ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

STUDY OF BIOAVAILABILITY AND BIOACTIVITY OF BLACK CARROT POLYPHENOLS USING DIGESTION MODELS COMBINED WITH A NOVEL CO-CULTURE MODEL OF INTESTINAL AND ENDOTHELIAL CELL LINES

Ph.D. THESIS

Senem KAMİLOĞLU BEŞTEPE

Department of Food Engineering

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SEPTEMBER 2016

İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

YENİ BİR BAĞIRSAK VE ENDOTELYAL HÜCRE KO-KÜLTÜR MODELİ İLE SİNDİRİM MODELLERİNİN BİRLİKTE KULLANILARAK KARA HAVUCUN BİYOYARARLILIK VE BİYOAKTİVİTESİNİN ÇALIŞILMASI

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Senem Kamiloğlu Beştepe, a Ph.D. student of ITU Graduate School of Science Engineering and Technology student ID 506122504, successfully defended the thesis entitled "STUDY OF BIOAVAILABILITY AND BIOACTIVITY OF BLACK CARROT POLYPHENOLS USING DIGESTION MODELS COMBINED WITH A NOVEL CO-CULTURE MODEL OF INTESTINAL AND ENDOTHELIAL CELL LINES", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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To my family and to all science buffs,

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September 2016

Senem KAMİLOĞLU BEŞTEPE
(Food Engineer, M.Sc.)

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ABBREVIATIONS

AAPH : 2,2′-azobis(2-methylpropionamidine) dihydrochloride
ABAP : 2,2′-azobis(2-methylpropionamidine) dihydrochloride
ABTS : 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt)
ACE : Angiotensin Converting Enzyme
ACN : Anthocyanin
ADI : Acceptable Daily Intake
Akt : Protein kinase B
ANOVA : Analysis of variance
ATCC : American Type Culture Collection
AWA : Acetone:Water:Acetic acid
BC : Black Carrot
BCP : Black Carrot Pomace
Caco-2 : Human colorectal adenocarcinoma cell line
Caco-2BBE : Human colorectal adenocarcinoma cell line
CD31 : Cluster of Differentiation 31
CD36 : Cluster of Differentiation 36
CD44 : Cluster of Differentiation 44
COX-2 : Cyclooxygenase-2
CUPRAC : Cupric ion reducing antioxidant capacity
C3G : Cyanidin-3-O-glucoside equivalent
DAD : Diode Array Detector
DMEM : Dulbecco's Modified Eagle's Medium
DMSO : Dimethyl sulfoxide
DMPD : N,N-dimethyl-p-phenylenediamine dihydrochloride
DPPH : 2,2-diphenyl-1-picrylhydrazyl
DSMZ : German collection of microorganisms and cell cultures
DW : Dry Weight
EA.hy926 : Endothelial hybrid cell line
EC : Enterochromaffin cell line
EDTA : Ethylenediaminetetraacetic acid
EFSA : European Food Safety Authority
ELISA : Enzyme Linked Immunosorbent Assay
eNOS : Endothelial Nitric Oxide Synthase
Erk : Extracellular signal regulated kinase
ESI : Electrospray ionization
ET : Electron Transfer
ET-1 : Endothelin-1
EtOH : Ethanol
FBS : Fetal Bovine Serum
FID : Flame Ionization Detector
FRAP : Ferric ion Reducing Antioxidant Power

GAE : Gallic Acid Equivalent
GC : Gas Chromatography
GLUT2 : Glucose transporter 2
GLUT4 : Glucose transporter 4
HAT : Hydrogen Atom Transfer
HBSS : Hank's Balanced Salt Solution
HCA : Hydroxycinnamic acid
HCT-116 : Human colorectal adenocarcinoma cell line
HepaRG : Human hepatic cell line
HepG2 : Human liver carcinoma cell line
HL-60 : Human promyelocytic leukaemia cell line
HMEC-1 : Immortalized human microvascular endothelial cell line
HMREC : Primary microvascular retinal endothelial cell line
HPLC : High Performance Liquid Chromatography
HS-GC : Headspace gas chromatography
HT-29 : Human colorectal adenocarcinoma cell line
HT-29-B6 : Mucus secreting human colorectal adenocarcinoma cell line
HT-29-MTX : Mucus secreting human colorectal adenocarcinoma cell line
HuTu-80 : Human duodenum adenocarcinoma cell line
HUVEC : Primary human umbilical vein endothelial cells
HUVEC-C : Immortalized human umbilical vein endothelial cells
ICAM-1 : Intercellular adhesion molecule-1
IEC-6 : Rat small intestinal cell line
IL-1β : Interleukin-1 beta
IL-6 : Interleukin-6
IL-8 : Interleukin-8
IPI-21 : Porcine small intestinal cell line
ISO-HAS : Human hemangiosarcoma cell line
JNK : c-Jun N-terminal kinase
KMBA : α-keto-γ-methiolbutyric acid
LC : Liquid Chromatography
LDL : Low Density Lipoprotein
LOD : Limit Of Detection
LOQ : Limit Of Quantification
LPS : Lipopolysaccharide
LS180EB3 : Human colon adenocarcinoma cell line
m-ICcl2 : Murine intestinal epithelial cell line
MAPK : Mitogen Activated Protein Kinase
MCP-1 : Monocyte Chemoattractant Protein-1
MCT : Monocarboxylic acid transporter
MeOH : Methanol
MKN-28 : Human gastric adenocarcinoma cell line
MMP : Matrix metalloproteinase
MS : Mass Spectrometry
MTT : 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide
NCM460 : Human colon mucosal epithelial cell line
NEAA : Non-Essential Amino Acids
NF-κβ : Nuclear Factor-κβ
NMR : Nuclear Magnetic Resonance
NO : Nitric Oxide

ORAC : Oxygen Radical Absorbance Capacity
PA : Phenolic Acid
PAI-1 : Plasminogen Activator Inhibitor-1
PAZ-6 : Human brown adipocyte cell line
PBS : Phosphate Buffered Saline
PCA : Principal Component Analysis
PC12 : Rat pheochromocytoma cell line
PDA : Photodiode array detector
PenStrep : Penicillin streptomycin
PGA : Phloroglucinaldehyde
PLS-DA : Partial Least Squares-Discriminant Analysis
ppm : Parts Per Million
QTOF : Quadrupole Time Of Flight
RAW264.7 : Murine macrophage cell line
RBL-2H3 : Rat basophilic leukemia cell line
ROO• : Peroxyl radical
ROS : Reactive Oxygen Species
RP : Reversed Phase
RT-PCR : Reverse Transcription Polymerase Chain Reaction
SCFA : Short Chain Fatty Acids
SGLT1 : Sodium dependent glucose transporter 1
SHIME : Simulator of Human Intestinal Microbial Ecosystem
SPE : Solid Phase Extraction
SW480 : Human colorectal adenocarcinoma cell line
TC-7 : Human colorectal adenocarcinoma cell line
TE : Trolox® Equivalent
TEAC : Trolox® Equivalent Antioxidant Capacity
TEER : Transepithelial electrical resistance
TFA : Trifluoroacetic acid
TFC : Total Flavonoid Content
THP-1 : Human leukemia monocytic cell line
TIM : TNO gastrointestinal digestion model
TK-6 : Human lymphoblastoid cell line
TNF-α : Tumor Necrosis Factor-alpha
TNO : Netherlands organisation for applied scientific research
TOF : Time Of Flight
TOSC : Total Oxyradical Scavenging Capacity
TPC : Total Phenolic Content
TPTZ : 2,4,6-tripyridyl-s-triazine
TRAP : Total peroxyl Radical trapping Antioxidant Parameter
Trolox® : 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
UPLC : Ultra high Performance Liquid Chromatography
VCAM-1 : Vascular Cell Adhesion Molecule-1
VE-cadherin : Vascular Endothelial-cadherin
VEGF : Vascular Endothelial Growth Factor
VSMC : Vascular Smooth Muscle Cell line
3-CQA : 3-*O*-caffeoylquinic acid, neochlorogenic acid
3T3-L1 : Mouse embryonic fibroblast adipose like cell line
4-CQA : 4-*O*-caffeoylquinic acid, cryptochlorogenic acid
5-CQA : 5-*O*-caffeoylquinic acid, chlorogenic acid

SYMBOLS

a*	**:** red/green (color parameter)
b*	**:** blue/yellow (color parameter)
C	**:** chroma (color parameter)
H°	**:** hue angle (color parameter)
L*	**:** lightness/darkness (color parameter)
α	**:** alpha (in TNF-α, α-amylase)
β	**:** beta (in IL-1β, β-carotene, β-phycoerythrin)
ε	**:** molar extinction coefficient
l	**:** pathlength
m	**:** milli (10^{-3})
μ	**:** micro (10^{-6})
n	**:** nano (10^{-9})
Ω	**:** ohm
Σ	**:** sum
p	**:** probability (statistical analysis)
R^2	**:** correlation coefficient (statistical analysis)

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STUDY OF BIOAVAILABILITY AND BIOACTIVITY OF BLACK CARROT POLYPHENOLS USING DIGESTION MODELS COMBINED WITH A NOVEL CO-CULTURE MODEL OF INTESTINAL AND ENDOTHELIAL CELL LINES

SUMMARY

Nowadays, black carrots, originating from Turkey and the Middle and Far East, have gained increasing interest due to their substantial content of polyphenols, especially anthocyanins. Black carrots are often not consumed as such, instead they are either processed into other products or used as food colorants. Processing of black carrots generates large amounts of by-products, which are often discarded as waste or, in the best case valorized in low-value applications such as animal feed. However, the plant processing by-products of black carrots have the potential to be used as relatively cheap but valuable resources of polyphenols, which could be used in the development of functional foods.

Considering the above, a research framework to study the bioavailability and bioactivity of black carrot polyphenols from various sources has been developed. The objectives of this Ph.D. thesis were (i) to determine the effect of food processing and storage on black carrot polyphenols; (ii) to investigate the bioaccessibility and intestinal transport of key polyphenols in black carrots, processed products and their agronomic by-products using various *in vitro* gastrointestinal digestion and absorption models; (iii) to valorize the by-products from black carrot via enrichment of food products; (iv) to develop an *in vitro* model that is able to combine absorption effects with changes in endothelial cell metabolism.

To achieve these goals, four different experiments (*Chapters 3-6*) were conducted. Firstly, black carrots were processed into jams and marmalades and stored in the dark and the changes in polyphenol content were monitored (*Chapter 3*). Then, black carrots, jams and marmalades, as well as plant processing by-products of black carrot, i.e., peel and pomace, were subjected to *in vitro* gastrointestinal digestion (*Chapters 3, 4*). Following that, black carrot pomace was used as a source of polyphenols to enrich cakes (*Chapter 5*). Finally, an *in vitro* co-culture of intestinal and endothelial cell model was developed to determine the ability of polyphenols from black carrots and by-products to modulate the inflammatory response in endothelial cells (*Chapter 6*).

In *Chapter 1,* research framework and objectives of this Ph.D. thesis are introduced. Following that, in *Chapter 2,* a comprehensive review on the bioavailability and bioactivity of polyphenols is presented, with a specific focus on black carrot polyphenols. Initially, black carrot polyphenols, i.e., anthocyanins and phenolic acids, and their related health effects have been introduced. Then, the studies investigating the effect of food processing and storage on black carrot polyphenols have been reviewed. Afterwards, factors affecting the bioavailability and the methods used to assess the bioavailability of polyphenols were discussed with an

emphasis on black carrot polyphenols. Lastly, the impact of polyphenols on endothelium and the trends and potential applications of cell culture models for polyphenol research were described.

In *Chapter 3*, the effects of food processing, i.e., jam and marmalade processing, storage conditions and *in vitro* gastrointestinal digestion on total and individual polyphenol contents and total antioxidant capacity of black carrots were examined. Black carrot jams and marmalades were prepared traditionally using either sugar or sweetener and stored in the dark at two different temperatures (4 °C and 25 °C) over a period of 20 weeks. Total polyphenol contents and total antioxidant capacity were determined using spectrophotometric methods, whereas individual polyphenols were quantified using HPLC–PDA. The major anthocyanins detected in black carrots used in all experimental chapters (Chapter 3–6) were cyanidin-based with different sugar moieties, among them two were non-acylated (cyanidin-3-xylosyl-glucosyl-galactoside and cyanidin-3-xylosyl-galactoside), and three were acylated with sinapic acid (cyanidin-3-xylosyl-sinapoyl-glucosyl-galactoside), ferulic acid (cyanidin-3-xylosyl-feruloyl-glucosyl-galactoside) and coumaric acid (cyanidin-3-xylosyl-coumaroyl-glucosyl-galactoside). The results showed that although processing of black carrots into jams and marmalades resulted in significant decreases in polyphenol content and total antioxidant capacity (49.5–96.7%) ($p < 0.05$), after digestion polyphenols from processed products were found to be more bioaccessible compared to the ones in raw material (0.8–31.5%). In addition, after 20 weeks of storage the reduction in polyphenol content of samples stored at 25 °C (26.4–92.2%) was higher than samples stored 4 °C (19.0–46.4%). In conclusion, this chapter showed that black carrot jams and marmalades provide considerable polyphenol intake, which are preserved to a certain degree after storage and digestion, and hence these products can serve as novel sources of functional foods. The HPLC method applied in this chapter was used in the succeeding chapters (*Chapters 4-6*).

Processing of foods of plant origin including black carrot generates large amounts of by-products. These by-products represent a major disposal problem for the industry concerned; however, they are also promising sources of bioactive compounds. Considering that, in *Chapter 4*, we focused on the changes in polyphenols and total antioxidant capacity from black carrot and its by-products, i.e., peel and pomace, during *in vitro* gastrointestinal digestion, which consisted of a three-step procedure simulating the digestion in the stomach, small intestine, and colon. The results showed that although the amount of polyphenols decreased significantly as a result of digestion (23−82%) ($p < 0.05$), the pomace anthocyanins released at all stages of *in vitro* gastrointestinal digestion were higher than black carrot anthocyanins, suggesting that pomace may be a better source of bioaccessible anthocyanins. Overall, this chapter highlighted black carrot by-products as substantial sources of polyphenols, which may be used to enrich food products. *In vitro* digestion method developed in this chapter is used later in *Chapter 6*.

Taking into account the findings of *Chapter 4*, in *Chapter 5*, black carrot pomace was used to enrich cake samples. We investigated the digestive stability of polyphenols from black carrot pomace enriched cakes and monitored changes in their total antioxidant capacity using a new standardized static *in vitro* digestion model. Results showed that for undigested samples enrichment of cakes with black carrot pomace at levels of 5%, 10% and 15% caused a dose-dependent increase in anthocyanins (72 to 267 μg/g dw), phenolic acids (49 to 148 μg/g dw), total

phenolics (54 to 202 mg GAE/100 g dw) and total antioxidant capacity (21−129 to 153−478 mg TE/100 g dw). During the *in vitro* digestion in the mouth and stomach the amount of polyphenols were reduced significantly (35−74%) ($p < 0.05$), whereas no anthocyanins were detected in the intestine after intestinal digestion. On the other hand, significant increases in total phenolics and total antioxidant capacity were obtained in the stomach and intestine (up to 5− and 12−fold respectively) ($p < 0.05$). Overall, this chapter demonstrated that black carrot pomace, which can serve as a functional ingredient might be utilized in the baking industry.

In *Chapter 6*, we determined the potential of polyphenols from black carrot and its by-products to modulate the inflammatory response in tumor necrosis factor α (TNF-α) stimulated endothelial cells co-cultured with differentiated intestinal cells. As the bioactivity of polyphenols depends on their bioavailability, gastrointestinal digestion as well as transepithelial intestinal absorption was also considered while evaluating the anti-inflammatory effects of polyphenols. The results indicated that after 4 h of treatment, the transport of polyphenols was higher for digested samples (1.3−7%) compared to undigested ones (0−3.3%). The transported polyphenols were able to regulate the secretion of pro-inflammatory markers, i.e., interleukin-8 (IL-8), monocyte chemoattractant protein-1 (MCP-1), vascular endothelial growth factor (VEGF) and intercellular adhesion molecule-1 (ICAM-1), under normal and TNF-α induced inflammatory conditions. The most pronounced effects were observed with digested samples under inflammatory conditions, which significantly decreased the secretion of all markers from 120−203% down to 34−144% ($p < 0.001$). Eventually, the results of this chapter suggest that polyphenols from black carrot and its by-products may function through an inhibitory regulation of the inflammatory cascade in endothelial cells, which can maintain a sustainable cardiovascular effect under pre-existing low-grade inflammation caused by Western-type diets.

Finally, in *Chapter 7*, based on the outcomes of the previous chapters, the general discussions and conclusion, as well as the future perspectives on the bioavailability and bioactivity of polyphenols is presented. The status and main outcomes of this thesis were discussed under the headings of characterization of black carrot polyphenols, impact of food processing and storage on black carrot polyphenols, black carrot by-products as sources of polyphenols, bioaccessibility of black carrot polyphenols, intestinal transport of black carrot polyphenols and bioactivity of black carrot polyphenols. During the discussion on the bioaccessibility of black carrot polyphenols, special attention has been paid to comparison of different *in vitro* gastrointestional digestion models and the effects of processing and other dietary compounds. Similarly, while referring to the bioactivity of black carrot polyphenols, different antioxidant capacity assays were compared and co-culture models to study anti-inflammatory effects of polyphenols were discussed.

YENİ BİR BAĞIRSAK VE ENDOTELYAL HÜCRE KO-KÜLTÜR MODELİ İLE SİNDİRİM MODELLERİNİN BİRLİKTE KULLANILARAK KARA HAVUCUN BİYOYARARLILIK VE BİYOAKTİVİTESİNİN ÇALIŞILMASI

ÖZET

Günümüzde, kökeni Türkiye ve Orta ve Uzak Doğu olan kara havuç antosiyaninler başta olmak üzere içerdiği önemli miktardaki polifenollerden ötürü artan bir ilgi kazanmıştır. Kara havuç genellikle ham olarak tüketilmek yerine başka ürünlere işlenerek veya gıda renklendiricisi olarak değerlendirilmektedir. Kara havucun işlenmesi sonucu oluşan büyük miktarlardaki yan ürünler, genellikle atık olarak ayrılmaktadır veya en iyi ihtimalle hayvan yemi gibi düşük değerli uygulamalarda kullanılmaktadır. Bununla birlikte, kara havucun işlenmesi sonucu ortaya çıkan yan ürünler, fonksiyonel gıdaların geliştirilmesinde kullanılabilecek, nispeten ucuz ama değerli polifenol kaynakları olarak kullanılma potansiyeline sahiptirler.

Yukarıdakiler dikkate alınarak kara havuç polifenollerinin biyoyararlılığını ve biyoaktivitesini çalışmak amacıyla bir araştırma çerçevesi oluşturulmuştur. Bu doktora tezinin hedefleri (i) gıda işleme ve depolamanın kara havuç polifenolleri üzerinde etkisini tespit etmek; (ii) çeşitli *in vitro* mide-bağırsak sindirim ve emilim modellerini kullanarak kara havuç, işlenmiş ürünler ve bunların tarımsal yan ürünlerinde bulunan başlıca polifenollerin biyoerişilebilirliğini ve bağırsak taşınımını araştırmak; (iii) gıda ürünlerinin zenginleştirilmesi vasıtasıyla kara havucun yan ürünlerini değerlendirmek ve (iv) emilim etkilerini endotelyal hücre metabolizmasındaki değişiklikler ile birleştiren bir *in vitro* model geliştirmektir.

Yukarıdaki hedeflere ulaşmak için dört farklı deneysel çalışma (*Bölüm 3–6*) yapılmıştır. Öncelikle kara havuç reçel ve marmelata işlenmiş, karanlıkta depolanmış ve polifenol içeriğinde meydana gelen değişimler izlenmiştir (*Bölüm 3*). Daha sonra, kara havuç, reçel ve marmelat, ve kara havucun yan ürünleri (kabuk ve posa), *in vitro* mide-bağırsak sindirimine tabi tutulmuştur (*Bölüm 3 ve 4*). Bunu takiben kara havuç posası keki zenginleştirmek için polifenol kaynağı olarak kullanılmıştır (*Bölüm 5*). Son olarak, kara havuç ve yan ürünlerdeki polifenollerin endotelyal hücrelerdeki anti-inflamatuar etkilerini tespit etmek amacıyla bir *in vitro* bağırsak ve endotelyal hücre ko-kültür modeli geliştirilmiştir (*Bölüm 6*).

İlk bölümde bu doktora tezinin araştırma çerçevesi ve hedefleri tanıtılmıştır. Bunu takiben *ikinci bölümde* kara havuç polifenollerinin biyoyararlılığı ve biyoaktivitesi ile ilgili kapsamlı bir derleme sunulmuştur. Başlangıç olarak kara havuç polifenolleri (antosiyaninler ve fenolik asitler) ve bunlarla ilgili sağlık etkilerinden bahsedilmiştir. Sonrasında gıda işleme ve depolamanın kara havuç polifenolleri üzerine etkilerini araştıran çalışmalar derlenmiştir. Daha sonra, biyoyararlılığı etkileyen faktörler, ve kara havuç polifenolleri ve diğer polifenollerin biyoyararlılığını ölçmek için kullanılan yöntemler ele alınmıştır. Son olarak, polifenollerin endotelyum üzerindeki etkileri ve polifenol araştırmaları için kullanılan hücre kültürü modellerindeki eğilimler ve potansiyel uygulamalardan bahsedilmiştir.

Üçüncü bölümde reçel ve marmelata işlemenin, depolama koşullarının ve *in vitro* mide-bağırsak sindiriminin kara havucun toplam ve bireysel polifenol içeriği ve toplam antioksidan kapasitesine olan etkileri incelenmiştir. Kara havuç reçel ve marmelatları geleneksel yöntemlerle şeker veya tatlandırıcı kullanılarak hazırlanmış ve karanlık ortamda iki farklı sıcakta (4 °C and 25 °C) 20 hafta süresince muhafaza edilmiştir. Toplam polifenol içeriği ve toplam antioksidan kapasite spektrofotometrik yöntemler kullanılarak, bireysel polifenoller ise HPLC–PDA kullanılarak tespit edilmiştir. Bu bölüm ve de diğer tüm deneysel bölümlerde (*Bölüm 3–6*) kullanılan kara havuç örneklerinde tespit edilen ana antosiyaninlerin hepsi siyanidin bazlı olup farklı şekerlere bağlanmışlardır. İçlerinden iki tanesi açillenmemiş (siyanidin-3-ksilosil-glükosil-galaktozid ve siyanidin-3 ksilosil-galaktozid), üç tanesi ise sinapik asit (siyanidin-3-ksilosil-sinapol-glükosil-galaktozid), ferulik asit (siyanidin-3-ksilosil-feruyol-glükosil-galaktozid) ve kumarik asit (siyanidin-3-ksilosil-kumarol-glükosil-galaktozid) ile açillenmiştir. Elde edilen sonuçlar kara havucun reçel ve marmelata işlenmesi sonucunda polifenol içeriğinde istatistiksel olarak önemli düşüşler olmasına rağmen (%49.5–96.7) ($p < 0.05$) sindirimden sonra işlenmiş ürünlerdeki polifenollerin hammaddeye kıyasla daha biyoerişilebilir olduğunu göstermiştir (%0.8–31.5). Ek olarak, 20 hafta depolama süresi sonucunda 25 °C'de depolanan örneklerin polifenol içeriğindeki azalmanın (%26.4–92.2) 4 °C'de depolanan ürünlerden (%19.0–46.4) daha yüksek olduğu görülmüştür. Sonuç olarak, bu bölümde sunulan deneysel çalışma sonuçları kara havuç reçel ve marmelatındaki polifenolerin depolama ve sindirim neticesinde belli bir dereceye kadar korunduğunu ve bu ürünlerin tüketimi ile dikkate değer bir miktarda polifenol alınımı sağlanabileceğini, ve dolayısıyla bu ürünlerin yeni bir fonksiyonel gıda kaynağı olabilecekleri göstermiştir. Bu bölümde uygulanan HPLC yöntemi, bu bölümü takip eden diğer bölümlerde de kullanılmıştır (Bölüm 4–6).

Kara havuç dahil olmak üzere bitkisel kökenli birçok gıdanın işlenmesi büyük miktarlarda yan ürünlerin oluşmasına neden olmaktadır. Oluşan bu yan ürünler gıda sanayisi için önemli bir bertaraf sorunu oluşturmakla beraber aynı zamanda biyolojik olarak aktif olan birçok bileşiği de içermektedirler. Bu durum dikkate alınarak bu tezin *dördüncü bölümünde* kara havuç ve kara havucun yan ürünlerinde (kabuk ve posa) bulunan polifenollerde *in vitro* mide-bağırsak sindirimi sırasında meydana gelen değişiklikler üzerinde durulmuştur. Bu bölümde uygulanan sindirim modeli sırasıyla mide, ince bağırsak ve kalın bağırsaktaki sindirimi taklit eden üç aşamalı bir prosedürden oluşmaktadır. Elde edilen sonuçlar sindirim sonucunda polifenol miktarlarında istatistiksel olarak önemli azalmalar (%23−82) ($p < 0.05$) olmasına rağmen posada bulunan antosiyaninlerin *in vitro* mide-bağırsak sindiriminin tüm aşamaları boyunca kara havuç antosiyaninlerinden daha biyoerişilebilir olduğunu göstermiştir. Sonuç olarak bu bölümde sunulan deneysel çalışma sonuçlarıyla beraber kara havucun yan ürünlerinin önemli polifenol kaynakları olabilecekleri ve diğer gıda ürünlerini zenginleştirmek için kullanılabilecekleri vurgulanmıştır. Bu bölümde uygulanan *in vitro* mide-bağırsak sindirim modeli daha sonra *altıncı bölümde* de kullanılmıştır.

Dördüncü bölümdeki bulgular da dikkate alınarak, *beşinci bölümde* kara havuç posası kek örneklerini zenginleştirmek için kullanılmıştır. Kara havuç posası ile zenginleştirilmiş keklerdeki polifenollerin sindirim sırasındaki stabiliteleri incelenmiş ve toplam antioksidan kapasitelerinde meydana gelen değişiklikler yeni standardize edilmiş bir *in vitro* sindirim modeli kullanılarak takip edilmiştir. Sonuçlar sindirilmemiş örnekler için keklerin %5, %10 ve %15 oranlarında kara

havuç posası ile zenginleştirilmesinin antosiyanin içeriği (72−267 μg/g kuru ağırlık), fenolik asit içeriği (49−148 μg/g kuru ağırlık), toplam fenolik madde içeriği (54−202 mg GAE/100 g kuru ağırlık) ve antioksidan kapasitede (21−478 mg TE/100 g kuru ağırlık) eklenen dozla doğru orantılı bir artışa neden olduğunu göstermiştir. Ağız ve midedeki *in vitro* sindirim sırasında polifenol miktarında istatistiksel olarak önemli miktarda azalmalar (%35−74) ($p < 0.05$) meydana gelmiştir. Bağırsak sindiriminden sonra ise hiçbir antosiyanin tespit edilememiştir. Diğer bir yandan mide ve bağırsakta toplam fenolik madde ve toplam antioksidan kapasite içeriklerinde önemli miktarda artışlar meydana gelmiştir (sırasıyla 5− ve 12−kat) ($p < 0.05$). Sonuç olarak, bu bölümde sunulan deneysel çalışma sonuçları fonksiyonel bir bileşen olan kara havuç posasının fırıncılık sanayisinde kullanılabileceğini göstermiştir.

Altıncı bölümde kara havuç ve kara havucun yan ürünlerinde bulunan polifenollerinin bağırsak hücreleri ile ko-kültürlenmiş ve tümör nekroz faktörü α (TNF-α) ile uyarılmış endotelyal hücrelerindeki inflamatuar tepkiyi etkileme potansiyali tespit edilmiştir. Polifenollerin biyoaktivitelerini göstermeleri biyoyararlılıklarına bağlı olduklarından dolayı, polifenollerin anti-inflamatuar etkilerinin değerlendirilmesinde mide-bağırsak sindirimi ve bağırsaktan geçişi de dikkate alınmıştır. Elde edilen sonuçlar uygulamadan 4 saat sonra sindirilmiş örneklerdeki polifenol geçişinin (%1.3−7) sindirilmemiş örneklere kıyasla (%0−3.3) daha yüksek olduğunu göstermiştir. Bağırsaktan geçen polifenoller hem normal hem de TNF-α ile uyarılmış edilmiş koşullarda proinflamatuar markörlerin (interlökin-8 (IL-8), monosit kemoatraktant protein-1 (MCP-1), vasküler endotelyal büyüme faktörü (VEGF) ve interselüler adhezyon molekülü-1 (ICAM-1)) salgılanmasını düzenleyebilmişlerdir. En belirgin etkiler, inflamatuar koşullar altında sindirilmiş örneklerde gözlenmiştir. Tüm markörlerin salgılanması %120−203'den %34−144%'de düşmüştür ($p < 0.001$). Sonuç olarak bu bölümde sunulan deneysel çalışma sonuçları kara havuç ve kara havucun yan ürünlerinde bulunan polifenollerin endotelyal hücrelerdeki inflamasyonda inhibitör olarak işlev görebileceğini göstermiştir. Bu durum batı tipi diyetle beraber önceden var olan düşük dereceli inflamasyona karşı sürdürülebilir bir kardiyovasküler etkinin oluşmasını sağlanabileceğini göstermektedir.

Son olarak, *yedinci bölümde*, önceki bölümlerde elde edilen veriler dikkate alınarak polifenollerin biyoyararlılığı ve biyoaktivitesi ile ilgili genel bir tartışma, sonuçlar ve gelecekteki araştırmalar için tavsiyeler verilmiştir. Bu tezden elde edilen sonuçlar kara havuç polifenollerinin karakterizasyonu, gıda işleme ve depolamanın kara havuç polifenolleri üzerine etkisi, polifenol kaynağı olarak kara havucun yan ürünleri, kara havuç polifenollerinin biyoulaşılabilirliği, kara havuç polifenollerinin bağırsaktan geçişi ve kara havuç polifenollerinin biyoaktivitesi başlıkları altında ele alınmıştır. Kara havuç polifenollerinin biyoulaşılabilirliği tartışılırken farklı *in vitro* mide-bağırsak sindirimi modellerinin karşılaştırılması ve gıda işleme ve diğer gıda bileşenlerinin etkisi konularına değinilmiştir. Benzer şekilde, kara havuç polifenollerinin biyoaktivitesi tartışılırken de farklı antioksidan kapasite ölçüm metotları karşılaştırılmış ve polifenollerin anti-inflamatuar etkilerini çalışmak için kullanılan ko-kültür modellerinden bahsedilmiştir.

1. INTRODUCTION

Hippocrates, one of the most outstanding figures in the history of medicine, is often quoted as saying, “Let your food be thy medicine and your medicine be thy food”. As such, we understand that the importance of healthy diet in preventing diseases has been stressed even many centuries ago. Today, epidemiological studies show that the high intake of fruits and vegetables is associated with a reduced risk of chronic diseases and the potential beneficial effects are attributed to the presence of bioactive compounds, in particular polyphenols. Polyphenols are plant secondary metabolites, which are important compounds for the sensory and nutritional quality of fruits and vegetables. However, the potential health-promoting effects of polyphenols from fruit and vegetables depends on their processing history as well as their absorption in the gastrointestinal tract.

Although *in vivo* trials are still being considered as the “gold standard” for addressing diet-related issues, *in vitro* models have the advantages of being more rapid and less expensive, and having no ethical restrictions. Over the last decade, many *in vitro* gastrointestinal models simulating the *in vivo* status have been developed, which have been shown to correlate well with the conclusions from human studies and animal trials. Furthermore, there is also an increasing interest in the co-cultivation of different cell lines to study cross-talk mechanisms between different tissues as an alternative for animal research. Therefore, use of *in vitro* gastrointestinal digestion and co-culture models to study polyphenols may provide new insights into their bioavailability and bioactivity.

Lately, black carrots, originating from Turkey and the Middle and Far East, have gained increasing interest due to their substantial content of polyphenols, especially anthocyanins. Black carrots are often not consumed as such, instead they are either processed into other products or used as food colorants. Processing of black carrots generates large amounts of by-products, which are often discarded as waste or, in the best case valorized in low-value applications such as animal feed. However, the plant processing by-products of black carrot have the potential to be used as relatively

cheap but valuable resources of polyphenols, which could be used in the development of functional foods.

Considering the above, a research framework to study the bioavailability and bioactivity of black carrot polyphenols from various sources has been developed. This Ph.D. thesis is a product of a joint collaboration between two institutions, namely Istanbul Technical University in Turkey and Ghent University in Belgium. Therefore, the experimental chapters of this thesis were partially carried out in Istanbul (*Chapter 3* and *5*) and partially in Ghent (*Chapter 4* and *6*).

Below are the major objectives of this Ph.D. thesis:

1. To evaluate the changes in polyphenol profile and antioxidant capacity of black carrots as a result of different processing and storage conditions (*Chapter 3*)
2. To investigate the digestive stability of polyphenols from black carrots, processed products and their agronomic by-products using *in vitro* gastrointestinal digestion models (*Chapter 3* and *4*)
3. To valorize the by-products of black carrot through enrichment of food products (*Chapter 5*)
4. To develop an *in vitro* model that is able to combine absorption effects with changes in endothelial cell metabolism (*Chapter 6*)

The schematic outline of this Ph.D. thesis is presented in Figure 1.1.

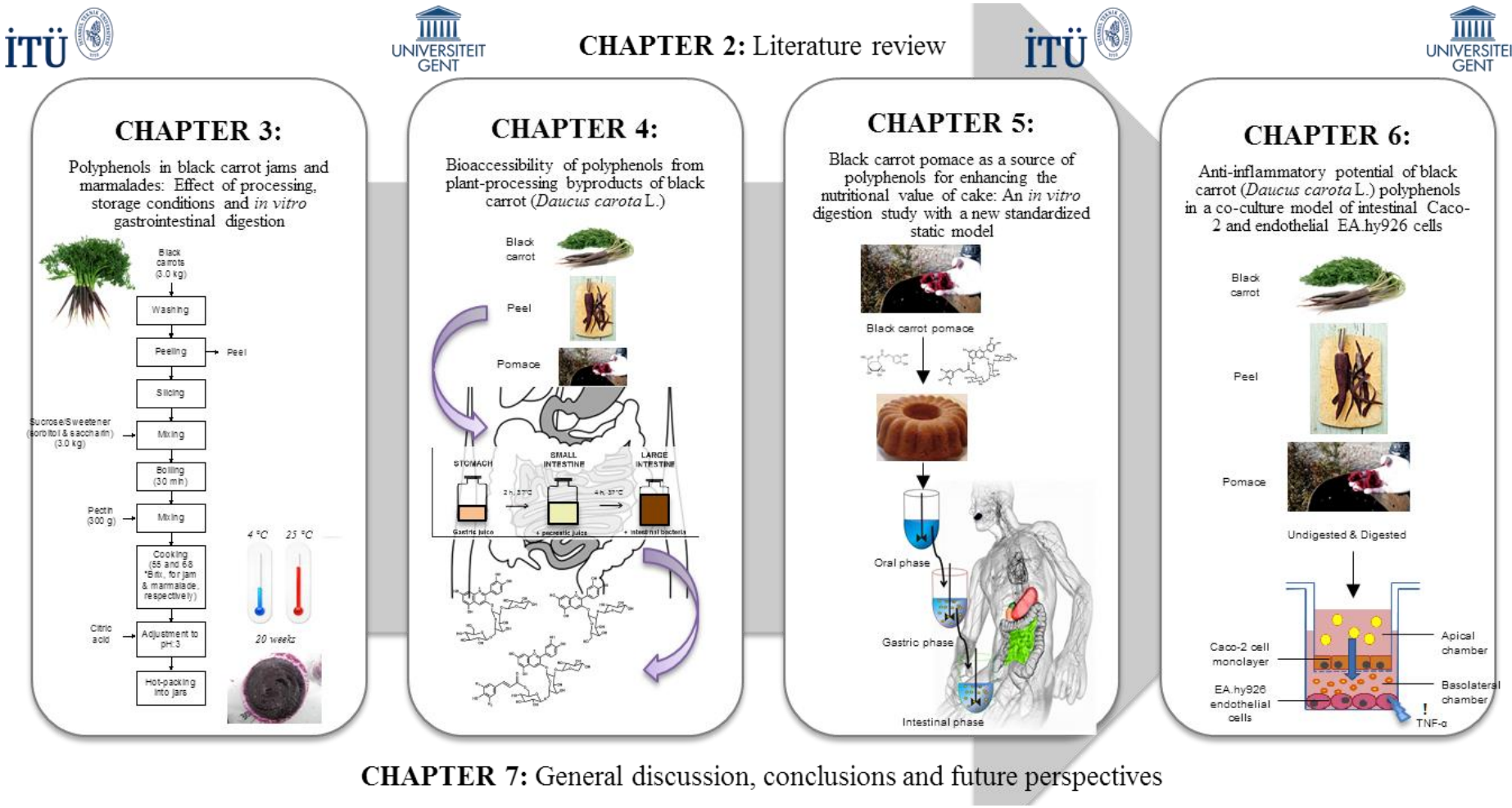


Figure 1.1 : Schematic outline of this Ph.D. research.

2. LITERATURE REVIEW

Redrafted from:

Kamiloglu, S., Capanoglu, E., Grootaert, C., and Van Camp, J. (2015). Anthocyanin absorption and metabolism by human intestinal Caco-2 cells—A review. *International Journal of Molecular Sciences, 16*, 21555-21574.

Grootaert, C.*, **Kamiloglu, S.***, Capanoglu, E., and Van Camp, J. (2015). Cell systems to investigate the impact of polyphenols on cardiovascular health. *Nutrients*, *7*, 9229-9255 (*shared first authorship).

2.1 Black Carrot Polyphenols and Related Health Effects

2.1.1 Black carrots

Carrots (*Daucus carota* L.), a member of Apiaceae (formerly Umbelliferae) family (Erten et al, 2008), constitute a valuable source of health-promoting ingredients and thus are important in human nutrition, with an annual worldwide production exceeding 35.6 million of tons (Carvalho et al, 2014). Cultivated carrots can be subdivided into two main groups, the western or carotene carrot (*Daucus carota* ssp. *sativus* var. *sativus*) and the eastern or anthocyanin carrot (*Daucus carota* ssp. *sativus* var. *atrorubens* Alef.) (Kammerer et al, 2004b). Although, orange carrot varieties account for the majority of this crop, black or purple carrots are thought to be much older than orange carrot varieties. In fact, black carrots, originating from oriental countries such as Turkey, Afghanistan, Egypt, Pakistan, India and the Far East (Kammerer et al, 2004a), have been cultivated for at least 3000 years (Schwarz et al, 2004). In Europe, black carrots have been cultivated from the Middle Ages to the 18th century until orange varieties became predominant (Kammerer et al, 2003). Nowadays, although orange carrots are more common, consumption of black carrots is also increasing (Algarra et al, 2014).

Black carrots have an attractive bluish-purple color with high levels of anthocyanins and can serve as a natural food colorant due to their high heat, light, and pH stability

(Montilla et al, 2011). Providing an excellent bright strawberry red shade at acidic pH values, black carrots are being used as a nutraceutical/functional ingredient in various food matrices including fruit juices and nectars, soft drinks, preserves, jellies and confectionary (Khandare et al, 2011; Murali et al, 2015). Apart from their colorant properties, black carrots also attracted attention due to their substantial nutrient content. Based on the information provided in Turkish food composition database, black carrots roots contain approximately 88% water, 1% protein, 8% carbohydrate, 0.14% fat, and 2.5% fiber. Black carrots are also significant sources of certain minerals and vitamins (Table 2.1).

Table 2.1 : Nutrient content of black carrots (per 100 g edible food).

Component	Unit	Mean	Minimum	Maximum
Energy	kcal	42	41	43
Energy	kJ	175	171	179
Water	g	87.66	87.29	88.02
Ash	g	0.84	0.76	0.92
Protein	g	0.87	0.75	1.00
Nitrogen	g	0.14	0.12	0.16
Fat, total	g	0.14	0.12	0.16
Carbohydrate	g	8.01	8.00	8.02
Fiber, total dietary	g	2.48	2.35	2.61
Fiber, water-soluble	g	0.90	0.84	0.97
Fiber, water-insoluble	g	1.58	1.51	1.65
Saccharose	g	0.00	0.00	0.00
Glucose	g	1.85	1.85	1.85
Fructose	g	0.14	0.14	0.14
Lactose	g	0.00	0.00	0.00
Maltose	g	0.00	0.00	0.00
Salt	mg	206	196	215
Iron, Fe	mg	0.26	0.24	0.28
Phosphorus, P	mg	29	21	38
Calcium, Ca	mg	33	31	35
Magnesium, Mg	mg	17	17	18
Potassium, K	mg	256	240	273
Sodium, Na	mg	82	78	86
Zinc, Zn	mg	0.15	0.14	0.17
Thiamin	mg	0.029	0.026	0.032
Riboflavin	mg	0.029	0.022	0.035
Niacin, preformed	mg	1.211	1.114	1.308
Vitamin B6, total	mg	0.072	0.064	0.079
Vitamin A	RE	0	0	0
Beta-carotene	µg	0	0	0
Lycopene	µg	0	0	0
Lutein	µg	0	0	0

2.1.2 Polyphenols

Polyphenols are divided into several classes according to the number of phenol rings that they contain and to the structural elements that bind these rings to each other. The main groups of polyphenols are flavonoids, phenolic acids, stilbenes, and lignans (D'Archivio et al, 2007) (Figure 2.1).

Flavonoids are low molecular weight compounds, comprising of fifteen carbon atoms, arranged in a $C_6{-}C_3{-}C_6$ configuration. Essentially, the structure consists of two aromatic rings, joined by a 3−carbon bridge, usually in the form of a heterocyclic ring. Variations in the substitution patterns of this heterocyclic ring result in six different subclasses, namely flavonols, flavones, flavanones, flavanols, isoflavones and anthocyanidins (Balasundram et al, 2006). Individual differences within each group arise from the variation in number and arrangement of the hydroxyl groups and their extent of alkylation and/or glycosylation (Pandey and Rizvi, 2009; Spencer et al, 2008). Major flavonols include quercetin, myricetin and kaempferol, which are abundantly present in onions, curly kale, leeks, broccoli, and blueberries. Flavones consist mainly of glycosides of apigenin and luteolin and their important sources are parsley and celery. The main flavanone aglycones are naringenin in grapefruit, hesperetin in oranges, and eriodictyol in lemons. Flavanols exist both in monomer (catechins) and polymer (proanthocyanidins) forms. Catechin and epicatechin are the main flavanols in fruits, chocolate, red wine, and tea. Soy and its processed products are the main dietary sources of isoflavones, which contain three main molecules namely genistein, daidzein, and glycitein. The most widespread anthocyanidins are cyanidin, delphinidin, malvidin, pelargonidin, and peonidin, which are found abundantly in colored fruits including blackberry, blueberry, strawberry, and cherry (Manach et al, 2004).

Phenolic acids consist of two subgroups of hydroxybenzoic and hydroxycinnamic acids. Hydroxybenzoic acids include gallic, *p*-hydroxybenzoic, protocatechuic, vanillic, and syringic acids having $C_6{-}C_1$ structure, whereas hydroxycinnamic acids are aromatic compounds with a three-carbon side chain ($C_6{-}C_3$), with caffeic, ferulic, *p*-coumaric and sinapic acids being the most common ones (Balasundram et al, 2006). Hydroxybenzoic acids are found in high concentrations in blackberry, raspberry, and tea, while blueberry and coffee are rich sources of hydroxycinnamic acids (D'Archivio et al, 2007).

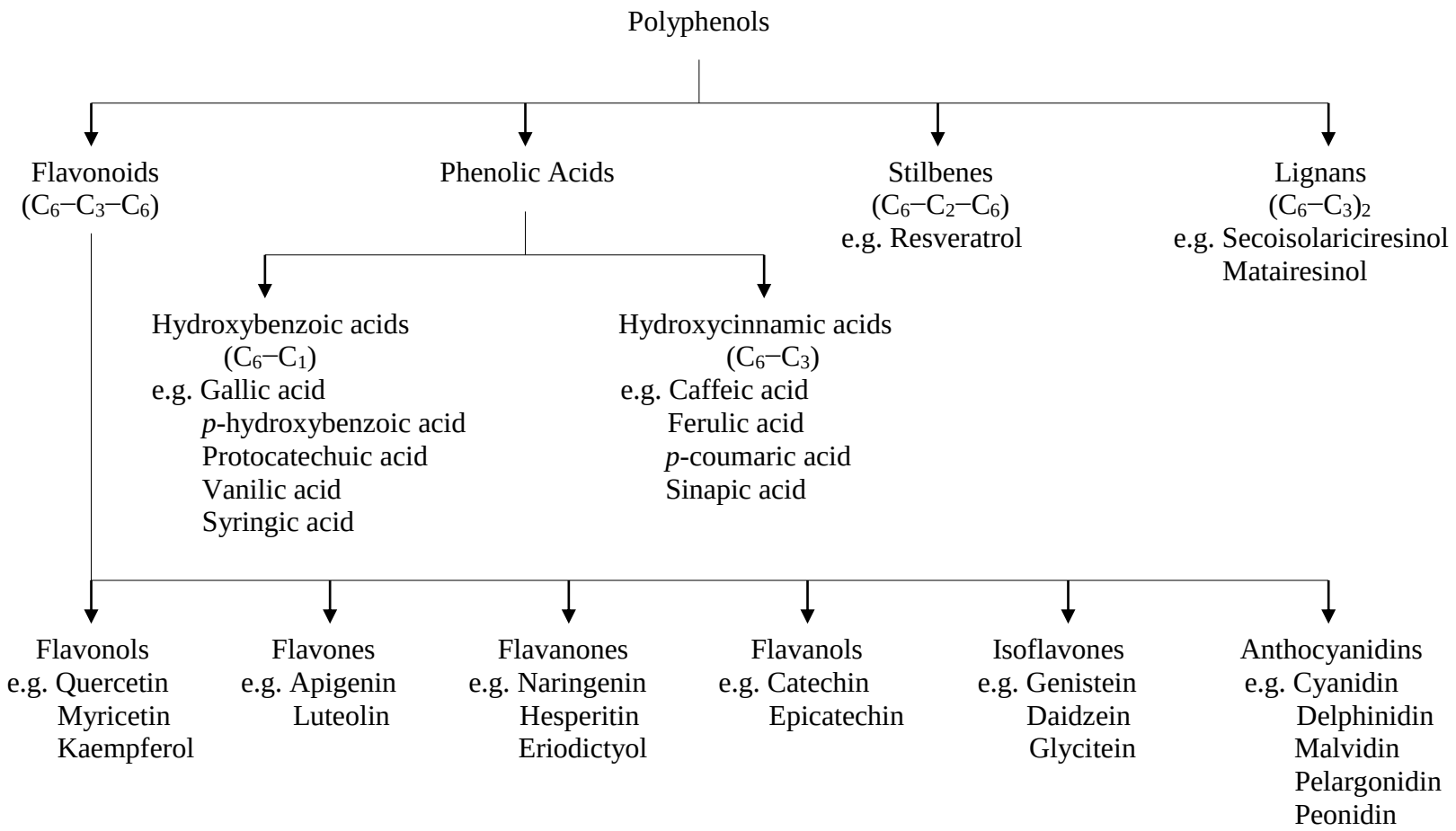


Figure 2.1 : Classification of major classes of dietary polyphenols.

Stilbenes contain two phenyl moieties connected by a two-carbon methylene bridge (Pandey and Rizvi, 2009). The main representative of stilbenes is resveratrol, which exists in more than 70 plant species, including grapes, berries, and peanuts (Ignat et al, 2011).

Lignans are produced by oxidative dimerization of two phenylpropane units. Linseed, containing secoisolariciresinol and low quantities of matairesinol, represents the main dietary source (D'Archivio et al, 2007; Manach et al, 2004).

Since anthocyanins and phenolic acids are the predominant polyphenols present in black carrots (Netzel et al, 2007), in the following sections these compounds will be discussed in detail.

2.1.2.1 Anthocyanins

Anthocyanins are water-soluble pigments responsible for the blue, purple, and red color of many plant tissues (Prior and Wu, 2009). The term anthocyanin is derived from the Greek words *anthos*, meaning flower, and *kyanos*, meaning blue (He and Giusti, 2010). Although they also occur in vegetables, roots, legumes, and cereals, these pigments are usually associated with fruits. In particular, berry fruits are rich sources of dietary anthocyanins (Alvarez-Suarez et al, 2011; Kamiloglu et al, 2013; Giampieri et al, 2014a; Howard et al, 2014) and can contribute tens to hundreds of milligrams of anthocyanins in a single serving. The daily intake of anthocyanins in the USA diet is estimated to be as much as 180–255 mg per day; a value that far exceeds the consumption of most other flavonoids (McGhie and Walton, 2007).

Anthocyanins have become increasingly important to the food industry, as their use as natural alternatives to synthetic dyes has become widespread (Wallace, 2011). According to the numbering system used by the Codex Alimentarius Commission, anthocyanins are listed as a natural colorant by the European Union legislation and coded as E163. With respect to the USA, the Food and Drug Administration has a different list of “natural” colors that do not require certification, and anthocyanins can be obtained either from “grape color extract”, “grape skin extract”, or “fruit or vegetable juices” (Mateus and de Freitas, 2008).

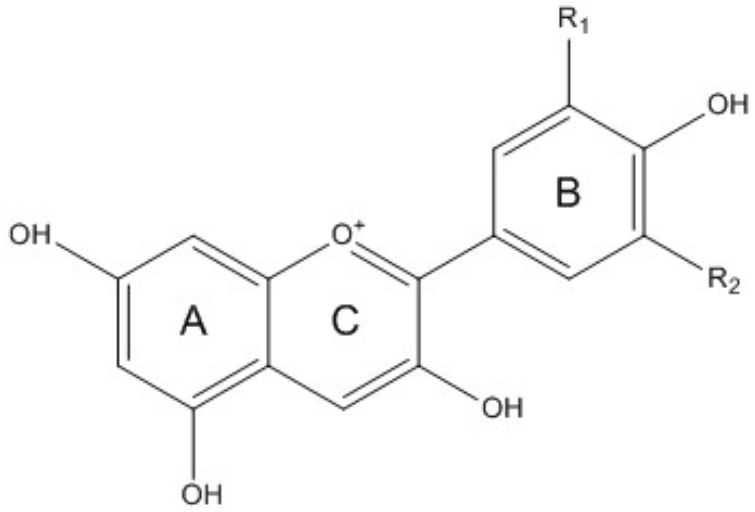
As mentioned above, anthocyanins belong to a large group of compounds collectively known as flavonoids, which are a subgroup of an even larger group of compounds known as polyphenols (McGhie and Walton, 2007). Chemically,

anthocyanins occur as glycosides of flavylium (2-phenylbenzopyrylium) salts but differ from them by structural variations in the number of hydroxyl groups, the degree of methylation of these hydroxyl groups, the nature and number of sugar moieties attached to the molecule, and the position of the attachment, as well as the nature and number of aliphatic or aromatic acids attached to the sugars (Faria et al, 2013).

Anthocyanins are found as glycosides of their respective aglycones, called anthocyanidins (Forbes-Hernandez et al, 2014). The anthocyanidins consist of an aromatic ring A bound to a heterocyclic ring C that contains oxygen, which is also bound by a carbon–carbon bond to a third aromatic ring B (Ignat et al, 2011). About 17 anthocyanidins have been identified, but only six of them are commonly distributed in nature: cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin (Figure 2.2). Despite there being only six common anthocyanidins, there are over 600 anthocyanins reported in plants (Wu et al, 2006). Glucose, galactose, arabinose, rutinose, rhamnose, and xylose are the most common sugars that are bound to anthocyanidins as mono-, di-, or trisaccharide forms (Fang, 2014a). The most widespread glycoside derivatives in nature are 3-monosides, 3-biosides, 3,5- and 3,7-diglucosides. The presence of the 3-glucoside derivatives is 2.5 times more frequent than the 3,5-diglucosides and the most common anthocyanin is cyanidin-3-glucoside (Castaneda-Ovando et al, 2009). In many cases, the sugar residues are acylated with *p*-coumaric, caffeic, ferulic, sinapic, *p*-hydroxybenzoic, malonic, oxalic, malic, succinic or acetic acid (De Pascual-Teresa et al, 2013).

Anthocyanins are highly instable and very susceptible to degradation. Oxygen, temperature, light, enzymes and pH are among the many factors that may affect the chemistry of anthocyanins and, consequently, their stability and color (Fernandes et al, 2014). The hue of anthocyanins may vary according to different substituent groups present on the B ring, and color saturation increases with increasing number of hydroxyl groups and decreases with the addition of methoxyl groups (Tsuda, 2012). In aqueous solution, anthocyanins undergo structural re-arrangements in response to changes in pH in four molecular structures: quinoidal base (blue), flavylium cation (red), carbinol (colorless) and chalcone (yellowish) forms (Figure 2.3). Anthocyanins are stable in acidic solutions (pH 1–3) where they exist primarily as flavylium cations. At pH > 4, anthocyanins adopt the forms of the carbinol and

chalcone. Chalcone can then undergo chemical degradations to produce phenolic acids (Fang, 2014a). The relative composition of the different molecular structures of anthocyanins coexisting in aqueous solution at any given time will depend on pH, temperature and time. This is particularly important as anthocyanins are exposed to different pH conditions through the gastrointestinal tract, which affects their bioavailability and hence their bioactivity (McGhie and Walton, 2007).



Anthocyanidin	R_1	R_2
Cyanidin	OH	H
Delphinidin	OH	OH
Malvidin	OCH_3	OCH_3
Pelargonidin	H	H
Peonidin	OCH_3	H
Petunidin	OH	OCH_3

Figure 2.2 : Anthocyanidin structures.

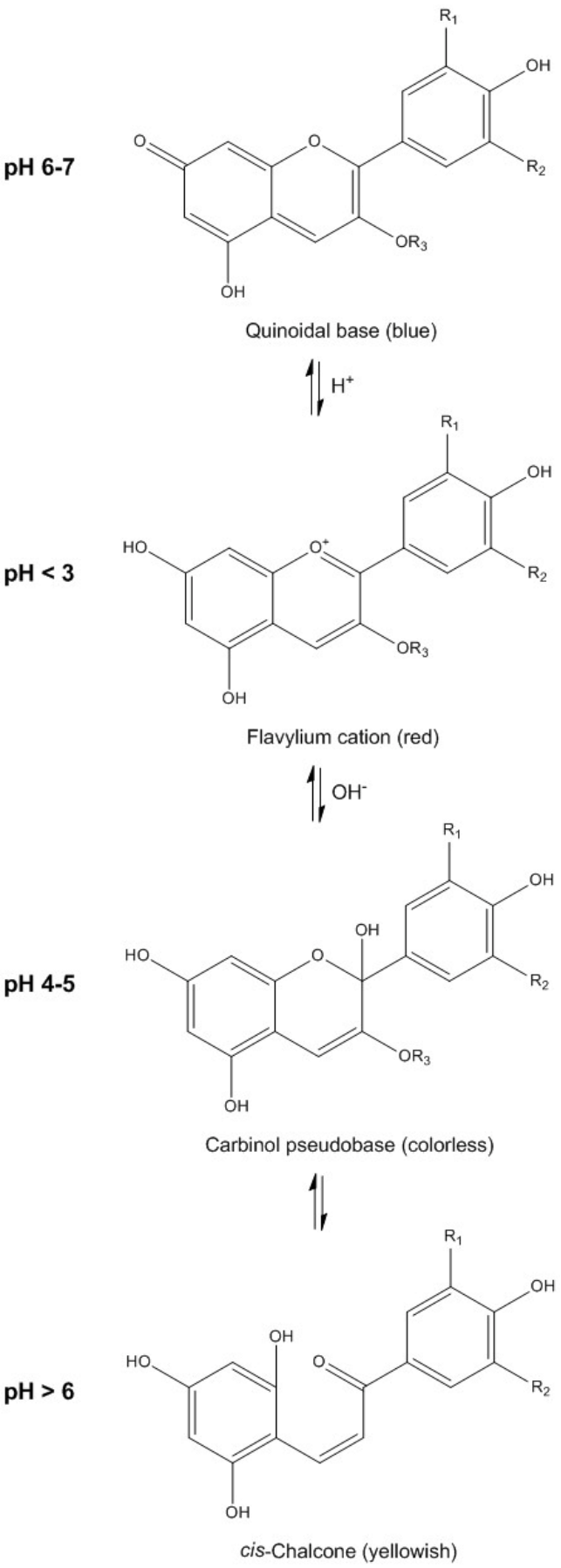


Figure 2.3 : Molecular structures of anthocyanins under different pH conditions.

The major anthocyanins identified in black carrots were cyanidin-based: cyanidin-3-xylosyl-glucosyl-galactoside, cyanidin-3-xylosyl-galactoside and the sinapic, ferulic and coumaric acids derivatives of cyanidin 3-xylosyl-glucosyl-galactoside (Wallace and Giusti, 2008; Montilla et al, 2011). Trace amount of pelargondin and peonidin glycosides have also been identified in black carrots (Figure 2.4) (Kammerer et al, 2003; Algarra et al, 2014). Total anthocyanin content in the roots of black carrots can vary widely between cultivars and even within a cultivar based on the degree of root coloring (Lazcano et al, 2001; Kammerer et al, 2004b). Black carrots are reported to contain up to 350 mg anthocyanins per 100 g fresh weight. For comparison, total anthocyanin concentration is approximately 113 mg/100 g in blueberries, 117 mg/100 g in cherries, and 48 mg/100 g in raspberries (Arscott and Tanumihardjo, 2010).

According to a report, anthocyanins accounted for about 42% of the total phenolics present in purple carrot extracts (Wallace and Giusti, 2008). Similarly, in another study, the level of anthocyanins corresponded to 50% of the total phenolic content of black carrots for Antonina variety (Algarra et al, 2014). Several studies are carried out to optimize the extraction of black carrot anthocyanins under different conditions (Gizir et al, 2008; Turker and Erdogdu, 2006; Guldiken et al, 2016). Shortening the extraction time by low pH and high temperature is found to be feasible for an industrial process (Turker and Erdogdu, 2006).

2.1.2.2 Phenolic acids

Phenolic acids, in general, describe phenols that possess one carboxylic acid functionality. However, when describing plant metabolites, it refers to a distinct group of organic acids. As described above, the naturally occurring phenolic acids contain two distinguishing constitutive carbon frameworks: the hydroxycinnamic and hydroxybenzoic structures. Although the basic skeleton remains the same, the numbers and positions of the hydroxyl groups on the aromatic ring create the variety. In many cases, aldehyde analogues are also grouped in with, and referred to as, phenolic acids (e.g., vanillin). Caffeic, *p*-coumaric, vanillic, ferulic, and protocatechuic are acids present in nearly all plants (Robbins, 2003).

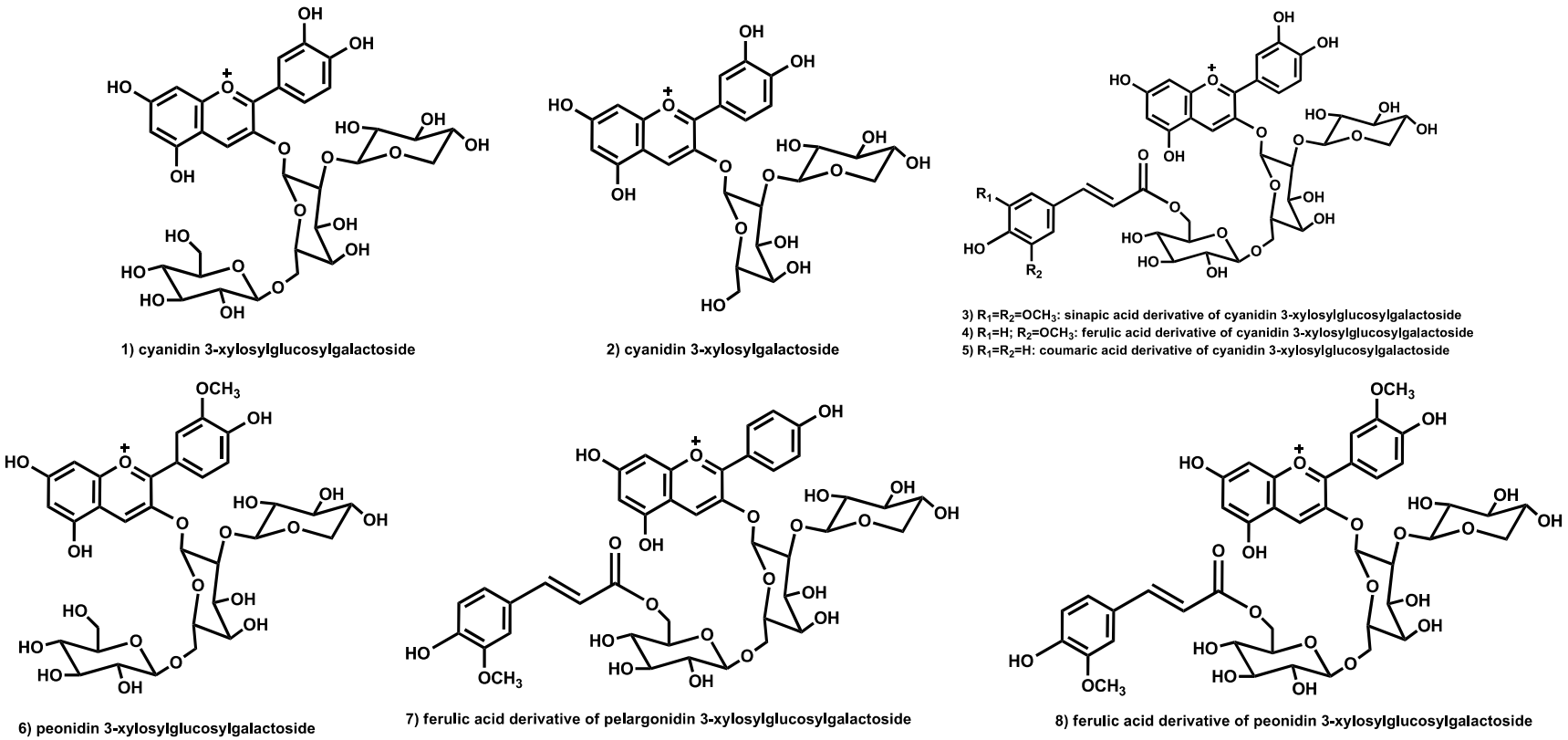


Figure 2.4 : Chemical structures of the anthocyanins detected in the black carrots.

Besides anthocyanins as the major polyphenols, black carrots also contain significant amounts of phenolic acids. The majority of the phenolic acids identified in black carrots were hydroxycinnamic acid derivatives. Chlorogenic acid (5-*O*-caffeoylquinic acid), an ester of caffeic and quinic acid (Figure 2.5), was found to be the predominant compound, amounting to 657 mg/kg in the roots of black carrot (Kammerer et al, 2004a). Studies showed that black/purple carrots contained higher phenolic content than roots of other colors (Grassmann et al, 2007; Sun et al, 2009; Leja et al, 2013; Koley et al, 2014).

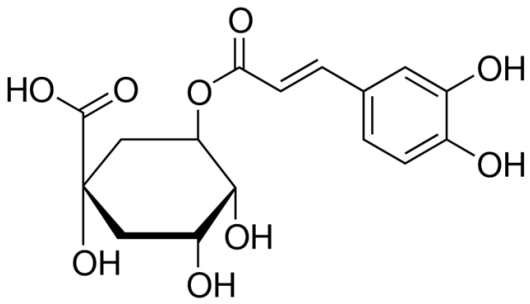


Figure 2.5 : Chemical structure of chlorogenic acid (5-*O*-caffeoylquinic acid).

2.1.3 Health effects

Several studies showed that black carrot polyphenols possess high *in vitro* antioxidant and antiproliferative activities. As an example, crude phenolic extract from black carrot concentrate showed strong radical scavenging activity, equivalent to or higher than that of other selected fresh fruits such as cherry, or berries: strawberry, raspberry and blueberry, that are considered to be the health-promoting fruits due to high polyphenol content (Figure 2.6) (Day et al, 2009). Furthermore, purified anthocyanin-rich polyphenol extract from black carrot inhibited proliferation of both HT-29 colorectal adenocarcinoma and HL-60 promyelocytic leukaemia cells in a dose-dependent manner (Netzel et al, 2007). Similarly, Olejnik et al (2016) indicated that purple carrot extract is capable of NCM460 colonic cells' protection against the adverse effects of oxidative stress including reactive oxygen species (ROS) production and DNA damage.

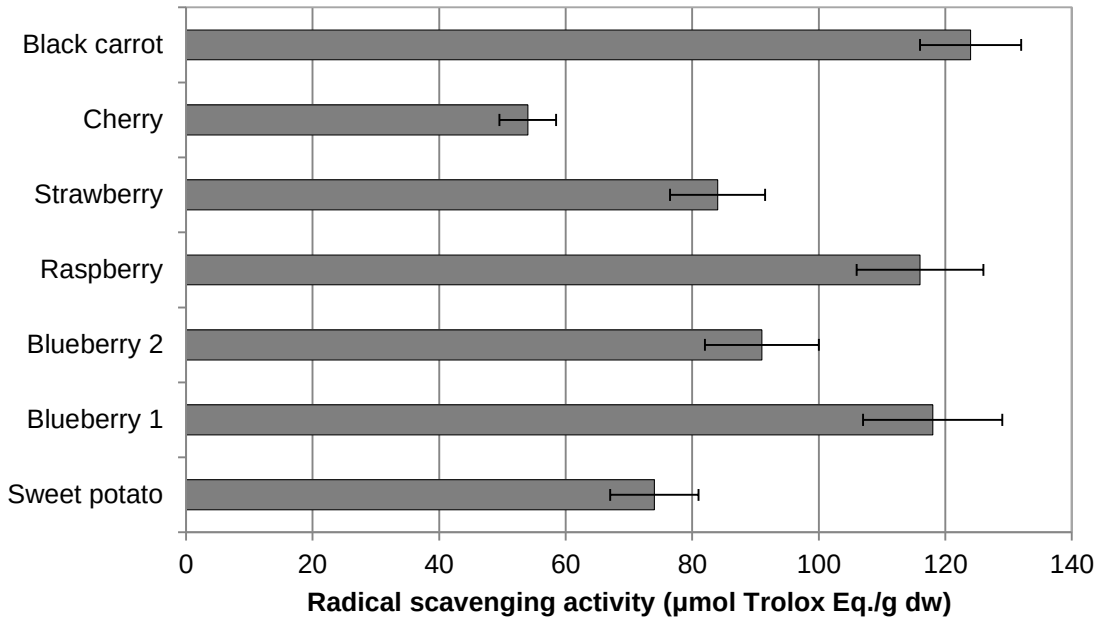


Figure 2.6 : Antioxidant activity of black carrot concentrate in comparison with other fruits (purchased at local market).

Anthocyanins and phenolic acids from black carrots were reported to be effective at reversing inflammation and metabolic alterations in animal models, potentially through inhibition of inflammatory pathways. For instance, purple carrot juice (5% of the diet) reduced abdominal obesity, systolic blood pressure, plasma lipids, hepatic steatosis, cardiac fibrosis, and inflammation along with improved glucose tolerance in high-carbohydrate, high-fat diet-fed rats (Poudyal et al, 2010). The authors reported that it is likely that the anthocyanins were responsible for the observed effects. Similarly, black carrot extracts fermented with *Aspergillus oryzae* prevented the impairment of energy, lipid and glucose metabolism in estrogen-deficient rats (Park et al, 2015). On the other hand, a human trial, which investigated the effects of dried purple carrot on body mass, body composition, blood pressure, lipids, inflammatory markers, liver function tests, and appetite in 16 volunteers with normal lipid and inflammatory markers, concluded that there was no evidence that 118.5 mg/day of anthocyanins and 259.2 mg/day of phenolic acids for 4 weeks resulted in statistically significant changes in body mass, body composition, appetite, dietary intake, low density lipoprotein, total cholesterol, blood pressure, or C-reactive protein in obese participants at the dose and length of intervention (Wright et al, 2013).

Black carrots also contain polyacetylene compounds such as falcarindiol, falcarindiol 3-acetate and falcarinol (Christensen and Kreutzmann, 2007). The polyacetylenes from purple carrots were reported to reduce the nitric oxide production in macrophage cells by as much as 65% without cytotoxicity. Considering that it has been suggested that polyacetylenes could also be responsible for anti-inflammatory activity of purple carrots (Metzger et al, 2008).

2.2 Effect of Food Processing and Storage on Black Carrot Polyphenols

Many fresh fruits and vegetables are subjected to some form of processing due to their seasonal and perishable nature. Effect of food processing on polyphenol content of fruits and vegetables has been studied extensively (Nicoli et al, 1999; Kalt, 2005; Kamiloglu et al, 2015d; Nayak et al, 2015; Rothwell et al, 2015). Although the impact of process differs widely according to the type of treatment, the conditions of the process applied, and also the variety, source or cultivation history of the fruit/vegetable used, for anthocyanins generally adverse effects have been reported (Patras et al, 2010).

The studies on the effect of processing on black carrot polyphenols are presented in Table 2.2. Heat treatment caused degradation of black carrot anthocyanins, which was more rapid at increased heating temperatures (Ersus and Yurdagel, 2007; Kirca et al, 2007; Murali et al, 2015). On the other hand, increased pH values caused only a small overall increase in anthocyanin degradation compared to values at low pHs (Iliopoulou et al, 2015). Thermal treatment resulted in a first-order reaction model of degradation for the monomeric anthocyanins in black carrots (Kirca et al, 2007; Reyes and Cisneros-Zevallos, 2007). Acylated anthocyanins were more resistant to thermal degradation compared to non-acylated anthocyanins. Upon severe heating, acylated anthocyanins are cleaved into the corresponding acyl-glycosides and the aglycones the latter being readily transformed into the intermediate chalcone, which is instantly cleaved into protocatechuic acid and phloroglucinaldehyde (Sadilova et al, 2007). In order to increase the stability of black carrot anthocyanins, microencapsulation methods have been employed. Studies showed that maltodextrin 20DE as the carrier material has proven to be better in retaining maximum anthocyanins (Ersus and Yurdagel, 2007; Murali et al, 2015). Sugar and ascorbic acid addition also resulted in a slight stabilizing effect of black carrot anthocyanins

(Sadilova et al, 2009). A study comparing the effect of different drying methods on anthocyanin content of various purple carrot roots revealed that for Deep Purple cultivar, the most advantageous drying method was microwave-convection drying, whereas convection drying caused the highest degradation. On the other hand, for roots of Purple Haze cultivar the most advantageous method was freeze-drying, while infrared convective drying was proved to be least advantageous (Witrowa-Rajchert et al, 2009). Pre-press maceration treatment with cell wall degrading enzyme pectinase (*Aspergillus niger* Teigh) significantly improved the total phenolic, flavonoid and anthocyanin content of black carrot juice (Khandare et al, 2011). Similarly, in other studies while depectinization resulted in increased content of anthocyanins (Turkyilmaz et al, 2012), total phenolics and hydroxycinnamic acids (Dereli et al, 2015), gelatine–kieselsol treatment led to reductions in polyphenols. Bentonite treatment and pasteurization resulted in varying effects, causing both reductions and enhancements in polyphenol content of black carrot juice (Turkyilmaz et al, 2012; Dereli et al, 2015). Processing black carrots into concentrate led to reductions in phenolics, flavonoids and anthocyanins (Suzme et al, 2014). Differences observed in degradation mechanisms of anthocyanins under solid and aqueous conditions. In particular, at high pHs anthocyanins at solid state were reported to be significantly more stable compared to aqueous conditions. Moreover, the stability of the acylated anthocyanins was markedly higher in the solid state than in the liquid form (Iliopoulou et al, 2015).

Although in general food processing decreased the anthocyanin content in black carrots, stability of anthocyanins to pH and thermal degradation was reported to be higher compared to anthocyanins from grape and purple-flesh potatoes (Reyes and Cisneros-Zevallos, 2007). Similarly, black carrot juice anthocyanins exhibited higher retention towards thermal treatment compared to elderberry and strawberry juices. Better stability may be attributed to the high ratio of acylated anthocyanins in black carrot samples compared to the non-acylated pigments (Sadilova et al, 2009).

Table 2.2 : Studies investigating the effect of processing on black carrot polyphenols.

Sample	Processing conditions	Major conclusions	Reference
Black carrot extract	Microencapsulation by spray drying (160–200 °C)	• Higher air inlet temperatures caused higher ACN losses. • 20–21 DE maltodextrin gave the highest ACN content.	(Ersus and Yurdagel, 2007)
Black carrot concentrate	Thermal treatment at 70–90 °C, 11–64 °Brix and pH 4.3, 6.0	• ACN degradation followed a first-order reaction model. • The degradation rate of ACNs increased with increased heating temperature and solid content.	(Kirca et al, 2007)
Purified black carrot anthocyanins	Heating at 95°C and pH 3.5 for 6 h	• Acylated ACNs were more resistant to degradation compared to non-acylated ACNs. • Acylated ACNs are cleaved into the corresponding acyl-glycosides and the aglycones the latter being readily transformed into the chalcone, which is cleaved into protocatechuic acid and phloroglucinaldehyde.	(Sadilova et al, 2007)
Black carrot juice	Sugar and ascorbic acid addition	• The retention of black carrot ACNs upon saccharide and ascorbic acid supplementation ranged from 50.4% to 58.1%, with the retention of unsupplemented juices being 48.3%.	(Sadilova et al, 2009)
Purple carrot roots	Convective drying (70 °C, 1.5 m/s), microwave-convective drying (40 °C, 300 W), infrared convective drying (7.875 kW/m^2, 1.2 m/s), and freeze drying (–70 °C, 63 Pa)	• For Deep Purple cultivar, the most advantageous drying method was microwave-convection drying (20% reduction in ACNs), whereas convection drying caused the highest degradation (about 50%). • For Purple Haze cultivar, the most advantageous method was freeze-drying (30% loss of ACNs), while infrared convective drying was the least advantageous.	(Witrowa-Rajchert et al, 2009)
Black carrot juice	Pre-press maceration treatment with pectinase	• Enzyme-assisted processing increased the TPC (27%), TFC (46%) and ACNs (100%).	(Khandare et al, 2011)

Table 2.2 (continued) : Studies investigating the effect of processing on black carrot polyphenols.

Sample	Processing conditions	Major conclusions	Reference
Black carrot juice	Clarification and pasteurization	• Depectinization and bentonite treatments resulted in increases (7% and 20%, respectively) in ACNs. • Gelatine–kieselsol treatment and pasteurization resulted in reduction (10% and 3–16%) in ACNs.	(Turkyilmaz et al, 2012)
Black carrot	Concentrate processing	• Processing black carrot into concentrate led to an overall reduction of 70%, 73% and 44% in TPC, TFC and ACNs, respectively.	(Suzme et al, 2014)
Black carrot juice	Clarification and pasteurization	• Depectinization (13 and 59%) and pasteurization (1.1- and 2.3-fold) led to increases in the TPC and HCAs. • Bentonite (10 and 7%) and gelatin–kieselsol (25 and 29%) treatments led to reductions in TPC and HCAs.	(Dereli et al, 2015)
Black carrot powder and solution	Freeze drying at pH 3.5 and 6.8 and thermal treatment at 180 °C for 1 h	• At pH 6.8, there was only a small overall increase in ACN degradation compared to pH 3.6. • For the samples at pH 3.6, the degradation properties of ACNs in the powder were similar to those of the solution, whereas at pH 6.8 powder was significantly more stable than in solution. • At pH 6.8, the stability of the acylated ACNs was markedly higher in the powder than in the solution.	(Iliopoulou et al, 2015)
Black carrot juice	Encapsulation with spray drying (150–225 °C) and freeze drying (–53 °C, 0.11–0.22 mbar)	• Maltodextrin 20DE as the carrier material has proven to be better in retaining ACNs compared to gum arabic and tapioca starch. • The best spray dried product, was obtained at 150 °C. • Freeze dried product was the most acceptable one with maximum ACNs.	(Murali et al, 2015)

ACN: anthocyanin; HCA: hydroxycinnamic acid; TFC: total flavonoid content; TPC: total phenolic content.

Studies investigating the stability of black carrot polyphenols during storage are presented in Table 2.3. In general, storage at higher temperatures resulted in faster anthocyanin degradation as compared to storage at lower temperatures (Turker et al, 2004; Ersus and Yurdagel, 2007; Kirca et al, 2007; Ozen et al, 2011; Turkyilmaz and Ozkan, 2012). In fact, anthocyanin content of black carrots did not change significantly during storage at cold temperatures (Lee et al, 2011; Zozio et al, 2011). Acylated antocyanins were significantly more stable than the non-acylated anthocyanins at all storage temperatures (Turker et al, 2004; Turkyilmaz and Ozkan, 2012). Pasteurization and sorbate treatment showed no significant positive or negative effects on total anthocyanin content or on the anthocyanin profile with respect to control at any storage temperature ($p < 0.05$) (Turker et al, 2004). The degradation of anthocyanins in purple carrots was particularly significant in the modified atmosphere packaging treatment of 95% O_2 and 5% CO_2 ($p < 0.05$) (Alasalvar et al, 2005). On the other hand, yogurt matrices containing higher fat contents showed increased stability of anthocyanins during two months of storage (Wallace and Giusti, 2008). Several studies confirmed that the degradation of black carrot anthocyanins during storage at high temperatures fitted a first-order reaction model (Kirca et al, 2007; Ozen et al, 2011; Turkyilmaz and Ozkan, 2012; Zozio et al, 2011; Wallace and Giusti, 2008). Although thermal storage reduced the amount of black carrot anthocyanins, the rate of degradation has been shown to be slower than açai and blackberry anthocyanins (Zozio et al, 2011).

To sum up, previous studies already examined the effect of several processing and storage conditions on black carrot polyphenols (Table 2.2, 2.3). However, no previous study investigated the influence of jam/marmalade processing and storage. Therefore, as a part of this research, this important topic is investigated (*Chapter 3*). Moreover, as a result of processing, large amounts of by-products are generated. These by-products are often discarded as waste or, in the best case valorized in low-value applications such as animal feed and bio-energy applications. However, these by-products have the potential to be used as relatively cheap but valuable resources for bioactive compounds. Considering that, in this study, black carrot processing by-products are characterized for the first time (*Chapter 4*).

Table 2.3 : Studies investigating the effect of storage on black carrot polyphenols.

Sample	Storage conditions	Major conclusions	Reference
Fermented black carrot beverage (Shalgam)	4, 25, and 40 °C for 90 days	• The effect of 40 °C storage temperature ACNs was significant, while there was no significant difference between 4 and 25 °C. • Acylated ACNs retained 7.9–48.9% of their initial level, whereas nonacylated ACNs retained only 0–11.1% of their initial level after storage at 40 °C. • There were no significant effects of pasteurization and sorbate treatment on the total ACN content or the ACN profile with respect to control at any storage temperature.	(Turker et al, 2004)
Ready-to-eat shredded purple carrots	Chilled temperature (5 ± 2 °C) in air, or in modified atmosphere packaging ($90\%N_2+5\%O_2+5\%CO_2$ and $95\%O_2+5\%CO_2$) for 13 days	• ACNs showed no significant decrease for all treatments over the entire storage period, except for modified atmosphere packaging treatment ($95\%O_2+5\%CO_2$), which significantly decreased on day 13 (≈10%).	(Alasalvar et al, 2005)
Microencapsulated pigments of black carrot	4 and 25 °C	• Storage at 4 °C increased half life of spray dried ACN pigments 3 times more compared to 25 °C storage temperature.	(Ersus and Yurdagel, 2007)
Black carrot concentrate	4, 20 and 37 °C and 30, 45 and 64 °Brix	• ACN degradation during storage fitted a to first-order reaction model. • Storage at 37 °C resulted in a much faster ACN degradation as compared to storage at 4 °C ($t_{1/2}$ = 4.0–4.5 and 71.8–215 weeks, respectively).	(Kirca et al, 2007)
Yoghurt with purple carrot extract	4 °C for 8 weeks	• A significant decrease in ACNs was noted in all yogurt fat matrices over the storage period ($p < 0.01$). • ACN degradation followed first order kinetics. • Yogurt matrices containing higher fat contents showed increased stability of ACNs.	(Wallace and Giusti, 2008)

Table 2.3 (continued) : Studies investigating the effect of storage on black carrot polyphenols.

Sample	Storage conditions	Major conclusions	Reference
Sliced purple carrots	2, 4 °C for 4 weeks	• ACNs did not change significantly at either temperature.	(Lee et al, 2011)
Turkish delight colored with black carrot juice concentrate	12, 20 and 30 °C for 5 months	• Kinetic data suggested a first-order reaction for the degradation of ACNs. • Degradation rates of ACNs increased with increasing temperature.	(Ozen et al, 2011)
Soft drink colored with black carrot extract	4, 20, 30 and 50 °C for 60 days	• No degradation was detected during the refrigerated storage (4 °C). • Degradation of the ACNs at higher temperatures followed a first-order kinetic model. • Black carrot ACNs degraded more slowly than açai and blackberry ACNs during thermal storage.	(Zozio et al, 2011)
Black carrot juice concentrate	−23, 5 and 20 °C for 319 days and at 30 °C for 53 days	• ACN degradation was fitted to a first-order reaction model. • Acylated ACNs were more stable during storage. • At sub-freezing temperatures ACN degradation was minimum.	(Turkyilmaz and Ozkan, 2012)

ACN: anthocyanin; $t_{1/2}$: time needed for 50% degradation of anthocyanins.

2.3 Bioavailability of Polyphenols: A Complex Field of Research

2.3.1 Factors affecting the bioavailability of polyphenols

Bioavailability is an important aspect when studying the role of polyphenols in human health. The interest in this aspect is increasing as food companies continuously develop new products, defined as "functional foods" by the presence of specific antioxidants or phytochemicals (Porrini and Riso, 2008).

The term "bioavailability" was originally used in pharmacology to define the "rate and extent to which a drug reaches its site of action" (Stahl et al, 2002; D'Archivio et al, 2007; Holst and Williamson, 2008). Although several other definitions of bioavailability have been stated, the most suitable one appears to be the fraction of an ingested nutrient or compound that reaches the systemic circulation and the specific sites where it can exert its biological action (Porrini and Riso, 2008). In other words, it shows how much of the ingested quantity of the nutrient is able to exert its beneficial effects in target tissues (D'Archivio et al, 2010). Another term that is commonly used is "bioaccessibility", which is defined as the amount of an ingested nutrient that is available for absorption in the gut after digestion (Tedeschi et al, 2009; Palafox-Carlos et al, 2011). When the amount of recovered nutrient after digestion is of concern then the term "bioaccessibility" is used. In a critical appraisal, the main factors recognized as affecting bioavailability in humans were discussed and gathered under four main categories: (i) factors related to the compound (e.g. chemical structure, molecular linkage); (ii) factors related to the food/preparation (e.g. matrix characteristics, processing); (iii) factors related to the host (e.g. enzyme activity, genetics) and (iv) external factors (e.g. food availability, different environmental factors such as climate) (Porrini and Riso, 2008).

Polyphenols are highly diverse compounds with varying bioavailability. For instance, while soy isoflavones are well absorbed through the gut barrier, proanthocyanidins in wine and cacao are hardly absorbed. Similarly, quercetin glucosides from onions are more efficiently absorbed than quercetin glycosides such as rutin present in tea or apple (Manach et al, 2005). Therefore it is not possible to generalize the outcomes obtained for one polyphenol to others.

2.3.2 Methods used to assess the bioavailability of polyphenols

Methods for determining bioavailability and/or bioaccessibility of polyphenols involve human (*in vivo*) or simulated experiments performed in a laboratory (*in vitro*). *In vivo* methods provide direct data of bioavailability and have been used for a great variety of nutrients (Hamdan et al, 2009). Generally a response is measured after consumption of a pure nutrient either by humans or animals, and compared to an equivalent nutrient dose found in a food source (Yeum and Russell, 2002). Therefore, definitive conclusions on the bioavailability of a single phenolic compound are difficult to obtain, because of the synergistic effects of the mixture of polyphenols contained in each food matrix (D'Archivio et al, 2010). Mostly, *in vivo* bioavailability studies refer to the consumption of certain dose of a nutrient and following changes of its concentration in the blood plasma and compared with time. The main drawbacks of *in vivo* data are the variability in physiological state of individuals and the possible interaction of the nutrient with other components in the diet (Parada and Aguilera, 2007). Moreover, such studies carried out in humans and/or animals are complicated, costly and raise ethical issues (McDougall et al, 2005).

In vitro methods for simulating the human digestive tract are being extensively used at present since they are rapid, safe, and do not have the same ethical restrictions as *in vivo* methods (Sannaveerappa et al, 2007; You et al, 2010; Liang et al, 2012). *In vitro* methods either simulate the digestion and absorption processes or only the digestion process and the response measured is the concentration of a nutrient in the final extract (Briones-Labarca et al, 2011). The digestion process is simulated under controlled conditions using commercial digestive enzymes such as pepsin and pancreatin, while the final absorption process is commonly assessed using dialysis bags or Caco-2 cell cultures (Parada and Aguilera, 2007; Ercan and El, 2012).

2.3.2.1 *In vitro* gastrointestinal digestion models

The methods that mimic the gastrointestinal digestion process under laboratory conditions are known as *in vitro* gastrointestinal digestion models. Generally, *in vitro* gastrointestinal digestion models are divided into two broad categories: Static models, where the products of digestion stay generally immobile and physical processes such as shear, mixing and hydration are not simulated, and dynamic

models which try to integrate physical and mechanical processes and temporal changes in luminal conditions to mimic parameters *in vivo* (Hamdan et al, 2009; Parada and Aguilera, 2007).

In static *in vitro* digestion models, the stomach and the upper part of the small intestine are simulated by addition of salts and enzymes, adjustment of pH and incubation at 37 °C. During the initial gastric phase, samples are incubated at gastric pH in the presence of gastric enzymes such as pepsin. The upper part of the small intestine, the duodenum, is then simulated by increasing the pH and addition of pancreatic enzymes and bile salts. Some models also contain an initial oral digestion phase, which mimics mastication in the presence of the enzyme amylase (Bengtsson et al, 2009). Moreover, there are also some models that include colon fermentation (Serrano et al, 2005).

The dynamic *in vitro* digestion models, such as the TNO gastrointestinal digestion model (TIM) and simulator of human intestinal microbial ecosystem (SHIME) have the same basic principles of static models, however they simulate better the conditions in the gastrointestinal tract. In general, dynamic models have advantages over static models including continuous removal of nutrients, simulation of peristalsis and more controlled, gradual changes in pH and enzyme levels (Svelander, 2011). The TIM model contains four serial sections simulating the stomach, duodenum, jejunum and ileum (Mitea et al, 2008). The SHIME consists of five reactors, which sequentially simulate the stomach, small intestine and the three regions of the large intestine, i.e. the ascending, transverse and descending colon (Molly et al, 1993). Careful control of the environmental parameters in these reactors allows obtaining complex and stable microbial communities, which are highly similar in both structure and function to the microbial community in the different regions of the human colon. A typical SHIME experiment consists of four stages: a stabilization period (2 weeks) to allow adaptation of the microbial community (high quality of the inoculum) to the environmental conditions in the respective colon regions; a basal period (2 weeks) in which the system reactor is operated under nominal conditions and basal parameters are measured; a treatment period (2/3 weeks) where the effect of a specific compound on the gastrointestinal microbial community is tested; and a washout period (2 weeks) to determine how long the

changes induced by the tested substance can still be measured in the absence of the substance itself.

So far, the *in vitro* digestion methods have already been tested for foods rich in polyphenols including orange juice (Gil-Izquierdo et al, 2001), pomegranate juice (Perez-Vicente et al, 2002), berries (McDougall et al, 2005; Bermudez-Soto et al, 2007; Liang et al, 2012), cherries (Fazzari et al, 2008; Toydemir et al, 2013b), grapes (Tagliazucchi et al, 2010) and apples (Bouayed et al, 2011), and it has been proven that the measurement of bioavailability by *in vitro* gastrointestinal digestion models can be well correlated with conclusions from human studies and animal models (Bouayed et al, 2011). To the best of our knowledge, current study is the first research reporting the fate of polyphenols from processed black carrots and their by-products during *in vitro* gastrointestinal digestion (*Chapters 3-5*).

2.3.2.2 Caco-2 cells

The Caco-2 cell line has been established by Fogh and co-workers in 1977 from a human colon adenocarcinoma, and originally used for the screening of cytotoxic effects of anti-tumor drugs and for the study of drug resistance mechanisms (Zucco et al, 2005). During the past few decades, this cell line has been extensively used for cellular permeability studies of polyphenols (Manna et al, 2000; Deprez et al, 2001; Konishi and Kobayashi, 2004; Manzano and Williamson, 2010). It has been well established that Caco-2 cells can undergo spontaneous differentiation in culture conditions and exhibit the characteristics of mature enterocytes. The cell surface facing the top medium develops a brush border that resembles the luminal membrane of the intestinal epithelium. The cell surface attaching to the permeable membrane and facing the bottom medium develops into the basolateral membrane (Yi et al, 2006; Zhang et al, 2014). Despite their colonic origin, Caco-2 cells express the morphological and functional characteristics of small intestinal cells. The Caco-2 monolayer houses multiple transporters, receptors and metabolic enzymes such as cytochrome P450 1A, sulfotransferases, UDP-glucuronosyltransferases, and glutathione S-transferases (Sun et al, 2008).

Transport experiments are generally carried out using filter-based inserts, where cells are seeded and allowed to grow and differentiate to confluent monolayers for approximately 21 days post seeding. Before performing the transport experiment, the

integrity of the Caco-2 monolayer is controlled by measuring the transepithelial electrical resistance (TEER), or, more reliably, by examining the permeability of paracellular markers such as mannitol, inulin, Dextran, PEG 4000, and Lucifer yellow (Sun et al, 2008). TEER is a non-invasive technique, which measures the impedance between the lumen and basolateral tissue. TEER measurements use a constant direct current applied by two electrodes, one connected with the lumen side and the other one with the basolateral side. By applying Ohm's law it is possible to measure the related cells resistance (Finotti et al, 2015). It was reported in the literature that an acceptable TEER value for Caco-2 cell monolayers should be from 200 to 1000 ohm per cm^2 (Sabboh-Jourdan et al, 2011). Since its original isolation, the Caco-2 cell line has been propagated in several laboratories around the world, producing Caco-2 cells of different "age", or number of passages in culture. The expressions of typical differentiation markers of intestinal enterocytes were shown to increase from early to late passages. Accordingly, the TEER value has also been demonstrated to increase in later passages of cell monolayer (Sambuy et al, 2005).

In order to reduce the heterogeneity of the Caco-2 parental cell line and to improve the performance and the stability of this cellular model, some clonal cell lines have been obtained from Caco-2. Among them, TC-7 is often used to simulate polyphenol transport (Barrington et al, 2009; Pereira-Caro et al, 2010; Soler et al, 2010). The TC-7 clone exhibited similar cell morphology to Caco-2 cells, displaying the presence of brush-border membrane and microvilli, and the formation of tight junctions. Similarly, on the basis of biochemical attributes and permeability characteristics, the TC-7 subclone appears to be similar to Caco-2 cells and presents a suitable alternative to parental cells for intestinal permeability studies (Balimane and Chong, 2005). The HT-29 cell line is another cell line from colorectal origin with epithelial morphology, and has been used as a model for absorption, secretion and transport by intestinal cells. Under standard culture conditions, these cells grow as a non-polarized, undifferentiated monolayer. However, altering culture conditions or treating the cells with different inducers results in a differentiated and polarized morphology, characterized by a redistribution of membrane antigens and development of an apical brush-border membrane (Zweibaum et al, 1985). Other human intestinal cell lines are less popular for the simulation of the human intestinal epithelium, such as the HCT-116 and SW480 cell lines, which are mainly used in

unraveling cancer-related mechanisms (Seeram et al, 2004), and the HuTu-80 cell line, a model for duodenal cells (Ackland et al, 2005).

As anthocyanins are the major polyphenols present in black carrots, here, we focused on anthocyanin transport and metabolism through Caco-2 cells.

Anthocyanin transport through Caco-2 cells

Studies investigating anthocyanin absorption by Caco-2 cells are presented in Table 2.4. The majority of these studies suggest that unlike other flavonoids, anthocyanins could be transported through Caco-2 monolayers in intact glycone forms, with the exceptions of black currant and some grape anthocyanins. Steinert et al (2008) demonstrated that anthocyanins from black currant, namely delphinidin-3-glucoside, delphinidin-3-rutinoside, cyanidin-3-glucoside and cyanidin-3-rutinoside, were not detected in any serosal solution. However, the authors showed that anthocyanins disappeared from the luminal side, not due to the anthocyanin degradation process but rather due to physiological actions of the cells. Similarly, delphinidin-3-glucoside from grape extract was not transported (Kuntz et al, 2015a). Diglucosylated malvidin-3,5-diglucoside and peonidin-3,5-diglucoside from grape/blueberry extract were also not transported in quantifiable concentrations (Kuntz et al, 2015b). On the other hand, studies that observed anthocyanin transport, reported very low transport efficiencies. The transport efficiency of anthocyanins from blueberry extracts averaged ca. 3%–4% (<1% in delphinidin-3-glucoside) (Yi et al, 2006). Similarly, only about 1% of the red grape skin anthocyanins passed through a Caco-2 cell monolayer and reached the basolateral side (Faria et al, 2009). The percentage of transported monomeric anthocyanin glycosides from açaí fruit ranged from 0.5% to 4.9% (Pacheco-Palencia et al, 2010), whereas according to Cardona et al (2015) the transport rate of açaí anthocyanins was 1.2%. Transport efficiencies of malvidin-3-glucoside and cyanidin-3-glucoside standards were found to be 4% and 0.8%–2.4%, respectively (Fernandes et al, 2012b; Zou et al, 2014). Moreover, cyanidin-3-glucosyl-rutinoside recovery from sour cherry fruit and nectar was ca. 0.5%–4% (Toydemir et al, 2013a). Trace amount of pelargonidin-3-glucoside, the predominant anthocyanin from strawberry extract, was found on the basolateral side of the epithelium (Kosinska-Cagnazzo et al, 2015). Transport efficiency of the major grape anthocyanin (malvidin-3-glucoside) was 0.35% (Kuntz et al, 2015a), while the absorption rates of malvidin-3-glucoside, peonidin-3-glucoside, petunidin-3-

glucoside, delphinidin-3-glucoside and cyanidin-3-glucoside from grape/blueberry extract were 0.005%–0.06% (Kuntz et al, 2015b). These results are in line with *in vivo* studies showing a very low bioavailability of anthocyanins, with <1% of the ingested amount reaching the plasma or being excreted in the urine (Netzel et al, 2001; Frank et al, 2003; Kurilich et al, 2005; Charron et al, 2009; Milbury et al, 2010)

Few studies compared the transport efficiency of anthocyanins across Caco-2 cells with other polyphenols. The transport of both malvidin-3-glucoside and catechin through Caco-2 cells was found to be time dependent and reached approximately the same value (4%) after 120 min of incubation (Fernandes et al, 2012b). Similarly, the recovery of epicatechin in the basolateral side (1%–4%) was also about the same with cyanidin-3-glucosyl-rutinoside (0.5%–4%) (Toydemir et al, 2013a). Reported transport of some other flavonoids through Caco-2 cells was 30% for quercetin, 17% for genistein and 6% for epicatechin (Tian et al, 2009).

The aglycone structure of anthocyanins is one of the many factors influencing their transport. For instance, delphinidin-3-glucoside from blueberry extract showed lower transport efficiency compared to malvidin-3-glucoside and peonidin-3-glucoside. This may be a result of the higher number of hydroxyl groups in delphinidin or the greater hydrophobic structure of malvidin that facilitated an increased partioning into cells and tissues. In addition, delphinidin has no OCH_3 group, while peonidin has one and malvidin has two OCH_3 groups (Figure 2.2), indicating that hydrophilic and hydrophobic groups affect the absorption of anthocyanins (Yi et al, 2006). Similarly, for black currant anthocyanins the loss of delphinidins was significantly higher than cyanidins. Thus, the structural features might be crucial for anthocyanin stability (Steinert et al, 2008).

Table 2.4 : Studies investigating anthocyanin absorption by Caco-2 cells.

Sample	Pre-treatment	Anthocyanins	Anthocyanin concentration	Cell origin	Cell differentiation	Incubation time	Key findings	Reference
Blueberry	Chemical extraction	Dp-3-Glu, Cy-3-Gal, Cy-3-Glu, Pt-3-Glu, Pn-3-Gal, Pn-3-Glu, Mv-3-Glu	50 μg/mL	ATCC	20-26 days	0-2 h	• Transport efficiency of ACNs averaged ca. 3-4% (<1% in Dp-3-Glu). • Glucose-based ACNs had higher bioavailability than galactose-based ACNs.	(Yi et al, 2006)
Black currant extract	-	Dp-3-Glu, Dp-3-Rut, Cy-3-Glu, Cy-3-Rut	180 μM	DSWZ	19-21 days	0-80 min	• ACNs were not detected in any serosal solution.	(Steinert et al, 2008)
Red grape skin	Chemical extraction	Dp-3-Glu, Cy-3-Glu, Pt-3-Glu, Pn-3-Glu, Mv-3-Glu	200 μg/mL	ATCC	25 days	4 days of pre-treatment + 6 min	• Only ca. 1% of ACNs are transported. • ACN transport significantly increased in the presence of ethanol. • Cells pre-treated with ACNs showed ca. 50% increased transport. • GLUT2 may be responsible for ACN transport.	(Faria et al, 2009)
Açaí pulp	Chemical extraction	Cy-3-Rut, Cy-3-Glu	50-500 μg/mL	ATCC	21 days	0.5-2 h	• Transport efficiency of ACNs was 0.5-4.9%. • Presence of polymeric ACNs decreased transport of monomeric ACN glycosides (up to 40.3%).	(Pacheco-Palencia et al, 2010)

Table 2.4 (continued) : Studies investigating anthocyanin absorption by Caco-2 cells.

Sample	Pre-treatment	Anthocyanins	Anthocyanin concentration	Cell origin	Cell differentiation	Incubation time	Key findings	Reference
Standard	-	Cat-Mv-3-Glu, Mv-3-Glu	100 μM	n/a	21 days	30-120 min	• Transport efficiency of Mv-3-Glu was 4%. • Absorption efficiency of Cat-Mv-3-Glu was lower than Mv-3-Glu (ca. 3%).	(Fernandes et al, 2012b)
Sour cherry fruit and nectar	Chemical extraction	Cy-3-Glu-Rut	55 μM	ATCC	23-24 days	6 h	• Cy-3-Glu-Rut recovery was ca. 0.5-4%. • Cy-3-Glu-Rut transported 3 times more efficiently from nectar than fruit. • Sucrose and citric acid enhanced the transport of Cy-3-Glu-Rut (ca. 5-fold). • SPE reduced the transport efficiency of Cy-3-Glu-Rut by 5-10 fold.	(Toydemir et al, 2013a)
Standard	Encapsulation	Cy-3-Glu	37.5 μM	n/a	20-26 days	1 h	• Nano-encapsulated Cy-3-Glu with apoferritin was more efficiently transported compared to free Cy-3-Glu.	(Zhang et al, 2014)
Standard	-	Cy-3-Glu	10-40 μmol/L	ATCC	13 days	30-120 min	• Transport efficiency of Cy-3-Glu was 0.8-2.4%. • Phloridzin and phloretin inhibited the absorption of Cy-3-Glu. • SGLT1 and GLUT2 are involved in the absorption of Cy-3-Glu.	(Zou et al, 2014)

Table 2.4 (continued) : Studies investigating anthocyanin absorption by Caco-2 cells.

Sample	Pre-treatment	Anthocyanins	Anthocyanin concentration	Cell origin	Cell differentiation	Incubation time	Key findings	Reference
Açaí concentrate	Chemical extraction	Cy-3-Glu, Cy-3-Rut	500 mg/L	ATCC	18-21 days	0-2 h	• Transport rate of ACNs was 1.22%. • Phospholipids from soy lecithin and terpenes from cold pressed citrus oil increased the transport of ACNs.	(Cardona et al, 2015)
Strawberry	Chemical extraction + *in vitro* digestion	Pg-3-Glu, Pg-3-Mal-Glu, Cy-3-Glu	16.3 mg/100 g	ATCC	21 days	120 min	• Trace amount of Pg-3-Glu was transported.	(Kosinska-Cagnazzo et al, 2015)
Grape	Chemical extraction	Mv-3-Glu, Pn-3-Glu, Pt-3-Glu, Cy-3-Glu, Dp-3-Glu	1766.1 mg/L	ATCC	21 days	30-240 min	• Mv-3-Glu, Pn-3-Glu, Pt-3-Glu and Cy-3-Glu were transported, whereas Dp-3-Glu was not transported. • Transport efficiency of major anthocyanin (Mv-3-Glu) was 0.35%.	(Kuntz et al, 2015a)
Grape/ blueberry extract	-	Mv-3-Glu, Pn-3-Glu, Pt-3-Glu, Dp-3-Glu, Cy-3-Glu, Mv-3,5-DGlu, Pn-3,5-DGlu	2613 µmol/L	ATCC	21 days	0-90 min	• Absorption rates of Mv-3-Glu, Pn-3-Glu, Pt-3-Glu, Dp-3-Glu and Cy-3-Glu were 0.005-0.06%. • Mv-3,5-DGlu and Pn-3,5-DGlu were not transported in quantifiable concentrations.	(Kuntz et al, 2015b)

ACN: anthocyanin; ATCC: American type culture collection; Cy-3-Gal: cyanidin-3-galactoside; Cy-3-Glu: cyanidin-3-glucoside; Cy-3-Glu-Rut: cyanidin-3-glucosylrutinoside; Cy-3-Rut: cyanidin-3-rutinoside; Dp-3-Glu: delphinidin-3-glucoside; Dp-3-Rut: delphinidin-3-rutinoside; DSMZ: German collection of microorganisms and cell cultures; GLUT2: glucose transporter 2; Mv-3-Glu: malvidin-3-glucoside; Mv-3,5-DGlu: malvidin-3,5-diglucoside; n/a: not available; Pg-3-Glu: pelargonidin-3-glucoside; Pg-3-Mal-Glu: pelargonidin-3-malonyl-glucoside; Pn-3-Gal: peonidin-3-galactoside; Pn-3-Glu: peonidin-3-glucoside; Pn-3,5-DGlu: peonidin-3,5-diglucoside; Pt-3-Glu: petunidin-3-glucoside; SGLT1: sodium-dependent glucose transporter 1, SPE: solid phase extraction.

Sugar moieties and polymeric structures may also have an influence on anthocyanin absorption by Caco-2 cells. For blueberry extracts, glucose-based anthocyanins had higher bioavailability than galactose-based anthocyanins. (Yi et al, 2006). On the other hand, for black currant anthocyanins no differences are shown between the respective glucose and rutinose sugar moieties indicating that sugar conjugates may have a minor effect on anthocyanin stability (Steinert et al, 2008). The presence of polymeric anthocyanins in açaí fruit decreased the transport of monomeric anthocyanins glycosides in a dose-dependent manner by up to 40.3% (Pacheco-Palencia et al, 2010). Similarly, the absorption efficiency of flavanol-anthocyanin dimer catechin-malvidin-3-glucoside, an anthocyanin derivative reported in grape skins and red wine, was lower than malvidin-3-glucoside (ca. 3%) (Fernandes et al, 2012b).

The presence of other food components has been shown to have a major impact on anthocyanin transport. Solid phase extraction (SPE) of sour cherry extracts reduced the transport efficiency of cyanidin-3-glucosyl-rutinoside by 5–10-fold (Toydemir et al, 2013a). Ethanol, one of the main constituents of red wine, improved anthocyanin transport through Caco-2 cells (Zou et al, 2014). However, this hypothesis is open to debate as there are some *in vivo* reports (Bub et al, 2001; Andlauer et al, 2003) claiming that ethanol has no influence on anthocyanin absorption. The ethanol concentration used in the cell culture study (1%), which was non-toxic to Caco-2 cells (Zou et al, 2014), is much lower than the actual ethanol concentration in red wine. Therefore the impact of ethanol on anthocyanin absorption and bioavailability may depend on the models as well as the doses used (Zou et al, 2014). Citric acid also enhanced anthocyanin transfer to the basolateral side of Caco-2 cells (Toydemir et al, 2013a). This may be linked to the fact that anthocyanins are more stable at low pH values (Fang, 2014a; Oliveira et al, 2015a) (Figure 2.3). This effect of pH on transport across Caco-2 cells may have some physiological relevance. Although the cellular interstices and blood have a pH of around 7.4, the pH in the upper intestinal tract under fasting conditions ranges from 5.0 to 6.5. In addition, the pH of the acidic microclimate just above the epithelial cell layer has been reported to be between 5.8 and 6.3 (Toydemir et al, 2013a; Yi et al, 2006). Furthermore, phospholipids from soy lecithin and terpenes from cold pressed citrus oil increased the transport of açaí anthocyanins in an *in vitro* cell monolayer model with Caco-2 cells, and a

combination of phospholipids and terpenes was found to be the most effective (Cardona et al, 2015).

In addition to the factors mentioned above, the physiological pH and temperature conditions (pH 7 and 37 °C) used in Caco-2 cell culture experiments may have a great influence on the stability of anthocyanins. In fact, a study on cyanidin-3-glucoside (Kay et al, 2009) showed that there was no significant difference between the Caco-2 cell and cell free incubations in terms of the losses of cyanidin-3-glucoside and the appearance of degradation products. These findings suggest that the loss of anthocyanins may be the result of chemical breakdown rather than Caco-2 cell induced enzymatic deglycosylation followed by chemical degradation.

Although the exact mechanism of anthocyanin absorption in the small intestine is still unclear, it has been proposed that anthocyanins could interfere with the transporters responsible for their own transport. The candidates for anthocyanin transporters were the glucose transporters, since anthocyanins possess a sugar moiety, in particular a glucose residue. Sodium-dependent glucose transporter 1 (SGLT1) and glucose transporter 2 (GLUT2) are the main hexose transporters described in Caco-2 cells. SGLT1 is an energy-dependent and sodium-dependent cotransporter, whereas GLUT2 is a facilitated transporter. SGLT1 is only present on the apical membrane and until a few years ago, GLUT2 was described to be present only in the basolateral membrane and in some pathologies on the apical membrane. Recently, it has been described and accepted that GLUT2 is present on the apical side and can be gathered to the membrane in the presence of a large amount of glucose, therefore becoming the main transporter responsible for glucose uptake (Faria et al, 2009; Zou et al, 2014). It was found that GLUT2 expression assessed by RT-PCR was increased in Caco-2 cells pretreated with red grape skin anthocyanins, by comparison with controls, indicating that chronic consumption of anthocyanins could be favorable for their own bioavailability. In addition, the tested red grape skin anthocyanins interfered with glucose uptake resulting in an inhibitory effect (about 60% decrease) (Faria et al, 2009). Similarly, peonidin-3-glucoside from strawberry extract was able to influence glucose uptake into the cells and transport to the basolateral side by inhibiting activities of the glucose transporters (Manzano and Williamson, 2010). Another study also confirmed that exposure to anthocyanin rich berry extract derived from blueberry, bilberry, cranberry, elderberry, raspberry seeds

and strawberry significantly reduce SGLT1 and GLUT2 expressions (Alzaid et al, 2013). Inhibition studies conducted using the pharmacological agents, phloridzin, an inhibitor of SGLT1, or phloretin, an inhibitor of GLUT2, revealed that the absorption of cyanidin-3-glucoside was significantly inhibited in the presence of these agents (Zou et al, 2014). These data suggest that anthocyanins may prevent hyperglycemia by decreasing glucose transporter expressions.

Since the high instability of anthocyanins has a direct impact on their potential health benefits, food processing technologies such as encapsulation may be used to improve their bioavailability (Betz et al, 2012; Oidtmann et al, 2012; Robert and Fredes, 2015). Accordingly, the nano-encapsulated cyanidin-3-glucoside with apoferritin was more efficiently transported through a Caco-2 cell monolayer compared to free cyanidin-3-glucoside (Zhang et al, 2014). In another study, processing of sour cherry fruit into nectar led to three times more efficient transport of cyanidin-3-glucosyl-rutinoside through a Caco-2 cell monolayer (Toydemir et al, 2013a).

Anthocyanin metabolism by Caco-2 cells

Polyphenols undergo Phases I and II transformations in the human body. Phase I transformations consist of oxidation, reduction and hydrolysis, but these transformations occur less frequently. Phase II biotransformations taking place in the liver and the intestine occur more intensively. These Phase II transformations consist of conjugation reactions where different metabolites are formed (methyl, glucuronic and sulfate derivatives) (Cardona et al, 2013). Spontaneous transformation of anthocyanins to phenolic acids and aldehydes is reported to occur under biological conditions (Vitaglione et al, 2007). Confirming that, under cell culture conditions, the main metabolites of cyanidin-3-glucoside and cyanidin are detected as protocatechuic acid and phloroglucinaldehyde, which are derived from the A and B rings of the parental compound (Figure 2.7). With action of enzymes, these metabolites can be further degraded to glucuronide and sulfate conjugates (Kay et al, 2009). Another important Phase II reaction of anthocyanins is the methylation, which alters the number of hydroxyl and methoxyl groups in ring B in comparison with the native compound. Although not so intense as genuine anthocyanins, methylated metabolites of cyanidin-3-glucoside, delphinidin-3-glucoside and petunidin-3-glucoside displayed some antiproliferative activity for the Caco-2 cell line (Fernandes et al, 2013). On the other hand, some other anthocyanin metabolites

including gallic acid, 3-*O*-methylgallic acid, and phloroglucinaldehyde reduced cell proliferation in Caco-2 cells more effectively compared to parental anthocyanins (Forester and Waterhouse, 2010). Therefore when assessing the health benefits of anthocyanins, potential effects of such metabolites should be taken into account.

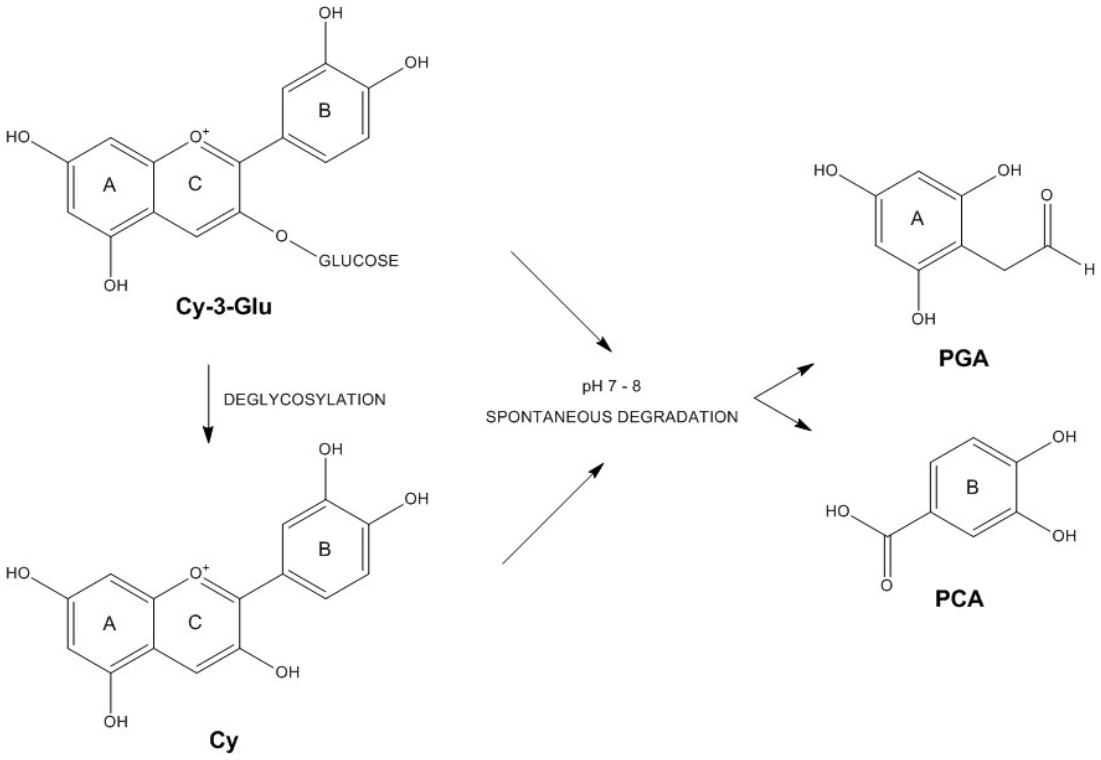


Figure 2.7 : Metabolites (PGA: phloroglucinaldehyde; PCA: protocatechuic acid) of cyanidin-3-glucoside (Cy-3-Glu) and cyanidin (Cy).

Future perspectives on anthocyanin absorption

Currently, the Caco-2 cell line is considered to be the most common *in vitro* model of the small intestine, despite some heterogeneity in its characteristics and some other limitations arising from its tumoral origin. A major difference between the Caco-2 cells and the intestinal enterocytes is that the Caco-2 cells do not have a mucus layer. Studies have been performed to co-culture a mucin secreting cell line (HT29-MTX) with the Caco-2 cell line, but they did not give the expected results. Another limitation of using Caco-2 cells is the poor reproducibility of results between different laboratories. Thus, a standardization of some important parameters such as cell origin, passage number and incubation time is necessary. On the other hand, there are also some benefits of using this cell line while evaluating anthocyanin absorption. It is a high throughput model, which allows the screening of a large

number of samples. Furthermore, the use of the Caco-2 cell model is also important to study molecular mechanisms of anthocyanin absorption. When the transport of anthocyanins is assessed using Caco-2 cells as intestinal models, in almost all studies cells are treated with pure standards or anthocyanin-rich extracts derived from plants and foods and data are reported at concentrations that showed a response. However, plasma and tissues are not exposed *in vivo* to anthocyanins in these forms. In this sense, the use of combined *in vitro* digestion and Caco-2 cells could be a better approach (*Chapter 6*). Also, the anthocyanin concentrations tested should be of the same order as the maximum plasma concentrations attained after a polyphenol-rich meal, which are in the range of 0.1–10 μmol/L (Kroon et al, 2004). In future studies, these conditions should be taken into account and the methodologies should be adopted accordingly.

Studies investigating anthocyanin absorption by Caco-2 cells reported very low transport of these compounds. The observed trends among different anthocyanins generally agreed with the published *in vivo* results. In spite of convincing observations in Caco-2 cell culture model, extrapolation of these *in vitro* findings for anthocyanins to the *in vivo* situation is difficult due to the unknown accumulation of these compounds at target tissues. Besides, the bioavailability of anthocyanins may be underestimated both *in vitro* and *in vivo* since the metabolites formed in the course of digestion could be responsible for the health benefits associated with anthocyanins. Recently, it was suggested that anthocyanins could also be absorbed from the stomach. In cell culture studies, anthocyanins were found to be able to cross MKN-28 cell monolayers (differentiated adenocarcinoma stomach cells) (Fernandes et al, 2012a; Fang, 2014b; Oliveira et al, 2015b). Therefore, the existing knowledge indicates that the observed low apparent bioavailability of anthocyanins could be due to their extensive presystemic metabolism, rather than poor absorption from the intestinal lumen. In addition, some anthocyanins can reach the colon in significant amounts and undergo microbial fermentation. The resultant microbial metabolites may also contribute to the health effects of anthocyanins. Eventually, the future studies should address the bioavailability of the anthocyanin metabolites to establish whether such metabolites could play a part in bioactivity.

2.3.3 Bioavailability of black carrot polyphenols

The majority of studies on the bioavailability of black carrot polyphenols have focused particularly on anthocyanins. Direct evidence on bioavailability of black carrot anthocyanins has been achieved by measuring the concentration of the compounds in biological fluids, mainly plasma and urine, after ingestion of black carrots in different matrices (e.g. raw, cooked, juice etc.). Research conducted on humans (Table 2.5) has shown that most of the black carrot anthocyanins were present in plasma and urine in intact form. Recovery of non-acylated anthocyanins was significantly higher than that of acylated anthocyanins (Kurilich et al, 2005; Netzel et al, 2007; Charron et al, 2009; Novotny et al, 2012). A kinetic study reported that the acylated anthocyanins exhibited a shorter half-life for gastrointestinal absorption than the non-acylated anthocyanins (Novotny et al, 2012). In a clinical feeding study that used both raw and cooked purple carrots as anthocyanin source, it has been shown that cooking increases the recovery of non-acylated anthocyanins, but not for acylated anthocyanins (Kurilich et al, 2005). Furthermore, increased dose of administration resulted in reduced recovery of both acylated and non-acylated anthocyanins (Kurilich et al, 2005; Charron et al, 2009). Only a single study was able to identify metabolites, namely cyanidin-monoglucuronide and cyanidin-monosulfate, in biological fluids. However, the authors noted that the concentrations of these metabolites were near or below the limit of quantification (Netzel et al, 2007).

As far as it is known, so far only a single research group conducted studies on *in vitro* gastrointestinal digestion of purple carrot juice polyphenols. The authors showed that during *in vitro* gastric and small intestinal digestion anthocyanins and phenolic acids in purple carrot juice bind to plant cell walls and, as a consequence, restrict the bioavailability (Padayachee et al, 2013). In the following chapters, various *in vitro* gastrointestinal digestion models were for the first time applied to polyphenols from black carrot jams and marmalades (*Chapter 3*), and black carrot peel and pomace (*Chapter 4*-*Chapter 6*).

Table 2.5 : Human studies on bioavailability of black carrot polyphenols.

Treatment	Dose	Design	Major conclusions	Reference
Raw and cooked purple carrot	250 g raw carrot (463 μmol ACNs); 250 and 500 g cooked carrot (357 and 714 μmol ACNs)	Blood sampling for 8 h Urine sampling for 24 h	• Four of the five carrot ACNs were found intact in plasma and urine. • Acylation of ACNs resulted in 11-14-fold and 8-10-fold decreases in ACN recovery in urine and plasma, respectively. • Cooking increased the recovery of non-acylated ACNs, but not acylated ACNs. • Increased dose reduced the recovery of both acylated and non-acylated ACNs.	(Kurilich et al, 2005)
Black carrot concentrate	100 mL concentrate (672.5 mg ACNs)	Urine sampling for 24 h	• Three acylated and two non-acylated ACNs were excreted in urine. • Cyanidin-monoglucuronide and and cyanidin-monosulfate were identified as metabolites. • Urine recovery of non-acylated ACNs was 8-fold higher than that of acylated ACNs.	(Netzel et al, 2007)
Purple carrot juice	50, 150, and 250 mL juice (76, 228, and 380 μmol ACNs)	Plasma sampling for 8 h	• Plasma concentrations of non-acylated ACNs were 4-fold higher than that for acylated ACNs. • Absorption efficiency declined across the doses administered.	(Charron et al, 2009)
Raw and cooked purple carrot	250 g raw carrot (463 μmol ACNs); 250 and 500 g cooked carrot (357 and 714 μmol ACNs)	Blood sampling for 8 h Urine sampling for 24 h	• Four of the five carrot ACNs were found intact in plasma and urine. • Absorption efficiencies of acylated ACNs were less than those for non-acylated ACNs. • The acylated ACNs exhibited a shorter half-life for gastrointestinal absorption than the non-acylated ACNs.	(Novotny et al, 2012)

ACN: anthocyanin

2.4 Cell Systems to Investigate the Impact of Polyphenols on Cardiovascular Health

2.4.1 Impact of polyphenols on endothelium

In this section, we will discuss the role of endothelium on cardiovascular health, which cell culture models have been used to model it with focus on those that have been used for polyphenol research so far, and how structurally different plant polyphenols influence cell behavior and cytokine expression in these models. The state-of-the-art about the effects of particular polyphenols on currently existing single cell culture models representing endothelium is summarized in Table 2.6.

The endothelium is a thin layer of cells that lines the interior surface of blood vessels and lymphatic vessels. Vascular endothelial cells line the entire circulatory system, and have distinct functions including (i) a barrier function; (ii) blood clotting; (iii) hormone trafficking; (iv) inflammation regulation; (v) angiogenesis; and (vi) vasoconstriction and dilatation.

Endothelial cells are a selective barrier (mediated by junction proteins such as vascular endothelial cadherin (VE-cadherin)) that contain fatty acids and glucose transporters (GLUT4, CD36) for the transport of nutrients, the latter are activated through protein kinase B (Akt). Endothelial dysfunction is a key event in the early stage of atherosclerosis and is often found in patients with coronary heart disease, type II diabetes, hypertension and hypercholesterolemia. Endothelial dysfunction is marked by increased reactive oxygen species (ROS) production and decreased nitric oxide (NO) production (result of activation of sirtuins and reduction of endothelial nitric oxide synthase (eNOS)). NO is involved in vasorelaxation, and may hence be a target to treat hypertension. Other molecular targets for vasodilatation are (i) proteins involved in the rennin-angiotensin-aldosterone system, such as angiotensin converting enzyme (ACE) and its receptors, that are involved in the regulation of blood pressure and water balances and (ii) endothelins (ET-1), which are proteins upregulated in response to hypoxia, oxidized low-density lipoprotein (LDL), pro-inflammatory cytokines, and bacterial toxins, and have an impact on blood pressure (Yamagata et al, 2015).

Table 2.6 : Impact of polyphenols on cell line systems from endothelial origin.

	Biomarkers	Compounds	Cell types	References
Transport	GLUT4, Akt	Silibinin, Xanthohumol	HUVEC	(Mojzis et al, 2008)
Vasorelaxation	NO, eNOS	Red wine polyphenols, Resveratrol, Sinapic acid	EA.hy926, HUVEC	(Duluc et al, 2012; Klinge et al, 2008; Silambarasan et al, 2014)
	ACE	Billberry anthocyanidins, Butein, Kaempferol, Oak polyphenols, Tannins, Tea polyphenols	ACE-test, HUVEC	(Dong et al, 2011; Rivas-Arreola et al, 2010; Persson et al, 2009; Olszanecki et al, 2008; Kang et al, 2003; Liu et al, 2003)
	ET-1	Quercetin	Isolated human umbilial chord veins	(Zhao et al, 1999)
Proliferation	MAPK, Erk, JNK	Apigenin, Catechins, Cocoa procyanidins, Genistein, Quercetin,	EC, VSMC, HMEC, HUVEC	(Negrao et al, 2013a; Negrao et al, 2013b; Kenny et al, 2004)
Migration	MMPs	Cyanidin, Delphinidin, Epigallocatechin-3-gallate, Green tea polyphenols, Hydroxytyrosol, Isoxanthohumol, Malvidin, Oleuropein, Pelargonidin, Peonidin, Petunidin, Quercetin, Resveratrol, Xanthohumol	HUVEC, HMEC-1	(Mojzis et al, 2008; Negrao et al, 2013a; Scoditti et al, 2012; Oku et al, 2003)
Tubulus formation		Hydroxytyrosol, Oleuropein, Quercetin, Resveratrol Xanthohumol	HUVEC and HMEC-1	(Negrao et al, 2013a; Scoditti et al, 2012; Elgass et al, 2012)
Inflammatory markers	NF-κB, TNF-α	Catechins, Isoxanthohumol, Silibinin	HUVEC, VSMC	(Mojzis et al, 2008; Negrao et al, 2013a; Negrao et al, 2013b)
	COX-2	Hydroxytyrosol, Oleuropein, Quercetin, Resveratrol	EC	(Scoditti et al, 2012; Zhao et al, 2013)

The low-grade inflammatory tone in metabolic syndrome patients has an impact on the endothelium, which is characterized by increased expression of transcription factors (NF-κB), enzymes (cyclo-oxygenase 2 (COX-2)) and cytokines (tumor necrosis factor alpha (TNF-α), intercellular adhesion molecule-1 (ICAM-1), monocyte chemoattractant protein-1 (MCP-1)) involved in the inflammation process.

Until now, most studies used primary HUVEC cells as a model for human endothelium. The HUV-EC-C cell line is similar to primary HUVEC cell lines, but these cells can be cultivated until 50 to 60 doubling times, which is at least ten times more than primary HUVEC cells. These cells are widely used to investigate the effect of drugs and nutrients on wound healing, angiogenesis (consisting of cell migration, differentiation and tubule formation), the production of NO, ROS, and monocyte/leukocyte attraction factors such as vascular cell adhesion molecule 1 (VCAM-1) and ICAM-1 and the expression and activity of ACE. Endothelial cells can be investigated as such, or stimulated with cytokines such as TNF-α, interleukin 1 beta (IL-1β), and vascular endothelial growth factor (VEGF) to mimic the inflammatory and hypoxic signaling in cardiovascular disease.

Other cell types that have been used to study vascular effects are primary microvascular retinal endothelial cells (HMREC), and immortalized human microvascular endothelial cells (HMEC-1) (Ades et al, 1992). The HMEC-1 cell line has a cobblestone morphology when grown in monolayer culture, expresses and secretes von Willebrand's Factor involved in coagulation, take up acetylated low-density lipoprotein, and rapidly form tubes when cultured on matrigel. They express cell-surface molecules typically associated with endothelial cells, including cluster of differentiation 31 (CD31) and CD36, and cell adhesion molecules such as ICAM-1 and CD44.

The ISO-HAS cell line has been established from tumor tissue of a human hemangiosarcoma, and has a life span of more than 100 passages (Masuzawa et al, 1999). This cell line has a cobble-stone morphology at confluency, contact-inhibited growth, active uptake of acetylated LDL and CD31 expression. Yet, because of the lack of the von Willebrand factor and tube-formation activity, as well as their high tumor-forming capacity in mice indicate that this is a malignant and poorly differentiated cell line. The EA.hy926 is a human somatic cell hybrid continuous cell line with endothelial properties. Compared to HMEC cells, this cell line is less

capable of tube formation and has significant differences in expression profiles (Ma et al, 2014). The EA.hy926 cell line has been used to investigate hyperglycemia induced stiffness and blood pressure mechanisms (Silambarasan et al, 2014; Targosz-Korecka et al, 2013).

These cell systems have been used to investigate the impact of a wide variety of polyphenols on nutrient transport, vasorelaxation, cell proliferation, tubulus formation, and inflammatory responses. Plant polyphenols including tea, cacao and bilberry polyphenols are shown to inhibit angiogenesis through regulation of multiple signaling pathways. Moreover, studies pointed out that resveratrol stimulate nitric oxide production, whereas tannins and sinapic acid exert antihypertensive effects (Table 2.6). Overall, studies indicated polyphenols as modulators of endothelium through different mechanisms.

One major concern for the use of cell cultures for the study of biomarkers triggered by polyphenols is the cancer-related origin of many commercially available cell cultures as many polyphenols selectively induce apoptosis in cancer cells by deregulation of the cell cycle, and are therefore considered as potential anticancer agents (Sak, 2014). Polyphenols can act as either antioxidant or prooxidant, depending on the dose, cell type and cell culture conditions. In general, most bioactive actions are related to the ROS scavenging potential of the polyphenols, including cardiovascular effects such as hypertension (Harrison and Gongora, 2009). In contrast, their anticancer effect has been shown to be mediated through their prooxidant properties, as cancer cells have higher and more persistent oxidative stress levels compared to normal cells, which makes them more sensitive towards the extra ROS levels generated by pro-oxidants. In a recent study by Sak (2014), the cytotoxicity, expressed as IC_{50} values, of flavonoids on more than 150 cell lines from bladder, blood, bone, breast, colon, liver, lung, melanoma, mouth, esophagus, ovary, pancreas, prostate, stomach and uterus origin, was reviewed. It was concluded that the toxicity effect was highly variable and dependent on flavonoid type, dose, cell line origin, and expression of estrogen receptors.

Besides the origin of the cells, one may also question whether the observed changes in biomarkers/cytokines in response to the polyphenol are considered to be beneficial or adverse. This is hard to assess, because this is strongly dependent on (i) the concentration of the added compound, (ii) the duration of incubation; (iii) the

intensity of the cellular response in terms of amount of "marker" that is produced and (iii) the pathways that are affected by the "marker", which is on itself also dependent on the amount of "marker" that is produced. So far, the relevant dose that needs to be added to a cell is still under discussion, and also the mode-of-action of the polyphenols, of which "anti-oxidant" activity is an example, is not fully understood. Besides that, only few proteomics, transcriptomics and metabolomics studies have been performed to have a full picture of the mechanisms. Therefore, although cell models provide a useful tool to perform mechanistic research, they should always be compared with *in vivo* data or primary cells.

2.4.2 Current cell culture research: Trends and potential application for polyphenol research

The past five years, cell culture research has evolved towards the development of more complex models, to obtain more relevant models that allow investigation of inter-cell signaling and cytokine expression. The Transwell® system, a static double well system separated by a filter membrane and generally used for transport experiments, and the collagen-embedded cell setup, are widely applied for indirect contact co-cultivation of multiple cell types. Nowadays, research groups and companies focus on the cultivation of one or more cell types on carriers and scaffolds containing extracellular matrix compounds to allow spatial organization and enhanced differentiation of the cells (Ou and Hosseinkhani, 2014). In some specific setups, low shear stress conditions are applied on cells adhered to carriers in rotating wall vessels, which may result in the differentiation of one cell line to multiple phenotypes (for instance HT-29 colonic cell line to the enterocyte and mucin producing phenotype). In other setups, dynamic conditions are applied to allow longer viability of the cells. In general, cell morphology and metabolism in these more advanced setups are now characterized and validated with tissue samples, and a first attempt to investigate the effects of highly characterized drugs was made. Yet, they are not widely applied for screening purposes of (digested) nutrients because of the specific expertise that is required.

As illustrated in Table 2.7, co-culture models of intestinal cell lines (mainly the Caco-2 cell line) with other intestinal cell lines, liver, endothelial, adipocyte, neuronal, fibroblast and a variety of immune cells have been developed. Co-cultures

of the Caco-2 and the intestinal HT29-MTX cell line has been used to investigate the impact of mucins on the bioavailability of curcumin nanoparticles (Guri et al, 2013). Especially co-cultures of intestinal cell lines with immune cells became very popular for the investigation of the effect of pathogens, probiotics, lipopolysaccharides, and a limited amount of environmental and food contaminants on intestinal behavior and general health. Less literature is available about co-cultures of intestinal cells with endothelial cells and only recently, the first publication about co-culture of intestinal with brown adipose tissue cell lines has appeared (Le Drean et al, 2014). A co-culture model of differentiated Caco-2 cells with primary HUVEC and HMEC-1 cells has been published in the context of ICAM-1 and VCAM-1 expression through a NF-κB-mediated mechanism (Maaser et al, 2001). In a publication of Zgouras et al (2003), undifferentiated Caco-2 cells were combined with HUVEC cells to investigate the effect of butyrate on tumor-derived angiogenesis. Only few of these systems have been used for the investigation of the impact of polyphenols on the cross-talk between intestinal and endothelial cell types in the context of cardiovascular diseases. In a publication of Kuntz et al (2015a), a mixture of Caco-2 cells with mucus secreting HT29-B6 cells was co-cultured with HUVEC cells, and it was shown that addition of an anthocyanin-rich grape extract had a beneficial effect on inflammation inhibition in the context of atherosclerosis, as measured by ICAM-1, VCAM-1, IL-6, IL-8 and E-selectin levels. The impact of polyphenols on other co-culture models, which are not including intestinal transport or modifications, have also been published before. In co-culture models of adipocyte cell lines with immune cells such as macrophages (Hirai et al, 2007; Reyes-Farias et al, 2015; Sakurai et al, 2010), as well as with endothelial cells have been more studied in the context of polyphenol research. Different crosstalk mechanisms including ROS, inflammatory markers, MCP-1 and Plasminogen activator inhibitor-1 (PAI-1) were influenced by wine, maqui, calafate and blueberry polyphenol extract as well as narigenin chalcone, in a mouse adipocyte-macrophage co-culture system. Although the primary objective of these models was related to the effect of polyphenols on obesity, these cross-talk mechanisms may have an indirect impact on endothelial function as well.

Table 2.7 : Co-culture models.

	Intestinal cell lines	Co-cultured cell line	Experimental setup	Applications	References
Intestine	Caco-2, Caco-2BBE	HT-29, HT-29-MTX, M-cells	Direct contact	Iron bioavailability, breast milk effects, nanoparticle uptake, curcumin bioavailability	(Guri et al, 2013; Woitiski et al, 2011; Nollevaux et al, 2006; Laparra et al, 2009; Yao et al, 2010; Bouwmeester et al, 2011)
Liver	Caco-2, Caco-2-TC7	HepG2, HepaRG, murine 3A	Transwell and continuous perfused fluidic system	Benzo(a)pyrene toxicity, β-carotene and retinoid transport	(Ouattara et al, 2011; Sakai et al, 2003; Rossi et al, 2012)
Neuronal	Caco-2, HT-29	PC12, glial cells, primary enteric neurocytes	Collagen-embedded system, Transwell system	Co-culture characteristics, LPS stimulation, pathogen invasion	(Satsu et al, 2001; Xiao et al, 2011; Flamant et al, 2010; Holland-Cunz et al, 2004)
Fibroblast	Caco-2, IEC-6, IPI-21, Caco-2BBE	Primary human and rat fibroblasts, Rat-2	Collagen-embedded, long term 3D	Co-culture characteristics	(Townley et al, 2012; Lahar et al, 2011; Yoshikawa et al, 2011; Viney et al, 2009)
Immune cells	Caco-2; HT-29, m-ICcl2	Whole blood cells, dendritic cells from isolated blood monocytes and bone marrow, lymphoblastoic TK6 cells, macrophage-like THP-1 and RAW264.7, murine lymphocytes of Peyers patches, Jurkat cells, RBL-2H3 (rat basophils), mast cells	Transwell system, floating filter system and direct contact, indirect micropattern surface	Co-culture characteristics, bioactivity of drugs, LPS, probiotica, benzo(a)pyrene, aflatoxin, fucoidan, immunoreactivity of ovalbumin	(Schmohl et al, 2012; Pozo-Rubio et al, 2011; Tiscornia et al, 2012; Zoumpopoulou et al, 2009; Rimoldi et al, 2005; Le Hegarat et al, 2012; Ishimoto et al, 2011; Tanoue et al, 2008; Chen et al, 2009; Stybayeva et al, 2009; Thierry et al, 2009; Wilcz-Villega et al, 2013)

Table 2.7 (continued) : Co-culture models.

	Intestinal cell lines	Co-cultured cell line	Experimental setup	Applications	References
3 or more cell types	Caco-2+HT29-MTX	Raji B, fibroblast + immunocytes, blood derived macrophages + dendritic cells	Transwell system, direct contact, collagen-embedded Transwell system	(Peptide) drug transport and permeability	(Antunes et al, 2013; Li et al, 2013; Leonard et al, 2012)
Adipocyte	Caco-2, HT29-19A	PAZ-6	Transwell system	Co-culture characteristics	(Le Drean et al, 2014)
Endothelium	Caco-2, HT29-6B, LS180EB3	Primary HMEC, immortalized isolated HMEC from lymph node, appendix, lung, skin and intestine microvessels, HUVEC, EA.hy926 cells	Transwell system, 3D dynamic model with decellularized jejunum segments, indirect contact	Co-culture characteristics, migration and adhesion of tumor cells, effect of anthocyanins of grape	(Pusch et al, 2011; Paprocka et al, 2008)
	Adipocyte cell lines	**Co-cultured cell line**	**Experimental setup**	**Applications**	**References**
Immune cells	Mouse preadipocytes, 3T3-L1	RAW264.7	Direct contact	Cross-talk grape, Maqui, calafate, blueberry polyphenol extracts, naringenin chalcone	(Hirai et al, 2007; Reyes-Farias et al, 2015; Sakurai et al, 2010)

To the best of our knowledge, current study is the first *in vitro* study that determined the potential of polyphenols from black carrot and its by-products to modulate the inflammatory response in TNF-α stimulated EA.hy 926 endothelial cells co-cultured with differentiated intestinal Caco-2 cells (*Chapter 6*).

3. POLYPHENOLS IN BLACK CARROT JAMS AND MARMALADES: EFFECT OF PROCESSING, STORAGE CONDITIONS AND *IN VITRO* GASTROINTESTINAL DIGESTION

Redrafted from:

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Kamiloglu, S., Pasli, A. A., Ozcelik, B., Van Camp, J. and Capanoglu, E. (2015). Colour retention, anthocyanin stability and antioxidant capacity in black carrot (*Daucus carota*) jams and marmalades: Effect of processing, storage conditions and *in vitro* gastrointestinal digestion. *Journal of Functional Foods*, *13*, 1-10.

3.1 Abstract

There is increasing interest in food use of black carrots due to the color stability of the anthocyanins and the substantial quantity of bioactive compounds. In the present study, the influence of jam and marmalade processing, storage conditions and *in vitro* gastrointestinal digestion on color attributes, total phenolic content, total and individual anthocyanin contents, phenolic acid content and antioxidant capacity of black carrots were examined. Total phenolic and anthocyanin contents, and antioxidant capacity were determined using spectrophotometric methods, whereas individual anthocyanins and phenolic acids were quantified using HPLC–PDA. Jam and marmalade processing significantly decreased total phenolics (89.2–90.5%), anthocyanins (87.6–95.6%), phenolic acids (49.5–96.7%) and antioxidant capacity (79.2–91.3%) ($p < 0.05$). After 20 weeks of storage, the reduction in total phenolics and anthocyanins in samples stored at 25 °C (26.4–48.0% and 30.7–92.2%, respectively) was higher than samples stored 4 °C (21.0–42.5% and 19.0–46.4%, respectively). In addition, jam and marmalade processing led to increases in the percent recovery of bioaccessible total phenolics (7.2–12.6%), anthocyanins (0.8–

10.3%) and phenolic acids (4.7–31.5%). In conclusion, the current study introduced a detailed understanding of the alterations in polyphenol content and antioxidant capacity of black carrot jams and marmalades, which can serve as novel sources of functional foods.

Keywords: *Daucus carota* L., anthocyanins, phenolic acids, antioxidant capacity, HPLC, bioaccessibility.

3.2 Introduction

Carrots have been ranked tenth in terms of their nutritional value among 38 other fruits and vegetables, and seventh for their contribution to nutrition (Alasalvar et al, 2005). Although orange carrots are more common, consumption of black carrots (*Daucus carota* L.) is currently increasing in Europe (Algarra et al, 2014). Black carrot was originated from Middle Asia, where it has been known for approximately 3000 years. It was not cultivated in Europe until the 12th century and is considered to be the archetype of all modern orange carrots, which were bred by Dutch growers around 1750 (Unal and Bellur, 2009).

Black carrot is indicated to play an important role in human nutrition, as it comprises a variety of health promoting components (Suzme et al, 2014). In addition to the presence of known antioxidants such as vitamins C and E, black carrots have also attracted attention due to the presence of polyphenols, which contribute significantly to the antioxidant capacity (Algarra et al, 2014). Besides anthocyanins as the major polyphenols, it has been shown that black carrot also contains significant amounts of phenolic acids, including hydroxycinnamates and caffeic acid (Kammerer et al, 2004a).

Many fruits and vegetables, including black carrots, are seasonal and perishable; therefore there is a need to apply preservation techniques such as jam/marmalade making. As a consequence of changes in consumption practices and the presence of alternative or new products on the market, the jam/marmalade industry needs to improve its competitiveness and develop new products, such as vegetable jams (Renna et al, 2013). In addition, black carrots are often underutilized and do not find much consumer acceptance as a vegetable (Khandare et al, 2011). Accordingly,

production of black carrot jams and marmalades may be a good alternative to fulfill these needs.

As a part of our diet, polyphenols are ingested as complex mixtures immersed in a food matrix, which undergo a digestion process in the gut. It is important to determine how the digestion process affects polyphenols and their stability as this, in turn, will affect their bioaccessibility for uptake as well as their possible beneficial effects on the cells lining the gut (Bermudez-Soto et al, 2007). Several factors including the chemical state of the compound, its release from the food matrix, possible interactions with other food components, presence of suppressors or cofactors affects the bioaccessibility of polyphenols (Parada and Aguilera, 2007). *In vitro* digestion methods have been developed as an alternative approach to *in vivo* studies and they are considered as simple, cheap and reproducible tools to assess the digestive stability of different food constituents (Rodriguez-Roque et al, 2013). There are some reports on the effect of *in vitro* digestion on dietary polyphenols in vegetables such as broccoli (Vallejo et al, 2004), red cabbage (McDougall et al, 2007) and pepper (Hervert-Hernandez et al, 2010). Nevertheless, no study to date evaluated the effect of *in vitro* gastrointestinal digestion on black carrot polyphenols.

The novel part of the current study is not only its focus on evaluation of the changes in polyphenol profile and antioxidant capacity of black carrots as a result of jam/marmalade processing and storage, but also the determination of the fate of black carrot polyphenols during *in vitro* gastrointestinal digestion. Given those, the aim of this study was to investigate the effect of processing, different storage conditions and *in vitro* gastrointestinal digestion on color attributes, total phenolic content, total and individual anthocyanin contents, phenolic acid content and antioxidant capacity of black carrots.

3.3 Materials and Methods

3.3.1 Chemicals and reagents

For the simulation of *in vitro* gastrointestinal digestion system, dialysis bags (Membra-Cel MD34-14 × 100 CLR) from Serva Electrophoresis GmbH (Heidelberg, Germany) were purchased. Cyanidin-3-*O*-glucoside (≥95%) was used for the quantification of anthocyanins, whereas chlorogenic acid (5-*O*-caffeoylquinic acid or

5-CQA) (≥98%), cryptochlorogenic acid (4-CQA) (≥98%), neochlorogenic acid (3-CQA) (≥98%) and caffeic acid (≥98%) were used for the quantification of phenolic acids. All chemicals and reagents used for the analyses were of analytical or high-performance liquid chromatography (HPLC) grade and obtained from Sigma–Aldrich Chemie GmbH & Co. KG (Steinheim, Germany), unless otherwise specified.

3.3.2 Carrots

Fresh black carrots (*Daucus carota* L. ssp. *sativus* var. *atrorubens* Alef.) used in this study were cultivated in Konya, Turkey and harvested as three different biological replicates in December 2012. In order to obtain the most representative results, jam and marmalade processing were performed in the following week after harvest. Carrots were stored at 4 °C until processing.

3.3.3 Jam and marmalade processing

Black carrot jams and marmalades were prepared traditionally in triplicate taking into account the information/limits described in Turkish Food Codex (Anonymous, 2002) (Figure 3.1). After washing, peeling and slicing, black carrots (3.0 kg for each batch) were mixed with commercial sucrose or sweetener (containing sorbitol and saccharin) at 1:1 (w/w) ratio. The mixtures were allowed to boil for 30 min, after which 300 g pectin solutions were added. Afterwards, they were heated until 55 and 68 °Brix of dry matters were achieved for jams and marmalades, respectively. All mixtures were adjusted to pH 3.0 with citric acid and hot-packed into jars. By the time jams and marmalades were cooled to room temperature they were divided into batches and stored in the dark at two different temperatures (4 °C and 25 °C) over a period of 20 weeks. Every 4 weeks, samples were taken, ground to a fine powder in liquid nitrogen using a pre-cooled grinder (IKA A11 basic, IKAWerke GmbH & Co., Germany), and stored at −80 °C until analysis.

3.3.4 Moisture content

The moisture content of samples were determined according to the guidelines of the official TS 1129-ISO 1026 method (Turkish Standard, 1998) at 70 °C using a vacuum oven (Gallenkamp, London, UK). All samples were analyzed in triplicate and average values were reported as percentage.

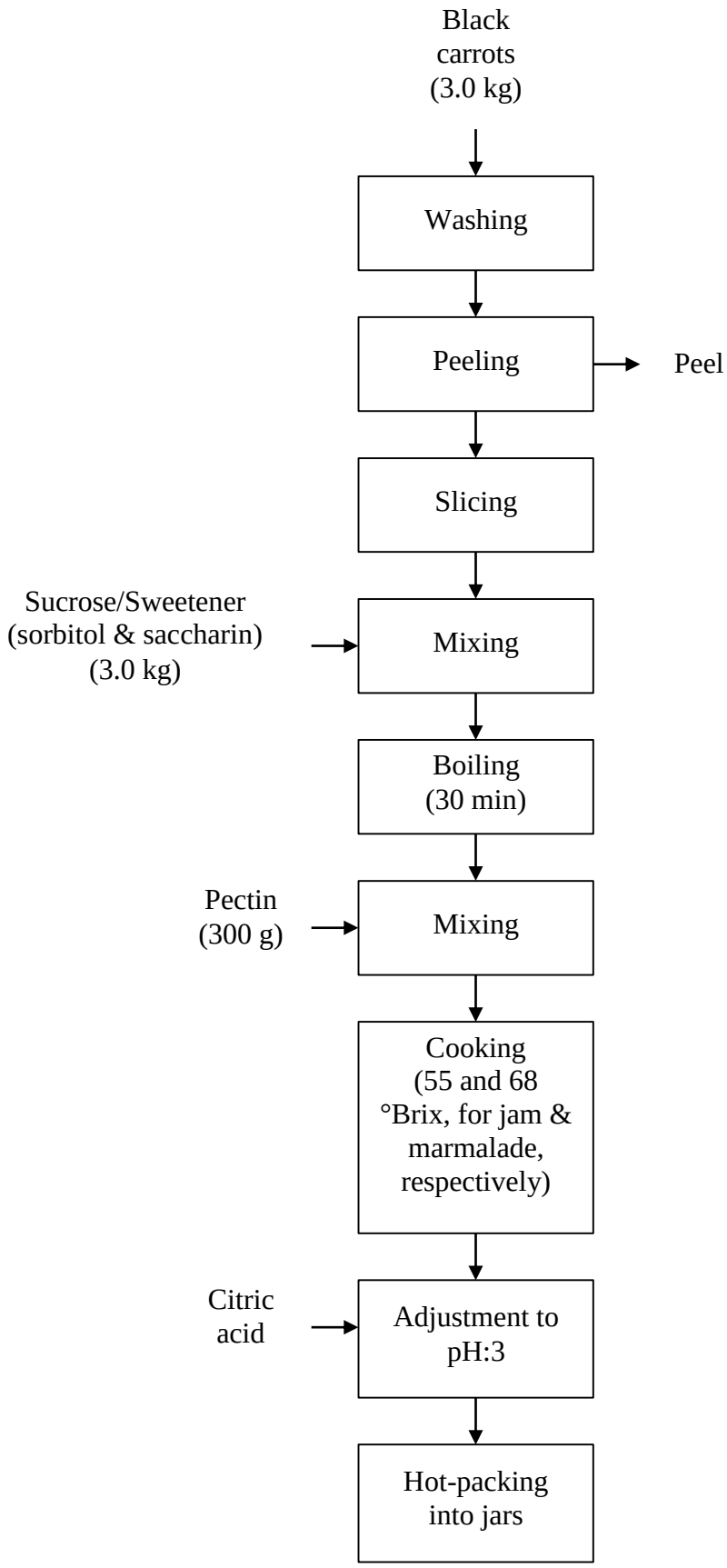

Figure 3.1 : Flow chart outlining the steps involved in production of black carrot jams and marmalades.

3.3.5 Color attributes

Color properties of black carrot jams and marmalades were measured using a CR-400 Minolta colorimeter (Tokyo, Japan) that was calibrated using a standard white plate. The measured parameters were L^* (lightness/darkness), a^* (red/green) and b^* (yellow/blue). Chroma (C), color intensity, and hue angle ($H°$), visual color appearance, were calculated by the following equations:

$$C = (a^{*2} + b^{*2})^{1/2} \quad (3.1)$$

$$H° = \tan^{-1}(b^*/a^*) \quad (3.2)$$

where 0° or 360° = red-purple, 90° = yellow, 180° = green, 270° = blue.

Samples of jams and marmalades were removed from different parts of the jars (bottom, middle and top) and transferred into Petri dishes for color measurements.

3.3.6 Chemical extraction

Three independent extractions for each sample were carried out as described previously by Capanoglu et al (2008) with some modifications. Briefly, 1 g of each sample was treated with 5 mL of two different solvents: 75% aqueous methanol containing 0.1% formic acid (v/v) and acetone:water:acetic acid (AWA, 70:29.5:0.5, v/v/v). The treated samples were sonicated in a cooled ultrasonic bath (USC900TH, VWR ultrasonic cleaner, Radnor, PA, USA) and centrifuged for 10 min (Universal 32R, Hettich Zentrifugen, Tuttlingen, Germany) at 4 °C, 2700 *g* and the supernatants were collected. This extraction procedure was repeated once more, and two supernatants were pooled to a final volume of 10 mL. Prepared extracts were stored at −20 °C. Since aqueous methanol with formic acid extracted significantly more phenolics than AWA (Appendix A, Figure A.1), 75% aqueous methanol containing 0.1% formic acid was used as the extraction solvent for all other subsequent analyses.

3.3.7 *In vitro* gastrointestinal digestion

The *in vitro* gastrointestinal digestion model adapted from McDougall et al (2005) was performed in triplicate for each sample. Black carrots, jams and marmalades were transferred to 250 mL beakers and mixed with 20 mL distilled water and 1.5 mL pepsin solution. The samples were acidified to pH 1.7–2.0 with 5 M HCl and

incubated at 37 °C in a shaking water bath (Memmert, Schwabach, Germany) at 100 rpm for 2 h. After gastric digestion, 2 mL aliquots of the postgastric (PG) digestion were collected for each sample. Afterwards, the pH was increased to 7.0 with 4.5 mL of pancreatin and bile salt mixture followed by the addition of dialysis bags filled with sodium bicarbonate. Samples were incubated in a shaking water bath (100 rpm) at 37 °C for another 2 h to complete the intestinal phase of the *in vitro* digestion process. After the intestinal phase, the solutions in the dialysis bags were taken as the IN sample representing the material that entered the serum, and the solution outside the dialysis bags were taken as the OUT sample representing material that remained in the gastrointestinal tract. The blank was also prepared with identical chemicals but without sample, and underwent the same conditions. Later, the collected samples (PG, IN and OUT) were assayed for total phenolics, total and individual anthocyanins, phenolic acids and antioxidant capacity (Figure 3.2).

3.3.8 Spectrophotometric assays

3.3.8.1 Total phenolic content

Total phenolic contents of the samples were quantified using Folin–Ciocalteu's method adapted to 96-well plate assay, as described by Sulaiman et al (2011). Ten microliters of extract was added to 25 μL of freshly prepared Folin–Ciocalteu reagent. The mixture was allowed to stand for 5 min and then 25 μL of 20% sodium carbonate solution was added to the mixture followed by addition of distilled water to a final volume of 200 μL per well. After 30 min of incubation at room temperature, the absorbance was read at 760 nm using a microplate reader (Synergy HT, BioTek Instruments Inc., Winooski, VT, USA). A standard curve was plotted using gallic acid and the results were expressed on dry weight basis as mg of gallic acid equivalent (GAE) per 100 g of dry weight (dw) of sample. All samples were analyzed in triplicate.

3.3.8.2 Total anthocyanin content

Total anthocyanin content was determined according to pH differential method (AOAC, 2006). Absorbance was measured using a microplate reader (Synergy HT) at 520 and 700 nm in buffers at pH 1.0 (KCl, 0.025M) and pH 4.5 (CH_3CO_2Na, 0.4 M) and calculated using Equation 3.3.

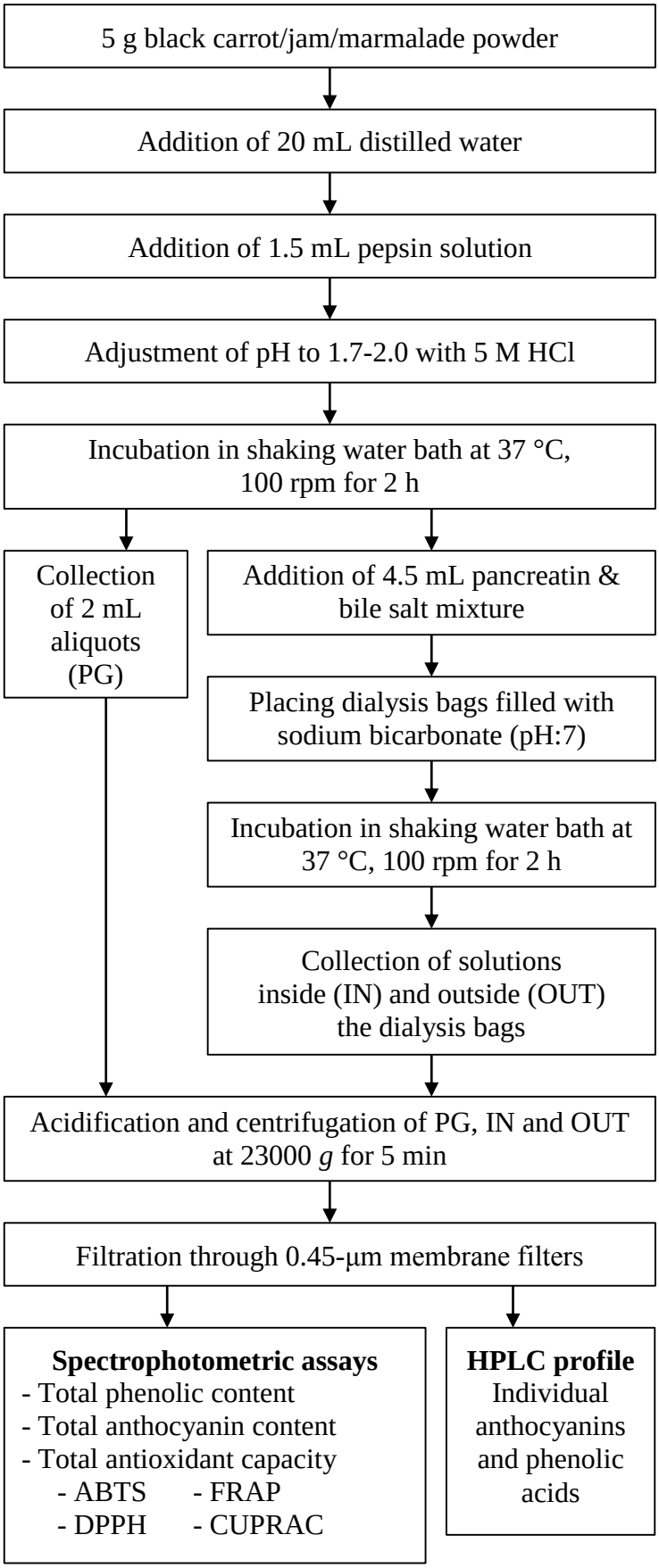

Figure 3.2 : Flow chart for the *in vitro* gastrointestinal digestion procedure and subsequent analyses of black carrot jams and marmalades.

$$\text{Total anthocyanin content} = (A \times MW \times DF \times 1000) / (\varepsilon \times l) \quad (3.3)$$

where $A = (A_{520nm} - A_{700nm})_{pH\ 1.0} - (A_{520nm} - A_{700nm})_{pH\ 4.5}$, MW = molecular weight of cyanidin-3-*O*-glucoside (C3G) (449.2 g/mol), DF = dilution factor, ε = molar extinction coefficient of C3G (26900 L/(mol.cm)), and l = pathlength (cm).

Total anthocyanin content was expressed as mg of C3G per 100 g of dw of sample. Samples were analyzed in triplicate.

3.3.8.3 Total antioxidant capacity

Total antioxidant capacity was estimated using four different assays (ABTS, DPPH, FRAP and CUPRAC). In all assays, Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as a standard and results were expressed in terms of mg of Trolox equivalent (TE) per 100 g dw of sample. Samples were analyzed in triplicate for each assay.

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

ABTS assay was performed according to Miller and Rice-Evans (1997). ABTS and potassium persulfate solutions were mixed and kept at room temperature in the dark for overnight. ABTS stock solution was diluted in 50 mM potassium phosphate buffer (pH 8.0) to an absorbance of 0.90 (±0.05) at 734 nm to prepare the ABTS-working solution. Then, 100 μL of sample was mixed with 1 mL of ABTS-working solution and the absorbance was measured at 734 nm exactly 1 min after initial mixing.

DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay

DPPH assay was performed as described by Kumaran and Karunakaran (2006). One hundred microliters of each sample extract was mixed with 2 mL of 0.1 mM DPPH in methanol. After 30 min of incubation at room temperature, the absorbance of the mixture was measured at 517 nm.

FRAP (ferric ion reducing antioxidant power) assay

FRAP assay was carried out according to the procedure of Benzie and Strain (1996). To perform the assay, a 900 μL aliquot of freshly prepared FRAP reagent (a mixture of acetate buffer (pH 3.6), 10 mM TPTZ solution and 20 mM ferric chloride in

proportions of 10:1:1 (v/v/v), respectively) was combined with 100 μL of extract. The absorbance of the reaction mixture was then recorded at 593 nm after 4 min.

CUPRAC (cupric ion reducing antioxidant capacity) assay

CUPRAC assay developed by Apak et al (2004) was used. One hundred microliters of extract was mixed with 1 mL of 10 mM copper (II) chloride, 7.5 mM neocuproine and 1 M ammonium acetate (pH: 7). Immediately, 1 mL of distilled water was added to the mixture so as to make the final volume 4.1 mL. After 30 min of incubation at room temperature, absorbance was measured at 450 nm.

3.3.9 HPLC-PDA analysis of polyphenols

Polyphenol profiles of samples were determined by comparison with a previous study performed by our group (Suzme et al, 2014), where the identification of polyphenols was carried out using an LC-PDA–QTOF–MS system. For the quantification of polyphenols, the method of Capanoglu et al (2008) was applied. Briefly, samples were passed through 0.45-μm-membrane filters and injected into a Waters 2695 HPLC (Waters Co., Milford, MA, USA) coupled with photodiode array (PDA) detector (Waters 2996). A Supelcosil LC-18 25 cm × 4.60 mm, 5 μm column (Sigma-Aldrich) was used as the stationary phase. The mobile phase was solvent A, Milli-Q water with 0.1% (v/v) TFA and solvent B, acetonitrile with 0.1% (v/v) TFA. The linear gradient was used as follows: at 0 min, 95% solvent A and 5% solvent B; at 45 min, 65% solvent A and 35% solvent B; at 47 min, 25% solvent A and 75% solvent B; and at 54 min returning to initial conditions. The flow rate of 1 mL/min and injection volume of 10 μL was used for spectral measurements at 312 and 520 nm. The content of anthocyanin glycosides was quantified using cyanidin-3-*O*-glucoside, whereas phenolic acids were quantified using their authentic standards. All analyses were performed in triplicates and the results were expressed as mg per 100 g dw of sample.

3.3.10 Statistical analysis

All experiments were conducted in triplicate and the obtained data were reported as mean ± standard deviation. Statistical analysis was applied using SPSS software (version 20.0, SPSS, Chicago, IL, USA). Mean values were compared by analysis of variance (ANOVA) followed by Tukey's post hoc test ($p < 0.05$). The correlation

coefficients (R^2) were calculated using Microsoft Office Excel 2011 software (Microsoft Corporation, Redmond, WA, USA).

3.4 Results and Discussion

3.4.1 Effect of jam and marmalade processing on color retention, polyphenol stability and antioxidant capacity of black carrots

After processing, the moisture content of fresh black carrots were decreased from 91.0% to 40.6% and 30.4% for jams and marmalades, respectively. To eliminate the differences in moisture contents, the results in current study are expressed on dry weight (dw) basis. Fresh black carrots showed lightness value (L^*) of 17.6 and hue angle ($H°$) of 306.8 (confirming the bluish purple color) (Table 3.1), which was darker than most of the data reported for black carrots in the literature, only Koley et al (2014) measured similar L^* (16.7) and $H°$ values (310.1) in Pusa Asita carrot cultivar. Jams and marmalades appeared to be significantly brighter and more intense than fresh carrots, as reflected by 25.6−58.0%, 89.8−96.1% and 134.4−158.1% increases in L^*, yellowness (b^*) and chroma (C), respectively ($p < 0.05$). Sadilova et al (2006) also observed a rapid increase in lightness for black carrots upon heating which was attributed to the transition of the colored flavylium cation into colorless and yellowish carbinol and chalcone forms, respectively. Therefore, a similar fading mechanism may be valid in the present work. Another reason of these changes might be ascribed to the native color of pectin gel. It has been suggested that the brownish hue of some pectins due to their high content of oxidized polyphenols may be a limiting factor regarding their use in certain products (Holzwarth et al, 2013). In addition, after processing a^* and $H°$ values significantly shifted towards red-purple color, which may be attributed to the formation of Maillard reaction products. In general, differences between the color properties of samples with sugar and sweetener were insignificant ($p > 0.05$).

Table 3.1 : Changes in color properties of black carrot jams and marmalades during 20 weeks of storage at 4°C and 25°C.

	Day 1	Week 4	Week 8	Week 12	Week 16	Week 20
Raw material						
*L**	17.6 ± 0.2					
*a**	9.6 ± 1.1					
*b**	-12.8 ± 0.8					
C	16.0 ± 1.1					
H°	306.8 ± 2.9					
			Storage at 4 °C			
Jam with sugar						
*L**	26.2 ± 1.0^{e}	27.7 ± 0.7^{de}	28.4 ± 0.3^{d}	34.0 ± 0.7^{c}	60.4 ± 1.2^{b}	65.6 ± 0.4^{a}
*a**	39.3 ± 0.3^{d}	39.9 ± 0.4^{d}	41.2 ± 0.2^{cd}	42.7 ± 1.1^{bc}	44.3 ± 0.9^{b}	48.4 ± 2.7^{a}
*b**	-1.2 ± 0.3^{a}	-1.2 ± 0.8^{a}	-1.7 ± 0.5^{ab}	-2.0 ± 0.1^{ab}	-2.8 ± 1.1^{bc}	-4.0 ± 0.7^{c}
C	39.3 ± 0.3^{d}	39.9 ± 0.4^{d}	41.3 ± 0.1^{cd}	42.8 ± 1.1^{bc}	44.4 ± 1.0^{b}	48.6 ± 2.7^{a}
H°	358.3 ± 0.4^{a}	358.3 ± 1.2^{a}	357.6 ± 0.8^{a}	357.3 ± 0.1^{a}	356.5 ± 1.4^{ab}	355.3 ± 0.6^{b}
Jam with sweetener						
*L**	27.8 ± 0.2^{d}	31.1 ± 0.2^{c}	34.1 ± 0.4^{b}	34.3 ± 0.9^{b}	58.3 ± 1.8^{a}	60.2 ± 1.8^{a}
*a**	41.3 ± 1.4^{c}	44.3 ± 2.1^{bc}	44.4 ± 0.8^{bc}	44.6 ± 0.5^{bc}	46.7 ± 3.1^{ab}	49.3 ± 0.2^{a}
*b**	-1.0 ± 0.1^{a}	-5.0 ± 0.2^{b}	-5.0 ± 1.5^{b}	-5.2 ± 0.5^{b}	-5.7 ± 0.2^{b}	-8.3 ± 0.4^{c}
C	41.3 ± 1.4^{c}	44.6 ± 2.1^{bc}	44.7 ± 0.9^{bc}	44.9 ± 0.6^{bc}	47.1 ± 3.0^{ab}	50.0 ± 0.1^{a}
H°	358.6 ± 0.1^{a}	353.6 ± 0.4^{b}	353.6 ± 1.9^{b}	353.3 ± 0.6^{b}	353.1 ± 0.7^{b}	350.4 ± 0.5^{c}
Marmalade with sugar						
*L**	26.8 ± 1.3^{d}	27.9 ± 0.1^{d}	32.2 ± 0.7^{c}	32.6 ± 0.5^{c}	50.5 ± 1.2^{b}	53.0 ± 0.3^{a}
*a**	37.6 ± 2.7^{c}	39.5 ± 0.3^{c}	40.6 ± 1.2^{c}	47.5 ± 1.9^{b}	54.4 ± 0.9^{a}	55.7 ± 1.4^{a}
*b**	-0.5 ± 0.1^{a}	-1.2 ± 0.1^{a}	-8.2 ± 1.2^{b}	-8.3 ± 0.5^{b}	-13.7 ± 0.2^{c}	-13.7 ± 1.0^{c}
C	37.6 ± 2.7^{c}	39.5 ± 0.3^{c}	41.4 ± 1.3^{c}	48.3 ± 1.8^{b}	56.2 ± 0.9^{a}	57.4 ± 1.4^{a}
H°	359.3 ± 0.1^{a}	358.2 ± 0.2^{a}	348.6 ± 1.4^{b}	350.1 ± 1.0^{b}	345.9 ± 0.2^{c}	346.2 ± 1.0^{c}
Marmalade with sweetener						
*L**	22.1 ± 0.5^{c}	23.1 ± 1.2^{c}	24.2 ± 0.5^{bc}	26.2 ± 0.1^{b}	43.5 ± 0.2^{a}	44.7 ± 1.5^{a}
*a**	37.5 ± 0.3^{d}	38.4 ± 0.4^{d}	38.2 ± 0.1^{d}	42.0 ± 0.4^{c}	53.2 ± 1.2^{b}	54.9 ± 0.2^{a}
*b**	-1.3 ± 0.5^{a}	-1.5 ± 0.2^{a}	-5.7 ± 0.1^{b}	-7.2 ± 1.3^{b}	-9.4 ± 0.7^{c}	-10.7 ± 0.3^{c}
C	37.5 ± 0.4^{d}	38.5 ± 0.4^{d}	38.6 ± 0.1^{d}	42.7 ± 0.2^{c}	54.0 ± 1.3^{b}	55.9 ± 0.3^{a}
H°	358.0 ± 0.7^{a}	357.7 ± 0.4^{a}	351.5 ± 0.1^{b}	350.3 ± 1.8^{bc}	350.0 ± 0.5^{bc}	348.9 ± 0.3^{c}

Table 3.1 (continued) : Changes in color properties of black carrot jams and marmalades during 20 weeks of storage at 4°C and 25°C.

	Day 1	Week 4	Week 8	Week 12	Week 16	Week 20
			Storage at 25 °C			
Jam with sugar						
*L**	26.2 ± 1.0^{c}	27.9 ± 0.1^{c}	33.5 ± 0.6^{b}	34.4 ± 2.4^{b}	69.8 ± 1.6^{a}	70.2 ± 0.5^{a}
*a**	39.3 ± 0.3^{e}	42.4 ± 0.2^{d}	46.6 ± 0.4^{c}	47.8 ± 0.6^{c}	51.4 ± 3.0^{b}	58.6 ± 2.0^{a}
*b**	-1.2 ± 0.3^{a}	-1.3 ± 0.6^{a}	-2.1 ± 0.5^{a}	-2.3 ± 0.2^{a}	-5.7 ± 1.3^{b}	-5.8 ± 1.1^{b}
C	39.3 ± 0.3^{e}	42.4 ± 0.2^{d}	46.7 ± 0.4^{c}	47.9 ± 0.7^{c}	51.8 ± 2.9^{b}	58.7 ± 2.0^{a}
H°	358.3 ± 0.4^{a}	358.3 ± 0.9^{a}	357.4 ± 1.6^{a}	357.3 ± 0.2^{a}	353.6 ± 1.8^{b}	354.4 ± 1.3^{b}
Jam with sweetener						
*L**	27.8 ± 0.2^{e}	31.2 ± 0.3^{de}	34.3 ± 0.3^{cd}	38.5 ± 0.4^{c}	55.5 ± 6.4^{b}	61.6 ± 0.2^{a}
*a**	41.3 ± 1.4^{c}	45.8 ± 0.7^{bc}	48.1 ± 0.1^{b}	49.4 ± 0.5^{b}	56.7 ± 2.4^{a}	58.9 ± 5.4^{a}
*b**	-1.0 ± 0.1^{a}	-5.6 ± 0.9^{b}	-7.2 ± 0.7^{c}	-7.7 ± 0.9^{cd}	-8.6 ± 0.3^{de}	-9.5 ± 0.5^{e}
C	41.3 ± 1.4^{c}	46.2 ± 0.8^{b}	48.7 ± 0.2^{b}	50.0 ± 0.4^{b}	57.4 ± 2.4^{a}	59.7 ± 5.4^{a}
H°	358.6 ± 0.1^{a}	353.1 ± 1.0^{b}	351.5 ± 0.8^{c}	351.1 ± 1.1^{c}	351.3 ± 0.7^{c}	350.9 ± 0.3^{c}
Marmalade with sugar						
*L**	26.8 ± 1.3^{d}	27.3 ± 0.1^{d}	32.7 ± 0.3^{c}	35.2 ± 0.2^{b}	51.3 ± 0.5^{a}	51.8 ± 1.7^{a}
*a**	37.6 ± 2.7^{d}	45.4 ± 0.4^{c}	46.3 ± 1.0^{bc}	49.9 ± 0.5^{bc}	53.9 ± 0.9^{ab}	54.5 ± 2.7^{a}
*b**	-0.5 ± 0.1^{a}	-1.4 ± 0.4^{a}	-7.7 ± 0.5^{b}	-10.6 ± 0.6^{c}	-11.0 ± 1.3^{c}	-11.7 ± 0.2^{c}
C	37.6 ± 2.7^{d}	49.9 ± 0.6^{c}	46.9 ± 1.0^{c}	51.0 ± 0.5^{bc}	55.0 ± 1.2^{ab}	55.8 ± 2.6^{a}
H°	359.3 ± 0.1^{a}	358.4 ± 0.5^{a}	350.6 ± 0.5^{b}	348.0 ± 0.7^{c}	348.4 ± 1.2^{c}	347.9 ± 0.8^{c}
Marmalade with sweetener						
*L**	22.1 ± 0.5^{e}	26.0 ± 0.4^{d}	26.7 ± 0.2^{d}	29.4 ± 0.6^{c}	53.2 ± 0.6^{b}	56.7 ± 2.1^{a}
*a**	37.5 ± 0.3^{d}	45.1 ± 0.1^{c}	46.2 ± 0.7^{c}	47.9 ± 0.5^{b}	54.3 ± 1.1^{a}	55.5 ± 0.1^{a}
*b**	-1.3 ± 0.5^{a}	-2.3 ± 0.5^{ab}	-2.9 ± 0.8^{b}	-5.1 ± 0.3^{c}	-5.9 ± 0.3^{c}	-8.7 ± 0.4^{d}
C	37.5 ± 0.4^{e}	45.2 ± 0.1^{d}	46.2 ± 0.6^{d}	48.2 ± 0.5^{c}	54.6 ± 1.0^{b}	56.2 ± 0.1^{a}
H°	358.0 ± 0.7^{a}	357.1 ± 0.7^{ab}	356.5 ± 1.0^{b}	354.0 ± 0.5^{c}	353.8 ± 0.4^{c}	351.1 ± 0.4^{d}

The data presented in this table consist of average values ± standard deviation of three independent samples. *L**, *a**, *b**, *C* and *H°* are color parameters: lightness/darkness, red/green, yellow/blue, chroma, and hue angle, respectively. Different letters in the rows represent statistically significant differences ($p < 0.05$).

Total phenolic content obtained for fresh black carrots in the current study (537 ± 57 mg GAE/100 g fw) was higher than most of the findings reported in the literature, only Algarra et al (2014) measured similar total phenolic content in Purple Haze cultivar (492.0 mg GAE/100 g fw). Jam and marmalade processing significantly decreased the total phenolic content (89.2–90.5%) ($p < 0.05$). The reduction in the total phenolic content was in agreement with the data reported for strawberry, cherry, apricot, fig and orange jams (68.6–93.2%) (Rababah et al, 2011). During jam processing, cell structure is disrupted and the raw material becomes prone to non-enzymatic oxidation, which could be one of the major causes for the loss in total phenolic content (Patras et al, 2011). Although in general, the use of sweetener instead of sugar in jams and marmalades did not lead to a significant difference in the total phenolic content, jam with sugar contained the highest amount of phenolics. This is in agreement with an earlier report (Scibisz and Mitek, 2009) showing that high-sugar jam contains higher total phenolic content compared with low sugar and light jams.

Total anthocyanin content of black carrots, jams and marmalades were determined using both spectrophotometric pH differential and HPLC methods. The level of total anthocyanin content in fresh black carrots determined spectrophotometrically (536 ± 25 mg C3G/100 g dw) was found be slightly higher than the previous study performed by our group (486 ± 43 mg C3G/100 g dw) (Suzme et al, 2014). The major anthocyanins detected were cyanidin-based with different sugar moieties, among them two were non-acylated (cyanidin-3-xylosyl-glucosyl-galactoside and cyanidin-3-xylosyl-galactoside), and three were acylated with sinapic acid (cyanidin-3-xylosyl-sinapoyl-glucosyl-galactoside), ferulic acid (cyanidin-3-xylosyl-feruloyl-glucosyl-galactoside) and coumaric acid (cyanidin-3-xylosyl-coumaroyl-glucosyl-galactoside). Compounds with an acylated structure constitute 77% of total anthocyanins contained in black carrots, whereas the predominant anthocyanin corresponded to cyanidin-3-xylosyl-feruloyl-glucosyl-galactoside represented approximately 57% of the total anthocyanins. The anthocyanin profile was consistent with those reported previously (Kammerer et al, 2003; Montilla et al, 2011; Algarra et al, 2014; Suzme et al, 2014). In addition to the anthocyanins identified in the current study, trace amounts of peonidin and pelargonidin glycosides have also been identified in black carrots (Kammerer et al, 2003; Montilla et al, 2011; Algarra et al,

2014). During jam and marmalade processing, 87.6–95.6% of the anthocyanins in black carrots were lost ($p < 0.05$). The loss in the anthocyanin content was in agreement with the data reported for strawberry, cherry, apricot, fig and orange jams (60.2–96.6%) (Rababah et al, 2011). Similarly, jam processing again reduced the total anthocyanin content of cherry, plum, raspberry (11–79%) (Kim and Padilla-Zakour, 2004) and blueberry (25–54%) (Scibisz and Mitek, 2009). Several factors are believed to affect the stability of anthocyanins in fruits and vegetables during processing, such as pH, temperature, light, oxygen, metal ions, enzymes, and sugars (Kim and Padilla-Zakour, 2004). Hydrolysis of the glycoside linkage is known to be the first step in anthocyanin degradation, where the elevated temperature during jam and marmalade processing shifts the anthocyanin equilibrium towards the colorless chalcones. The chalcones might be exposed to degradation through oxidation reactions generating brown compounds or pigments with high molecular weights. Additionally, flavylium salts, which are stable in highly acidic conditions, may also cause reduction in anthocyanin content. Furthermore, salts can lose a proton at higher pH and be transformed into an unstable pigment (quinoidal base) that is bonded to water and form a colorless compound called chromenol (Rababah et al, 2011). Nevertheless, compared to jams and marmalades with sweetener, samples prepared with sugar, retained higher amount of individual anthocyanins, with jam with sugar retaining the highest among all. In jam and marmalade processing this might be due to reduced water activity and acting as partial oxygen barrier, which is in agreement with an earlier report (Scibisz and Mitek, 2009). Moreover, after processing a highly linear relationship was obtained between the total anthocyanin content and color properties (a^*, b^*, C, $H°$) of black carrots ranging from $R^2 = 0.9660$ to $R^2 = 0.9891$.

Four major phenolic acids were detected in the analyzed samples, namely chlorogenic acid (5-*O*-caffeoylquinic acid or 5-CQA), cryptochlorogenic acid (4-CQA), neochlorogenic acid (3-CQA) and caffeic acid. In accordance with previous reports (Kammerer et al, 2004a; Suzme et al, 2014), chlorogenic acid was identified as the predominant compound in black carrots. Chlorogenic acid content of black carrots in the current study was higher than the mean value of 657 mg/kg fw reported for black carrots in the literature (Kammerer et al, 2004a). Processing black carrots into jams and marmalades led to significant decreases in phenolic acid contents (49.5–96.7%) ($p < 0.05$). This reduction was comparable to the chlorogenic acid

results reported by Howard et al (2010) for blueberry jams. Although jam and marmalades were prepared using the same procedure, the level of losses of phenolic acids depended on ingredients. Marmalade with sugar contained relatively higher amounts of individual phenolic acids (except for caffeic acid), unlike the trend observed for total phenolic content, where jam with sugar included the highest amounts.

The antioxidant capacity of samples was determined using four different assays, namely ABTS, DPPH, FRAP and CUPRAC assays. Similar to total phenolic content, the antioxidant capacity of fresh black carrots were also found to be higher than most of the data reported previously. For example, the results achieved by (Algarra et al, 2014), who used DPPH and FRAP methods in their study, were low (17.6–240 and 86.4–182 μM TE/100 g fw for DPPH and FRAP, respectively) compared to our observations (1720 ± 149 and 5617 ± 357 mg TE/100 g dw for DPPH and FRAP, respectively). On the other hand, in a study (Koley et al, 2014), where ABTS and CUPRAC assays were applied, higher antioxidant activity was measured for Pusa Asita carrot cultivar (57.53 and 77.80 μmol Trolox/g for ABTS and CUPRAC, respectively) compared to our results (9976 ± 923 and 14,152 ± 2100 mg TE/100 g dw for ABTS and CUPRAC, respectively). The reason of varying antioxidant capacity values could be associated with several factors related to the plant material (e.g., cultivar, growing conditions, harvesting time) as well as the protocols used for the measurements. During jam and marmalade processing, 79.2–91.3% of antioxidant capacity in black carrots was lost ($p < 0.05$). It appears that some of the polyphenols present in the fresh black carrots are destroyed or converted to non-antioxidant forms during jam and marmalade preparation. These results were consistent with the data reported previously by Kim and Padilla-Zakour (2004) who demonstrated that the antioxidant capacity of raspberry and plum measured using ABTS radical decreased significantly after jam processing. Similarly, jam processing also reduced the antioxidant capacity of *Rubus coreanus* Miquel berry (Lee et al, 2013), and strawberry, cherry, apricot and fig (Rababah et al, 2011). Additionally, similar to anthocyanin content, jam with sugar possessed the highest antioxidant activity among the analyzed samples. Furthermore, total antioxidant capacity and total anthocyanin content correlated well ($R^2 = 0.9882–0.9910$) after processing.

3.4.2 Effect of storage on color retention, polyphenol stability and antioxidant capacity of black carrots

During 20 weeks of storage, *L**, *a** and *C* values increased significantly (93.3–167.9%, 19.4–49.1% and 21.1–52.7%, respectively) ($p < 0.05$) (Table 3.1). On the other hand, *b** and *H*° values declined after 20 weeks of storage (2.3–26.4 fold and 0.8–3.6%, respectively). Although, these values were slightly better retained when jams and marmalades were stored at 4 °C instead of 25 °C, at the end of storage, almost all samples exhibited comparable color properties.

Storage time and temperature also had effects on total phenolic content (Figure 3.3). The changes in total phenolic contents of jams and marmalades in time during storage varied. After 20 weeks of storage, the decrease in total phenolic content in samples stored at 25 °C (26.4–48.0%) was slightly higher than the samples stored at 4 °C (21.0–42.5%). This finding of higher degradation of total phenolic content during storage at higher temperature is consistent with previously reported data for blueberry jams (Scibisz and Mitek, 2009).

The total and individual anthocyanin contents were highly influenced by different storage conditions (Figure 3.4, Table 3.2). At the end of storage period, anthocyanin retention in jams and marmalades stored at 4 °C and 25 °C ranged from 53.4–81.0 to 7.8–69.3%, respectively. This finding of storage at lower temperatures resulting in better anthocyanin preservation is in agreement with previously reported data for blueberry (Scibisz and Mitek, 2009; Howard et al, 2010) and strawberry (Wicklund et al, 2005; Holzwarth et al, 2013) jams. These losses may be a result of hydrolytic reactions causing conversion of anthocyanin glycosides to chalcones, which spontaneously degrade into phenolic acids and aldehydes. It is also possible that heat stable forms of polyphenol oxidase or peroxidase may play a role in the reduction of anthocyanins (Howard et al, 2010). The amount of loss in individual anthocyanins of black carrots was generally higher compared to malvidin and pelargonidin derivatives present in blueberry and strawberry jams (Scibisz and Mitek, 2009; Holzwarth et al, 2013). This was expected since cyanidin based anthocyanins are known to be more prone to degradation due to the presence of vicinal hydroxyl groups (Holzwarth et al, 2013).

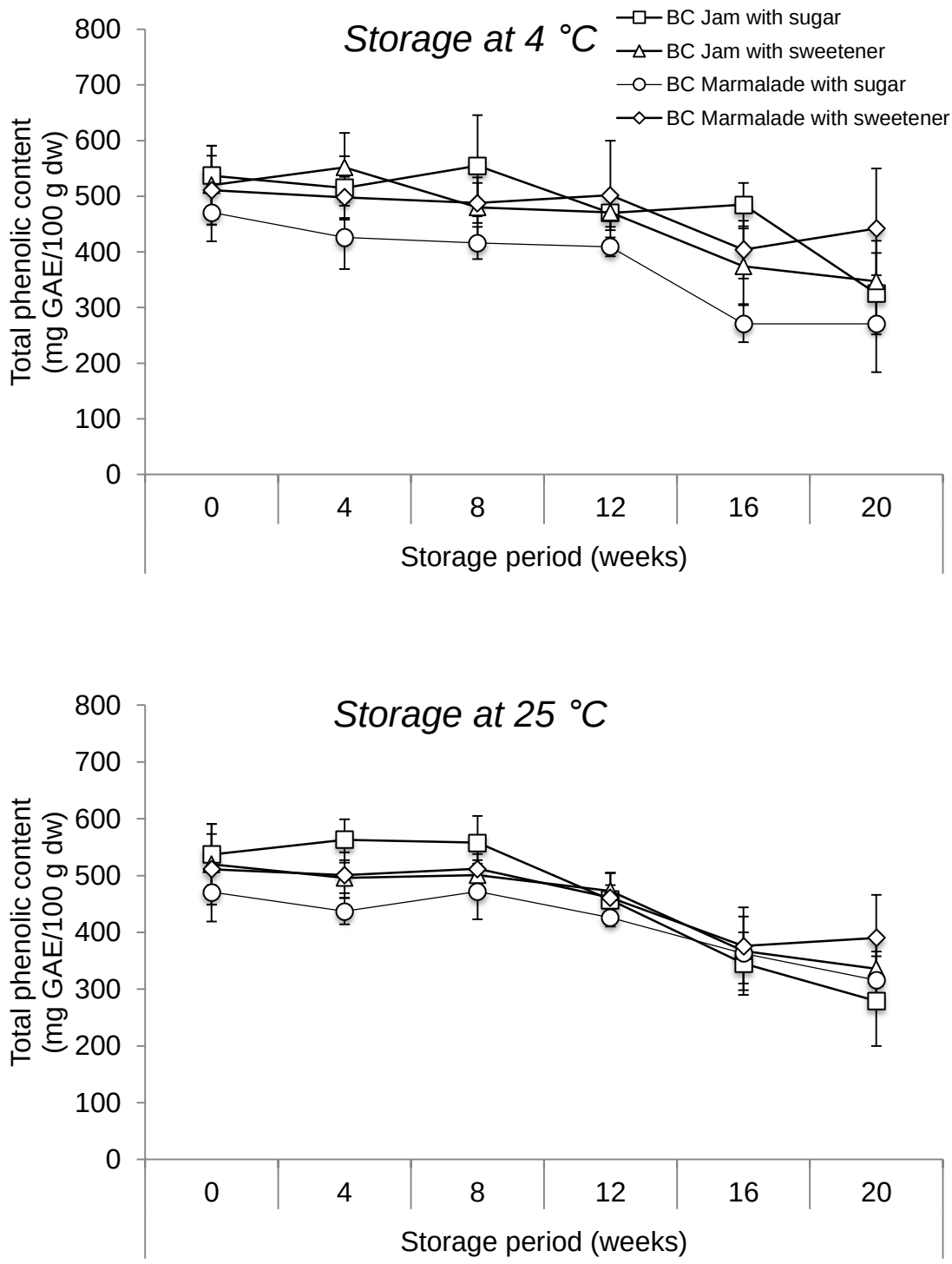

Figure 3.3 : Changes in the total phenolic content of black carrot (BC) jams and marmalades during 20 weeks of storage at 4 °C and 25 °C.

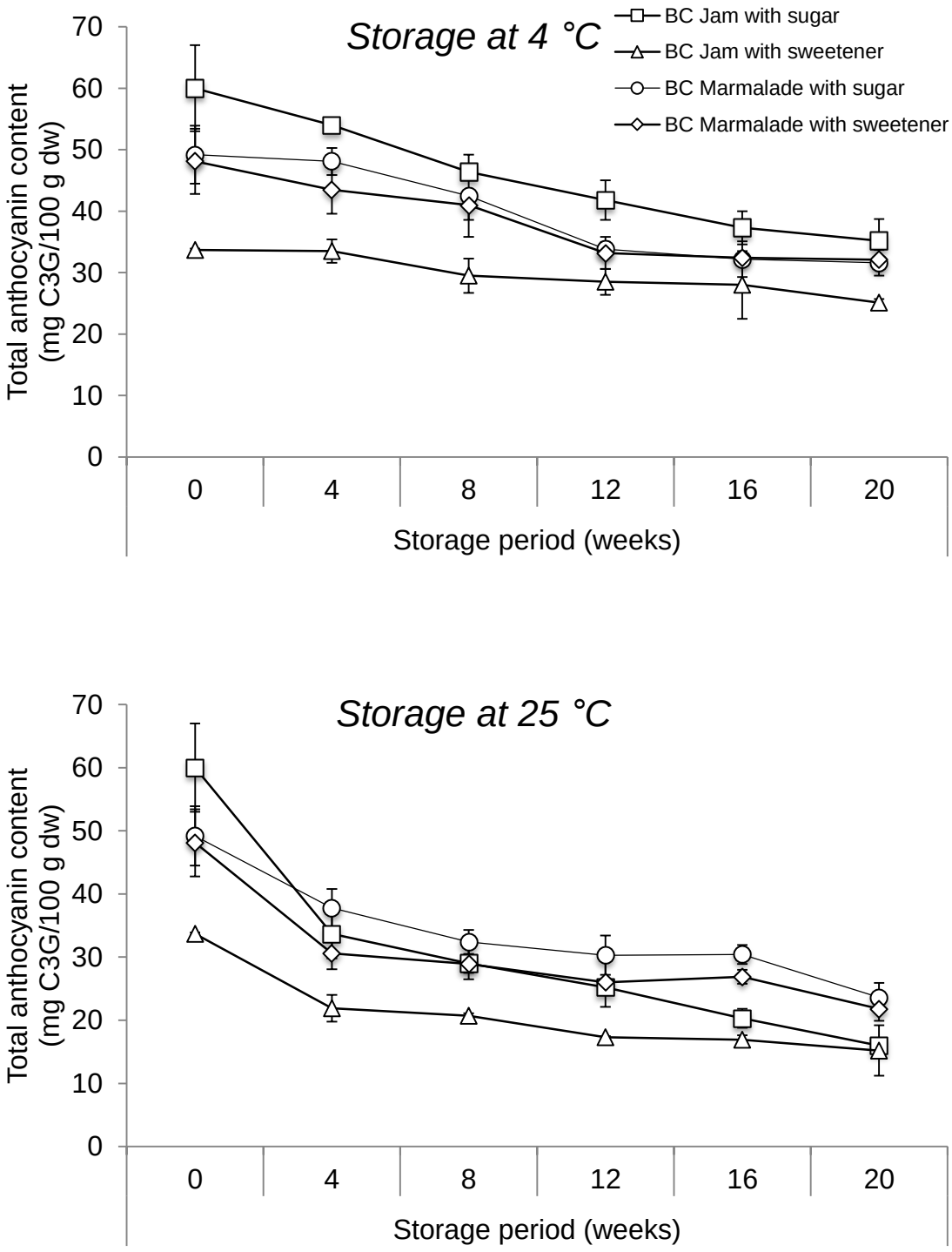

Figure 3.4 : Changes in the total anthocyanin content of black carrot (BC) jams and marmalades during 20 weeks of storage at 4 °C and 25 °C.

Table 3.2 : Changes in anthocyanins of black carrot jams and marmalades during 20 weeks of storage at 4 °C and 25 °C.

	Day 1	Week 4	Week 8	Week 12	Week 16	Week 20
Raw material						
Cyd-3-xyl-gluc-gal	102.3 ± 5.1					
Cyd-3-xyl-gal	143.1 ± 8.1					
Cyd-3-xyl-sin-gluc-gal	109.7 ± 9.8					
Cyd-3-xyl-fer-gluc-gal	596.9 ± 27.6					
Cyd-3-xyl-coum-gluc-gal	97.3 ± 3.9					
			Storage at 4 °C			
Jam with sugar						
Cyd-3-xyl-gluc-gal	10.7 ± 0.4^{a}	9.3 ± 1.2^{ab}	9.1 ± 1.3^{ab}	8.7 ± 0.3^{ab}	8.1 ± 1.4^{ab}	6.9 ± 1.3^{b}
Cyd-3-xyl-gal	14.8 ± 1.4^{a}	13.5 ± 1.7^{ab}	13.2 ± 1.8^{ab}	12.1 ± 0.5^{ab}	11.9 ± 2.2^{ab}	10.0 ± 1.9^{b}
Cyd-3-xyl-sin-gluc-gal	8.0 ± 0.9^{a}	7.8 ± 0.4^{a}	7.2 ± 1.0^{a}	7.2 ± 0.5^{a}	6.7 ± 0.9^{a}	5.7 ± 1.1^{a}
Cyd-3-xyl-fer-gluc-gal	46.0 ± 4.0^{a}	45.8 ± 4.7^{d}	42.6 ± 2.0^{a}	41.9 ± 6.1^{a}	40.1 ± 6.3^{a}	33.3 ± 6.5^{a}
Cyd-3-xyl-coum-gluc-gal	8.4 ± 0.9^{a}	8.2 ± 0.7^{ab}	7.7 ± 0.7^{ab}	7.2 ± 1.0^{ab}	7.0 ± 1.2^{ab}	5.8 ± 1.1^{b}
Jam with sweetener						
Cyd-3-xyl-gluc-gal	4.9 ± 0.2^{a}	4.5 ± 0.1^{ab}	3.8 ± 0.2^{bc}	3.8 ± 0.4^{c}	3.7 ± 0.2^{c}	3.3 ± 0.3^{c}
Cyd-3-xyl-gal	8.0 ± 0.2^{a}	7.0 ± 0.3^{ab}	6.2 ± 0.5^{bc}	6.0 ± 0.5^{bc}	6.0 ± 0.8^{bc}	5.4 ± 0.5^{c}
Cyd-3-xyl-sin-gluc-gal	5.2 ± 0.2^{a}	5.0 ± 0.2^{ab}	4.3 ± 0.4^{abc}	4.3 ± 0.5^{abc}	4.3 ± 0.3^{bc}	3.6 ± 0.3^{c}
Cyd-3-xyl-fer-gluc-gal	29.4 ± 1.2^{a}	28.0 ± 1.1^{ab}	25.5 ± 3.0^{abc}	25.4 ± 1.9^{abc}	23.9 ± 2.0^{bc}	21.5 ± 2.1^{c}
Cyd-3-xyl-coum-gluc-gal	7.1 ± 0.2^{a}	6.8 ± 0.3^{ab}	6.3 ± 0.2^{ab}	6.3 ± 0.8^{abc}	5.7 ± 0.6^{bc}	5.1 ± 0.3^{c}
Marmalade with sugar						
Cyd-3-xyl-gluc-gal	7.9 ± 0.7^{a}	7.6 ± 0.7^{a}	7.0 ± 0.8^{a}	7.0 ± 1.1^{a}	6.8 ± 0.3^{a}	6.4 ± 1.0^{a}
Cyd-3-xyl-gal	17.7 ± 1.4^{a}	17.2 ± 0.5^{a}	16.5 ± 1.7^{a}	16.4 ± 1.3^{a}	15.5 ± 2.7^{a}	14.1 ± 4.4^{a}
Cyd-3-xyl-sin-gluc-gal	7.0 ± 0.4^{a}	6.2 ± 0.6^{ab}	5.2 ± 0.3^{b}	5.1 ± 0.7^{b}	5.0 ± 0.7^{b}	5.0 ± 0.3^{b}
Cyd-3-xyl-fer-gluc-gal	34.5 ± 1.4^{a}	30.4 ± 2.4^{ab}	26.8 ± 1.6^{ab}	26.4 ± 2.9^{b}	25.5 ± 3.1^{b}	24.9 ± 4.4^{b}
Cyd-3-xyl-coum-gluc-gal	6.9 ± 0.2^{a}	5.8 ± 0.7^{ab}	5.2 ± 0.5^{b}	5.2 ± 0.8^{b}	5.1 ± 0.5^{b}	5.1 ± 0.3^{b}
Marmalade with sweetener						
Cyd-3-xyl-gluc-gal	5.8 ± 0.8^{a}	5.2 ± 0.1^{a}	5.1 ± 0.8^{a}	5.1 ± 0.5^{a}	4.5 ± 0.2^{a}	4.1 ± 1.3^{a}
Cyd-3-xyl-gal	9.8 ± 1.4^{a}	8.6 ± 0.1^{a}	8.6 ± 0.9^{d}	8.3 ± 1.1^{a}	7.7 ± 0.3^{ab}	5.6 ± 0.4^{b}
Cyd-3-xyl-sin-gluc-gal	6.0 ± 0.1^{a}	5.8 ± 1.1^{a}	5.7 ± 0.6^{a}	5.6 ± 1.0^{a}	5.0 ± 0.1^{a}	4.2 ± 1.1^{a}
Cyd-3-xyl-fer-gluc-gal	26.4 ± 3.7^{a}	26.3 ± 0.9^{a}	25.4 ± 3.0^{a}	24.2 ± 3.3^{a}	22.2 ± 1.0^{a}	19.2 ± 6.4^{a}
Cyd-3-xyl-coum-gluc-gal	5.8 ± 0.8^{a}	5.7 ± 0.1^{a}	5.5 ± 0.7^{a}	5.2 ± 0.7^{a}	5.0 ± 0.2^{a}	3.1 ± 0.1^{b}

Table 3.2 (continued) : Changes in anthocyanins of black carrot jams and marmalades during 20 weeks of storage at 4 °C and 25 °C.

	Day 1	Week 4	Week 8	Week 12	Week 16	Week 20
			Storage at 25 °C			
Jam with sugar						
Cyd-3-xyl-gluc-gal	10.7±0.4^{a}	6.3±0.6^{b}	4.6±0.4^{b}	2.4±1.4^{c}	1.8±0.1^{c}	1.2±0.3^{c}
Cyd-3-xyl-gal	14.8±1.4^{a}	8.5±1.3^{b}	6.0±0.4^{b}	2.6±1.3^{c}	2.2±0.3^{c}	1.6±0.4^{c}
Cyd-3-xyl-sin-gluc-gal	8.0±0.9^{a}	6.8±0.4^{ab}	6.4±1.0^{ab}	4.7±0.6^{b}	4.4±0.2^{b}	4.2±2.0^{b}
Cyd-3-xyl-fer-gluc-gal	46.0±4.0^{a}	39.4±2.2^{a}	37.9±4.7^{a}	27.7±3.9^{b}	25.6±1.1^{b}	23.7±4.9^{b}
Cyd-3-xyl-coum-gluc-gal	8.4±0.9^{a}	6.7±1.0^{ab}	6.7±0.7^{ab}	4.3±0.4^{bc}	3.9±0.3^{c}	3.6±1.7^{c}
Jam with sweetener						
Cyd-3-xyl-gluc-gal	4.9±0.2^{a}	2.8±0.2^{b}	1.6±0.1^{c}	0.7±0.1^{d}	0.4±0.1^{d}	0.4±0.2^{d}
Cyd-3-xyl-gal	8.0±0.2^{a}	4.3±0.2^{b}	2.2±0.1^{c}	1.0±0.2^{d}	0.7±0.1^{d}	0.7±0.2^{d}
Cyd-3-xyl-sin-gluc-gal	5.2±0.2^{a}	4.5±0.6^{ab}	3.8±0.3^{bc}	2.9±0.2^{cd}	2.6±0.1^{d}	2.4±0.7^{d}
Cyd-3-xyl-fer-gluc-gal	29.4±1.2^{a}	26.4±1.3^{ab}	21.5±2.4^{bc}	17.2±1.3^{cd}	15.1±0.5^{d}	14.4±2.9^{d}
Cyd-3-xyl-coum-gluc-gal	7.1±0.2^{a}	6.5±0.1^{a}	5.0±0.6^{b}	3.8±0.4^{bc}	3.1±0.2^{c}	3.0±0.7^{c}
Marmalade with sugar						
Cyd-3-xyl-gluc-gal	7.9±0.7^{a}	7.2±1.0^{a}	5.2±0.3^{b}	4.3±0.3^{b}	2.6±0.1^{c}	2.2±0.5^{c}
Cyd-3-xyl-gal	17.7±1.4^{a}	16.9±2.1^{a}	10.9±0.1^{b}	9.4±0.5^{b}	5.4±0.1^{c}	4.5±1.0^{c}
Cyd-3-xyl-sin-gluc-gal	7.0±0.4^{a}	5.3±0.1^{b}	5.2±0.1^{b}	4.7±0.4^{b}	4.0±0.1^{c}	4.0±0.1^{c}
Cyd-3-xyl-fer-gluc-gal	34.5±1.4^{a}	26.8±0.8^{ab}	25.6±1.6^{b}	24.7±6.1^{b}	24.6±2.2^{b}	21.9±1.2^{b}
Cyd-3-xyl-coum-gluc-gal	6.9±0.2^{a}	5.2±0.5^{b}	5.2±0.3^{b}	5.1±0.2^{b}	4.9±1.0^{b}	3.9±0.1^{b}
Marmalade with sweetener						
Cyd-3-xyl-gluc-gal	5.8±0.8^{a}	3.4±0.3^{b}	2.0±0.2^{c}	0.9±0.2^{cd}	0.8±0.5^{cd}	0.5±0.1^{d}
Cyd-3-xyl-gal	9.8±1.4^{a}	5.3±0.4^{b}	3.1±0.4^{c}	1.6±0.2^{cd}	1.3±0.7^{cd}	0.7±0.2^{d}
Cyd-3-xyl-sin-gluc-gal	6.0±0.1^{a}	5.7±1.0^{b}	5.1±0.2^{ab}	4.9±0.7^{ab}	4.5±0.3^{ab}	4.0±0.3^{ab}
Cyd-3-xyl-fer-gluc-gal	26.4±3.7^{a}	23.5±2.4^{a}	21.3±1.1^{a}	20.5±2.6^{a}	19.9±0.4^{a}	18.3±5.2^{a}
Cyd-3-xyl-coum-gluc-gal	5.8±0.8^{a}	4.8±0.5^{ab}	4.4±0.4^{ab}	3.9±0.2^{b}	3.9±0.6^{b}	3.4±0.9^{b}

The data represent average values ± standard deviation of three independent samples. All contents are expressed as mg per 100 g dry weight (dw). Different letters in the rows represent statistically significant differences ($p < 0.05$). Cyd-3-xyl-gluc-gal: cyanidin-3-xylosyl-glucosyl-galactoside; Cyd-3-xyl-gal: cyanidin-3-xylosyl-galactoside; Cyd-3-xyl-sin-gluc-gal: cyanidin-3-xylosyl-sinapoyl-glucosyl-galactoside; Cyd-3-xyl-fer-gluc-gal: cyanidin-3-xylosyl-feruloyl-glucosyl-galactoside; Cyd-3-xyl-coum-gluc-gal: cyanidin-3-xylosyl-coumaroyl-glucosyl-galactoside.

During storage at 25 °C, acylated anthocyanins were significantly more stable than non-acylated anthocyanins. This finding was also not surprising since acylated anthocyanins are known to be more stable under heat, light and other environmental condition as compared to non-acylated anthocyanins (Koley et al, 2014). Moreover, anthocyanins in jams and marmalades containing sugar preserved better than samples prepared with sweetener. Thus, higher sugar content associated with lower water activity assisted anthocyanin stability, as discussed previously. Low correlations were found between total anthocyanin content and color properties of jams and marmalades during storage, with the correlation coefficients of R^2 = 0.3981–0.4419 at 4 °C and R^2 = 0.3054–0.6870 at 25 °C.

The changes in phenolic acid content of jams and marmalades during storage varied (Table 3.3). As a result of 20 weeks of storage, samples stored at 4 °C retained higher levels of phenolic acids than in samples stored at 25 °C (60.9–81.3% and 33.3–76.2%, respectively). This is in line with the results achieved in an earlier report (Scibisz and Mitek, 2009) indicating that chlorogenic acid in blueberry jams stored at 6 °C degraded less than the ones stored at 22 °C.

Although storage of jams and marmalades tend to reduce the antioxidant capacity, for most samples this reduction was statistically insignificant ($p > 0.05$) (Figure 3.5 and Figure 3.6). At the end of storage period, samples prepared with sugar retained the highest antioxidant capacity following the trend observed for total phenolic and total anthocyanin contents. After 20 weeks of storage at 4 °C and 25 °C, losses in antioxidant capacity of samples accounted for 7.6–54.8% and 12.8–60.9%, respectively. These findings of more extensive reduction of antioxidant capacity during storage of jams at a higher temperature are compatible with the previously reported data (Wicklund et al, 2005; Scibisz and Mitek, 2009).

Table 3.3 : Changes in phenolic acids of black carrot jams and marmalades during 20 weeks of storage at 4 °C and 25 °C.

	Day 1	Week 4	Week 8	Week 12	Week 16	Week 20
			Chlorogenic acid (5-CQA)			
Raw material	1086.1 ± 63.6					
Storage at 4°C						
Jam with sugar	87.7 ± 8.9^{a}	87.7 ± 6.1^{a}	86.5 ± 2.4^{a}	76.3 ± 11.6^{ab}	75.2 ± 10.6^{ab}	60.9 ± 11.2^{b}
Jam with sweetener	127.8 ± 4.6^{a}	123.6 ± 5.3^{ab}	112.0 ± 7.9^{abc}	111.6 ± 13.9^{abc}	103.6 ± 8.6^{bc}	100.6 ± 3.3^{c}
Marmalade with sugar	137.6 ± 5.0^{a}	120.1 ± 9.7^{ab}	107.1 ± 5.9^{b}	105.4 ± 8.4^{b}	98.9 ± 12.6^{b}	97.4 ± 18.3^{b}
Marmalade with sweetener	90.2 ± 17.3^{a}	85.5 ± 12.0^{a}	82.0 ± 9.6^{a}	78.3 ± 10.0^{a}	74.2 ± 2.3^{a}	60.8 ± 19.1^{a}
Storage at 25°C						
Jam with sugar	87.7 ± 8.9^{a}	84.5 ± 7.0^{a}	79.3 ± 4.5^{a}	73.9 ± 10.0^{ab}	73.2 ± 10.0^{ab}	43.2 ± 11.3^{b}
Jam with sweetener	127.8 ± 4.6^{a}	123.7 ± 7.2^{a}	111.5 ± 13.9^{ab}	100.6 ± 3.3^{b}	96.8 ± 7.3^{b}	95.7 ± 9.4^{b}
Marmalade with sugar	137.6 ± 5.0^{a}	108.4 ± 4.2^{ab}	105.8 ± 24.5^{ab}	102.2 ± 13.9^{ab}	92.2 ± 16.5^{b}	66.9 ± 19.1^{b}
Marmalade with sweetener	90.2 ± 17.3^{a}	84.6 ± 8.7^{a}	77.2 ± 6.0^{a}	74.4 ± 1.8^{a}	70.7 ± 2.9^{a}	55.1 ± 25.0^{a}
			Cryptochlorogenic acid (4-CQA)			
Raw material	75.6 ± 3.3					
Storage at 4°C						
Jam with sugar	16.8 ± 0.5^{a}	16.8 ± 0.5^{a}	15.9 ± 1.4^{a}	15.1 ± 0.9^{ab}	14.1 ± 2.0^{ab}	11.4 ± 2.2^{b}
Jam with sweetener	20.2 ± 1.2^{a}	20.1 ± 0.8^{a}	18.4 ± 1.2^{ab}	17.8 ± 2.3^{ab}	16.5 ± 1.3^{ab}	15.3 ± 1.4^{b}
Marmalade with sugar	21.1 ± 0.9^{a}	18.6 ± 1.5^{ab}	16.3 ± 1.1^{b}	15.3 ± 1.2^{b}	14.5 ± 1.9^{b}	14.4 ± 2.8^{b}
Marmalade with sweetener	14.0 ± 2.4^{a}	13.5 ± 1.9^{a}	13.4 ± 1.6^{a}	13.0 ± 1.7^{a}	11.2 ± 0.4^{a}	9.5 ± 3.2^{a}
Storage at 25°C						
Jam with sugar	16.8 ± 0.5^{a}	16.6 ± 1.7^{a}	14.3 ± 2.2^{a}	14.2 ± 1.6^{a}	13.8 ± 1.8^{a}	5.6 ± 0.8^{b}
Jam with sweetener	20.2 ± 1.2^{a}	19.9 ± 0.9^{a}	18.3 ± 2.2^{ab}	16.2 ± 0.4^{b}	16.0 ± 0.6^{b}	15.4 ± 1.4^{b}
Marmalade with sugar	21.1 ± 0.9^{a}	16.6 ± 0.8^{ab}	15.4 ± 3.5^{abc}	15.1 ± 2.0^{abc}	11.7 ± 2.4^{bc}	9.3 ± 3.4^{c}
Marmalade with sweetener	14.0 ± 2.4^{a}	13.4 ± 1.3^{a}	11.7 ± 3.1^{ab}	11.7 ± 0.2^{ab}	11.7 ± 0.5^{ab}	7.9 ± 1.4^{b}

Table 3.3 (continued) : Changes in phenolic acids of black carrot jams and marmalades during 20 weeks of storage at 4 °C and 25 °C.

	Day 1	Week 4	Week 8	Week 12	Week 16	Week 20
			Neochlorogenic acid (3-CQA)			
Raw material	29.3 ± 8.0					
Storage at 4°C						
Jam with sugar	12.9 ± 0.8^{a}	12.6 ± 0.3^{a}	12.3 ± 1.2^{a}	11.5 ± 0.7^{a}	10.7 ± 1.5^{ab}	8.1 ± 1.4^{b}
Jam with sweetener	13.4 ± 0.5^{a}	13.2 ± 0.7^{a}	12.0 ± 1.7^{a}	11.8 ± 1.4^{a}	11.3 ± 0.2^{a}	10.9 ± 0.8^{a}
Marmalade with sugar	14.8 ± 0.6^{a}	12.9 ± 1.2^{ab}	11.5 ± 0.6^{bc}	10.8 ± 1.0^{bc}	10.1 ± 1.5^{bc}	9.8 ± 1.5^{c}
Marmalade with sweetener	9.9 ± 0.9^{a}	9.6 ± 1.4^{a}	9.1 ± 1.2^{a}	8.8 ± 0.5^{a}	8.3 ± 0.2^{a}	6.6 ± 2.2^{a}
Storage at 25°C						
Jam with sugar	12.9 ± 0.8^{a}	11.7 ± 1.0^{ab}	10.6 ± 1.2^{ab}	10.4 ± 1.6^{ab}	10.3 ± 1.5^{ab}	7.4 ± 3.2^{b}
Jam with sweetener	13.4 ± 0.5^{a}	12.9 ± 0.5^{ab}	11.9 ± 0.7^{abc}	11.0 ± 0.9^{bc}	10.9 ± 0.3^{c}	10.0 ± 0.9^{c}
Marmalade with sugar	14.8 ± 0.6^{a}	11.6 ± 0.4^{ab}	11.4 ± 2.8^{ab}	10.7 ± 1.5^{ab}	10.1 ± 1.9^{ab}	6.7 ± 2.4^{b}
Marmalade with sweetener	9.9 ± 0.9^{a}	9.5 ± 1.3^{a}	8.8 ± 0.5^{a}	8.2 ± 0.2^{ab}	7.9 ± 0.4^{ab}	6.2 ± 0.8^{b}
			Caffeic acid			
Raw material	55.2 ± 1.9					
Storage at 4°C						
Jam with sugar	2.8 ± 0.5^{a}	2.8 ± 0.2^{a}	2.5 ± 0.2^{ab}	2.3 ± 0.2^{ab}	2.1 ± 0.1^{ab}	1.8 ± 0.3^{b}
Jam with sweetener	2.1 ± 0.1^{a}	2.0 ± 0.3^{a}	2.0 ± 0.1^{a}	1.9 ± 0.1^{a}	1.7 ± 0.3^{a}	1.6 ± 0.1^{a}
Marmalade with sugar	1.8 ± 0.1^{a}	1.6 ± 0.1^{ab}	1.4 ± 0.1^{b}	1.3 ± 0.1^{b}	1.3 ± 0.3^{b}	1.3 ± 0.2^{b}
Marmalade with sweetener	1.8 ± 0.2^{a}	1.8 ± 0.2^{a}	1.6 ± 0.1^{a}	1.6 ± 0.1^{a}	1.5 ± 0.5^{a}	1.4 ± 0.1^{a}
Storage at 25°C						
Jam with sugar	2.8 ± 0.5^{a}	2.5 ± 0.1^{a}	2.3 ± 0.3^{a}	2.0 ± 0.2^{a}	1.9 ± 1.0^{a}	1.8 ± 0.7^{a}
Jam with sweetener	2.1 ± 0.1^{a}	2.0 ± 0.1^{ab}	1.8 ± 0.2^{abc}	1.8 ± 0.1^{abc}	1.7 ± 0.1^{bc}	1.5 ± 0.1^{c}
Marmalade with sugar	1.8 ± 0.1^{a}	1.4 ± 0.1^{ab}	1.4 ± 0.1^{bc}	1.3 ± 0.1^{bc}	0.9 ± 0.2^{cd}	0.8 ± 0.3^{d}
Marmalade with sweetener	1.8 ± 0.2^{a}	1.6 ± 0.2^{a}	1.6 ± 0.1^{a}	1.6 ± 0.3^{a}	1.5 ± 0.1^{a}	1.3 ± 0.5^{a}

Data represent average values ± standard deviation of three independent samples. All contents are expressed as mg per 100 g dry weight (dw). Different letters in the rows represent statistically significant differences ($p < 0.05$).

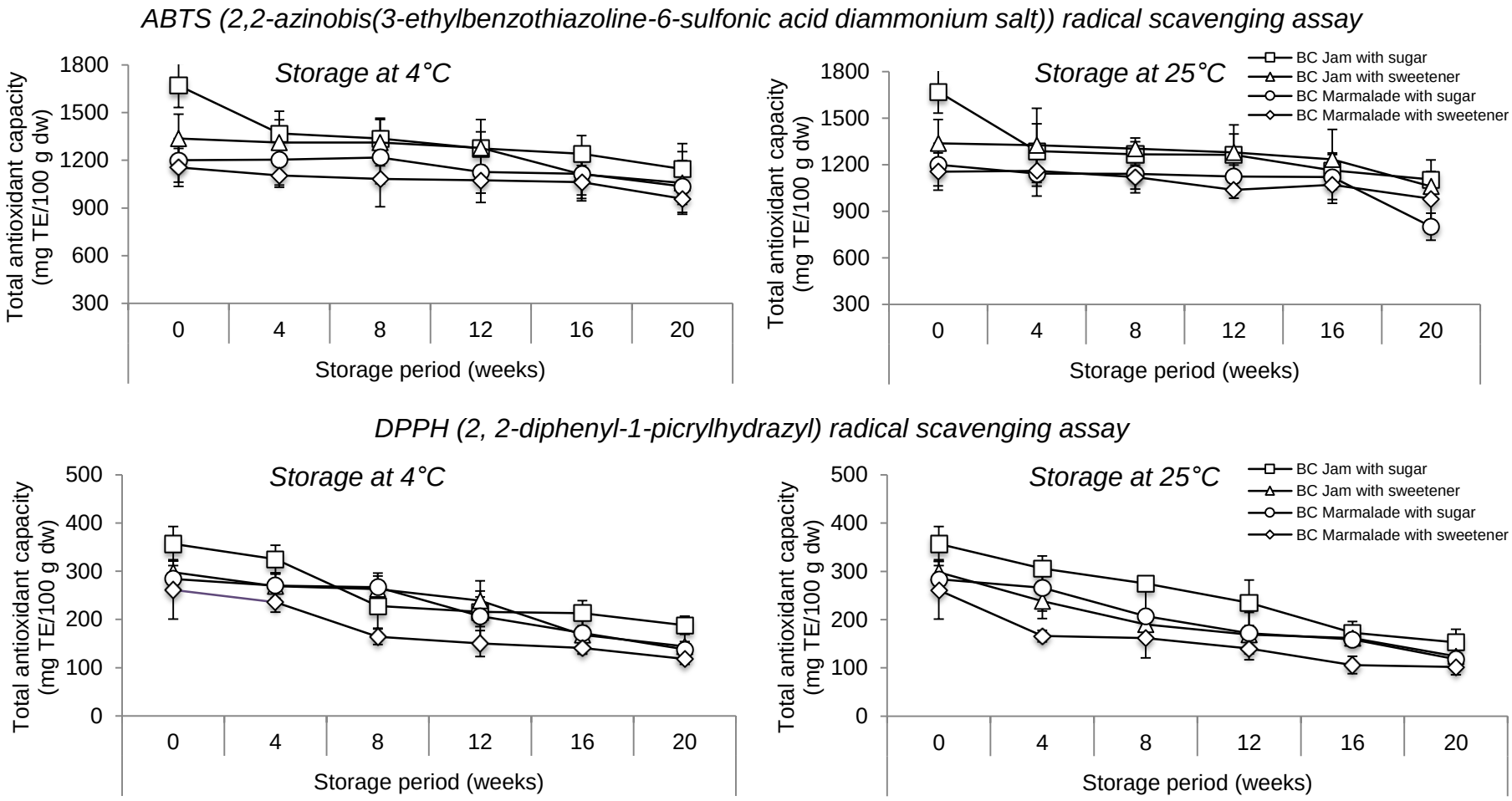

Figure 3.5 : Changes in the total antioxidant capacity (determined using ABTS and DPPH assays) of black carrot (BC) jams and marmalades during 20 weeks of storage at 4 °C and 25 °C.

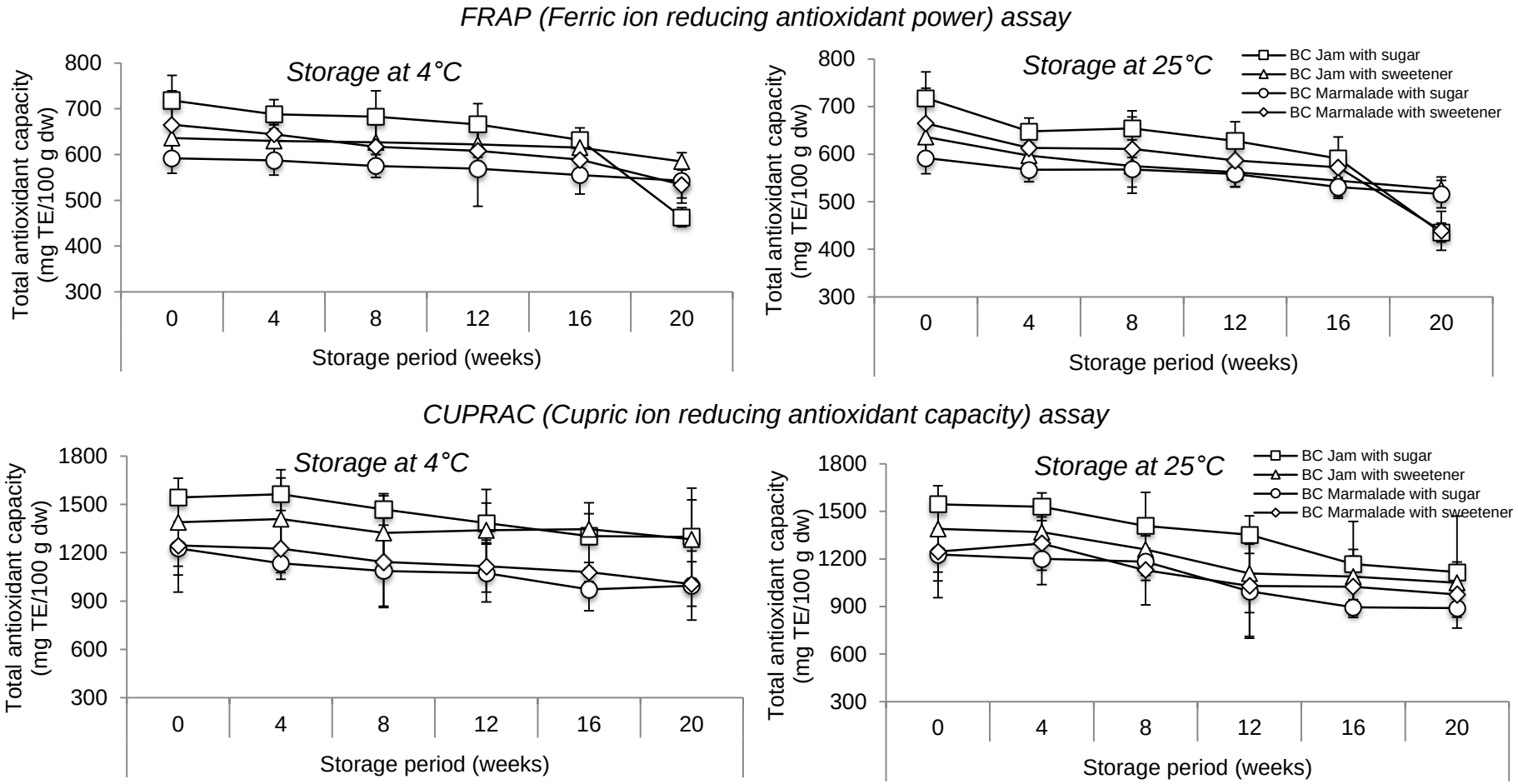

Figure 3.6 : Changes in the total antioxidant capacity (determined using FRAP and CUPRAC assays) of black carrot (BC) jams and marmalades during 20 weeks of storage at 4 °C and 25 °C.

3.4.3 Effect of *in vitro* gastrointestinal digestion on polyphenol stability and antioxidant capacity of black carrot jams and marmalades

Figure 3.7 shows the effect of *in vitro* gastrointestinal digestion on total phenolic content of black carrots, jams and marmalades. After gastric digestion (PG), significantly lower total phenolic contents (51.7–68.2%) were observed for all samples ($p < 0.05$). Our findings are comparable with the results noted by Bouayed et al (2011) who determined that total phenolics in apples after gastric phase was ca. 35% lower compared to values obtained by chemical extraction. Similarly, only 60.4% of the total phenolics initially present in mulberries were recovered after gastric digestion (Liang et al, 2012). The dialyzed phenolic fraction (IN) represented 4.9–17.5% of the initial total phenolic content of the samples. In previous studies, bioaccessible total phenolics within this range was obtained for sour cherry nectar (11.6%) (Toydemir et al, 2013b), dried tomatoes (12%) (Kamiloglu et al, 2014) and pomegranate (14.2%) (Sengul et al, 2014). In addition, jam and marmalade processing led to increases in percent recovery of bioaccessible total phenolic content (7.2–12.6%). Heat treatment applied during jam and marmalade processing might yield structural changes that would end up with higher bioaccessibility of phenolics. Previously, a similar situation where heat treatment increased the bioaccessible phenolics, was observed in case of tomatoes (Kamiloglu et al, 2014). Non-dialyzed phenolic fraction (OUT) accounted for 33.8–52.3% of the initial total phenolic content. Compared to PG values, slight increases in OUT values (0.7–9.5%) were observed. This could be explained by the additional time of extraction and/or the effect of intestinal digestive enzyme on the complex food matrix, facilitating the release of phenolics bound to the matrix (Bouayed et al, 2011).

It is well documented that the Folin–Ciocalteu assay is highly influenced by the interferences of enzymes used for the *in vitro* gastrointestinal digestion procedure (Tagliazucchi et al, 2010) and therefore this assay alone may not be sufficient to reflect the changes in phenolics during *in vitro* digestion. In order to obtain more accurate results, HPLC–PDA analysis of individual phenolic acids was also performed in the present study.

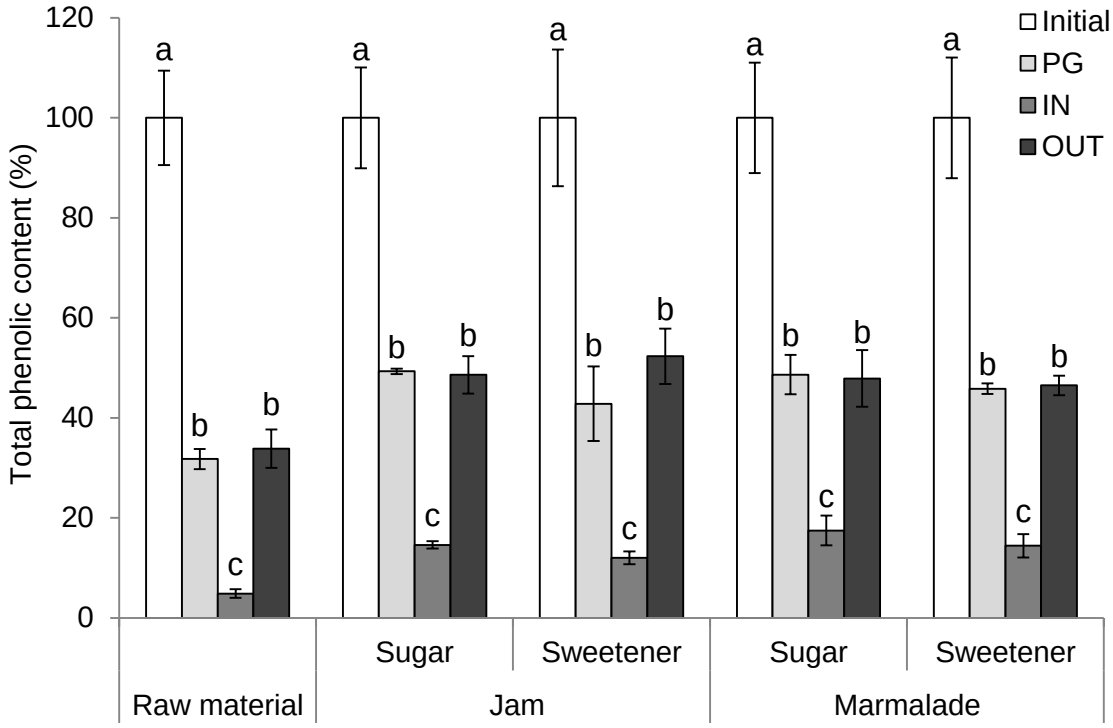

Figure 3.7 : Changes in the total phenolic content of black carrot jams and marmalades during *in vitro* gastrointestinal digestion. Different letters above bars represent statistically significant differences ($p < 0.05$).

The influence of gastrointestinal digestion on total and individual anthocyanin contents of black carrots, jams and marmalades are shown in Figure 3.8 and Figure 3.9. After gastric digestion (PG), significantly lower total anthocyanin content (20.4–65.0%) was observed for all samples ($p < 0.05$). Our findings were comparable with the results noted by Bobrich et al (2014) who determined that 34% and 38% of anthocyanins in Queen Garnet and Black Diamond plum varieties were released after gastric digestion. On the other hand, slight increases (5.9–10.3%) in PG values of non-acylated anthocyanins of marmalades were detected. These increases after gastric digestion were comparable with the results noted for anthocyanins in pomegranate juice (10%) (Perez-Vicente et al, 2002), chokeberry juice (7%) (Bermudez-Soto et al, 2007) and Jonagold apple variety (7%) (Bouayed et al, 2011). The dialyzed anthocyanin fraction (IN) represented only 0.1–10.6% of the initial anthocyanin content of the samples. In previous studies, bioaccessible anthocyanins within this range were obtained for raspberry (5.3% on average) (McDougall et al, 2005), mulberry (0.34%) (Liang et al, 2012), purple figs (0–5%) (Kamiloglu and Capanoglu, 2013) and sour cherry (2.8%) (Toydemir et al, 2013b). In addition, jam and marmalade processing led to increases on the percent recovery of bioaccessible

individual anthocyanins (0.8–10.3%). Confirming the results of an *in vivo* study conducted with 10 healthy adults consuming purple carrot juice (Charron et al, 2009), non-acylated anthocyanins in jams and marmalades were found to be more bioavailable than acylated anthocyanins. Nondialyzed phenolic fraction (OUT) accounted for 0.1–58.1% of the initial anthocyanin content. Nevertheless, anthocyanins that reach the colon may be subjected to microbial degradation, and the metabolites obtained may play a role in the beneficial health effects.

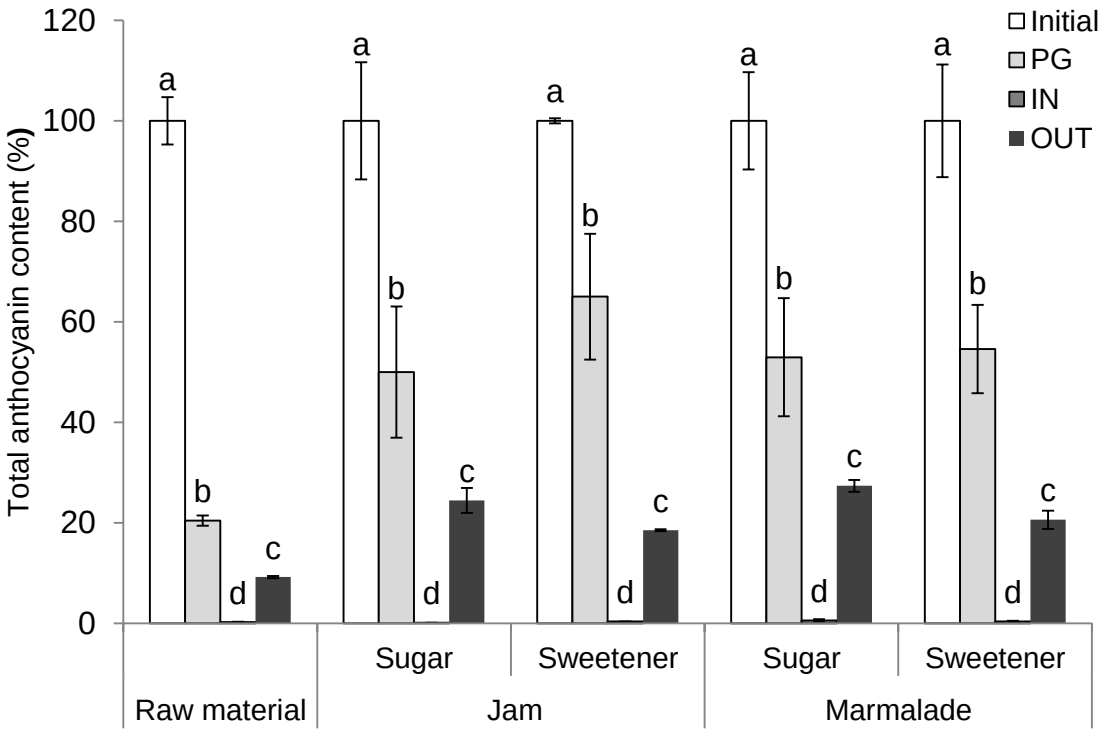

Figure 3.8 : Changes in the total anthocyanin content of black carrot jams and marmalades during *in vitro* gastrointestinal digestion. Different letters above bars represent statistically significant differences ($p < 0.05$).

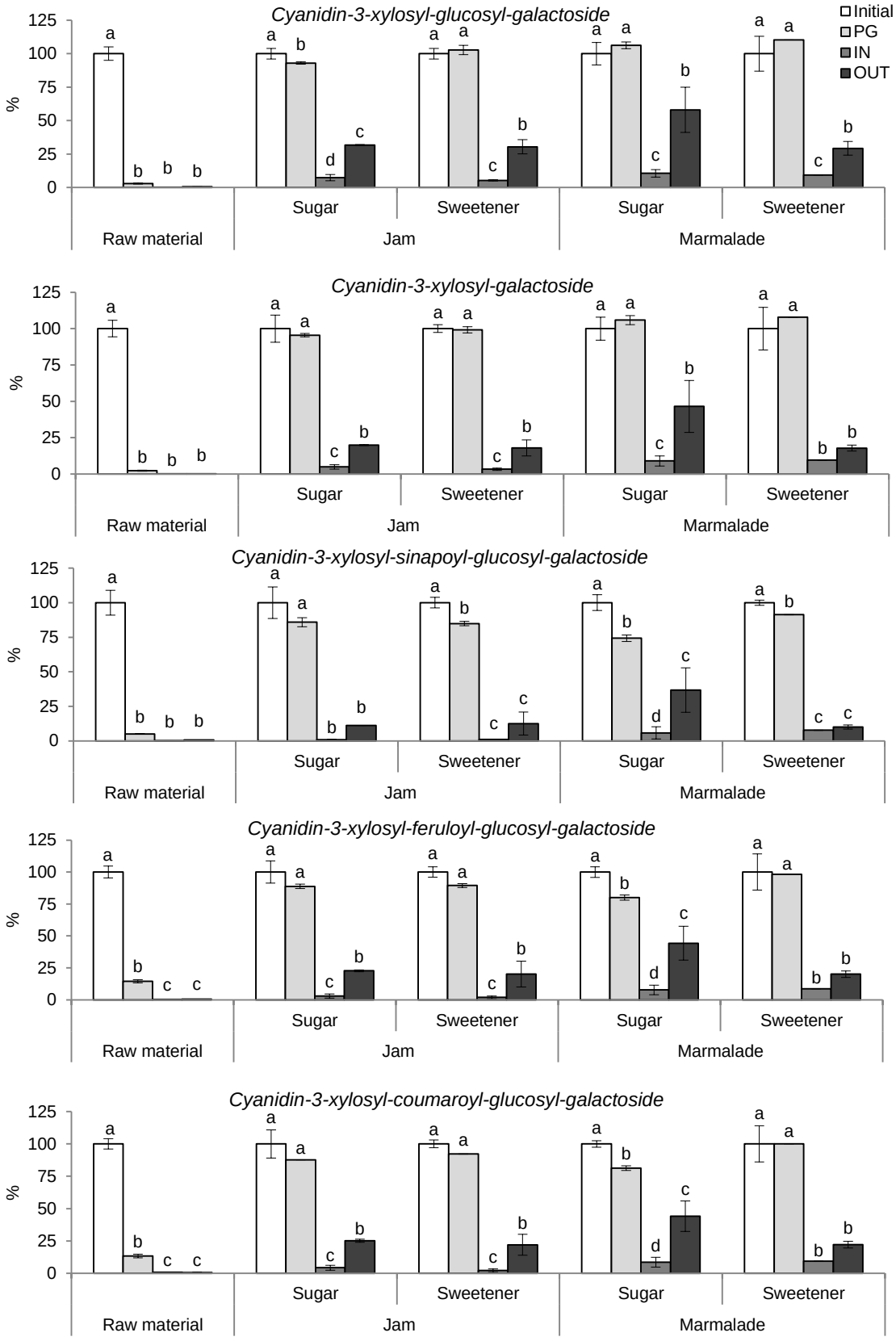

Figure 3.9 : Changes in individual anthocyanin content of black carrot jams and marmalades during *in vitro* gastrointestinal digestion. Different letters above bars represent statistically significant differences ($p < 0.05$).

The impact of gastrointestinal digestion on phenolic acid content of black carrots, jams and marmalades is shown in Figure 3.10. Gastric digestion (PG) significantly decreased the caffeoylquinic acid content of all samples except for marmalade with sweetener (14.6–87.7%) ($p < 0.05$). This reduction was comparable to the results reported by Vallejo et al (2004) who obtained 24.3% loss in caffeoylquinic acid content of broccoli inflorescence after gastric digestion. On the other hand, gastric digestion did not have a significant effect on caffeic acid content of jams and marmalades ($p > 0.05$), which is in accordance with the results of Bermudez-Soto et al (2007) who determined no alteration in levels of caffeic acid derivatives in chokeberry juice during stomach treatment. When the amount of phenolic acids after intestinal digestion was analyzed, 0.7–44.3% of the initial values was determined in the dialyzed fraction (IN). Previously, similar low bioaccessible phenolic acid contents were reported for chlorogenic acid and neochlorogenic acid in sweet cherries (33.8–36.9% and 26.2–31.5%, respectively) (Fazzari et al, 2008) and chlorogenic acid in fresh figs (1–36%) (Kamiloglu and Capanoglu, 2013). These high losses could be associated with the instability of the caffeoylquinic acids in aqueous solution. In addition, the pH value of 7 and the bile salts could contribute to increase this loss (Vallejo et al, 2004). Moreover, the high sugar content in our samples could also interfere the diffusion of phenolic acids during *in vitro* gastrointestinal digestion. It has been noted that high sugar content of samples enables the diffusion of water from the dialysis tubing to the food phase, therefore decreasing the volume of dialyzed fraction (Gil-Izquierdo et al, 2002). Nevertheless, verifying the results acquired for total phenolic content and total antioxidant capacity, jam and marmalade processing also induced higher percent recovery of bioaccessible phenolic acids in all samples except for marmalade with sweetener (4.7–31.5%). In our previous study, a similar increase was observed for figs, where the heat applied during sun-drying resulted with an increase in the bioaccessible chlorogenic acid content (Kamiloglu and Capanoglu, 2013). Non-dialyzed fraction (OUT) represented 0.7–10.4% and 25.6–120.7% of the initial phenolic acid content of fresh black carrots and processed products (jams and marmalades), respectively. These findings show that jam and marmalade processing also increased the colon available phenolic acids.

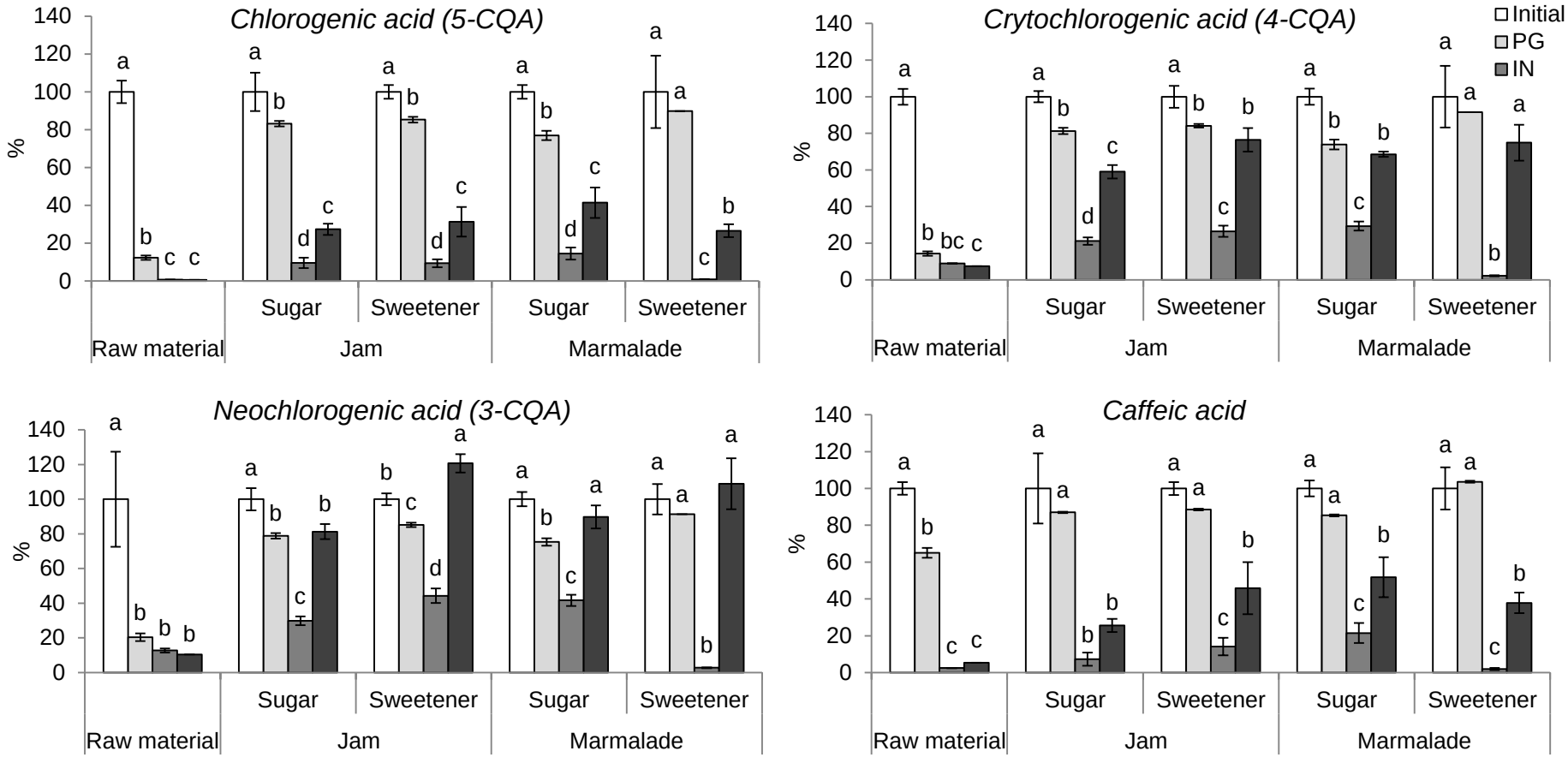

Figure 3.10 : Changes in phenolic acids of black carrot jams and marmalades during *in vitro* gastrointestinal digestion. Different letters above bars represent statistically significant differences ($p < 0.05$).

Polyphenols that reach the colon may be subjected to microbial degradation, and those degradation products derived from the ingested polyphenols may play a role on the beneficial health effects. Despite the suggestions for polyphenol absorption and metabolism, there is still limited information and much of the detail is missing. Further research is needed for a greater understanding of how polyphenols are absorbed, how molecular structures in a consumed food material vary, and how the new structures generated *in vivo* contribute to the health benefits.

The influence of gastrointestinal digestion on antioxidant capacity of black carrots, jams and marmalades is shown in Figure 3.11. Similarly to the outcomes observed in the case of phenolics and anthocyanins, for most of the sample, values obtained after gastric digestion (PG) were significantly lower (30.3–85.4%) compared to the initial values ($p < 0.05$), indicating incomplete release or degradation. Studies carried out on Jonaprinz and Golden apple varieties (Bouayed et al, 2011) and Tesco tomato juice (Wootton-Beard et al, 2011) also revealed that fruits and vegetables might show lower antioxidant activity after gastric digestion. The dialyzed fraction (IN) of the analyzed samples stood for only 0.3–19.1% of the initially determined total antioxidant capacity, which is quite low compared to IN values obtained for total phenolic content as well as the results reported for other fruits in the literature (Liang et al, 2012; Kamiloglu et al, 2014). On the other hand, confirming the results obtained for phenolics and anthocyanins, jam and marmalade processing also caused increases on the percent recovery of bioaccessible antioxidants (1.4–8.1%). As discussed earlier, this increase could be related to the release of bound antioxidants as a result of heat treatment applied during jam and marmalade processing. Non-dialyzed antioxidant fraction (OUT) indicated that 6.1–60.7% of the antioxidants reached the colon. In addition, correlations between phenolics, anthocyanins and total antioxidant capacity were also determined. Before the *in vitro* gastrointestinal digestion as well as after the gastric phase of digestion, a highly linear relationship was obtained between total phenolic content and total antioxidant capacity (R^2 = 0.9519–0.9951 and R^2 = 0.9700–0.9967, respectively). On the other hand, correlations between phenolics and antioxidants after intestinal digestion were generally found to be poor for both IN (R^2 = 0.2667–0.6206) and OUT values (R^2 = 0.3091–0.3257).

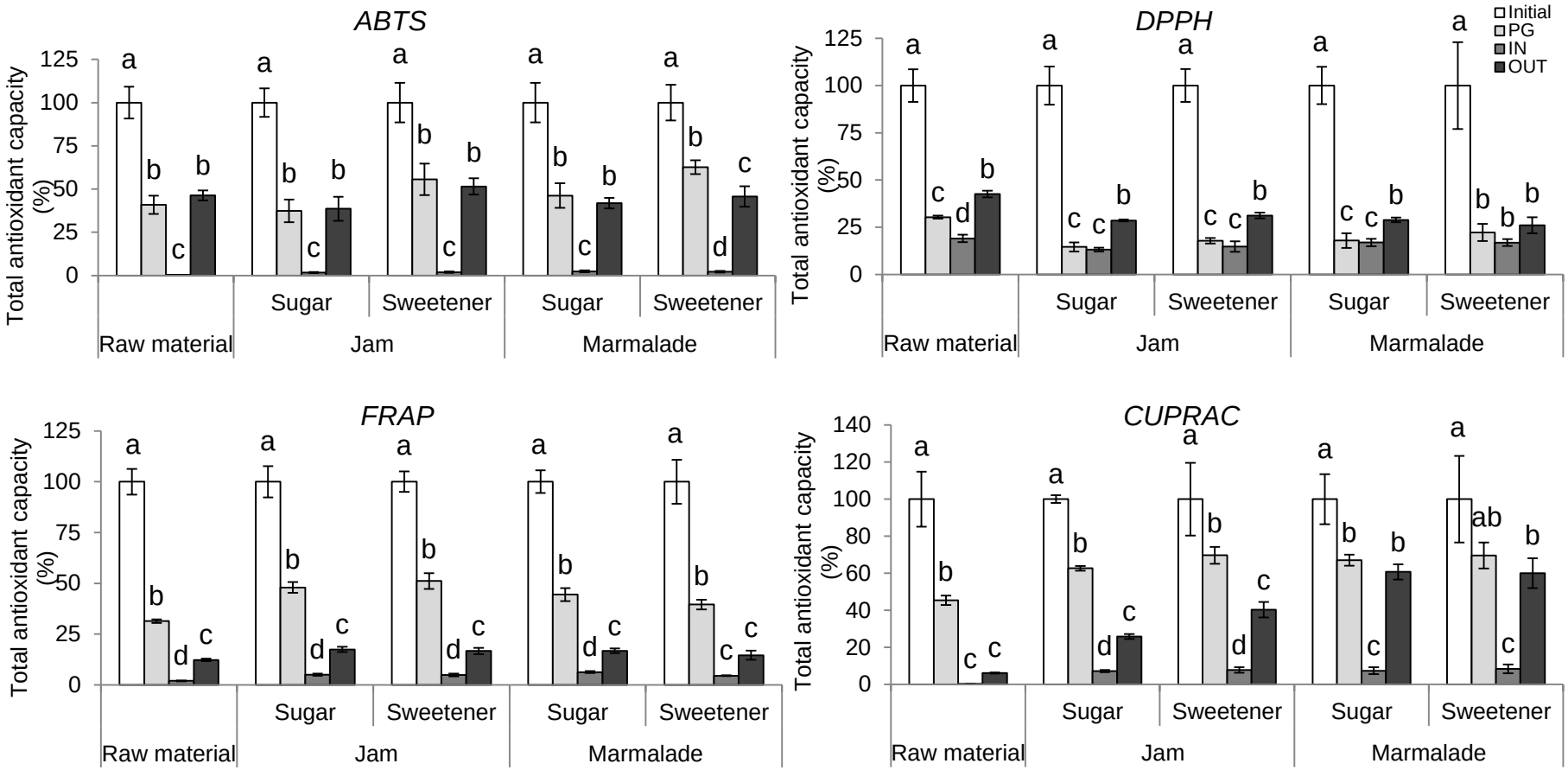

Figure 3.11 : Changes in the total antioxidant capacity of black carrot jams and marmalades during *in vitro* gastrointestinal digestion. Different letters above bars represent statistically significant differences ($p < 0.05$).

Similarly, correlations between anthocyanins and total antioxidant capacity were generally found to be poor for PG (R^2 = 0.4153–0.4349), IN (R^2 = 0.0585–0.0957) and OUT (R^2 = 0.2094–0.2162) values. Lack of correlation between anthocyanins and total antioxidant capacity may be related to the antioxidant activity of colourless phenolic compounds present in black carrots. Theoretically, alterations in the structure of antioxidants following digestion may affect their reactivity, leading to the underestimation of total antioxidant capacity (Wootton-Beard et al, 2011). Moreover, it is well known from the literature that the antioxidant activity of polyphenols is strongly pH-dependent. In addition to the dependency on pH, chemical structure of the phenolic compounds also plays an important role regarding that aglycones are known to show a higher antioxidant activity than their glycosides. Furthermore, interaction of phenolic compounds with other dietary compounds released during digestion, e.g., dietary fiber or proteins, is known to affect the polyphenol solubility and availability, and hence the antioxidant potential (Bouayed et al, 2011). Antioxidant activity occurs by different mechanisms, which means employing a method depending on one mechanism may not reflect the true antioxidant capacity (Karadag et al, 2009). Consequently, it is recommended that at least two of the antioxidant capacity measurement assays should be combined to ensure a reliable picture as was done in the current study.

3.5 Conclusions

To our knowledge, the current study is the first research describing the stability of color properties, polyphenols and antioxidant capacity of black carrot jams and marmalades during processing, storage and *in vitro* gastrointestinal digestion. The current study showed that black carrot jams and marmalades provide considerable polyphenol intake, which are preserved to a certain degree after storage and digestion. The high losses of anthocyanins observed during jam and marmalade processing and storage bring challenges to the food industry. In order to minimize the loss of beneficial compounds and to achieve a good colored with high antioxidant capacity, these products should be stored at 4 °C. Even though the conclusions drawn with the static model used for the simulation of *in vitro* gastrointestinal digestion cannot directly predict the human *in vivo* conditions, we propose that this model is useful for investigating the effect of food matrix and enzymes on polyphenol

bioaccessibility. Nevertheless, *in vitro* gastrointestinal digestion models can be considered as novel techniques, and therefore in future studies *in vivo* feeding trials should also be carried out in order to support the findings of the present study. In further studies, we are planning to focus on digestion of black carrot polyphenols using a model that includes a colon microbial digestion, coupled together with cellular models such as Caco-2 in order to obtain qualitatively well-correlated results with human studies. In addition, more information about the effect of storage on the preservation of microbial safety in jams and marmalades could be necessary before the production of these products at industrial scale.

Processing of foods of plant origin including black carrot generates large amounts of by-products (e.g. peel). Considering that, in the following chapter (*Chapter 4*), polyphenols from black carrot by-products were investigated. Furthermore, the HPLC method applied here was used for the characterization of polyphenols in by-products, as well as to investigate their bioavailability and cellular metabolism in the succeeding chapters (*Chapter 4, 5* and *6*).

4. BIOACCESSIBILITY OF POLYPHENOLS FROM PLANT-PROCESSING BYPRODUCTS OF BLACK CARROT (*DAUCUS CAROTA* L.)

Redrafted from:

Kamiloglu, S., Capanoglu, E., Bilen, F. D., Gonzales, G. B., Grootaert, C., Van de Wiele, T., and Van Camp, J. (2016). Bioaccessibility of polyphenols from plant-processing byproducts of black carrot (*Daucus carota* L.). *Journal of Agricultural and Food Chemistry*, *64*, 2450-2458.

4.1 Abstract

Plant-processing byproducts of black carrot represent an important disposal problem for the industry; however, they are also promising sources of polyphenols, especially anthocyanins. The present study focused on the changes in polyphenols from black carrot, peel, and pomace during *in vitro* gastrointestinal digestion. Total phenolic content, total monomeric anthocyanin content, and total antioxidant capacity were determined using spectrophotometric methods, whereas identification and quantification of polyphenols were carried out using UPLC-ESI-MS^E and HPLC-DAD, respectively. Total phenolic content, total monomeric anthocyanin content, and total antioxidant capacity significantly decreased (23−82%) as a result of *in vitro* gastrointestinal digestion. Nevertheless, the amount of pomace anthocyanins released at all stages of *in vitro* gastrointestinal digestion was higher than black carrot anthocyanins, suggesting that pomace may be a better source of bioaccessible anthocyanins. Overall, the current study highlighted black carrot byproducts as substantial sources of polyphenols, which may be used to enrich food products.

Keywords: peel, pomace, anthocyanins, phenolic acids, antioxidant capacity, HPLC, *in vitro* gastrointestinal digestion, short-chain fatty acids.

4.2 Introduction

Black carrots originate from Turkey and the Middle and Far East, where they have been cultivated for at least 3000 years. They have an attractive bluish-purple color with high levels of anthocyanins and can serve as a natural food colorant due to their high heat, light, and pH stability (Montilla et al, 2011). Apart from their colorant properties, anthocyanins may serve an important role in promoting health by reducing the risk of atherosclerosis and cancer, preventing inflammation, and acting as antioxidants (Kurilich et al, 2005). Besides anthocyanins as the major polyphenols, black carrots also contain significant amounts of phenolic acids, including hydroxycinnamates and caffeic acid (Kammerer et al, 2004a).

Like many fruits and vegetables, black carrots are seasonal and perishable and difficult to preserve as a raw material. Therefore, they are processed into various products such as juice (Khandare et al, 2011), concentrate (Suzme et al, 2014), jam (Kamiloglu et al, 2015c) and shalgam, a traditional lactic acid fermented beverage (Turker et al, 2004). As a result of processing, large amounts of byproducts including peel and pomace are generated. Black carrot pomace production in Turkey is estimated as 2700 and 6900 tons in 2009 and 2010, respectively. This trend is expected to continue and even to accelerate in coming years (Agcam and Akyildiz, 2015). Byproducts of plant food processing represent a major disposal problem for the industry concerned; however, they are also promising sources of bioactive compounds (Schieber et al, 2001). In fact, several byproducts, especially the ones from wine industry (Kammerer et al, 2004c; Makris et al, 2007), have been shown to be rich sources of polyphenols.

Bioaccessibility is defined as the amount of a food constituent that is released from a complex food matrix in the lumen of the gastrointestinal tract and, therefore, could potentially be available for absorption into the body. Only polyphenols released from the food matrix by the action of digestive enzymes and bacterial microflora are bioaccessible in the gut and therefore potentially bioavailable (Saura-Calixto et al, 2007). *In vitro* methods simulating digestion processes are widely used to study the gastrointestinal behavior of food components. Although human nutritional studies are still being considered as the “gold standard” for addressing diet-related questions, *in vitro* methods have the advantages of being more rapid, less expensive, and less labor intensive and do not have ethical restrictions (Minekus et al, 2014). *In vitro*

gastrointestinal digestion methods have already been used to study the release of polyphenols from byproducts of grape (Wang et al, 2013), mango (Blancas-Benitez et al, 2015), pomegranate (Mosele et al, 2015), and cauliflower (Gonzales et al, 2015). Nevertheless, to the best of our knowledge, this is the first study that has focused on the changes in polyphenols from black carrot peel and pomace during *in vitro* gastrointestinal digestion.

Given the above, the aim of this study was to investigate the digestive stability of the total phenolics, total monomeric anthocyanins, and some abundant phenolic acids and anthocyanins from black carrot and its byproducts as well as changes in their antioxidant activity using an *in vitro* model that simulates the conditions in the stomach, small intestine, and colon. In addition, short-chain fatty acids formed as a result of microbial metabolic activity were also monitored.

4.3 Materials and Methods

4.3.1 Plant material

Black carrot (*Daucus carota* L. spp. *sativus* var. *atrorubens* Alef.) and its industrial byproduct pomace were collected in triplicate from an established processing plant (Erkon Konsantre Co.) in Konya, Turkey, in April 2014. Peel was obtained manually from whole black carrots. All samples were ground to a fine powder in liquid nitrogen using a precooled grinder (IKA A11 basic, IKA-Werke GmbH & Co., Staufen, Germany) and subsequently lyophilized (Christ Alpha 1-2 LD plus, Martin Christ Gefriertrocknungsanlagen, Osterode am Harz, Germany) for 24 h and transported in frozen state to Belgium, where they were stored at −20 °C until further analysis.

The flowchart of the black carrot juice processing line, where the pomace samples were collected, is provided in Figure 4.1.

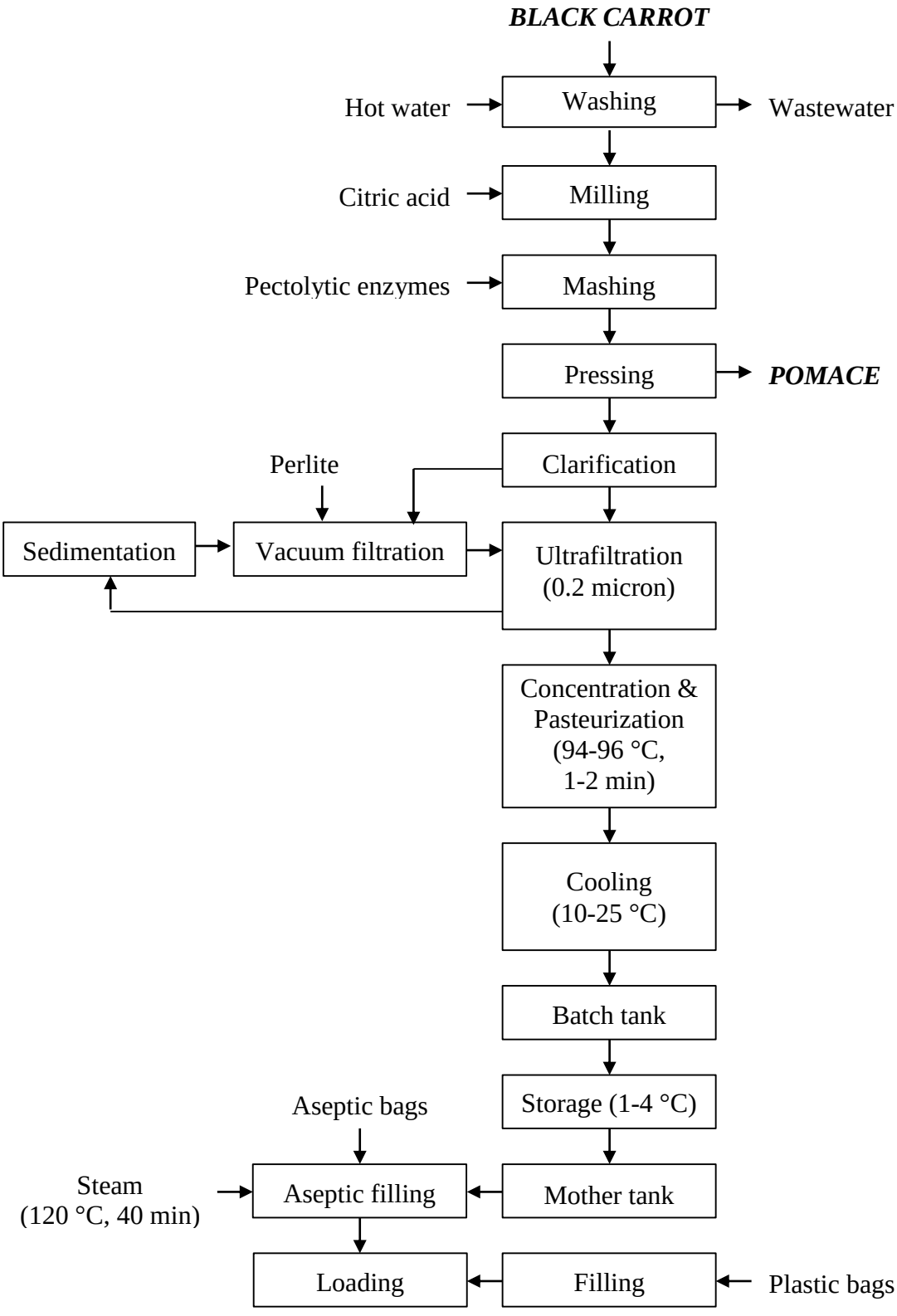

Figure 4.1 : Flow chart outlining the steps involved in black carrot juice production.

4.3.2 Chemicals

For simulation of *in vitro* digestion, mucin, bovine serum albumin (BSA), pepsin, pancreatin, lipase, and bile from Sigma-Aldrich Chemie GmbH (Steinheim, Germany) and urea from Chem-Lab NV (Zedelgem, Belgium) were purchased. The following standards were used for the quantification of major polyphenols: neochlorogenic acid (≥98%), caffeic acid (≥98%), and cyanidin-3-*O*-glucoside (≥95%) from Sigma-Aldrich; chlorogenic acid (≥98%) and ferulic acid (≥99%) from Fluka Chemie AG (Buchs, Switzerland).

4.3.3 Simulated *in vitro* gastrointestinal digestion

The *in vitro* gastrointestinal digestion model applied in this study consisted of a three-step procedure, which sequentially simulated the digestion in the stomach, small intestine, and colon. The compositions of gastric, duodenal, and bile solutions are presented in detail in Table 4.1. Briefly, 3 g of black carrot, peel, and pomace samples were weighed into penicillin bottles and mixed with 47 mL of distilled water and 10 mL of gastric solution. The samples were acidified to pH 3 and incubated at 37 °C in a shaker (Edmund Bühler TH 15, Hechingen, Germany) at 100 rpm for 2 h. After gastric digestion, 15 mL aliquots were collected for each sample. Afterward, the pH was increased to 7 with the addition of 30 mL of duodenal and 15 mL of bile solutions. The samples were incubated in a shaker at 37 °C and 100 rpm for another 4 h to complete the small intestine phase of the *in vitro* gastrointestinal digestion. After the small intestine phase, again samples were collected. Following sampling, 30 mL of colon microbial suspensions from the descending colon compartment of the Simulator of the Human Intestinal Microbial Ecosystem (SHIME) were incubated into closed bottles for 48 h at 37 °C. The descending colon compartment of SHIME contained *in vitro* cultured microbiota that harbored a reproducible human microbial community representative for the *in vivo* conditions, in both composition and metabolic activity (Van den Abbeele et al, 2010; Sanchez-Patan et al, 2015). Samples were taken at 24 h and 48 h with a needle that extends beyond the rubber stoppers that seal off the incubation bottles. The blank (without the added black carrot, peel, and pomace samples) was also incubated under the same conditions and used for the correction of interferences from the digestive fluids and colon microbiota. Samples collected from the stomach, small intestine, and colon phases were centrifuged at 3000 *g* and 4 °C for 10 min (Sigma Laboratory Centrifuge 4K15,

Osterode am Harz, Germany), and the supernatants were kept at −20 °C until further analysis.

Table 4.1 : Composition of gastric, duodenal and bile solutions applied during *in vitro* gastrointestinal digestion.

Constituent (g/L)	Gastric solution pH 3	Duodenal solution pH 7	Bile solution pH 8
Salts			
NaCl	2.75	7.01	5.26
NaH_2PO_4	0.35	-	-
KCl	0.82	0.56	0.38
$CaCl_2.2H_2O$	0.40	0.20	0.22
$NaHCO_3$	-	5.61	5.79
KH_2PO_4	-	0.08	-
$MgCl_2.6H_2O$	-	0.50	-
Host factors			
Urea	0.09	0.10	0.25
Mucin	0.35		
Bovine serum albumin	1.00	1.00	1.80
Pepsin	1.00	-	-
Pancreatin	-	3.00	-
Lipase	-	0.50	-
Bile	-	-	6.00

All solutions were prepared with distilled water to a total volume of 1 L. The enzymes and other compounds assigned in the table were mixed just before use, and pH was adjusted using 1 M NaOH or concentrated HCl in cases where it was necessary.

4.3.4 Chemical extraction of undigested samples

For each sample, three independent extractions were carried out as described previously in Section 3.3.6. Lyophilized powder (0.1 g) was treated with 5 mL of two different solvents, 75% aqueous methanol or ethanol, both containing 0.1% (v/v) formic acid. The treated samples were sonicated in an ultrasonic bath (Elma S60H elmasonic, Singen, Germany) for 15 min and subsequently centrifuged at 3000 *g* and 4 °C for 10 min (Sigma), and the supernatants were collected. This extraction protocol was repeated once more for the pellet, and the two supernatants were pooled to a final volume of 10 mL. Prepared extracts were stored at −20 °C until analysis. Because methanol extracted significantly more total phenolics and total monomeric anthocyanins than ethanol (Appendix B, Figure B.1), methanol was selected as the extraction solvent for all other subsequent analyses.

4.3.5 Spectrophotometric assays

Total phenolic content, total monomeric anthocyanin content, and total antioxidant capacity assays were performed using a Varian Cary 50 Bio UV−visible spectrophotometer (Varian Inc., Palo Alto, CA, USA). For each spectrophotometric assay, samples were analyzed in triplicate, and the average values were reported.

4.3.5.1 Total phenolic content

Total phenolic content of samples was estimated using Folin−Ciocalteu reagent as described before (Velioglu et al, 1998). One hundred microliters of extract was mixed with 0.75 mL of Folin−Ciocalteu reagent. The mixture was allowed to stand for 5 min, and then 0.75 mL of 6% Na_2CO_3 solution was added to the mixture. After 90 min of incubation, the absorbance was measured at 725 nm. Total phenolic content was expressed as milligrams of gallic acid equivalent (GAE) per 100 g of dry weight (dw) of sample.

4.3.5.2 Total monomeric anthocyanin content

Total monomeric anthocyanin content was determined according to the pH differential method described in Section 3.3.8.2. Results were expressed as milligrams of cyanidin-3-*O*-glucoside (C3G) per 100 g of dw of sample.

4.3.5.3 Total antioxidant capacity

Total antioxidant capacity was evaluated using three different assays: 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric ion reducing antioxidant power (FRAP), and cupric ion reducing antioxidant capacity (CUPRAC) as described in Section 3.3.8.3. For all total antioxidant capacity assays, results were expressed in terms of milligrams of Trolox equivalent (TE) per 100 g of dw of sample.

4.3.6 Identification of anthocyanins and phenolic acids using UPLC-ESI-MS^E

Before LC-MS analysis, samples from the *in vitro* gastrointestinal digestion were purified using a solid-phase extraction (SPE) method. Initially, 500 mg/4 mL C18 SPE cartridges (Grace Davison Discovery Sciences, Deerfield, IL, USA) were conditioned by rinsing with 6 mL of formic acid/methanol (1:100, v/v) followed by 4 mL of formic acid/MQ water (1:100, v/v). Aliquots (1.5 mL) of samples were acidified with 30 μL of formic acid and centrifuged (Labnet Spectrafuge 16M,

Labnet International Inc., Woodbridge, NJ, USA) at 16000 *g* for 10 min. Afterward, the supernatants were loaded to activated cartridges, which were subsequently washed with 5 mL of formic acid/MQ water (1:100, v/v). Samples were eluted with formic acid/methanol (1:100, v/v) and then dried using nitrogen. Prior to LC-MS analysis, samples were redissolved in DMSO/MQ water (1:10, v/v) and filtered through 0.45 μm membrane filters.

LC-MS analysis was performed with a Waters Acquity UPLC system (Waters Co., Milford, MA, USA) connected to a Waters Synapt HDMS TOF mass spectrometer. For chromatographic separation, a Waters Acquity BEH C18 column (2.1 mm × 150 mm, 1.7 μm particle size) attached to a Waters VanGuard pre-column (2.1 mm × 5 mm) was used. Five microliters of each filtered sample was injected into the system using formic acid/MQ water (1:1000, v/v; eluent A) and formic acid/methanol (1:1000, v/v; eluent B) at a flow rate of 250 μL/min. The gradient was as follows: 0 min, 5% B; 0−10 min, 5−15% B, linear; 10−15 min, 15% B, isocratic; 15−23 min, 15−95% B, linear; 23−28 min, 95% B, isocratic; 28−30 min, 95−5%B, linear; 30−32 min, 5% B, isocratic. The column temperature was maintained at 40 °C, whereas the temperature of the autosampler was held at 10 °C. ESI-MS analysis was performed in both positive and negative modes. Collision energies of 6 V (for low energy) and 45 V (for high energy) were used for full-scan LC-MS in the m/z range 100−1500. Leucine enkephalin was used for online mass calibration. For the acquisition of the data, MassLynx 4.1 software (Waters Co.) was used.

4.3.7 Quantification of anthocyanins and phenolic acids using HPLC-DAD

Anthocyanins and phenolic acids were quantified following the method of Capanoglu et al (2008) described in Section 3.3.9. Samples purified using SPE were passed through 0.45 μm membrane filters and injected into a Thermo Dionex Ultimate 3000 HPLC (Thermo Fischer Scientific, Landsmeer, The Netherlands) coupled with a diode array detector (DAD). A Grace Smart RP C18 column (250 × 4.6 mm, 5 μm) was used as the stationary phase. Matrix-matched calibration curves were prepared by spiking blank control samples of *in vitro* gastrointestinal digestion at concentration levels from 0.1 to 100 ppm. Curves were plotted as the chromatographic peak area of the standards versus nominal concentrations. Slope, intercept, R^2, limit of detection (LOD), and limit of quantification (LOQ) of the

standard curves for each stage of the digestion are provided in Appendix B, Table B.1. All results were expressed as milligrams per 100 g dw of sample.

4.3.8 Analysis of short-chain fatty acids using GC-FID

Short-chain fatty acids were extracted from colon samples of *in vitro* gastrointestinal digestion and analyzed as described previously by De Weirdt et al (2010). Briefly, 500 μL of extracts was treated with 500 μL of H_2SO_4 (1:1, v/v) followed by the addition of 400 μL of 2-methylhexanoic acid as an internal standard. Subsequently, 0.4 g of NaCl and 2 mL of diethyl ether were added and centrifuged at 3000 *g* for 3 min (Sigma). The supernatants were injected into a GC-2014 gas chromatograph (Shimadzu, 's-Hertogenbosch, The Netherlands), equipped with a capillary fatty acid-free EC-1000 Econo-Cap column (25 × 0.53 mm, 1.2 mM; Alltech, Laarne, Belgium), a flame ionization detector (FID), and a split injector. The temperature profile was set from 110 to 160 °C, with a temperature increase of 6 °C/min. Nitrogen was used as a carrier gas, the injection volume was 1 μL, and the temperatures of the injector and detector were maintained at 100 °C and 220 °C, respectively. Results were expressed as milligrams per liter of extracts.

4.3.9 Statistical analysis

All analyses were performed with three technical and three biological replicates. Data were subjected to statistical analysis 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. $p < 0.05$ was considered significant. Slope, intercept, R^2, LOD, and LOQ of the standard curves were calculated using the LINEST function in Microsoft Office Excel 2011 software (Microsoft Co., Redmond, WA, USA).

4.4 Results and Discussion

4.4.1 Spectrophotometric assays

The impact of gastrointestinal digestion on total phenolic content, total monomeric anthocyanin content, and total antioxidant capacity of samples is shown in Table 4.2. For undigested samples, although byproducts contained respectively 10−28 and 12−31% lower total phenolic content and total antioxidant capacity than black carrot,

statistically there was no significant difference ($p > 0.05$). The lower total phenolic content and total antioxidant capacity of pomace may be attributed to citric acid treatment, which previously has been shown to reduce the total phenolic content and total antioxidant capacity of black carrots (Suzme et al, 2014) as a result of low pH values, which may affect the sensitivity of the methods used. On the other hand, total monomeric anthocyanin content of pomace was found to be slightly higher (3%) than that of black carrots. This may be explained by the facilitated extraction of compounds through the macerated tissue, due to increased membrane permeability with applied temperature and/or release of bound compounds with the breakdown of cellular constituents as a consequence of applied temperature and enzyme treatment (Suzme et al, 2014). In the stomach, lower total phenolic content (43−71%), total monomeric anthocyanin content (51−74%), and total antioxidant capacity (31−69%) were observed for all samples. Studies carried out on black mulberry (Tomas et al, 2015) and purple tomato (Li et al, 2014) also revealed lower total phenolic content (28%), total monomeric anthocyanin content (35−82%), and total antioxidant capacity (28−79%) after gastric digestion. The small intestine phase gave further increases in total phenolic content, total monomeric anthocyanin content, and total antioxidant capacity of the byproducts (<28−30%, 6−12%, and 26−46%, respectively) compared to amounts released in the stomach; however, the values were still lower compared to the undigested samples. A similar trend was also observed in the case of apple polyphenols, and the authors explained this increase after the small intestine phase by the additional contact time of the food material with the intestinal fluids (plus 4 h) and/or the effect of intestinal digestive enzymes (lipase and pancreatin, with the latter also having amylase and protease activity) on the complex food matrix, facilitating the release of polyphenols bound to the matrix (Bouayed et al, 2011). Furthermore, higher antioxidant activity could also be explained by the fact that enzyme action and pH change might generate new compounds with higher total antioxidant capacity than the ones found in the stomach. From the small intestine to the colon after 24 h of incubation, although mostly additional reductions in total phenolic content (4−37%), total monomeric anthocyanin content (18−22%), and total antioxidant capacity (8−46%) occurred, there was no statistical difference ($p > 0.05$). Further incubation in the colon (t = 48 h) resulted in varying results, showing both increases and decreases. However, in general, these variations were statistically not significant ($p > 0.05$). Correlations

between total phenolic content, total monomeric anthocyanin content, and total antioxidant capacity were also determined. For both the undigested and digested samples, generally a highly linear relationship was obtained between total phenolic content and total antioxidant capacity, with the CUPRAC assay being the highest with R^2 = 0.8873−0.9712. On the other hand, weak correlations were obtained between total monomeric anthocyanin content and total antioxidant capacity, ranging between R^2 = 0.0068 and 0.6867. These results imply that phenolic compounds other than anthocyanins may be the major contributors to the antioxidant activity. The correlations within different total antioxidant capacity assays were quite high, especially correlations between FRAP and CUPRAC assays (R^2 = 0.8577−0.9503). Taking into account the pH conditions of the total antioxidant capacity assays applied in this study, it can be hypothesized that the FRAP assay conducted at pH 3.6 could be more suitable for assessing antioxidant activity in the stomach, whereas DPPH and CUPRAC assays could be more appropriate to evaluate total antioxidant capacity in the small intestine and colon with a working pH of 7.0−8.0. In addition to the pH dependency, the chemical structure of the polyphenol and interaction of polyphenols with other dietary compounds released during digestion, for example, dietary fiber or proteins, are also known to affect the polyphenol accessibility and, thus, the antioxidant activity (Bouayed et al, 2011). Eventually, it is recommended that at least two of the total antioxidant capacity assays should be combined to ensure a reliable picture as was done in the current study.

4.4.2 Anthocyanins

LC-MS analysis of black carrots and byproducts led to the identification of five major anthocyanins. The detected anthocyanins were all cyanidin-based with different sugar moieties; among them, two were non-acylated (cyanidin-3-xylosyl-glucosyl-galactoside and cyanidin-3-xylosylgalactoside) and three were acylated with sinapic acid (cyanidin-3-xylosyl-sinapoyl-glucosyl-galactoside), ferulic acid (cyanidin-3-xylosyl-feruloyl-glucosyl-galactoside), and *p*-coumaric acid (cyanidin-3-xylosyl-coumaroyl-glucosyl-galactoside) (Table 4.3).

Table 4.2 : Changes in the total phenolic content, total monomeric anthocyanin content and total antioxidant capacity of the bioaccessible fraction of black carrot and by-products during *in vitro* gastrointestinal digestion.

Sample	Undigested	Stomach	Small Intestine	Colon t = 24h	Colon t = 48h
			Total phenolic content (mg GAE/100 g dw)		
Black carrot	5743.0 ± 910.8^{aA}	3296.0 ± 751.5^{abA}	2768.5 ± 711.8^{bA}	2653.0 ± 697.2^{bA}	3669.3 ± 964.4^{abA}
Peel	5170.1 ± 620.4^{aA}	2234.4 ± 351.2^{bAB}	3761.2 ± 796.3^{abA}	3218.6 ± 345.7^{bA}	3136.7 ± 214.0^{bA}
Pomace	4151.3 ± 224.8^{aA}	1202.9 ± 142.6^{dB}	2987.2 ± 223.0^{bA}	1883.3 ± 293.0^{cdA}	2215.8 ± 577.9^{bcA}
			Total monomeric anthocyanin content (mg C3G/100 g dw)		
Black carrot	1653.8 ± 183.1^{aA}	804.0 ± 62.8^{bA}	337.5 ± 54.9^{cB}	451.1 ± 38.7^{cA}	304.6 ± 50.9^{cC}
Peel	1221.1 ± 236.2^{aA}	324.3 ± 36.0^{bB}	470.0 ± 64.0^{bAB}	367.9 ± 32.3^{bA}	577.9 ± 13.9^{bB}
Pomace	1703.4 ± 164.1^{aA}	446.1 ± 52.3^{cB}	545.6 ± 54.3^{bcA}	449.7 ± 58.9^{cA}	730.0 ± 69.8^{bA}
			Total antioxidant capacity (mg TE/100 g dw)		
DPPH (2,2-diphenyl-1-picrylhydrazyl)					
Black carrot	7198.7 ± 1027.3^{aA}	3583.3 ± 469.3^{bA}	3095.7 ± 1136.6^{bA}	2686.4 ± 605.8^{bAB}	2443.6 ± 630.1^{bA}
Peel	4943.9 ± 1316.3^{abA}	2702.5 ± 260.1^{bB}	4995.6 ± 1188.6^{aA}	3750.8 ± 450.8^{abA}	3058.9 ± 338.7^{abA}
Pomace	5247.4 ± 770.4^{aA}	1868.8 ± 151.5^{cC}	4064.5 ± 300.0^{abA}	2187.9 ± 518.6^{cB}	2534.5 ± 903.8^{bcA}
FRAP (Ferric ion reducing antioxidant power)					
Black carrot	9257.2 ± 1537.0^{aA}	4605.6 ± 1231.4^{bA}	3548.1 ± 1170.4^{bA}	3183.8 ± 1094.8^{bA}	3489.3 ± 629.1^{bA}
Peel	7641.7 ± 913.0^{aA}	2719.6 ± 272.9^{cAB}	4761.2 ± 1055.1^{bA}	3468.2 ± 533.8^{bcA}	3818.4 ± 389.3^{bcA}
Pomace	6937.5 ± 587.7^{aA}	2167.1 ± 250.5^{cB}	4372.3 ± 695.8^{bA}	2893.9 ± 617.3^{bcA}	3197.5 ± 803.0^{bcA}
CUPRAC (Cupric ion reducing antioxidant capacity)					
Black carrot	17426.1 ± 2935.5^{aA}	11992.6 ± 1474.7^{abA}	7817.9 ± 2291.5^{bA}	7195.6 ± 1828.3^{bAB}	7647.3 ± 1301.4^{bA}
Peel	15324.0 ± 1802.4^{aA}	7311.3 ± 944.9^{bB}	11343.5 ± 2655.6^{abA}	9250.8 ± 961.8^{bA}	8583.1 ± 337.0^{bA}
Pomace	12960.7 ± 852.6^{aA}	4447.4 ± 785.1^{cB}	8919.1 ± 959.5^{bA}	5542.2 ± 1012.1^{bcB}	6924.4 ± 2066.4^{bcA}

The data presented in this table consist of average values ± standard deviation of three independent batches. Total phenolic content, total monomeric anthocyanin content and total antioxidant capacity are expressed as mg of gallic acid equivalent (GAE), cyanidin-3-*O*-glucoside equivalent (C3G) and Trolox® equivalent (TE) per 100 g of dry weight (dw) of sample, respectively. Different capital letters in the columns or small letters in the rows represent statistically significant differences ($p < 0.05$).

Table 4.3 : Mass spectrometric data and identification of the polyphenols detected in the black carrot and byproducts by LC-MS.

Retention time (min)	Identity	λ (nm)	Mass (m/z)	MS^2 main fragment (m/z)
Anthocyanins				
17.57	Cyanidin-3-xylosyl-glucosyl-galactoside	500	(+) 743	287
18.34	Cyanidin-3-xylosyl-galactoside	500	(+) 581	287
18.98	Cyanidin-3-xylosyl-sinapoyl-glucosyl-galactoside	500	(+) 949	287
19.61	Cyanidin-3-xylosyl-feruloyl-glucosyl-galactoside	500	(+) 919	287
19.67	Cyanidin-3-xylosyl-coumaroyl-glucosyl-galactoside	500	(+) 889	287
Phenolic Acids				
8.97	Neochlorogenic acid	312	(-) 353	191
12.40	Chlorogenic acid	312	(-) 353	191
15.42	Caffeic acid	312	(-) 179	135
21.76	Ferulic acid	312	(-) 193	178

The identification of each anthocyanin was carried out in positive mode, based on the UV−visible characteristics, MS, and fragmentation pattern, as well as by comparison with the data already reported in the literature. The anthocyanin profile of black carrot was in agreement with those reported previously (Montilla et al, 2011; Suzme et al, 2014; Algarra et al, 2014; Kamiloglu et al, 2015b). The influence of *in vitro* gastrointestinal digestion on major anthocyanins from black carrot and its byproducts is presented in Table 4.4, whereas representative chromatograms recorded at 520 nm corresponding to the anthocyanin profile of black carrot at different stages of gastrointestinal digestion are displayed in Figure 4.2. Among the undigested samples, pomace contained the highest amount of total anthocyanins, which was consistent with the results obtained spectrophotometrically (Table 4.2). The predominant anthocyanin was cyanidin-3-xylosyl-feruloyl-glucosyl-galactoside for all undigested samples, representing about 40−69% of the total anthocyanins, whereas anthocyanins with acylated structure constituted 61−88% of the total anthocyanins. It has been shown that acylated anthocyanins are more stable to pH and temperature changes than non-acylated ones due to intramolecular copigmentation effects of acylated anthocyanins (Kammerer et al, 2004b). Therefore, byproducts from black carrot as sources of acylated anthocyanins may provide the desirable stability for food applications. After gastric digestion in the stomach, a significantly lower amount of anthocyanins was observed (37−89%) for almost all samples ($p < 0.05$). Our findings were comparable with the results of an *in vitro* study on purple figs, in which 35% cyanidin-3-rutinoside was recovered after gastric digestion (Kamiloglu and Capanoglu, 2013). In the small intestine, although there was no significant change in the anthocyanin content of black carrot and peel, additional loss of anthocyanins (11−32%) was observed for pomace. The high loss of anthocyanins may be related to the fact that they are metabolized to some noncolored forms, oxidized, or degraded into other chemicals, which may not be detected under the present conditions (Perez-Vicente et al, 2002). In addition, it has been reported that due to ionic interactions, approximately 65% of the anthocyanins from black carrots were bound to plant cell walls and overwhelmingly remain bound after *in vitro* gastric and small intestinal digestion (Padayachee et al, 2013).

Table 4.4 : Changes in the anthocyanins of the bioaccessible fraction of black carrot and by-products during *in vitro* gastrointestinal digestion.

Sample	Undigested	Stomach	Small Intestine	Colon t = 24h	Colon t = 48h
			cyanidin-3-xylosyl-glucosyl-galactoside (mg C3G/100 g dw)		
Black carrot	44.1 ± 7.4^{aB}	11.0 ± 3.1^{bB}	12.4 ± 5.5^{bB}	33.1 ± 11.5^{aB}	33.2 ± 8.2^{aB}
Peel	10.5 ± 4.8^{bC}	13.8 ± 3.2^{bB}	10.9 ± 2.0^{bB}	31.7 ± 13.0^{aB}	32.0 ± 2.2^{aB}
Pomace	81.0 ± 8.7^{aA}	50.8 ± 6.7^{bA}	24.6 ± 4.4^{cA}	82.4 ± 3.9^{aA}	96.2 ± 12.9^{aA}
			cyanidin-3-xylosyl-galactoside (mg C3G/100 g dw)		
Black carrot	176.9 ± 54.9^{aB}	19.2 ± 2.5^{bB}	19.2 ± 8.3^{bB}	41.2 ± 13.8^{bB}	44.9 ± 17.2^{bB}
Peel	75.8 ± 30.7^{aB}	23.2 ± 6.8^{bB}	11.1 ± 1.1^{bB}	44.8 ± 20.5^{abB}	52.6 ± 14.8^{abB}
Pomace	507.8 ± 38.2^{aA}	165.1 ± 31.4^{bA}	64.3 ± 10.7^{cA}	179.8 ± 4.2^{bA}	220.4 ± 41.6^{bA}
			cyanidin-3-xylosyl-sinapoyl-glucosyl-galactoside (mg C3G/100 g dw)		
Black carrot	282.2 ± 92.1^{aA}	31.7 ± 7.7^{bB}	22.9 ± 10.4^{bB}	24.3 ± 12.0^{bB}	27.1 ± 9.7^{bB}
Peel	96.9 ± 22.3^{aB}	29.5 ± 9.1^{bB}	12.2 ± 1.9^{bB}	26.6 ± 8.6^{bB}	26.6 ± 5.3^{bB}
Pomace	250.6 ± 34.7^{aA}	110.8 ± 10.9^{bA}	75.5 ± 15.1^{bA}	82.9 ± 13.2^{bA}	66.1 ± 10.6^{bA}
			cyanidin-3-xylosyl-feruloyl-glucosyl-galactoside (mg C3G/100 g dw)		
Black carrot	844.9 ± 181.1^{aA}	110.8 ± 35.6^{bB}	50.4 ± 20.1^{bB}	62.8 ± 28.5^{bB}	46.1 ± 19.9^{bB}
Peel	519.5 ± 62.7^{aB}	98.2 ± 29.3^{bB}	41.2 ± 4.6^{bB}	70.5 ± 30.3^{bB}	49.7 ± 13.4^{bAB}
Pomace	610.6 ± 43.0^{aAB}	265.2 ± 37.5^{bA}	198.5 ± 16.6^{bcA}	143.3 ± 0.6^{cdA}	86.5 ± 11.1^{dA}
			cyanidin-3-xylosyl-coumaroyl-glucosyl-galactoside (mg C3G/100 g dw)		
Black carrot	97.5 ± 3.5^{aA}	35.0 ± 15.9^{bA}	19.6 ± 6.8^{bA}	17.9 ± 5.7^{bA}	31.0 ± 14.0^{bA}
Peel	46.5 ± 9.1^{aC}	28.4 ± 11.6^{abA}	13.8 ± 3.4^{bA}	33.3 ± 18.2^{abA}	31.1 ± 11.6^{abA}
Pomace	77.4 ± 5.1^{aB}	38.3 ± 6.4^{bA}	25.2 ± 6.3^{cA}	17.5 ± 1.1^{cA}	24.3 ± 2.8^{cA}

The data presented in this table consist of average values ± standard deviation of three independent batches. Different capital letters in the columns or small letters in the rows represent statistically significant differences ($p < 0.05$).

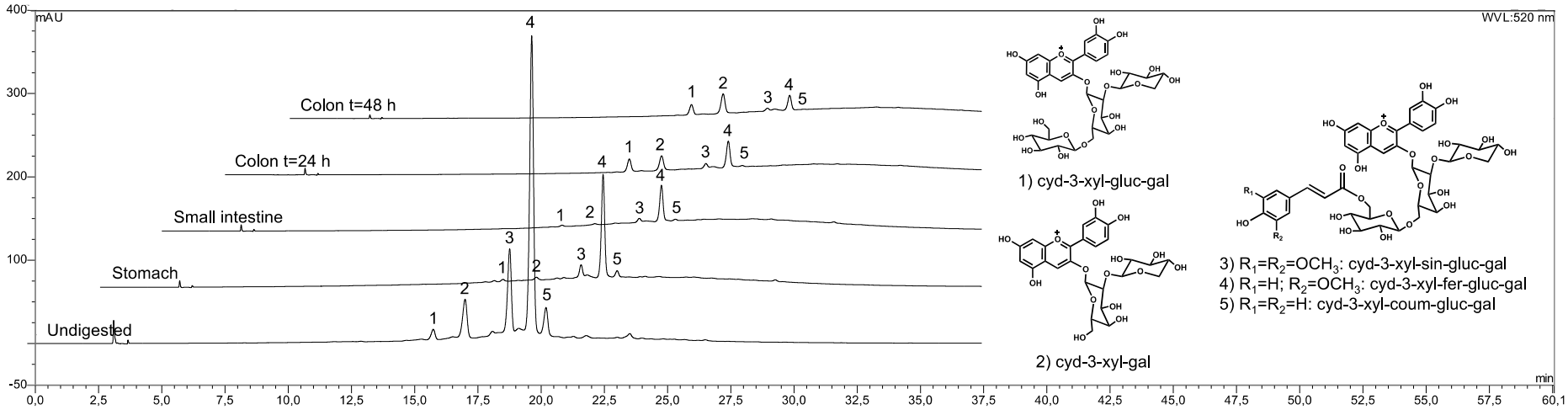

Figure 4.2 : HPLC chromatograms (DAD, recorded at 520 nm) of black carrot anthocyanins during *in vitro* gastrointestinal digestion.

The fermentation in the colon for 24 h resulted in elevated anthocyanin levels (up to 2−fold) compared to amounts released in the small intestine, whereas extra incubation (48 h) did not cause a significant difference ($p < 0.05$). The human gut lacks enzymes such as pectinases and cellulases, allowing the transit of pectin and cellulose components through the gastrointestinal tract. Therefore, food matrix interactions between anthocyanins and these carbohydrate molecules may significantly enhance the bioaccessibility of anthocyanins in the colon, where these macrocomplexes are degraded by the residing microbiota (Correa-Betanzo et al, 2014). The differences in the absorptivity of the black carrot anthocyanins in relation to cyanidin-3-glucoside might also be the cause of the increase in individual anthocyanin concentrations. Furthermore, the percent recovery of non-acylated anthocyanins in the colon (up to 3-fold) was found to be higher than acylated anthocyanins (up to 72%). An *in vivo* study conducted with 12 healthy volunteers consuming purple carrots revealed that plasma and urine recoveries of non-acylated anthocyanins were greater than recovery of acylated anthocyanins (8−10- and 11−14-fold, respectively) (Kurilich et al, 2005). The authors proposed that the acylations might have been cleaved to produce cyanidin-3-xylosyl-glucosyl-galactoside, which may also be valid for our study. Overall, the amount of pomace anthocyanins released at all stages of *in vitro* gastrointestinal digestion was higher than black carrot anthocyanins, suggesting that pomace may be a better source of bioaccessible anthocyanins.

4.4.3 Phenolic acids

Four major phenolic acids, namely, neochlorogenic acid (3-*O*-caffeoylquinic acid), chlorogenic acid (5-*O*-caffeoylquinic acid), caffeic acid, and ferulic acid, were detected in black carrot and byproducts using LC-MS in negative mode (Table 4.3). Table 4.5 shows the impact of *in vitro* gastrointestinal digestion on major phenolic acids present in black carrot and byproducts. In accordance with previous black carrot reports (Kammerer et al, 2004a; Suzme et al, 2014; Kamiloglu et al, 2015c) chlorogenic acid was identified as the most abundant compound for all samples, accounting for up to 86% of total phenolic acids, whereas caffeoylquinic acids represented 82−89% of the phenolic acids.

Table 4.5 : Changes in the phenolic acids of the bioaccessible fraction of black carrot and by-products during *in vitro* gastrointestinal digestion.

Sample	Undigested	Stomach	Small Intestine	Colon t = 24h	Colon t = 48h
			Neochlorogenic acid (mg/100 g dw)		
Black carrot	9.5 ± 3.5^{aB}	4.4 ± 1.0^{bAB}	1.6 ± 1.1^{bB}	3.7 ± 0.5^{bA}	5.1 ± 0.4^{abA}
Peel	6.8 ± 0.9^{aB}	3.3 ± 1.2^{bcB}	0.9 ± 0.4^{cB}	3.3 ± 0.9^{bcA}	5.3 ± 0.8^{abA}
Pomace	16.2 ± 0.9^{aA}	7.0 ± 1.3^{bA}	4.3 ± 0.4^{cdA}	2.9 ± 0.7^{dA}	5.7 ± 0.4^{bcA}
			Chlorogenic acid (mg/100 g dw)		
Black carrot	335.5 ± 141.6^{aAB}	37.0 ± 11.0^{bB}	26.0 ± 12.0^{bB}	39.6 ± 4.7^{bA}	25.8 ± 0.7^{bA}
Peel	170.2 ± 60.0^{aB}	43.0 ± 16.3^{bB}	34.5 ± 4.1^{bB}	35.3 ± 12.3^{bA}	27.0 ± 4.0^{bA}
Pomace	475.7 ± 43.4^{aA}	277.1 ± 27.3^{bA}	156.9 ± 6.3^{cA}	51.4 ± 8.2^{dA}	26.1 ± 7.1^{dA}
			Caffeic acid (mg/100 g dw)		
Black carrot	2.2 ± 0.5^{cB}	8.9 ± 2.2^{abA}	5.9 ± 0.5^{bcA}	12.8 ± 1.2^{aB}	11.8 ± 2.4^{aB}
Peel	2.0 ± 0.2^{bB}	8.8 ± 2.9^{abA}	5.4 ± 0.8^{bA}	16.1 ± 5.1^{aB}	14.4 ± 2.5^{aB}
Pomace	19.1 ± 2.2^{bcA}	8.8 ± 0.8^{cA}	4.3 ± 0.9^{cA}	28.6 ± 4.0^{bA}	46.1 ± 12.3^{aA}
			Ferulic acid (mg/100 g dw)		
Black carrot	63.3 ± 13.7^{bA}	68.5 ± 15.3^{bA}	98.4 ± 7.4^{abA}	93.7 ± 23.1^{abAB}	108.0 ± 1.0^{aAB}
Peel	38.2 ± 7.6^{bB}	52.8 ± 11.3^{bA}	79.0 ± 15.3^{abA}	128.7 ± 49.8^{aA}	129.7 ± 21.6^{aA}
Pomace	41.5 ± 7.3^{bAB}	24.8 ± 1.1^{bB}	34.0 ± 4.2^{bB}	32.2 ± 3.9^{bB}	72.1 ± 16.5^{aB}

The data presented in this table consist of average values ± standard deviation of three independent batches. Different capital letters in the columns or small letters in the rows represent statistically significant differences ($p < 0.05$).

Similar to anthocyanin results, for undigested samples the highest content of total phenolic acids was determined in pomace. Especially the amounts of neochlorogenic and caffeic acids were significantly higher (70% and 8-fold, respectively) than those found in black carrot ($p < 0.05$). Some studies described the influence of processing on the conversion of caffeoylquinic acids into other isomers. Besides isomerization, caffeoylquinic acids can also undergo hydrolysis during processing, resulting in a consequent increase in caffeic acid (Van Boekel et al, 2010). Accordingly, the higher neochlorogenic and caffeic acid contents in pomace may be related to these factors. Gastric digestion in the stomach significantly decreased the caffeoylquinic acid content of the samples (42−89%) ($p < 0.05$). Previous studies have also noted low stability of phenolic acids under gastric conditions (Kamiloglu and Capanoglu, 2013; Mosele et al, 2015). Pancreatic digestion in the small intestine caused further loss of caffeoylquinic acids (3−35%). This additional loss could be associated with the instability of the caffeoylquinic acids in aqueous solution. In addition, the effect of pH (pH 7) and bile salts could also contribute to the yield with a higher loss (Vallejo et al, 2004). Moreover, phenolic acids from black carrots were shown to stay bound to plant cell walls during *in vitro* gastric and small intestinal digestion (Padayachee et al, 2013). On the other hand, increases in ferulic acid content were observed as a result of small intestinal digestion. This may be attributed to cleavage of the major anthocyanin cyanidin-3-xylosyl-feruloyl-glucosyl-galactoside to ferulic acid. At the end of the *in vitro* fermentation in the colon, a 27% further decrease in the content of chlorogenic acid was observed for pomace, along with the formation of caffeic acid. This was an expected outcome of microbial fermentation because caffeic acid has been reported as the major product resulting from the hydrolysis of chlorogenic acid by the colon microbiota (Correa-Betanzo et al, 2014).

4.4.3 Short chain fatty acids (SCFAs)

To investigate whether the release of anthocyanins and phenolic acids in colon conditions can be correlated with changes in microbial activity, SCFAs as end products of fermentation have been investigated. Although there are other indicators of microbial activity (e.g., ammonium production), measurement of SCFAs production is also often used as an indicator of microbial activity when colonic metabolism is studied. SCFAs produced from black carrot, byproducts, and control (without the food matrix) samples during colonic fermentation are composed mainly

of acetic, propionic, and butyric acids (Figure 4.3). The proportional productions of major SCFAs (acetate/propionate/butyrate) after 24 h were similar for black carrot (74:23:3), peel (74:22:4), and pomace (79:18:3). In a previous study, Aura et al (2013) also showed production of similar proportions of SCFAs at the end of a 24 h *in vitro* microbial fermentation of digested grape by a human fecal inoculum. After 48 h, butyric acid concentrations increased twice as much, whereas the changes in acetic and propionic acid were not significant ($p > 0.05$). At both time points, the total amount of SCFAs produced from pomace samples was significantly higher (15−24%) than that from black carrot and peel ($p < 0.05$). This is probably due to the higher sugar content of free sugars in the pomace compared to the peel and black carrot. The amount of acetate present in the colon after 24 h was correlated well with the amount of cyanidin-3-xylosyl-galactoside released ($R^2 = 0.8415$). Similarly, after 48 h a high correlation was obtained between acetate and ferulic acid ($R^2 = 0.8742$).

4.5 Conclusions

To the best of our knowledge, this is the first study that has focused on the changes in bioaccessible polyphenols from black carrot by-products during *in vitro* gastrointestinal digestion. The current study pointed out that black carrot peel and pomace provide considerable polyphenol intake, which are preserved to a certain degree after digestion; therefore they may be used to enrich food products. Even though the conclusions achieved with this static model used for the simulation of *in vitro* gastrointestinal digestion cannot directly estimate the human *in vivo* conditions, we suggest that this model is useful for studying the effect of the food matrix, enzymes and colon microbiota on polyphenol bioaccessibility. In future studies, to investigate the bioavailability of the phenolic compounds and their uptake mechanisms, cell culture systems simulating the epithelial barrier such as Caco-2 cells may be used.

Considering the above, in the following chapter (*Chapter 5*) black carrot pomace was used enriched cakes. Furthermore, *in vitro* digestion method developed here is combined with intestinal Caco-2 cells to study the absorption of polyphenols from black carrot and its byproducts (*Chapter 6*).

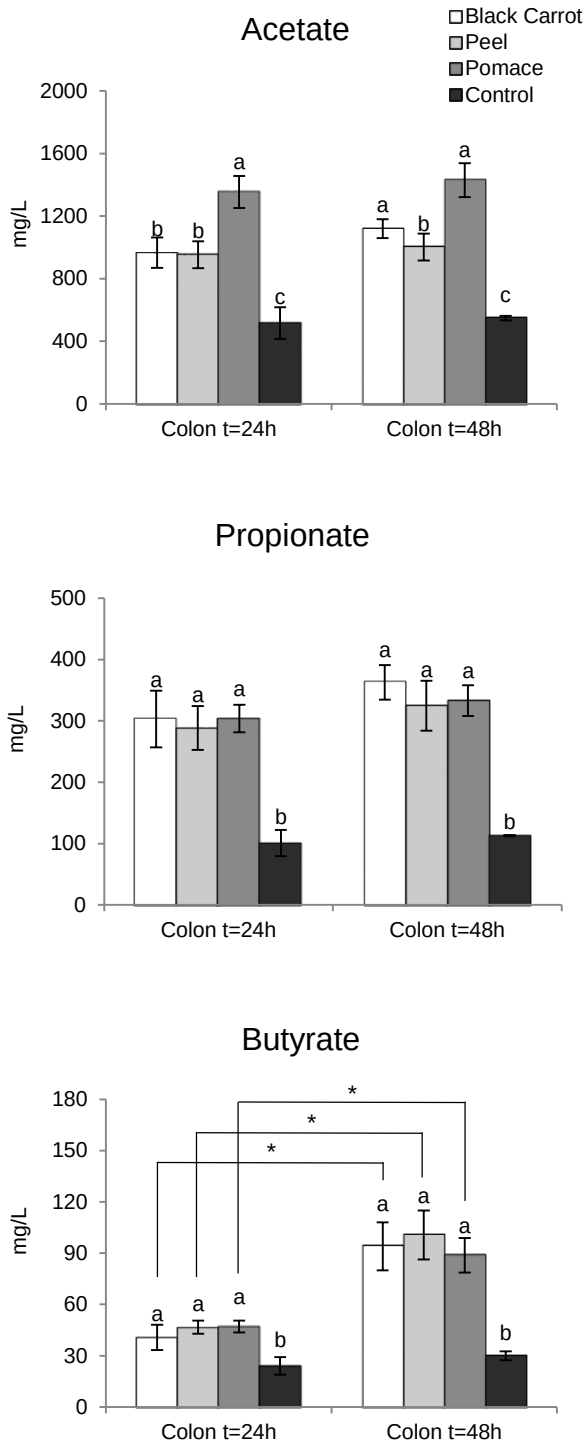

Figure 4.3 : Short chain fatty acids (SCFAs) produced during colon fermentation. Different letters above bars represent statistically significant differences between samples ($p < 0.05$), whereas differences between time points are indicated as $*p < 0.05$.

5. BLACK CARROT POMACE AS A SOURCE OF POLYPHENOLS FOR ENHANCING THE NUTRITIONAL VALUE OF CAKE: AN *IN VITRO* DIGESTION STUDY WITH A NEW STANDARDIZED STATIC MODEL

Redrafted from:

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5.1 Abstract

Black carrot pomace, an industrial by-product of juice processing, is a promising source of polyphenols, in particular anthocyanins. In the present study, we investigated the digestive stability of polyphenols from black carrot pomace enriched cakes and monitored changes in their antioxidant capacity using a new standardized static *in vitro* digestion model. Results showed that although enrichment of cakes with black carrot pomace at levels of 5%, 10% and 15% caused a dose-dependent increase in anthocyanins (72 to 267 μg/g dw), phenolic acids (49 to 148 μg/g dw), total phenolics (54 to 202 mg GAE/100 g dw) and total antioxidant capacity (21−129 to 153−478 mg TE/100 g dw) for undigested samples, there was no statistical difference between the 10% and 15% black carrot pomace enriched sample ($p > 0.05$) after digestion. During the *in vitro* digestion in the mouth and stomach the amount of anthocyanins and phenolic acids were reduced significantly (46−74% and 35−65%, respectively) ($p < 0.05$), whereas no anthocyanins were detected in the intestine after intestinal digestion. On the other hand, significant increases in total phenolic content and total antioxidant capacity were obtained in the stomach and intestine (up to 5− and 12−fold respectively). Overall, the present study demonstrated that black carrot pomace, which can serve as a functional ingredient might be utilized in the baking industry.

Keywords: *Daucus carota* L., by-product, anthocyanins, phenolic acids, antioxidant capacity, bioaccessibility.

5.2 Introduction

Black carrots (*Daucus carota* ssp. *sativus* var. *atrorubens* Alef.) have an appealing bluish-purple color with significant levels of anthocyanins, which show high heat, light, and pH stability. Recently, they have received increasing interest as a source of natural food colorant due to the legal restrictions for synthetic colorants and increasing consumer demand for natural pigments (Ekici et al, 2015). In addition to their colorant features, anthocyanin rich black carrots are indicated to exhibit health promoting properties including antioxidant (Koley et al, 2014; Kamiloglu et al, 2015b), anti-inflammatory (Mizgier et al, 2016) and anticancer (Sevimli-Gur et al, 2013) activities.

Due to the seasonal and perishable nature, many fresh fruits and vegetables are subjected to some form of processing. Processing of foods of plant origin generates large amounts of by-products. Disposal of these by-products creates both a cost to the food manufacturer and a potential negative impact on the environment. Research over the past 20 years has revealed that many of these by-products could potentially serve as a source of valuable bioactive compounds (Wijngaard et al, 2012; Huang et al, 2013). In Turkey, around 2,700 and 6,900 tons of black carrot pomace, an industrial by-product of juice processing, was produced in 2009 and 2010, respectively. This trend is expected to continue and even to accelerate in upcoming years (Agcam and Akyildiz, 2015). Black carrot pomace is considered as a potential food ingredient and has been used to improve the functional properties of cookies (Turksoy et al, 2011) and fermented black carrot juice (Tatoglu, 2014).

Lately, more attention has been given to the bioavailability of polyphenols, a prerequisite for further physiological functions in the body. As human studies are time consuming, costly, and restricted by ethical concerns, *in vitro* models for investigating the effects of digestion have been developed and employed to predict the release of polyphenols from the food matrix and assess changes in their profiles prior to absorption. However, the diversity of *in vitro* digestion model conditions has limited the ability to compare results across different studies (Alminger et al, 2014). To overcome this issue, a standardized and practical static digestion model based on

physiologically relevant conditions was proposed (Minekus et al, 2014). This model has already been used to study the bioaccessibility of certain polyphenols including anthocyanins (Pineda-Vadillo et al, 2016).

In a previous study performed by our group (*Chapter 4*), we showed that polyphenols initially present in black carrots are largely preserved in black carrot pomace. Furthermore, the bioaccessibility of anthocyanins from black carrot pomace was found to be higher than black carrot (Kamiloglu et al, 2016). Considering the above, we suggest that black carrot pomace, a cheap and substantial source of polyphenols may be used to enrich food products. Bakery products including cakes are considered to be a good source of energy, however they have low antioxidant capacity. Hence, in order to increase the nutritional value of cake and to valorize black carrot pomace, in the present study we investigated the digestive stability of polyphenols from black carrot pomace enriched cakes and monitored changes in their antioxidant capacity using the new standardized static *in vitro* digestion model of Minekus et al (2014).

5.3 Materials and Methods

5.3.1 Plant material and cake formulations

Black carrot pomace is collected as three independent biological replicates from an established juice processing plant (Erkon Konsantre Co.) in Konya, Turkey, in April 2014. Collected black carrot pomace samples were ground to a fine powder in liquid nitrogen using a precooled grinder (IKA A11 basic, IKA-Werke GmbH & Co., Staufen, Germany) and subsequently freeze-dried (Christ Alpha 1-2 LD plus, Martin Christ Gefriertrocknungsanlagen, Osterode am Harz, Germany) for 24 h.

All formulations used for cake preparations are reported in Table 5.1. Cake flour was enriched with black carrot pomace powder at levels of 0%, 5%, 10% and 15%. In a bowl, premixed eggs were combined with sugar and mixed for 3 min using a blender (Tefal Smart Pro Silver, Tefal Co., Istanbul, Turkey). Afterwards, sunflower oil and yoghurt were inserted, followed by the addition of baking powder, cake flour and black carrot pomace powder. The pooled mixtures were baked in an oven (Luxell LX-3580, Kumtel Co., Istanbul, Turkey) at 180 °C for 30 min (Figure 5.1). After baking, cakes were allowed to cool down and later finely ground using a laboratory

grinder (Premier PRG 259, Stil Electronics Co., Istanbul, Turkey), lyophilized (Christ Alpha) and stored at −80 °C until analysis.

Table 5.1 : Ingredients of cakes prepared using 5%, 10% and 15% black carrot pomace (BCP) powder[a].

Ingredients (g)	Black carrot pomace powder enrichment			
	Control	5%	10%	15%
Eggs[b]	60	60	60	60
Sugar	60	60	60	60
Sunflower oil[c]	30	30	30	30
Yoghurt[d]	90	90	90	90
Baking powder[e]	10	10	10	10
Cake flour[e]	120	120	120	120
BCP powder[f]	-	6	12	18

[a]Based on the flour content, cakes were enriched with black carrot pomace powder by 5%, 10% and 15%.
[b]Whole eggs were opened, shells discarded, pooled, mixed and weighed.
[c]Yudum, Yudum Food Co., Balikesir, Turkey.
[d]Mis, Mis Milk Co., Istanbul, Turkey.
[e]Piyale, Gidasa Co., Izmir, Turkey.
[f]Erkon, Erkon Konsantre Co., Konya, Turkey.

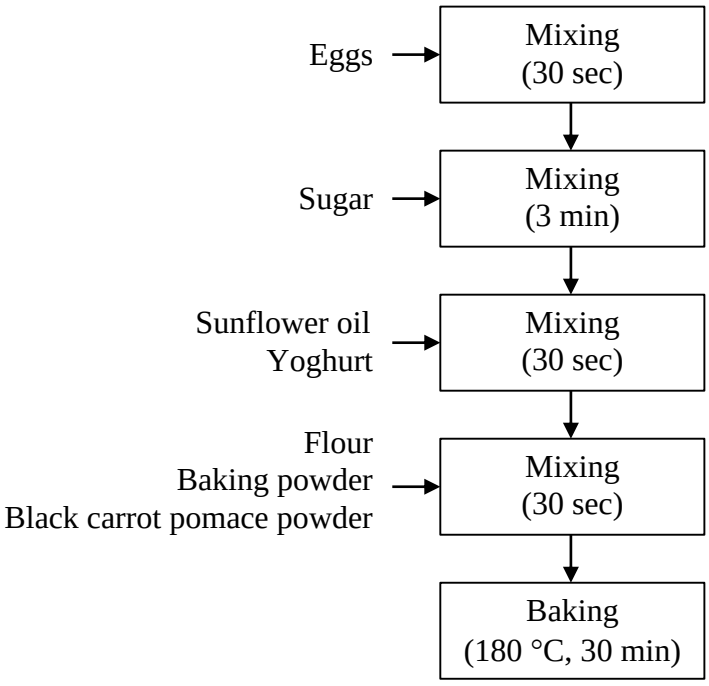

Figure 5.1 : Flow chart outlining the steps involved in production of black carrot pomace enriched cakes.

5.3.2 Chemicals

For simulation of *in vitro* digestion, α-amylase, pepsin, pancreatin and bile from Sigma-Aldrich Chemie GmbH (Steinheim, Germany) were purchased. The following standards were used for the quantification of major polyphenols: cyanidin-3-*O*-glucoside (≥95%), neochlorogenic acid (≥98%) and caffeic acid (≥98%) from Sigma-Aldrich; chlorogenic acid (≥98%) from Fluka Chemie AG (Buchs, Switzerland).

5.3.3 Extraction of polyphenols from undigested samples

For each cake sample, three independent extractions were performed as described previously in Section 3.3.6. Lyophilized powder (1 g) was treated with 75% aqueous methanol containing 0.1% (v/v) formic acid. The treated samples were sonicated in an ultrasonic bath (VWR USC900TH ultrasonic cleaner, VWR Int., Radnor, PA, USA) and subsequently centrifuged (Hettich Universal 32R, Hettich Zentrifugen GmbH & Co., Tuttlingen, Germany), and the supernatants were collected. This extraction protocol was repeated several times for the pellet, and the supernatants were pooled. Prepared extracts were stored at −20 °C until analysis.

5.3.4 *In vitro* digestion model

The *in vitro* digestion model adapted from Minekus et al (2014) consisted of a three-step procedure, which sequentially simulated the digestion in the mouth, stomach and small intestine. The compositions of the salivary, gastric and intestinal juices were described in Table 5.2. To simulate oral digestion, 0.4 g freeze-dried cake samples (≈ 5 g fresh sample) were mixed with 3.5 mL of salivary juice, 0.5 mL of α-amylase solution (1,500 U/mL), 25 µL of 0.3 M $CaCl_2$, and 0.975 µL of distilled water to attain a final volume of 5 mL. The mixture was incubated at 37 °C in a shaking water bath (Memmert SV 1422, Memmert GmbH & Co., Nürnberg, Germany) for 2 min. After oral digestion, 2 mL aliquots were collected for each sample.

To simulate gastric digestion, 6 mL of gastric juice, 1.28 mL of pepsin solution (25,000 U/mL), 4 µL of 0.3 M $CaCl_2$ were added to the remaining fluid and the pH was adjusted to 3.0 using 1 M HCl. Afterwards, the total volume of the mixture was completed to 8 mL with the addition of distilled water and the mixture was incubated in a shaking water bath (Memmert) at 37 °C for 2 h. After gastric digestion, 2 mL

aliquots were collected for each sample. To simulate intestinal digestion, the remainder of the mixture was mixed with 7.7 mL of duodenal juice, 3.5 mL of pancreatin (800 U/mL), 1.75 mL 160 mM bile and 28 μL 0.3 M $CaCl_2$. The pH of the mixture was adjusted to 7.0 using 1 M NaOH. Then, the total volume was completed to 14 mL using distilled water and the mixture was incubated in a shaking water bath (Memmert) at 37 °C for another 2 h. After intestinal digestion, again 2 mL aliquots were collected for each sample (Figure 5.2). The blank (without the added cake samples) was also incubated under the same conditions described above and used for the correction of interferences from the digestive fluids. Samples collected from the mouth, stomach and small intestine phases were centrifuged (Hettich) at 23,000 *g* and 4 °C for 5 min, and the supernatants were kept at −20 °C until further analysis.

Table 5.2 : Simulated salivary, gastric and intestinal fluids.

Constituents	Stock concentration (M)	Volume of stock (mL)		
		Salivary fluid (pH 7)	Gastric fluid (pH 3)	Intestinal fluid (pH 7)
KCl	0.5	15.1	6.9	6.8
KH_2PO_4	0.5	3.7	0.9	0.8
$NaHCO_3$	1	6.8	12.5	42.5
NaCl	2	-	11.8	9.6
$MgCl_2(H_2O)_6$	0.15	0.5	0.4	1.1
$(NH_4)_2CO_3$	0.5	0.06	0.5	-
HCl	6	0.09	1.3	0.7

All solutions were prepared with distilled water to a total volume of 400 mL.

5.3.5 Solid phase extraction (SPE)

Aliquots (1.5 mL) of digested samples were acidified with formic acid (30 μL) and centrifuged (Labnet Spectrafuge 16M, Labnet International Inc., Woodbridge, NJ, USA) at 16000 *g* for 10 min. 500 mg/4 mL C18 SPE cartridges (Grace Davison Discovery Sciences, Deerfield, IL, USA) were conditioned by rinsing with 6 mL of formic acid/methanol (1:100, v/v) followed by 4 mL of formic acid/MQ water (1:100, v/v). Later the centrifuged samples were loaded to activated cartridges, which were subsequently washed with 5 mL of formic acid/MQ water (1:100, v/v). Samples were eluted with formic acid/methanol (1:100, v/v).

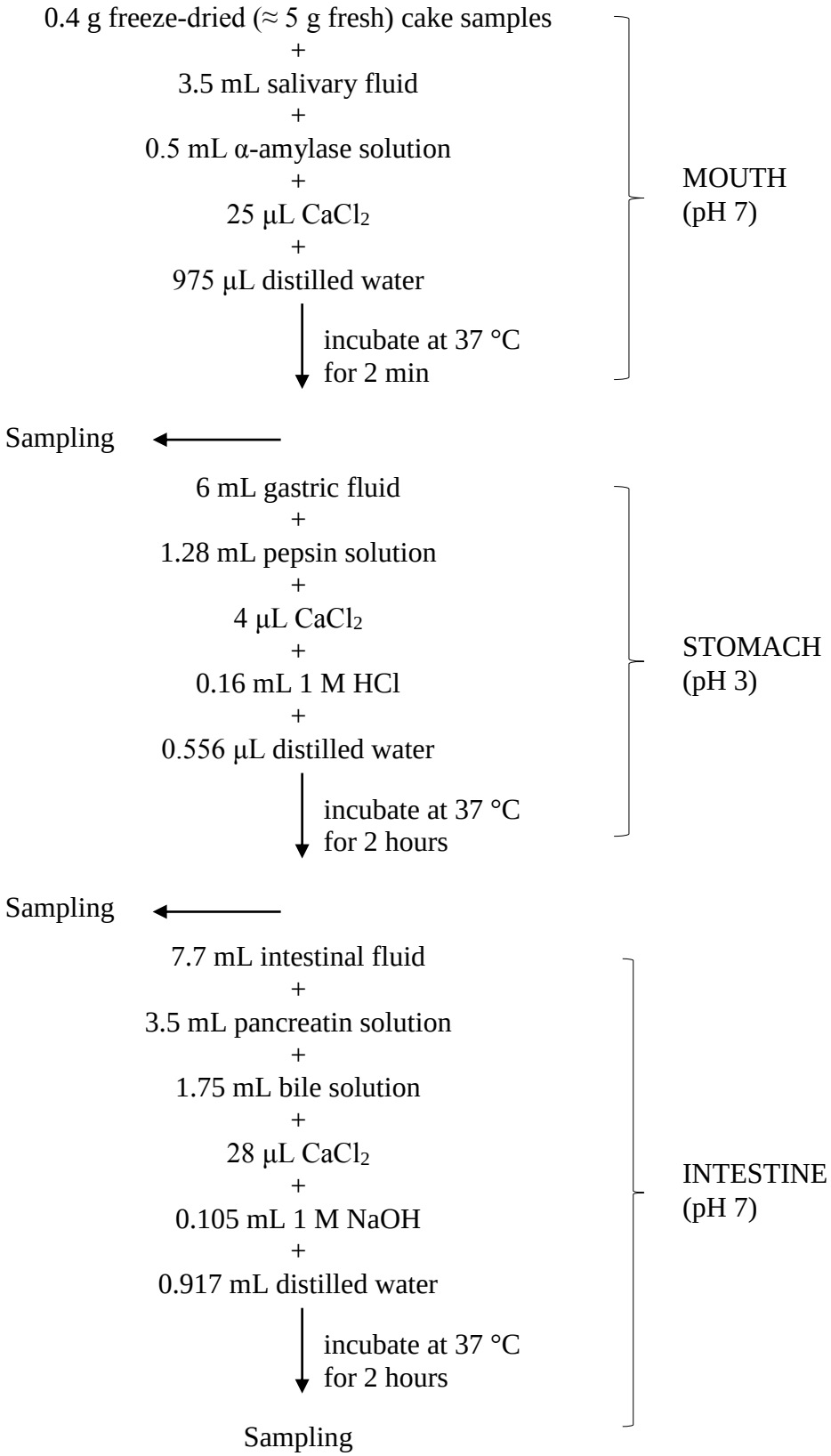

Figure 5.2 : Flow chart for the *in vitro* gastrointestinal digestion model.

5.3.6 HPLC-DAD analysis of anthocyanins and phenolic acids

Anthocyanin and phenolic acid profiles of samples were determined by comparison with a previous study performed by our group (Kamiloglu et al, 2016) where the identification of black carrot pomace polyphenols was carried out using an UPLC-ESI-MS^E system (Section 4.3.6). For the quantification of anthocyanins and phenolic acids, the method of Capanoglu et al (2008) was applied (Section 3.3.9). Samples purified using SPE were passed through 0.45 µm membrane filters and injected into a Thermo Dionex Ultimate 3000 HPLC (Thermo Fischer Scientific Inc., Landsmeer, The Netherlands) coupled with a diode array detector (DAD). The quantification of anthocyanin glycosides was done using cyanidin-3-*O*-glucoside, whereas phenolic acids were quantified using their authentic standards. All analyses were performed in triplicate and the results were expressed as µg per g dw of sample.

5.3.7 Spectrophotometric assays

Total phenolic content was determined using Folin−Ciocalteu reagent as described previously in Section 4.3.5.1. The results were expressed as mg gallic acid equivalent (GAE) per 100 g dw sample. Total antioxidant capacity was estimated by four different assays. The ferric reducing antioxidant power (FRAP), cupric ion reducing antioxidant capacity (CUPRAC), 2,2-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays were performed according to Benzie and Strain (1996), Apak et al (2004), Miller and Rice-Evans (1997), and Kumaran and Karunakaran (2006), respectively (Section 3.3.8.3). In all assays, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox®) was used as a standard and the results were expressed in terms of mg Trolox equivalent (TE) per 100 g dw of sample.

5.3.8 Statistical analysis

All analyses were performed with three biological and three technical replicates, and the obtained data were reported as mean ± standard deviation. 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 2011 software (Microsoft Co., Redmond, WA, USA).

5.4 Results and Discussion

5.4.1 Anthocyanins

The five major anthocyanins detected in black carrot pomace enriched cakes were all cyanidin-based with different sugar moieties, two were non-acylated, cyanidin-3-xylosyl-glucosyl-galactoside (cyd-3-xyl-gluc-gal) and cyanidin-3-xylosyl-galactoside (cyd-3-xyl-gal), and three were acylated with phenolic acids, cyanidin-3-xylosyl-sinapoyl-glucosyl-galactoside (cyd-3-xyl-sin-gluc-gal), cyanidin-3-xylosyl-feruloyl-glucosyl-galactoside (cyd-3-xyl-fer-gluc-gal) and cyanidin-3-xylosyl-coumaroyl-glucosyl-galactoside (cyd-3-xyl-cou-gluc-gal), whereas no anthocyanins were detected in control samples. The predominant anthocyanin, representing 43% of the total anthocyanins corresponded to cyd-3-xyl-fer-gluc-gal (Peak 4 in Figure 5.3), which is also reported to be the most abundant anthocyanin in black carrots (Suzme et al, 2014; Guldiken et al, 2016). On the other hand, in undigested black carrot pomace enriched cakes, compounds with an acylated structure constituted approximately 65% of total anthocyanins, while in black carrots this amount was reported to be higher (77−85%) (Gras et al, 2015; Kamiloglu et al, 2015b). The influence of *in vitro* digestion on anthocyanins from black carrot pomace enriched cakes is presented in Figure 5.4, and a representative chromatogram corresponding to the anthocyanin profile of 15% black carrot pomace sample at different stages of *in vitro* digestion is given in Figure 5.3. Compared to undigested samples, significantly lower amount of total anthocyanins (46−74%) were released after the oral digestion in the mouth ($p < 0.05$). Some polyphenols, especially those with a high number of hydroxyl groups have been reported to strongly bind to proteins such as albumins, both from the food matrix and also from saliva, resulting in complexes that reduce polyphenol absorption (Bohn, 2014). From the mouth to the stomach the amount of anthocyanins did not change significantly ($p > 0.05$), whereas no anthocyanins were detected in the intestine. Although the enrichment of cakes with black carrot pomace resulted in proportional increase in anthocyanin content for undigested samples (72 to 267 μg/g dw), when digested there was no statistical difference between the 10% and 15% black carrot pomace enriched sample ($p > 0.05$). Furthermore, while the percentage of acylated anthocyanins remained unaltered in the mouth, in the stomach it was increased up to 85%. This was an expected outcome as the stability of acylated anthocyanins towards pH change is shown to be higher than non-acylated

counterparts (Kammerer et al, 2004b). The degradation of anthocyanins during intestinal digestion is in line with previously published studies (Correa-Betanzo et al, 2014; Sun et al, 2015). It is well known from the literature that anthocyanins are not stable under neutral and/or basic pH conditions as the red flavylium cation is being transformed to colourless chalcone (Kamiloglu et al, 2015a). The high loss of anthocyanins may also be related to the fact that they are metabolized to some non-colored forms, oxidized, or degraded into other chemicals, which may not be detected under the present conditions (Perez-Vicente et al, 2002). Nevertheless, the health beneficial effects of anthocyanins have been questioned due to their instability, and it has been suggested that under *in vivo* conditions, anthocyanins could be absorbed across the stomach as well (Fang, 2014a). Therefore, the existing knowledge indicates that the observed low apparent bioavailability of anthocyanins could be due to their extensive presystemic metabolism, rather than poor absorption from the intestinal lumen.

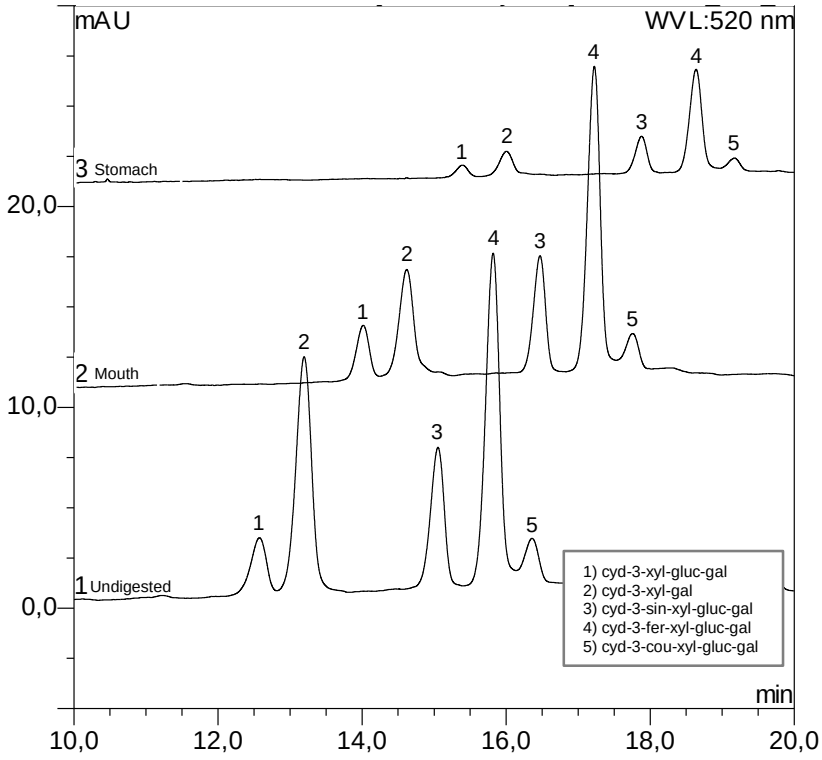

Figure 5.3 : HPLC-DAD chromatograms of cake sample (enriched with 15% black carrot pomace) during *in vitro* digestion. Chromatograms for the mouth and stomach phases were shifted to right to obtain a better image.

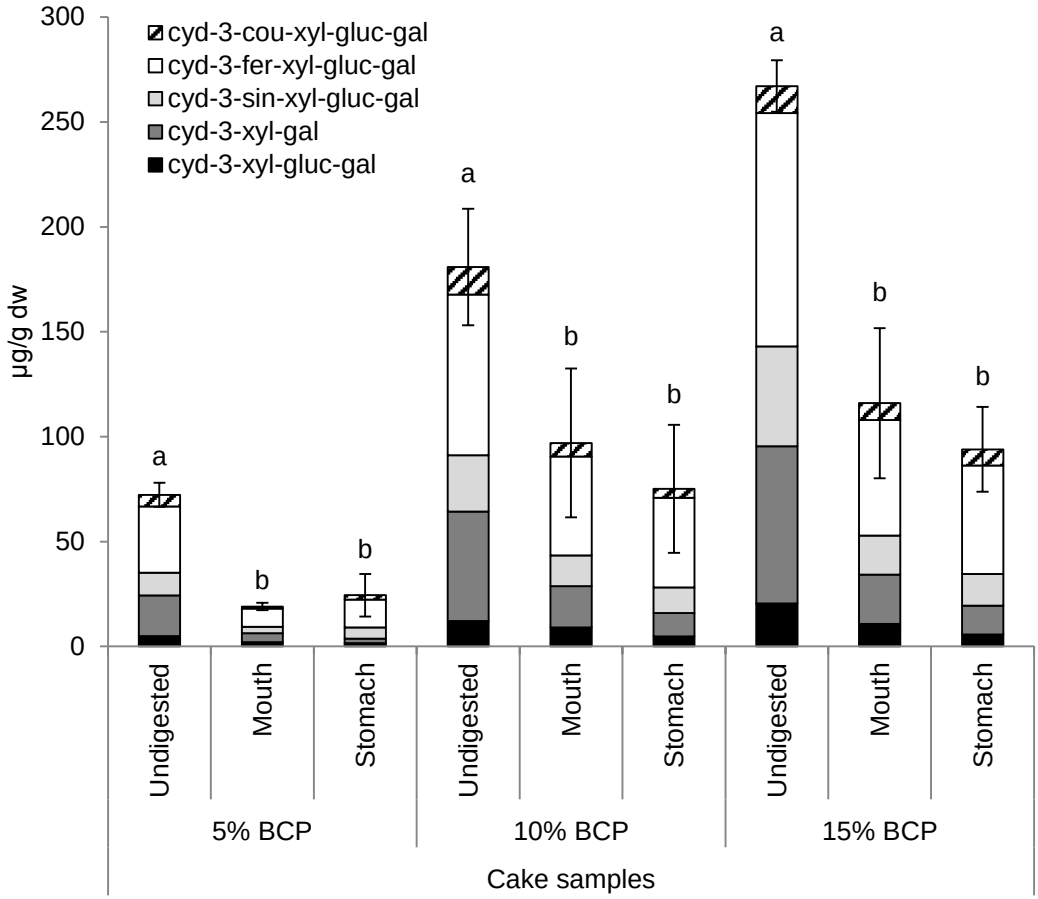

Figure 5.4 : Changes in anthocyanin content of black carrot pomace (BCP) enriched cakes during *in vitro* digestion. Different letters above bars represent statistically significant differences ($p < 0.05$).

5.4.2 Phenolic acids

Neochlorogenic acid, chlorogenic acid and caffeic acid were the three major phenolic acids identified in black carrot pomace enriched cakes. As in black carrots (Suzme et al, 2014; Kamiloglu et al, 2016), chlorogenic acid, accounting for approximately 90% of the total phenolic acids, was the most abundant compound in black carrot pomace enriched cakes. Similar to anthocyanins, no phenolic acids were determined in control samples. Figure 5.5 shows the impact of *in vitro* digestion on major phenolic acids present in black carrot pomace enriched cakes. For undigested samples, from 5% black carrot pomace sample to 15% black carrot pomace sample, a dose-dependent increase in phenolic acid content (49 to 148 µg/g dw) was observed. Oral digestion in the mouth caused significant decreases in total phenolic acid content for all samples (35−40%) ($p < 0.05$). Although the total phenolic acid content

reduced in the mouth, when individual compounds are investigated, it has been shown that there was an increase in the neochlorogenic acid content (up to 52%), compared to undigested samples. Some studies demonstrated the isomerization of chlorogenic acid to other caffeoylquinic acids in the course of digestion (Tagliazucchi et al, 2012), which may explain the increase in neochlorogenic acid content. In the stomach, the amount of total phenolic acids decreased further compared to the mouth, which was statistically significant for 15% black carrot pomace sample (46%) ($p < 0.05$). In the intestine, significantly lower amounts of chlorogenic acid were found compared to the stomach (74−85%) ($p < 0.05$), whereas neither neochlorogenic acid nor caffeic acid was detected. Previous studies also reported dramatic decreases of phenolic acids during *in vitro* digestion (Sengul et al, 2014; Celep et al, 2015). These reductions in phenolic acids could be associated with changes in pH and the presence of bile salts. The instability of the caffeoylquinic acids in aqueous solutions could also contribute to these losses (Vallejo et al, 2004). Furthermore, phenolic acids from black carrots were shown to remain bound to plant cell walls during *in vitro* digestion (Padayachee et al, 2013), which may partially explain the low recovery of phenolic acids from black carrot pomace enriched cake samples. In addition, cake matrix containing protein rich ingredients (e.g. yoghurt, eggs) may lead to possible protein-polyphenol interactions resulting in reduced availability of phenolic acids as in an earlier study, in which the amount of phenolic acids was decreased when orange juice was consumed with yoghurt (Roowi et al, 2009). Additional effects of other matrix components such as the dietary fibers from cake flour and black carrot pomace should also be taken into consideration as it is hypothesized that dietary fiber decreases the availability of polyphenols, mainly due to factors such as physical entrapment, and increased viscosity and bulk. On the other hand, dietary lipids (e.g. sunflower oil) could have a limited influence as the majority of the polyphenols present in black carrot pomace are water-soluble (Bohn, 2014).

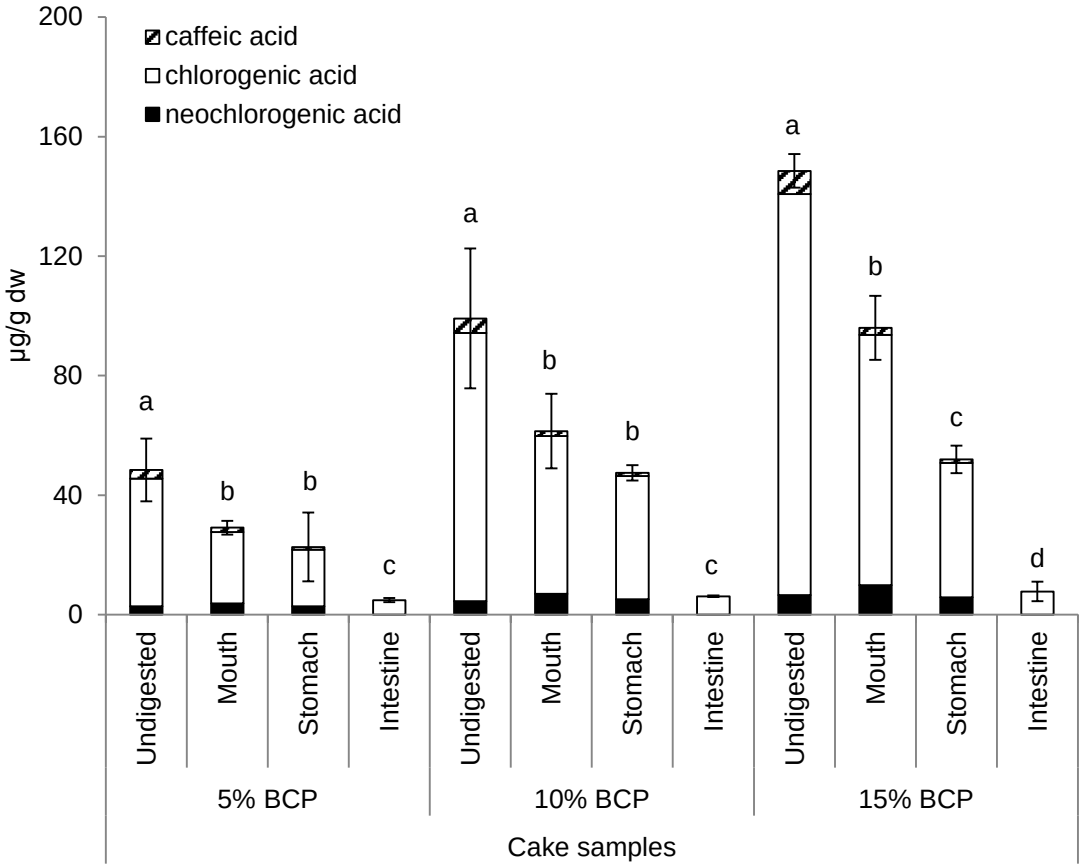

Figure 5.5 : Changes in phenolic acid content of black carrot pomace (BCP) enriched cakes during *in vitro* digestion. Different letters above bars represent statistically significant differences ($p < 0.05$).

5.4.3 Spectrophotometric assays

The effect of *in vitro* digestion on total phenolic content and total antioxidant capacity of black carrot pomace enriched cakes is shown in Table 5.3. For undigested samples, addition of black carrot pomace caused a dose-dependent increase in total phenolic content (54 to 202 mg GAE/100 g dw) and total antioxidant capacity (21−129 to 153−478 mg TE/100 g dw), with 10% and 15% black carrot pomace enriched samples being statistically significant for all assays (up to 6−fold) ($p < 0.05$). In general, while the same trend continued at different stages of the digestion, there was no statistical difference between the 10% and 15% black carrot pomace enriched sample ($p > 0.05$), suggesting that addition of 10% black carrot pomace to cakes is a favorable condition to obtain a product having significantly high total phenolic content and total antioxidant capacity. In the mouth, total phenolic content and total antioxidant capacity determined using FRAP and

CUPRAC assays did not change significantly ($p > 0.05$), whereas the results of ABTS and DPPH assays were significantly lower (58−97%) compared to undigested samples ($p < 0.05$). After the gastric digestion in the stomach, significant increases in total phenolic content and total antioxidant capacity (in case of FRAP and CUPRAC) were obtained compared to amounts released in the mouth (up to 5− and 12−fold, respectively) ($p < 0.05$). From the stomach to the small intestine further significant increases in total antioxidant capacity (75−159%) were observed ($p < 0.05$). On the other hand, total phenolic content remained stable with the exception of 15% black carrot pomace sample, which resulted in additional 116% increase. These results are in line with the study carried out on strawberry and peach enriched yoghurts, in which the antioxidant activity increased greatly (up to 5.5−fold) during the gastric and intestinal phases of *in vitro* digestion (Oliveira and Pintado, 2015). The increase in total antioxidant capacity in the stomach can be attributed to acidic hydrolysis of the phenolic glycosides to their aglycons during simulated gastric digestion, whereas in the intestine formation of new oxidation products with a higher antioxidant capacity than that of their precursors may be the cause of the increase. Another reason could be the unmasking of a previously sterically impeded pool of hydroxyl radicals as a consequence of conformational changes undergone by polyphenols (Pineda-Vadillo et al, 2016). Furthermore, the release of the phenolics bound to the matrix as a result of the interaction of polyphenols with gastric and intestinal enzymes has also been described previously (Bouayed et al, 2011).

Correlations between total phenolic content and total antioxidant capacity assays were determined. For both the undigested and digested samples, moderate to high correlations were obtained, with correlation between FRAP and CUPRAC assay being the highest (R^2 = 0.7492−0.9704). FRAP and CUPRAC assays are electron transfer (ET)-based antioxidant capacity assays, whereas ABTS and DPPH assays are mixed mode (ET- and hydrogen atom transfer (HAT)-based) assays. Recently criticisms have been directed towards ABTS and DPPH assays on the grounds that these assays use sterically hindered, N-centered free radicals as targets to antioxidants rather than biologically active short-lived radicals and that their action as radical scavenger should be irrelevant *in vivo* (Schaich et al, 2015). Consequently, these assays may lead to the underestimation of the total antioxidant capacity during digestion. Considering the pH conditions of the total antioxidant capacity assays

performed in this study, it can be presumed that the FRAP assay (pH 3.6) could be more appropriate for evaluating total antioxidant capacity in the stomach, while CUPRAC assay (pH 7.0) could be more suitable to assess total antioxidant capacity in the mouth and small intestine. Eventually, it is clear that the measurement of antioxidant capacity of food products, multifunctional or complex multiphase systems, cannot be evaluated satisfactorily using a single antioxidant assay due to the many variables, which can influence the results. Therefore, it is highly recommended to apply variety of assays with different mechanisms to obtain the full picture (Apak et al, 2016).

Table 5.3 : Changes in the total phenolic content and total antioxidant capacity of black carrot pomace (BCP) enriched cakes during *in vitro* digestion.

Sample	Undigested	Mouth	Stomach	Intestine
	Total phenolic content (mg GAE/100 g dw)			
Control	54 ± 13 bC	21 ± 6 bB	259 ± 6 aB	263 ± 34 aC
5% BCP	87 ± 24 bBC	39 ± 5 bB	527 ± 22 aA	529 ± 15 aB
10% BCP	146 ± 33 bAB	217 ± 15 bA	548 ± 67 aA	556 ± 15 aB
15% BCP	202 ± 24 cA	261 ± 46 cA	600 ± 91 bA	1294 ± 16 aA
	Total antioxidant capacity (mg TE/100 g dw)			
FRAP				
Control	63 ± 17 cC	70 ± 22 cB	364 ± 104 bB	709 ± 45 aC
5% BCP	143 ± 19 cB	165 ± 61 cAB	472 ± 17 bAB	824 ± 16 aC
10% BCP	225 ± 40 cA	179 ± 25 cA	533 ± 110 bAB	987 ± 80 aB
15% BCP	270 ± 39 cA	255 ± 22 cA	616 ± 108 bA	1139 ± 57 aA
CUPRAC				
Control	129 ± 25 cB	220 ± 17 bcB	464 ± 85 bB	1201 ± 193 aC
5% BCP	236 ± 41 cB	345 ± 38 cAB	726 ± 88 bA	1464 ± 128 aBC
10% BCP	417 ± 52 cA	417 ± 72 cA	765 ± 35 bA	1749 ± 26 aAB
15% BCP	478 ± 70 cA	481 ± 69 cA	828 ± 77 bA	1868 ± 116 aA
ABTS				
Control	75 ± 21 B	-	-	-
5% BCP	143 ± 30 aAB	4 ± 1 bB	10 ± 3 bB	21 ± 5 bB
10% BCP	168 ± 21 aA	14 ± 4 cA	36 ± 11 cA	84 ± 25 bA
15% BCP	178 ± 15 aA	15 ± 1 dA	37 ± 4 cA	87 ± 10 bA
DPPH				
Control	21 ± 8 B	-	-	-
5% BCP	56 ± 28 abAB	15 ± 5 bC	33 ± 5 bB	96 ± 18 aA
10% BCP	126 ± 51 aA	44 ± 7 bB	103 ± 21 abA	102 ± 29 abA
15% BCP	153 ± 33 aA	65 ± 14 bA	135 ± 35 abA	127 ± 36 abA

The data presented in this table consist of average values ± standard deviation of three independent batches. Total phenolic content and total antioxidant capacity is expressed as mg of gallic acid equivalent (GAE), and Trolox equivalent (TE) per 100 g of dry weight (dw) of sample, respectively. Different capital letters in the columns or small letters in the rows represent statistically significant differences ($p < 0.05$).

5.5 Conclusions

The present study focused on the digestive stability of anthocyanins, phenolic acids and total phenolics from black carrot pomace enriched cakes and monitored the

changes in their total antioxidant capacity using the new standardized static *in vitro* digestion protocol. Although the total phenolic content and total antioxidant capacity increased significantly as a result of digestion, high losses in anthocyanins and phenolic acids were observed. However, the bioavailability of polyphenols may be underestimated since the metabolites formed in the course of digestion could be responsible for the health benefits associated with these bioactive compounds. Therefore, we suggest that future studies should address the bioavailability of the polyphenol metabolites to establish whether such metabolites could play a part in bioactivity. In addition, further information about sensory characteristics and other related quality parameters could be necessary before any industrial application of these products.

6. ANTI-INFLAMMATORY POTENTIAL OF BLACK CARROT (*DAUCUS CAROTA* L.) POLYPHENOLS IN A CO-CULTURE MODEL OF INTESTINAL CACO-2 AND ENDOTHELIAL EA.HY926 CELLS

Redrafted from:

Kamiloglu, S., Grootaert, C., Capanoglu, E., Ozkan, C., Smagghe, G., Raes, K., and Van Camp, J. (2016). Anti-inflammatory potential of black carrot (*Daucus carota* L.) polyphenols in a co-culture model of intestinal Caco-2 and endothelial EA.hy926 cells. *Molecular Nutrition & Food Research* (Accepted for publication, DOI:10.1002/mnfr.201600455).

6.1 Abstract

The present study was developed to determine the ability of polyphenol-rich black carrot and its by-products, i.e., peel and pomace, to modulate the inflammatory response in tumor necrosis factor-α (TNF-α) treated endothelial cells after gastrointestinal digestion and in a co-culture of intestinal Caco-2 and endothelial EA.hy926 cell model. The results indicated that after 4 h of treatment, the transport of anthocyanins and phenolic acids was higher for digested samples (1.3−7%) compared to undigested ones (0−3.3%). The transported polyphenols were able to down-regulate the secretion of pro-inflammatory markers, i.e., interleukin-8 (IL-8), monocyte chemoattractant protein-1 (MCP-1), vascular endothelial growth factor (VEGF) and intercellular adhesion molecule-1 (ICAM-1), under normal and TNF-□ induced inflammatory conditions. The most pronounced protective effects were observed with digested samples under inflammatory conditions, which significantly decreased the secretion of all markers from 120−203% down to 34−144% ($p < 0.001$). Overall, these results show that the polyphenol-rich black carrot products may function through an inhibitory regulation of the inflammatory cascade in endothelial cells.

Keywords: anthocyanins, *in vitro* digestion, phenolic acids, pro-inflammatory markers, transepithelial transport.

6.2 Introduction

Over the past few decades, several epidemiological and clinical studies indicated that chronic inflammation is a significant risk factor in the development of various human diseases, in particular cardiovascular diseases (Pan et al, 2010), which is the leading cause of morbidity and mortality in the world regardless of race, ethnicity, or sex (Widmer et al, 2015). Endothelial cells that line the internal lumen of blood vessels play a crucial role in the regulation of inflammation through the expression of cytokines (e.g. TNF-α), chemokines (e.g. IL-8, MCP-1), growth factors (e.g. VEGF), adhesion molecules (e.g. ICAM-1) and inflammatory mediators (e.g. NO) (Yamagata et al, 2015).

Polyphenols are widely present in the average diet especially in fruits and vegetables, and have been demonstrated to exhibit a broad spectrum of biological activities for human health including protective effects on endothelial cells (Forbes-Hernandez et al, 2014; Giampieri et al, 2014b; Pistollato et al, 2015). Black carrots contain high levels of polyphenols, in particular anthocyanins and phenolic acids (Olejnik et al, 2016; Padayachee et al, 2013; Kamiloglu et al, 2015c), of which the anti-inflammatory properties were demonstrated recently (Mizgier et al, 2016). Black carrots are often consumed after processing into various products, which results in the generation of large amounts of by-products, including peel and pomace. In our previous work (Kamiloglu et al, 2016), we showed that the polyphenols initially present in black carrots are largely preserved in these by-products.

To obtain any influence in a specific tissue or organ, polyphenols must be bioavailable, i.e., effectively absorbed from the gut into the circulation and transferred to the appropriate location within the body while still maintaining their bioactivity. One of the key factors affecting the bioavailability of polyphenols is their transport through the gut epithelium. The Caco-2 cell line, a human intestinal epithelial cell model derived from a colon carcinoma, has been proven to be a good alternative to animal studies for predicting intestinal absorption of polyphenols including anthocyanins (Kamiloglu et al, 2015a). Although anthocyanins can be absorbed directly in the intestine without any chemical alterations, they can also be

further metabolized into methyl, glucuronide, or sulfate conjugates in the small intestine and liver by the action of phase II enzymes and the resultant metabolites may also have an endothelium protecting effect (Kuntz et al, 2015a).

To date, only few studies (Kuntz et al, 2015a; Toaldo et al, 2016) investigated the anti-inflammatory effects of polyphenols in co-cultured intestinal epithelial and endothelial cells and yet application of this model is important due to the cross-talk mechanisms between the two cell lines, which may have an indirect impact on endothelial function (Grootaert et al, 2015). Considering the above, the aim of the current study was to determine the potential of polyphenols from black carrot and its by-products to modulate the inflammatory response in TNF-α stimulated EA.hy926 endothelial cells co-cultured with differentiated intestinal Caco-2 cells. As the bioactivity of polyphenols depends on their bioavailability, gastrointestinal digestion as well as transepithelial intestinal absorption was also considered while evaluating the anti-inflammatory effects of polyphenols. To the best of our knowledge, this is the first *in vitro* study that introduced the interaction of the transported polyphenols from black carrot and its by-products with the cellular inflammation response system in stimulated endothelial cells.

6.3 Materials and Methods

6.3.1 Plant material

Black carrot (*Daucus carota* L. spp. *sativus* var. *atrorubens* Alef.) and its industrial by-product pomace were collected from an established processing plant (Erkon Konsantre Co.) in Konya, Turkey. Peel was obtained manually from whole black carrots. All samples were ground to a fine powder in liquid nitrogen using a precooled grinder (IKA A11 basic, IKA-Werke GmbH & Co., Staufen, Germany) and subsequently freeze-dried (Christ Alpha 1-2 LD plus, Martin Christ Gefriertrocknungsanlagen, Osterode am Harz, Germany) for 24 h and stored at −20 °C.

6.3.2 Chemical extraction of undigested samples

Extractions were performed as described previously in Section 3.3.6. Freeze-dried powder (0.1 g) was treated with 5 mL of 75% aqueous methanol containing 0.1% formic acid. The treated samples were sonicated in an ultrasonic bath (Elma S60H

elmasonic, Singen, Germany) for 15 min and subsequently centrifuged at 3000 *g* and 4 °C for 10 min (Sigma Laboratory Centrifuge 4K15, Osterode am Harz, Germany), and the supernatants were collected. This extraction protocol was repeated once more for the pellet, and the two supernatants were pooled to a final volume of 10 mL (Capanoglu et al, 2008). Prepared extracts were dried under nitrogen and stored at −20 °C.

6.3.3 *In vitro* gastrointestinal digestion

In vitro gastrointestinal digestion (Kamiloglu et al, 2016) was carried out as described previously (Section 4.3.3). Each sample was subjected to simulated gastric and intestinal conditions in a shaker (Edmund Bühler TH 15, Hechingen, Germany) at 37 °C. Briefly, black carrot, peel, and pomace samples were mixed with distilled water and gastric solution and acidified to pH 3. After 2 h of incubation the pH was increased to 7 with the addition of duodenal and bile solutions. The samples were incubated for another 4 h to complete the small intestine phase of the *in vitro* gastrointestinal digestion. The blank (without the added samples) was also incubated under the same conditions and used for the correction of interferences from the digestive fluids. Samples collected after gastrointestinal digestion were centrifuged at 3000 *g* and 4 °C for 10 min (Sigma Laboratory Centrifuge 4K15), and the supernatants were dried and stored at −20 °C.

6.3.4 Maintenance of cell cultures

The human colon adenocarcinoma cell line Caco-2 (HTB37™) and endothelial cell line EA.hy926 (CRL2922™) were obtained from American-type culture collection (ATCC, Manassas, VA, USA). The cells were grown separately in 25 cm^2 canted neck tissue culture flasks (Sarstedt Co., Essen, Belgium) using high glucose Dulbecco's modified eagle's medium (DMEM) supplemented with Glutamax (Gibco, Life Technologies, Carlsbad, CA, USA), 10% heat-inactivated fetal bovine serum (FBS, Greiner Bio One, Wemmel, Belgium), 1% non-essential amino acids (NEAA, Gibco, Life Technologies) and 1% penicillin-streptomycin (Pen Strep, Gibco, Life Technologies). Cells were maintained in a humidified atmosphere of 10% CO_2 in air at 37 ºC (Memmert CO_2 incubator, Memmert GmbH & Co., Nurnberg, Germany). Medium was replaced three times per week and cells were sub-cultured at 80-90% confluence. For sub-culturing, the cells washed with 3 mL

phosphate buffered saline (PBS, Gibco, Life Technologies), were detached from the bottom of the flask with 2 mL of 0.25% trypsin-EDTA wash (Sigma-Aldrich) and subsequently incubated at 37 ºC and 10% CO_2 in air for 3-5 min. A volume of suspension, depending on the cell concentration and passage number, was seeded to new flasks, and the final volume was made up to 4 mL with the DMEM medium with Glutamax, 10% FBS, 1% NEAA and 1% Pen Strep.

6.3.5 Transport experiments

For transport experiments, Caco-2 cells in flasks, having a passage number <50, were harvested using the trypsinization protocol described above and resuspended in DMEM medium with Glutamax, 10% FBS, 1% NEAA and 1% Pen Strep. One hundred microliter of this cell suspension was mixed with 100 µL trypan blue stain (0.4%) and the number of viable cells was determined using a 0.1 mm depth Burker counting chamber under a AE30/31 inverted microscope (MOTIC, VWR, Leuven, Belgium). Caco-2 cells were seeded in 6-well Transwell® plates (0.4 µm pore diameter, 24 mm insert, Corning Costar Co., Elscolab, Kruibeke, Belgium) at a concentration of approximately 6.0 x 10^5 cells per well. Culture medium with cells was added to the apical compartment and without cells to the basolateral compartment in volumes of 2 mL and 2.5 mL, respectively. Cells were allowed to grow and differentiate to confluent monolayers for 21 days post seeding. Culture medium was replaced two or three times a week. Experiments were carried out using undigested and digested black carrot, peel and pomace samples dissolved in Hank's Balanced Salt Solution (HBSS, Gibco Life Technologies), which was selected as transport medium based on preliminary studies. In order to use sample concentrations that do not negatively influence the monolayer integrity of Caco-2 cells, studies were performed in which different concentrations were analyzed during several hours of exposures to cells and the maximum non-toxic concentrations were determined. On the day of the transport experiment (day 21), to ensure that the monolayers exhibit the properties of a tight biological barrier, transepithelial electrical resistance (TEER) values were monitored before, during and after (t = 0, 4, 24 h) the incubation experiments using an automated tissue resistance measurement system (REMS, World Precision Instruments, Hertfordshire, UK). The transport medium was replaced with HBSS, and preincubated for 1 h. The pH of the samples in HBSS were adjusted to 6.5, which is the pH of the duodenum, sterilized and

loaded to the apical compartment of the culture wells (2 mL). HBSS alone was adjusted to pH 7.5, sterilized and loaded to the basolateral compartment (2.5 mL). The Caco-2 cells were incubated with samples for 4 h at 37 °C and 10% CO_2. Apical and basolateral compartments were sampled every 2 h, stabilized by adjusting the pH to 2 with formic acid, and stored at −20 °C until further analysis. Caco-2 cells were harvested and used for the measurement of cytotoxicity.

6.3.6 Measurement of cytotoxicity

To analyse the cytotoxic effects of undigested and digested black carrot, peel and pomace samples on Caco-2 cells, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide) assay was applied. Briefly, 200 μL of MTT (Sigma-Aldrich) dissolved in PBS (5 mg/mL) was added to cells and incubated for 2 h at 37 °C to convert MTT to formazan. After 2 h, the medium was removed, the formazan crystals were dissolved in dimethyl sulfoxide (DMSO) and the absorbance was recorded at 570 nm with a Bio-Rad multiplate reader (Bio-Rad Laboratories, Hercules, CA, USA). The results were expressed as percentage compared to untreated cells.

6.3.7 Identification and quantification of polyphenols

Polyphenol profiles of samples were determined based on a previous study performed by our group (Kamiloglu et al, 2016) where the identification of black carrot polyphenols was carried out using an UPLC-ESI-MS^E system (Section 4.3.6). For the quantification of polyphenols, the method of Capanoglu et al (2008) was applied (Section 3.3.9). Samples were passed through 0.45 μm membrane filters and injected into a Thermo Dionex Ultimate 3000 HPLC (Thermo Fischer Scientific, Landsmeer, The Netherlands) coupled with a diode array detector (DAD). A Grace Smart RP C18 column (250 × 4.6 mm, 5 μm) was used as the stationary phase. The content of anthocyanin glycosides was quantified using cyanidin-3-*O*-glucoside (≥95%, Sigma-Aldrich, Steinheim, Germany), whereas phenolic acids were quantified using neochlorogenic acid (≥98%, Sigma-Aldrich), chlorogenic acid (≥98%, Fluka Chemie AG, Buchs, Switzerland) and caffeic acid (≥98%, Sigma-Aldrich). The calibration curves of polyphenol standards showed good linearity (R^2 > 0.99) within the established range (0.1-100 ppm). Limits of detection (LOD) and quantification (LOQ) ranged from 0.02 to 0.09 ppm, and from 0.05 to 0.29 ppm,

respectively. The results were expressed as percentage compared to initial polyphenol contents of the samples.

6.3.8 Co-culture experiments

A co-culture model consisting of a differentiated 21-day-old intestinal Caco-2 cell monolayer, located on the apical compartment and confluent 3-day-old culture of endothelial EA.hy926 cells, adhered to the surface of the basolateral compartment of the Transwell® plates (Corning Costar Co.) was used to evaluate the anti-inflammatory potential of the undigested and digested black carrot, peel and pomace samples following intestinal transepithelial transport (Figure 6.1).

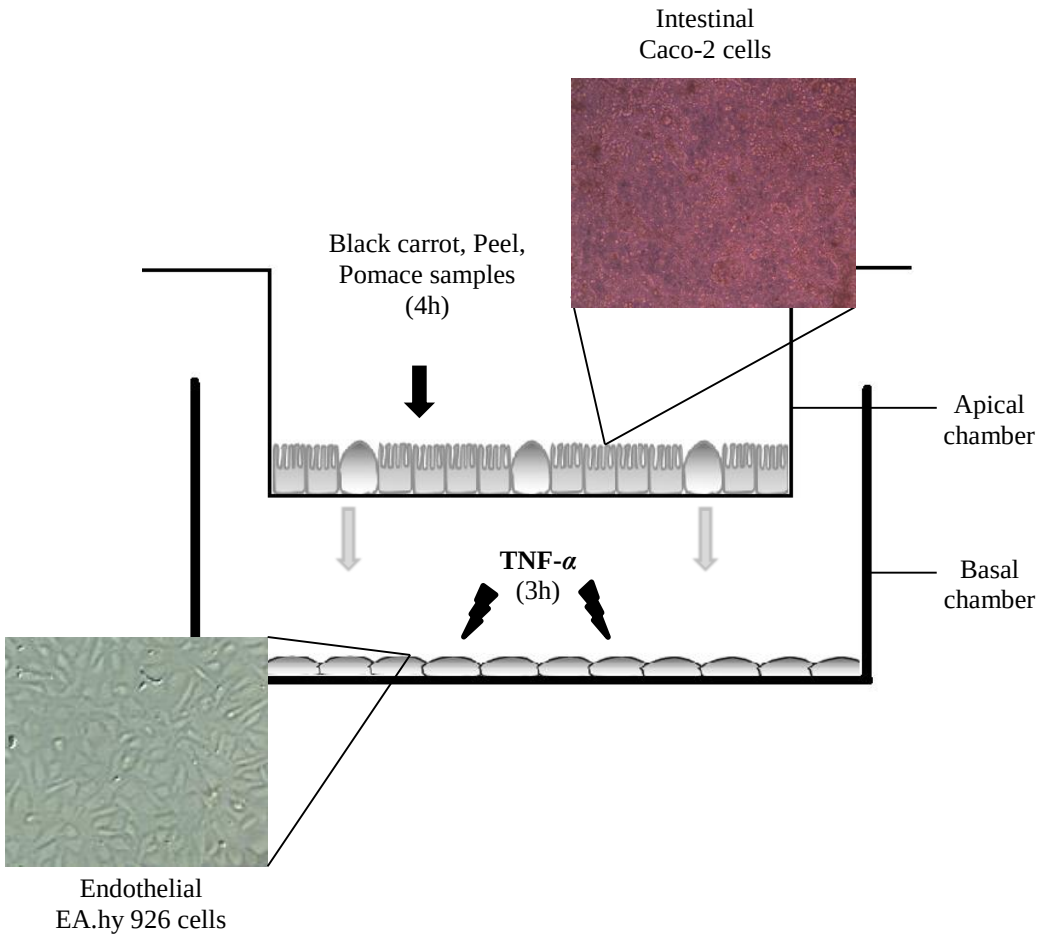

Figure 6.1 : Determination of the anti-inflammatory effects of undigested and digested black carrot, peel and pomace samples in an *in vitro* epithelial-endothelial co-culture cell system.

In order to ensure an intact intestinal monolayer during the time of the experiment, TEER was monitored before and after the incubation experiments. Only monolayers exerting TEER values above 850 $\Omega.cm^2$ were used. The experiment was initiated by loading the samples prepared in HBSS (as described above in Section 6.3.5) to the apical compartment of the co-culture system. Prior to this treatment, basolateral compartment of the co-culture system was stimulated with 1 ng/mL TNF-α for 3 h in order to induce inflammation. During the TNF-α treatment, to avoid a possible interaction with the Caco-2 cell monolayer, the inserts of the Transwell® plates were incubated separately. After 4 h incubation with the samples at 37 °C and 10% CO_2 (Memmert CO_2 incubator), the basal media were collected and assayed for NO, IL-8, MCP-1, VEGF and ICAM-1 production.

6.3.9 Measurement of NO production

The production of NO in endothelial cells was monitored using the Griess colorimetric assay (Toaldo et al, 2016). For this purpose, equal volumes of the Griess reagent (Sigma-Aldrich) and endothelial cell culture supernatant were mixed and incubated at room temperature for 15 min. The absorbance was measured at 540 nm using a multiplate reader (Bio-Rad Laboratories). A standard curve of sodium nitrite ($NaNO_2$) was plotted (0-20 µM) to quantify the amount of NO produced. The results were expressed as percentage compared to untreated cells.

6.3.10 Measurement of IL-8, MCP-1, VEGF and ICAM-1 production

The cellular secretion of IL-8, MCP-1, VEGF and ICAM-1 by endothelial cells was determined with the enzyme-linked immunosorbent (ELISA) assay kits (Peprotech, London, UK) following the manufacturer's instructions. The results were expressed as percentage compared to untreated cells.

6.3.11 Statistical analysis

All analyses were performed with four biological (cell monolayers/co-cultures treated with extracts/digests) and three technical (measurements per polyphenol analysis and/or assays) replicates. Data were subjected to statistical analysis 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 with significance levels of $p < 0.05$, $p < 0.01$ and $p < 0.001$.

6.4 Results and Discussion

6.4.1 Transport of polyphenols through intestinal Caco-2 cells

Before performing the transport assays in the co-culture setup, experimental conditions were optimized. When optimizing transport conditions, polyphenol stability as measured by HPLC-DAD, Caco-2 cell monolayer integrity as measured by TEER, and cytotoxicity as measured by MTT assay were investigated. Several concentrations of samples (1−10 mg/mL) were incubated in different media (DMEM and HBSS) under varying pH conditions (with and without a gradient). Preliminary data showed that the highest transport efficiency that does not negatively influence the monolayer integrity of Caco-2 cells was observed when the samples were incubated in HBSS in the presence of a pH gradient (pH 6.5 and 7.5 for apical and basolateral compartments, respectively). The polyphenol contents of the maximum non-toxic concentrations of samples applied on Caco-2 cells are presented in Table 6.1.

The major polyphenols detected in undigested and digested forms of black carrots and by-products were anthocyanins and phenolic acids (Table 6.1). Anthocyanins were cyanidin based; among them two were non-acylated (cyanidin-3-xylosyl-glucosyl-galactoside and cyanidin-3-xylosyl-galactoside) and three were acylated with phenolic acids (cyanidin-3-xylosyl-sinapoyl-glucosyl-galactoside, cyanidin-3-xylosyl-feruloyl-glucosyl-galactoside and cyanidin-3-xylosyl-coumaroyl-glucosyl-galactoside). Neochlorogenic acid, chlorogenic acid and caffeic acid were identified as the three main phenolic acids. As in previous studies (Kamiloglu et al, 2016; Suzme et al, 2014), the predominant anthocyanin, representing 39−77% of the total anthocyanins corresponded to cyanidin-3-xylosyl-feruloyl-glucosyl-galactoside (Peak 4 in Figure 6.2), whereas chlorogenic acid, accounting for approximately 75−92% of the total phenolic acids, was the most abundant phenolic acid in all samples except for digested black carrot and peel, in which caffeic acid was determined to be the most prominent compound (55%). In general for both undigested and digested samples, phenolic profile of black carrot and peel were similar, while pomace showed a different trend. In particular, the acylated anthocyanins constituted approximately 61% of total anthocyanins in undigested pomace, whereas in other samples this amount was significantly higher (85−91%).

Table 6.1 : Polyphenol contents of undigested and digested black carrot, peel and pomace samples applied on Caco-2 cells.

	Undigested (μM)			Digested (μM)		
	Black carrot	Peel	Pomace	Black carrot	Peel	Pomace
Anthocyanins (ACNs)						
Cyanidin-3-xylosyl-glucosyl-galactoside	3.9 ± 0.3	1.3 ± 0.2	10.5 ± 0.2	0.2 ± 0.1	0.3 ± 0.1	3.1 ± 0.1
Cyanidin-3-xylosyl-galactoside	11.0 ± 0.5	5.0 ± 0.3	46.4 ± 0.3	0.12 ± 0.04	0.4 ± 0.2	2.3 ± 0.3
Cyanidin-3-xylosyl-sinapoyl-glucosyl-galactoside	12 ± 1	7.97 ± 0.04	23.8 ± 0.4	0.10 ± 0.02	0.20 ± 0.02	5 ± 1
Cyanidin-3-xylosyl-feruloyl-glucosyl-galactoside	63 ± 1	53 ± 1	56.8 ± 0.5	2.0 ± 0.3	3.72 ± 0.02	28 ± 3
Cyanidin-3-xylosyl-coumaroyl-glucosyl-galactoside	8.1 ± 0.4	5.5 ± 0.4	10.1 ± 0.3	0.13 ± 0.01	0.37 ± 0.02	3 ± 1
ΣACNs	**98.3**	**72.3**	**147.5**	**2.6**	**5.0**	**41.7**
Phenolic acids (PAs)						
Neochlorogenic acid	0.56 ± 0.02	0.024 ± 0.003	2.0 ± 0.2	0.35 ± 0.02	0.12 ± 0.02	5 ± 1
Chlorogenic acid	25.8 ± 0.2	4.77 ± 0.04	62.3 ± 0.3	1.0 ± 0.4	1.4 ± 0.1	18.1 ± 0.4
Caffeic acid	2.9 ± 0.2	0.55 ± 0.01	3.4 ± 0.3	1.71 ± 0.04	1.81 ± 0.04	1.47 ± 0.01
ΣPAs	**29.3**	**5.3**	**67.6**	**3.1**	**3.3**	**24.2**

The data presented in this table consist of average values ± standard deviation of four biological and three technical replicates.

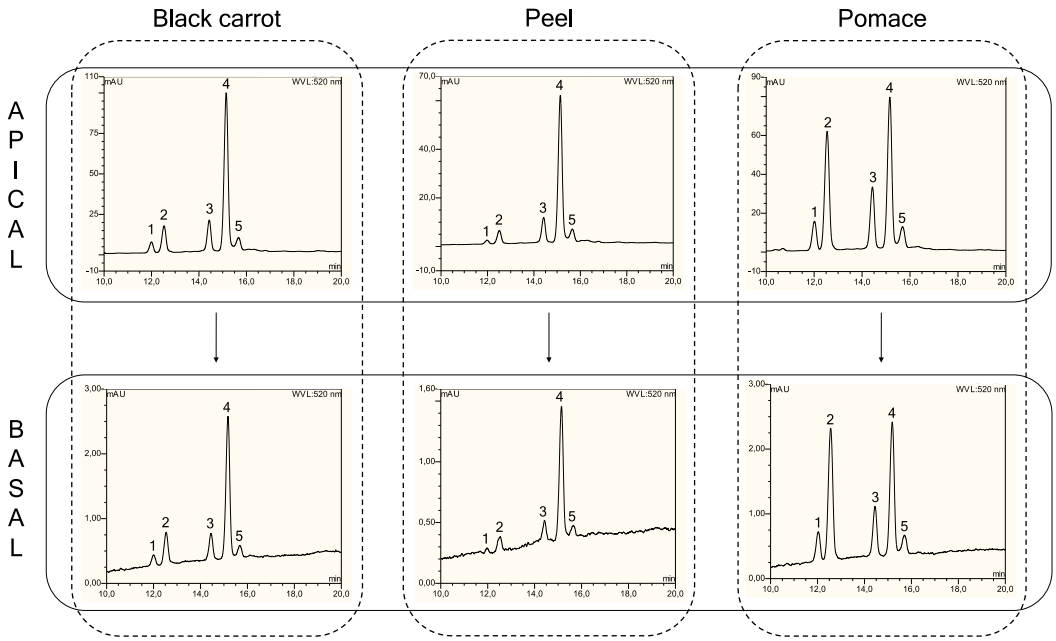

Figure 6.2 : Representative HPLC-DAD chromatograms (recorded at 520 nm) of black carrot, peel and pomace anthocyanins in the apical (upper panel) and basolateral (lower panel) compartments after 4 h incubation.

The TEER measurements performed to investigate the monolayer integrity demonstrated that initial TEER values were largely preserved (≥93%) after pre-incubation of Caco-2 cells with HBSS alone for 1 h (Table 6.2). On the other hand, treatment of cells with the samples for 4 h led to significant decreases in TEER values (15−31%) ($p < 0.05$), which was partially recovered after 24 h (increased up to 74−93% of initial values), indicating that the reduction in cell layer integrity was reversible. Furthermore, although the TEER values decreased after transport, they were still considerably above 200 $\Omega.cm^2$, which is the recommended minimum level in the literature (Toydemir et al, 2013a). In addition to TEER measurements, to make sure that the selected sample concentrations were not toxic to Caco-2 cells, MTT assay, measuring the cellular viability based on mitochondrial activity, was also applied after 4 hours of treatment (Figure 6.3).

Table 6.2 : TEER ($\Omega.cm^2$) measurements performed in growth medium (DMEM medium with Glutamax, 10% FBS, 1% NEAA and 1% Pen Strep) and during incubations in HBSS.

Samples	t = 0 h (Growth medium)	t = 1 h (HBSS)	t = 1+4 h (HBSS + Samples)	t = 24 h (Growth medium)
Undigested				
Blank	1408 ± 113^{A}	1390 ± 180^{A} (99%)[b]	1198 ± 77^{A} (85%)	1285 ± 124^{A} (91%)
Black carrot	1272 ± 62^{AB}	1349 ± 50^{A} (106%)	1065 ± 40^{C} (84%)	1178 ± 56^{B} (93%)
Peel	1547 ± 50^{A}	1631 ± 69^{A} (105%)	1111 ± 14^{C} (72%)	1232 ± 47^{B} (80%)
Pomace	1577 ± 99^{A}	1466 ± 75^{AB} (93%)	1091 ± 38^{C} (69%)	1286 ± 119^{BC} (82%)
Digested				
Blank	1382 ± 60^{A}	1401 ± 37^{A} (101%)	965 ± 66^{C} (70%)	1138 ± 12^{B} (82%)
Black carrot	1464 ± 61^{A}	1453 ± 41^{A} (99%)	1080 ± 53^{B} (74%)	1143 ± 13^{B} (78%)
Peel	1592 ± 49^{A}	1536 ± 31^{A} (96%)	1106 ± 14^{C} (69%)	1196 ± 36^{B} (75%)
Pomace	1656 ± 42^{A}	1578 ± 31^{A} (95%)	1165 ± 43^{B} (70%)	1222 ± 12^{B} (74%)

The data presented in this table consist of average values ± standard deviation of 4 biological and 3 technical replicates. Different letters in the rows represent statistically significant differences ($p < 0.05$), TEER: transepithelial electrical resistance, DMEM: Dulbecco's modified eagle's medium, FBS: fetal bovine serum, NEAA: non-essential amino acids, HBSS: Hank's Balanced Salt Solution. [b] Numbers in brackets represent the TEER values relative to the initial TEER value at t = 0 h, expressed as percentages.

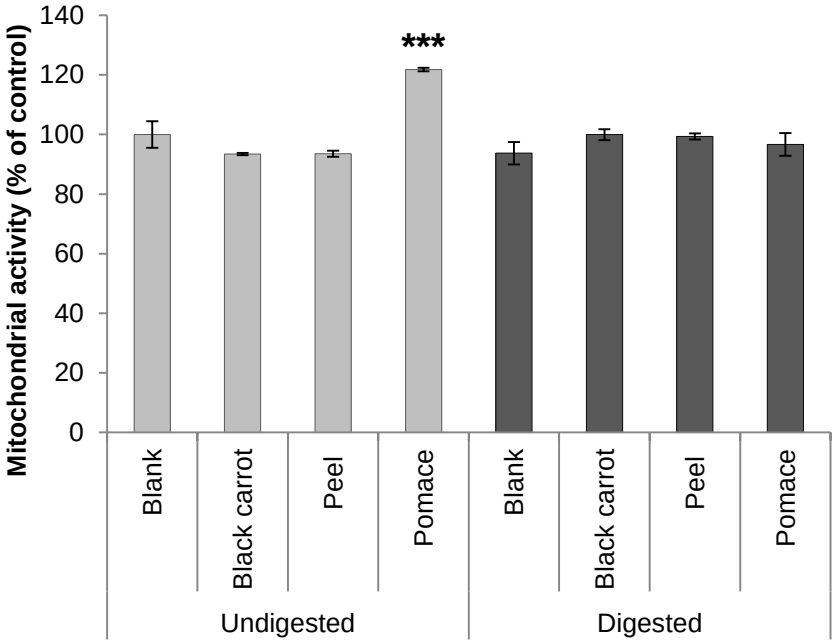

Figure 6.3: Mitochondrial activity of differentiated Caco-2 cells exposed to undigested and digested forms of black carrot, peel, pomace and no treatment (blank). Statistical differences compared to undigested blank are denoted as ***$p < 0.001$.

The results of MTT assay showed that for all samples except for undigested pomace, there was statistically no difference in cell viability compared to untreated cells (blank) ($p > 0.001$), whereas in case of undigested pomace cellular viability was increased by 22%. This finding may be related to the high sugar content of pomace, which may be taken up by the cell rapidly, leading to an increased mitochondrial activity.

The recoveries of anthocyanins (Table 6.3) and phenolic acids (Table 6.4) on the apical and basolateral compartments at different time points (t = 2 h and t = 4 h) were expressed as the percentage of the initial amount of anthocyanin/phenolic acid loaded to the apical compartment at t = 0 h. Figure 6.2 shows representative chromatograms recorded at 520 nm corresponding to the anthocyanins from undigested black carrot, peel and pomace after 4 h of treatment. Anthocyanin recoveries in the apical compartment after 2 h of exposure to samples ranged between 72 to 106%, which was in general not significantly different among samples for each compound ($p > 0.05$). On the other hand, after 4 h of treatment with the samples, apical recoveries of acylated anthocyanins obtained for digested samples (52−79%) were significantly lower compared to undigested samples (86−98%) ($p < 0.05$). This observation may be explained by the chemical breakdown of acylated anthocyanins in the presence the digestive enzymes (Kamiloglu et al, 2016). As majority of studies in the literature (Yi et al, 2006; Pacheco-Palencia et al, 2010; Fernandes et al, 2012b; Toydemir et al, 2013a; Kosinska-Cagnazzo et al, 2015) suggested, unlike other flavonoids, anthocyanins could be transported through Caco-2 monolayers in intact glycone form. It has been shown that the transport of intact anthocyanins at the basolateral compartment was very low after 2 h of treatment (0.09−1.2%), in fact even not detected for many compounds in digested form of black carrot and peel. However, after 4 h, the amount of compounds transported to the basolateral compartment increased significantly (0.8−5.3%), which was comparable with the transport results reported for anthocyanins in blueberry (3−4%) (Yi et al, 2006), açai (0.5−4.9%) (Pacheco-Palencia et al, 2010) and sour cherry (0.5−3.9%) (Toydemir et al, 2013a). Moreover, for most of the samples, digestion significantly increased the anthocyanin transport to the basolateral compartment ($p < 0.05$). This finding may be related to higher anthocyanin concentrations in undigested samples as large doses have been shown to reduce the recovery of

anthocyanins in plasma and urine, suggesting saturation of absorption mechanisms (Kurilich et al, 2005; Charron et al, 2009). Previous studies of anthocyanin dose response have had varying results with respect to saturation. A study of strawberry anthocyanins (Carkeet et al, 2008) reported no change in absorption efficiency over three dose levels ranging from 15 to 60 μmol. Another study on red cabbage anthocyanins (Charron et al, 2007) showed that absorption of anthocyanins increased with increasing dose, but with decreasing absorption efficiency, over doses ranging from 138 to 414 μmol (Charron et al, 2009). Although the exact mechanism of anthocyanin absorption in the small intestine is still unclear, it has been proposed that anthocyanins could interfere with the transporters responsible for their own transport. The candidates for anthocyanin transporters were the glucose transporters, since anthocyanins possess a sugar moiety, in particular a glucose residue. Sodium-dependent glucose transporter 1 (SGLT1) and glucose transporter 2 (GLUT2) are the main hexose transporters described in Caco-2 cells (Faria et al, 2009; Zou et al, 2014). Considering that, it may be hypothesized that the higher concentration of sodium in digested samples could also contribute to their transport to the basolateral compartment. In addition, for each sample the recoveries of non-acylated anthocyanins on the basolateral compartment after 4 h were higher than the acylated anthocyanins. These results were in line with *in vivo* studies conducted with volunteers consuming raw and cooked purple carrots (Kurilich et al, 2005) and purple carrot juice (Charron et al, 2009) showing that the plasma and urine concentrations of non-acylated anthocyanins were significantly higher than that for acylated anthocyanins. Overall transport efficiencies of anthocyanins were 0.001−0.015 and 0.008−0.074 at t = 2 h and t = 4 h, respectively. Previously, results within this range were obtained for other anthocyanins including cyanidin-3-glucosyl-rutinoside (0.011−0.034) (Toydemir et al, 2013a) and malvidin-3-glucoside (0.035) (Kuntz et al, 2015a). Recent studies suggest that the rapid appearance of anthocyanins in plasma after consumption could result from their absorption through the gastric wall. In cell culture studies, anthocyanins were found to be able to cross MKN-28 differentiated adenocarcinoma stomach cell monolayers (Fang, 2014b; Oliveira et al, 2015b). Therefore, the existing knowledge indicates that the observed low apparent bioavailability of anthocyanins could be due to their extensive presystemic metabolism, rather than poor absorption from the intestinal lumen.

Table 6.3 : Apical and basal side recoveries and transport efficiencies of anthocyanins from undigested and digested black carrot, peel and pomace samples.

Samples	t = 2 h			t = 4 h		
	Apical recovery (%)[b]	Basal recovery (%)[b]	Transport efficiency[c]	Apical recovery (%)	Basal recovery (%)	Transport efficiency
			Cyanidin-3-xylosyl-glucosyl-galactoside			
Undigested						
Black carrot	103 ± 3^{A}	0.5 ± 0.2^{AB}	0.005 ± 0.002^{AB}	101 ± 2^{A}	2.1 ± 0.4^{CD}	0.021 ± 0.004^{BC}
Peel	95 ± 6^{AB}	0.3 ± 0.1^{B}	0.003 ± 0.001^{B}	96 ± 8^{A}	1.3 ± 0.8^{D}	0.013 ± 0.008^{C}
Pomace	96 ± 1^{AB}	0.6 ± 0.1^{AB}	0.006 ± 0.001^{AB}	95 ± 1^{A}	2.5 ± 0.3^{BCD}	0.027 ± 0.003^{BC}
Digested						
Black carrot	100 ± 17^{AB}	nd[d]	-	84 ± 15^{AB}	4.6 ± 0.2^{A}	0.056 ± 0.010^{A}
Peel	78 ± 16^{B}	Nd	-	72 ± 6^{B}	2.7 ± 0.5^{BC}	0.037 ± 0.006^{B}
Pomace	90 ± 11^{AB}	0.8 ± 0.3^{A}	0.009 ± 0.004^{A}	69 ± 7^{B}	4 ± 1^{AB}	0.053 ± 0.009^{A}
			Cyanidin-3-xylosyl-galactoside			
Undigested						
Black carrot	100 ± 3^{A}	0.5 ± 0.2^{BC}	0.005 ± 0.002^{BC}	96 ± 2^{A}	2.4 ± 0.4^{CD}	0.024 ± 0.003^{B}
Peel	94 ± 4^{A}	0.2 ± 0.1^{C}	0.002 ± 0.001^{C}	95 ± 4^{A}	1.1 ± 0.4^{D}	0.011 ± 0.004^{B}
Pomace	96 ± 1^{A}	0.6 ± 0.1^{B}	0.006 ± 0.001^{AB}	93 ± 1^{A}	2.7 ± 0.3^{BC}	0.029 ± 0.004^{B}
Digested						
Black carrot	100 ± 8^{A}	Nd	-	93 ± 10^{A}	5.3 ± 0.1^{A}	0.058 ± 0.007^{A}
Peel	91 ± 24^{A}	Nd	-	83 ± 21^{AB}	3.9 ± 0.1^{AB}	0.050 ± 0.011^{A}
Pomace	88 ± 24^{A}	1.1 ± 0.2^{A}	0.013 ± 0.004^{A}	64 ± 17^{B}	4 ± 2^{A}	0.068 ± 0.018^{A}
			Cyanidin-3-xylosyl-sinapoyl-glucosyl-galactoside			
Undigested						
Black carrot	94 ± 1^{A}	0.4 ± 0.2^{A}	0.005 ± 0.002^{A}	94 ± 2^{A}	1.8 ± 0.3^{C}	0.019 ± 0.003^{C}
Peel	101 ± 2^{A}	0.5 ± 0.1^{A}	0.004 ± 0.001^{A}	98 ± 1^{A}	1.1 ± 0.2^{D}	0.011 ± 0.002^{C}
Pomace	94 ± 2^{A}	0.3 ± 0.1^{A}	0.004 ± 0.001^{A}	92 ± 3^{AB}	1.8 ± 0.3^{C}	0.019 ± 0.003^{C}

Table 6.3 (continued): Apical and basal side recoveries and transport efficiencies of anthocyanins from undigested and digested black carrot, peel and pomace samples [a].

Samples	t = 2 h			t = 4 h		
	Apical recovery (%)[a]	Basal recovery (%)[a]	Transport efficiency[b]	Apical recovery (%)	Basal recovery (%)	Transport efficiency
			Cyanidin-3-xylosyl-sinapoyl-glucosyl-galactoside			
Digested						
Black carrot	87 ± 19^{A}	Nd	-	79 ± 11^{BC}	2.7 ± 0.1^{B}	0.034 ± 0.005^{B}
Peel	82 ± 7^{A}	Nd	-	63 ± 8^{D}	3.1 ± 0.1^{AB}	0.049 ± 0.007^{A}
Pomace	82 ± 5^{A}	0.4 ± 0.1^{A}	0.004 ± 0.001^{A}	71 ± 5^{CD}	3.4 ± 0.5^{A}	0.048 ± 0.004^{A}
			Cyanidin-3-xylosyl-feruloyl-glucosyl-galactoside			
Undigested						
Black carrot	94 ± 2^{A}	0.4 ± 0.1^{BC}	0.004 ± 0.001^{BC}	95 ± 2^{A}	1.7 ± 0.3^{C}	0.018 ± 0.003^{C}
Peel	97 ± 2^{A}	0.4 ± 0.1^{C}	0.004 ± 0.001^{C}	94 ± 2^{A}	1.0 ± 0.2^{C}	0.011 ± 0.002^{C}
Pomace	94 ± 4^{A}	0.5 ± 0.1^{BC}	0.005 ± 0.001^{BC}	93 ± 2^{A}	2.1 ± 0.4^{BC}	0.022 ± 0.004^{C}
Digested						
Black carrot	75 ± 14^{B}	0.9 ± 0.5^{AB}	0.012 ± 0.006^{AB}	64 ± 4^{BC}	5 ± 1^{A}	0.074 ± 0.020^{A}
Peel	76 ± 4^{B}	1.2 ± 0.1^{A}	0.015 ± 0.001^{A}	61 ± 2^{C}	3 ± 1^{AB}	0.054 ± 0.014^{AB}
Pomace	85 ± 8^{AB}	0.4 ± 0.1^{BC}	0.005 ± 0.002^{BC}	71 ± 7^{B}	3.4 ± 0.5^{AB}	0.049 ± 0.008^{B}
			Cyanidin-3-xylosyl-coumaroyl-glucosyl-galactoside			
Undigested						
Black carrot	92 ± 4^{AB}	0.2 ± 0.1^{A}	0.002 ± 0.001^{A}	86 ± 4^{AB}	1.3 ± 0.4^{AB}	0.015 ± 0.005^{B}
Peel	106 ± 6^{A}	0.3 ± 0.2^{A}	0.003 ± 0.002^{A}	98 ± 11^{A}	0.8 ± 0.2^{B}	0.008 ± 0.003^{B}
Pomace	94 ± 10^{AB}	0.3 ± 0.1^{A}	0.004 ± 0.001^{A}	98 ± 4^{A}	1.8 ± 0.5^{A}	0.018 ± 0.005^{B}
Digested						
Black carrot	83 ± 8^{B}	Nd	-	69 ± 15^{BC}	1.6 ± 0.3^{AB}	0.023 ± 0.003^{B}
Peel	85 ± 12^{AB}	Nd	-	65 ± 4^{C}	1.3 ± 0.6^{AB}	0.019 ± 0.009^{B}
Pomace	72 ± 15^{B}	0.09 ± 0.01^{A}	0.001 ± 0.000^{A}	52 ± 7^{C}	2.1 ± 0.4^{A}	0.040 ± 0.005^{A}

[a] The data presented in this table consist of average values ± standard deviation of four biological and three technical replicates. Different letters in the columns within each compound represent statistically significant differences ($p < 0.05$). [b] Apical and basal recovery (%) = (concentration after transport) / (initial concentration) X 100. [c] Transport efficiency = (basal recovery, %) / (apical recovery, %). [d] nd: not detected.

Table 6.4 : Apical and basal side recoveries and transport efficiencies of phenolic acids from undigested and digested black carrot, peel and pomace samples.

Samples	t = 2 h			t = 4 h		
	Apical recovery (%)[b]	Basal recovery (%)[b]	Transport efficiency[c]	Apical recovery (%)	Basal recovery (%)	Transport efficiency
			Neochlorogenic acid			
Undigested						
Black carrot	102 ± 3^{A}	nd[d]	-	98 ± 7^{A}	2.1 ± 0.5^{B}	0.022 ± 0.004^{B}
Peel	98 ± 15^{A}	Nd	-	94 ± 14^{A}	nd	-
Pomace	102 ± 1^{A}	0.4 ± 0.2^{B}	0.004 ± 0.002^{A}	101 ± 1^{A}	3.3 ± 0.5^{AB}	0.033 ± 0.005^{AB}
Digested						
Black carrot	96 ± 12^{A}	nd	-	89 ± 14^{A}	5 ± 1^{A}	0.057 ± 0.027^{A}
Peel	93 ± 6^{A}	nd	-	86 ± 5^{A}	4 ± 2^{AB}	0.046 ± 0.016^{AB}
Pomace	94 ± 2^{A}	0.8 ± 0.2^{A}	0.008 ± 0.003^{A}	81 ± 13^{A}	4.0 ± 0.2^{AB}	0.050 ± 0.008^{AB}
			Chlorogenic acid			
Undigested						
Black carrot	96 ± 2^{A}	0.7 ± 0.2^{AB}	0.007 ± 0.002^{A}	95 ± 3^{A}	2.6 ± 0.3^{C}	0.027 ± 0.003^{B}
Peel	100 ± 4^{A}	0.7 ± 0.5^{AB}	0.007 ± 0.005^{AB}	95 ± 3^{A}	2.1 ± 0.5^{C}	0.022 ± 0.005^{B}
Pomace	96 ± 1^{A}	0.8 ± 0.1^{A}	0.008 ± 0.001^{A}	93 ± 1^{A}	3.2 ± 0.5^{C}	0.034 ± 0.005^{B}
Digested						
Black carrot	89 ± 15^{A}	0.16 ± 0.04^{B}	0.002 ± 0.001^{B}	80 ± 14^{AB}	6.1 ± 0.3^{A}	0.077 ± 0.011^{A}
Peel	85 ± 7^{A}	0.24 ± 0.03^{AB}	0.003 ± 0.000^{B}	83 ± 8^{AB}	5.2 ± 0.2^{AB}	0.063 ± 0.007^{A}
Pomace	83 ± 17^{A}	0.7 ± 0.3^{AB}	0.008 ± 0.002^{A}	72 ± 10^{B}	4 ± 1^{B}	0.062 ± 0.007^{A}
			Caffeic acid			
Undigested						
Black carrot	90 ± 5^{A}	0.5 ± 0.1^{B}	0.006 ± 0.001^{B}	85 ± 5^{A}	1.6 ± 0.4^{B}	0.018 ± 0.004^{B}
Peel	85 ± 14^{A}	0.2 ± 0.1^{B}	0.003 ± 0.002^{B}	73 ± 6^{A}	2.3 ± 0.2^{B}	0.032 ± 0.003^{B}
Pomace	99 ± 3^{A}	0.3 ± 0.1^{B}	0.003 ± 0.001^{B}	91 ± 7^{A}	3 ± 1^{B}	0.028 ± 0.009^{B}

Table 6.4 (continued) : Apical and basal side recoveries and transport efficiencies of phenolic acids from undigested and digested black carrot, peel and pomace samples [a].

Samples	t = 2 h			t = 4 h		
	Apical recovery (%)[a]	Basal recovery (%)[a]	Transport efficiency[b]	Apical recovery (%)	Basal recovery (%)	Transport efficiency
			Caffeic acid			
Digested						
Black carrot	97 ± 12 [A]	2 ± 1 [A]	0.023 ± 0.005[A]	89 ± 9[A]	7 ± 1[A]	0.079 ± 0.015[A]
Peel	86 ± 2 [A]	2 ± 1 [A]	0.025 ± 0.010[A]	70 ± 7[A]	6 ± 1[A]	0.092 ± 0.025[A]
Pomace	96 ± 15 [A]	2.2 ± 0.1 [A]	0.023 ± 0.005[A]	78 ± 20[A]	5 ± 1[A]	0.072 ± 0.026[A]

[a] The data presented in this table consist of average values ± standard deviation of four biological and three technical replicates. Different letters in the columns within each compound represent statistically significant differences ($p < 0.05$). [b] Apical and basal recovery (%) = (concentration after transport) / (initial concentration) X 100. [c] Transport efficiency = (basal recovery, %) / (apical recovery, %). [d] nd: not detected.

The recoveries of phenolic acids on the apical compartment after 2 h and 4 h of exposures (83−102% and 70−101%, respectively) were in general not significantly different among samples ($p > 0.05$) (Table 6.4). Similar to the results obtained for anthocyanins, the basolateral recoveries of phenolic acids after 2 h of treatment were also quite low except for caffeic acid, which showed significantly higher absorption efficiency (up to 2.2%) in digested samples compared to chlorogenic and neochlorogenic acids (0−0.8%) ($p < 0.05$). These results were in line with a previous study (Konishi and Kobayashi, 2004), in which considerably higher amount of caffeic acid standard (1.57%) was transported into the basolateral side compared to chlorogenic acid standard (0.06%). The latter authors reported that caffeic acid is likely to be taken up and transported into the basolateral side according to a pH gradient, whereas chlorogenic acid seems to be restricted by the tight junction. Furthermore, the authors also indicated that the transport was mainly via paracellular diffusion, although caffeic acid was absorbed to a lesser extent by the monocarboxylic acid transporter (MCT) (Konishi and Kobayashi, 2004). Confirming the results obtained for anthocyanins, digestion also significantly increased the transport of all phenolic acids, except for neochlorogenic acid to the basolateral compartment (4−7% and 1.6−3.2% for digested and undigested samples at t = 4 h, respectively) ($p < 0.05$). Transport efficiency results recorded at t = 4 h, were also in line with this observation, showing significantly higher values for digested samples (0.046−0.092) compared to undigested samples (0.018−0.034) ($p < 0.05$). However, it should also be noted that chlorogenic acid was partially hydrolyzed into caffeic acid in the course of digestion (Kamiloglu et al, 2016) and the transport was more efficient only in relative numbers possibly due to saturation kinetics but in absolute numbers the concentration in basolateral compartment was lower.

6.4.2 Anti-inflammatory effects of transported polyphenols on endothelial EA.hy 926 cells

In order to study the anti-inflammatory potential of black carrot polyphenols from different matrices, we have used an in-house developed *in vitro* co-culture model using differentiated intestinal Caco-2 cells and endothelial EA.hy926 cells (Toaldo et al, 2016). In the current study, we preferred to induce low-grade inflammation (1 ng TNF-α/mL) to endothelial cells to investigate the anti-inflammatory effects, as a previous study in the literature showed that anthocyanins possessed no effects under

high-grade inflammatory conditions (Kuntz et al, 2015a). In addition, chronic low-grade inflammation has been proposed as an underlying condition of various diseases including obesity, insulin resistance, metabolic syndrome, cardiovascular diseases and cancer (Pereira and Alvarez-Leite, 2014; Emanuela et al, 2012; Rodriguez-Hernandez et al, 2013). Given the increasing prevalence of these Western-type diseases, we expect a high percentage of the population to be exposed to low-grade inflammation, and therefore, may benefit from anti-inflammatory food supplements.

The effect of undigested and digested forms of black carrot, peel and pomace samples on cellular NO production is presented in Figure 6.4.

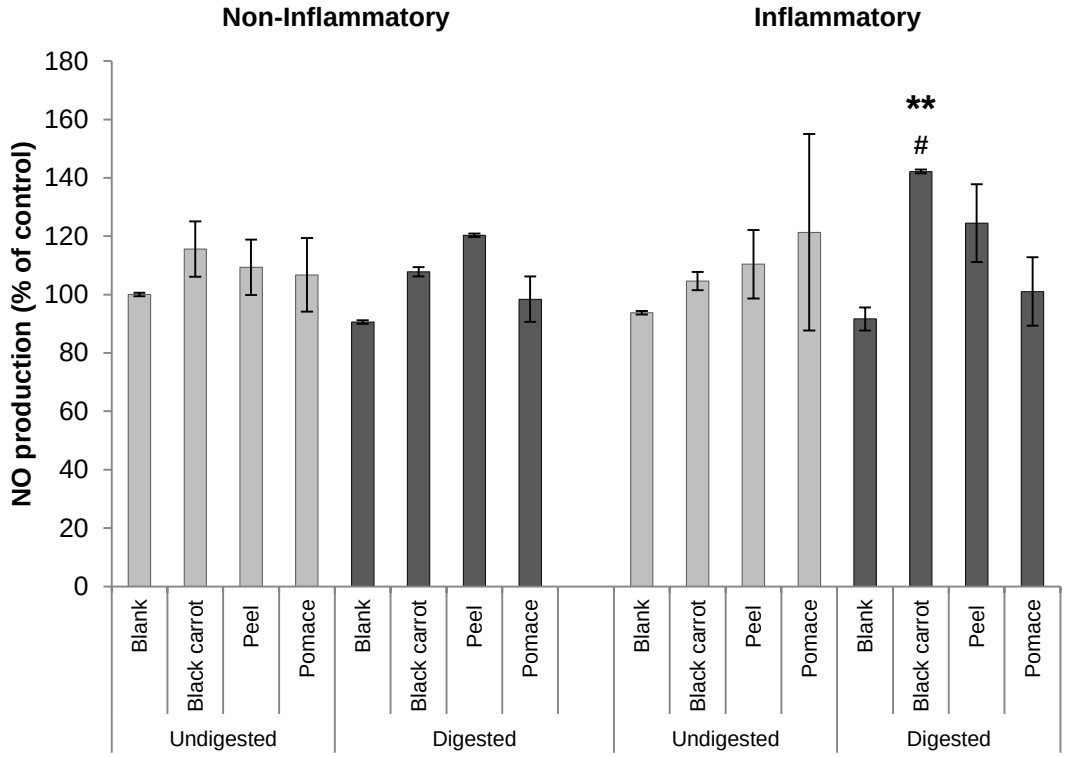

Figure 6.4 : Changes in NO production of endothelial EA.hy 926 cells in response to 4 h treatment of undigested and digested forms of black carrot, peel and pomace, under non-inflammatory and inflammatory conditions. Statistical differences compared to non-inflammatory undigested blank are denoted as $**p < 0.01$, whereas the differences to inflammatory undigested blank are indicated as $\#p < 0.05$.

Pre-treatment of cells with TNF-α resulted in insignificant change (6% decrease) in untreated cells (blank). Similarly, digestion fluid (digested blank) also caused insignificant changes (8−9% decreases) under both non-inflammatory and inflammatory conditions. However, after 4 h of treatment with samples, NO production increased (7−20% and 1−42% under non-inflammatory and inflammatory conditions, respectively), which was significant for digested black carrot sample under inflammatory condition ($p < 0.01$). This particular effect may be related to the higher amount of polyphenols, in particular anthocyanins, transported to the basolateral compartment for digested black carrot sample compared to other samples (Table 6.3). This finding is consistent with literature reporting that a cyanidin-based anthocyanin, which is also the prominent anthocyanin pigment in black carrots, increases endothelial nitric oxide synthase (eNOS) expression in bovine aortic endothelial cells, which is an important factor in modulating blood pressure and endothelial dysfunction (Metzger et al, 2008).

Figure 6.5 shows the effect of transported polyphenols on secretion of pro-inflammatory markers, i.e., IL-8, MCP-1, VEGF, ICAM-1, under normal and TNF-α induced inflammatory conditions. TNF-α treatment (undigested blank) caused increased secretion of all markers except ICAM-1 (20−103%). Similar to the outcomes of NO, digestion fluid (digested blank) also induced effects on cellular response, resulting in significantly increased secretion of IL-8, MCP-1 and ICAM-1 (43−125%). In general, treatment of cells with either undigested or digested forms of black carrot, peel and pomace reduced the concentrations of IL-8, MCP-1, VEGF and ICAM-1 under both non-inflammatory and inflammatory conditions. The most pronounced effects were observed with digested samples under inflammatory conditions, which significantly decreased the secretion of all markers from 120−203% down to 34−144% ($p < 0.001$). As mentioned above, digestion significantly enhanced the transport of polyphenols, which may explain these marked decreases in secretion of pro-inflammatory markers. Furthermore, although the monolayers exerted acceptable TEER values before and after the co-culture experiments, produced inflammatory mediators may have influenced the permeability of Caco-2 cells in the course of incubation.

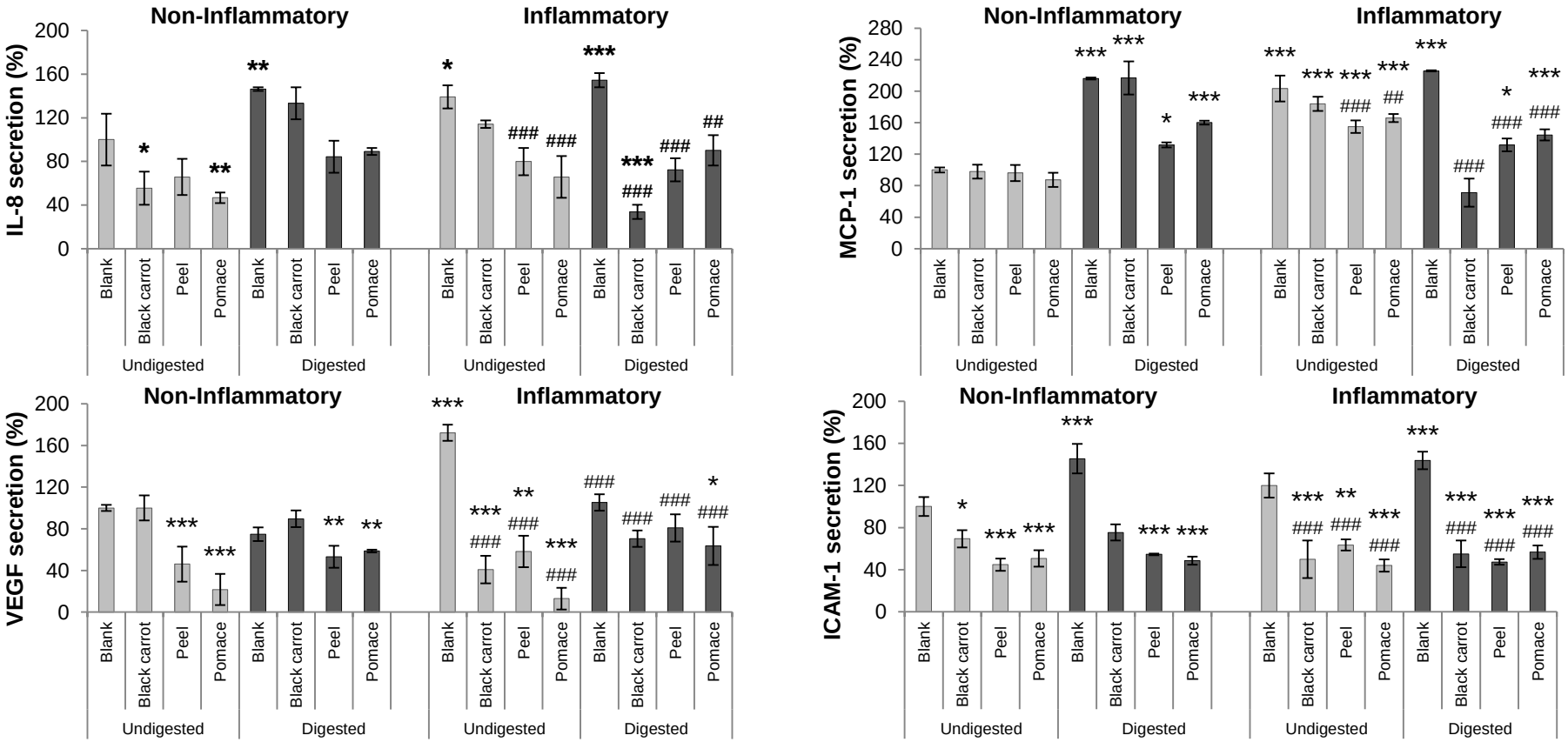

Figure 6.5 : Secretion of IL-8, MCP-1, VEGF and ICAM-1 in endothelial EA.hy 926 cells in response to 4 h treatment of undigested and digested forms of black carrot, peel and pomace, under non-inflammatory and inflammatory conditions. Statistical differences compared to non-inflammatory undigested blank are denoted as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, whereas the differences to inflammatory undigested blank are indicated as $\#\#p < 0.01$, $\#\#\#p < 0.001$.

Previous studies also reported that anthocyanins from various sources could inhibit the production of IL-8 (Chao et al, 2013), MCP-1 (Huang et al, 2014), VEGF (Nizamutdinova et al, 2009) and ICAM-1 (Kim et al, 2006) in TNF-α-stimulated endothelial cells. In addition, it is possible that the digestion conditions release other food matrix compounds with direct or indirect impact on cell signaling cascades involved in these responses. In particular, black carrots are good sources of dietary fiber, which may play a role in reducing the secretion of relevant biomarkers of inflammation (Basu et al, 2006). Moreover, besides polyphenols, black carrots also contain polyacetylene compounds such as falcarindiol, falcarindiol 3-acetate and falcarinol, which could also be responsible for anti-inflammatory activity of black carrots (Metzger et al, 2008). Overall, these results suggest that transported polyphenols from black carrot and its by-products function through an inhibitory regulation of the inflammatory cascade in cells, which can maintain a sustainable effect under pre-existing low-grade inflammation.

The co-culture model that we developed in-house may be improved in certain aspects. Caco-2 cell line has some limitations, e.g. lack of mucus layer, which can affect the transport dynamics and hence the observed effects on endothelial cells. To overcome this issue, attempts have been done to co-culture a mucin secreting cell line with Caco-2 cells. A recent study on grape anthocyanins (Kuntz et al, 2015a) showed that anti-inflammatory effects were only observed using 100% Caco-2 cells, whereas mixtures of Caco-2 and mucus secreting HT29-B6 cells failed to induce an effect. Furthermore, the use of HUVEC cells instead of EA.hy926 cells may rule out the carcinoma effect and hence may improve the model. Still, our set-up is a good example of a physiologically relevant model, which consider the cross-talk mechanisms between the two cell lines and the digestion as suggested in the literature (Brown et al, 2016).

6.5 Conclusions

The present study is the first *in vitro* study that addressed the interaction of the intestinally transported polyphenols from black carrot and its by-products with the cellular inflammation response system in normal and TNF-α treated cells. By measuring the cellular NO production and secretion of pro-inflammatory markers after the *in vitro* gastrointestinal digestion and transepithelial transport, we have

provided biologically relevant data detailing the anti-inflammatory properties of the tested samples. This study demonstrated that polyphenols from black carrots and by-products markedly down regulated the secretion of certain pro-inflammatory markers, i.e., IL-8, MCP-1, VEGF and ICAM-1, especially in TNF-α treated endothelial cells. The applied methodology may therefore open perspectives for the development of functional foods improving cardiovascular and even general health under sub-optimal physiological states characterized by low-grade inflammation.

7. GENERAL DISCUSSION, CONCLUSIONS AND FUTURE PERSPECTIVES

7.1 Status and Main Outcomes of This Thesis

7.1.1 Characterization of black carrot polyphenols

Black carrots represent a valuable source of polyphenols, in particular anthocyanins and phenolic acids, and has attracted the attention of the scientific community especially due to the unique profile of anthocyanin compounds which are well distinguished for their role in health promotion and prevention of chronic diseases.

The first step in establishing the bioactive potential of polyphenols from agricultural sources is the characterization of the bioactive target. Accordingly, as a start of this thesis, polyphenols from black carrots have been characterized. The anthocyanin profile of the black carrots used in the experimental chapters (*Chapters 3, 4* and *6*) of this thesis was consistent. In all chapters, five anthocyanins were identified which were all cyanidin-based: cyanidin-3-xylosyl-glucosyl-galactoside, cyanidin-3-xylosyl-galactoside and the sinapic, ferulic and coumaric acid derivatives of cyanidin 3-xylosyl-glucosyl-galactoside. On the other hand, the phenolic acid profile varied between the chapters. Neochlorogenic (3-CQA), chlorogenic (5-CQA) and caffeic acids were detected in all chapters, whereas in addition to these three compounds in *Chapter 3* and *Chapter 4* cryptochlorogenic (4-CQA) and ferulic acids were also identified, respectively. We know from the literature that several factors related to the plant material (e.g., variety, degree of ripeness, agricultural treatments, harvesting time) as well as the protocols used for extraction may affect the polyphenol composition and content of agricultural products (Capanoglu et al, 2010). The black carrots used in *Chapter 3* were harvested in December 2012, whereas the ones used in other chapters (*Chapters 4* and *6*), were obtained in April 2014. Furthermore, in *Chapters 4* and *6* lyophilized powder was used for the extraction while in *Chapter 3* extractions were carried out on fresh powder. In *Chapter 6*, extracts were dried and re-dissolved in Hank's balanced salt solution (HBSS), which

has higher pH than the original extraction solvent, i.e., 75% aqueous methanol containing 0.1% formic acid. It has been reported that, at high pHs 5-CQA can undergo isomerization to 3-CQA and 4-CQA, and a methyl caffeate (Dawidowicz and Typek, 2011). Moreover, the presence of ferulic acid in *Chapter 4*, may be attributed to cleavage of cyanidin-3-xylosyl-feruloyl-glucosyl-galactoside to ferulic acid. Although the phenolic composition of black carrots varied slightly in all chapters, the predominant anthocyanin corresponded to cyanidin-3-xylosyl-feruloyl-glucosyl-galactoside, whereas chlorogenic acid was identified as the major phenolic acid.

In several studies in literature, fruit and vegetable samples are collected from local markets and analyzed with respect to their polyphenol content. However, for such samples, information on their history (variety, source, age, treatment) is generally missing. Here we showed that even the same variety of product (*Daucus carota* ssp. *sativus* var. *atrorubens* Alef.) collected from the same location (Konya, Turkey) could have differences in polyphenol content and factors such as harvesting time and pre-treatments may have an influence on these bioactive compounds. Thus, it is recommended to be careful while comparing the polyphenol content of fruits and vegetables when the associated metadata is incomplete or unavailable. Preferentially, only materials with a full, known history should be used.

7.1.2 Impact of food processing and storage on black carrot polyphenols

The need for food processing arises from various reasons: (i) to prolong the shelf-life of fresh produce; (ii) to provide products out of season; (iii) to produce convenient products for home consumption; (iv) to supply novel food products with alternative flavor, texture and color; or (v) to provide improved nutritional properties. It has been shown that food processing often results in oxidation, thermal degradation and other conditions that lead to lower levels of polyphenols in processed food compared with fresh material. Hence, attention should be given to minimize the detrimental effects of food processing and the subsequent storage conditions used.

Black carrots grown in Turkey do not find much consumer acceptance as a vegetable and therefore they are often processed into products such as juice, concentrate and shalgam, a traditional lactic acid fermented beverage. To the best of our knowledge, this thesis is the first study that investigated the effect of jam and marmalade

processing on black carrot polyphenols (*Chapter 3*). Jams and marmalades were prepared using either sugar or sweetener and stored in the dark at two different temperatures (4 °C and 25 °C) over a period of 20 weeks. Our experimental data revealed that processing of black carrots into jams and marmalades significantly decreased total phenolics, total and individual anthocyanins, phenolic acids and total antioxidant capacity (≥50%). It is noted that during jam and marmalade processing, cell structure is disrupted and the raw material becomes prone to non-enzymatic oxidation, which could be one of the major causes for these losses. Regarding the reduction in anthocyanin content, it is well known that at high temperatures anthocyanins are converted into colorless chalcones, which spontaneously degrade into phenolic acids and aldehydes (Kay et al, 2009). Storage time and temperature also affected polyphenol content and total antioxidant capacity of black carrot jams and marmalades. After 20 weeks of storage, the decrease in polyphenol content and antioxidant capacity of samples stored at 25 °C was found to be higher than the samples stored at 4 °C. During storage, acylated anthocyanins were significantly more stable than non-acylated anthocyanins, which was an expected outcome, as acylated anthocyanins are known to be more stable under heat, light and other environmental conditions as compared to non-acylated anthocyanins. Although in general, the use of sweetener instead of sugar in jams and marmalades did not lead to a statistically significant difference in polyphenol content, jam with sugar retained the highest amount of polyphenols after both processing and storage, which might be attributed to the reduced water activity and acting as partial oxygen barrier. Based on these results, it can be concluded that in order to reduce the loss of polyphenols and to achieve a product with high antioxidant capacity, black carrot jams should be prepared with sugar and stored at 4 °C.

In the literature, studies investigating the effect of processing and storage conditions on black carrot polyphenols mainly focused on anthocyanins (Tables 2.2 and 2.3 in *Chapter 2*). On the other hand, there is limited information on the changes in phenolic acids during processing and storage of black carrots. Considering the high amount of phenolic acids in black carrots, it is also important to investigate these compounds in order to better understand the fate of all health-associated compounds during processing and storage, as was done in this thesis.

Here we demonstrated that polyphenol content and hence health-promoting capacity of black carrots also depends on the processing history. This aspect should also be considered in future studies. We suggest that understanding the mechanisms occurring in the tissue during processing and storage can lead to innovations in the food industry by controlling and optimizing technical and environmental parameters.

7.1.3 Black carrot by-products as sources of polyphenols

The fast growing food industry generates by-products obtained during production. These by-products are usually disposed both in environmental and economical terms. They are often discarded as waste or, in the best case valorized in low-value applications such as animal feed and bio-energy applications. However, these by-products have the potential to be used as relatively cheap but valuable resources for bioactive compounds. In many cases by-products contain the same valuable constituents as the starting products and possibly yet uncharacterized active compounds that could lead to higher value applications.

We observed that processing of black carrots into jams and marmalades created significant amounts of peel (*Chapter 3*). In addition, it has been reported that at industrial scale, black carrot juice processing results in generation of up to almost 7000 tons of pomace per year (Agcam and Akyildiz, 2015). Considering that, in this thesis we characterized the plant processing by-products of black carrot, i.e., peel and pomace, for the first time (*Chapter 4*). Although peel and pomace contained a lower amount of total phenolics and total antioxidant capacity than black carrot, individual anthocyanin and phenolic acid contents of pomace was found to be higher than that of black carrots. This observation may be explained by the release of bound compounds with the breakdown of cellular constituents (i.e., cellulose and cellulose-pectin composites) a consequence of applied temperature and enzyme treatment during black carrot juice processing. Considering the amount of polyphenols retained in black carrot pomace, next we proposed that this by-product can be considered as a potential food ingredient and that it can be used to improve the functional properties of cakes (*Chapter 5*). Accordingly, cake flour was enriched with black carrot pomace powder at levels of 5%, 10% and 15%, and the changes in total phenolics and antioxidant capacity were compared with the control cake containing no pomace. Results showed that addition of pomace caused a dose-dependent increase in total

phenolic content and total antioxidant capacity, with 10% and 15% black carrot pomace enriched samples being statistically significant compared to the control.

Studies on the by-products of black carrot processing are very limited. Considering the results obtained in this thesis, we believe that these by-products deserves extra attention as they represent a potentially valuable source of functional ingredients for the food industry providing not only a high amount of polyphenols but also a considerable amount of dietary fiber.

7.1.4 Bioaccessibility of black carrot polyphenols

7.1.4.1 Comparison of *in vitro* gastrointestinal digestion models

Bioaccessibility of polyphenols is a prerequisite for further physiological functions in the body. Considering the fact that *in vivo* studies are time consuming, costly, and restricted by ethical concerns, *in vitro* models investigating the effects of digestion have been developed and employed to predict the release of polyphenols from the food matrix and assess changes in their profiles prior to absorption.

In this thesis, three different *in vitro* gastrointestinal digestion models were applied. The model applied in *Chapter 3*, involved the simulation of gastric and intestinal digestion with the help of digestive enzymes and a dialysis bag. This model was rather simple with a lack of a number of essential components of digestion such as electrolyte solutions. Furthermore, use of a dialysis bag to estimate the availability of polyphenols for absorption may have some limitations. For instance, differences in sugar concentrations of the samples can affect the dialysation of polyphenols, which cannot be truly associated with absorption of these compounds *in vivo*. Considering all these restrictions, in *Chapter 4*, we developed a better model, which sequentially mimicked the digestion in stomach, small intestine and colon via digestion enzymes, salts and intestinal bacteria. This model was later combined with a Caco-2 cell transport model in *Chapter 6*. Although the model developed in *Chapter 4*, was far more advanced than the model of *Chapter 3*, further amendments of the conditions may be needed, for example to simulate digestion in different target groups (e.g. infants or elderly). The diversity of *in vitro* digestion model conditions has limited the ability to compare results across different studies. To overcome this issue, a standardized and practical static digestion model based on physiologically relevant conditions was proposed by the COST action INFOGEST consortium (Minekus et al,

2014). This standardized static *in vitro* digestion model, which simulated the digestion in the mouth, stomach and small intestine, was applied in *Chapter 5*.

Regardless of the *in vitro* digestion model used, the amount of black carrot polyphenols released during digestion was lower than what was initially present in undigested samples. These observations may be related to the fact that polyphenols, in particular anthocyanins may be metabolized to some non-colored forms (i.e. chalcones), oxidized, or degraded into other compounds (phenolic acids and aldehydes), which may not be detected under the conditions used in this thesis. In addition, a large amount (65%) of polyphenols from black carrots, i.e., anthocyanins and phenolic acids, was reported to be bound to plant cell walls and overwhelmingly remain bound after *in vitro* gastric and small intestinal digestion (Padayachee et al, 2013). Another finding in common for all digestion models was that the availability of non-acylated anthocyanins was higher than acylated anthocyanins, which was also in line with *in vivo* studies on purple carrot polyphenols (Kurilich et al, 2005; Netzel et al, 2007; Charron et al, 2009; Novotny et al, 2012).

One of the major problems to be resolved considering the *in vitro* digestion models is that the majority of these models do not consider the fact that polyphenols may be transformed by the gut microbiota (to ring fission products) and by intestinal cells (to conjugates such as sulfated, glucuronidated and methylated forms). Although in this thesis, while evaluating the bioactivity of black carrot polyphenols, we tried to employ these conditions by using SHIME suspension (*Chapter 4*) and intestinal Caco-2 cells (*Chapter 6*), we could not have the chance to identify all the gut and intestinal metabolites of black carrot. Therefore, in future studies it would be interesting to pay attention to these metabolites, which may be responsible from the observed biological activities.

7.1.4.2 Food processing affects the bioaccessibility of black carrot polyphenols

Since most food products consumed on a daily basis are processed foods, an understanding of how food processing changes the food matrix and composition is important, as such changes also often alter the bioaccessibility of polyphenols and hence their possible beneficial effects on human health. In this context, the results of *Chapter 3* revealed that processing of black carrots into jam and marmalade led to increases in percent recovery of bioaccessible polyphenols. Similarly, in *Chapter 4*,

the amount of pomace anthocyanins released at all stages of *in vitro* gastrointestinal digestion was higher than black carrot anthocyanins. Heat and enzyme treatments applied during processing might yield structural changes that would end up with higher bioaccessibility of polyphenols. Overall, these results suggest that processing of black carrots may be desirable to obtain better sources of bioaccessible polyphenols.

7.1.4.3 Dietary compounds affect the bioaccessibility of black carrot polyphenols

Interaction of polyphenols with other dietary compounds (e.g. proteins, lipids, dietary fiber, etc.) can remarkably affect the bioaccessibility, uptake, and further metabolism of these bioactive compounds. In particular, in *Chapter 5*, we observed that cake matrix considerably influenced the release of polyphenols from black carrot pomace. Protein rich ingredients in cake matrix (e.g. yoghurt, eggs) may lead to possible protein-polyphenol interactions resulting in reduced availability of polyphenols. Some polyphenols, especially those with a high number of hydroxyl groups have been reported to strongly bind to proteins such as albumins, both from the food matrix and also from saliva, resulting in complexes that reduce polyphenol absorption. Additional effects of other matrix components such as the dietary fibers from cake flour and black carrot pomace should also be taken into consideration as it is hypothesized that dietary fiber decreases the availability of polyphenols, mainly due to factors such as physical entrapment, and increased viscosity (Bohn, 2014). On the other hand, dietary lipids (e.g. sunflower oil) could have a limited influence, as the majority of the polyphenols present in black carrot pomace are water-soluble.

There has been comparatively little effort into assessing the effects of combinations of foodstuffs or realistic meals on the bioaccessibility of polyphenols. However, as shown in this thesis, meal components can interact with polyphenols and affect their bioaccessibility. Determining the most optimal form of consumption can allow us to benefit more from health promoting effects of polyphenols.

7.1.5 Intestinal transport of black carrot polyphenols

Despite the heterogeneity in its characteristics and limitations arising from its tumoral origin, Caco-2 cell lines are considered to be the most common *in vitro* model of the small intestine. When the transport of polyphenols is assessed using Caco-2 cells as intestinal models, in almost all studies cells are treated with pure

standards or polyphenol-rich extracts derived from plants. However, plasma and tissues are not exposed *in vivo* to polyphenols in these forms. Considering that, we proposed that the use of combined *in vitro* digestion and Caco-2 cells could be a better approach.

In this thesis, we studied the intestinal transport of polyphenols from undigested and digested black carrots and by-products using differentiated intestinal Caco-2 cells (*Chapter 6*). We observed that unlike other flavonoids, anthocyanins could be transported through Caco-2 monolayers in intact glycone form. The results further indicated that the transport of polyphenols was higher for digested samples compared to undigested ones. We proposed that this outcome might be related to higher polyphenol concentrations in undigested samples, which may result in reduced recovery of polyphenols, in particular anthocyanins, due to a saturation mechanism. This theory was based on an *in vivo* study on purple carrot anthocyanins, which demonstrated no increase in total anthocyanin absorption when dose was doubled (Kurilich et al., 2005). Moreover, we also hypothesized that higher concentrations of sodium in digested samples could contribute to anthocyanin transport to the basolateral compartment whereby the sodium-dependent glucose transporter 1 (SGLT1) and glucose transporter 2 (GLUT2) are proposed as anthocyanin transporters. Although the transport of anthocyanins was higher in the digested form, still the amount of transported compounds was found to be very low, which is in line with the published *in vivo* studies (Kurilich et al, 2005; Netzel et al, 2007; Charron et al, 2009; Novotny et al, 2012). In spite of convincing observations in the Caco-2 cell culture model, extrapolation of these *in vitro* findings to the *in vivo* situation is difficult due to the unknown accumulation of these compounds in the target tissues. Furthermore, recent studies suggest that the high content of intact anthocyanins detected in plasma a few minutes after intake could be possibly due to their absorption through the gastric wall (Fernandes et al, 2012a; Fang, 2014b; Oliveira et al, 2015b). Absorption of anthocyanins in the stomach is still a new phenomenon; therefore more research should be done in order to increase the current knowledge on this topic.

One of the major differences between the Caco-2 cells and the intestinal enterocytes is the lack of an intestinal mucus layer. To overcome this issue, attempts have been done to co-culture a mucin secreting cell line (HT29-MTX) with Caco-2 cells.

However HT29-MTX cells produces a gastric type of mucin instead of the intestinal type, therefore, researchers should seek for better cell line models for intestinal transport (e.g. HT29-B6). Another limitation of using Caco-2 cells is the poor reproducibility of results between different laboratories (Zucco et al., 2005). Hence, a standardization of some important parameters such as cell origin, passage number and incubation time is necessary. On the other hand, there are also some advantages of using Caco-2 cell line while evaluating polyphenol absorption. It is a high throughput model, which allows the screening of a large number of samples at the same time. In addition, the use of the Caco-2 cell model is also important to study the molecular mechanisms of polyphenol absorption. Overall, we can conclude that the interpretation of *in vitro* results needs to be carefully done and should be confirmed with *in vivo* findings.

7.1.6 Bioactivity of black carrot polyphenols

7.1.6.1 Comparison of antioxidant assays

Over the past few decades, the role of dietary antioxidants including polyphenols have gained increased interest, especially because of their associated health beneficial effects against a number of oxidative stress related diseases. Due to the complex composition of foods, isolation of each antioxidant compound and investigating it separately is costly and inefficient, notwithstanding the possible synergistic interactions among the antioxidant compounds in a food mixture. Therefore, it is important for researchers to have a convenient method to quantify the antioxidant activity/capacity of foods.

Considering the chemical reactions involved, antioxidant activity/capacity measurement assays may be classified as hydrogen atom transfer (HAT)-based, electron transfer (ET)-based and mixed mode (ET- and HAT-based) assays. HAT-based antioxidant activity assays involve oxygen radical absorbance capacity (ORAC), total peroxyl radical trapping antioxidant parameter (TRAP), total oxyradical scavenging capacity (TOSC) and crocin bleaching assays and measure the capability of an antioxidant to quench free radicals by hydrogen atom donation. ET-based total antioxidant capacity assays include the following assays: Folin−Ciocalteu, ferric reducing antioxidant power (FRAP), cupric reducing antioxidant capacity (CUPRAC) and reducing power assays. ET-based assays are

relatively slower than HAT-based assays and are both solvent- and pH-dependent. Mixed mode assays involve 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)/Trolox equivalent antioxidant capacity (ABTS/TEAC), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and *N*,*N*-dimethyl-*p*-phenylenediamine dihydrochloride (DMPD) methods (Apak et al, 2016). An overview of the most widely used antioxidant activity/capacity measurement assays are presented in Table 7.1.

In this thesis we used ABTS, DPPH, FRAP and CUPRAC methods to study the antioxidant capacity of black carrots, processed products and their agronomic by-products (*Chapters 3-5*). We have also applied the Folin−Ciocalteu assay to measure the total phenolic content, however this assay not only measures phenolic compounds but also the total reducing capacity of a sample, and therefore some researchers consider this assay as an antioxidant capacity test. From our experience it was clear that the measurement of antioxidant capacity of food products could not be evaluated satisfactorily using a single antioxidant assay due to the many variables, which can influence the results. Therefore, we highly recommend applying a variety of assays with different mechanism to obtain the full picture. Considering the outcomes of this thesis as well as the recommendations in the literature (Apak et al., 2016), below are given a number of remarks as to how, in the future, one might approach to this topic.

- One of the important parameters of antioxidant assay selection is the working pH. There are assays working in acidic (FRAP), neutral (CUPRAC) and alkaline (Folin−Ciocalteu) conditions. Phenolic antioxidants do not dissociate, partially dissociate and predominantly dissociate in acidic, neutral and alkaline media, respectively, resulting in a possible under- (FRAP) or over-estimation (Folin−Ciocalteu) of the antioxidant capacity compared to physiological conditions (CUPRAC, DPPH). Taking this into account, we can presume that while evaluating the antioxidant capacity of polyphenols during digestion, the FRAP assay could be more suitable for assessing the activity in the stomach, whereas CUPRAC or DPPH assay could be more appropriate to evaluate total antioxidant capacity in the small intestine and colon.

Table 7.1 : Most widely used antioxidant activity/capacity measurement assays.

Assay	Oxidant	Probe	Detection	Reference
HAT-based assays				
ORAC	ROO• generated by AAPH	*β*-phycoerythrin, fluorescein	Fluorometer	(Cao et al, 1993; Ou et al, 2001)
TRAP	ROO• generated by ABAP	ABTS	Spectrophotometer	(Bartosz et al, 1998)
TOSC	ROO•	KMBA	HS-GC	(Winston et al, 1998)
Crocin bleaching	ROO• generated by AAPH	Crocin	Spectrophotometer	(Bors et al, 1990)
ET-based assays				
Folin−Ciocalteu	Phenolic compounds	Folin−Ciocalteu reagent	Spectrophotometer	(Singleton et al, 1999)
FRAP	Iron(III)	TPTZ	Spectrophotometer	(Benzie and Strain, 1996)
CUPRAC	Copper(II)	Neocuproine	Spectrophotometer	(Apak et al, 2004)
Reducing power	Iron(III)	Ferricyanide	Spectrophotometer	(Oyaizu, 1986)
Mixed mode (ET- and HAT-based) assays				
ABTS/TEAC	ABTS	ABTS	Spectrophotometer	(Miller and Rice-Evans, 1997)
DPPH	DPPH	DPPH	Spectrophotometer	(Brand-Williams et al, 1995)
DMPD	DMPD	DMPD	Spectrophotometer	(Fogliano et al, 1999)

AAPH: 2,2′-azobis(2-methylpropionamidine) dihydrochloride; ABAP: 2,2′-azobis(2-amidinopropane) dihydrochloride; ABTS: 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); CUPRAC: cupric reducing antioxidant capacity; DMPD: *N*,*N*-dimethyl-*p*-phenylenediamine dihydrochloride; DPPH: 2,2-diphenyl-1-picrylhydrazyl; FRAP: ferric reducing antioxidant power; HS-GC: headspace gas chromatography, KMBA: α-keto-γ-methiolbutyric acid; ORAC: oxygen radical absorbance capacity; ROO•: peroxyl radical; TEAC: Trolox equivalent antioxidant capacity; TOSC: total oxyradical scavenging capacity; TPTZ: 2,4,6-tripyridyl-s-triazine; TRAP: total peroxyl radical trapping antioxidant parameter.

- Applicability of the antioxidant assay to both hydrophilic and lipophilic antioxidants is an important factor. While ABTS and CUPRAC assays can measure both hydrophilic and lipophilic antioxidants, some methods measure only the hydrophilic antioxidants (FRAP and Folin−Ciocalteu), whereas others are only applicable to hydrophobic systems (DPPH).
- The background color from the food matrix (e.g. anthocyanins from black carrots) may cause absorbance interruptions, which shows maximal adverse effects in case of color-fading reactions (ABTS, DPPH) compared to color forming reactions (FRAP, CUPRAC).
- Reaction time required to approach an end point is another important parameter. Antioxidants having slow kinetic behaviours may not be fully oxidized within the protocol time of the certain assays (FRAP).

7.1.6.2 Co-culture models to study anti-inflammatory effects of polyphenols

As introduced in *Chapter 2* (Section 2.4.2), current trends in the study of polyphenols involve the use of co-culture systems to better simulate physiological conditions in the human body. Yet, only few studies investigated the anti-inflammatory effects of polyphenols in co-cultured intestinal epithelial and endothelial cells. The work presented in *Chapter 6* represents the first study that introduced the interaction of the polyphenols from black carrot and its by-products with the cellular inflammation response system in stimulated endothelial cells. With this set up, we have explored the direct and indirect effect of these polyphenols on cardiovascular markers in a more relevant setup that combines absorption effects with changes in endothelial cell metabolism. The results revealed that polyphenols were able to regulate the secretion of pro-inflammatory markers, i.e., IL-8, MCP-1, VEGF and ICAM-1, under both normal and inflammatory conditions. The most pronounced effects were observed with digested samples under inflammatory conditions. As explained earlier, digestion significantly enhanced the transport of polyphenols, which may explain the marked decreases in secretion of pro-inflammatory markers. Furthermore, it is possible that the digestion conditions released other food matrix compounds with direct or indirect impact on cell signaling cascades involved in these responses. In particular, black carrots are good sources of dietary fiber, which may play a role in reducing the secretion of relevant biomarkers

of inflammation (Basu et al, 2006). Overall, we believe that the applied methodology in *Chapter 6* may open perspectives for the development of functional foods improving cardiovascular and even general health under sub-optimal physiological states characterized by low-grade inflammation.

Of course the co-culture model that we developed in-house have some missing links compared to *in vivo* situation. As mentioned above, the Caco-2 cell line has some limitations (e.g. lack of mucus layer), which can affect the transport dynamics and hence the observed effects on endothelial cells. Moreover, when we look at the absorption pathway of anthocyanins after ingestion (Figure 6.1), we see that although anthocyanins can directly enter the systemic circulation in the intestine, they may also first reach the liver where they are metabolized to methyl, glucuronic and sulfate derivatives. In the future, these conditions should also be taken into account and methodologies should be adopted accordingly.

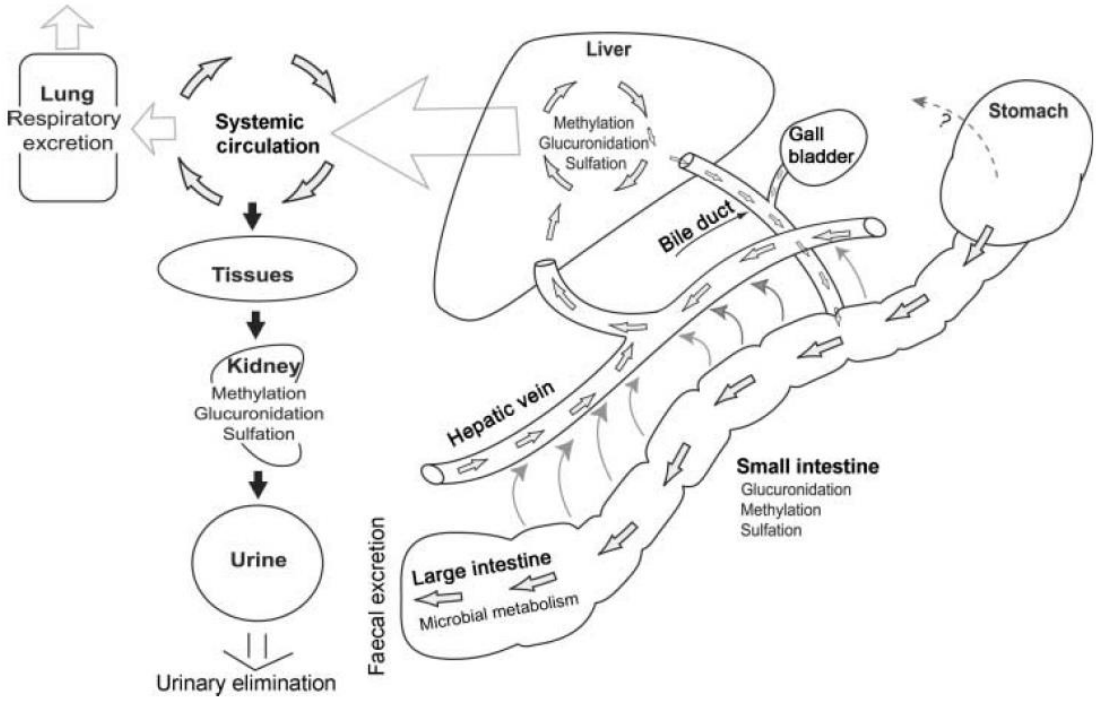

Figure 7.1 : Schematic representation of the potential route for anthocyanin absorption, metabolism and elimination.

7.2 Future Perspectives

In addition to the research performed in this thesis, new strategies need to be addressed to gain additional information. Below, a number of important points are highlighted as to how, in the future, we might approach this topic differently, in order to maximize the knowledge gained from this research.

- ***Subcritical water for green extraction of polyphenols:*** As in many studies in the literature, in this thesis, we used an organic solvent, i.e., methanol, to extract polyphenols from an agricultural product. In addition to their negative impact on human health and environment, organic solvents are also often costly to purchase and dispose (Mustafa and Turner, 2011). Subcritical water extraction is a cheap, efficient and consumer-friendly technique for extraction of valuable compounds; therefore it can be classified in the group of efficient "green" processing techniques for production of health-beneficial ingredients (Monrad et al, 2010). It has been shown that under optimized conditions, subcritical water might be a good substitute to organic solvents such as methanol and ethanol to extract polyphenols from plant sources (Singh and Saldana, 2011). Hence, for the sake of both the environment and human health, in future studies, "green solvent" aspects should be taken into account.
- ***Metabolomics as a novel, more integrated analytical approach:*** Metabolomics is the study of metabolites present in biological samples such as biofluids, tissue/cellular extracts and culture media. Using the latest liquid chromatography (LC) and gas chromatography (GC) separation approaches together with mass spectrometry (MS), or nuclear magnetic resonance (NMR) detection methods, so called untargeted analyses (or fingerprints) are performed to give a deep insight into the composition of these complex mixtures. Combining metabolomics data with multivariate data analysis tools (e.g. principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA)) allowed researchers to study alterations in metabolic pathways (Capanoglu et al, 2010; Brennan, 2013). Nowadays, the use of metabolomics in polyphenol research has attracted great attention. In particular, this technique is now being used to study the inter-individual variations in the metabolism of dietary polyphenols. Experts in polyphenol research propose that metabolomics can contribute to a better understanding

of the complex interactions between polyphenol intake and human health. Considering that, we expect to see more nutrimetabolomics research, especially on inter-individual differences, in the near future.

- ***Improving the bioavailability of polyphenols by means of encapsulation:*** Encapsulation technology can be used to enhance the bioavailability of polyphenols or to achieve controlled release of these compounds during digestion. Spray drying, coacervation, liposome entrapment, inclusion complexation, cocrystallization, nanoencapsulation, freeze drying, yeast encapsulation and nanoemulsion are some of the current technologies that are used to encapsulate polyphenols (Fang and Bhandari, 2010). Although there are few reports in the literature that studied the encapsulation of black carrot polyphenols, in particular anthocyanins (Ersus and Yurdagel, 2007; Murali et al, 2015), the effect of this processing technique on the human body is not established yet. Therefore, in the future it would be interesting to do some research on this topic.
- ***Gastric absorption of anthocyanins – a new approach***: Recent studies suggest that the rapid appearance of anthocyanins in plasma after consumption could result from their absorption through the gastric wall. In fact, research conducted with rats confirmed that anthocyanins are rapidly absorbed from the stomach (Felgines et al, 2006; Talavera et al, 2003; Passamonti et al, 2003). An alternative to animal studies for predicting stomach absorption of anthocyanins could be the use of a gastric cell culture model. A critical feature of such a model is that it has to work in the presence of a reduced pH. In a recent work (Fernandes et al, 2012a) moderately differentiated adenocarcinoma stomach cells (MKN-28) were used as a gastric barrier model to study the absorption of anthocyanins at different pH conditions (pH of 3.0 and 5.0). The authors concluded that anthocyanin standards, namely delphinidin-3-glucoside, cyanidin-3-glucoside and malvidin-3-glucoside could cross the gastric epithelium in a time dependent manner with no statistical differences in their transport efficiency according to the pH. In the future, this model can be improved by co-culturing the gastric MKN-28 cell line with the mucin secreting cell line HT29-MTX to better simulate the stomach conditions, which then can be used to study the gastric absorption of black carrot anthocyanins.

- ***Launch of a functional food:*** Development of a functional food product requires identification of functional compounds and assessment of their physiological effects, product formulation and development of a suitable food matrix, clinical trials on product efficacy in order to get approval for the health claims and market and public acceptance (Siro et al, 2008). In this thesis, we proposed that black carrot pomace can be used as a functional food ingredient due its high anthocyanin content and we evaluated the possible effects of the cake matrix. However, before introducing this product to clinical trials, there is another important issue that needs to be resolved, which is the organoleptic properties of the product. Once the sensorial properties of this product are approved, then a double-blind, randomized, placebo-controlled design should be performed. One of the important factors in clinical trials is to decide the dose of administration. According to the scientific opinion of EFSA on the use of anthocyanins as a food additive (EFSA Panel on Food Additives and Nutrient Sources added to Food, 2013), the currently available toxicological database is inadequate to establish a numerical ADI for anthocyanins. Therefore, further research on toxicological data of anthocyanins is necessary. Finally, although often ignored, the consumer acceptance of such product also needs to be investigated.
- ***Low-grade inflammation and health claims***: The EFSA guidance on scientific requirements for health claims related to gut and immune function (EFSA Panel on Dietetic Products Nutrition and Allergies, 2011) states that chronic inflammation is associated with the development of a number of diseases, and that altering levels of markers of inflammation might indicate a beneficial physiological effect in the context of a reduction of disease risk claim, if it can be demonstrated that altering the levels of inflammatory markers is accompanied by a reduced incidence of a disease for a specific dietary intervention. Currently, EU health claim register does not contain any authorized or non-authorized health claims that specifically address the health benefit area of suppression or control of low-grade inflammation. The lack of health claims is probably due to the fact that, although many biologically relevant mechanisms have been established to explain inflammation–disease associations, no single biomarker or group of biomarkers of inflammation has yet been strongly demonstrated to be sufficiently predictive of future disease.

A suggested strategy for building an EFSA health claim on this topic comprises (i) a description of product composition; (ii) a well grounded selection of the target population; (iii) the selection of a clinically relevant composite biomarker panel representing inflammation as well as the selected health benefit endpoints; and (iv) several sufficiently powered and well-controlled human studies assessing the effect of the test material on the relevant biomarkers in the relevant target population (Minihane et al, 2015). Overall, in the future to build health claims on black carrot polyphenols for improving inflammation, researchers should first focus on the need for well-validated biologically relevant markers that reflect the inflammatory state.

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APPENDICES

APPENDIX A: Chapter 3 Supporting Information

APPENDIX B: Chapter 4 Supporting Information

APPENDIX A: Chapter 3 Supporting Information

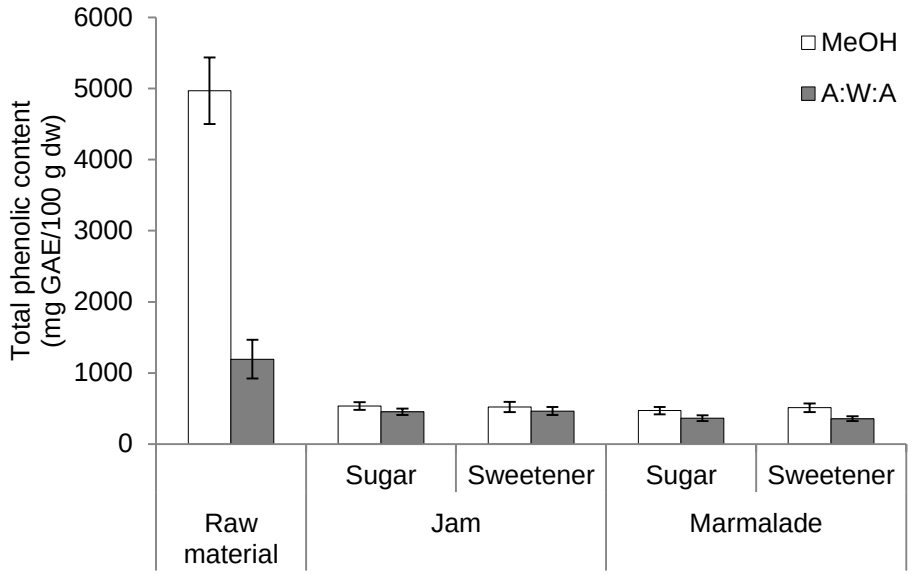

Figure A.1 : Total phenolic content of black carrot, jams and marmalades extracted with methanol (MeOH) and acetone:water:acetic acid (AWA).

APPENDIX B: Chapter 4 Supporting Information

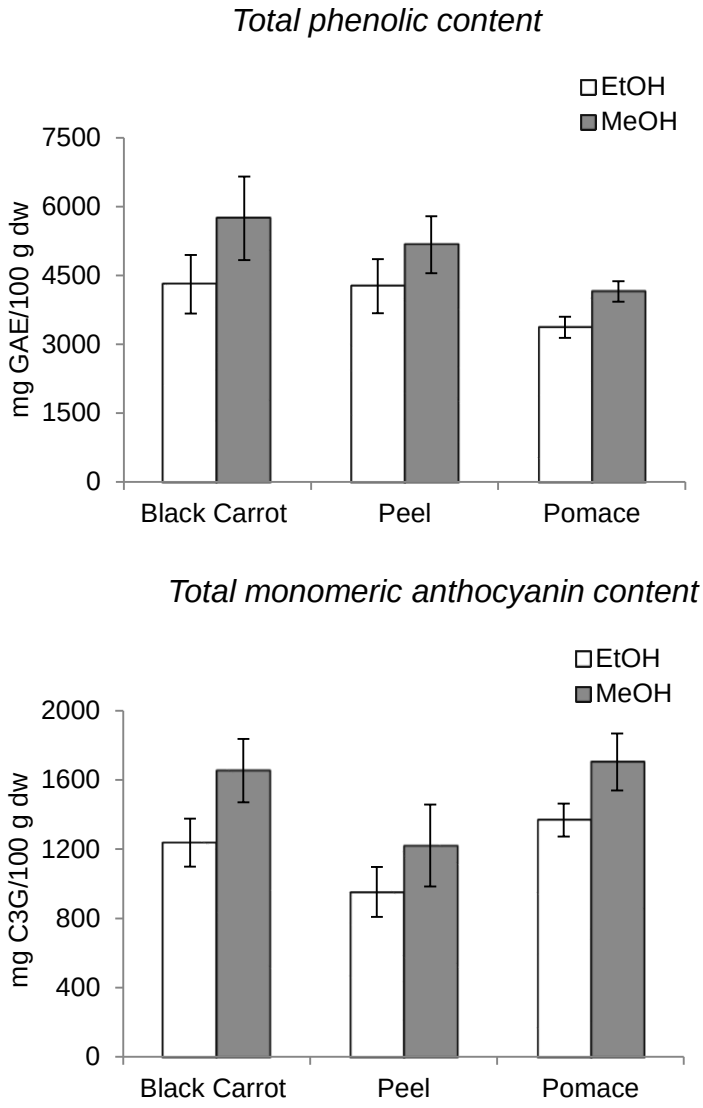

Figure B.1 : Total phenolic and total monomeric anthocyanin contents of black carrot and its by-products extracted with ethanol (EtOH) and methanol (MeOH).

Table B.1 : Standards used for the quantification of polyphenols during *in vitro* gastrointestinal digestion.

Standard		Undigested	Stomach	Small Intestine	Colon t=24h	Colon t=48h
Neochlorogenic acid	LOD (ppm)	0.02	0.12	0.15	0.06	0.08
	LOQ (ppm)	0.08	0.40	0.51	0.19	0.26
	Slope	0.34	0.37	0.38	0.40	0.43
	Intercept	-0.14	0.17	0.17	0.15	-0.09
	R^2	0.994	0.994	0.997	0.997	0.999
Chlorogenic acid	LOD (ppm)	0.09	0.17	0.10	0.18	0.10
	LOQ (ppm)	0.29	0.57	0.32	0.60	0.34
	Slope	0.39	0.37	0.44	0.43	0.42
	Intercept	-0.01	-0.01	-0.13	-0.03	-0.06
	R^2	0.998	0.999	0.997	0.999	0.999
Caffeic acid	LOD (ppm)	0.02	0.03	0.09	0.04	0.07
	LOQ (ppm)	0.05	0.10	0.30	0.12	0.23
	Slope	0.78	0.71	0.72	0.73	0.73
	Intercept	-0.12	-0.05	0.00	-0.40	-0.37
	R^2	0.999	0.998	0.998	0.994	0.998
Ferulic acid	LOD (ppm)	0.03	0.06	0.17	0.26	0.06
	LOQ (ppm)	0.10	0.22	0.55	0.86	0.19
	Slope	0.79	0.72	0.77	0.75	0.74
	Intercept	-0.04	-0.05	-0.64	-0.51	-0.30
	R^2	0.999	0.998	0.996	0.996	0.996
Cyanidin-3-*O*-glucoside	LOD (ppm)	0.06	0.11	0.12	0.07	0.13
	LOQ (ppm)	0.22	0.38	0.41	0.25	0.45
	Slope	0.67	0.70	0.75	0.76	0.85
	Intercept	0.09	0.15	-0.03	-0.69	-2.00
	R^2	0.998	0.997	0.999	0.998	0.991

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