by using the SAS technique with PVP as the polymeric carrier. The results obtained in terms of morphology and dissolution rate for the tested flavonoids using the SAS technique are very promising.

The effect of the main parameters of the SAS process on the mean size, on the particle size distribution as well as the dissolution rate in water was studied. The operating conditions were chosen in order to obtain well-separated microparticles by means of an accurate selection of the type of solvent, of the operating pressure, of the total concentration of solutes and of the polymer/FC ratio. As indicated by the results, amorphous microparticles using solute concentrations greater than 20 mg/mL and high polymer/flavonoid ratios were obtained. Indeed, microspheres with mean diameters in the range of 8.33–9.52 μm for the system PVP/quercetin and 1.15–8.17 μm for the system PVP/rutin were obtained under optimized conditions. Furthermore, release studies confirmed an improvement of the flavonoids dissolution rates: 10 and 3.19 times faster dissolution rate than unprocessed quercetin and rutin, respectively were obtained. Besides, the high entrapment efficiencies, up to 99.8%, were achieved for quercetin and rutin coprecipitated particles using DMSO, which was the solvent chosen to coprecipitate the flavonoid compounds with PVP by the SAS process.

Consequently, the characteristics of the powders and the high entrapment efficiencies of PVP/quercetin and PVP/rutin systems obtained by SAS could allow the production of supplements with antioxidant and anticancer benefits for pharmaceutical and nutraceutical applications, in which the flavonoid compounds have high stability and bioavailability.

6. COMBINING *IN VITRO* DIGESTION/CACO-2 CELL CULTURE MODEL: EVALUATION OF THE SUPERCRITICAL ANTISOLVENT PROCESS AND FOOD MODELS ON ANTIOXIDANT EFFECTS, BIOACCESSIBILITY AND TRANSPORT DYNAMICS OF FLAVONOL-LOADED MICROPARTICLES[5]

6.1 Abstract

In the present study, the effects of Supercritical Anti-Solvent (SAS) process and food models on the antioxidant capacity, bioaccessibility and transport dynamics of flavonol-loaded microparticles were investigated using a combined *in vitro* gastrointestinal digestion/Caco-2 cell culture model. SAS-processed and unprocessed bioactives were supplied in two different food models: 10 % ethanol for aqueous hydrophilic food simulant and 3 % acetic acid for acidic food simulant. The SAS processing of quercetin and rutin resulted in a much higher recovery of those bioactives as well as a greater retention of antoxidant capacity after gastrointestinal digestion in both hydrophilic and acidic food models. The present study also demonstrates that SAS coprecipitation has a positive effect on the stability and the transport of the bioactives across the epithelial cell layer.

Key words: quercetin, rutin, microparticles, *in vitro* digestion, Caco2 cell model, bioavailability, supercritical antisolvent.

6.2 Introduction

Flavonoids are polyphenolic compounds, which are present in a wide variety of foods such as fruits, vegetables, flowers and leaf of plants (Yao et al., 2004). Quercetin and its glycoside form, namely rutin, have been reported to exhibit a wide range of biological activities, including anticancer, antioxidant, anti-inflammatory and antiviral activities, as well as prevention of cardiovascular, pancreas and liver diseases (Huang

[5] This chapter is based on the paper "Ozkan, G., Franco, P., De Marco, I., Capanoglu, E., and Esatbeyoglu, T. Combining in vitro digestion/Caco-2 cell culture model: Evaluation of the supercritical antisolvent process and food models on antioxidative effects, bioaccessibility and transport dynamics of flavonol-loaded microparticles, Submitted."

et al., 2017; Rashad et al., 2015; Russo et al., 2012). However, those flavonoids undergo several chemical changes during food processing and storage. Besides, there are also other drawbacks to consider before the incorporation of quercetin and rutin into functional foods, cosmetic and pharmaceutical industries; e.g., their crystalline structure at both ambient and body temperatures, poor water solubility, chemical instability in gastrointestinal tract and, thus, a reduced bioavailability (Record & Lane, 2001; Wang et al., 2016).

Therefore, a wide variety of delivery systems have been investigated to improve the solubility, stability, bioaccessibility and bioavailability of phenolic compounds, including lipid-based formulations (i.e., emulsion/nanoemulsion, solid lipid nanoparticles, nanostructured lipid carriers, liposomes, etc.) (Ozkan et al., 2020) as well as techniques assisted by supercritical fluids (e.g., RESS, rapid expansion from supercritical solutions; SAS, supercritical antisolvent process; SAA, supercritical assisted atomization process, etc.) (Peng et al., 2019).

Considering the above, the purpose of this study was to determine whether quercetin and/or rutin could be successfully protected and released by polyvinylpyrrolidone (PVP) based microparticles obtained by SAS process. Specifically, we have investigated (i) the retention and the antioxidant capacity of pure or SAS-processed quercetin and rutin; (ii) the influence of SAS processing on the apical and basolateral recovery as well as the transport efficiency of those bioactives; (iii) the effects of acidic and hydrophilic food conditions on the stability and bioavailability of the bioactives of interest, using *in vitro* simulated gastrointestinal digestion, coupled with the human intestinal Caco-2 epithelial cell system as a model. To the best of our knowledge, this is the first *in vitro* study with stimulated human intestinal epithelial cells performed on the flavonols (quercetin and its glycosylated form rutin) embedded into PVP-based matrix by SAS coprecipitation.

6.3 Materials and Methods

6.3.1 Materials

Quercetin-polyvinylpyrrolidone (Q-PVP) and rutin-polyvinylpyrrolidone (R-PVP) coprecipitates were fabricated by SAS process at the University of Salerno. The Q-

PVP and R-PVP microparticles employed in this study were produced using the optimum process conditions described by Ozkan et al. (2019), which were as follows: 90 bar, 40 °C, 40 mg/mL as overall concentration of solutes (Q/R and PVP) in dimethyl sulfoxide (DMSO), flavonol/PVP weight ratio equal to 1/20 for Q/PVP and 1/10 for R/PVP.

Polyvinylpyrrolidone (PVP, average molecular weight 10,000 g/mol), quercetin dihydrate (purity 98%), rutin hydrate (purity 95%), pepsin (EC 3.4.23.1, from porcine gastric mucosa), pancreatin (8x USP, EC 232.468.9, from porcine pancreas, contains trypsin, amylase and lipase) and bile salt were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Caco-2 human colon adenocarcinoma cell line was obtained from German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin, non-essential amino acids (MEM NEAA 100x), trypsin/EDTA (10x) were purchased from Pan Biotech (Aidenbach, Germany) and trypan blue was obtained from Carl Roth (Karlsruhe, Germany). Ultrapure water was used (Purelab flex 3; Veolia Water Technologies, Celle/Germany). All other reagents were of analytical or HPLC grade.

6.3.2 Food models

The *in vitro* digestion experiments were carried out for selected unprocessed flavonols or flavonol-loaded microparticles in two different media, 3% acetic acid and 10% ethanol, which are considered as food simulants for acidic and hydrophilic food products, respectively, according to the Commission Regulation 10/2011 EU (10/2011/EC) (Atay et al., 2018).

6.3.3 *In vitro* simulated gastrointestinal digestion

The *in vitro* digestion procedure was adapted from Sessa et al. (2011) and Ozkan et al. (2021) with some modifications. Due to the daily intake of quercetin is in the range of 0 -30 mg (Böhm et al., 1998; Knekt et al., 2002), 5 mg active compound was selected as initial dose. Briefly, 5 mg unprocessed quercetin/rutin or 5 mg quercetin/rutin equivalent microparticles (105 mg for Q/PVP and 55 mg for R/PVP) were dissolved in 5 mL media (acidic or hydrophilic), individually. A sample of 5 mL from each was placed in a test tube and incubated in a water bath at 37 °C for 15 min. To simulate

gastric digestion, the pH was adjusted to 2 using 1 M HCl and porcine pepsin was then added to a final concentration of 1.3 mg/mL. The samples were incubated at 37 ℃ in a shaking water bath (GFL 1092, Burgwedel, Germany) at 100 rpm for 2 h. To simulate intestinal digestion, the pH of the gastric chyme was raised to pH 5.8 with 1 M $NaHCO_3$ dropwise, and 2.5 mL of pancreatin and bile salts mixture were added to final concentrations of 0.175 and 1.1 mg/mL, respectively. Afterwards, the pH was adjusted to pH 6.5 with 1 M $NaHCO_3$, and samples were incubated at 37 ℃ in a shaking water bath at 100 rpm for 2 h. A blank without bioactive compound was incubated under the same conditions for correcting the interferences from the digestive enzymes and buffers. After gastrointestinal digestion, the samples were cooled by immersing in an ice bath and then centrifuged at 10,000 rpm for 30 min at 4 ℃ (Megafuge 8R; Thermo Scientific, Darmstadt, Germany) to separate the soluble or bioaccessible fraction and the residual fraction. Bioaccessible fractions of the digests were kept at -80 ℃ until further analysis. All experiments were done in triplicates.

6.3.4 Antioxidant capacity during *in vitro* digestion

All spectrophotometric assays were performed using an Infinite M200 UV–visible spectrophotometer (Tecan, Crailsheim, Germany). The antioxidant capacity was estimated by using the cupric ion reducing antioxidant capacity (CUPRAC) (Apak et al., 2004) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Molyneux, 2004) assays. In both assays, 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was used as a standard. All samples (quercetin or rutin in free or microparticle form) were measured in triplicates and results were expressed as mmol Trolox equivalents (TE) per g of sample.

6.3.5 *In vitro* bioaccessibility calculations

The bioaccessibility of quercetin or rutin was defined as the amount of bioactive released into the mixed micelles formed by bile salts after the intestinal phase (Chen et al., 2020). The bioaccessibility was calculated using equation (6.1) and expressed as percentage.

$$\text{Bioaccessibility (\%)} = (\text{BCdigested}/\text{BCnon} - \text{digested}) * 100 \qquad (6.1)$$

where $BC_{digested}$ was the amount of bioactive compounds (quercetin or rutin) recovered in the supernatants of the centrifuged final digesta (BF; bioaccessible fraction) and $BC_{initial}$ was the initial amount of bioactive compounds in the stock solution.

6.3.6 Cell culture

Caco-2 cells were cultured in Dulbecco's modified eagle's medium including 4.5 g/L glucose and stable glutamine supplemented with 20% fetal bovine serum, 1% non-essential amino acids, 100 IU/mL penicillin and 100 µg/mL streptomycin. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. Cells were subcultured at 70–80% confluence using trypsinization. For the transport experiment, Caco-2 cells were seeded in inserts of 6-well transwell plates (0.4 µm pore diameter, 24mm insert, Sarstedt, Nümbrecht, Germany) at a density of $4*10^4$ cell/well, with 2 mL of medium in the apical side and 2.5 mL of medium in the basolateral side. Cells were allowed to grow and differentiate to confluent monolayers for 21 days post-seeding. Cell monolayer integrity during differentiation of Caco-2 cells was monitored by measuring the transepithelial electrical resistance, TEER value, using a Millicell© ERS-2 Volt-Ohm Meter (Millipore, Bedford, MA, USA). Only differentiated enterocytes displaying unchanged transepithelial electrical resistance values were used in the experiment. Medium was changed three times per week. Passage numbers of the cells used in this study were less than 30.

6.3.7 Cytotoxicity test

Caco-2 cells were seeded in 96 well plates, allowed to differentiate for 14 days after confluency and treated with digests at a dilution of 1/2, 1/5 and 1/10 (v/v) in HBSS. After 4 h of treatment, the cytotoxic effect of samples on Caco-2 cells was evaluated using the sulforhodamine B (SRB) assay as described in Longo-Sorbello et al. (2006). For the SRB assay, differentiated cells were first fixed by the addition of 50 µL of 50 % TCA (trichloroacetic acid in Milli-Q water) and kept at 4 °C for 45 min. The plates were rinsed with tap water and air-dried. Afterward, the cells were stained with 70 µL SRB solution (0.4% in 1% glacial acetic acid) and left for 15 min. The plates were then rinsed with 1% glacial acetic acid in Milli-Q water, air-dried and the stain was re-suspended in 200 µL of 10 mM Tris-buffer. Thereafter, the absorbance was determined at 490 nm, subtracting the background measurement at 620 nm.

6.3.8 Transport experiment in CaCo-2 cells

Transport experiment was carried out according to Wu et al. (2017). Briefly, the medium in the transwells was changed to HBSS and preincubated for 1 h and then removed. BF used for the Caco-2 experiments was diluted 1:5 (v:v) with HBSS, since cell viability after 4 h of exposure to the samples in such conditions, assessed by SRB assay, was higher than 85–90%. Incubation was performed at 37 ℃ with 5% CO_2 for 4 h. During incubation, 200 µL samples were collected from both the apical and basolateral compartments every 2 h, after which 200 µL of HBSS was added to adjust the volume of the media. TEER value of the cells was detected before treatment and immediately after the last sampling to ensure the integrity of monolayer. Then the HBSS was replaced by growth medium, and the monolayers were incubated for another 20 h to measure irreversible damage to the cell monolayer. Therefore, after 24 h, monolayer integrity was tested using TEER measurement. Samples were stored at -80 ℃ until further analysis.

6.3.9 Identification and quantification of flavonoids by UPLC

The method for determination of flavonoids by UPLC was adapted from Ozkan et al. (2021) with minor modifications. Briefly, samples (unprocessed flavonols and microparticles) were passed through 0.20 µm membrane filters and injected into a UPLC (Agilent 1290 Infinity II, Germany). The UPLC system is equipped with a binary pump with vacuum degasser, autosampler, photodiode array detector and a column oven. A Phenomenex Luna C18(2) analytical column (250*4.6 mm, 5 µm) with a C18 guard column (Phenomenex, Torrance, CA) was used for the separation. A mobile phase consisting of TFA/MQ water (trifluoroacetic acid in Milli-Q water) (1 mL/L; eluent A) and TFA/acetonitrile (1 mL/L; eluent B) was used. The linear gradient was as follows: 0 min, 5% B; 0-6.48 min, 35% B; 6.48-6.77 min, 100% B; 6.77-8.00 min, 100% B; 8.00-8.10 min, 5% B. The injection volume was 10 µL, the flow rate was 0.6 mL/min and spectral measurements were at 369 and 257 nm for quercetin and rutin, respectively. For calibration curves, chromatographic peak area of the standards versus nominal concentrations was plotted. All samples were measured in triplicates and results were expressed as milligrams per g sample.

6.3.10 Statistical analysis

Experiments were conducted at least in triplicate. Results were reported as mean ± standard deviation. Error bars on figures represented standard deviations. Statistical analysis was applied using SPSS software (version 20.0, SPSS Inc. Chicago, IL, USA). Treatments were compared using one-way analysis of variance (ANOVA) followed by a Tukey post hoc test ($p < 0.05$).

6.4 Results and Discussion

6.4.1 *In vitro* bioaccessibility of bioactives

The use of polyphenols is currently limited due to its relatively low water solubility, chemical instability in the GI tract, and thus, reduced oral bioavailability (2–20% absorption) (Chen et al., 2016; Hu, 2007; Zou et al., 2014). Therefore, it is essential to investigate the effects of digestion on their stability to reveal the mechanism of absorption and metabolism. It was assumed that the bioaccessible fraction of a compound incorporated within the micelles would be available for adsorption by epithelial cells in the small intestine (Pool et al., 2013). For these reasons, effects of precipitation process, physical state of the quercetin/rutin and different food models on the bioaccessibility were studied. Bioaccessibility was quantified by measuring the amount of quercetin/rutin within the micelle phase collected at the end of the simulated small intestine phase.

Quercetin had a low bioaccessibility value of 0.21±0.09 % when simply suspended in hydrophilic food simulant, which can be related to its low solubility and crystalline state in water (Figure 6.1). Moreover, there was no triacylglycerol present that could be digested and form mixed micelles to solubilize the hydrophobic component. According to the literature, quercetin was chemically unstable because of hydroxyl groups and the unstable pyrone structure (Lv et al., 2017). The degradation of quercetin was found to be sensitive to medium pH, especially at alkaline pH values (pH > 7). Increasing the medium pH value from 6.0 to 6.8 or 7.5 increased the k values by 2- and 12-fold, respectively (Wang & Zhao, 2016). On the other hand, the bioaccessibility of quercetin in Q-PVP microparticles was found to be significantly higher (19.8±2.02 %) than that of unprocessed one ($p < 0.05$). These results suggest that the use of PVP

could achieve an amorphization and increased water solubility; thus enhanced bioaccessibility. Concerning increasing quercetin bioaccessibility, different attempts have been conducted. Aditya et al. (2014) obtained 60% bioaccessibility of quercetin within nanostructured lipid carriers and lipid nanoemulsions, and 35% with solid lipid nanoparticles, which were much higher than free quercetin solution (~7 %). Similarly, the quercetin bioaccessibility was increased from <5 % in bulk water to 53 % within the nanoemulsion (Pool et al., 2013). Ni et al. (2015) reported a bioaccessibility of 33.6% for quercetin in nanostructured lipid carriers, which was higher than that obtained in water (<2 %). Additionally, Eudragit, an anionic copolymer based on methacrylic acid and ethyl acrylate, based delivery system was also used to increase the stability and solubility of quercetin in the GI tract. After the simulated GI digestion, quercetin release was enhanced from 7% (for free quercetin dispersed in water) to 22% for quercetin loaded polymeric nanoparticles (Pool et al., 2012). Besides, Chen et al. (2020) reported the bioaccessibility of quercetin as 12.7% by incorporating it into the rice bran protein-stabilized nanoemulsions, whereas the bioaccessibility of free quercetin was found to be 1.40%.

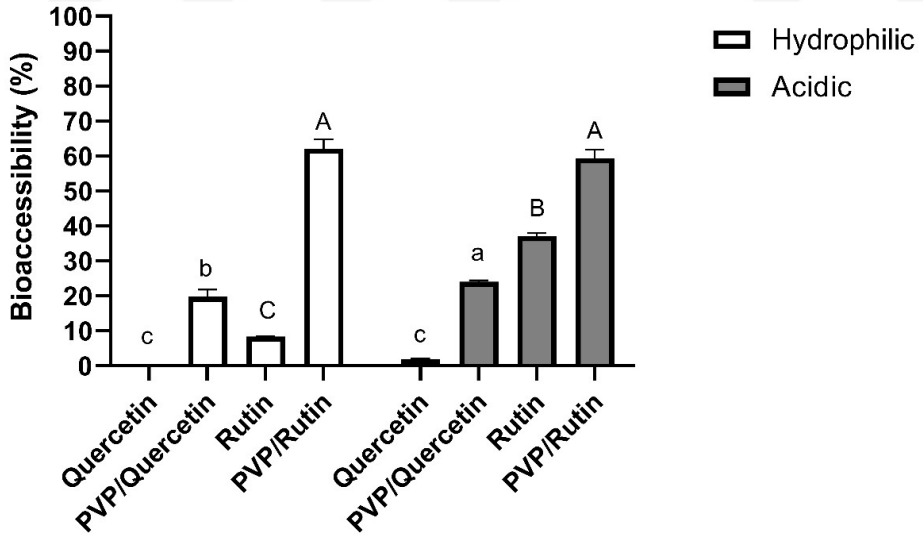


Figure 6.1 : The bioaccessibility of unprocessed and SAS processed quercetin or rutin using PVP in two different food stimulants.

As for the acidic food model, while a lower bioaccessibility value (1.91±0.13%) was obtained with unprocessed quercetin, there was a significant improvement ($p < 0.05$) in quercetin within Q-PVP microparticles (24.03±0.44 %). Strong acidic conditions may result in the degradation of quercetin to phenolic acids (e.g., protocatechuic acid) and losing the skeleton structure of quercetin (Wang et al., 2016). It can be deduced

from the results that the PVP matrix could protect the bioactive compound in acidic conditions which was selected as the worst-case scenario in which the release was expected to be faster due to the quick swelling or even dissolution of the polymeric matrices in acidic conditions (Atay et al., 2018).

The bioaccessibility of rutin in hydrophilic food model was obtained as 8.38±0.10 %. It has been reported that phenolics are highly sensitive to alkaline conditions and may degrade during gastrointestinal digestion (Bermúdez-Soto et al., 2007). On the other hand, the recovery of rutin was statistically enhanced ($p < 0.05$) to 62.1±2.66% when it was coprecipitated with PVP. These results are in agreement with Bermúdez-Soto et al. (2007) who showed that rutin exhibited a high bioaccessibility (3% loss) after pancreatic digestion. In line with the results of the rutin bioaccessibility in acidic food model, recovery of unprocessed rutin was increased from 37.1±0.84 and 59.4±2.45%. Results indicated a protective effect of PVP matrix for rutin in both hydrophilic and acidic conditions during *in vitro* digestion due to its pH-stable properties (Franco & De Marco, 2020). It can also be deduced from the results that the stability of rutin in both free and microparticle form was found to be higher than that of quercetin ones. Indeed, previous studies have noticed a stronger stability of rutin rather than quercetin in hydrophilic conditions, indicating stabilization effect of glycoside linkage present in rutin (Dechene, 1951).

6.4.2 Changes in the *in vitro* antioxidant capacity during digestion

Changes in antioxidant capacity of the samples in different food models, measured by both CUPRAC and DPPH assays, during the *in vitro* GI digestion are shown in Table 6.1.

From the results, bioactive-free microparticles (PVP matrix) showed almost no antioxidant capacity (data not shown) in all conditions. In line with the antioxidant capacity results obtained for unprocessed bioactives after *in vitro* digestion, values of CUPRAC antioxidant capacity were found to be quite lower than that of PVP microparticles in all food models due to crystalline structure of these compounds under these conditions. Therefore, findings indicated preservative and supportive effects of SAS process on the antioxidant capacities of bioactives. In CUPRAC assay, Cu(II) is reduced to Cu(I) through the action of electron-donating (ET) antioxidants (Apak et

al., 2004). It could be stated that the use of PVP as a carrier has favored the electron-donating ability of the bioactive loaded PVP-based microparticles (higher reducing power) when compared with unprocessed ones. Samborska et al. (2019) indicated that CUPRAC antioxidant activities of spray-dried honey powders were increased by the use of maltodextrin as a carrier. CUPRAC method has superiority over other ET−based assays in terms of working pH (Apak et al., 2007). Thus, it could be deduced from the results that the decrease in CUPRAC values in hydrophilic and acidic food simulants may arise from the reduced stability of bioactives in those conditions.

Table 6.1 : Changes in the antioxidant activities of the samples during GI digestion*.

Sample	Before digestion	Hydrophilic		Acidic	
		After digestion	Increase (fold)	After digestion	Increase (fold)
Cupric ion reducing antioxidant capacity (CUPRAC)					
Quercetin					
Q	17.2±1.64^{a}	0.10±0.02^{c}	---	4.63±0.63^{b}	0.27
Q-PVP	321±8.87^{a}	205±16.7^{b}	0.64	206±23.6^{b}	0.64
Rutin					
R	11.3±1.32^{a}	0.66±0.12^{c}	---	4.32±0.28^{b}	0.38
R-PVP	147±2.75^{a}	61.8±3.61^{b}	0.42	59.3±6.61^{b}	0.40
2,2-Diphenyl-1-picrylhydrazyl (DPPH)					
Quercetin					
Q	6.28±0.07^{a}	0.1±0.00^{c}	---	0.76±0.32^{b}	0.12
Q-PVP	6.40±0.27^{c}	58.6±5.20^{a}	9.16	37.1±4.78^{b}	5.80
Rutin					
R	2.41±0.16^{a}	0.60±0.05^{c}	0.25	1.10±0.31^{b}	0.46
R-PVP	2.59±0.26^{c}	18.1±0.56^{a}	6.99	12.3±1.97^{b}	4.75

*The data presented in this table consist of average values ± standard deviation of three independent batches. Different small letters in the same rows represent statistically significant differences ($p < 0.05$) between quercetin and rutin samples, individually. Total antioxidant capacity is expressed as mmol trolox equilavent/g sample.

Similarly, DPPH radical scavenging capacity of bioactives within PVP matrix were found to be much higher than that of unprocessed ones. In addition to this, antioxidant activities of unprocessed quercetin and rutin decreased after digestion ($p < 0.05$), whereas it was increased for SAS processed Q-PVP and R-PVP microparticles in all food models ($p < 0.05$). DPPH is a mixed mode assay which attributed to the scavenging of a radical by antioxidants with both hydrogen atom transfer and electron transfer based mechanisms (Capanoglu et al., 2017). It is well known that the radical scavenging capacity of polyphenols is strongly pH-dependent. Higher pH values lead to enhancement in this capacity. The increase in the radical scavenging capacity based

on the deprotonation of the hydroxyl moieties present on the aromatic rings of the phenolic compounds (Mukai et al., 1997; Tyrakowska et al., 1999). The transition from the stomach to the intestinal environment may induce structural changes in the phenolic molecules by ionization of the hydroxyl groups (Tagliazucchi et al., 2010). Similar results were also obtained by Tagliazucchi et al. (2010). It was found that radical scavenging activity of gallic acid, caffeic acid, catechin, quercetin and resveratrol were ascended after pancreatic digestion.

Regarding the effect of food models on the antioxidant capacity of the samples after *in vitro* digestion, there was a significant change ($p < 0.05$) in the DPPH antiradical scavenging abilities of the samples. The results present that statistically higher antioxidant capacity of bioactives in PVP matrix is obtained in hydrophilic media. Similar to the results obtained in the present work, Pekal & Pyrzynska (2015) found that there was a significant variation in DPPH inhibition of tea infusions in the measuring system in buffered (methanol with acetate buffer at pH 5.5) and non-buffered reagent (methanol). Indeed, higher antioxidant capacity of tea infusions was determined in less acidic media.

6.4.3 Determination of the cytotoxicity in Caco-2 cells

Before transport experiments, it is necessary to determine the maximum non-toxic sample concentration by a cytotoxicity test. Thus, SRB assay has been subjected to determine the cellular viability based on cellular protein content. Cells were allowed to differentiate for 14 days after confluency. Then, Caco-2 cells were treated with bioactive free and bioactive containing digests at different dilution ratios. 1/5 dilution ratios of the digests with Q, Q/PVP, R or R/PVP did not result in a major (i.e. biologically relevant) cytotoxic effect (cell viability was never < 85%), indicating no cell release from the monolayer. Therefore, dilution of 1/5 was used in further Caco-2 transport experiments.

6.4.4 Transport experiments

The TEER values before and after 4 and 24 h of treatment for all tested digests have been monitored. Before treatment, TEER values of all conditions were found to be higher than 300 Ωcm^2. TEER values did not change more than 10% of the original values (not shown) after 4 and 24 h, which are considerably above the recommended

minimum level (200 Ωcm^2) needed for monolayer integrity (Palm et al., 1996). Therefore, we conclude that the monolayer integrity was not irreversibly damaged during transport experiments in the presence of a 1/5 diluted digestive matrix and paracellular transport was not affected, which could influence bioavailability calculations.

One of the aims of this study was to determine the effect of SAS process and different food models on the transport dynamics of quercetin and rutin, in the form of free or bioactive-loaded PVP microparticles, in the human digestive system using a recognized cell-based assay. Although there have been a series of studies conducted related with the absorption of bioactive compounds, the effects of food models with different pH and SAS process on uptake of phenolic compounds are largely unknown. In the present research, the combined *in vitro* digestion/Caco-2 cell culture system was used to screen the transport of bioactives – quercetin and rutin – across the gut epithelium.

The digests of quercetin and rutin in the form of free or bioactive-loaded PVP microparticles from different food simulants were added to the apical (top) side of the Caco-2 cells growing in transwells. After, samples were collected from the apical compartment after 0, 2 and 4 h and from the basolateral (bottom) compartment after 2 and 4 h of incubation. At the end of 2 and 4 h exposure, recoveries on the apical and basolateral sides were quantified for bioactives, as a percentage of the amount of bioactives loaded to the apical side at 0 h (Table 6.2).

According to the results, free quercetin from hydrophilic or acidic food simulants could not be detected at the apical or basolateral sides. After *in vitro* gastrointestinal digestion, quite lower amount of unprocessed quercetin was detected in the both hydrophilic and acidic food models as described above. Before the cell culture study, digests were diluted with HBSS at a pH of 7.8. Due to the fact that intact quercetin in the digests may be degraded at this pH. Considering the recoveries of quercetin within PVP matrix in the apical compartment after 2 h of exposure were found be 66 and 81 % for hydrophilic and acidic food simulants, respectively. Furthermore, after 4 h of treatment with the samples, apical recovery of quercetin was decreased to 61 and 65% for hydrophilic and acidic food simulants, respectively. While the basolateral side recovery of intact quercetin was found to be 0.98 and 1.38% after 2 h of treatment,

after 4 h, the amount of compounds transported to the basolateral compartment increased to 1.93 and 3.89 %, for hydrophilic and acidic food models, respectively. The basolateral side recovery (indicating transport) was corrected for the apical side recovery (indicating stability), defining as transport efficiency (Toydemir et al., 2013). The transport efficiency of quercetin in the acidic food model was greater than that of from hydrophilic food model, indicating higher retention. What has become evident from this research is that the basolateral recovery and transportation of quercetin is altered in a positive manner with SAS processing. While quercetin in free form could not be detected in the basolateral compartment after 2 or 4 h exposure, relatively higher transportation could be achieved with quercetin in the form of PVP-based microparticles.

As for transportation of rutin in both free or microparticle forms, while apical recoveries (t = 4 h) in hydrophilic food simulants were statistically higher than that of acidic food simulant, basolateral recoveries (t= 4 h) in acidic food simulant were statictically higher than hydrophilic ones. The major outcome of this work was that transport of the rutin was enhanced by SAS processing. In detail, after 4 h exposure, transport efficiency (t= 4 h) was found to be 1.2 and 1.5 times higher for the rutin within PVP matrix from hydrophilic and acidic food models, respectively, compared to the unprocessed ones.

The reason for greater retention of bioactive compounds within PVP matrix could be explained by the physical state of the bioactives. Amorphous structure for both bioactives have been achieved by SAS coprecipitation (Ozkan et al., 2019) and, thereby, enhanced water solubility resulted in an increased bioavailability.

The transepithelial transport of quercetin and rutin have been studied before using Caco-2 cells. In the small intestine, most flavonoid glycosides –like rutin – degraded to their corresponding aglycone by deglycosylation using luminal lactase phloridzin hydrolase (LPH) or β-glucosidase (Németh et al., 2003). Thereafter, flavonoid aglycones, such as quercetin, enter enterocytes and transepithelial flux originate from passive diffusion followed by various conjugation reactions including sulphation and glucuronidation (Erdman et al., 2000). Moreover, methylation was also obtained as the main metabolism of quercetin in buckwheat samples after intestinal transport through Caco-2/HepG2 coculture models (Yao et al., 2020). Similar to the results obtained

here, it was reported that 0.3–6.4% of the quercetin and its conjugates have been detected after the consumption of quercetin-rich foods such as apples, onions, buchwheat and tomato products (Kahle et al., 2011; Manach et al., 2005). The results of this study were in line with *in vivo* studies conducted with volunteers, in which the fate of the flavonols in lightly fried onions has been investigated (Mullen et al., 2006). According to the results, about 4% recovery of the ingested flavonol glucosides as metabolites were present in plasma and urine samples. Besides, rutin metabolites in urine collected from healthy human subjects were studied after the consumption of tomato juice fortified with rutin (quercetin-3-*O*-rutinoside). It was reported that excretion of the metabolites ranged from 0.02% to 2.8% of intake (Jaganath et al., 2006).

6.5 Conclusion

The aim of this study was to investigate the effect of SAS processing on the absorption of flavonoids, namely quercetin and rutin, in the human digestive system using a recognized combined gastrointestinal digestion/cell-based assay. Moreover, aqueous hydrophilic and acidic conditions were simulated to analyze food-related factors that could have an impact on the transport of these compounds across the gut epithelium. SAS processing enhanced the recovery of the quercetin (94 and 13 times in hydrophilic and acidic conditions, respectively) and rutin (7 and 2 times in hydrophilic and acidic conditions, respectively) after *in vitro* digestion. Besides, transepithelial transportation of these flavonoids were also affected positively. Thereby, it can be deduced from the results that SAS processing can be a useful tool in the pharmaceutical and nutraceutical applications with high stability, bioaccessibility, bioavailability and thus enhanced nutritional value. On the other hand, current studies could be enriched with colonic fermentation in order to examine the fate of bioactives in an entire approach. Because, majority of the phenolic substances reach the colon and are consecutively metabolized by colonic microbiota. Furthermore, owing to the significant effects on the transport of the bioactive compounds across the gut epithelium, food-related factors like matrix effect could be also included in future studies.

Table 6.2 : Apical and basolateral side recovery and transport efficiency of bioactives in free and microparticle forms.

Food model[A]	t= 2h			t= 4h		
	Apical side recovery (%)[B]	Basolateral side recovery (%)[C]	Transport efficiency[D]	Apical side recovery (%)[B]	Basolateral side recovery (%)[C]	Transport efficiency[D]
			Quercetin			
Hydrophilic	n.d.	n.d.	–	n.d.	n.d.	–
Acidic	n.d.	n.d.	–	n.d.	n.d.	–
			Quercetin loaded PVP based microparticles			
Hydrophilic	66±7.69^{b}	0.98±0.03^{c}	0.015±0.004^{c}	61±9.96^{b}	1.93±0.71^{b}	0.032±0.008^{b}
Acidic	81±5.71^{a}	1.38±0.36^{bc}	0.017±0.005^{c}	65±2.63^{b}	3.89±0.38^{a}	0.060±0.006^{a}
			Rutin			
Hydrophilic	73.2±3.21^{c}	1.17±0.00^{c}	0.010±0.000^{c}	69.0±2.944^{c}	3.52±0.77^{b}	0.011±0.001^{c}
Acidic	88.4±4.10^{ab}	0.72±0.01^{c}	0.013±0.000^{c}	85±5.00^{b}	0.74±0.77^{c}	0.016±0.000^{c}
			Rutin loaded PVP based microparticles			
Hydrophilic	87.8±2.91^{ab}	1.42±0.03^{c}	0.014±0.000^{c}	82.5±3.49^{b}	4.65±0.67^{a}	0.048±0.006^{b}
Acidic	89±4.56^{a}	1.21±0.17^{c}	0.017±0.002^{c}	87±2.26^{ab}	1.35±0.67^{c}	0.058±0.005^{a}

[A]The data presented in this table consist of average values ± standard deviation of three independent batches. Different small letters represent statistically significant differences ($p < 0.05$) between quercetin or rutin samples, individually. n.d.: not detected. [B]Apical side recovery percentages were calculated as (bioactive concentration at the apical side after transport)/(bioactive concentration at the apical side at 0 h of incubation) × 100. [C]Basolateral side recovery percentages were calculated as (bioactive concentration at the basolateral side after transport)/(bioactive concentration at the apical side at 0 h of incubation) × 100. [D]Transport efficiency was calculated as (basolateral side recovery, %)/(apical side recovery, %).

7. GENERAL DISCUSSION AND CONCLUSIONS

7.1 Status and Main Outcomes of This Thesis

7.1.1 Fate of the polyphenols after application of novel non-thermal food processing techniques

Novel non-thermal technologies such as HHP and PEF provide high physico-chemical quality of food products with the retention of the freshness equivalent. Considering the studies conducted to highlight the high pressure treatments, it is obviously seen that HPP is a superior processing technique rather than conventional thermal food processing techniques due to the production of "fresh-like" products. Additionally, the application of PEF also results in a higher juice yield as well as higher retention of bioactive compounds when compared to the thermal treatments. Hence, PEF processing can promote the cellular antioxidant defence system against oxidative stress.

The first step in evaluating the effect of non-thermal food processing on the bioactive potential of polyphenols from agricultural sources is the characterization of the bioactive target. Accordingly, as a start of this thesis, polyphenols from cranberrybush have been characterized. The polyphenol profile of the cranberrybush used in the experimental chapters (*Chapters 3 and 4*) of this thesis was consistent. Three flavan-3-ols; catechin, epicatechin, epi(catechin) hexoside; three proanthocyanidins; procyanidin dimer, procyanidin trimer, procyanidin dimer monoglycoside, six flavonols; quercetin, quercetin-deoxyhexose, quercetin-*3*-*O*-glucoside, quercetin pentoside hexoside, rutin, isorhamnetin-*3*-*O*-rutinoside, a flavone; diosmetin-rhamnosylglucoside, four phenolic acids; caffeic acid, chlorogenic acid, coumaric acid and coumaroyl-quinic acid as well as three anthocyanins (cyanidin-3-glucoside, cyanidin-3-rutinoside and cyanidin-3-xylosyl-rutinoside) were detected in cranberrybush samples.

Later, the effect of high pressure processing and pulsed electric field treatment on physicochemical properties, bioactive compounds, antioxidant capacities and

polyphenol oxidase activity of cranberrybush samples were evaluated immediately after processing. Furthermore, due to the fact that chlorogenic acid is the major compound of *V. opulus*, in order to screen the effects of HPP and PEF treatments on the individual phenolic compounds, chlorogenic acid content was investigated. It was found that chlorogenic acid content was increased at elevated pressure levels and extended treatment times for HPP; and increasing the specific energy input for PEF.

On the other hand, the selection of HHP process parameters including pressure, temperature and duration as well as PEF process parameters such as electric field strength, treatment time, number and type of pulse waveform, their width and frequency should be optimized for each product individually according to the composition of the product of interest. Besides, more researches should be needed to operate large scale production systems and adapted to various food processes. More research is still necessary to carefully evaluate the advantages and disadvantages, as well as the efficiency of these novel methods to fully understand their effect and action of mechanism on the antioxidants of foods.

At the second step (*Chapter 4*), final absorption and metabolism of cranberrybush antioxidants were assessed to obtain the full picture on the effects of non-thermal treatments. In this contenxt, the *in vitro* digestion/Caco-2 cell system was conducted to study the effect of HPP and PEF treatments on the transportation of cranberrybush polyphenols across the gut epithelium. We observed that catechin and epicatechin were detected at the basoletaral compartment of all samples, whereas none of the oligomeric procyanidins could be detected. As for flavonoids, while quercetin-deoxyhexose and quercetin-3-*O*-glucoside were found in the basolateral compartment of all the samples, other flavonoids present in the cranberrybush purée could not be detected. On the other hand, none of the anthocyanins could be detected in the basolateral compartment after 4 h exposure. This finding may be related to the low anthocyanin amount of the samples and chemical instability in the alkali conditions. Majority of the phenolic acids, namely, caffeic acid, chlorogenic acid and coumaric acid were detected at the basolateral compartment of all the samples. Addition to these, quinic acid was also detected due to possible degradation of chlorogenic acid, present in the cranberrybush, by the cleavage of the chlorogenic acid moiety into quinic acid and caffeic acid by small intestine mucosal esterases (Dupas et al.,2006a). It could be concluded that the

cranberrybush derived phenolic acids are relatively stable in the small intestine. Moreover, a glance at the results revealed that both HPP and PEF enhanced or did not have negative effect on the recovery of the bioactives. To conclude, the results of these part of the thesis provided promising perspectives for the use of HPP and PEF as novel non-thermal treatments to enhance nutritional quality of cranberrybush polyphenols.

7.1.2 Effects of encapsulation on the food phenolics

In line with the outcomes of previous studies, it was reported that some of the phenolic compounds could not be detected at the basolateral compartment of Caco2 cells after transport experiments with cranberrybush samples. Therefore, encapsulation technology was used to improve the stability and to enhance the bioavailability of polyphenols during digestion.

Effects of microencapsulation techniques on food antioxidants including the changes in the antioxidant capacity, stability, solubility and retention of bioactive compounds should be evaluated together with the advantages, disadvantages and potential applications of each method. Based on the effects of encapsulation on the food antioxidant, it may be concluded that:

- The protection of the bioactive compounds to be encapsulated or coprecipitated could be improved in the case of using carrier agents.
- In fact, the technique and wall material (type, hydrophilicity, ratio between active and wall material etc.) have a great impact on the encapsulation characteristics including core retention, stability, solubility and the antioxidant power of the processed food antioxidants.
- Thermal stability of the polymer matrix is also effective on the bioactivities of core material against detrimental conditions.
- The parameters related with the physico-chemical functions of encapsulated material should be optimized for each encapsulation technique, core and wall material; thus enables to obtain narrower size distributions and that prevents high product loss and allows enhanced nutritional value.
- Each method has several advantages and disadvantages on different aspects. However, selection of the microencapsulation process is mainly related with the thermosensitivity and solubility of the active compounds.

- It should be taken into consideration while implementing the microencapsulation technique that whether the post-encapsulation steps such as separation, solvent removal or purification are necessary for the resulting product.
- More comprehensive studies on the precipitation, co-precipitation and encapsulation of food antioxidants, assessing the effects of process parameters on the antioxidant activities via different assays should be performed. Besides investigating the effects on antioxidant properties, the chemical, physical and sensory quality of the products should also be considered during the application of these techniques.

Considering the above, optimum conditions for supercritical antisolvent process in terms of the type of solvent, operating pressure, overall concentration and polymer/flavonoid ratio for the formation of spherical composite microparticles were determined (*Chapter 5*). In this part of the thesis, we demonstrated that it is possible to coprecipitate the poorly water soluble flavonoid compounds with PVP as the polymeric carrier by using the SAS technique. Flavonol-entrapped microparticles in amorphous structure were obtained with the solute concentrations greater than 20 mg/mL and high polymer/flavonoid ratios.

One of the main aspect of microencapsulation is to improve the bioavailability of food antioxidants. Thus, to get better approach about the final absorption of SAS processed antioxidant substances, *in vitro* digestion/Caco-2 cell system was carried out (*Chapter 6*). Besides, aqueous hydrophilic and acidic conditions were also simulated to analyze food-related factors that could have an impact on the transport of these compounds across the gut epithelium. SAS processing enhanced the recovery of the quercetin and rutin after *in vitro* gastrointestinal digestion as well as affect transepithelial transportation of these flavonoids positively. It could be deduced from the results that SAS processing can be a useful tool in the pharmaceutical and nutraceutical applications with high stability, bioaccessibility, bioavailability and thus enhanced nutritional value of the bioactives.

As a future aspect, it could be suggested that more sophisticated *in vitro* as well as *in vivo* tests should be conducted to stimulate the physicochemical change and digestion process of the formulation in the entire digestive tract. The bioaccessibility and

bioavailability of many dietary phenolic compounds are not well defined. The potential biological activity of each compound and their metabolites should be investigated and compared to get a better approach to assess the effects of encapsulated bioactives on the human digestive system.

7.1.3 Interactions of phenolics and food matrix

The increase or decrease in the bioaccessibility or bioavailability may vary by a number of factors such as the concentration of the substances in the food matrix, temperature, pH, type of substances, and hydrophilic/hydrophobic characteristics. Increasing number of *in vitro* and particularly *in vivo* studies on the bioavailability of phenolic substances in contact with various food matrixes will guide the design of functional foods enriched with phenolic substances.

In particular, in *Chapter 4*, effect of milk food matrix on the bioavailability of the cranberybush juice polyphenols were investigated. We observed that among other flavonoids, quercetin-deoxyhexose and quercetin-3-*O*-glucoside were transported to the the basolateral compartment for all the samples. On the other hand, catechin/epicatechin were transported through the Caco-2 cells in only cranberrybush juice+water samples. Besides, none of the oligomeric procyanidins could be detected. Most of the phenolic acids including caffeic acid, chlorogenic acid and coumaric acid were detected at the basolateral compartment of all the samples. Similar to the results of processing effect on the recovery of phenolic acids, quinic acid was also detected in all the juice samples. Unlike other flavonoids, none of the anthocyanins could be detected in the basolateral compartment after 4 h exposure. Particularly important from a food matrix perspective is that addition of milk appear to have a positive effect on transepithelial transportation of chlorogenic acid. On the other hand, majority of the polyphenols reach the colon and are consecutively metabolized by colonic microbiota, thus, future studies involving the combination of *in vitro* digestion models and Caco-2 cell culture studies could also be included with the colonic fermentation. In this regard, sulfated, glucuronidated, or methylated metabolites of polyphenols could also be evaluated to present overall consideration of the fate of the polyphenols to be released from the food matrix. Furthermore, it would be interesting to conduct advanced investigations on the transport dynamics of polyphenols from different food matrices that will be tretaed by non-thermal food processing methods.

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PUBLICATIONS, PRESENTATIONS AND PATENTS ON THE THESIS:

- **Ozkan, G.**, Guldiken, B., and Capanoglu, E. (2019). Effect of Novel Food Processing Technologies on Beverage Antioxidants. *Processing and Sustainability of Beverages: Volume:2*, Woodhead Publishing, 413-449. (Published)

- **Ozkan, G.**, Franco, P., De Marco, I., Xiao, J., and Capanoglu, E. (2019). A review of microencapsulation methods for food antioxidants: Principles, advantages, drawbacks and applications. *Food Chemistry*, 272, 494-506. (Published)

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- **Ozkan, G**., Franco, P., Capanoglu, E., and De Marco, I. (2020). PVP/quercetin coprecipitation by supercritical antisolvent (SAS) process: preparation, characterization and *in vitro* study. 4th International Symposium on Phytochemicals in Medicine and Food (4-ISPMF), November 30-December 5, 2020, Xi'an, China. (Oral presentation)

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- **Ozkan, G**., Franco, P., De Marco, I., Capanoglu, E., and Esatbeyoglu, T. Combining *in vitro* digestion/Caco-2 cell culture model: Evaluation of the supercritical antisolvent process and food models on antioxidative effects, bioaccessibility and transport dynamics of flavonol-loaded microparticles. (Submitted).

OTHER PUBLICATIONS, PRESENTATIONS AND PATENTS:

<u>International Articles:</u>

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- COST Action FA1403-POSITIVe (Interindividual variation in response to consumption of plant food bioactives and determinants involved), COST, 11/12/2014 - 10/12/2018. (Researcher)

- Research Center for Food Additives / Auxiliaries from Local Resources and Wastes, Ministry of Development Turkey, 11/12/2014 - 10/12/2018. (Researcher)

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