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THE EFFECTS OF NECTAR PROCESSING ON SOUR CHERRY ANTIOXIDANT COMPOUNDS: CHANGES IN METABOLITE PROFILE AND BIOAVAILABILITY

Ph.D. THESIS

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NEKTAR İŞLEME PROSESİNİN VİŞNEDEKİ ANTİOKSİDAN BİLEŞİKLER ÜZERİNDEKİ ETKİSİ: METABOLİT PROFİLİ ve BİYOYARARLILIKTAKİ DEĞİŞİMLERİN İNCELENMESİ

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Annem, babam ve ağabeyime,

FOREWORD

Sour cherry is an important fruit in Turkey, which is the world's leading producer. Turkish sour cherries are highly esteemed for their high quality juice (called "nectar"). The objective of this thesis was to investigate the effects of industrial-scale processing of sour cherry fruit into nectar on the composition of the end product and bioavailability, paying specific attention to the phenolic antioxidant, particularly anthocyanins. I hope this research will contribute to the limited literature on sour cherry and its health promoting bioactive components.

First of all, I would like to express my special thanks and gratitude to my supervisor and mentor, Prof. Dr. Dilek BOYACIOĞLU who taught me the academic life, constituted and shaped my scientific vision, encouraged me in several ways to further improve myself, and supervised me to carry out this study. I also would like to thank Prof. Dr. Beraat ÖZÇELİK and Prof. Dr. Reşat APAK with whom it was a pleasure to work with and I do appreciate for their valuable contributions.

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March, 2013 **Gamze TOYDEMİR**

(Food Engineer)

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ABBREVIATIONS

AA : Ascorbic Acid
AAG : Ascorbic Acid Glucoside
ABTS : 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
ANOVA : Analysis of Variance
AOAC : Association Official of Analytical Chemists
ATCC : American-Type Culture Collection
Bx : Brix
C3G : Cyanidin-3-glucoside
C3GR : Cyanidin-3-(2^G-glucosylrutinoside)
C3R : Cyanidin-3-rutinoside
C3S : Cyanidin-3-sophoroside
CA : Citric Acid
CE : Catechin Equivalent
CJ : Concentrated Juice
CJ-APF : Concentrated Juice-After Paper Filtration
CJ-BPF : Concentrated Juice-Before Paper Filtration
ClJ : Clarified Juice
CUPRAC : Copper Reducing Antioxidant Capacity
DAD : Diode Array Detector
DHAA : Dehydroascorbic acid
DMEM : Dulbecco's Modified Eagle's Medium
DNP : Dictionary of Natural Products
DP : Degree of Polymerization
DPPH : 1,1-diphenyl-2-picrylhydrazyl
DTPA : Diethylene Triamine Pentaacetic Acid
DTT : Dithiothreitol
EDTA : Ethylene Diamine Tetraacetic Acid
EnJ : Enzyme-treated Juice
ESI-MS : Electrospray Ionization Mass Spectrometry
FBS : Fetal Bovine Serum
FF : Fresh Fruit
FF-St : Fresh Fruit without Stalk
FtJ : Filtered Juice
FR : Filtration Residue
GAE : Gallic Acid Equivalent
GI : Gastrointestinal
GLM : General Linear Model
HBSS : Hank's Balanced Salt Solution
HEPES : (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HPLC : High Performance Liquid Chromatography
IN : Serum availabile, dialyzable fraction
LC-MS : Liquid Chromatography-Mass Spectrometry

MC **:** Moisture Content
MH **:** Mash Heating
MP **:** Mash Pressing
MPEX-I **:** Mash Press Extraction I
MPEX-II **:** Mash Press Extraction II
MPEX-III **:** Mash Press Extraction III
MQ **:** Milli-Q (ultrapure)
N **:** Nectar
PrJ **:** Pressed Juice
PsJ **:** Pasteurized Juice
PC **:** Press Cake
PDA **:** Photodiode Array
OUT **:** Non-dialyzable fraction
QTOF **:** Quadropole Time Of Flight
RP-HPLC **:** Reversed Phase High Performance Liquid Chromatography
Sd **:** Seed
SGLT-1 : Sodium-dependent Glucose Transporter 1
SPE **:** Solid Phase Extraction
SSN **:** Sucrose Syrup-added Nectar
SUC **:** Sucrose
PCA **:** Principal Component Analysis
PN **:** Pasteurized Nectar
St **:** Stalk
TEAC **:** Trolox Equivalent Antioxidant Capacity
TEER **:** Transepithelial Electrical Resistance
TFA **:** Trifluoroacetic Acid
Trolox **:** 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
WF **:** Whole Fruit
WN **:** Whole Nectar

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THE EFFECTS OF NECTAR PROCESSING ON SOUR CHERRY ANTIOXIDANT COMPOUNDS: CHANGES IN METABOLITE PROFILE AND BIOAVAILABILITY

SUMMARY

Sour cherry (*Prunus cerasus* L.) is an important fruit in Turkey, who is the world's leading producer. Sour cherries are mostly consumed after processing into various products, including nectar as the major one. The relation between fruit and vegatable intake and reduced risk of human diseases (i.e., cancer, cardiovascular disease, obesity, and diabetes, etc.) has been attributed to the bioactivity of specific food components, including phytochemicals. Sour cherry is a rich source phenolic antioxidants, especially anthocyanins. The nutritional properties of sour cherry, considering the quantity and bioavailability of its bioactive antioxidant components, can largely be affected during processing.

The steps included in industrial-scale sour cherry nectar production are washing and selection, separation of stalks, mash heating, mash pressing, press cake extraction, pasteurization of the pressed juice, enzymation, clarification, filtration, evaporation to concentrated juice, which is subsequently processed to the final nectar through performing the further steps of water, sucrose syrup, and citric acid addition and pasteurization. In this thesis, each step of nectar processing was studied to evaluate the changes in quantity and profile of sour cherry antioxidants, paying specific attention to anthocyanins. Samples from industrial-scale sour cherry nectar processing were collected as five different batches. The variety of sour cherry fruit, used as raw material, was the local variety "Kütahya". In order to monitor the process, a total of 22 samples, each with 5 independent replicates, were collected from 18 steps of nectar processing for analysis. The analyses performed on the sour cherry samples were moisture content analysis by gravimetric method, total phenolic, total flavonoid, total anthocyanin contents, and total antioxidant activity by spectrophotometric methods, the identification and quantification of individual anthocyanins, procyanidins, flavonols, and phenolic acids by HPLC, the contribution of individual antioxidants to the total antioxidant capacity by on-line antioxidant detection using HPLC, and untargeted metabolomics approach by LC-QTOF-MS. In addition, the bioavailability of sour cherry anthocyanins was assessed using *in vitro* simulated gastrointestinal digestion model and *in vitro* Caco-2 cell model. In *in vitro* Caco-2 cell experiments the effects of nectar ingredients, which were sucrose and citric acid, as well as the food matrix on anthocyanin bioavailability were also examined.

The most pronounced change in moisture content was obtained in concentration step that led to a 2-fold reduction, from 89.6% in filtered juice to 43.4% in concentrated juice. Further analyses were performed using equal amounts of dry-weight for each sample, since changes in wet-weight were much more dramatic than changes in dry-weight.

The total phenolic, total flavonoid, and total anthocyanin contents of sour cherry fruit (without stalk) sample were 747.0 ± 100.3 mg GAE, 3526.8 ± 604.7 mg (+)CE, and 145.7 ± 26.2 mg C3G per 100 g dry-weight sample, respectively. These values for final nectar sample were 477.6 ± 34.4 mg GAE, 1586.3 ± 216.2 mg (+)CE, and 64.9 ± 4.6 mg C3G per 100 g dry-weight sample, respectively. Two of the applied treatments during nectar processing resulted in significant changes in the contents ($p<0.05$). First, the steps leading from fruit to pressed juice consistently led to a 2- to 3-fold increases in all three assays, on dry-weight basis. This was linked to the press-cake removal, which was mainly relevant to the removal of dry-weight (29% of total dry-weight) poor in antioxidants, resulted in an overall increase in the relative representation of antioxidants in the pressed juice, on dry-weight basis. The lower contents of antioxidant compounds in press-cake was linked to the efficient compound recovery into the juice fraction by the facilitated extraction with the repeated press-cake extraction steps. Second, processing of concentrated juice into nectar gave rise to a 2- to 4-fold reduction in the measured values. This reduction could be related to the addition of sucrose to the concentrated juice during the production of nectar, which led to a more than 50% sucrose containing in the dry matter of the nectar sample. Among the waste samples other than press cake, seed had the lowest values (4%, 2%, and 1% of fruit phenolics, flavonoids, anthocyanins, respectively), whereas stalks were found to be rich in flavonoids (160% of fruit flavonoids), although they were also poor in anthocyanins (1% of fruit anthocyanins).

The total antioxidant capacities of sour cherry fruit samples were determined to be 2123.8 ± 445.8, 3974.0 ± 861.0, 820.1 ± 85.8 mg TEAC/100 g dry-weight with the applied *in vitro* tests, ABTS, CUPRAC and DPPH, respectively. Among the three different assays CUPRAC method gave the highest values, while the lowest values were obtained with DPPH method. ABTS and CUPRAC were highly correlated, by considering the changes obtained for each individual processing step.

The HPLC based targeted analysis of individual sour cherry antioxidants revealed that the major anthocyanin component was cyanidin-3-(2^G-glucosylrutinoside) in sour cherry samples, composed around 75% of total anthocyanins, with amounts of 195.1 ± 38.0 mg C3G eq./100 g dry-weight in fruit and 89.6 ± 25.7 mg C3G eq./100 g dry-weight in final nectar samples. The other anthocyanins present were cyanidin-3-rutinoside, cyanidin-3-sophoroside, and cyanidin-3-glucoside. When overlooking the total effect of processing from fruit to nectar, the anthocyanin content (based on the content of cyanidin-3-(2^G-glucosylrutinoside) in the final nectar was about 35-45% of the content in fruit on dry-weight basis. However, when correcting for the addition of sucrose, the anthocyanin levels in the final nectar were around 99-133% of those in the fruit. Noteworthy, anthocyanins were not very dominant in the processing waste materials. This indicates that anthocyanin losses during the total process are remarkably modest, suggesting that the repeated extraction permits efficient cumulative removal of the anthocyanin compounds present in the fruit into the final nectar product.

Sour cherry fruit and nectar samples were found to contain a total flavan-3-ol content of 308.3 mg and 105.2 mg per 100 g dry-weight sample. The contents of epicatechin, as the most abundant procyanidin, in fruit and final nectar samples were 283.0 ± 66.7 mg/100 g dry-weight and 92.7 ± 24.7 mg/100 g dry-weight. Targeted HPLC analysis of procyanidins pointed out that procyanidins were over-represented in waste materials including stalks, and to a lesser extent, in press cake as compared to the

fruit. The degree of polymerization (DP) of procyanidins was low (<3) in all samples. All waste materials including stalks, seeds, press-cake, and filtration residue samples contained the highest chain-length procyanidins (DP between 2.5 and 3) and hence their removal caused a reduction of DP value from 2.1 in fruit to 1.7 in the final nectar.

The procyanidins (represented by epicatechin) behaved differently from the anthocyanins (represented by cyanidin-3-(2^{G}-glucosylrutinoside)). While most anthocyanin was recovered from the press cake by the repeated mash press extractions, procyanidins were still present in the third mash press extraction, and a considerable portion of procyanidins (around 40% of the total) remained in the press cake.

Neochlorogenic acid was the major phenolic acid, constituting >60% of total phenolic acid content in sour cherry samples, followed by *p*-coumaroylquinic acid, chlorogenic acid, and rosmarinic acid; whereas the most abundant flavonol component was rutin followed by quercetin-3-glucoside and kaempferol-3-rutinoside. Changes in phenolic acid and flavonol contents during processing followed similar trends as observed for anthocyanins, with the exception of stalks and press cake, which were found to contain relatively high concentrations of flavonols. Indeed, the extracts recovered from the repeated mash-press extraction steps displayed a strong over-representation (per dry-weight) of phenolic compounds, such as quercetin derivatives, chlorogenic acids, as in procyanidins and anthocyanins.

Vitamin C measurements using HPLC figured out that the fruit contained only the oxidized form of ascorbic acid (AA), dehydroascorbic acid (DHAA) with contents varying between 8.3-16.0 mg/100 g dry-weight, whereas there was no AA or DHAA detected in the final nectar sample.

The most abundant individual antioxidants contributing to the total antioxidant activity of the samples, determined by HPLC based on-line antioxidant analysis, were found to be the anthocyanins which comprised 61% and 57% of the total of antioxidant peaks in fruit and nectar, respectively. For samples other than wastes, the total contribution of phenolic acids and flavonols (expressed as quercetin derivatives) revealed consistent results with values ranging from 10-15% of the total antioxidant activity. In addition, highly polar antioxidants, including putative ascorbic acid derivative, showed a relative minor contribution to the total antioxidant activity (1-6%).

LC-QTOF-MS based untargeted metabolomics analysis enabled the identification of 38 compounds, included four anthocyanins, five flavan-3-ols, eight phenylpropanoids, eight flavonols, four flavanones, four organic acids, and two carbohydrates. The principal component analysis applied to the LC-MS data indicated consistent effects of the treatments on metabolite composition. Stalk and press cake waste materials differentiated substantially from the other samples. Compounds over-represented in the press cake relative to the fruit included catechin and a number of flavonols, while anthocyanins and phenolic esters such as neochlorogenic acid were under-represented, meaning that they were retained in the juice fraction and not lost in the waste material. Apparently, repeated press-cake washes add to the recovery of these compounds into the juice fraction.

Metabolites that strongly distinguished nectar from the fruit were dominated by polar compounds, like sucrose and citric acid, that had been added as a supplement during

the last production steps. Apart from sucrose and citric acid, the separation of nectar could be ascribed to the additional nine compounds, including neochlorogenic acid, which were higher in nectar on dry-weight basis. Noteworthy, anthocyanins were absent in this list of significantly different metabolites between fruit and nectar, suggesting that this class of compounds were recovered well.

Another important finding of metabolomics analysis was the putative identification of a conjugated stable form of AA, ascorbic acid-glucoside (C12H18O11, calculated [M-H]- = 337.0776) in sour cherry samples, for the first time to our knowledge. Ascorbic acid-glucoside concentrations in sour cherry were calculated as 2.2 mg/100 g dry-weight and 1.3 mg/100 g dry-weight in fruit and nectar samples, respectively.

The major finding obtained in *in vitro* simulated gastrointestinal digestion model was that the total anthocyanin recovery obtained for dialyzable fraction (IN) of nectar sample (39%) was 13-fold higher than the recovery obtained for the same fraction of fruit sample (3%). This may indicate a higher *in vitro* anthocyanin bioavailability in the nectar sample.

Prior to the Caco-2 cell based assays performed for the assessment of the *in vitro* bioavailability of sour cherry anthocyanins, optimum cell medium for transport experiments was investigated. The stability of the sour cherry anthocyanins and the integrity of the Caco-2 cell monolayers were evaluated in selected four different mediums, including HBSS, HBSS with 100 mM HEPES, DMEM, and DMEM with 9.1% FBS. The stability of anthocyanins was determined by monitoring the changes in cyanidin-3-(2^G-glucosylrutinoside) concentrations, using HPLC, as the representative anthocyanin compound. The stability of anthocyanins in HBSS medium was found to be significantly ($p < 0.05$) higher than in the other media tested which was very likely linked to the lower pH values measured for samples diluted in this medium. Although the recovery of anthocyanins in HBSS was higher, the low pH of samples in HBSS was not appropriate to mimic the human intestinal conditions. As the second parameter investigated for the integrity of the Caco-2 cell monolayers, the tight junctions of the cell monolayers in different mediums were evaluated with TEER measurements. The TEER was negatively affected by HBSS media resulting in significant decreases in TEER values. The TEER values were high (> 200 $\Omega.cm^2$) in DMEM media. Because of the fact that the proteins present in DMEM supplemented with 9.1% FBS could potentially have interactions with sour cherry phenolic compounds, DMEM with no FBS was decided to be used as the transport medium for further transport experiments.

In the transport experiments performed with whole fruit and whole nectar samples, the recovery of anthocyanins on the apical side of Caco-2 cell monolayers were low for whole fruit and whole fruit with sucrose-added (45%), whereas higher recoveries were obtained for whole nectar (77%), whole fruit with citric acid-added (78%) and whole fruit with citric acid-and-sucrose-added (82%) samples. The higher recoveries in the apical solutions could be linked to the lower pH values obtained for the citric acid including samples. In general, samples those provided higher recovery of anthocyanins on the apical side of cell monolayers, were also found to have higher transport efficiencies to the basolateral side of cell monolayers (except for whole fruit with sucrose-added). The transport efficiencies (%) of anthocyanins to the basolateral side were determined to have the highest values for the samples including both citric acid and sucrose added to their contents, which were whole fruit with citric acid-and-sucrose-added and whole nectar, with 3.9% and 2.6% of transport

efficiency percentages, respectively. Both citric acid and/or sucrose inclusion in the content of the samples resulted in an enhanced transport of anthocyanins. Citric acid could contribute this higher transport efficiency (%) values through enhanced anthocyanin stability as a result of lower pH. On the other hand, sucrose inclusion had no effect on recovery (%) values, but contributed more significantly to the transport with transport efficiency values of 2.4% in whole fruit with sucrose-added sample.

In further transport experiments, sour cherry fruit and nectar samples were subjected to solid phase extraction (SPE) procedure in order to purify anthocyanins and other flavonoids and to eliminate the possible effects of the other food matrix ingredients. The anthocyanin recovery on the apical side of cells were not statistically different ($p > 0.05$) in fruit-SPE and nectar-SPE samples. Citric acid inclusion resulted in higher anthocyanin recoveries, which was consistent with the results obtained for whole samples. Fruit-SPE sample was found to have the lowest transport efficiency (0.30%) of anthocyanins, whereas citric acid-added nectar-SPE sample had the highest transport efficiency (0.58%) values. The transport efficiencies of anthocyanins in fruit-SPE and nectar-SPE samples were not statistically different ($p > 0.05$). The anthocyanins in fruit-SPE and nectar-SPE samples were found to have lower efficiencies in transport through the Caco-2 cell monolayers in comparison to the transport efficiencies obtained for the whole fruit and whole nectar samples (≈ 2-fold lower in fruit samples and ≈ 5-fold lower in nectar samples).

NEKTAR İŞLEME PROSESİNİN VİŞNEDEKİ ANTİOKSİDAN BİLEŞİKLER ÜZERİNDEKİ ETKİSİ: METABOLİT PROFİLİ ve BİYOYARARLILIKTAKİ DEĞİŞİMLERİN İNCELENMESİ

ÖZET

Vişne (*Prunus cerasus* L.), dünyanın da birinci sıradaki üreticisi olan Türkiye'nin önemli bir meyve ürünüdür. Vişne meyvesi çoğunlukla değişik ürünlere işlenerek tüketilmektedir, ve bu ürünlerin başında da vişne nektarı gelmektedir. Meyve ve sebze tüketimi ile çeşitli rahatsızlıklara (kanser, kalp-damar hastalıkları, obezite, diabet, vb.) yakalanma riskinin azaltılması, fitokimyasallar grubunu da içeren spesifik gıda bileşenlerinin biyoaktif özellikleri ile ilişkilendirilmektedir. Vişne, fenolik antioksidanlar, özellikle de antosiyaninler açısından zengin bir meyvedir. Gıda işleme prosesleri sırasında vişnenin besinsel özellikleri, içerdiği biyoaktif bileşenlerinin miktarı ve biyoyararlılığı bakımından ele alındığında, büyük ölçüde etkilenebilmektedir.

Endüstriyel boyuttaki vişne nektarı üretim basamakları yıkama ve ayıklama, sap ayırma, mayşe ısıtma, mayşe presleme, posanın ekstraksiyonu, pres suyunun pastörizasyonu, enzimasyon, durultma, filtrasyon ve evaporasyon ile konsantre vişne suyuna işleme olarak sıralanabilir. Konsantre vişne suyu daha sonra su, sukroz şurubu ve sitrik asit ilavesi ile nektara işlenmekte ve bunu takiben yapılan pastörizasyon işlemi ile son ürün olan pastörize vişne nektarı elde edilmektedir. Bu tez kapsamında, vişne nektarı prosesindeki tüm üretim basamaklarının vişne antioksidanlarının, özellikle de antosiyaninlerin, miktarı ve profili üzerindeki etkisi incelenmiştir. Örnekler, endüstriyel skaladaki vişne nektarı üretim basamaklarından 5 bağımsız üretim sırasında toplanmıştır. Hammadde olarak "Kütahya" yerel vişne cinsi kullanılmıştır. Proses sırasında, analizlenmek üzere, 18 farklı nektar işleme basamağından toplamda 22 örnek, 5 tekrarlı olarak, alınmıştır. Örnekler üzerinde gerçekleştirilen analizler, gravimetrik metot ile nem tayini, spektrofotometrik yöntemler ile toplam fenolik, toplam flavonoid ve toplam antosiyanin içeriği ile toplam antioksidan aktivite analizleri, antosiyanin, prosiyanidin, flavonol ve fenolik asit bileşenlerinin HPLC yöntemi ile kalitatif ve kantitatif analizleri, on-line HPLC antioksidan tespiti yöntemi ile herbir antioksidan bileşeninin toplam antioksidan kapasiteye katkısının belirlenmesi ve LC-QTOF-MS yöntemi esaslı metabolomik yaklaşımı ile hedef-dışı metabolit analizleridir. Bunun yanı sıra, *in vitro* mide-bağırsak sistemi simülasyonu modeli ve *in vitro* Caco-2 hücre modeli kullanılarak vişne antosiyaninlerinin *in vitro* biyoyararlığı çalışılmıştır. *In vitro* Caco-2 hücre modeli analizlerinde, nektar ingredientleri olan sukroz ve sitrik asitin ve gıda matriksinin antosiyanin biyoyararlılığı üzerindeki etkisi de incelenmiştir.

Nem miktarındaki en belirgin değişim, yaklaşık 2 kat azalmaya neden olan konsantreye işleme basamağında görülmüş, ve filtrasyon basamağından elde edilen filtre vişne suyunun nem miktarı % 89.6'dan konsantre vişne suyunda % 43.4'e

düşmüştür. Örneklerin yaş maddeleri arasındaki farklılık büyük ölçüde değiştiğinden, analizler ve hesaplamalar kuru madde bazında gerçekleştirilmiştir.

Vişne meyvesinin (saplarından ayrılmış) toplam fenolik, toplam flavonoid ve toplam antosiyanin içerikleri, 100 g kuru-madde bazında, sırasıyla, 747 ± 100.3 mg GAE, 3526.8 ± 604.7 mg (+)CE ve 145.7 ± 26.2 mg C3G olarak bulunmuştur. Nektar işleme basamaklarından iki tanesi içeriklerde istatistiksel olarak önemli değişimlere neden olmuştur ($p<0.05$). İlki, meyvenin pres suyuna işlenmesi ile miktarlarda (kuru-madde bazında) saptanan 2-3 kat artışlardır. Bu artış, düşük antioksidan madde içeriğine sahip olan posanın presleme aşamasından sonra uzaklaştırılması ile açıklanmaktadır. Toplam kuru maddenin % 29'unu oluşturan ve antioksidanlarca fakir olan posanın ayrılması, pres suyundaki miktarlarda kuru-madde bazında artışa neden olmuştur. Posada antioksidan madde içeriğinin düşük olması, posanın ekstraksiyonu için tekrarlanan presleme basamaklarının antioksidan bileşenlerin pres suyuna kazanımını arttırması ile ilişkilendirilmektedir. İkinci önemli değişim, konsantre vişne suyunun nektara işlenmesi ile miktarlarda tespit edilen 2-4 kat azalıştır. Kuru-madde bazındaki bu azalış, nektara işleme sırasında ilave edilen sukroz şurubunun nektarın kuru maddesinin % 50'sinden daha fazlasının sukrozdan oluşmasına neden olmasıyla açıklanmaktadır. Posa dışındaki diğer proses atıkları içerisinde, çekirdek en düşük miktarlara (meyvedeki toplam fenolik, toplam flavonoid ve toplam antosiyanin içeriğinin, sırasıyla, % 4, % 2 ve % 1'i oranında) sahiptir. Saplarda toplam flavonoid madde içeriği yüksek (meyvedeki içeriğin % 160'ı oranında) iken, toplam antosiyanin madde içeriği düşüktür (meyvedeki içeriğin % 1'i oranında).

Vişne meyvesinin toplam antioksidan kapasitesi, ABTS, CUPRAC ve DPPH *in vitro* antioksidan tayini yöntemleri ile, sırasıyla, 2123.8 ± 445.8, 3974.0 ± 861.0 ve 820.1 ± 85.8 mg TEAC/100 g kuru-madde olarak belirlenmiştir. Uygulanan 3 farklı yöntem arasında CUPRAC metodu en yüksek değerleri verirken, DPPH metodu ile en düşük değerler elde edilmiştir. Proses basamakları arasındaki değişimi ortaya koyarken ABTS ve CUPRAC metotları arasındaki korelasyon en yüksektir.

Vişnedeki başlıca antosiyanin bileşeni, HPLC yöntemi ile siyanidin-3-(2^G-glukozilrutinozit) olarak belirlenmiştir. Örneklerdeki toplam antosiyanin içeriğinin (HPLC yöntemi ile belirlenen) % 75'ini oluşturan siyanidin-3-(2^G-glukozilrutinozit), meyvede 195.1 ± 38.0 mg C3G ekuvalenti/100 g kuru-madde ve nektarda 89.6 ± 25.7 mg C3G ekuvalenti/100 g kuru-madde miktarlarında bulunmaktadır. Vişnedeki diğer antosiyaninler siyanidin-3-rutinozit, siyanidin-3-soforozit ve siyanidin-3-glukozit olarak tespit edilmiştir. Meyveden nektara işlemenin toplam etkisine bakıldığında, nektarın antosiyanin içeriğinin (kuru madde bazında siyanidin-3-(2^G-glukozilrutinozit) miktarındaki değişime göre yorumlanan) meyvedekinin % 35-45'i arasında olduğu bulunmuştur. Ancak, nektarın kuru maddesindeki sukroz miktarı (> % 50) dikkate alındığında nektardaki değerlerin meyvedekilerin % 99-133'ü oranında değiştiği sonucuna varılmaktadır. Proses atıklarında antosiyanin içeriği düşüktür. Bu durum, proses sırasındaki antosiyanin kayıplarının önemli ölçüde az olduğunu göstermektedir. Bunun yanı sıra, posaya uygulanan ilave pres ektraksiyonu basamaklarının meyvedeki antosiyaninlerin son ürün olan nektara etkin olarak kazanımına imkan sağladığı söylenebilir.

Meyve ve nektardaki toplam flavan-3-ol içeriği, 100 g kuru-madde için, 308.3 mg ve 105.2 mg olarak ölçülmüştür. Vişnedeki başlıca prosiyanidin olan epikateşin miktarı ise, meyvede 283.0 ± 66.7 mg/100 kuru-madde, nektarda 92.7 ± 24.7 mg/100 kuru-

madde olarak belirlenmiştir. Prosiyanidinlerin HPLC ile (hedeflenmiş) analizi proses atıklarından sapların ve -saplara oranla daha düşük miktarlarda- posanın prosiyanidinlerce zengin olduğunu göstermiştir. Bütün örneklerde prosiyanidinlerin polimerizasyon derecesi (DP) düşüktür (< 3). Nektar işleme örnekleri arasında en yüksek zincir uzunluğuna (DP 2.5 – 3) sahip olan prosiyanidinler proses atıklarında (sap, çekirdek, posa, filtrasyon kalıntısı) tespit edilmiş ve atıkların uzaklaştırılması ile DP değeri meyveden (DP 2.1) nektara (DP 1.7) düşüş göstermiştir.

Prosiyanidinlerdeki değişim (epikateşin esas alınmıştır) antosiyaninlerdeki değişimden [(siyanidin-3-(2^G-glukozilrutinozit) esas alınmıştır] farklılık göstermektedir. Posa üzerinde tekrar edilen pres ekstraksiyonu basamakları antosiyaninlerin büyük ölçüde meyve suyu fraksiyonuna kazanımını sağlarken, üçüncü ve son pres ekstraksiyonu basamağından sonra önemli miktarlarda prosiyanidinin (toplam içeriğin % 40'ı) posada kaldığı belirlenmiştir.

Vişnedeki başlıca fenolik asit, toplam fenolik asit içeriğinin > % 60'ını oluşturan neoklorojenik asit olarak tespit edilmiştir. Bunu sırasıyla, *p*-kumaroylkuinik asit, klorojenik asit ve rozmarinik asit takip etmektedir. Başlıca flavonol bileşenleri ise, sırasıyla, rutin, kuersetin-3-glukozit ve kaempferol-3-rutinozit olarak belirlenmiştir. Proses sırasında fenolik asit ve flavonol içeriğindeki değişimler antosiyaninler ile benzerlik göstermektedir. Ancak, antosiyaninlerden farklı olarak, sap ve posa proses atıklarında falavonol içeriğinin daha yüksek konsantrasyonlarda bulunduğu saptanmıştır. Prosiyanidinler ve antosiyaninler için elde edilen bulgular ile tutarlı olarak, posanın tekrar edilen ekstraksiyonu ile edilen ekstraktlarda kuersetin türevi ve klorojenik asit gibi fenolik bileşenler yüksek oranlarda bulunmaktadır.

C vitamini analizleri (HPLC) ile meyvede sadece okside olmuş formdaki askorbik asit, dehidroaskorbik asit bulunduğu belirlenmiş (8.3 – 16.0 mg/100 g kuru-madde), son ürün olan nektarda ise AA veya DHAA tespit edilmemiştir.

On-line antioksidan tespiti analizi (HPLC) ile örneklerdeki toplam antioksidan aktiviteye katkıda bulunan başlıca antioksidan bileşenlerin, meyvede % 61, nektarda ise % 57 seviyesindeki oranlarla antosiyaninler olduğu belirlenmiştir. Proses atıkları dışındaki örneklerde fenolik asit ve flavonoller (kuersetin türevi olarak tanımlanmıştır) toplam antioksidan aktivitenin % 10 – 15'ini oluşturmaktadırlar. Buna ek olarak, askorbik asit türevini de içeren polar antioksidanların antioksidan aktiviteye olan katkıları % 1 – 6 oranında değişmektedir.

LC-QTOF-MS yöntemi esaslı (hedef-dışı) metabolomik analizleri ile vişnede 38 bileşik tanımlanmıştır. Bu bileşikler içerisinde 4 antosiyenin, 5 flavan-3-ol, 8 fenilpropanoid, 8 flavonol, 4 flavanon, 4 organik asit ve 2 karbonhidrat bileşeni bulunmaktadır. LC-MS datası üzerinde yapılan temel bileşen analizleri proses basamaklarının vişnedeki metabolit kompozisyonu üzerindeki etkisini ortaya çıkarmıştır. Temel bileşen analizi, sap ve posa proses atıklarının diğer örneklerden farklılaştığını ortaya koymuştur. Posada, kateşin ve çeşitli flavonol bileşenleri meyveye oranla daha yüksek oranlarda bulunurken, antosiyaninler ve fenolik asitlerin (neoklorojenik asit, vb.) meyveye oranla daha düşük oranlarda olduğu tespit edilmiştir. Bu durum antosiyaninler ile fenolik asitlerin meyve suyu fraksiyonuna etkili bir şekilde kazandırıldığını ve atıklar ile kayıpların önemli ölçüde az olduğunu göstermektedir.

Temel bileşen analizleri, nektarı meyveden ayıran metabolitlerin büyük ölçüde polar bileşenlerden oluştuğunu göstermektedir. Bunların başında, nektara işleme sırasında kullanılan sukroz ve sitrik asit bileşenleri gelmektedir. Sukroz ve sitrik asit dışında,

neoklorojenik asidi de içeren toplam 9 metabolitin nektarda kuru madde bazında daha yüksek oranlarda bulunduğu ve nektar ile meyvenin metabolit profilleri arasındaki farklılığı oluşturduğu belirlenmiştir. Bu metabolitler içerisinde antosiyanin bileşenlerinin bulunmaması diğer analiz sonuçları ile de tutarlı olarak meyvedeki antosiyaninlerin nektara etkili bir şekilde kazandırıldığını bir kez daha ortaya koymaktadır.

Metobolomik analizleri ile elde edilen önemli bulgulardan biri de vişne örneklerinde, incelenen literatür doğrultusunda, ilk defa askorbik asitin konjüge stabil bir formu olan askorbik asit-glukozitin ($C_{12}H_{18}O_{11}$, $[M-H]^-$ = 337.0776) tespitidir. Vişne meyvesi ve nektarında askorbik asit-glukozit konsantrasyonu, sırasıyla, 2.2 mg/100 g kuru-madde ve 1.3 mg /100 g kuru-madde olarak belirlenmiştir.

In vitro mide-bağırsak sistemi simülasyonu modeli ile elde edilen başlıca bulgu, nektarda seruma geçebilir (diyalize olabilir) (IN) fraksiyondaki toplam antosiyanin içeriğinin (% 39) meyve için aynı fraksiyonda tespit edilen toplam antosiyanin içeriğine (% 3) oranla 13 kat daha fazla olmasıdır. Bu durum, nektarda antosiyaninlerin *in vitro* biyoyararlılığının daha yüksek olduğu şeklinde yorumlanabilir.

Vişne antosiyaninlerinin *in vitro* biyoyararlılığının belirlenmesi amacı ile kullanılan diğer bir yöntem olan Caco-2 hücre modeli analizlerinden önce ilk basamak olarak antosiyanin taşınımı denemelerinde kullanılacak optimum hücre ortamının belirlenmesine çalışılmıştır. Seçilen 4 farklı hücre ortamında –HBSS, 100mM HEPES ilaveli HBSS, DMEM ve % 9.1 FBS ilaveli DMEM- vişne antosiyaninlerinin stabilitesi ve Caco-2 tek katmanlı hücre tabakalarının bütünlüğü test edilmiştir. Antosiyanin stabilite testlerinde, vişnedeki başlıca antosiyanin olan siyanidin-3-(2^G-glukozilrutinozit) konsantrasyonundaki değişimler, HPLC yöntemi ile, incelenmiştir. HBSS hücre ortamı ile hazırlanan örneklerde daha düşük pH değerlerinin elde edilmesi, bu ortamdaki antosiyanin stabilitesinin diğer hücre ortamlarına göre istatistiksel olarak önemli derecede yüksek ($p < 0.05$) olmasını sağlamıştır. Ancak, HBSS ortamında antosiyaninlerin stabilitesinin yüksek olmasına rağmen, düşük pH değerleri insan bağırsağındaki koşulların taklit edilmesi için uygun değildir. Uygun hücre ortamının seçilmesi için incelenen diğer bir parametre olan Caco-2 hücre katmanının bütünlüğünün belirlenmesi için, hücreler arasındaki bağlantıların sıkılığı TEER ölçümleri ile analizlenmiştir. HBSS ortamları TEER değerlerini negatif yönde etkileyerek azalmaya neden olmuştur. DMEM ortamlarında yüksek TEER değerleri (> 200 $\Omega.cm^2$) ölçülmüştür. DMEM ortamları arasında seçim yapıldığında % 9.1 oranında FBS ilaveli DMEM ortamındaki proteinlerin vişne fenolikleri ile etkileşime girebilecekleri göz önünde bulundurularak, FBS ilave edilmemiş DMEM ortamının antosiyanin taşınımı denemelerinde kullanılmak üzere seçilmiştir.

Bütün meyve ve bütün nektar örnekleri ile gerçekleştirilen antosiyanin taşınımı analizlerinde, meyve ve sukroz ilaveli meyve örnekleri için Caco-2 hücre katmanının üst tarafındaki ortamda antosiyanin stabilitesi (%) için belirlenen değerler % 45 iken, bu değerler nektar, sitrik asit ilaveli meyve ve sukroz ile sitrik asit ilaveli meyve örnekleri için sırasıyla % 77, % 78 ve % 82'dir. Sitrik asit içeren örneklerde stabilitenin daha yüksek olması düşük pH değerleri ile ilişkilendirilebilir. Genel olarak, Caco-2 hücre katmanının üst tarafındaki ortamda daha yüksek antosiyanin stabilitesinin görüldüğü örneklerde hücre katmanının alt tarafına olan antosiyanin taşınımının (%) de daha yüksek olduğu tespit edilmiştir (sukroz ilaveli meyve örneği hariç). Sitrik asit ve sukroz ingredientlerinin her ikisini birlikte içeren sukroz ile

sitrik asit ilaveli meyve ve nektar örneklerinde antosiyanin taşınımının (%), sırasıyla, % 3.9 ve % 2.6 değerleri ile en yüksek değerleri aldığı saptanmıştır. Sitrik asit ve/veya sukroz ilavesinin her ikisinin de taşınımını arttırmada rolü olduğu söylenebilir. Sitrik asit ilavesinin, sağladığı daha düşük pH değerleri ile antosiyanin stabilitesini arttırarak, daha yüksek antosiyanin taşınımı (%) değerlerininin elde edilmesine katkıda bulunduğu söylenebilir. Öte yandan, sukroz ilavesi antosiyanin stabilitesi üzerinde pozitif/negatif bir etkiye neden olmazken antosiyanin taşınımını önemli ölçüde arttırmış ve sukroz ilaveli meyve örneğinde antosiyanin taşınımı (%) değerlerinin % 2.4 oranında omasını sağlamıştır.

Gıda matriksinin *in vitro* antosiyanin biyoyararlılığı üzerindeki etkisinin incelenmesi amacıyla, meyve ve nektar örneklerindeki fenolik bileşenler solid faz ekstraksiyonu ile matriksten ekstrakte edilmiş ve elde edilen ekstraktlar antosiyanin taşınımı analizlerinde kullanılmıştır. Meyve ve nektardan elde edilen ekstraktlardaki antosiyaninler stabilite değerleri açısından istatistiksel olarak farklı bulunmamıştır ($p > 0.05$). Bütün meyve ve bütün nektar örnekleri için elde edilen bulgularla tutarlı olarak, sitrik asit ilavesinin ekstraktlarda da stabiliteyi arttırdığı belirlenmiştir. Meyve ekstraktındaki antosiyaninler en düşük taşınım değerlerine (% 0.30) sahipken, sitrik asit ilave edilmiş nektar ekstraktındaki antosiyaninler en yüksek değerleri (% 0.58) vermiştir. Meyve ve nektar ekstraktlarındaki antosiyaninler iletim değerleri açısından istatistiksel olarak farklı bulunmamıştır ($p > 0.05$). Meyve ve nektardan ekstrakte edilerek hücrelere verilen antosiyaninler için belirlenen taşınım değerleri, bütün meyve ve bütün nektar örneklerinin hücrelere verilmesiyle elde edilen antosiyanin iletim değerlerinden daha düşüktür. Meyve örneği için 2 kat, nektar örneği için 5 kat düşük değerler ölçülmüştür.

1. INTRODUCTION

Sour cherry (*Prunus cerasus* L.) is an important fruit in Turkey, who is the world's leading producer generating almost 200,000 metric tonnes in 2010 (FAOSTAT, 2010). Approximately 85 % of Turkish sour cherry production is used for processing into various products, such as jams and frozen fruits but it is predominantly used in the fruit juice industry (Turkey Stone Fruit Annual Report, 2011). For the Turkish juice industry, sour cherry is an important fruit with 25-40% of the fruit being processed into nectar (Akdag, 2011). The report "Fruit/Vegetable Juice in Turkey" indicated that the sour cherry nectar was the second leading flavor with 25-30% of retail volume after peach nectar having approximately 35% of retail volume (Euromonitor International, 2012).

Production of sour cherry juice (called 'nectar') involves several steps such as heating, pressing, and filtration. These can potentially determine the fate of phenolic antioxidants during processing. However, the effects of cherry processing on antioxidant compounds has hardly been studied yet (Kim and Padilla-Zakour, 2004; Kirakosyan *et al.*, 2009). In food science, metabolomics has recently become established as a tool for the analyses of quality, processing and safety of both raw materials and commercial products (Stewart *et al.*, 2011). Both targeted and untargeted metabolite analyses have been used to study the effect of processing berries, tomatoes and tea on the phenolic compound composition of the final products (Woodward *et al.*, 2011; Capanoglu *et al.*, 2008; Pongsuwan *et al.*, 2008). Targeted analyses allow the quantification of those major compounds, such as anthocyanins, while untargeted metabolomics analyses allow the identification of processing steps which have a major effect on the overall composition of the product (Capanoglu *et al.*, 2008; Capanoglu *et al.*, 2010).

Sour cherry (*Prunus cerasus* L.) fruit contains substantial quantities of phenolic antioxidants, including specifically, anthocyanins. In addition, other groups, such as hydroxycinnamates, flavonols, and flavan-3-ols (procyanidins) are present (Bonerz *et al.*, 2007; Capanoglu *et al.*, 2011). Anthocyanins, a group in flavonoids, are naturally

occurring plant secondary metabolites that are present in a number of fruits and vegetables, and commonly consumed *via* our daily diets. Dietary consumption of anthocyanins has been reported to be higher than that for other flavonoid groups such as flavonols (e.g. quercetin) (Hertog *et al.*, 1993; Clifford, 2000; Scalbert and Williamson, 2000). This has been attributed to their widespread distribution and occurrence in fruits and vegetables (Scalbert and Williamson, 2000).

Anthocyanins have been related with the prevention of a broad range of human diseases (He and Giusti, 2010), like certain cancers (Butelli *et al.*, 2008; Hou *et al.*, 2004; Seeram *et al.*, 2004), cardiovascular diseases (Renaud and Lorgeril, 1992; Toufektsian *et al.*, 2008), age-related degenerative diseases (Joseph et al., 1999), obesity (Titta *et al.*, 2010; Tsuda *et al.*, 2003) and diabetes (Sugimoto *et al.*, 2003; Tsuda *et al.*, 2003). In rats, oral administration of sour cherry anthocyanins reduced the severity of inflammatory symptoms such as oedema, gout and arthritis (Tall *et al.*, 2004). In mice, supplementing the diet with sour cherry material led to fewer and smaller caecal adenomas (Kang *et al.*, 2003). Consumption of sour cherry juice has been reported to decrease body weight, reduce blood pressure and improve blood lipid profiles of diabetic patients (Ataie-Jafari *et al.*, 2008). These health enhancing properties of anthocyanins have mostly been linked to their high antioxidant activities (Chun *et al.*, 2003), as well as their ability to modulate mammalian cell signaling pathways (Meiers *et al.*, 2001; Williams *et al.*, 2004).

Although anthocyanins have been reported to have several potential health benefits, there are many questions still needed to be answered in relation with the points that have been shown with *in vitro* studies performed so far and the points that are aimed to be achieved with *in vivo* conditions. It is known that anthocyanins are consumed in relatively high amounts, but it is more important to know how much is bioavailable (Srinivasan, 2001). Little is yet known about how anthocyanins survive in the digestive tract, how their stability in lumen and bodyfluids affected, how they are taken up into the body and metabolized by gut epithelial cells, and how food processing and product matrix influence this process (McGhie *et al.*, 2003; Yi *et al.*, 2006). It is apparent that more information is needed for improving the association between anthocyanin intake and human health and that improved knowledge can support the development of functional food with an increased bioavailability and bioactivity (Arts and Hollman, 2005).

There are many studies performed, using both *in vivo and in vitro* strategies, to assess the bioavailability of anthocyanins (Bub *et al.*, 2001; Cao and Prior, 1999; Cao *et al.*, 2001; Charron *et al.*, 2009; Cooney *et al.*, 2004; Faria *et al.*, 2009; Fazzari *et al.*, 2008; Felgines *et al.*, 2003; Felgines *et al.*, 2010; Hassimotto *et al.*, 2008; Ichiyanagi *et al.*, 2006; Matsumoto *et al.*, 2001; McGhie *et al.*, 2003; Milbury *et al.*, 2002; Miyazawa *et al.*, 1999; Perez-Vicente *et al.*, 2002; Steinert *et al.*, 2008; Yi *et al.*, 2006). However, many of those studies revealed conflicting results. Anthocyanins are known to have a complex biochemistry, influenced by several factors including oxygen, enzymes, light, temperature, and specifically pH (Jackman *et al.*, 1987). These compounds can exist in several interconvertable forms in aqueous solutions depending on pH, temperature, and time. Since anthocyanins are subjected to different pH conditions during food processing, storage, or during passage through the gastrointestinal (GI) tract, the chemical forms of these compounds can be altered from red flavylium cation form, at low pH values (pH < 2), to other molecular forms (i.e. quinonoidal bases, hemiketals, and chalcones) at neutral pH values. Due to this great variation, different molecular forms of anthocyanins can be responsible for the bioactivity observed in different conditions which leads to complications in understanding the health effects of anthocyanins (McGhie and Walton, 2007). Additionally, *in vivo* human and animal models which are highly complex and are influenced by several factors, such as chemical instability, make this anthocyanin bioavailability issue more complicated (Balimane *et al.*, 2000). Thus, little is known yet about the details of this issue, and much remains to be investigated and explained in order to contribute to the limited information on absorption and metabolism (McGhie and Walton, 2007; Yi *et al.*, 2006).

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

of *in vitro* GI digestion on the stability of polyphenols has already been tested in several foods such as raspberry (McDougall *et al.*, 2005a), mulberry (Liang *et al.*, 2012), and apple (Bouayed *et al.*, 2011). However, no information is available particularly on the effect of *in vitro* GI digestion on sour cherry fruit and its processed nectar.

The intestinal cells in the gut are exposed to and interact with the food we consume. They are primarily responsible for nutrient uptake via inter- and intracellular processes. The Caco-2 cell line, a human intestinal epithelial cell line derived from a colon carcinoma, is known to be able to represent many small intestinal functions and commonly used for bioavailability studies (Boyer *et al.*, 2005; Glahn *et al.*, 1998). Good correlations were reported between Caco-2 cell permeability and the *in vivo* absorption rates in rat (Conradi *et al.*, 1993) and human (Yee, 1997). The bioavailability of various phytochemicals, such as quercetin from red onions (Walgren *et al.*, 1998) and the tea flavonoid (-)-epicatechin (Vaidyanathan and Walle, 2001), were investigated using *in vitro* Caco-2 cell model system, providing much valuable information. However, we do not consume phytochemicals solely but as e.g. fruits and vegetables. In products, phytochemicals highly interact with each other and with the food matrix (Parada and Aguliera, 2007). In addition, fruits and vegetables are mostly consumed after processing to a wide range of products.

Cherry fruits are still under-researched compared to the other fruits, and in particular, the current information about the fate of cherry antioxidants during processing, influenced by several factors, is very limited. The first objective of this research was to investigate the effects of industrial-scale processing of sour cherry fruit into nectar on the composition of the end product paying specific attention to the phenolic antioxidant components by using both targeted and untargeted metabolite analyses, both allowed identification of a large number of phenolic compounds in sour cherry. Untargeted metabolomics approach also enabled to monitor the changes in metabolite profile during processing. As well as, spectrophotometric methods were used to investigate the changes in antioxidant capacity, and HPLC coupled to on-line antioxidant detection was used to determine the contribution of different classes of compounds to the antioxidant activity.

Secondly, in this thesis we aimed to assess the bioavailability of sour cherry anthocyanins using the *in vitro* simulated GI digestion model and the human Caco-2

intestinal cell model system. The recovery of sour cherry antioxidants in the human digestive system was studied with *in vitro* GI digestion. In the cell based assays, the bioavailability of sour cherry anthocyanins was evaluated by introducing whole sour cherry fruit and whole sour cherry nectar samples to the Caco-2 cells. The influence of nectar processing on anthocyanin availability was investigated by considering also how the additional sucrose and citric acid ingredients included in nectar during processing change the biovailability. As well as, the effect of food matrix on bioavailability was examined, for which solid phase extracted anthocyanins from fruit and nectar matrices were used to eliminate the matrix effect, with further in comparisons with whole fruit and nectar.

This thesis represents the research performed based on the specific objectives described above as follows: "literature" section gives a summary of the literature on sour cherry fruit, the health-enhancing properties and bioavailability of the specific fruit antioxidants; "materials and methods" section describes the fruit materials collected at an industrial-scale nectar production and the methods applied – including spectrophotometric methods, HPLC based targeted analysis, LC-QTOF-MS based untargeted metabolomics approach, *in vitro* bioavailability assays; "results and discussion" section presents the data obtained in a detailed way using tables and figures and evaluates all of the observations in the view of the current literature; and "conclusion" section provides the significant impact of the findings and suggestions those would be contributing to the current literature by enlightening needs for future antioxidant research.

2. LITERATURE

2.1 Sour Cherry

Cherries (*Prunus* spp.) belong to the stone fruit family, Rosaceae, genus *Prunus*, with the most important species included in the subgenus Cerasus, those are known as sweet cherry (*Prunus avium* L.) and sour cherry or tart cherry (*Prunus cerasus* L.) (Ferretti *et al.*, 2010).

Cherry fruit quality is specified by fruit colour, firmness, sweetness, and sourness. Sweet taste in the cherry fruit is determined by glucose and fructose containing, and sour taste is associated with the presence of malic acid (organic acid) (Bernalte *et al.*, 2003; Esti *et al.*, 2002). Sour cherries are lower in sugar content (8 g/100 g) compared to the sweet cherries (13 g/100 g), while malic acid content of sour cherries are higher (approximately 2%) (Ferretti *et al.*, 2010; Filimon *et al.*, 2011). Cherries are reported to include hydrophilic (C, B) and lipophilic (A, E, K) vitamins, as well as minerals such as calcium, magnesium, phosphorus and potassium (Ferretti *et al.*, 2010). Especially, cherry fruits are important for their high polyphenol contents, in which sour cherries are characterized by higher contents (Ferretti *et al.*, 2010; Kim *et al.*, 2005).

Turkey has been reported to be among the top sour cherry producing and exporting countries in the world, specifically in the last 15 years (Turkey Stone Fruit Annual Report, 2011). The countries other than Turkey include Russian Federation, Ukraine, Poland, and Iran (Table 2.1) (FAOSTAT, 2010). Sour cherries are mostly grown (43% of total production) in Central Anatolia, mainly in Kütahya and Afyon regions (Durmus and Yigit, 2003). The industry sources points out that approximately 85% of sour cherries produced in Turkey are consumed after processing into various products such as canned products, marmalades, frozen fruits, and fruit juices. Turkish sour cherries are highly esteemed for their high quality juice (called “nectar”) (Turkey Stone Fruit Annual Report, 2011). For the Turkish juice industry, sour

cherry is an important fruit, with 25-40% of the produced fruit being processed into juice (Table 2.1) constituting 7-9 % of the processed raw material in juice industry (Akdag, 2011). Sour cherry fruit can not be processed into 100% fruit juice because of its characteristic sour taste. Since the sour cherry juice is not palatable in its natural state, certain amounts of water and sugar addition is needed to obtain a drinkable juice, which is classified as "nectar". The report of Euromonitor International (2012) on "Fruit/Vegetable Juice in Turkey" pointed out that the off-trade sales of nectar (25-99% juice) in Turkey comprised around 65% of total fruit/vegetable juice sales in years 2006-2011 (Table 2.2). The same report also indicated that the sour cherry nectar was the second leading flavor with 25-30% of retail volume after peach nectar having approximately 35% of retail volume (Table 2.3). It has also been reported in 2012 Liquid Market Report of European Fruit Juice Association (AIJN) (European Fruit Juice Association, 2012) that cherry nectar is the second mostly consumed nectar in Turkey with 19.7% in year 2011, following peach nectar which has 32.4% of consumption rate.

Table 2.1 : World's leading sour cherry producers by years between 2008 – 2010 (FAOSTAT, 2010; Akdag, 2011).

Country	**2010**[a]	**2009**[a]	**2008**[a]
Turkey	194989 (73500)[b]	192705 (49700)[b]	185435 (54600)[b]
Russian Federation	165000	190000	183000
Ukraine	154500	115800	129200
Poland	147238	189220	201681
Iran	103232	106000	106461

[a] Data represent the production values in tonnes.
[b] The values in brackest are the sour cherry quantities in Turkey those processed into the juice (Akdag, 2011).

Table 2.2 : Off-trade sales of fruit/vegetable juice in Turkey by category (Euromonitor International, 2012).

	2006[a]	**2007**[a]	**2008**[a]	**2009**[a]	**2010**[a]	**2011**[a]
100% juice	34.4	46.3	59.0	63.7	68.8	74.5
Juice drinks (up to 24% juice)	20.0	22.0	32.4	36.0	38.7	41.4
Fruit flavoured drinks (not juice content)	119.2	119.5	129.5	140.6	151.3	162.2
Nectars (25-99% juice)	303.8	405.1	427.3	467.0	503.4	541.2
Total	477.4	592.9	648.2	707.3	762.3	819.3

[a] Data represent the sales by volume in million litres.

Table 2.3 : Leading flavours for nectars (25-99% juice) in Turkey (Euromonitor International, 2012).

	2006[a]	**2007**[a]	**2008**[a]	**2009**[a]	**2010**[a]	**2011**[a]
Peach	34.5	34.6	35.6	36.0	36.2	36.0
Sour cherry	29.0	29.0	29.1	25.5	25.3	25.3
Apricot	16.7	16.8	16.9	16.0	15.8	15.7
Mixed fruits	5.7	6.5	7.0	11.3	11.8	11.8
Orange	5.5	5.2	5.0	5.0	5.1	5.2
Apple	3.5	3.2	3.0	3.0	3.0	3.0
Others	5.1	4.7	3.4	3.2	2.9	3.0

[a] Data represent the leading flavours of nectars by % retail volume.

2.2 Nutritional Properties of Sour Cherries

In recent years an increasing interest on a new diet-health paradigm has placed more emphasis on the positive aspects of diet, which has led to the nutritional studies those are examining foods for their protective and disease preventing potential (Nicoli *et al.*, 1999). Accordingly, fruits and vegetables have gained an important status in human diet as "functional foods", which are capable of providing additional physiological benefits, including preventing or delaying onset of several chronic diseases, due to their phytochemical contents (Kaur and Kapoor, 2001; Nicoli *et al.*, 1999). The 5-a-Day program was developed in 1989 with the report by National Academy of Sciences, recommending the consumption of 5 or more servings of fruit and vegetables daily in order to reduce the risk of certain chronic diseases (National Academy of Sciences, 1989).

Pyhtochemicals are bioactive non-nutrient plant compounds those are known to have an antioxidative effect through oxidative stress caused by free oxygen radicals in the body. Free radicals and reactive oxygen species, which are the products of normal oxygen metabolism or are induced by exogenous damage, continuously threaten the body cells and tissues (Groot and Rauen, 2009). Antioxidant phytochemicals, with their free radical scavenging activity, stabilize the reactive oxygen species. They react with the reactive compound of the radical, through their highly reactive hydroxyl groups, resulting in a more stable and less-reactive radical (Hanasaki *et al.*, 1994). The modulation of biological oxidative stress protects the cellular lipids, proteins, and DNA from damaging. Intake of these biologically active components via fruits and vegetables have been significantly correlated with the prevention and lower incidence of several degenerative diseases, i.e. cardiovascular diseases, and

certain types of cancers (Dillard and German, 2000; Garcia-Closas *et al.*, 1999; Hollman *et al.*, 1999; Steinmetz and Potter, 1996; Visioli *et al*., 2000; Yao *et al.*, 2004). Phytochemicals are classified into five groups, as carotenoids, phenolics, alkaloids, nitrogen-containing compounds, and organosulfur compounds (Table 2.4), in which the most studied are the phenolics and carotenoids (Liu, 2004).

Phenolics are the products of secondary metabolism in plants and they are derivatives of benzene (cyclic derivatives in the case of polyphenols) possessing one or more aromatic rings with one or more hydroxyl groups (Dey and Harborne, 1989; Liu, 2004). They range from low-molecular-weight simple molecules such as phenolic acids to complex polymerised compounds (e.g. proanthocyanidins) (Manach *et al.*, 2009; Rice-Evans *et al.*, 1996). Plant phenolics are mainly synthesized from carbohydrates through two main pathways, including the shikimate and phenylpropanoid pathways (Figure 2.1) (Ferretti *et al.*, 2010).

Flavonoids constitute the major subclass of polyphenols, being the most abundant one in the human diet with more than 5000 (accounts for approximately two-thirds of the dietary phenols) compounds identified so far (Harborne, 1986; Perez-Jimenez *et al.*, 2010; Sampson *et al.*, 2002). Flavonoids are all derivatives of the 2-phenylchromone parent compound, having the characteristic C6 – C3 – C6 carbon skeleton. Their generic structure consists of two aromatic rings (A and B rings) linked by 3 carbons that are mainly in an oxygenated heterocycle ring, or C ring, all of which exhibit various levels of hydroxylation and methoxylation (Figure 2.2). They vary in the structure of the heterocyclic oxygen ring (C ring), and are classified due to these differences as flavonols, flavones, flavanols (catechins), flavanones, anthocyanidins, and isoflavonoids (Table 2.4) (Liu, 2004).

Fruits are indicated as substantial sources of flavonoids, but with the fact that there are significant differences in content, composition, and bioavailability (Crozier *et al.*, 2009; Prior *et al.*, 2005; Szajdek and Borowska, 2008). The classification of fruits is also based on the quantity of dominant flavonoids in them; for example, flavonols in apples and berries, catechins in red wine and tea, and anthocyanins in berries and cherries. (Burton-Freeman, 2010; Nijveldt *et al.*, 2001).

Sour cherry, *Prunus cerasus* L. (Rosaceae), is an autochthonous edible species with white flowers and tart red fruits. These heart-shaped drupes' colors range from light

to dark red, in which anthocyanin compounds present in fruit are responsible. Sour cherries are important sources of phenolics, specifically anthocyanins as the major flavonoids (Table 2.5). In addition, other flavonoid groups such as flavonols, and flavan-3-ols (procyanidins), as well as phenolic acids other than flavonoids have also been quantified in sour cherries (Bonerz *et al.*, 2007; Capanoglu *et al.*, 2011; Kim *et al.*, 2005; Kirakosyan *et al.*, 2010).

Table 2.4 : Classification of dietary phytochemicals (Dillard and German, 2000; Liu, 2004).

Class	**Subclass**	**Sub-subclass**	**Examples**
Carotenoids			α-carotene, β-carotene, β-cryptoxanthin, lutein, zeaxanthin, astaxanthin, lycopene
Phenolics	Phenolic acids	Hydroxy-benzoic acids	gallic, protocatechuic, vanillic, syringic
		Hydroxy-cinnamic acids	p-coumaric, caffeic, ferulic, sinapic
	Flavonoids	Flavonols	quercetin, kaempferol, myricetin, galangin, fisetin
		Flavones	apigenin, chrysin, luteolin
		Flavanols (Catechins)	catechin, epicatechin, epigallocatechin, epicatechin gallate, epigallocatechin gallate
		Flavanones	eriodictyol, hesperetin, naringenin
		Anthocyanidins	cyanidin, pelargonidin, delphinidin, peonidin, malvidin
		Isoflavonoids	genistein, daidzein, formononetin
	Stilbenes	no subclass	
	Coumarins	no subclass	
	Tannins	no subclass	
Alkaloids			amaryllidaceae, betalain, diterpenoid, indole, isoquinoline, lycopodium, monoterpene, sesquiterpene, peptide, pyrrolidine and piperidine, pyrrolizidine, quinoline, quinolizidine, steroidal, tropane compounds
Nitrogen-containing compounds			non-protein amino acids, amines, cyanogenic glycosides, glucosinolates, purines and pyrimidines
Organosulfur compounds			isothiocyanates, indoles, allylic sulfur compounds

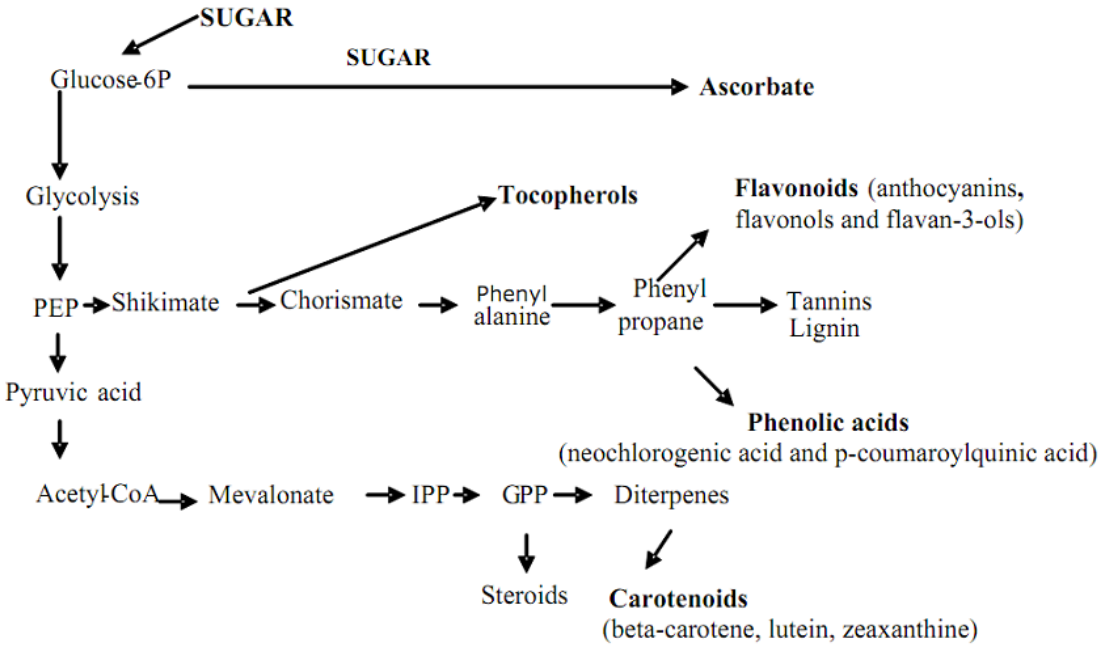

Figure 2.1 : Schematic representation of the two major pathways used for the production of plant phenolics (Ferretti *et al.*, 2010).

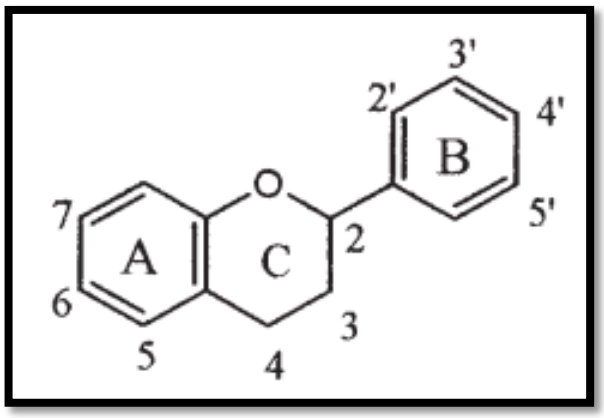

Figure 2.2 : The generic structure of flavonoids (Liu, 2004).

Table 2.5 : Total phenolics and total anthocyanins quantified in sour cherries in the literature.

Cultivar	Total phenolics [a]	Total anthocyanins [b]	Reference
Danube	161.7	65.5	Kim *et al.*, 2005
Balaton	146.1; 254.2	49.1; 45.0	Kim *et al.*, 2005; Kim and Padilla-Zakour, 2004
Schattenmorelle	295.5	72.4	Kim *et al.*, 2005
Sumadinka	312.4	109.2	Kim *et al.*, 2005
Karneol	366.0	56.8	Kim and Padilla-Zakour, 2004
Kroeker	398.5	67.1	Kim and Padilla-Zakour, 2004
Montmorency	407	8.7	Chaovanalikit and Wrolstad, 2004a
Northstar	335.2	66.8	Kim and Padilla-Zakour, 2004

[a] Total phenolics are represented as mg gallic acid equivalent (GAE)/100 g fresh fruit.
[b] Total anthocyanins are represented as mg cyanidin-3-glucoside equivalent/100 g fresh fruit.

2.2.1 Anthocyanins

Anthocyanins are flavonoids formed from phenylalanine through phenylpropanoid metabolism (Figure 2.1) (Ferretti *et al.*, 2010). As a flavonoid, they are polyhydroxy or polymethoxy derivatives of 2-phenylbenzopyrylium, containing A and B benzoyl rings seperated by a heterocyclic C ring (Figure 2.3) (Clifford, 2000). These water-soluble natural pigments provide the scarlet, magenta, purple, blue, and red colours of the fruits, flowers, as well as leaves of many plants (Glover and Martin, 2012). The anthocyanidin aglycones rarely exist in fresh plant material; instead all are found in nature in glycosylated form on the 3-carbon of the C-ring through an O-linkage to increase the stability in an aqueous solution (Figure 2.3) (Clifford, 2000; Glover and Martin, 2012). The variations in the number and the degree of methylation of the hydroxyl groups in the molecule; the nature, the number, and the position of the attached sugar moieties existing in the aglycone molecule; and the nature and the number of aromatic and aliphatic acids bound to the sugar molecules determine the structural differences in anthocyanin molecules (Mazza and Miniati, 1993).

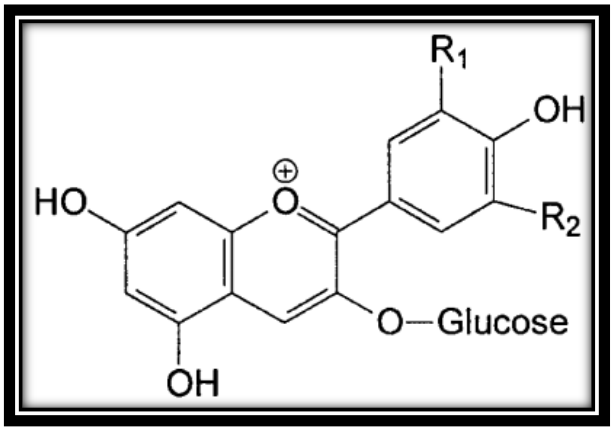

Figure 2.3 : Anthocyanin skeleton (Clifford, 2000; He and Giusti, 2010).

R_1	R_2	Anthocyanin	Color
H	H	Pelargonidin-3-glucoside	Red
OH	H	Cyanidin-3-glucoside	Magenta
OH	OH	Delphinidin-3-glucoside	Purple
OCH_3	H	Peonidin-3-glucoside	Magenta
OCH_3	OH	Petunidin-3-glucoside	Purple
OCH_3	OCH_3	Malvidin-3-glucoside	Purple

Anthocyanins differentiate from other flavonoids with their complex biochemistry. Their stability is influenced by several factors, such as temperature, light, and especially pH (Jackman *et al.,* 1987). Different pH values in aqueous solutions give rise to the existence of different molecular forms of anthocyanins. Anthocyanins are in their relatively stable flavinium cation form at pH values below 2, while between the pH values of 3 and 6 the colourless carbinol (hemiketal) pseudobase occurs. Quinonoidal bases with purple and violet colors are predominant at higher pH values (Glover and Martin, 2012; McGhie and Walton, 2007).

Cherry fruits have been reported among the richest sources of anthocyanins (Koponen *et al.*, 2007; Pantelidis *et al.*, 2007; Wang *et al.*, 1997; Wu *et al.*, 2006a). Anthocyanin contents of some fruits are presented in Table 2.6. Sour cherry varieties was found to contain higher amounts of anthocyanins compared to the sweet cherry varieties (Ferretti *et al.*, 2010). Various anthocyanin compounds have been identified in several products of sour cherry fruit including cyanidin-3-glucosylrutinoside, cyanidin-3-rutinoside, cyanidin-3-sophoroside, cyanidin-3-glucoside (Bonerz *et al.*, 2007; Chandra *et al.*, 1992; Chaovanalikit and Wrolstad, 2004b; Kim *et al.*, 2005; Kirakosyan *et al.*, 2009; Wang *et al.*, 1997). The anthocyanin composition of raw sour cherry fruit and various sour cherry products are given in Table 2.7.

Table 2.6 : Anthocyanin contents in common fruits (Koponen *et al.*, 2007).

Fruit	Total Anthocyanins (mg/100 g fresh weight)
Cherry	66.4
Grape, red	37.6
Plum, dark	25.1
Grapefruit, red	5.9
Peach	4.2
Nectarine	2.4
Apple, Red Delicious	1.7

Table 2.7 : Anthocyanin composition of sour cherry fruit and its products.

Product	Cya-3-soph	Cya-3-gluc-rut	Cya-3-gluc	Cya-3-rut	Total anthocyanins
Raw fruit[a]		89.0 – 227.7	1.4 – 2.8	15.5 – 22.0	49.1 – 109.2
Frozen fruit[b]	1.6 – 19.3	434.3 – 1258.7	5.8 – 49.1	218.1 – 688.2	533 - 1741
IQF powder[b]	4.1 – 14.5	375.7 – 487.4	7.1 – 19.2	226.1 – 342.9	482 - 1063
Dried fruit[b]	13.9 – 15.7	64.8 – 203.6	3.6 – 7.6	24.9 – 95.8	173 - 564
Juice concentrate[b]	2.2 – 15.5	105.7 – 350.9	1.7 – 13.3	46.6 – 130.3	213 – 722
Juice[c]	39 – 185	361 – 515		125 – 213	569 – 858

Cya-3-soph = Cyanidin-3-sophoroside; Cya-3-gluc-rut = Cyanidin-3-glucosyl-rutinoside; Cya-3-gluc = Cyanidin-3-glucoside; Cya-3-rut = Cyanidin-3-rutinoside

[a] Values are represented as mg/100 g fresh fruit for four different varieties (Kim *et al.*, 2005)

[b] Values are represented as µg/g dry-weight for two different varieties (Kirakosyan *et al.*, 2009)

[c] Values are represented as mg C3G/L juice for five different varieties (Bonerz *et al.*, 2007)

2.2.2 Other flavanoids

Sour cherry fruit has been pointed out to be an important source of flavonoids other than anthocyanins. Flavan-3-ols (i.e. catechin, epicatechin) and flavonols (i.e. quercetin-3-rutinoside, quercetin-3-glucoside, kaempferol-3-rutinoside) are the two subclasses that are identified and quantified in sour cherry (Bonerz *et al.*, 2007; Capanoglu *et al.*, 2011; Chaovanalikit and Wrolstad, 2004b; Ferretti *et al.*, 2010; Kim *et al.*, 2005; Kirakosyan *et al.*, 2010).

Flavanols are a flavonoid subclass which refers to the phenolic compounds called proanthocyanidins or condensed tannins. These flavonoid polymers determine the flavor and astringency in teas, wines, and fruit juices (Gawel, 1998; Porter, 1989). Flavan-3-ol subunits, including (+)-catechin and (-)-epicatechin, are the building blocks of most proanthocyanidins (Figure 2.4). The extension units of proanthocyanidins turn into the colored anthocyanidins through acid hydrolysis, which is used for their analysis (Kennedy and Jones, 2001; Porter, 1989). While

proanthocyanidins represent the oligomers or polymers of flavan-3-ol subunits, anthocyanins are specified as monomeric molecules with glycosidic moieties (Capanoglu *et al.*, 2011).

There are two types of chromatographic methods used for the analysis of proanthocyanidins, one type of methodology analyzes proanthocyanidins in their intact forms, and the second analyzes the proanthocyanidins after their acid-catalyzed cleavage (Matthews *et al.,* 1997). Proanthocyanidins are depolymerized under acidic conditions, resulting in the release of their terminal subunits as flavan-3-ol monomers and their extension subunits as electrophilic flavan-3-ol intermediates (Figure 2.4). Nucleophilic reagents (i.e. phloroglucinol, benzyl mercaptan, etc.) can be used to trap the electrophilic intermediates in order to convert them into analyzable adducts. This method also enables to determine the degree of polymerization (DP) of the different-sized oligomeric fractions (Kennedy and Jones, 2001).

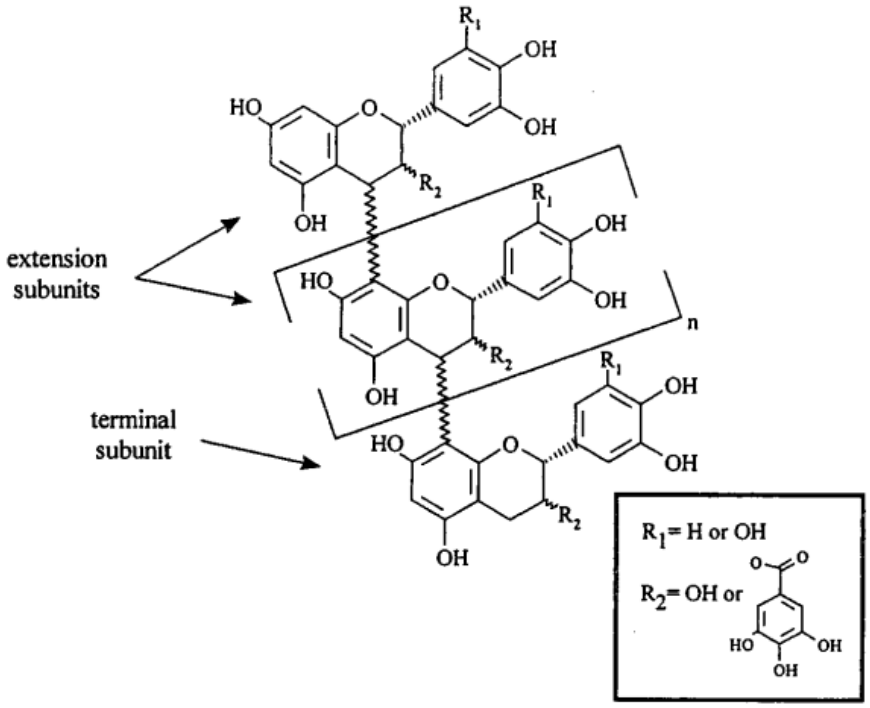

Figure 2.4 : General proanthocyanidin structure (Kennedy and Jones, 2001).

Cherry fruits have been reported to contain substantial amounts of proanthocyanidins (Auger *et al.*, 2004; Capanoglu *et al.*, 2011; Goncalves *et al.*, 2004). Fruits that are known to be rich sources of proanthocyanidins are listed in Table 2.8. Epicatechin is the most abundant flavanol in cherries and cherry products followed by catechin

(Table 2.9) (Auger *et al.*, 2004). One of the distinctive characteristics of sour cherries from the other cherry varieties is their short-chain procyanidins with low degree of polymerization values (DP ≤ 3). There is an apparent relation between DP values of procyanidins, which can range from two subunits to several hundred units, and their absorption in the intestinal tract (Deprez *et al.*, 2001; Khanal *et al.*, 2009). Khanal *et al.* (2009) pointed out that long-chain procyanidin oligomers and polymers were poorly absorbed, while short-chain procyanidins (monomers, dimers, and trimers) (DP value up to 3) absorbed more efficiently.

Table 2.8 : (+)- Catechin and (-)- epicatechin contents of common fruits [a] (Arts *et al.*, 2000).

	mg catechin/kg fresh edible weight		
Fruit	**(+)- catechin**	**(-)- epicatechin**	**total catechins**
Grape, black	89.4±91.8	86.4±71.2	203.9
Blackberry	6.6±0.58	180.8±21.4	187.4
Cherry	21.7±9.2	95.3±24.8	117.1
Apricot	49.5±43.7	60.6±78.5	110.1

[a] Data include seasonal and regional variations.

Table 2.9 : Procyanidin contents of sour cherry fruit and its juice.

Fruit	**(+)- catechin**	**(-)- epicatechin**	**total catechins**
Raw fruit[a]	15.4	312.5	327.9
Juice[b]		24 – 336	

[a] Flavan-3-ol contents in mg/100 g dry-weight (Capanoglu *et al.*, 2011).
[b] Values are represented as mg/L juice (Bonerz *et al.*, 2007).

Flavonol glycosides, including the rutinosides and glucosides of quercetin and kaempferol, are another important group of sour cherry phenolic phytochemicals, among which quercetin-3-rutinoside is identified as the major flavonol by several studies (Bonerz *et al.*, 2007; Chaovanalikit and Wrolstad, 2004b; Kim *et al.*, 2005; Kirakosyan *et al.*, 2009; Wang *et al.*, 1999). Chaovanalikit and Wrolstad (2004b) compared the sour and sweet cherry flavonols in their study and concluded that sour cherry had a higher flavonol glycoside content (11.2 mg/100 g fresh-weight). Qeurcetin-3-rutinoside, quercetin-3-glucoside, and kaempferol-3-rutinoside contents of four different sour cherry cultivars were determined to range between the values of 0.97 – 4.36, 0.21 – 0.44, 0.30 – 1.29 mg/100 g fresh-weight sample, respectively (Kim *et al.*, 2005). The contents of these flavonol compounds were found to be in between 18 – 59, 3 – 8, 4 – 13 mg/L in processed juice of five different sour cherry cultivars, respectively (Bonerz *et al.*, 2007).

2.2.3 Phenolic acids

The term phenolic acids is described as simple phenols that possess one carboxylic acid functionality and refer to a specific group of organic acids while describing plant metabolites. These naturally occurring plant metabolites are classified into two groups as hydroxycinnamic (Xa in Figure 2.5) and hydroxybenzoic acids (Xb in Figure 2.5) (Robbins, 2003).

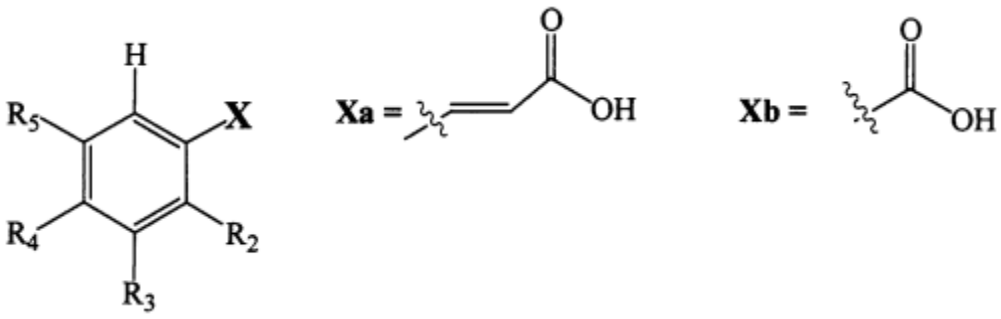

Figure 2.5 : General structure of the naturally occurring phenolic acids (Robbins, 2003).

Phenolics acids account for almost all of the remaining one-third of the phenolics, following flavonoids. Sour cherries are listed among the fruit products those possess substantial amounts of hydroxycinnamates and also with relatively higher amounts than sweet cultivars. Especially, neochlorogenic acid, chlorogenic acid, and *p*-coumaroylquinic acid were reported to be the predominant phenolic acids in sour cherry (Bonerz *et al.*, 2007; Chaovanalikit and Wrolstad, 2004b; Kim *et al.*, 2005; Robbins, 2003). Chlorogenic acid derivatives composed 75.5 to 92.5% of total hydroxycinnamic acids with the values of neochlorogenic acid ranging between 6.7 to 27.8 mg and chlorogenic acid from 0.6 to 5.8 mg per 100 g fresh sample in four different sour cherry cultivars investigated by Kim *et al.* (2005). On the other hand, Bonerz *et al.* (2007), who investigated the phenolic profiles of sour cherry juices, reported that neochlorogenic acid (212 – 998 mg/L juice), 3-coumaroylquinic acid (191 – 999 mg/L juice), and chlorogenic acid (119 – 268 mg/L) were the most abundant colorless phenolics.

2.3 Effects of Processing on Sour Cherry Antioxidants

The natural antioxidants are suggested to be consumed through the consumption of foods those are naturally rich in these bioactive compounds, instead of consumption in the form of diet supplements (Southon, 2000). On the other hand, it is pointed out that food composition tables, which are tools used for epidemiological and nutritional studies, are generally only referring to the raw state consumption of foodstuffs; whereas it is not taken into consideration that nutritional properties and biological activities may be largely affected by several factors, including processing as one of the major ones (Greenfield and Southgate, 2003). The evaluation of the influence of food processing on naturally occurring antioxidants is of great importance since foods are mostly consumed after processing to various products in terms of several quality and safety aspects, and for economic reasons. The understanding of the consequences of food processing on food composition is vital in order to preserve and/or improve the antioxidant activity and bioavailability of these functional food components (Nicoli *et al.*, 1999).

Fruit processing have been indicated, for a long time, to result in lower bioactivity in processed products compared to the fresh ones, since the fact that only the interested and barely stable antioxidant compounds (i.e. vitamin C) have been studied to evaluate the effects of processing (Lund, 1979; Miller *et al.*, 1995). Processing, such as maceration, separation steps, can lead to a loss of antioxidants in processed foods compared with their fresh counterparts, as a result of oxidation, thermal degradation, leaching, etc. These conclusions are particularly true in the case of more sensitive compounds such as ascorbic acid and phenolics (Kalt, 2006). However, now in current years, it is well known that food processing not always negatively affect the functional properties of food components (Nicoli *et al.*, 1999). There are several recent studies reported that compounds possessing antioxidative effects, including lycopene or β-carotene, may have result in increased quantities and bioavailability as a result of food processing techniques such as moderate heating or the enzymatic disruption of cell wall (Capanoglu, 2008; Graziani *et al.*, 2003; Shi and Le Maguer, 2000). Blanching also helps in retaining the original antioxidant activity properties of raw material through preventing enzymatic oxidations (Nicoli *et al.*, 1999).

Fruit antioxidants are localized in various parts of plant materials which becomes important while processing these foods into specific products such as juice and wine, in which the separation of peels and seeds is necessary. Certain antioxidant compounds, such as anthocyanins, are typically localized in peel, although there exists differences between species and cultivars in this aspect (Kalt, 2006). Thus, processing can result in reduced levels of these compounds in processed juices and yield by-products those are rich sources of related compounds (Skrede *et al.*, 2000).

Sour cherry fruit, a substantial food source of anthocyanins, is commonly consumed after being processed into various products, predominantly fruit drinks (nectar), instead of consumption in fresh form. Sour cherry nectar processing includes several steps, including mash heating, mash pressing, clarification, filtration, sucrose syrup addition, etc. (Figure 3.1, Table 3.1), which can have drastic effects on anthocyanin pigment stability, and so in quantity and bioactivity. Anthocyanin stability has been reported to be strongly influenced from the processing parameters as well as the intrinsic properties of the product such as pH, temperature, light, oxygen, enzymes, proteins, sugar, metallic ions, and the chemical structure and the concentration of anthocyanins present (Francis, 1989; Rein, 2005).

There are only a few studies those investigated and evaluated the effects of food processing on cherry antioxidants, which mostly focused on anthocyanins as the major antioxidant compound in this fruit (Chaovanalikit and Wrolstad, 2004a; Chaovanalikit and Wrolstad, 2004b; Kim and Padilla-Zakour, 2004; Kirakosyan *et al.*, 2009). Canning of sweet cherries was found to result in approximately 50% transfer of cherry anthocyanins and phenolics from the fruits into the syrup. Heat processing applied during canning was reported not to lead a loss, instead an apparent slight increase in total anthocyanin and total phenolic contents, which was partly linked to the increased efficiency of extraction in the softened fruits (Chaovanalikit and Wrolstad, 2004a). Macerated peel tissue could have an increased membrane permeability at high temperatures, facilitating the extraction of compounds (Spanos *et al.*, 1990). Additionally, bound phenolics may also be released with the breakdown of cellular constituents with temperature (Dewanto *et al.*, 2002). The analyses of various sour cherry products, including frozen and dried (dried with or without sugar) cherries, powders from individually quick frozen (IQF) cherries, and cherry juice concentrate revealed that the highest total anthocyanin

levels were in frozen cherries followed by IQF powder, juice concentrate, dried without sugar and dried with sugar (added in quantities of 15% of fresh weight) products (Kirakosyan *et al*, 2009). In contrast to this finding, there are several studies reported the stabilizing effect of sugar on anthocyanins (Rubinskiene *et al.*, 2005; Tsai *et al.*, 2004; Wrolstad *et al.*, 1990). Sucrose concentratios at more than 20% were pointed out to increase pigment stability which could be linked to the reduced water activity (a_w) and/or increased ability of binding water molecules (Rubinskiene *et al.*, 2005; Tsai *et al.*, 2004). In another study processing of sour cherries into jam was determined to result in hardly any change in total phenolic content, while up to 79% losses obtained in total anthocyanins. Boiling step applied to grinded fruit during jam making was suggested to give rise to the destruction of anthocyanin pigments (Kim and Padilla-Zakour, 2004). Thermal degradation of anthocyanis have been determined to follow 1^{st}-order reaction kinetics, and longer heating times at higher temperatures may cause higher anthocyanin losses (Cemeroglu *et al.*, 1994; Wang and Xu, 2007). Jam processing involves long boiling times at 104-105ºC to reach to the final brix (ºBx) values of 65 – 68ºBx (Kim and Padilla-Zakour, 2004), which can lead to higher degradation rates of anthocyanins.

Although there are no studies in literature evaluating the effects of juice processing on sour cherry anthocyanins and other polyphenolics, various anthocyanin-rich fruits other than sour cherry were evaluated for the changes in their phenolics, predominantly anthocyanins, during processing into juice. Bayberry (*Myrica rubra* Sieb. et. Zucc.) juice processing gave rise to more than 69% anthocyanin loss during crushing, depectinization, and centrifugation steps, followed by approximately 5 – 10%, 2 – 5%, and 1 – 6% losses during subsequent gelatin-bentonite fining step, filtration step, and ultra-high temperature operation, respectively. On the other hand, application of heat by additional blanching of fruit (90ºC, 2 min) and/or pasteurization (90ºC, 1 min) of crushed pulp were determined to result in 1.5 – 2.0 times higher anthocyanin contents in final juice, which pointed out decreased anthocyanin degradation -through the inactivation of native enzymes- or increased anthocyanin extraction -through cellular breakdown and/or increased membrane permeability- with these applications (Fang *et al.*, 2006). Similar observations were also reported by Lee *et al.* (2002) and Rossi *et al.* (2003) with the application of heat treatment in blueberry juice processing. In addition, only 16 – 26% of the total berry

polyphenolics were determined to remain in the final UHT juices, as a result of losses of 57 – 74% in crushing, depectinization, and centrifugation steps, 13 – 17% losses through fining, and 3 – 9% losses during filtration. Remarkably, 52 – 58% of fresh fruit anthocyanins and 30 – 35% of fresh berry polyphenolics left in the centrifuged cakes (Fang *et al.*, 2006).

The processing of highbush blueberries into juice was determined to result in approximately 50% losses in anthocyanins when the content remained in the press cake (18% of the anthocyanins in starting fruit material) was not taken into account. The most significant losses in individual anthocyanins occurred during processing to the initial pressed juice which were related to the enzymatic degradation subsequent to initial pressing, since there was no any heat treatment applied before pressing. On the other hand, comparison of the proportions of the individual blueberry anthocyanins to the total anthocyanins revealed that while the proportion of the malvidin derivatives increased from 44% (in fruit) to 63% (in pasteurized juice), these proportions were determined to decrease from 12% to 5% for delphinidin glycosides which indicated the role of different chemical structures on stability. Among the other blueberry polyphenolics, chlorogenic acid released easily with pressing with very little amounts (1%) remained in the press cake. This was related to the higher water solubility of chlorogenic acid and localization in the cell vacuole, whereas anthocyanins were reported to have relatively lower solubility in water and to be associated with cell-wall material in epidermal tissue. However, approximately 50% of chlorogenic acid was lost due to the enzymatic degradation during processing. Flavonol glycosides in blueberry were reported to follow a different trend compared to anthocyanins and chlorogenic acid. They were not as susceptible to enzymatic degradation as the anthocyanins and the chlorogenic acid, existing in higher proportions in the initial pressed juice and remaining in lower concentrations in the press cake. Procyanidin levels in highbush blueberry juice was 40% of those found in the raw fruit material. Relatedly, press cake involved little (3%) of the fruit procyanidins. (Skrede *et al.*, 2000). On the other hand, in contrast to the finding of Skrede *et al.* (2000), procyanidins have been reported to be bound easily to the cell-wall polysaccharides through hydrogen-bonding and/or hydrophobic interactions (Le Bourvellec *et al.*, 2004; Renard *et al.*, 2001). Accordingly, in another study blueberry juice processing resulted in the removal of 24% of total procyanidins with press

cake, due to the strong affinity of these compounds to the cell wall polysaccharides (Brownmiller *et al.*, 2009).

Degradation of anthocyanins during black currant nectar processing occurred during the 1 h standing period (at ≈ 35ºC) of raw juice applied after the mash treatment (Iversen, 1999). Since anthocyanins exist only in the skin fraction of black currant berries, pectolytic mash enzymes are used in order to release the anthocyanin compounds into the raw juice through disintegrated cell walls of the skin, by the action of these enzymes (Wightman and Wrolstad, 1996). However, these mash enzymes have been reported to have much higher anthocyanin degrading activity in the raw juice, after the pulp removal (Wightman and Wrolstad, 1995). Therefore, leaving the raw juice in storage tanks for hours, for stabilization before clarification, results in substantial losses in anthocyanins (Iversen, 1999).

Woodward *et al.* (2011) reported non-significant losses of anthocyanins during pre-press blackcurrant fruit processing and post-press blackcurrant juice processing. Pre-press fruit processing included the milling, mash heating (50ºC), and sodium bisulphite addition with less than 13% anthocyanin loss compared to the initial whole berry fruit. On the other hand, post-press juice processing resulted in 17% total anthocyanin loss through the pasteurization, decantation, filtration, and concentration steps applied. However, there was a significant loss of anthocyanins observed between the pre-press mash and post-press juice samples which was indicated to be most likely due to the suboptimal extraction of anthocyanin components from the berry pomace, leading to the removal of some anthocyanins with the berry skins.

In conclusion, fruit phenolics are diversely affected during food processing by several factors, resulting in either loss or increase in contents. The processing parameters (temperature, time, processing aids, etc.) as well as the chemical structure and the localization of those structures in fruit determine their fate in final products.

2.4 Bioavailability of Sour Cherry Phenolics

2.4.1 Dietary intake of flavonoids

The relation between fruit and vegetable intake and reduced risk of diseases in humans has been attributed to the bioactivity of specific food components included vitamins, minerals, fibre, and more recently, phytochemicals, particularly phenolic

compounds (Knekt *et al.*, 2002; Mennen *et al.*, 2004). Fruits, as natural vehicles of phenolics, are known to differ substantially in content, composition, and bioavailability (Crozier *et al.*, 2009; Prior *et al.*, 2005). Estimates of dietary intake of phenolic compounds can range up to several folds depending on individual dietary patterns, as well as methods used for assessment (Burton-Freeman, 2010). For instance, the amount of flavonoid consumption in USA has been suggested to be 20 mg/day (Sampson *et al.*, 2002) based predominantly on flavonol and flavone intake, while this suggestion is approximately 190 mg/day (Chun *et al.*, 2007) based on intake of flavan-3-ols, flavanones, flavonols, anthocyanidins, flavones, and isoflavones (Burton-Freeman, 2010). Flavonoid intakes of individuals for several countries have been reported to be estimated to range from about 20 mg/day (USA, Denmark, Finland) to more than 70 mg/day (Holland) (Beecher, 2003).

Anthocyanins are widespread in food plants, occurring in 27 families (Timberlake, 1988), with the potential dietary intake among the greatest of the various classes of flavonoids (Wu *et al.*, 2006a). Edible anthocyanin sources in nature include coloured fruits such as berries, cherries, grapes, pomegranates, and plums, as well as many dark-coloured vegetables such as red onion, eggplant, red cabbage, and black bean (Eder, 2000; Wu *et al.*, 2006a). Anthocyanins most likely constitute the most important subclass of flavonoids with regard to the mass consumed (He and Giusti, 2010), i.e. whose annually global consumption from black grapes has been estimated to be 10000 tons (Clifford, 2000). Kühnau (1976) previously estimated daily consumption of anthocyanins as 180-215 mg/day per person but in a recent report published by the USDA, the estimation was 12.5 mg/day per person in the USA (Wu *et al.*, 2006a).

It is well known that in addition to the varying contents of total anthocyanins in foods, the abundance of the six common individual anthocyanidins in the edible parts of plants also varies greatly (He and Giusti, 2010; Wu *et al.*, 2006a). Previously, Kong *et al.* (2003) estimated the abundance order as cyanidin (50%), pelargonidin (12%), peonidin (12%), delphinidin (12%), petunidin (7%), and malvidin (7%), while, in a more recent study, the abundance order was estimated to be cyanidin (30%), delphinidin (22%), pelargonidin (18%), peonidin (7.5%), malvidin (7.5%), and petunidin (5%) (Andersen and Jordheim, 2006). The three nonmethylated anthocyanidins (cyanidin, delphinidin, pelargonidin) were pointed out to be more

widespread than the three methylated anthocyanidins (peonidin, malvidin, petunidin), in both reports (Andersen and Jordheim, 2006; Kong *et al.*, 2003). These different anthocyanidin aglycons with different sugar moieties attached have recently been indicated to have different responses in terms of their bioavailability and potential health effects (Felgines *et al.*, 2003; Marko *et al.*, 2004; Wu *et al.*, 2002; Wu *et al.*, 2004; Wu *et al.*, 2006b).

2.4.2 Health-promoting effects of sour cherries

Sour cherry contains several phenolic compounds whose biological activities - including antioxidant, anti-inflammatory, and anticancer properties- have recently been investigated in different experimental models.

The antioxidant and anti-inflammatory activities of sour cherry extracts has been studied using different methodological approaches. Blando *et al.* (2004) determined the *in vitro* antioxidant capacities of different sour cherry cultivars to range between 1.1-1.9 μmol Trolox equivalents (TE)/100g, using an ORAC (oxygen radical absorbance capacity) assay. Remarkably, these values are comparable to those found in some berry fruits (i.e. strawberry). In another study, sour cherry phenolics were determined to have a protective effect on neuronal PC 12 cell lines (derived from a transplantable rat pheochromocytoma) from cell-damaging oxidative H_2O_2 toxicity *in vitro*. In addition, since anthocyanins comprise the most abundant flavonoid group in cherry cultivars (> 70% of total phenolics), the role of anthocyanins individually was also investigated in this *in vitro* cytoprotection process. The PC 12 cell viability showed highly positive correlation (r^2 0.68-0.87) to anthocyanins, suggesting that cherry anthocyanins played a more important role than the other phenolics present (Kim *et al.*, 2005). The antioxidative and anti-inflammatory effects of sour cherry phenolics have also been shown by *in vivo* experiments. Mice having diet supplementation with sour cherry juice feeding *in vivo*, were shown to have increased superoxide dismutase antioxidative enzyme activity in liver and blood, and gluthathione peroxidase antioxidative enzyme activity liver tissues, respectively. Sour cherry juice consumption also provided cyclooxygenase-2 inhibitory activity in the mice peritoneal macrophages, proving the anti-inflammatory effect of sour cherry, which has been most likely linked to the abundance of anthocyanins (Saric *et al.*, 2009). Cyclooxigenases are pro-inflammatory enzymes that have an important

role in processes such as inflammation, carcinogenesis, apoptosis, cell proliferation, and angiogenesis (Mulabagal *et al.*, 2009; Wang *et al.*, 1999). Sour cherry consumption was also reported to reduce inflammation-induced thermal hyperalgesia, mechanical hyperalgesia, and paw edema in rat, related with the anti-inflammatory and antioxidant properties of anthocyanins (Tall *et al.*, 2004). In a study on human subjects, 12 volunteer older adults (6 men and 6 women, age 69±4 year) consumed either sour cherry juice or placebo (240 mL twice daily for 14 days) in random order, and the capacity to resist oxidative damage was determined with the measurements on the changes in plasma F_2-isoprostane levels, a marker of oxidative damage, in response to forearm ischemia-reperfusion. Sour cherry juice intake was associated with a reduced I/R-induced F_2-isoprostane response, suggesting that the sour cherry juice consumption enhances antioxidative activity *in vivo* in older adults (Traustadottir *et al.*, 2009). Sour cherry anthocyanins were also pointed out to play an effective role in the inhibition of other inflammation related diseases, such as obesity and diabetes. *In vivo* animal models (Zucker fatty rat model of obesity and metabolic syndrome and Dahl Salt-Sensitive rat) revealed that diets enriched with sour cherry resulted in reduced plasma and tissue inflammation. Dietary intake of sour cherries led to significant reductions in levels of proinflammatory molecules in plasma (interleukin-6, TNF-α) and increases peroxisome proliferator-activated receptors (PPARs) in abdominal fat and in hepatic tissues. These modifications were linked to the decreases observed in serum glucose, triglycerides, and cholesterol levels (Seymour *et al.*, 2008; Seymour *et al.*, 2009). In addition, Ataie-Jafari *et al.* (2008) indicated that concentrated sour cherry juice consumption with amounts of 40 g/day for 6 weeks led to a decreased body weight, blood pressure, and hemoglobin A1c in diabetes type 2 women and improved blood lipids in diabetic patients with hyperlipidemia.

Anthocyanins have been shown to possess multiple anticarcinogenic effects using several *in vitro* cell culture systems, including colon, endothelial, liver, breast and leukemic cells, and keratinocytes (Bomser *et al.*, 1996; Bomser *et al.*, 1999; Kamei *et al.*, 1995; Meiers *et al.*, 2001; Nagase *et al.*, 1998; Wang and Stoner, 2008). Sour cherry anthocyanins were reported to inhibit intestinal tumor development in Apc^{Min} mice and growth of human colon cancer cell lines HT 29 and HCT 116, suggesting that sour cherry anthocyanins have a substantial potential to reduce the risk of colon

cancer (Kang *et al.*, 2003). Moreover, Bobe *et al.* (2006) investigated the cancer chemoprevention effect of a therapy including the simultaneous utilization of anthocyanin-rich sour cherry extract (as dietary phytochemical source) with an anti-inflammatory drug to Apc^{Min} mice for 19 weeks. This combination was determined to result in fewer tumors in the small intestine when compared to mice fed with the drug alone.

2.4.3 Absorption mechanisms and metabolism of anthocyanins

The bioavailability of the intact phenolic phytochemicals is a fundamental factor for their physiological functions (Keppler and Humpf, 2005). To unravel mechanisms of action of dietary flavonoids in their potential role in disease prevention and to validate their prominent health-promoting effects, it is crucial to know the factors that determine their release from foods and to consider their bioavailability (He and Giusti, 2010; Hollman, 2004). It is not only important to know how much of a nutrient is present in a food or dietary supplement, but even more important to know how much of that present is bioavailable. The commonly accepted definition of bioavailability is "the proportion of a nutrient that is digested, absorbed, and metabolized through normal pathways" (Srinivasan, 2001).

Anthocyanins are known to differ from the other flavonoid groups in terms of their apparent low bioavailability (less than 0.1% of the ingested dose), quick bioabsorption (t_{max} in plasma is 15-60 min, and excretion is complete within 6-8 h), and absorption and distribution into the circulatory system in their intact glycosidic forms without undergoing extensive metabolism of the parent glycosides to glucurono, sulfo or methyl derivatives (McGhie *et al.*, 2003; McGhie and Walton, 2007; Miyazawa *et al.*, 1999).

Feeding studies performed using animals and humans reported that typically 0.1% or even much less of the ingested anthocyanins were detected in urine (McGhie *et al.*, 2003; Wu *et al.*, 2005). Reported bioavailability of some other polyphenols was 1-5% for quercetin, 10-30% for flavanones and flavanols, 30-50% for isoflavones, and gallic acid (Scalbert and Williamson, 2000). Although anthocyanin bioavailability have been determined to appear low, it could likely be underestimated for two main reasons; some important metabolites might have been ignored or the methods used might need to be optimized for the analysis of the anthocyanin metabolites. In most

studies, anthocyanins are measured with UV-visible light detection, relying on the conversion of all the chemical forms of anthocyanins into the red coloured flavylium cation with acidification, whereas there is still a possibility of the existence of the other chemical forms at neutral pH (Manach *et al.*, 2005). On the other hand, in a study performed by Felgines *et al.* (2003) the metabolites of strawberry anthocyanins, except for the native glucoside, were found to be very unstable and degraded to a large degree when acidified samples were stored in frozen state, making the detection of these metabolites with the current technology impossible.

To the current knowledge in the literature, anthocyanins are also seemed to differentiate from the other flavonoids in their metabolism. While flavonoids other than anthocyanins are generally detected in plasma and urine as their glucuronidated and/or sulphated derivatives, anthocyanins are detected in their unchanged glycosidic forms in most studies (Manach *et al.*, 2005). However, there are also some recent studies those identified the glucuronides and sulfates of anthocyanins in human urine with HPLC/MS-MS approach (Felgines *et al.*, 2003; Wu *et al.*, 2002). Figure 2.6 shows the absorption and metabolism of anthocyanins based on the current knowledge (McGhie and Walton, 2007).

After ingestion, anthocyanin glycosides can be rapidly absorbed from the stomach with a process including bilitranslocase, which is a plasma membrane organic anion carrier expressed both in the basolateral side of the liver plasma membrane and in the gastric epithelium (Passamonti *et al.*, 2002; Passamonti *et al.*, 2003). Methylation and glucuronidation reactions may take place to a some degree to metabolise a portion of anthocyanins, which are subsequently transported to the intestine as bile. In the higher pH of the small intestine, anthocyanin glycosides (that are not absorbed in the stomach) may be converted to the other chemical forms (hemiketal, chalcone, and quinonoidal forms). Further absorption appears to take place in the jejunum. Absorbed anthocyanins passes through the liver and enters the systemic circulation, where they may be metabolised. Anthocyanins those reach to the colon may be subjected to a microbial degradation, and those degradation products derived from the ingested anthocyanins, may play a role on the health effects of anthocyanins (McGhie and Walton, 2007). Despite these suggestions for anthocyanin 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 anthocyanins are

absorbed, how the variation of molecular structures consumed in food, and how the forms generated *in vivo* contribute to the health benefits.

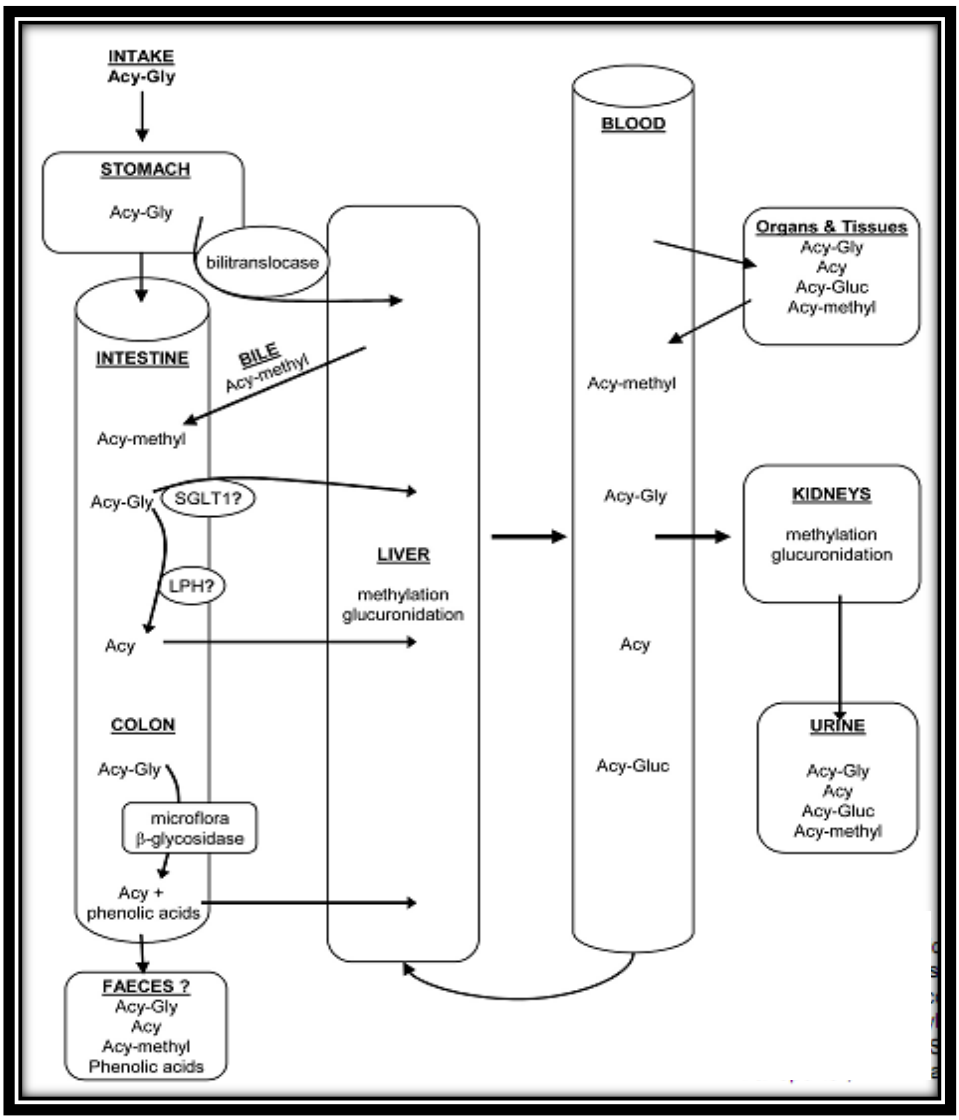

Figure 2.6 : Schematic representation of the absorption and metabolism of anthocyanins based on the current literature. Acy-Gly, anthocyanin glycosides; Acy-methyl, methylated metabolite; Acy-Gluc, glucuronidated metabolite; SGLT-1, sodium dependent glucose transporter; LPH, lactase phloridzin hydrolase (McGhie and Walton, 2007).

The represented protective role of anthocyanins, i.e. against oxidation (Ghosh *et al.*, 2006), tumorogenesis (Chen *et al.*, 2006; Zhang *et al.*, 2005), and inflammation (Munoz-Espada and Watkins, 2006), on mammalian cells are good indicators of potential interaction of anthocyanins with cells directly (McGhie and Walton, 2007). Anthocyanins have been reported to cross the cell membrane and can be detected in the cells (Youdim *et al.*, 2000). Accordingly, Yi *et al.* (2006) determined the *in vitro* absorption of blueberry anthocyanins into Caco-2 human intestinal cell monolayers.

The structure of the anthocyanidin aglycone and the nature of the sugar moiety attached have been pointed out to be the determinants of the absorption and the excretion phenomena of anthocyanins (McGhie *et al.*, 2003; Wu *et al.*, 2005). Yi *et al.* (2006) reported a higher transport efficiency of malvidin anthocyanidin than delphinidin, as well as the glucoside structure of the same aglycon had a higher transport efficiency in comparison to the galactoside structure.

To assess the true bioavailability of bioactive phytochemicals, essential data is required on their absorption, metabolism, tissue and organ distribution, and excretion (van de Waterbeemd *et al.*, 2004). Since the studies carried out using *in vivo* human and animal models are known to be highly complex, expensive, raise moral and ethical questions, and may be confounded by various factors, i.e., chemical instability and inadequate analytical methodology, it is easier to control these parameters in an *in vitro* model, which is simple, more convenient, and less expensive (McDougall *et al.*, 2005b; Yi *et al.*, 2006). In the current literature, the most commonly used *in vitro* models to assess the bioavailability of bioactive food components, including anthocyanins, are *in vitro* simulated gastrointestinal digestion model and *in vitro* Caco-2 cell model.

2.4.3.1 *In vitro* gastrointestinal (GIT) digestion model

Studies conducted to mimick the gastrointestinal tract can provide a simple predictive instrument to assess the stability and to investigate the potential bioavailability of dietary compounds. Moreover, these studies enable monitoring and comparing a set of samples, providing data about the effect of different food matrices on the recovery of individual components (Fazzari *et al.*, 2008). The first *in vitro* digestion was performed by Miller *et al.* (1981) determining the iron content in IN (serum available, dialyzable) fraction which was closely correlated to the serum iron bioavailability *in vivo*. This methodology was further adapted for assessing the potential bioavailability of anthocyanins and other phenolics in various fruit products (Gil-Izquierdo *et al.*, 2001; Gil-Izquierdo *et al.*, 2002; McDougall *et al.*, 2005a; McDougall *et al.*, 2005b; Perez-Vicente *et al.*, 2002). The low recovery of anthocyanins in the IN fraction from the *in vitro* digestion procedure was pointed out to match the low bioavailability of serum anthocyanins observed in *in vivo* animal and human feeding trials (Bub *et al.*, 2001; Lapidot *et al.*, 1998).

In vitro GIT digestion model mimics the physicochemical and biochemical conditions encountered in the upper GIT (McDougall *et al.*, 2005b). The gastric digestion is simulated with pepsin-HCl digestion at pH 2 and 37ºC for 1h, and small intestine conditions are simulated with pancreatin-bile digestion at pH 6.9 and 37ºC for 2 h. The samples are collected after gastric digestion and after small intestinal digestion. From the latter, two fractions are obtained as IN fraction that represents the "serum available or dialyzable" fraction and OUT fraction that represents the "colon-available or undialyzable" fraction (Fazzari *et al.*, 2008; Liu *et al.*, 2004). The knowledge on relative stability of phytochemicals under GIT conditions is necessary for the assessment of the bioavailability of these compounds, as it will influence the possible active mechanisms in the stomach (Passamonti *et al.*, 2003) or the small intestine (Gee *et al.*, 1998) after which the transport of anthocyanins into the blood stream occurs (McDougall *et al.*, 2005b).

Fazzari *et al.* (2008) investigated the *in vitro* bioavailability of sweet cherry (*Prunus avium* L.) anthocyanins using *in vitro* GIT digestion model. Acid conditions of the pepsin digestion (pH 2) stabilized and even favoured the formation of some coloured anthocyanin derivatives resulting in a slight increase in anthocyanins, which was also observed for pomegranate juice (Perez-Vicente *et al.*, 2002) and raspberry extracts (McDougall *et al.*, 2005a). The % recovery of cherry anthocyanins in IN fractions were determined to be around 19-21%. Gil-Izquierdo *et al.* (2002), Perez-Vicente *et al.* (2002), and McDougall *et al.* (2005a,b) also reported significant decreases after pancreatic digestion, giving the % anthocyanin recovery values in IN samples of 12.4%, 1.5%, 5%, and 4% in frozen strawberries, pomegranate juice, raspberry, and red wine samples, respectively. The major factor that determines these low recovery values of anthocyanins in the small intestinal digestion is the pH shift to neutral pH value, which leads to the irreversible breakdown (McDougall *et al.*, 2005b). The measured bioavailability of frozen cherries was found to be higher than those measured in the other mentioned food products above. However, a true comparison between these *in vitro* studies is not possible since the results may differentiate substantially due to the starting material as well as the procedure used (Fazzari *et al.*, 2008).

Although the low recovery of anthocyanins in the IN (serum available) fraction from the *in vitro* digestion procedure approaches the low serum bioavailability of

anthocyanins in *in vivo* animal and human feeding trials (i.e., Talavera *et al.*, 2006) and can indicate which compounds survive GIT conditions, it cannot completely mimic the active transport processes carried out in the stomach (Passamonti *et al.*, 2003), the transport of flavonoids through interaction with the sodium dependent glucose transporter (SGLT1) in the small intestine (Gee *et al.*, 1998) or the structural changes those are suggested to accompany anthocyanin transport from the small intestine (McDougall *et al.*, 2007). On the other hand, while anthocyanins are similar to other flavonoids in their structure, it is not clear yet that whether their transport carried out via SGLT1 or they are liable to attack by the glycosidases (McDougall *et al.*, 2005a).

2.4.3.2 *In vitro* Caco-2 cell model

The intestines are always in contact with food substances orally taken which are modulators of the physiological functions of the body. Relatedly, the intestinal epithelial cells (IECs), covering the internal surface of the intestines, are always exposed to high concentrations of nutrients, which differentiates them from the other cells in the body. IECs are responsible for the absorption mechanism of large amounts of nutrients derived from diet, so the interaction between IECs and food substances is an important issue (Shimizu, 2010). The other reported functions of these cells are barrier function and signal recognition and transduction (Figure 2.7) (Shimizu, 2010).

The IECs have been reported to have various transport mechanisms for the absorption of nutrients and non-nutrients those possess different structural and physicochemical properties (Shimizu, 2010). The apical cell membrane of these cells has several transporters which transport nutrients (i.e., glucose, amino acids, peptides, vitamins, minerals) and sometimes non-nutrient food components. Subsequently, the same or different transporters located in the basolateral membrane of IECs provide the excretion of the nutrients (those are apically taken into the cells) from the cells to the blood circulation (Tsuji and Tamai, 1996).

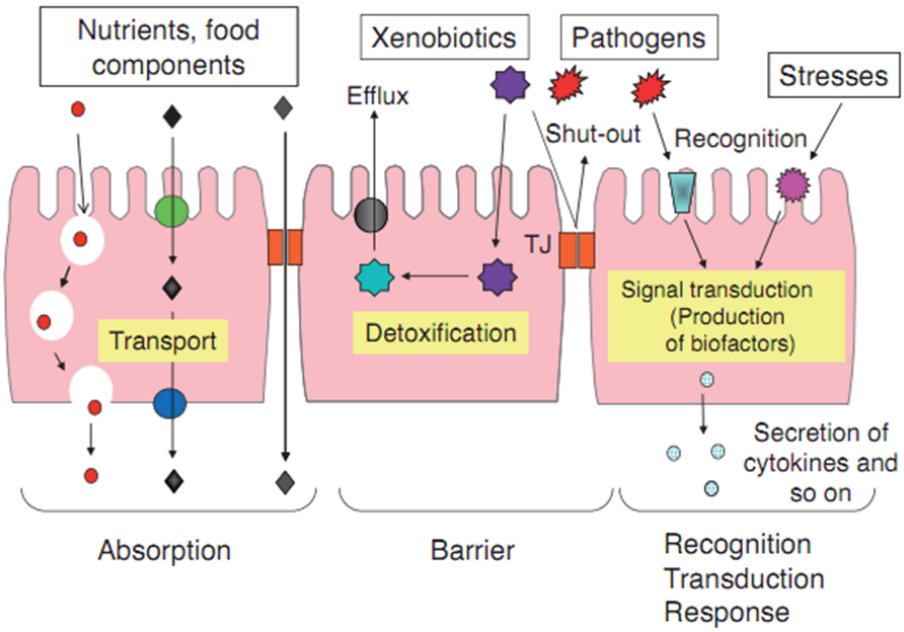

Figure 2.7 : Three major functions of the intestinal epithelial cell (IEC) monolayer; absorption, detoxification (barrier function), signal transduction (Shimizu, 2010).

The slow growth rate of IECs and the limited information about the differentiation process of the small intestinal cells make it difficult to work with primary culture of IECs with full functions. Therefore, to analyse the food-intestine interactions at the cellular and molecular levels, various cell-based assays have been conducted using cell lines of IEC those are mostly derived from human intestinal tissues, including colon cancer tissues (Zweibaum, 1991). Some of the cell lines derived from colon cancer possess specific characteristics, despite their origin. Among them, a human IEC line, named “Caco-2”, is known to have the ability of expressing many small intestinal functions. These special cells, when cultured on plastic plates and on semipermeable membranes for about 3 weeks, form a monolayer and are spontaneously differentiated to mimic the small intestine. Tight junctions are formed between the cells, microvillus structures are formed on the apical cell surface, a variety of brush-border digestive enzymes, transporters, and receptors are expressed (Figure 2.8) (Hashimoto and Shimizu, 1993; Hidalgo *et al.*, 1989; Pinto *et al.*, 1983). Good correlations were reported to be observed between Caco-2 cell permeability and the absorption rates in rat (Conradi *et al.*, 1993) and human (Yee, 1997) intestines.

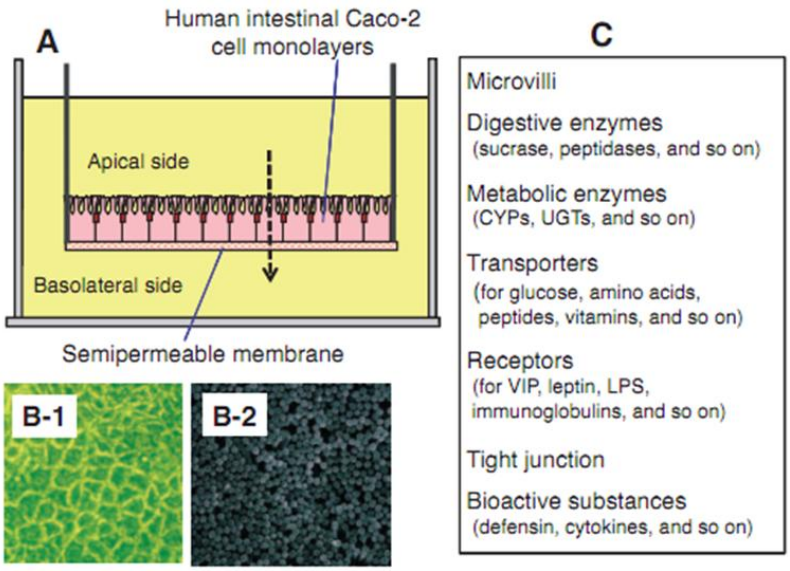

Figure 2.8 : Characteristics of the Caco-2 human intestinal epithelial cell line. (A) Differentiated cells in confluent Caco-2 monolayers on semipermeable membranes. (B-1) Light microscopic picture have a microvillus structure. (B-2) Scanning electron microscopic picture. (C) A variety of molecules expressed in the cell monolayers, those enable to examine many IEC functions *in vitro*. CYP, cytochrome P450 enzymes; UGT, uridine 5'-diphospho-glucuronosyltransferase; VIP, vasoactive intestinal peptide; LPS,lipopolysaccharides. (Shimizu, 2010).

There is much valuable information, obtained using the differentiated Caco-2 cell monolayers, on absorption/transport mechanisms of phytochemicals, such as quercetin (Boyer *et al.*, 2004; Murota *et al.*, 2000), epicatechin (Vaidyanathan and Walle, 2003), proanthocyanidins (Deprez *et al.*, 2001), anthocyanins (Steinert *et al.*, 2008; Yi *et al.*, 2006), as well as other flavonoids (Kaldas *et al.*, 2002; Walle *et al.*, 1999), and carotenoids (Liu *et al.*, 2004).

Anthocyanins from blueberries were shown to be transported through the Caco-2 cell monolayers in their intact glycosidic forms, although the transport/absorption efficiency was reported to be relatively low compared to some other polyphenols (Yi *et al.*, 2006). The transport efficiency of blueberry anthocyanins across Caco-2 cell monolayers was approximately 3-4% (Yi *et al.*, 2006), whereas in another study by Steensma *et al.* (2004) 30-40% of genistein and daidzein isoflavones at the apical side were found to be transported to the basolateral side of the cells. The low absorption rate of anthocyanins is most likely to be resulted from their poor lipophilic properties. The hydrophilic/hydrophobic nature of the anthocyanidin aglycone influences their bioavailability. Accordingly, among blueberry anthocyanins, while the greater number of hydroxyl groups in delphinidin led to the

lowest transport/absorption efficiency; the greater hydrophobic nature of malvidin (having two OCH_3 groups) facilitated increased portioning into cells and provided the highest transport/absorption efficiency (Yi *et al.*, 2006). Free hydroxyl groups included in flavonoid structure can hinder transport in Caco-2 cell monolayers (Tammela *et al.*, 2004). Hydrogen-bond formation between the flavonoids' hydroxyl groups and polar groups of the lipid molecules at the lipid/water interface is the most probable mechanism that causes longer retention delays of polyhydroxylated flavonoids in cell membranes (Ollila *et al.*, 2002).

Sugar moieties attached to the anthocyanidin aglycone have also been considered to influence the absorption of anthocyanins. Cyanidin-glucoside and peonidin-glucoside were found to show significantly higher transport efficiencies through Caco-2 cell monolayers in comparison to their galactoside counterparts. A higher absorption rate was also observed for anthocyanin glucosides than rutinosides (Nielsen *et al.*, 2003). From the latter observation, it has been suggested that the absorption of anthocyanins in their intact glycosidic forms may involve the glucose transport receptors (SGLT1, sodium dependent glucose transporter) (Milbury *et al.*, 2002), since monoglucosides of flavonoids/quercetin can be transported by these receptors (Gee *et al.*, 1998; Walgren *et al.*, 2000), and since quercetin and anthocyanidins share a similar basic flavonoid structure (Yi *et al.*, 2006).

The degradation and low stability of anthocyanins under neutral pH of cell culture conditions *in vitro*, could be another reason of low absorption and excretion of these components compared to the other flavonoids (Wu *et al.*, 2002), which could also be observed under *in vivo* conditions.

2.4.4 Food Matrix and Bioavailability

Nutrients are often located in natural cellular compartments from which they need to be released during digestion, subsequently they can be absorbed in the gut. Recent studies have pointed out that in the case of certain nutrients the state of the food matrix of natural foods or the microstructure of processed foods may favour or hinder their nutritional response *in vivo* (Parada and Aguliera, 2007).

The full absorption process of a particular nutrient is indicated to be most likely dependent on interactions within food matrix, including other food components (Parada and Aguliera, 2007). Due to the synergistic and additive effects of

compounds in whole food matrices, the bioavailability of the same compounds may differ when they are included in food or when they are in the form of pure compounds (Boyer *et al.*, 2004). Relatedly, the exposure of raspberry anthocyanins (extracts) to differences in pH, oxygen, and temperature *in vitro* was found to result in a significant reduction in anthocyanin availability to the serum fraction, whereas codigestion with common foodstuffs (i.e., bread, breakfast cereal, ice cream, and cooked minced beef) protected the labile anthocyanins from extensive degradation, providing higher levels in the serum available fraction (McDougall *et al.*, 2005a).

The physical state of the food matrix plays a key role in the release, accessibility, and biochemical stability of many food components (Aguliera, 2005). Food processing, including grinding, fermentation, and/or mild heating may improve bioavailability through disruption of the cell walls of plant tissues, dissociation of the nutrient-matrix complexes, or transformation into more active molecular structures. It is well known that cooking process can enhance the bioavailability of *β*-carotene through increasing its extractability from the plant matrix. Significantly more *β*-carotene was found to be absorbed from cooked and pureed carrots (65%) than from the raw vegetable (41%) (Livny *et al.*, 2003) as well as from homogenized (up to 25%) and heat applied (21%) tomatoes than non-homogenized raw tomatoes (2.5%) (van Het Hof *et al.*, 2000). Charron *et al.* (2009) evaluated the overall role of the plant matrix in anthocyanin absorption by comparing the absorption rates between black carrot fruit and black carrot juice. Their results suggested that the total absorption of anthocyanins were similar for carrot juice and whole carrots those provided equivalent amounts of anthocyanins with amounts of 32 nmol h/L and 27 nmol h/L, respectively. On the other hand, anthocyanin absorption from juice was more rapid, indicating that more time is needed in digestive processes to liberate anthocyanins from the plant matrix.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

The standards of chlorogenic acid, +(-) catechin, (-)- epicatechin, quercetin-3-glucoside, rutin (quercetin-3-rutinoside), kaempferol-3-rutinoside, rosmarinic acid and ascorbic acid were purchased from Sigma (St. Louis, USA), and cyanidin-3-glucoside was obtained from Extrasynthese (Genay, France). The solvents of methanol and acetonitrile were of HPLC supra gradient quality and were obtained from Biosolve (Valkenswaard, The Netherlands). Formic acid (98-100%) was purchased from Merck-Schuchardt (Hohenbrunn, Germany). Metaphosphoric acid, diethylene triamine pentaacetic acid (DTPA), sodium phosphate, dithiothreitol (DTT) and leucine enkaphaline were obtained from Sigma (St. Louis, USA). The OASIS HLB (3 cc column) cartridges, for solid phase extraction, were purchased from Waters Corporation (Milford, MA, USA).

For bioavailability assays, using *in vitro* simulated gastrointestinal digestion model, pepsin (P7000), pancreatin (P7545), bile salt (B8756) sodium bicarbonate (S6014) were purchased from Sigma Aldrich (St. Louis, USA); and dialysis tubing (MD 34 14 x 100CLR) was purchased from Membra-Cel (Viskase Corporation, Chicago, USA). For bioavailability assays, using *in vitro* Caco-2 cell model, Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, 42430-082; with 4.5 g/L glucose, no pyruvate, 4 mM L-glutamine, and 25 mM HEPES), Hanks' Balanced Salt Solution (HBSS) (Invitrogen, 14170; no calcium, no magnesium, no phenol red), 0,25% Trypsin with 0.38 g/L EDTA.4Na (sodium ethylene diamine tetraacetic acid) (Invitrogen, 25200-072) were purchased from Invitrogen (California, USA). Fetal Bovine Serum (FBS, Hyclone Perbio) (Fischer Scientific CH 30160.03; heat inactivated at 56ºC for 45 min) was purchased from Fisher Scientific (USA). HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) was obtained from Sigma

(USA). Caco-2 human colorectal adenocarcinoma cell line was purchased from the American-Type Culture Collection (ATCC HTB-37-TM; USA).

3.1.2 Nectar processing samples

Samples from industrial-scale sour cherry nectar processing were collected at a Turkish fruit juice factory (Aroma Bursa Fruit Juice and Food Industry Inc.,). On five different days in July 2011, five different, independent batches were obtained representing five series of biological replicates. The variety of sour cherry fruit, used as raw material, was the local variety "Kütahya". The fruits were gathered from the regions of Afyon, Isparta, Kütahya and Kayseri, and fruits from different origin were mixed before processing. Fresh fruit was first processed to concentrated juice in an industrial facility in Karaman and this concentrated juice was shipped to a facility in Bursa, for the production of the pasteurized nectar.

In order to monitor the process, 18 steps towards nectar processing (Figure 3.1) were identified and defined and this resulted with a total of 22 samples for analysis, each with 5 independent replicates. Samples were snap-frozen in liquid nitrogen and ground to a fine powder in Turkey. These were subsequently transported on dry ice to The Netherlands, where the samples were stored at -80˚C until analysis. All samples were then freeze-dried at -80ºC, < 1 mP pressure for 20 hrs using a freeze-dryer (Snijders Scientific, LY-5FM) in order to perform the further analyses on dry-weight basis.

The steps included in industrial-scale sour cherry nectar production are summarized in processing order in Table 3.1, where also the specific objective of each step is mentioned and the samples collected and coded for analysis are also given. Raw sour cherry material was first processed to the concentrated juice in Aroma – Karaman facility. Fresh sour cherry fruit sample was taken after washing and selection step, to get the whole undamaged fruit. After separation of stalks, mash heating and mash pressing steps took place which provided enzyme inactivation and obtaining the first juice, respectively. In mash pressing unit, press cake was the waste material, which was subsequently subjected to three further press cake extraction steps with certain amounts of water addition, in order to increase the juice yield. The whole juice obtained from these mash pressing and press cake extraction steps was collected in press outlet collecting tanks, which was further pasteurized, enzyme treated,

clarified, filtered and evaporated to concentrated juice. Paper filtration was applied to the concentrated juice to eliminate harmful bacteria (*Alycyclobacillus* spp.). The concentrated juice was then shipped to Aroma – Bursa facility and stored in this concentrated form, which was subsequently processed to the nectar with water, sucrose syrup and citric acid addition. Pasteurization of the nectar was the last step after which the final pasteurized nectar sample was obtained.

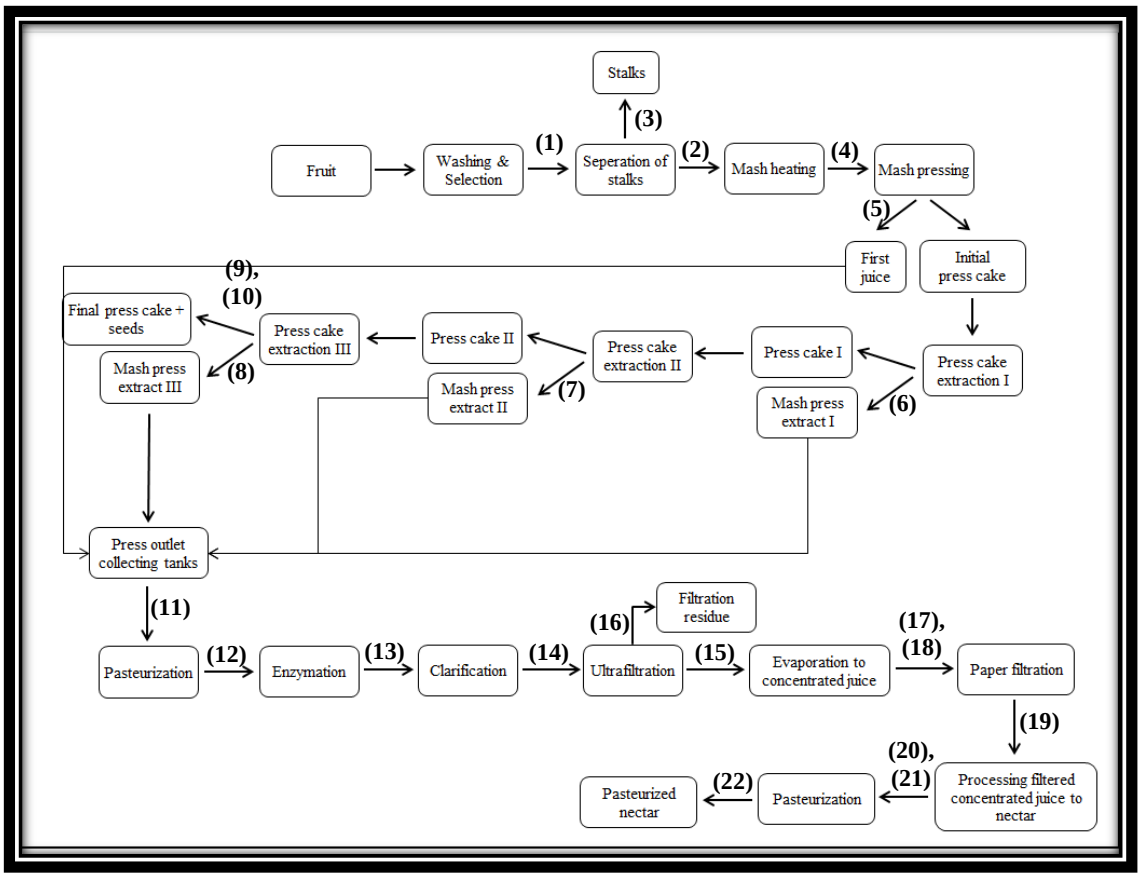

Figure 3.1 : Schematic representation of industrial-scale sour cherry nectar processing with the sampling points identified with numbers.

Table 3.1 : Description of industrial-scale sour cherry nectar processing steps with numbers in processing order and with codes of samples collected.

No	Sample & Code	Treatment & Conditions	Aim	Weight Data
1	Fresh fruit (FF)	Washing and selection	Removal of unwanted material	3.5% reduction in wet-weight (318 kg waste from 9094 kg)
2, 3	Fresh fruit without stalk (FF-St) (2) Stalk (St) (3)	Separation of stalks	Obtaining the original fruit for processing by excluding the stalk	8600 kg original fruit and 176 kg stalk giving 2% and 4% reduction in wet- and dry-weight bases, respectively
4	Mash (MH)	Mash heating; 80˚C-90s[a]	Enzyme inactivation	No change
5	Mash press (MP)	Mash pressing; 110 bar	Obtaining the juicy part	6313 kg juice from 8600 kg mash (73% juice yield)
6	Mash press cake extract-I (MPEX-I)	Mash press extraction-I (first pressing to press cake with water)	Increasing the yield of juice	2287 kg PC extracted with 1000 kg water: 1785 kg MPEX-I and 1502 kg PC (juice yield increased to 83%)
7	Mash press cake extract-II (MPEX-II)	Mash press extraction-II (second pressing to press cake with water)	Increasing the yield of juice	1502 kg PC extracted with 1250 kg water: 1368 kg MPEX-II and 1384 kg PC (juice yield increased to 84%)
8	Mash press cake extract-III (MPEX-III)	Mash press extraction-III (third pressing to press cake with water)	Increasing the yield of juice	1384 kg PC extracted with 1500 kg water: 1572 kg MPEX-III and 1312 kg final PC (juice yield increased to 85%)
9, 10	Press cake (PC) (9) with seeds (Sd) (10)	Press cake resulting after MPEX-III	Removal insoluble fruit parts	1312 kg final PC with Sd giving 15% reduction in wet-weight and 29% reduction in dry-weight
11	Pressed juice (PrJ)	Combined juices: MP, MPEX-I, MPEX-II, MPEX-III.	Mixing all juice obtained	≈ 11000 kg of pressed juice
12	Pasteurized juice (PsJ)	Pasteurization of pressed juice; 95˚C-90s	Microbial inactivation	No change
13	Enzyme treated juice (EnJ)	Enzymation; 50˚C-2 h	Degradation of pectic substances and starch	37.5 mL pectolytic enzyme[b] / ton juice 6.25 mL amylolytic enzyme[c] / ton juice
14	Clarified juice (ClJ)	Clarification; 50˚C-1 h	Precipitating potential haze precursors	780 g gelatin[d] / ton juice 1.2 kg bentonite[e] / ton juice
15, 16	Filtered juice (FtJ) (15) Filtration residue (FR) (16)	Ultrafiltration	Obtaining the clear juice by removing precipitates	60 kg FR / ton juice giving 6% and 7% reduction in wet- and dry-weight bases, respectively
17	Concentrated juice (CJ)	Evaporation to 65˚Brix(Bx); 65-80˚C	Volume reduction for storage	≈ 10400 kg FtJ at 12.5˚Bx evaporated to 2000 kg CJ at 65˚Bx

Table 3.1 (continued) : Description of industrial-scale sour cherry nectar processing steps with numbers in processing order and with codes of samples collected.

No	Sample & Code	Treatment & Conditions	Aim	Weight Data
18, 19	Non paper-filtered CJ (CJ-BPF). Paper-filtered CJ (CJ-APF)	Paper filtration	Elimination of *Alycyclobacillus* bacteria (resistant to pasteurization temperatures)	Filter residue on paper represents negligible weight
20	Nectar (N)	Diluting concentrate to nectar: addition of sucrose and citric acid	Production of nectar	56% sucrose on dry-weight basis with; (a) 2000 kg CJ (65˚Bx) diluted to 10000 kg with water to 12.5 ˚Bx (b) 2450 kg sucrose syrup (65˚Bx) addition to 22.8˚Bx € 10260 kg water addition to 12.5˚Bx (d) ≈27 l citric acid (50%, w/v) addition to 8 g/l (pH 3.5) € 22737 kg nectar in total
21	Sucrose syrup added nectar (SSN)	Additional supply of sucrose syrup	Obtaining most proper nectar for consumption	4012 kg sucrose syrup (65˚Bx) addition to 22737 kg nectar giving 66% sucrose concentration in dry-weight basis (23.7˚Bx, 3.4 pH, 6 g/l acidity)
22	Pasteurized nectar (PN)	Pasteurization of final nectar; 95˚C, 45 s	Microbial inactivation	≈ 26750 kg

***** It takes 15-20 min to transfer the mash from mash heating step to mash pressing step
[1] Fructozym ®PFA from ERBSLOH – Germany
[2] Fructamyl ®HT from ERBSLOH – Germany
[3] Narmada Gelatines Ltd. – India
[4] Gokmenoglu Mine Petrol Food Construction Incorporated Company – Turkey

3.2 Methods

Some of the treatments applied during processing from fruit to nectar had significant effects on both dry-weight and wet-weight (**Table 3.1**). Firstly, the removal of stalks, press cake and filtration residue wastes reduced the total dry-weight by about 4%, 29% and 7%, respectively. Secondly, sucrose syrup addition at the end of processing the concentrated juice into nectar, strongly increased the total dry-weight, which more than doubled, resulting in a sucrose content of more than 50% in the dry matter of the nectar (Table 3.1). Thirdly, the evaporation step converting filtered juice into concentrated juice resulted in a reduction of 81% in wet-weight, without affecting the dry-weight. Fourthly, a modest change in wet-weight (11% increase of moisture content) was observed during mash pressing, where press cake was removed and

water was added to extract the press cake (**Table 3.1**). Consequently, further analyses were performed using equal amounts of dry-weight for each sample, since changes in wet-weight were much more dramatic than changes in dry-weight, and since quantitative information was available on alterations of the total dry-weight, resulting from the removal of waste materials such as stalks, press cake and filtration residue and sucrose syrup addition.

3.2.1 Moisture content analysis

To determine the moisture content of the sour cherry samples, the AOAC method 925.10 (AOAC, 1990) was deployed. For each sample, 2 grams of fresh powdered material was dried in an oven (Marius Instrumenten, Utrecht, The Netherlands) with air-drying at 105˚C for 18 hrs, after which the dry-weight was calculated and recorded.

3.2.2 Spectrophotometric assays

3.2.2.1 Extract preparation

For the estimation of the total phenolic (TP), total flavonoid (TF), total monomeric anthocyanin (TA) contents, as well as the total antioxidant capacity (TAC), by applying the spectrophotometric assays, 1 g of fresh powder of each sample was freeze-dried and extracted with 5 mL of 75% methanol with 0.1% formic acid. After ultrasonification for 30 min, centrifugation at 2500 rpm for 25 min was performed. The collected supernatant (if necessary) was used for the analyses. A VWR UV-1600 PC spectrophotometer and 1-cm path length disposable cells were used for measurements.

3.2.2.2 Analysis of total phenolics

The total phenolic content (TP) of the sample extracts was estimated using Folin−Ciocalteau reagent as described before (Karahalil and Sahin, 2011), using 100 µL of extract for all samples diluted with either 900 µL or 5000 µL of pure water. To diluted extracts, 0.5 mL of 0.2 N Folin−Ciocalteau reagent was added. After 3 min incubation, 1.5 mL of Na_2CO_3 (2%) solution was added and the mixture was incubated for 2 hr at room temperature with intermittent shaking. At the end of incubation, the absorbance was measured at 760 nm. Two different standard curves

were obtained by using 0.01–0.60 mg/mL gallic acid, diluted with either 900 µL or 5000 µL of pure water, and data were calculated and expressed in milligrams of gallic acid equivalents (GAE) per 100 g dry-weight. The two calibration curves –the first one is with 900 µL water dilution, and the second one is with 5000 µL water dilution- obtained for gallic acid are shown in Appendix, Figure A.1 and Figure A.2, respectively.

3.2.2.3 Analysis of total flavonoids

The flavonoid content (TF) of methanolic extracts was measured using the method explained by Piccolella *et al.* (2008). For each sample extract or for standard solution of catechin (0.01 – 1.0 mg / mL), 0.5 mL sample was diluted with distilled water to make a volume of 5 mL. At 0 time, 0.3 mL of $NaNO_2$ (5%, w/v) was added and after 5 min, 0.6 mL of $AlCl_3$ (10%, w/v) was added. After 6 min, 2 mL of NaOH (1.0 M) was added to the mixture followed by the addition of 2.1 mL of distilled water. Absorbance was read at 510 nm against the blank (water), and flavonoid content was expressed as milligrams of (+)catechin equivalents (CE) per 100 g of dry-weight. The calibration curve is shown in Appendix, Figure A.3.

3.2.2.4 Analysis of total anthocyanins

The total monomeric anthocyanin content (TA) was determined using the pH-differential method (Giusti and Wrolstad, 2001). Two dilutions (with an appropriate dilution factor) of each sample were prepared, one with potassium chloride buffer (0.025 M, pH 1.0) and the other with sodium acetate (0.4 M, pH 4.5) and incubated in the dark for 15 min. Absorbance of each dilution was measured at the $\lambda_{vis\text{-}max}$ and at 700 nm against a blank of distilled water. Pigment content was calculated as milligrams cyanidin-3-glucoside (C3G)/100 g dry-weight using an extinction coefficient of 26900 L/cm/mol and molecular weight of 449.2 g/mol.

3.2.2.5 Analysis of total antioxidant capacity

Total antioxidant capacity (TAC) of the methanolic extracts were estimated using three different *in vitro* tests in parallel. Trolox (0.01-0.2 mg / mL) was used as the reference compound in all assays, and results were expressed in terms of mg of Trolox Equivalent Antioxidant Capacity (TEAC) per 100 g dry-weight. The

calibration curves obtained for Trolox in three different methods applied are shown in the Appendix, Figures A.4-A.6.

The ABTS [2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt] method was performed according to Miller and Rice-Evans (1997) with some modifications. Briefly, 100 µL of sample extract or standard was mixed with 1 mL of ABTS solution with 0.05 M potassium phosphate (final pH of the mixture was 7.4). After 30 s-1 min, absorbance was measured at 734 nm.

The DPPH (1,1-diphenyl-2-picrylhydrazyl) method (Kumaran and Karunakaran, 2006) was performed by mixing 100 µL of sample extract or standard solution with 2 mL of 0.1 mM DPPH in methanol (not buffered). The absorbance of the mixture was measured at 517 nm at the end of 30 min incubation at room temperature, in the dark.

The CUPRAC (Cupric Reducing Antioxidant Capacity) method was utilized using the method described by Apak *et al.* (2004) First, 1 mL of 0.01 M copper (II) chloride ($CuCl_2$), 1 mL of 0.0075 M neocuproine (Nc), 1 mL of ammonium acetate (NH_4Ac) buffer (pH 7.0) was mixed in a test tube. Subsequently, 100 µL of sample extract or Trolox was added to this mixture. Lastly, 1 mL of MQ water was included to make the final volume 4.1 mL. After 1 h reaction time, absorbance was measured at 450 nm.

3.2.3 Targeted HPLC analysis of anthocyanins, flavonols, and phenolic acids

For determination of anthocyanin, flavonol and phenolic acid contents, 28 mg freeze-dried sample was extracted with 2 mL of 75% methanol in MQ water with 0.1% formic acid followed by 15 min sonification. Samples were then centrifuged at 2500 rpm for 10 min with successive filtering of supernatants through 0.45µm filters (Minisart SRP4, Biotech GmbH, Germany). Filtered extracts were analysed using a W600 Waters HPLC system coupled to a Waters 996 photodiode array (PDA) detector as described previously (Bino *et al.*, 2005). Compounds were separated using a Luna C18 column (150 x 4.6 mm, 3µ; Phenomenex, Torrance, CA, USA) heated to 40˚C and applying a gradient from 95% to 25% MQ and a 5-75% acetonitrile, both in 0.1% trifluoroacetic acid (TFA) (1 mL min^{-1} flow rate) across a period of 50 min. Eluting compounds were monitored continuously between 240 and 600 nm using a PDA detector. Waters MILLENIUM software was used for data analysis. Anthocyanins were detected at 512 nm, flavonols at 360 nm and phenolic

acids at 312 nm. For identification of some peaks, absorbance spectra and retention times of eluting peaks were compared with available standards, including (+)-catechin, chlorogenic acid, cyanidin-3-glucoside, (-)- epicatechin, rutin (quercetin-3-rutinoside), quercetin-3-glucoside, kaempferol-3-rutinoside, and rosmarinic acid. The other identified peaks were determined with data from the literature (Bonerz *et al.*, 2007; Chandra *et al.*, 2001; Kirakosyan *et al.*, 2009). For quantification, dose-response curves of available pure standards (0-100 μg/mL) were used as reference (Figures A.7-A.9).

3.2.4 Targeted HPLC analysis of procyanidins.

For procyanidin analysis, the depolymerization of procyanidins to generate their terminal subunits (flavan-3-ol monomers) and electrophilic extension subunits (flavan-3-ol intermediates), was carried out using the phloroglucinol acid hydrolyzation method as described before by Capanoglu *et al.* (2011) with slight modifications. To 28 mg freeze-dried material, 1.2 mL ice-cold phloroglucinol reagent (50 g/L phloroglucinol, 10 g/L vitamin C, 8 mL/L HCl (37%) diluted in methanol) was added. These phloroglucinol-treated samples were incubated at 50˚C for 30 min, shaking every 10 min, for hydrolyzation and they were taken into ice immediately after incubation. Then, 6 mL 40 mM ice-cold sodium acetate was added and the samples were centrifuged at 2500 rpm for 10 min. After filtering through 0.45μm filters, samples were analysed by HPLC. The detection of (+)-catechin and (-)-epicatechin was done with the HPLC system coupled to a Waters 2475 Flourescence Detector with excitation at 275 nm and emission at 310 nm. For the detection of free flavan-3-ol monomers in the unhydrolyzed samples another 28 mg freeze-dried sample was extracted with 2 mL 75% methanol in MQ water with 0.1% formic acid. While this unhydrolyzed sample gave the originally free flavan-3-ol monomers; the hydrolyzed sample was used to determine the terminal subunits plus the originally free flavan-3-ols both as flavan-3-ol monomers. The extension units were observed as phloroglucinol conjugates in the hydrolyzed sample. The degree of polymerization (DP) was calculated with the determined concentrations of terminal subunits and extension units, using the following equation: DP = [(terminal subunits + extension units) / terminal subunits]. Calibration curves obtained for (+)-catechin and (-)-epicatechin (0-20 μg/mL) (Figure A.10 and Figure A.11) were used for quantification.

3.2.5 HPLC analysis of reduced and non-reduced forms of vitamin C

Vitamin C measurements, reduced form; ascorbic acid (AA) as well as its partially oxidized form; dehydroascorbic acid (DHAA), were performed as described by Tulipani *et al.* (2008) using initial original fruit (FF-St) and final pasteurized nectar (PN) samples. The extraction solution was prepared, at least one day in advance, by dissolving 5% (w/v) meta-phosphoric acid (Sigma ACS, 35%) and 1mM diethylene triamine pentaacetic acid (DTPA) (Sigma, D-1133) in MQ with continuous stirring and sonication until a homogeneous solution was obtained. This solution was kept at 4ºC until analysis.

Vitamin C extraction was performed with 0.5 g fresh frozen powder (at -80ºC) in 2 mL of ice-cold extraction solution, followed by 15 min sonication, 10 min centrifugation at 2500 rpm, and filtering through 0.45µm filters. The reduced AA was directly determined on this extract.

For determination of total ascorbic acid (including both AA and DHAA), 200 µL of the filtered extract was mixed with 0.4 mL of a 0.5 M sodium phosphate solution (pH 8.9) (Sigma S0876) containing 0.1% (w/v) dithiothreitol (DTT) (Sigma D0632) to increase its pH to pH 6.0 in order to enable the reduction of DHAA to AA. After 15 min incubation at room temperature, 0.4 mL extraction solution was added to stop the reaction by lowering the pH again to around pH 3.

The samples were run on an HPLC-PDA system with a YMC-Pro C18 150 mm x 4.6 mm, 5µ column. The mobile phase used was 100% 50 mM sodium dihydrogen phosphate (pH 4.5) at a flow rate of 0.5 mL/min. Compounds were separated using these isocratic conditions for15 min, after which the column was washed with 50% acetonitrile for 5 min for regeneration at initial isocratic conditions before the next injection. The detection and quantification of the reduced form of AA and total AA was done at 262 nm by means of a calibration curve using ascorbic acid (0-100 µg/mL) as a standard which is given in the Appendix, Figure A.12. The amount of DHAA was determined using the equation: DHAA (mg/mL) = Total AA (mg/mL) – reduced form of AA (mg/mL).

3.2.6 On-line antioxidant analysis

The contribution of each identified antioxidant compound to the total antioxidant capacity of the extracts was determined with the same HPLC-PDA system (described in **section 3.2.3**) which was coupled to post-column on-line antioxidant detection (Beekwilder *et al.*, 2005; Capanoglu *et al.*, 2008). Briefly, 28 mg freeze-dried powder was extracted with 2 mL of 75% methanol containing 0.1% formic acid, followed by 15 min sonication, 10 min centrifugation at 2500rpm, and filtration through 0.45μm filters. Filtered extracts were then loaded on HPLC. The HPLC system comprised a Waters 600 controller, a Waters 996 PDA detector, and a column incubator at 40 °C. The column used was C18 250 mm × 4.6 mm, 5μ (Phenomenex, CA). Separation of compounds was conducted in a 65 min run with a gradient from 95% solution A (MQ with 0.05% TFA) and 5% solution B (acetonitrile with 0.05% TFA) to 35% solution B at a flow rate of 1 mL/min. Eluted compounds passed first through the PDA detector (set at an absorbance range of 240-600 nm) and subsequently, through a post-column reaction loop (3 m stainless steel tube (internal diameter 0.508 mm) at 40 °C), where they reacted online for 30 s with a buffered solution of ABTS cation radicals (pH 7.4). The ABTS solution was prepared by dissolving 110 mg of ABTS in 100 mL of MQ, followed by the addition of potassium permanganate (19 mg/mL). After 16 h of incubation in the dark, the radical solution was diluted further in three volumes of 0.1 M Na-phosphate buffer, pH 8.0. Then, the decreased absorption of the mixture -with ABTS reacting with antioxidants- was monitored through a second detector (Waters 2487, dual-wavelength UV-vis detector), where the amount of ABTS cation radicals was measured at 412 nm. The percent contribution of each antioxidant compound to the total antioxidant capacity of the extracts was calculated by integrating the main antioxidant peaks on the ABTS chromatogram.

3.2.7 Untargeted metabolomics analysis using LC-QTOF-MS

An LC-PDA-QTOF-MS system was used to perform the untargeted metabolomics analysis of sour cherry samples. Fifty mg of freeze-dried powder was extracted with 1.5 mL of 75% methanol in MQ water acidified with 0.1% formic acid. Extracts were sonicated for 15 min, centrifuged at 2500 rpm for 10 min and filtered through

0.45µm filters (Minisart SRP4, Biotech GmbH, Germany). Chromatographic and mass spectrometric conditions were as described by Moco *et al.* (2006). In summary, for chromatographic separation, a Waters Alliance 2795 HT system with a Luna C18(2) pre-column (2.0 x 4 mm) and an analytical column (2.0 x 150 mm, 100Å, particle size 3µm) from Phenomenex (Torrance, CA, USA) were used. Five microliters of each filtered sample were injected into the system for LC-PDA-MS analysis using formic acid : water (1:1000, v/v; eluent A) and formic acid : acetonitrile (1:1000, v/v; eluent B) as elution solvents. Flow was set at 0.19 mL/min with the gradient from 95% eluent A and 5% eluent B to 65% Eluent A and 35% eluent B across a period of 45 min. The column temperature was maintained at 40°C and the samples at 20°C. UV absorbance was measured using a Waters 2996 PDA (λ range from 240 to 600 nm) and ESI-MS analysis was performed using a QTOF Ultima V4.00.00 mass spectrometer (Waters-Corporation, MS technologies) in negative mode. A collision energy of 10 eV was used for full-scan LC-MS in the m/z range 100 to 1,500. Leucine enkephalin, $[M - H]^- = 554.2620$, was used for online mass calibration (lock mass).

3.2.8 Processing of LC-MS data

Acquisition and visualization of the LC-PDA-QTOF data were performed using MassLynx 4.0 software (Waters). The MetAlign software package (www.metAlign.nl; Lommen, 2009) was used for baseline correction, noise estimation, and spectral alignment. Baseline and noise eliminations were performed from scan numbers 60 (retention begin) to 2,340 (retention end). The maximum amplitude was 35,000 and peaks having slope factor below two times the local noise and threshold factor below three times the local noise were discarded. The aligned mass signals that were <3 times the local noise (below a signal intensity of 50) were filtered out, resulting in 1,588 filtered mass signals from a total of 2,709 original mass signals. The aligned and filtered peak table was subjected to redundancy removal using MSClust software (Tikunov *et al.*, 2012), by which mass peaks originating from the same metabolite (including isotopes, adducts, and fragments) were clustered. This analysis resulted in a total of 193 reconstructed metabolites (centrotypes) of which for each cluster the highest signal per metabolite was chosen as a representative for the respective cluster and was used for further (statistical) analysis. For annotation of the reconstructed metabolites, UV spectra, mass accuracy

and (in-source) fragments were used in combination with different metabolite databases, such as the MotoDB (http://appliedbioinformatics.wur.nl/moto) (Moco *et al.*, 2006), the Dictionary of Natural Products (http://dnp.chemnetbase.com), KnapSAcK (http://kanaya.naist.jp/KNApSAcK/), as well as the information on the compounds of *Prunus* spp. reported previously in publications (Wagner *et al.*, 1969; Bonerz *et al.*, 2007). In addition, we checked for phenolic compounds that were previously unambiguously identified in other plant sources analysed (Iijima *et al.*, 2008; Moco *et al.*, 2006; Tikunov *et al.*, 2010; van der Hooft *et al.*, 2012) but were not present in the above-mentioned *Prunus* metabolite databases. For an observed accurate mass, a list of possible molecular formulas was obtained, selected for the presence of C, H, O, and N, S, P.

Comparison and visualization of the main features of the LC-MS data were performed by loading the data matrix into GeneMaths XT 1.6 software (www.applied-maths.com). Metabolite intensities were normalized using log2 transformation and standardized using range scaling (autoscaling normalization). Principal Components Analysis (PCA) was performed for unsupervised comparison of samples and metabolites.

3.2.9 Bioavailability analysis to determine the effect of nectar processing on bioavailability of sour cherry anthocyanins

3.2.9.1 Sour cherry samples

Whole sour cherry fruit and whole sour cherry nectar samples

Bioavailability analysis were performed on sour cherry fruit material and its industrial-scale processed nectar, those were obtained from Aroma Bursa fruit juice factory. Fruit and nectar samples were snap-frozen in liquid nitrogen and ground to a fine powder in Turkey, which were subsequently transported on dry ice to The Netherlands, where they were stored at -80˚C until analysis.

Solid phase extracts of fruit and nectar samples

The phenolic compounds, including anthocyanins, in fruit and nectar samples were purified using solid phase extraction (SPE) procedure. Initially, the OASIS HLB cartridges (Waters OASIS HLB 3 cc column, Waters Corp., Milford, MA) were conditioned by rinsing first with 6 mL 100% methanol followed by rinsing with 4

mL MQ water. Sour cherry fruit and nectar samples to be extracted were prepared by dissolving 0.5 g fresh weight powder in 1.6 mL 5% methanol in MQ water (v:v), followed by 10 min sonication and 10 min centrifugation at 2500 rpm. To obtain the solid phase extracted fruit (fruit-SPE) and the solid phase extracted nectar (nectar-SPE) samples, these methanol-water extracts were loaded to activated HLB cartridges which were subsequently washed with 4 mL MQ water. All cartridges were then eluted with 1 mL 75% methanol in MQ water (v:v), and 0.4 mL 100% methanol. The solvent was removed from the eluates using a speed vacuum concentrator (Christ RVC 2-18) until totally dry-pellets of fruit-SPE and nectar-SPE samples were obtained, which were stored at -20ºC until further analysis.

3.2.9.2 Bioavailability analysis using simulated *in vitro* gastrointestinal (GI) digestion model

In order to simulate the *in vivo* GI digestion conditions, and to determine the amount of free soluble polyphenols potentially available for further uptake, the procedure adapted from McDougall *et al.* (2005a) was followed. This *in vitro* bioavailability determination method was applied only on whole sour cherry fruit (WF) and whole sour cherry nectar (WN) samples. Release of phytochemicals (phenolics, flavonoids and anthocyanins) from WF and WN matrices was analyzed at different stages of digestion. These stages represented the aliquots from gastric digesta (post gastric, PG) and from GI digesta, including IN (representing the material that entered the serum; dialyzable fraction) and OUT (representing the material that remained in the GI tract, undialyzable fraction). Post gastric (PG), IN and OUT samples were stored at -20ºC until further analysis. These were thawed and centrifuged at 18000 rpm prior to analysis. TP, TF, and TA contents, and TAC were determined for each of these PG, IN, and OUT samples using the methods described in sections between 3.2.2.2 and 3.2.2.5, respectively.

Gastric Phase. In a 250 mL glass beaker, 5 g of homogenized fruit and nectar fractions were mixed with 20 mL distilled water and 1,5 mL pepsin solution. The pH was adjusted to 1.7 by adding 5 M HCl and the beaker was sealed with parafilm. The mixture was incubated for 2 h in a shaking water bath at 37°C and 100 rpm. After 2 h, 2 mL aliquots of the post gastric digestion were collected and stored at -20^{0}C until

further analysis. A blank was prepared with identical chemicals but without the fruit/nectar matrix, and subjected to the same treatment as the samples.

Intestinal Phase. 4.5 mL of 4 mg/mL pancreatin and 25 mg/mL bile salt mixture was added to the remainings from the gastric phase in the glass beaker. Segments of dialysis bags were cut to a specified length, and filled with a sufficient amount of $NaHCO_3$ (20 mL) to neutralize the samples' titratable acidity and then the beaker was again sealed with parafilm. After 2 h incubation in a shaking water bath at 37°C and 100 rpm, the solution in the dialysis tubing was taken as the IN sample representing the material that entered the serum and the solution outside the dialysis bag was taken as the OUT sample representing the material that remained in the gastrointestinal tract. IN and OUT samples were stored at -20^0C until further analysis.

3.2.9.3 Bioavailability analysis using *in vitro* Caco-2 cell model

Maintaining Caco-2 cell germ line

Caco-2 human colon cancer cell line was obtained from the American-Type Culture Collection (ATCC HTB-37TM; USA). The cells were routinely grown in 75 cm^2 tissue culture flasks (with canted neck and 0.2 μm vented cap, Corning, 430641) using Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 9.1% Fetal Bovine Serum (FBS). Cells were maintained in a humidified atmosphere of 5% CO_2 in air at 37ºC. Medium was changed 3 times (every 2 days) per week and cells were sub-cultured every 7 days (at 80-90% confluence).

For sub-culturing, the cells, formerly washed with 10 mL HBSS (without Ca^{+2} and Mg^{+2}), were detached from the bottom of the flask by a 1 mL 0.25% trypsin-EDTA wash for 5-10 min at 37ºC and 5% CO_2 in air. Then, 5 mL DMEM with 9.1% FBS was added to reach the total volume of 6 mL. A volume of suspension (between 1-3 mL; depending on the cell concentration and passage number) was seeded to new flasks, and the volume was made up to 20 mL with DMEM medium with 9.1% FBS. This sub-culturing procedure enabled to split the cells in culture flask to the new flask with concentrations of 1:6 to 1:2 (1mL to 3 mL volume of suspension) of original concentrations those in culture flask.

Cell culture

For transport experiments, Caco-2 cells in flasks, having a passage number between 40-51, were harvested using the tyriptinisation protocol described above and resuspended in DMEM with 9.1% FBS. One hundred microliter of this cell suspension was diluted 1:10 (v/v) in DMEM with 9.1% FBS at 37ºC and cell density was determined using a heamocytometer (cell counter; W. Schreck, Hofheim/TS, Germany) under an inverted phase tissue culture microscope (4x, PL10x, CPL20x inverted phase microscope; Olympus, CK, USA) . They were seeded in 6-well tissue culture plate inserts (Greiner bio-one 657640; translucent, 0.4 μm pores, $1x10^8$ pores/cm, 452.4 mm^2 surface area for cell growth) at a concentration of (approx.) $6.0*10^5$ cells/mL per well. Culture medium (DMEM containing 9.1% FBS) with cells was added to the apical chamber and without cells to the basal chamber in volumes of 1.5 mL and 2.5 mL, respectively. Cells were allowed to grow and differentiate to confluent monolayers for 23-24 days post seeding. Culture medium was replaced three times a week. To ensure that the monolayers exhibit the properties of a tight biological barrier, transepithelial electrical resistance (TEER) was monitored using a MilliCell-ERS voltohmmeter (Millipore Co., United States). Monolayers with TEER values exceeding 200 $\Omega.cm^2$ were used exclusively for transport experiments since TEER values above this value are generally considered as acceptable (Palm *et al.*, 1996). TEER values were also obtained after completion of the transport experiments.

Determination of the optimum cell medium for transport experiments

The most proper cell medium, for use in transport experiments, was investigated on the basis of anthocyanin stability and cell integrity. Analyses were performed with four different mediums, including HBSS, HBSS buffered with 100 mM HEPES, DMEM supplemented with 9.1% FBS, and DMEM without FBS in order to select the most proper medium giving a high anthocyanin stability and a good cell integrity.

Stability of sour cherry anthocyanins in four different cell mediums analysed with HPLC

HPLC-based targeted analysis was carried out in order to determine the stability of sour cherry anthocyanins in different cell mediums tried out. For this purpose, freeze-dried powder forms of fruit and nectar samples were diluted first in MQ water

for a sample concentration of 100 mg dry-weight / mL water. After centrifugation for 10 min at 2500 rpm, the supernatants were collected, which were subsequently subjected to further 1:6 (v/v) dilution using one of the selected cell mediums, resulting in final concentration of 16.7 mg dry-weight / mL medium. Since the simulated intestinal conditions should be similar to the conditions in human body, fruit and nectar samples, diluted to this final concentration in different mediums, were checked for their pH values. Then, these samples were incubated for 6 h in a humidified atmosphere of 5% CO_2 in air at 37ºC (without Caco-2 cells). At 0 h and 6 h of incubation, 100 µL sample was taken from each medium extract, as 3 parallels, and diluted 1:2 (v:v) with 100 µL methanol (adjusted to pH 2 with formic acid) addition. These methanolic extracts were stored at -20ºC until further HPLC analysis. Before HPLC measurements, the samples were sonicated for 10 min, centrifuged for 5 min at 13200 rpm and filtered through 0.45 µm filters (Minisart SRP4, Biotech GmbH, Germany) to vials. Anthocyanin measurements were performed, as described previously in section 3.2.3, in order to determine the changes in concentrations (degradation rate/recovery/stability) during 6 h incubation time. For quantification, dose-response curve of cyanidin-3-glucoside (0-50 µg/mL) was used as a reference.

Cell monolayer integrity with different cell mediums

The effects of different cell mediums on the integrity of cells were assessed with TEER measurements performed using the protocol below. Initially, first TEER measurement was performed with Caco-2 cells in their culture medium, before the replacement of the culture medium with one of the four mediums -including HBSS, HBSS with 100 mM HEPES, DMEM with 9.1% FBS, and DMEM (no FBS)- selected. This first value, measured in culture medium, was recorded as initial TEER value. Secondly, the culture medium was removed and cell monolayers were rinsed with one of the four mediums tried out. After rinsing, 2.0 mL and 2.5 mL of this new medium was added to the apical and the basolateral compartments of cell monolayers, respectively. Following the replacement of the culture medium with the new medium, 1 h of pre-incubation time allowed at 37ºC with 5% CO_2, and then the TEER value was measured for the second time and recorded as TEER at 0 h. The third and final TEER measurement was performed after 6 h from the 0 h measurements and recorded as TEER at 6 h of incubation. At the end of 6 h

incubation in the replaced medium, the mediums giving TEER values above the value of 200 $\Omega.cm^2$ were defined as proper mediums for further experiments.

Transport experiments with fruit and nectar samples

Transport experiments were carried out with whole fruit (WF) and whole nectar (WN) samples, as well as their solid phase extracted forms (fruit-SPE and nectar-SPE). Wet-weight powders of WF and WN were diluted in transport medium, which was determined as DMEM (no FBS), in order to have the concentrations of approximately 35 mg dry-weight / mL medium (≈ 40 - 45 μg C3GR / mL medium) for WF and 55 mg dry-weight / mL medium (≈ 40 - 45 μg C3GR / mL medium) for WN. The fruit and nectar samples were diluted in transport medium in different dry-weight concentrations since nectar included more than 50% sucrose added to its dry content (Table 3.1).

Fruit-SPE and nectar-SPE pellets, which were obtained by applying the procedure detailed in section 3.2.9.1, were diluted in transport medium in order to obtain the anthocyanin concentrations equal to the concentrations obtained for WF and WN samples when they were diluted in transport medium (≈ 40 - 45 μg C3GR / mL medium) (in order to make these calculations, all samples were initially analysed with HPLC for their anthocyanin levels).

Furthermore, the possible effects of sucrose and/or citric acid, the nectar ingredients providing the most significant differentiation between nectar and fruit samples, on transport through Caco-2 cells were also assessed. These additional ingredients of WN matrix were added to the WF sample and also to the fruit-SPE and nectar-SPE samples. Citric acid was added to the samples to lower the pH to the values recorded for nectar in transport medium. In addition, sucrose was included into samples with approximately the same concentrations (on dry-weight basis) as in nectar.

Caco-2 cells used in transport experiments were seeded and differentiated on 6-well transwell inserts due to the protocol given above. Culture medium was replaced with transport medium. After 1 h of pre-incubation in transport medium, the medium was removed and freshly prepared WN, WF, fruit-SPE and nectar-SPE samples, as well as WF, fruit-SPE, and nectar-SPE samples with their sucrose and/or citric acid added forms in transport medium were added to the apical side of the cells (in 2.0 mL volume) and the transport medium only was added to the basolateral compartment

(in 2.5 mL volume). Before adding to the cells, 0 h samples were taken from the freshly prepared samples in transport medium, and extracted in methanol (adjusted to pH 2 with formic acid) with 1:2 (v/v) dilution ratio. TEER values were measured just after the addition of the samples to the wells, and recorded as 0 h values. Transepithelial transport was allowed through 6 h incubation time at 37ºC with 5% CO_2 in air. At the end of the incubation time, TEER measurements were performed again, to assess the integrity of the cell layer, and recorded as 6 h values. Subsequently, apical and basolateral sides for each sample were collected (as three replicates at least), stabilized in 1:1 (v:v) methanol (adjusted to pH 2 with formic acid), and stored at -20ºC until HPLC analysis. The analysis of anthocyanins was carried out on both apical and basal side methanolic extracts by a RP-HPLC as described in section 3.2.3. The HPLC results obtained for apical side samples were used to evaluate the recovery of anthocyanins on cell layer and the results obtained for samples from basolateral compartments were used to assess the passage of anthocyanins through cell layer during 6 h incubation.

3.2.10 Statistical analysis

Statistical analyses were conducted using the general linear model (GLM) followed by Duncan's new multiple range test, using the data of all five biological replicates for each nectar processing sample ($\alpha = 0.05$). Pairwise comparisons between the treatments were done using Tukey test with a 95% confidence level. Student's *t*-test was used to determine the differences in relative mass signal intensities, obtained by LC-MS data, between processing steps. Comparison and visualization of the main features of the LC-MS data were performed by loading the data matrix into GeneMaths XT 1.6 software (www.applied-maths.com). Metabolite intensities were normalized using log2 transformation and standardized using range scaling (autoscaling normalization). Principal Components Analysis (PCA) was performed for unsupervised comparison of samples and metabolites. Tables for statistical analysis are given in the Appendix Table B.1.

4. RESULTS AND DISCUSSION

In this study, industrial-scale sour cherry nectar processing was monitored for five different nectar production batches, in which18 different processing steps were addressed, leading to 22 samples per fruit batch and a total of 110 samples. Each sample was analyzed for their moisture, total phenolic, total flavonoid, and total monomeric anthocyanin contents; total antioxidant capacities and on-line antioxidant determinations; profiles and quantities of anthocyanins, flavonols, flavan-3-ols, phenolic acids; vitamin C contents; and metabolomics. Additionally, fruit and nectar samples were subjected to bioavailability analyses performed using simulated *in vitro* gastrointestinal digestion procedure and *in vitro* Caco-2 cell model.

4.1 Moisture Contents of Sour Cherry Samples

The average values for moisture contents of 22 samples taken from 5 different independent batches of sour cherry processing are given in Table 4.1. The most pronounced effect was obtained with evaporation (concentration) step which led to a ≈ 2-fold reduction in moisture content, from 89.6% in filtered juice (FtJ) to 43.4% in concentrated juice (CJ).

The waste materials, including stalk, seed and press cake, were lower in moisture content compared to the other processing samples. A modest change in wet-weight, with 11% increase of moisture content, was observed during mash pressing, where press cake was removed and water was added to extract the press cake.

Table 4.1 : Moisture content (%) values of all 22 samples taken from sour cherry processing.

Sample	Moisture content (%) [a]
FF	78.6 ± 3.7 de
FF-St	78.2 ± 3.8 de
St	66.8 ± 0.6 fg
MH	76.8 ± 5.0 e
MP	86.2 ± 1.1 cd
MPEX-I	92.8 ± 2.5 abc
MPEX-II	96.7 ± 0.9 ab
MPEX-III	98.4 ± 0.2 a
PrJ	88.0 ± 1.1 c
PC	57.4 ± 6.9 g
Sd	26.7 ± 0.0 i
PsJ	86.1 ± 1.5 cd
EnJ	87.4 ± 1.6 c
ClJ	87.5 ± 2.4 c
FtJ	89.6 ± 1.8 bc
FR	89.2 ± 2.7 bc
CJ	43.4 ± 0.7 h
CJ-BPF	66.5 ± 10.1 fg
CJ-APF	70.0 ± 2.6 ef
N	88.0 ± 2.4 c
SSN	86.6 ± 5.0 cd
PN	86.7 ± 5.2 cd

[a] Values given for moisture content (%) in this table represent the average values ± standard deviation of five biological replications for each sample. Different letters represent statistically significant differences ($p<0.05$).

4.2 Spectrophotometric Assays

Six different spectrophotometric analyses were performed on methanolic extracts of the samples, and values were compared on a dry-weight basis.

4.2.1 Total phenolics, total flavonoids, and total anthocyanins

Total phenolic contents (TP), total flavonoid contents (TF), and total monomeric anthocyanin contents (TA) of sour cherry processing samples are shown in Table 4.2 and the percent changes, in comparison to the contents in fruit (without stalk) sample (represented as 100%), during each nectar processing step are given in Figure 4.1.

Table 4.2 : Total phenolic, total flavonoid, and total anthocyanin contents of nectar processing samples from 5 batches of sour cherry fruit.

Sample	Total phenolics (mg GAE / 100 g dw)[a]	% of (FF-St)	Total flavonoids (mg (+)CE / 100 g dw)[a]	% of (FF-St)	Total anthocyanins (mg C3G / 100 g dw)[a]	% of (FF-St)
FF	921.8 ± 184.3 cdef		440.6 ± 81.3 cdefghi		169.8 ± 28.4 bcde	
FF-St	747.0 ± 100.3 cdefg	100	410.5 ± 58.5 defghi	100	145.7 ± 26.2 cdef	100
St	759.2 ± 60.4 cdefg	102	610.7 ± 91.3 cdefgh	149	2.0 ± 0.2 fg	1
MH	1053.4 ± 139.8 cdef	141	642.2 ± 114.8 cdefg	156	244.0 ± 55.8 abc	167
MP	1252.3 ± 68.0 cd	168	665.9 ± 68.3 cdef	162	266.5 ± 72.1 abc	183
MPEX-I	2041.3 ± 305.7 ab	273	1375.9 ± 282.0 b	335	343.8 ± 90.0 a	236
MPEX-II	2130.0 ± 5.3 a	285	1634.2 ± 389.1 ab	398	371.4 ± 92.1 a	255
MPEX-III	2155.5 ± 835.7 a	289	2081.3 ± 416.3 a	507	238.0 ± 5.9 abcd	163
PrJ	1458.5 ± 189.8 abc	195	798.8 ± 177.4 cd	195	332.0 ± 28.0 a	228
PC	273.1 ± 43.5 fg	37	158.6 ± 39.8 hi	39	19.3 ± 4.8 fg	13
Sd	29.9 ± 4.6 g	4	25.1 ± 4.3 i	6	1.8 ± 0.4 g	1
PsJ	1336.0 ± 160.8 bcd	179	807.9 ± 147.4 cd	197	298.7 ± 23.8 ab	205
EnJ	1407.6 ± 266.5 abc	188	717.5 ± 28.6 cdef	175	324.6 ± 28.6 a	223
ClJ	1165.4 ± 28.4 cde	156	762.5 ± 114.7 cde	186	309.2 ± 67.6 ab	212
FtJ	1501.6 ± 230.0 abc	201	828.6 ± 39.8 cd	202	247.8 ± 40.0 abc	170
FR	1490.3 ± 473.4 abc	200	868.8 ± 191.3 cd	212	276.3 ± 97.3 abc	190
CJ	303.9 ± 43.3 efg	41	787.3 ± 60.2 cd	192	233.7 ± 6.6 abcd	160
CJ-BPF	674.9 ± 51.7 cdefg	90	867.7 ± 81.8 cd	211	230.1 ± 25.6 abcd	158
CJ-APF	668.7 ± 89.8 defg	90	898.1 ± 107.3 c	219	266.8 ± 24.8 abc	183
N	363.5 ± 40.0 efg	49(113)[b]	293.5 ± 44.0 efghi	72(164)[b]	108.6 ± 3.0 cdef	75(173)[b]
SSN	316.6 ± 51.3 efg	42(122)[c]	175.0 ± 1.3 ghi	43(124)[c]	46.4 ± 1.4 efg	32(93)[c]
PN	477.6 ± 34.4 efg	64(186)[c]	251.4 ± 6.1 fghi	61(178)[c]	64.9 ± 4.6 defg	45(131)[c]

[a] Data given in this table represents average amounts ± standard deviation of five biological replications for each sample. Different letters in the columns represent statistically significant differences ($p<0.05$).

[b] The values in the brackets represent the original percent multiplied by factor 2.3 to compensate for the additional sucrose in dry-weight composition of the initial nectar (N) sample.

[c] The values in brackets represent the original percent multiplied by a factor 2.9 to compensate for the additional sucrose in dry-weight composition of the sucrose syrup added nectar (SSN) and final pasteurized nectar (PN) samples.

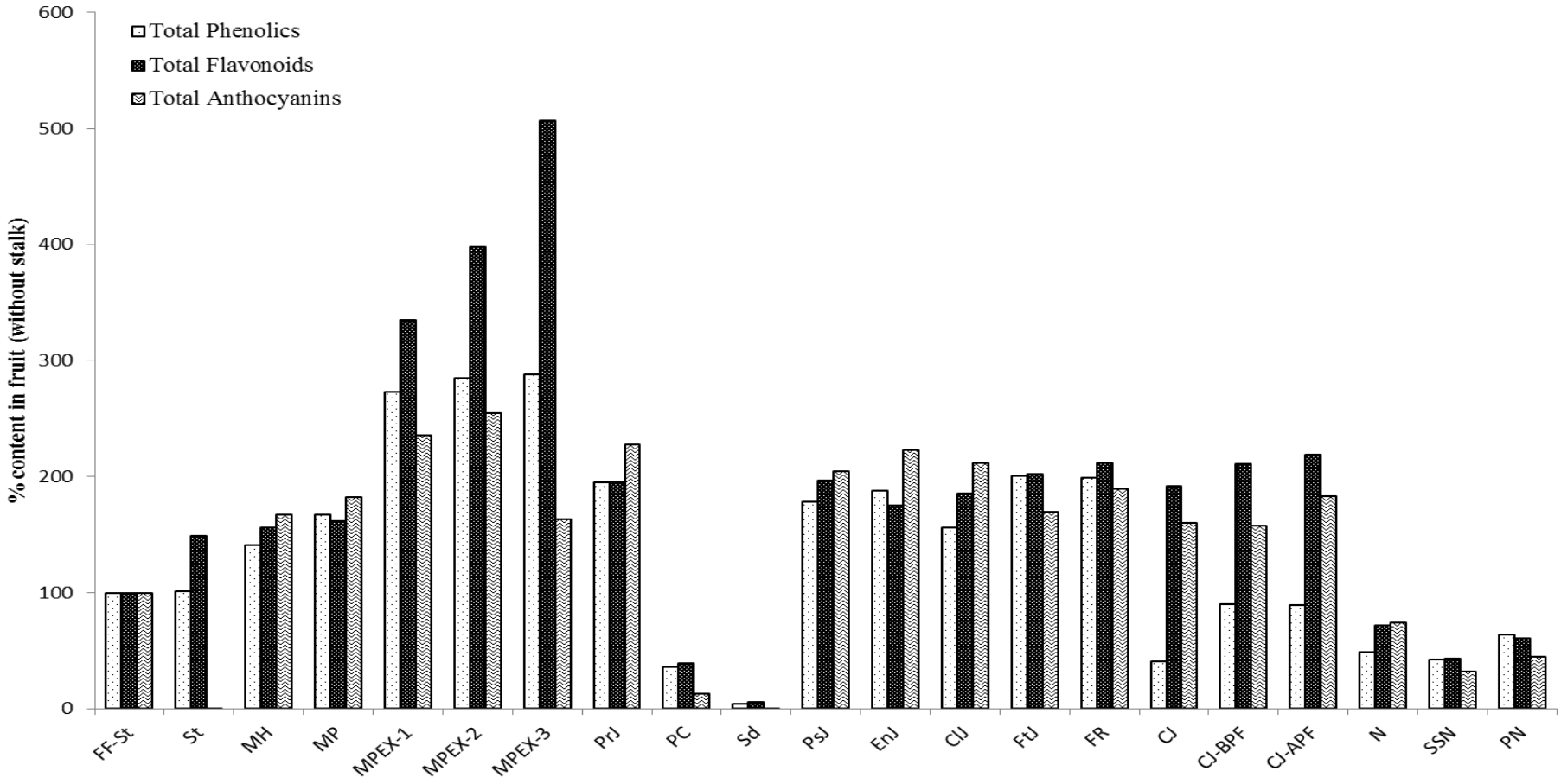

Figure 4.1 : The percent changes in total phenolics, total flavonoids, and total anthocyanins contents of sour cherry nectar processing samples collected from 5 different batches. The contents of each component in fruit (without stalk) sample was represented as 100%.

The phenolic contents of sour cherry fruit (without stalk) and final pasteurized nectar samples were determined to be 747.0 ± 100.3 mg GAE/100 g dry-weight (162.8 mg GAE/100 g fresh weight) and 477.6 ± 34.4 mg GAE/100 g dry-weight (635 mg GAE/L), respectively. The total phenols recorded for fruit sample was much lower than the reported values by Capanoglu *et al.* (2011) as 3371.1 mg GAE/100 g dry-weight. On the other hand, the average TP value of fruit was in the range of TP values measured for four different sour cherry cultivars by Kim *et al.* (2005) who reported the lowest TP value as 146.1 mg GAE/100 g fresh weight for Balaton cultivar and the highest value as 312.4 mg GAE/100 g fresh weight for Sumadinka cultivar. Our findings for TP of sour cherry fruit were also similar to the results of Kirakosyan *et al.* (2009) who reported 674,2 mg GAE/100 g dry-weight and 1266,5 mg GAE/100 g dry-weight for Balaton cherry (frozen) and Montmorency cherry (frozen), respectively. Additionally, Piccollella *et al.* (2008) have found similar results for TP with a value of 141.9 mg GAE/100 g fresh-weight. In literature, the TP of sour cherry nectar and sour cherry juice was based on a (+) catechin calibration and expressed as 475.7 mg catechin /L by Tosun and Ustun (2003) and 2704 – 4998 mg catechin/L by Bonerz *et al.* (2007), respectively. This replacement of the recommended gallic acid reference standard (Singleton and Rossi, 1965) with catechin equivalents results in lack of standardization of methods which may yield several orders of magnitude difference in detected phenol contents (Prior *et al.*, 2005).

Total flavonoid content of fruit samples was determined to be 410.5 ± 58.5 mg (+)CE/100 g dry-weight (89.5 mg (+)CE/100 g fresh-weight). These results were in consistence with the results obtained for TF of sour cherry fruit as 420.5 mg (+)CE/100 g dry-weight by Capanoglu *et al.* (2011) and 138.6 mg (+)CE/100 g fresh-weight by Marinova *et al.* (2005). Additionally, Piccollella *et al.* (2008) estimated the TF content of sour cherry as 3.9 mg (+)CE/100 g fresh-weight. Apparently, there could be variations between the studies in literature for TF values determined using different fruit material.

Total anthocyanin contents of sour cherry fruit and nectar samples were determined to be 145.7 ± 26.2 mg C3G/100 g dry-weight (31.8 mg C3G/100 g fresh-weight) and 64.9 ± 4.6 mg C3G/100 g dry-weight (86 mg C3G/L), respectively. Kim *et al.* (2005) reported TA values of four different sour cherry cultivars ranging between

49.1 – 109.2 mg C3G / 100 g fresh cherry. Chaovanalikit and Wrolstad (2004a) reported TA for Montmorency variety as 8.7 mg C3G / 100 g fresh weight. The TA contents of different sour cherry cultivars investigated by Kim and Padilla-Zakour (2004) were reported to be in the range of 45.0 – 67.1 mg C3G / 100 g fresh weight. Kirakosyan *et al.* (2009) measured the TA values of Montmorency and Balaton sour cherries as 53.3 and 174.1 mg C3G/100 g dry-weight, respectively. Our results are in aligned with those findings reported in the literature. Total anthocyanin content for sour cherry nectar was found to be 24.6 mg C3G/L by Tosun and Ustun (2003) which was lower than TA content obtained for nectar samples in our study.

The TP, TF, and TA contents of all nectar processing samples, monitored using spectrophotometric assays during the nectar-making process, revealed significant changes in two of the applied treatments. Firstly, the steps leading from original fruit (FF-St) to pressed juice (PrJ) consistently led to an increase up to 2-3 fold in all three assays, as determined on a dry-weight basis (Table 4.2 and Figure 4.1). These substantial increases obtained during processing the original fruit (FF-St) to the initial pressed juice (PrJ) was linked to the removal of dry-weight in the form of press-cake (29% of total dry-weight), which was found to be relatively poor in these compounds. The TP, TF, and TA contents of press-cake sample were found to be 37%, 39%, and 13% of the contents determined for original fruit (FF-St) sample, respectively. Apparently, press-cake removal is mainly relevant to the removal of dry-weight which is low in antioxidant compounds, and the net result is an increase in the relative representation of antioxidants on dry-weight basis. The lower contents of antioxidant compounds in PC samples also indicates the efficiency of compound recovery into the juice fraction, which may be facilitated by the repetitions of the press-cake extraction (Figure 3.1 and Table 3.1). In addition, mash heating step (80ºC, 90s) applied before pressing likely gave rise to an increased phenolic content through several ways: preventing the further oxidation of these compounds by inactivating the native enzymes (Auw *et al.*, 1996); and/or facilitating the extraction of compounds through macerated peel tissue, with increased membrane permeability at high temperatures, obtained during mash heating (Spanos *et al.*, 1990); and/or releasing the bound phenolics with the breakdown of cellular constituents with temperature (Dewanto *et al.*, 2002). Therefore, mash heating could be indicated as an effective step which helped retaining original antioxidant properties. Secondly,

processing of concentrated juice (CJ) into nectar (N) led to a 2 to 4-fold reduction in the measured values (except for TP content assay). However, this reduction could be related to the addition of sucrose to the concentrate (CJ) during the production of nectar (N), which led to an addition of more than 50% sucrose to the total dry-weight of the nectar samples (Table 3.1). When the values obtained for final pasteurized nectar (PN) were corrected for the added sucrose, TP, TF, TA contents were around 131-186% of those in the original fruit (FF-St). Moreover, wet-weight basis calculations revealed that the TP, TF, TA concentrations in PN sample were 39%, 37%, and 27% of those obtained for FF-St sample, respectively. When the recovery was calculated on a total input / output basis (i.e. total amount of components present in one nectar processing batch starting from original fruit material (FF-St) compared to the total amount present in the final nectar (PN)), we observed that 121%, 116%, and 84% of fruit phenolics, flavonoids, and anthocyanins ended up in the final nectar, respectively. These values can all be linked to the high extraction efficiency from the repeated press-cake extractions, which increased the juice yield from 73% to 85% (Table 3.1) and the stability of the processing method. Press-cake residues were determined to be relatively poor in fruit antioxidants, specifically anthocyanins: 11%, 12%, and 4% of fruit phenolics, flavonoids, and anthocyanins retained in the press-cake (on a total input/output basis).

On the other hand, the steps between pressed juice (PrJ) and concentrate (CJ) show less consistent results: strong (± 4-fold) reductions occurred in the TP assay, while modest losses or even increases were observed in the other assays. In addition, the re-calculated TP values in PN samples to compensate for the added-sucrose on dry-weight basis were higher (186%) than the values obtained for TF and TA (178% and 131%, respectively). The lack of the specificity of the Folin Ciocalteau (F – C) method for phenolic compounds (Capanoglu *et al.*, 2008) could be a reason for this method not to reflect the changes consistently in TP during nectar processing. The F – C method was reported to be suffering from a number of interfering substances, including specifically sugars, aromatic amines, sulfur dioxide, ascorbic acid, organic acids, and Fe(II) (Box, 1983), and it was strongly suggested that corrections for those interfering substances should be made in order to establish a uniformly acceptable method of TP to compare the obtained results rationally (Prior *et al.*, 2005). The high amounts of sucrose (66% of dry-matter) (see Table 3.1), ascorbic acid derivative (see

Table 4.14), and citric acid (see Table 3.1) containing in nectar samples could be related to the higher nectar:fruit ratios obtained for TP using Folin – Ciocalteau method compared to the ratios calculated for TF and TA in nectar.

Among the waste samples other than press-cake, seed had the lowest values (4%, 6%, and 1% of TP, TF, TA contents of FF-St) in all assays. Stalks were significantly poor in TA content (1% of FF-ST), whereas they gave higher values in the other assays (102% and 149% of TP and TF contents of those in FF-St).

The high degree of extraction of health-associated antioxidant components from sour cherry as reported here, differs significantly from the losses that have been reported for other fruit juice processes. In bayberry juice processing, 21% and 16% of bayberry phenolics and anthocyanins were determined to be recovered respectively, in the juice, even though the juice yield ranged from 73 to 78% (w/w). The centrifuged bayberry cake residues were still found to contain a substantial amount of the fresh fruit polyphenolics (30-35%) and anthocyanins (≈ 55%) (Fang *et al.*, 2006). Additionally, Lee *et al.* (2002) reported poor extraction efficiencies of frozen blueberry polyphenolics and anthocyanins into the pressed juice (75-83% (w/w) yield), with ratios of < 22% and 35-40%, respectively. This was related to the degradation of the compounds during the process. Furthermore, 15-20% of frozen berry polyphenolics and 55% of the anthocyanins were retained in the press-cake waste (Lee *et al.*, 2002). These findings suggest that repeated press-cake extraction in sour cherry nectar processing permits efficient cumulative transfer of the phenolic compounds present in the fruit into the final product. In addition, another factor relevant to anthocyanin recovery from cherry fruit could be the location of these pigments in the fruit tissue. As press-cake is mainly composed of relatively intact fruit skins, fruits such as cherry with higher anthocyanin contents in their flesh (as opposed to in the skin such as blackcurrants) may yield greater levels of anthocyanins in their processed juices.

4.2.2 Total antioxidant capacity

Three different *in vitro* tests including ABTS, CUPRAC, and DPPH methods were performed for the antioxidant capacity measurements of sour cherry nectar processing samples (Table 4.3 and Figure 4.2). Among the three different assays used for measuring the TAC, CUPRAC method gave the highest values, while the

lowest values were obtained with DPPH method. ABTS and CUPRAC were highly correlated, by considering the changes obtained for each individual processing step, whereas the trend of change in antioxidant capacity measured with DPPH method was slightly different. DPPH method was reported to have several drawbacks which could adversely affect the correct determination of TAC by measuring the ability of antioxidant compounds to react with DPPH. The color loss in DPPH method can be obtained either with radical reaction (by hydrogen atom transfer) or reduction reaction (by single electron transfer). Additionally, one of the major determinant factors of this reaction is known to be steric accessibility which facilitates a better accesss of small molecules to the radical site that ends up with higher apparent TAC of these small molecules with this test (Prior *et al.,* 2005). DPPH method has been reported as a method more suitable for samples with lipophilic antioxidants or with high lipid contents (Ozgen *et al.,* 2006).

The total antioxidant capacities of sour cherry fruit samples were determined to be 2123.8 ± 445.8, 3974.0 ± 861.0, 820.1 ± 85.8 mg TEAC/100 g dry-weight (8485.4, 15877.6, 3276.6 µmol TEAC/100 g dry-wieght; 18.2, 34.0, and 7.0 µmol TEAC/g fresh-weight) with the applied *in vitro* tests, ABTS, CUPRAC and DPPH, respectively. The values obtained for TAC of sour cherry fruit samples were in accordance with the results of Chaovanalikit and Wrolstad (2004a) who determined the TAC of four sour cherry fruit cultivars ranging between 2.92 – 37.56 µmol TE/g fresh-weight. However, the recorded average values for TAC of fruit samples with ABTS and CUPRAC methods were 2-fold lower than the values reported by Capanoglu *et al.* (2011). On the other hand, TAC values determined with ABTS and CUPRAC methods were higher than the results of Kirakosyan *et al.* (2009) who recorded those values to be 9.6 – 9.8 mM TEAC for frozen Balaton and Montmorency sour cherry varieties.

Table 4.3 : Total antioxidant capacities of sour cherry samples determined using 3 different analytical methods.

Sample	ABTS (mg TEAC/100g dw)[a]	% of (FF-St)	CUPRAC (mg TEAC/100g dw)[a]	% of (FF-St)	DPPH (mg TEAC/100g dw)[a]	% of (FF-St)
FF	2668.9 ± 583.6 cdefg		6367.0 ± 1287.3 abcde		1349.1 ± 293.1 bc	
FF-St	2123.8 ± 445.8 defgh	100	3974.0 ± 861.0 def	100	820.1 ± 85.8 bc	100
St	3079.8 ± 440.1 bcde	145	5606.0 ± 673.5 bcde	141	1100.8 ± 120.6 bc	134
MH	3331.3 ± 477.0 bcd	157	6393.8 ± 912.9 abcde	161	1169.3 ± 164.4 bc	143
MP	3489.2 ± 269.7 abcd	164	6449.2 ± 564.8 abcde	162	1302.8 ± 96.1 bc	159
MPEX-I	4295.5 ± 500.0 abcd	202	9856.6 ± 957.0 a	248	3870.4 ± 693.1 a	472
MPEX-II	5273.1 ± 93.3 ab	248	9863.5 ± 229.4 a	248	3936.5 ± 971.1 a	480
MPEX-III	5655.4 ± 2149.6 a	266	9661.8 ± 3279.9 ab	243	4214.6 ± 1832.8 a	514
PrJ	4033.4 ± 710.6 abcd	190	7706.5 ± 1407.5 abc	194	1199.6 ± 144.0 bc	146
PC	780.3 ± 165.2 fgh	37	1217.7 ± 214.5 f	31	287.5 ± 43.3 bc	35
Sd	102.0 ± 24.7 h	5	147.4 ± 23.8 f	4	80.3 ± 3.9 c	10
PsJ	3998.5 ± 633.4 abcd	188	7554.9 ± 1290.4 abcd	190	1456.0 ± 180.5 bc	178
EnJ	4215.1 ± 750.7 abcd	198	7889.4 ± 1650.1 abc	199	1547.2 ± 280.1 bc	189
ClJ	3779.6 ± 566.3 abcd	178	8154.7 ± 1878.3 abc	205	1466.4 ± 269.3 bc	179
FtJ	3662.8 ± 1268.0 abcd	172	7618.1 ± 1473.3 abc	192	1630.6 ± 263.4 bc	199
FR	4436.1 ± 861.9 abc	209	8270.6 ± 1246.7 abc	208	1696.7 ± 513.1 b	207
CJ	2954.5 ± 208.2 cdef	139	5845.7 ± 373.0 abcde	147	1619.6 ± 389.8 bc	197
CJ-BPF	3080.2 ± 228.1 bcde	145	5441.7 ± 241.9 cde	137	1483.5 ± 145.2 bc	181
CJ-APF	3450.0 ± 497.9 abcd	162	6217.5 ± 897.0 abcde	156	1787.1 ± 239.3 b	218
N	1006.7 ± 464.2 efgh	47(109)[b]	2684.5 ± 286.1 ef	68(156)[b]	997.8 ± 158.5 bc	122(281)[b]
SSN	684.5 ± 69.3 gh	32(93)[c]	1223.1 ± 120.2 f	31(90)[c]	498.2 ± 92.9 bc	61(177)[c]
PN	1026.9 ± 55.2 fgh	48(140)[c]	1795.9 ± 84.6 f	45(131)[c]	727.9 ± 64.0 bc	89(258)[c]

[a] Data represent average amounts ± standard deviation of five processing events for each sample. Different letters in the columns represent statistically significant differences ($p<0.05$).

[b] The values in the brackets represent the original percentage multiplied by factor 2.3 to compensate for the additional sucrose in dry-weight composition of the initial nectar (N) sample.

[c] The values in brackets represent the original percentage multiplied by factor 2.9 to compensate for the additional sucrose in dry-weight composition of the sucrose syrup added nectar (SSN) and final pasteurized nectar (PN) samples.

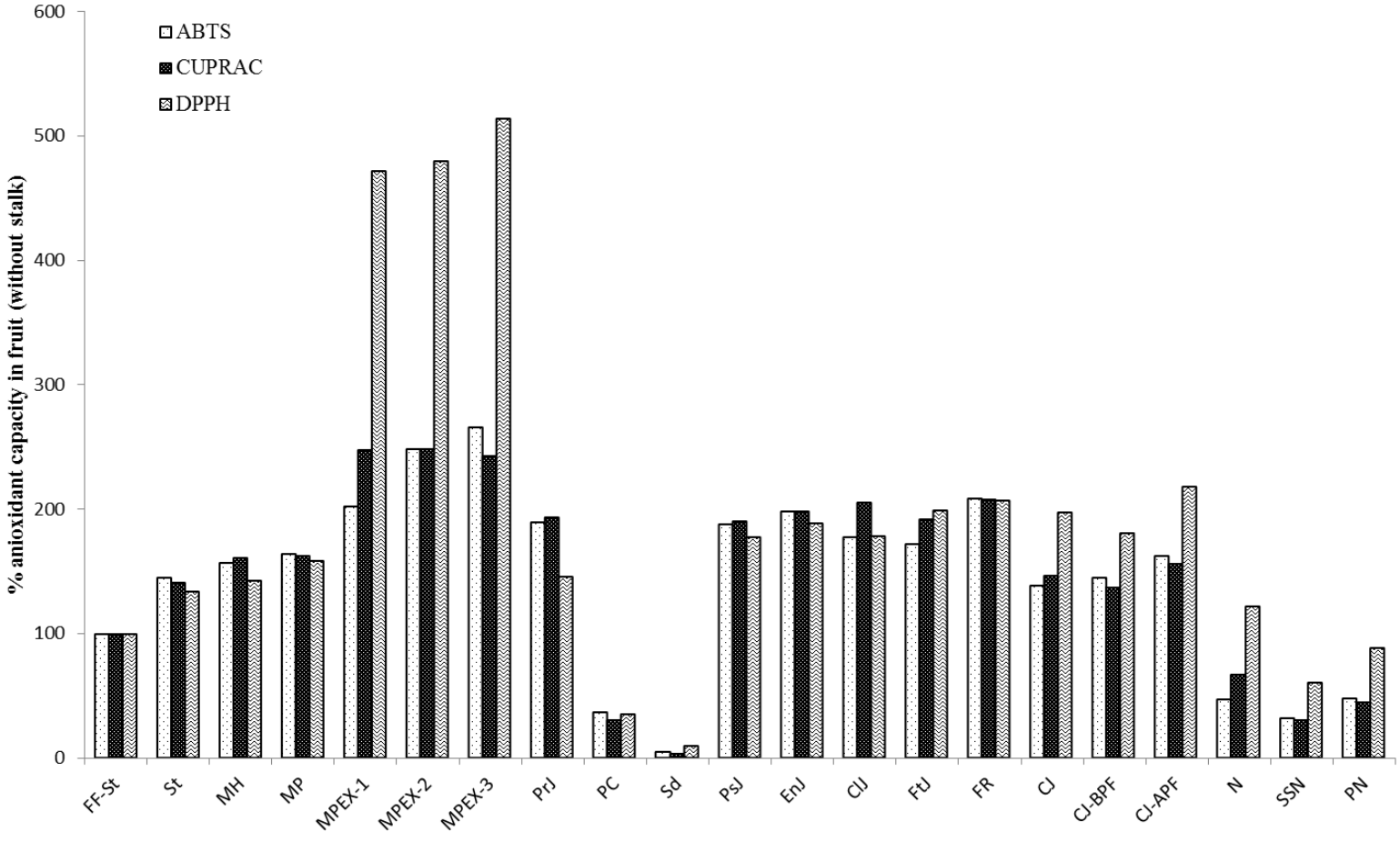

Figure 4.2 : The percent changes in total antioxidant capacities of sour cherry samples obtained from 5 independent nectar processing batches. The antioxidant capacities, measured with 3 different *in vitro* assays, determined for fruit (without stalk) sample was represented as 100%.

The TAC values measured for final nectar samples were 1026.9 ± 55.2, 1795.9 ± 84.6, 727.9 ± 64.0 mg TEAC/100 g dry-weight for ABTS, CUPRAC, and DPPH methods, respectively, which were corresponding to the values of between 3.9 – 9.5 mmol TEAC/L nectar for those applied 3 tests. Those findings were lower than the values determined by Bonerz *et al.* (2007) as 27.5 – 54.6 mmol/L Trolox equivalents for sour cherry juices of five different sour cherry cultivars, including *Schattenmorelle, Gerema, Ungarische Traubige, Cigany, Stevnsbaer Birgitte*.

The processing of original fruit (FF-St) to pressed juice (PrJ) gave rise to approximately 2-fold increase in TAC in all assays. On the other hand, while the three additional MPEX steps applied after the initial mash pressing step (MP) increased the TAC up to 2-3 fold in ABTS and CUPRAC methods, these steps did result in approximately 5 fold increase in DPPH method. In addition, the processing of the PrJ to the CJ led to modest decreases in both ABTS and CUPRAC methods, but a modest increase was obtained in DPPH method (Table 4.3). Similarly to the trend obtained for TP, TF, and TA analyses results, processing of concentrated juice (CJ) into nectar (N) led to a 2 to 3-fold reduction in the measured values, which was explained with the addition of sucrose to the dry-weight of nectar. The re-calculated TAC values in PN samples by multiplying with the factor, which was determined to compensate for the added sucrose in nectar samples, led to TAC in PN samples ranging between 131% and 258% of those in original fruit (FF-St) sample. In TAC assays, wet-weight values for the PN samples were in between 27-54% of the values for FF-St sample. Equivalent recoveries for a total input / output basis calculations in TAC assays were 92%, 86%, and 168% in ABTS, CUPRAC, and DPPH methods, respectively. The poor antioxidant capacity, determined on a total input / output basis to be ranging between 9% and 19% of TAC in FF-St sample, in PC could explain these high recoveries into the nectar.

The total antioxidant capacity measured in press-cake (PC) and seed (Sd) waste samples were found to have lower values (app. 35%, and ≤ 10% of values in original fruit (FF-St) sample in PC and Sd samples, respectively), whereas the values determined for stalk (St) and filtration residue (FR) wastes were 1.5-2-fold higher than the TAC of original fruit sample. High amounts of flavonoids in St sample, and high amounts of all phenolics, flavonoids, and anthocyanins in FR sample could be the result of high TAC values in these waste samples.

Many different methods have been required for the evaluation of the effectiveness of antioxidants in foods and in biological systems since a total antioxidant capacity assay based on a single chemical reaction is thought to be rather unrealistic and difficult to come by (Huang *et al.*, 2005). *In vitro* testing of natural antioxidants may need to consider the composition of the system analyzed, inhibition of the oxidation of a suitable substrate, the mode of oxidation initiation, the extent of oxidation (an end-point), and the method of quantification of the antioxidant activity (Frankel and Meyer, 2000; Sanchez-Moreno, 2002). The combination of these factors are numerous and various analytical strategies are possible even with the same analytical techniques (Arnao *et al.*, 1999). For this reason, it has been pointed out that it is not sufficient to use one-dimensional methods in evaluation of the multifunctional food and biological system antioxidants (Frankel and Meyer, 2000). Hence, it has been reported to be more appropriate to perform several *in vitro* tests based on different principles in order to be able to evaluate the antioxidants in a food or biological system in a broader aspect (Antolovich *et al.*, 2002).

4.3 Targeted HPLC Analysis for Phenolic Identification and Quantification

The individual sour cherry antioxidant components were identified and quantified using targeted HPLC analysis performed on the 22 samples from each of the 5 replicates. The representative HPLC chromatograms for sour cherry fruit and nectar samples recorded using PDA detector at 280 nm are given in Figure 4.3.

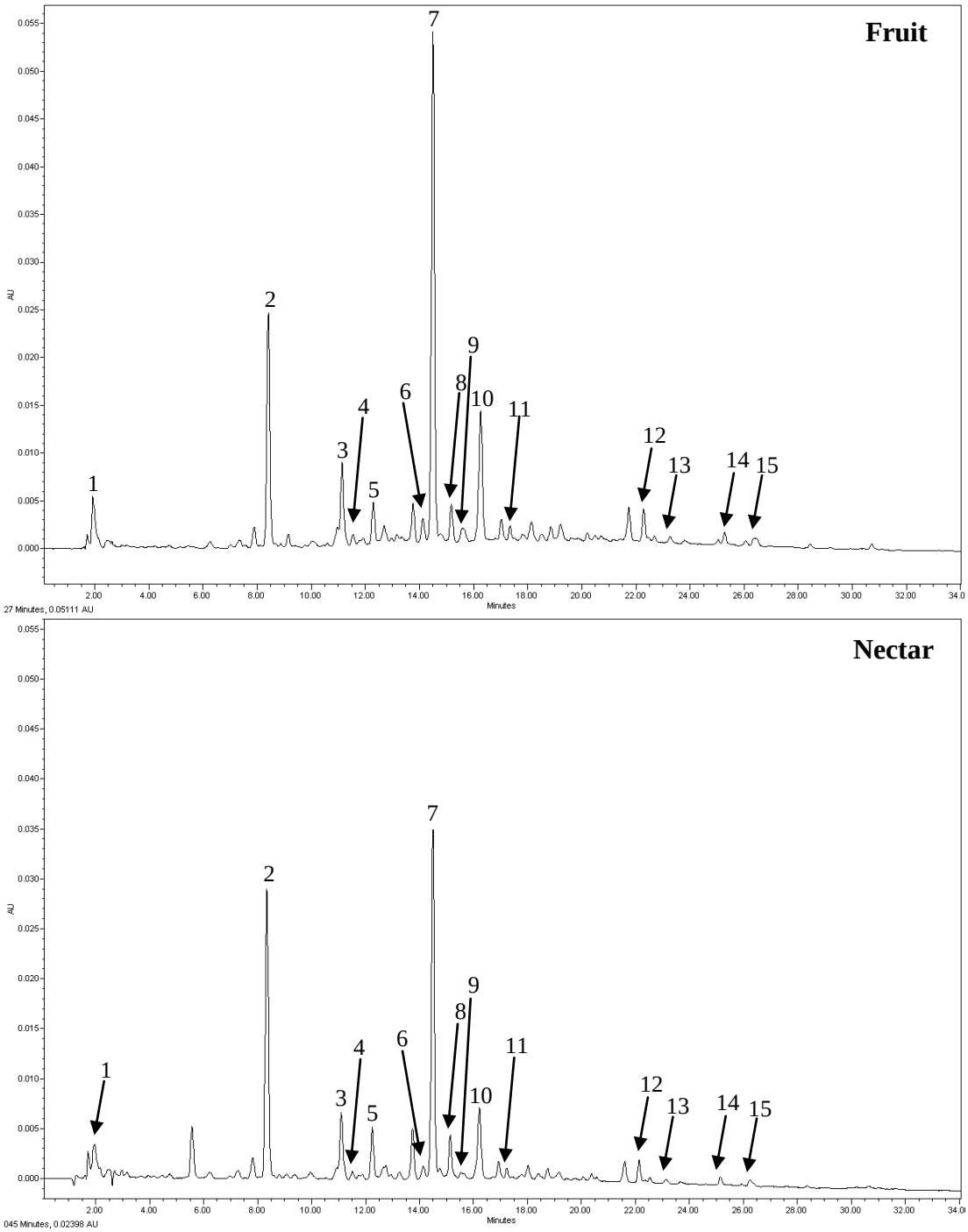

Figure 4.3 : Representative HPLC chromatograms of sour cherry original fruit (FF-St) (upper panel) and final nectar (PN) (lower panel) extracts recorded by PDA, 280 nm. Chromatograms are on the same scale. Numbers refer to the main peaks identified: 1, putative ascorbic acid derivative (Capanoglu *et al.*, 2008); 2, neochlorogenic acid (Bonerz *et al.*, 2007); 3, 3-coumaroylquinic acid (Bonerz *et al.*, 2007); 4, (+)-catechin (standard); 5, chlorogenic acid (standard); 6, cyanidin-3-sophoroside (Bonerz *et al.*, 2007); 7, cyanidin-3-(2^G-glucosylrutinoside) (Bonerz *et al.*, 2007; Chandra *et al.*, 2001); 8, (-)-epicatechin (standard); 9, cyanidin-3-glucoside (standard); 10, cyanidin-3-rutinoside (Bonerz *et al.*, 2007; Chandra *et al.*, 2001); 11, quercetin-rutinoside-glucoside (Bonerz *et al.*, 2007); 12, rutin (quercetin-3-rutinoside) (standard); 13, quercetin-3-glucoside (standard); 14, kaempferol-3-rutinoside (standard); 15, rosmarinic acid (standard).

4.3.1 Anthocyanins

The sour cherry anthocyanin components, identified using HPLC-PDA detection at 512 nm, in nectar processing samples were cyanidin-3-sophoroside (C3S), cyanidin-3-(2^G-glucosylrutinoside) (C3GR), cyanidin-3-glucoside (C3G), and cyanidin-3-rutinoside (C3R) (Figure 4.3). The major anthocyanin component was C3GR in sour cherry samples, composed around 75% of total anthocyanins, with amounts of 195.1 ± 38.0 mg C3G eq./100 g dry-weight (42.5 mg C3G eq./100 g fresh-weight) in original fruit (FF-St) samples and 89.6 ± 25.7 mg C3G eq./100 g dry-weight (119 mg C3G eq./L nectar) in final nectar (PN) samples (Table 4.4 and Figure 4.4). The second most abundant anthocyanin component was determined to be C3R with contents of 49.3 ± 7.9 mg C3G eq./100 g dry-weight (10.7 mg C3G eq./100 g fresh-weight) in FF-St and 19.9 ± 5.8 mg C3G eq./100 g dry-weight (26 mg C3G eq./L nectar) in PN samples.

The cyanidin-3-(2^G-glucosylrutinoside) and cyanidin-3-rutinoside contents in fruit samples were ≥ 2-fold lower than the contents in sour cherry varieties studied by Kim *et al.* (2005) who measured the amount of these anthocyanin compounds in four different sour cherry varieties ranging between 88.95 – 227.66, and 15.95 – 21.97 mg C3G eq./100 g fresh-weight, respectively. On the other hand, Kirakosyan *et al.* (2009) determined the C3GR and C3R contents in frozen sour cherry varieties changing between 43.4 – 125.9 mg/100 g dry-weight, and 21.8 – 68.8 mg/100 g dry-weight, which were consistent with our findings. In addition, C3R content was more than 2-fold higher than the C3R contents reported by Kirakosyan *et al.* (2010) as 226.1 μg/g dry-weight.

In final nectar (PN) samples, the values of C3GR and C3R were found to be lower than the values reported by Bonerz *et al.* (2007) ranging between 361–515 mg C3G eq./L for C3GR, and 125–213 mg C3G eq./L for C3R in sour cherry juices produced from five different sour cherry varieties.

Table 4.4 : Changes in concentrations of identified anthocyanins during nectar processing.

Sample	Cyanidin-3-(2^{G}-glucosylrutinoside)[a]	% of (FF-St)	Cyanidin-3-rutinoside[a]	% of (FF-St)	Cyanidin-3-sophoroside[a]	% of (FF-St)	Cyanidin-3-glucoside[a]	% of (FF-St)
FF	197.0±24.7 def		54.2±11.6 defgh		8.1±0.8 def		5.6±1.5 bc	
FF-St	195.1±38.0 efg	100	49.3±7.9 defgh	100	8.2±2.7 def	100	6.4±1.6 bc	100
St	3.1±1.0 i	2	1.1±0.6 h	2	0.2±0.1 g	2	0.0±0.0 c	0
MH	233.8±73.4 cde	120	69.4±22.7 cdefg	141	9.8±3.1 cde	119	8.1±2.7 bc	127
MP	354.5±28.5 ab	182	88.0±10.4 bcde	179	15.2±2.0 bc	185	11.3±1.9 bc	178
MPEX-I	431.7±67.8 a	221	134.1±24.1 b	272	17.5±2.2 b	212	14.6±2.7 b	230
MPEX-II	391.3±55.2 ab	201	130.4±18.4 bc	265	16.4±3.5 b	200	14.5±2.6 b	228
MPEX-III	335.2±40.8 abc	172	119.0±25.0 bc	242	15.1±2.8 bc	183	14.5±3.8 b	228
PrJ	356.9±31.7 ab	183	98.9±11.5 bcd	201	15.2±2.0 bc	184	11.4±2.9 bc	180
PC	17.5±5.8 hi	9	7.4±1.6 gh	15	0.8±0.2 g	9	0.0±0.0 c	0
Sd	3.4±0.3 i	2	1.8±0.4 h	4	0.0±0.0 g	0	0.0±0.0 c	0
PsJ	390.6±58.6 ab	200	107.8±20.5 bcd	219	16.2±2.8 b	197	13.2±2.9 bc	208
EnJ	359.5±36.9 ab	184	96.5±12.4 bcd	196	15.3±2.8 bc	186	10.7±2.0 bc	169
ClJ	347.2±34.0 ab	178	89.2±12.4 bcde	181	14.6±2.3 bc	178	10.7±2.4 bc	169
FtJ	350.8±51.7 ab	180	80.8±12.0 bcdef	164	13.5±2.9 bcd	163	7.2±1.0 bc	113
FR	301.1±59.9 bcd	154	314.8±127.0 a	639	30.3±8.4 a	368	71.0±30.0 a	1123
CJ	337.2±3.1 abc	173	78.2±1.7 bcdef	159	13.9±0.4 bcd	168	8.2±1.2 bc	128
CJ-BPF	320.5±70.9 bc	164	74.8±17.0 bcdef	152	12.7±3.3 bcd	154	7.3±1.8 bc	115
CJ-APF	331.7±33.8 bc	170	77.06±7.0 bcdef	158	13.4±1.8 bcd	163	8.3±1.0 bc	130
N	113.6±28.8 fgh	58(133)[b]	25.8±7.0 efgh	52(127)[b]	4.2±1.1 efg	51(117)[b]	2.5±0.9 bc	39(90)[b]
SSN	96.6±18.9 ghi	50(145)[c]	21.8±4.6 fgh	44(128)[c]	3.7±0.8 efg	45(131)[c]	2.3±0.4 bc	36(104)[c]
PN	89.6±25.7 hi	46(133)[c]	19.9±5.8 fgh	40(116)[c]	3.4±1.0 fg	42(122)[c]	2.2±0.8 bc	34(99)[c]

[a] Data given in this table represent average amounts ± standard deviation of five biological replications of each sample. All concentrations in terms of (mg /100 g dry-weight) are expressed as cyanidin-3-glucoside equivalents. Different letters in the columns represent statistically significant differences ($p<0.05$).

[b] The original per cent is multiplied by a factor 2.3 to compensate for the additional sucrose in dry-weight composition of the initial nectar (N) sample.

[c] The original per cent is multiplied by a factor 2.9 to compensate for the additional sucrose in dry-weight composition of the sucrose syrup added nectar (SSN) and final pasteurized nectar (PN) samples.

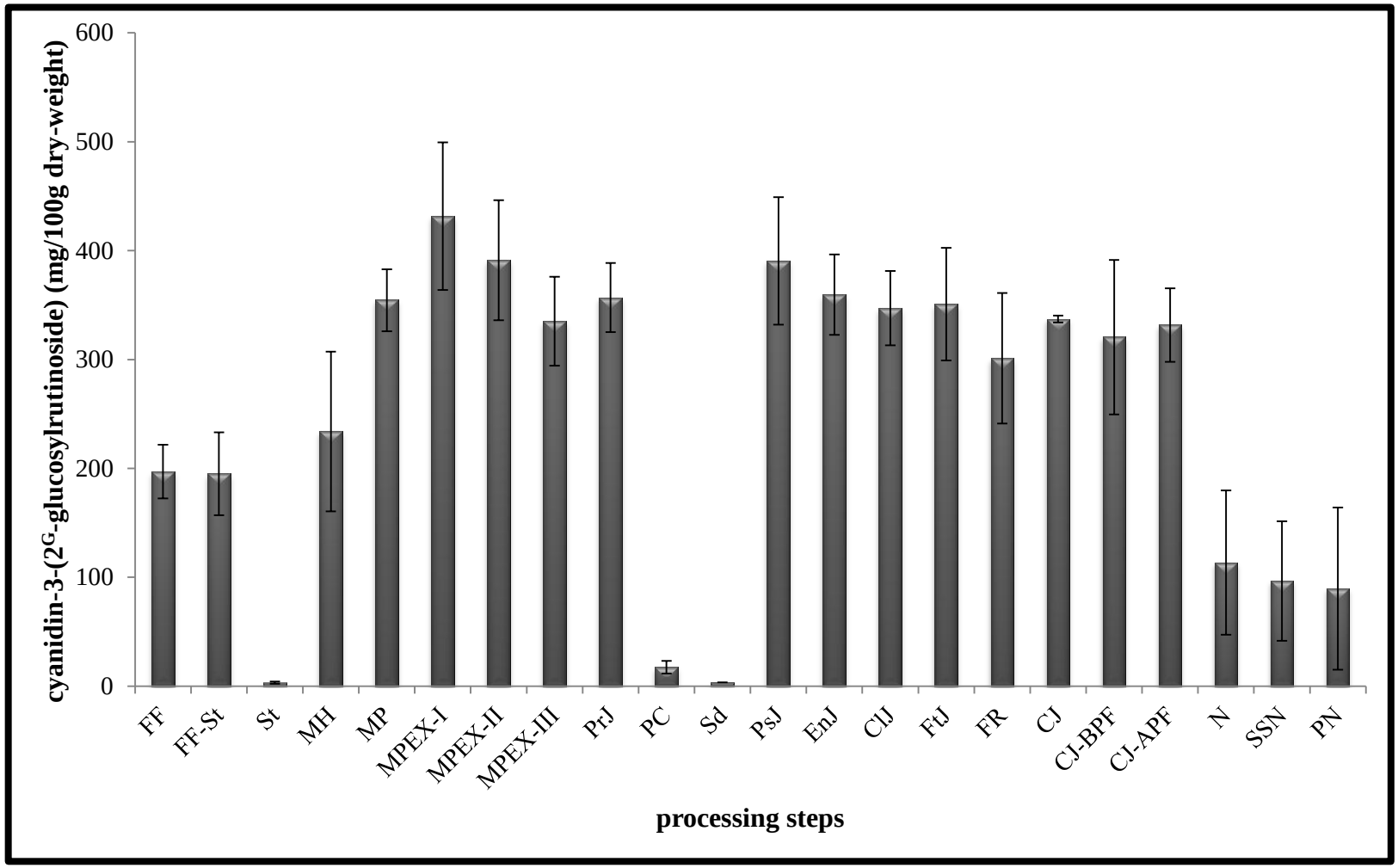

Figure 4.4 : Variation in cyanidin-3-(2^{G}-glucosylrutinoside) contents from fruit to nectar among five replicate processing events.

Two of the applied treatments exerted a relatively strong effect on anthocyanin content. Firstly, mash pressing steps gave rise to a substantial (≈ 2-fold) increase in the contents of all individual anthocyanin components on a dry-weight basis in the pressed juice (PrJ), relative to the original fruit (FF-St). This can be explained by the removal of dry-weight in the form of press cake, which has relatively low anthocyanin content (9 – 15% of the contents in FF-St). Secondly, processing of concentrated juice (CJ) to nectar (N) led to around a three-fold reduction in anthocyanins, corresponding to the addition of sucrose to the dry-weight. When overlooking the total effect of processing from fruit to nectar, the anthocyanin content in the final nectar (PN) was about 35-45% of the content in original fruit (FF-St) on dry-weight basis. When correcting for the addition of sucrose, the anthocyanin levels in the final nectar (PN) samples were around 99-133% of those in the original fruit (FF-St). This indicates that anthocyanin losses during the total process are remarkably modest, which was also confirmed by calculations per batch on fresh-weight basis (Figure 4.5(A)).

When the variation in content of C3GR during processing was calculated on a wet-weight basis, the concentration of this anthocyanin in final nectar (PN) is 28% of that in fruit, which is relevant for the consumer. On the other hand, when the recovery is calculated on a total input / output basis on a batch-scale (i.e. total amount of anthocyanin present in the batches (in grams per batch) of starting fruit material (FF-St) (representing 8600 kg of fresh-weight fruit) compared to the total amount present in the final nectar (PN) (representing 26750 kg pasteurized nectar)) we observe that 87% of fruit anthocyanins is present in the final nectar (Figure 4.5(A)). This value shows a high efficiency of the extraction process.

Remarkably, anthocyanins were not very dominant in the processing waste materials. The main sour cherry anthocyanin C3GR, was significantly lower in stalks, seed and press cake samples (St, Sd and PC), compared to the original fruit (FF-St) (2%, 2% and 9% of FF-St, respectively; Table 4.4 and Figure 4.4). Filtration was the only step that showed high concentrations of anthocyanins in the waste fraction. Depending on the anthocyanin species, the concentration was slightly (1.5 times for C3GR) or strongly (11-fold for C3G) higher in the filtration residue compared to the fruit sample. But the removal of filtration residue did not change the anthocyanin content

significantly, since this waste sample only removed the 6% of dry-weight from the process.

The extraction of anthocyanins from sour cherry compares favourably to the losses of anthocyanins that are reported for other fruit juice processes. Several factors are known to influence the recovery of phenolic compounds from plant materials. Anthocyanins are often considered to need relatively long times, or the presence of organic solvents like alcohols, for efficient extraction from fruit. For instance, on making wine from red grapes, the fruit skins need to ferment with the pomace for several days during initial fermentation. This is essential in order to extract a large portion of the anthocyanins into the final product, wine, as these are predominately located in the skins of the fruit (Sims and Bates, 1994). In sour cherry processing, no fermentation step is involved (Table 3.1 and Figure 3.1), and no alcohol is being formed. A glycosidic enzymatic treatment is however included in the process, which could negatively affect the stability of anthocyanins (step 13; Table 3.1). Nevertheless, hardly any loss of anthocyanins by either of these treatments was observed.

Anthocyanin recovery analyses have recently been performed for the production of black-currant juice in an industrial setting (Woodward *et al.,* 2011). For black-currant, it has been reported that significant losses of anthocyanins occurred between the pre-press mash and the post-press juice material (Woodward *et al.,* 2011), since anthocyanins are known to be associated with cell-wall material in the epidermal tissue (Skrede *et al.,* 2000). Such losses have not been observed here in the process used for sour cherry nectar production. In the cherry system, this may be partly due to the repetitions of the press cake extraction (Figure 4.5(B)). This suggests that repeated extraction permits efficient cumulative removal of the phenolic compounds present in the fruit into the final nectar product. In addition, mash heating applied just before pressing step could be another factor that helped in retaining the original fruit components. For anthocyanins, another factor for recovery from berries could be the exact chemical structure of the anthocyanins involved. In sour cherry, the main anthocyanin is cyanidin-3-(2^{G}-glucosylrutinoside) while in black-currant a significant portion of the anthocyanins is in the form of delphinidin-rutinoside (Woodward *et al.,* 2011). Delphinidin is known to be less stable under higher pH values than is cyanidin (Cabrita *et al.,* 2000), for which reason it may be less stable

upon processing. Delphinidin glycosides were reported to have a greater lability compared to cyanidin derivatives (Skrede *et al.*, 2000). The varying anthocyanin composition in different fruits may affect the susceptibility during processing (Skrede *et al.*, 2000; Wang and Xu, 2007). A third aspect that could influence anthocyanin recovery is the exact location of these compounds within the fruit. In contrast to black grapes and black-currants, where the skins contain most of the anthocyanins, the fruit pulp (pericarp) of several sour cherry varieties, including the "Kütahya" variety investigated here in this study, is strongly pigmented (Yarilgac *et al.*, 2005). In grape berries, most pigment resides in the fruit skin, and needs a few days of fermentation before efficient extraction of its anthocyanins into the liquid phase can be achieved (Netzel *et al.,* 2003). In tomato, during the processing of tomato fruits into tomato puree, major losses of phenolic compounds was clearly associated with the step involving the removal of the skin fraction from the pulp (Capanoglu *et al.,* 2008). Tomato skins are known to contain nearly all the phenolic components present in tomato fruit and the industrial process used failed to break open the epidermal cells and hence little was released to go forward into the final product. In sour cherry, advantageously, the anthocyanins are predominantly located also in the fruit flesh, which is more readily disrupted and hence it is proposed that this permits a more efficient extraction to give an end product rich in potentially health-promoting phenolic antioxidants.

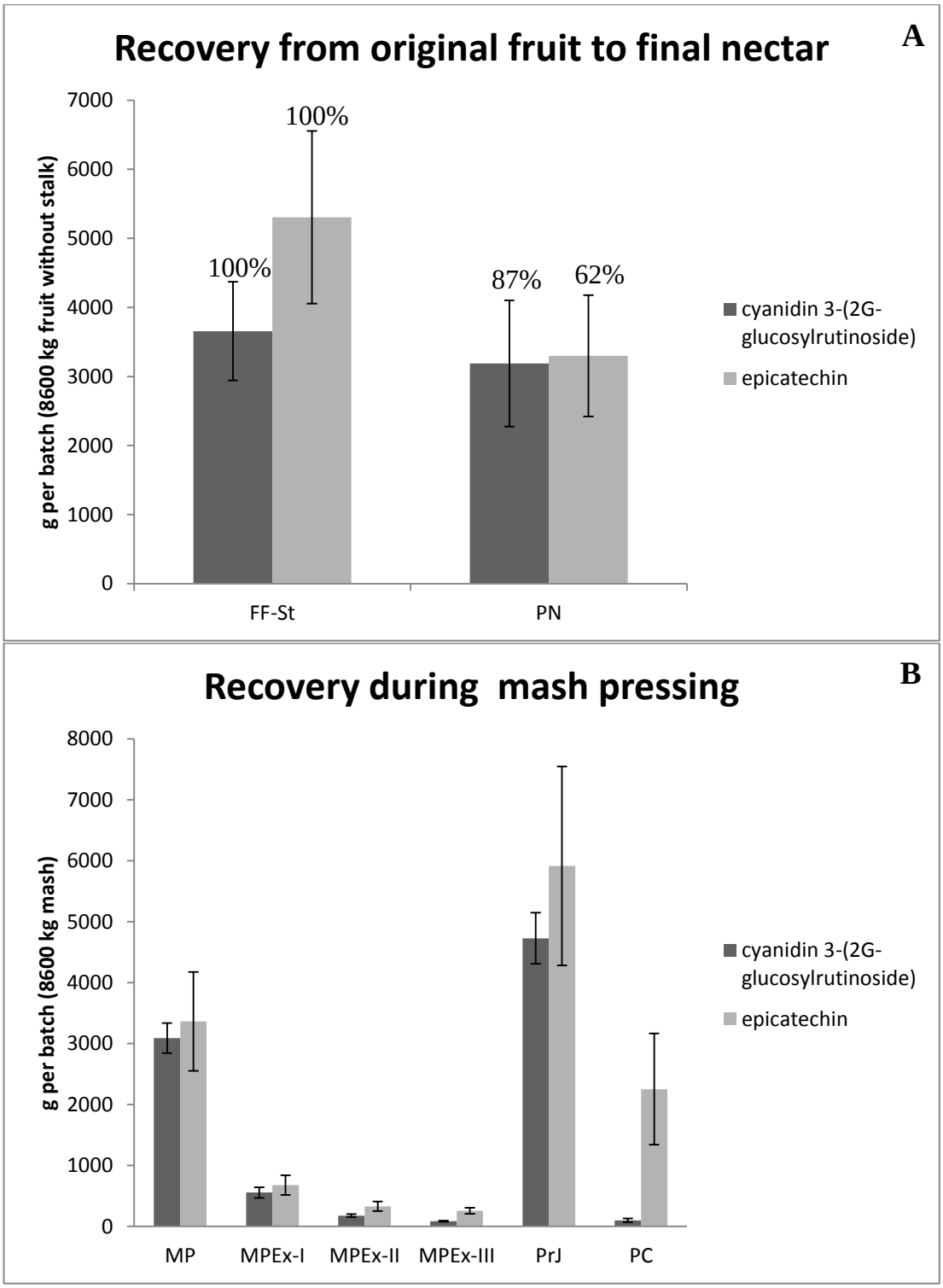

Figure 4.5 : Recovery of cyanidin-3-(2^{G}-glucosylrutinoside) (dark bars) and epicatechin (light bars) per batch. (**A**) recovery from original fruit (FF-St) and final nectar (PN). (**B**) recovery from press cake during mash pressing (MP) and press extraction steps (MPEX-I, II and III). The values were based on g compound per batch starting from 8600 kg fresh weight fruits without stalks.

4.3.2 Procyanidins

Targeted analysis using HPLC with fluorescence detection revealed that sour cherry fruit as well as nectar processing samples included substantial amounts of procyanidin components, specifically epicatechin.

Sour cherry fruit and nectar samples were found to contain a total flavan-3-ol content of 308.3 mg and 105.2 mg per 100 g dry-weight sample, corresponding to 67.2 mg/100 g fresh weight fruit and 140 mg/L nectar. Epicatechin contents in original fruit and final nectar samples were 283.0 ± 66.7 mg/100 g dry-weight (61.7 mg/100 g fresh-weight) and 92.7 ± 24.7 mg/100 g dry-weight (123 mg/L nectar). On the other hand, catechin contents were found to be much more lower with values of 25.3 ± 6.6 mg/100 g dry-weight (5.5 mg/100 g fresh-weight) in original fruit samples, and 12.5 ± 3.3 mg/100 g dry-weight (17 mg/L nectar) in final nectar samples.

The values determined for total flavan-3-ol content in fruit sample were in accordance with the amounts reported by Capanoglu *et al.* (2011) as 328 mg/100 g dry-weight sour cherry fruit. Similar contents of (-)-epicatechin and (+)-catechin were determined in sour cherry fruit samples by Capanoglu *et al.* (2011) with the values of 312.5 mg epicatechin and 15.4 mg catechin/100 g dry-weight sample. These values are also comparable to the levels of other well known rich sources of procyanidins including grape seeds (17-250 mg/100 g) (Guendez *et al.*, 2005) and dark chocolate (31-37 mg/100 g) (Miller *et al.*, 2009). On the other hand, the values reported by Tsanova-Savova *et al.* (2005) were much lower than our findings which were determined to be 6.8 and 3.0 mg/kg fresh-weight for epicatechin and catechin, respectively, in sour cherry fruits. Epicatechin content of *Montmorency* sour cherry variety investigated by Chaovanalikit and Wrolstad (2004b) was also lower than our *Kütahya* local sour cherry variety with values of 19.6 mg/100 g fresh-weight sample. Bonerz *et al.* (2007) reported the contents of (-)-epicatechin and (+)-catechin in sour cherry juices, obtained from five different sour cherry cultivars, ranging between 49 – 336 mg/L and 1 – 14 mg/L, respectively. These results were in accordance with the results recorded for nectar samples investigated in this study.

Targeted HPLC analysis of procyanidins pointed out that procyanidins were over-represented in waste materials including stalks (St) and filtration residue (FR), and to

a lesser extent, in press cake (PC) as compared to the original fruit (Table 4.5 and Figure 4.6). Although the procyanidin content was high in St and FR samples, their removal did not have a strong effect on the procyanidin content of the nectar since these waste materials made up only a small portion (3.5% for St; 7% for FR) of the total dry-weight. The degree of polymerization (DP) of procyanidins was low (<3) in all samples (Table 4.5). All waste materials including St, Sd, PC and FR samples contained the highest chain-length procyanidins (DP between 2.5 and 3; Table 4.5) and their removal hence caused a reduction of DP value from 2.1 in original fruit to 1.7 in the final nectar.

When the recovery of procyanidins were assessed on a batch-scale by calculating the procyanidin contents in grams per batch (representing 8600 kg of fresh-weight fruit), calculated for both fruit and final nectar (Figure 4.5(A)), by taking into account the moisture content (Table 4.1) and the total mass of the cherry fractions during the specific processing stages (Table 3.1), the recovery of the major procyanidin, epicatechin, was determined to be 62% in the final nectar.

Table 4.5 : Changes in total catechin and total epicatechin contents and the average degree of polymerization (DP) values during processing.

Sample	Total catechin [a]	% of FF-St	Total epicatechin [a]	% of FF-St	DP
FF	49.3±31.0 def		352.6±194.0 def		2.1 de
FF-St	25.3±6.6 efg	100	283.0±66.7 efg	100	2.1 de
St	243.3±61.6 a	962	625.8±110.3 cd	221	2.5 bc
MH	32.8±7.6 defg	129	328.0±68.9 defg	116	2.0 def
MP	38.2±8.4 defg	151	386.1±93.3 def	136	1.9 defg
MPEX-I	73.3±18.7 cd	290	526.6±125.5 cde	186	1.8 efg
MPEX-II	99.0±23.3 bc	391	725.8±174.2 bc	256	1.9 defg
MPEX-III	132.3±28.7 b	523	1015.0±188.0 b	359	2.2 cd
PrJ	46.0±8.2 defg	182	446.4±123.1 cde	158	1.9 defg
PC	57.3±31.2 cdef	226	402.9±163.4 def	142	2.6 ab
Sd	3.3±0.28 g	13	49.5±2.3 g	18	2.5 b
PsJ	43.9±3.3 defg	174	399.4±66.4 def	141	1.8 efg
EnJ	43.9±7.8 defg	174	397.9±96.0 def	141	1.9 defg
ClJ	42.5±5.5 defg	168	381.1±60.8 def	135	1.8 efg
FtJ	44.2±7.6 defg	175	361.4±94.7 def	128	1.7 fg
FR	65.2±9.2 cde	258	1380.5±356.0 a	488	2.9 a
CJ	46.4±4.5 defg	183	394.8±64.5 def	139	1.7 fg
CJ-BPF	42.0±11.5 defg	166	341.0±119.7 defg	120	1.7 fg
CJ-APF	40.2±7.2 defg	159	331.3±71.2 defg	117	1.7 fg
N	14.9±3.4 fg	59(136)[b]	112.6±33.1 fg	40(92)[b]	1.7 fg
SSN	13.6±4.2 fg	54(157)[c]	103.6±36.7 fg	37(107)[c]	1.7 fg
PN	12.5±3.3 fg	49(142)[c]	92.7±24.7 fg	33(96)[c]	1.7 g

[a] Data represent average quantities ± standard deviation of five biological replications for each sample. Total catechin and total epicatechin contents are expressed as mg / 100 g dry-weight basis. Different letters in the columns represent statistically significant differences ($p<0.05$).

[b] The original per cent is multiplied by a factor 2.3 to compensate for the additional sucrose in dry-weight composition of the initial nectar (N) sample.

[c] The original per cent is multiplied by a factor 2.9 to compensate for the additional sucrose in dry-weight composition of the sucrose syrup added nectar (SSN) and final pasteurized nectar (PN) samples.

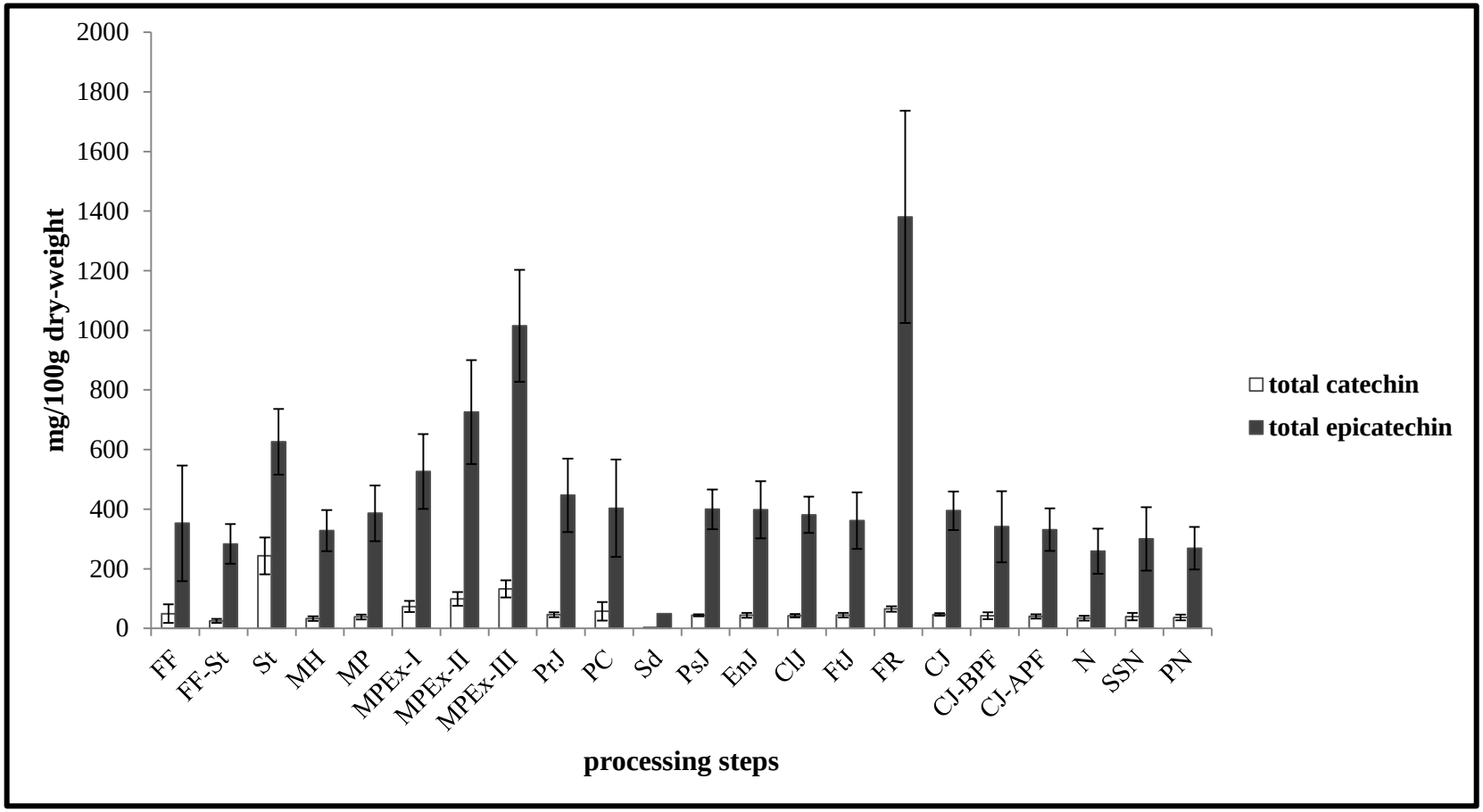

Figure 4.6 : Variation in total catechin (open bars) and total epicatechin (filled bars) contents from fruit to nectar among five replicate processing events.

Mash pressing appeared to be an important treatment for the recovery of phenolic compounds. The level of epicatechin was also calculated on a per batch basis for the fractions of the mash pressing part of the process (Figure 4.5(B)). The procyanidins (represented by epicatechin) behaved differently from the anthocyanins (represented by cyanidin-3-(2^{G}-glucosylrutinoside)). While most anthocyanin was recovered from the press cake by the repeated mash press extractions, procyanidins were still present in the third mash press extraction (MPEX-III), and a considerable portion of procyanidins (around 40% of the total) remained in the press cake. Procyanidins have been reported to be bound easily to the cell-wall polysaccharides through hydrogen-bonding and/or hydrophobic interactions (Le Bourvellec *et al.*, 2004; Renard *et al.*, 2001). Brownmiller *et al.* (2009) also determined that 24% of total procyanidins were retained in the press cake during blueberry juice processing, which was linked to the strong affinity of these compounds to the cell wall polysaccharides.

The analysis of procyanidins did also reveal a slight shift in the degree of polymerization, from 2.1 in the fruit to 1.7 in the final nectar (Table 4.5). Accordingly, waste materials (stalks, seeds, press cake and filtration residue) contain procyanidins with a relatively high degree of polymerization (DP ≈ 3; Table 4.5). This indicated the loss of longer chain procyanidins with waste materials through sour cherry nectar processing, which also agrees with the results of Brownmiller *et al.* (2009) who reported that the mono- and dimers were well-retained in juice fraction of blueberries, whereas larger oligomers were lost to a greater extent. The recovery of epicatechin calculated for the total quantity of fruit and nectar produced was lower than for anthocyanins, but still more than half of epicatechin (62%) was recovered.

4.3.3 Phenolic acids and flavonols

Neochlorogenic acid was the major phenolic acid, constituting >60% of total phenolic acid content in sour cherry samples, followed by *p*-coumaroylquinic acid, chlorogenic acid, and rosmarinic acid; whereas the most abundant flavonol component was rutin (quercetin-3-rutinoside) followed by quercetin-3-glucoside and kaempferol-3-rutinoside. The contents of phenolic acids and flavonols in samples for 5 batches are shown in Table 4.6 and Table 4.7, respectively.

Table 4.6 : Changes in phenolic acid contents during nectar processing.

Sample	Neochlorogenic acid [a,b]	% of FF-St	*p*-Coumaroylquinic acid [a,b]	% of FF-St	Chlorogenic acid [a,b]	% of FF-St	Rosmarinic acid [a]	% of FF-St
FF	262.3±119.5 abcdefg		70.5±34.3 defg		35.6±14.7 defgh		2.3±1.4 efg	
FF-St	187.0±52.7 defg	100	77.0±18.4 cdef	100	36.9±7.8 defgh	100	2.6±1.0 efg	100
St	73.9±33.4 g	40	70.3±14.6 defg	91	51.3±5.6 cdefg	139	0.0±0.0 g	0
MH	252.9±87.9 bcdefg	135	87.6±36.5 bcdef	114	52.4±16.3 cdefg	142	4.3±0.8 cdef	163
MP	408.5±134.8 abcdef	219	135.8±30.4 abcde	176	70.3±17.6 cdef	190	2.7±0.6 efg	103
MPEX-I	532.1±282.4 a	285	167.1±46.7 a	217	124.0±43.6 ab	336	7.2±1.6 c	277
MPEX-II	516.8±204.4 ab	276	168.0±25.9 a	218	134.5±25.7 a	364	11.1±1.8 b	424
MPEX-III	480.4±167.4 abc	257	163.7±26.2 a	213	140.1±24.1 a	379	16.2±3.5 a	620
PrJ	385.0±33.6 abcdef	206	134.6±24.3 abcde	175	74.2±10.8 cd	201	3.4±0.6 ef	132
PC	41.8±36.8 g	22	17.2±8.6 fg	22	15.9±10.2 gh	43	7.0±3.5 cd	268
Sd	5.1±0.2 g	3	3.0±0.4 g	4	2.5±0.1 h	7	0.0±0.0 g	0
PsJ	439.9±45.9 abcd	235	150.3±44.2 ab	195	80.2±27.6 bcd	217	4.2±1.4 cdef	160
EnJ	413.4±73.0 abcde	221	138.2±21.4 abcd	179	78.1±13.3 cd	211	4.1±0.7 cdef	158
ClJ	407.6±90.1 abcdef	218	131.7±29.2 abcde	171	70.9±22.4 cde	192	3.5±1.1 ef	134
FtJ	446.3±127.2 abcd	239	146.8±34.8 abc	191	84.3±20.5 bc	228	3.8±1.2 def	144
FR	209.7±202.9 cdefg	112	63.8±62.9 efg	83	38.6±38.0 cdefgh	104	5.1±1.4 cde	196
CJ	438.6±85.9 abcde	235	147.3±21.4 abc	191	82.9±13.2 bcd	224	4.1±0.3 cdef	156
CJ-BPF	409.3±40.5 abcdef	219	133.6±24.9 abcde	174	78.1±10.5 cd	211	3.2±0.7 efg	122
CJ-APF	440.3±107.1 abcd	236	144.1±29.4 abc	187	83.2±17.6 bc	225	3.8±1.3 def	146
N	167.7±46.4 efg	90(207)[c]	47.4±14.0 fg	62(143)[c]	28.6±9.0 efgh	78(179)[c]	1.4±0.4 fg	53(122)[c]
SSN	140.9±25.8 fg	75(218)[d]	39.7±4.0 fg	52(151)[d]	25.1±4.0 fgh	68(197)[d]	1.2±0.4 fg	46(133)[d]
PN	138.3±37.4 fg	74(215)[d]	39.5±8.2 fg	51(148)[d]	24.5±6.1 gh	66(191)[d]	1.2±0.3 fg	47(136)[d]

[a] Values given represent average quantities ± standard deviation of five biological replications for each sample. Contents are expressed as mg / 100 g dry-weight basis. Different letters in the columns represent statistically significant differences ($p<0.05$).

[b] Concentrations were quantitated via a calibration curve determined with chlorogenic acid.

[c] The original percent is multiplied by a factor 2.3 to compensate to the additional sucrose in dry-weight composition of the initial nectar (N) sample.

[d] The original percent is multiplied by a factor 2.9 to compensate to the additional sucrose in dry-weight composition of the sucrose syrup added nectar (SSN) and final pasteurized nectar (PN) samples.

Table 4.7 : Changes in flavonol contents in nectar processing samples.

Sample	Rutin	% of FF-St	Quercetin-3-glucoside	% of FF-St	Kaempferol-3-rutinoside	% of FF-St
FF	29.1±7.2 de		1.5±0.6 de		8.2±2.7 cd	
FF-St	17.6±3.6 def	100	0.6±0.2 e	100	2.9±2.9 cd	100
St	109.0±16.9 a	619	21.0±5.2 a	3569	65.3±3.6 a	2228
MH	24.5±7.0 def	139	1.1±0.1 de	182	6.4±2.2 cd	218
MP	24.0±4.9 def	136	0.7±0.1 de	112	4.4±1.1 cd	149
MPEX-I	62.2±26.9 bc	353	3.1±0.8 bcd	527	18.2±8.4 b	620
MPEX-II	66.4±20.9 b	378	4.1±0.6 bc	687	20.5±7.3 b	700
MPEX-III	67.9±20.5 b	386	5.2±1.1 b	875	22.9±7.8 b	780
PrJ	28.4±9.0 de	161	1.1±0.1 de	192	5.9±2.5 cd	202
PC	13.3±7.9 def	76	1.9±0.9 cde	328	6.1±2.9 cd	206
Sd	1.0±0.0 f	6	0.0±0.0 e	0	0.0±0.0 d	0
PsJ	38.8±7.7 cd	220	1.3±0.3 de	226	8.6±2.0 c	294
EnJ	36.5±6.0 cde	207	1.3±0.1 de	217	8.2±1.4 cd	280
ClJ	35.5±7.1 de	202	1.2±0.3 de	203	8.0±1.8 cd	272
FtJ	38.5±9.7 cd	219	1.3±0.2 de	219	8.6±2.2 c	292
FR	25.7±18.4 def	146	1.1±0.4 de	181	5.0±4.1 cd	170
CJ	36.3±0.4 cde	207	1.2±0.1 de	208	8.4±1.2 cd	287
CJ-BPF	34.9±6.4 de	199	1.3±0.3 de	213	7.6±1.6 cd	258
CJ-APF	37.2±8.3 cde	212	1.3±0.2 de	218	8.4±2.3 c	288
N	13.4±3.7 def	76(175)[a]	0.5±0.2 e	80(184)[a]	3.0±1.0 cd	101(232)[a]
SSN	11.3±1.5 ef	64(186)[b]	0.4±0.1 e	75(218)[b]	2.5±0.4 cd	85(247)[b]
PN	10.9±2.4 ef	62(180)[b]	0.4±0.2 e	72(209)[b]	2.4±0.6 cd	81(235)[b]

Values given represent average quantities ± standard deviation of five biological replications for each sample. Contents are calculated via different calibration curves obtained for each compound separately, and expressed as mg / 100 g dry-weight basis. Different letters in the columns represent statistically significant differences ($p<0.05$).

[a] The original percent is multiplied by a factor 2.3 to compensate to the additional sucrose in dry-weight composition of the initial nectar (N) sample.

[b] The original percent is multiplied by a factor 2.9 to compensate to the additional sucrose in dry-weight composition of the sucrose syrup added nectar (SSN) and final pasteurized nectar (PN) samples.

The contents of neochlorogenic acid, *p*-coumaroylquinic acid, and chlorogenic acid in sour cherry nectar samples were calculated to be 184, 53, and 33 mg/L nectar. The neochlorogenic acid content was similar to the minimum values reported by Bonerz *et al.* (2007) as 212-998 mg/L nectar, whereas the quantities determined for *p*-coumaroylquinic acid and chlorogenic acid were much more lower (reported as 191-999, and 119-268 mg/L, respectively, by Bonerz *et al.* (2007)).

The flavonol glycoside content of nectar samples was constituted of 14.5 mg rutin/L nectar, 3,2 mg kaempferol-3-rutinoside/L nectar, and 0.5 mg quercetin-3-glucoside/L nectar. These values were slightly lower than the minimum values recorded by Bonerz *et al.* (2007) as 18, 4, and 3 mg/L nectar for the respective compounds.

In sour cherry fruit samples, the total amounts of phenolic acids and flavonol glycosides were approximately 65.6 mg chlorogenic acid/100 g fresh-weight, and 4.5 mg/100 g fresh-weight, respectively. Chaovanalikit and Wrolstad (2004b) recorded these values for the edible portion of *Montmorency* sour cherry variety as 58.2 mg chlorogenic acid/100 g fresh-weight, and 11.2 mg rutin/100 g fresh-weight. The difference in flavonol glycoside quantities could be linked to the differences in terms of expressions. The values for flavonol glycoside contents in our work were calculated using different calibration curves obtained separately for each compound, whereas Chaovanalikit and Wrolstad (2004b) reported these values in terms of rutin equivalents using only one calibration curve. The neochlorogenic acid, *p*-coumaric acid, and chlorogenic acid contents were ranging between 6.74-27.79, 0.89-4.06, 0.58-5.77 mg/100 g fresh weight in four different sour cherry varieties investigated by Kim *et al.* (2005). These values were much lower than the quantities obtained for our *Kütahya* sour cherry variety having 40.8, 16.8, 8.0 mg/100 g fresh weight for the related components, respectively. On the other hand, the contents calculated for flavonol glycosides were similar, reported by Kim *et al.* (2005) as 0.97-4.36, 0.21-0.44, and 0.30-1.29 mg/100 g fresh weight for rutin, quercetin-3-glucoside, and kaempferol-3-rutinoside, respectively, and obtained as 3.8, 0.1, and 0.6 mg/100 g fresh weight, respectively, in this present study.

Changes in phenolic acid and flavonol contents during processing followed similar trends as observed for anthocyanins, with the exception of stalks and press cake, which were found to contain relatively high concentrations of flavonols (**Table 4.7**).

Indeed, the extracts recovered from the repeated mash-press extraction steps displayed a strong over-representation (per dry-weight) of phenolic compounds, such as quercetin derivatives, chlorogenic acids, as in procyanidins and anthocyanins (Table 4.6 and Table 4.7).

4.4 Vitamin C Measurements Using HPLC

Dedicated vitamin C extraction and analysis for reduced (AA) and non-reduced (DHAA) by HPLC-PDA revealed that the total ascorbic acid content in the original fruit (FF-St) sample contained only the oxidized form of AA, dehydroascorbic acid (DHAA). Contents varied between 1.6-3.7 mg/100 g fresh-weight (8.3-16.0 mg/100 g dry-weight) in five biological replicates. There was no AA or DHAA detected in the final nectar (PN) sample. The level of AA in our original sour cherry sample appears relatively low as compared to those reported for sour-cherry fruit and its juice in literature as 10 mg/100 g fresh weight fruit (NutritionData, 2012) and 43 – 177 mg /L nectar (Bonerz *et al.*, 2007), which may be due to differences in cherry varieties, juice processing and/or analysis methods (HPLC-PDA in this study versus potentiometric analysis in Bonerz *et al.*, 2007).

4.5 On-line Antioxidant Analysis for Individual Antioxidants

On-line HPLC antioxidant detection, based on post-column reaction with ABTS cation radicals, was used to determine the relative contributions of the identified individual phenolic components to the total antioxidant activity (Beekwilder *et al.*, 2005). The most abundant antioxidant peaks were found to be the anthocyanins (Figure 4.7, lower panel; Table 4.8), which comprised 41%-62% of the total antioxidant activity (based on total peak area) of all samples excluding the waste samples. The contributions of anthocyanins in fruit (FF-St) and nectar (PN) were 61% and 57% of the total of antioxidant peaks, respectively. The major anthocyanin, identified as cyanidin-3-(2^{G}-glucosylrutinoside) (peak 7), provided the highest antioxidant activity, being responsible for approximately 50% and 40% of the total antioxidant peak area in fruit and nectar extracts, respectively. In addition, including the contribution of other identified cyanidin derivatives in our samples – which were cyanidin-3-glucoside and cyanidin-3-rutinoside – these provided 61% and 57% of the total antioxidant activity in fruit and nectar samples, respectively. These results

are in accordance with those of Kirakosyan *et al.* (2009) who compared the antioxidant activities of several single constituents those were present in various sour cherry products, including frozen and dried cherries, powders from individually quick-frozen cherry and juice concentrates.

For samples other than wastes, the total contribution of phenolic acids and flavonols (expressed as quercetin derivatives) revealed consistent results with values ranging from 10-15% of the total antioxidant activity.

On the other hand, in stalk and press cake wastes, anthocyanins contributed only 7% and 11% of the total antioxidant peak area, respectively. In stalks, total flavonols were found to have the highest percent contribution (17%) in comparison to total anthocyanins and total phenolic acids. Values were also higher than those obtained for total flavonols in other samples.

In all samples, highly polar antioxidants, i.e. compounds including putative ascorbic acid derivative eluting within the first 5 minutes under the chromatographic conditions applied (Beekwilder *et al.*, 2005), showed a relative minor contribution to the total antioxidant activity (1-6%, except for seed sample with 10% contribution).

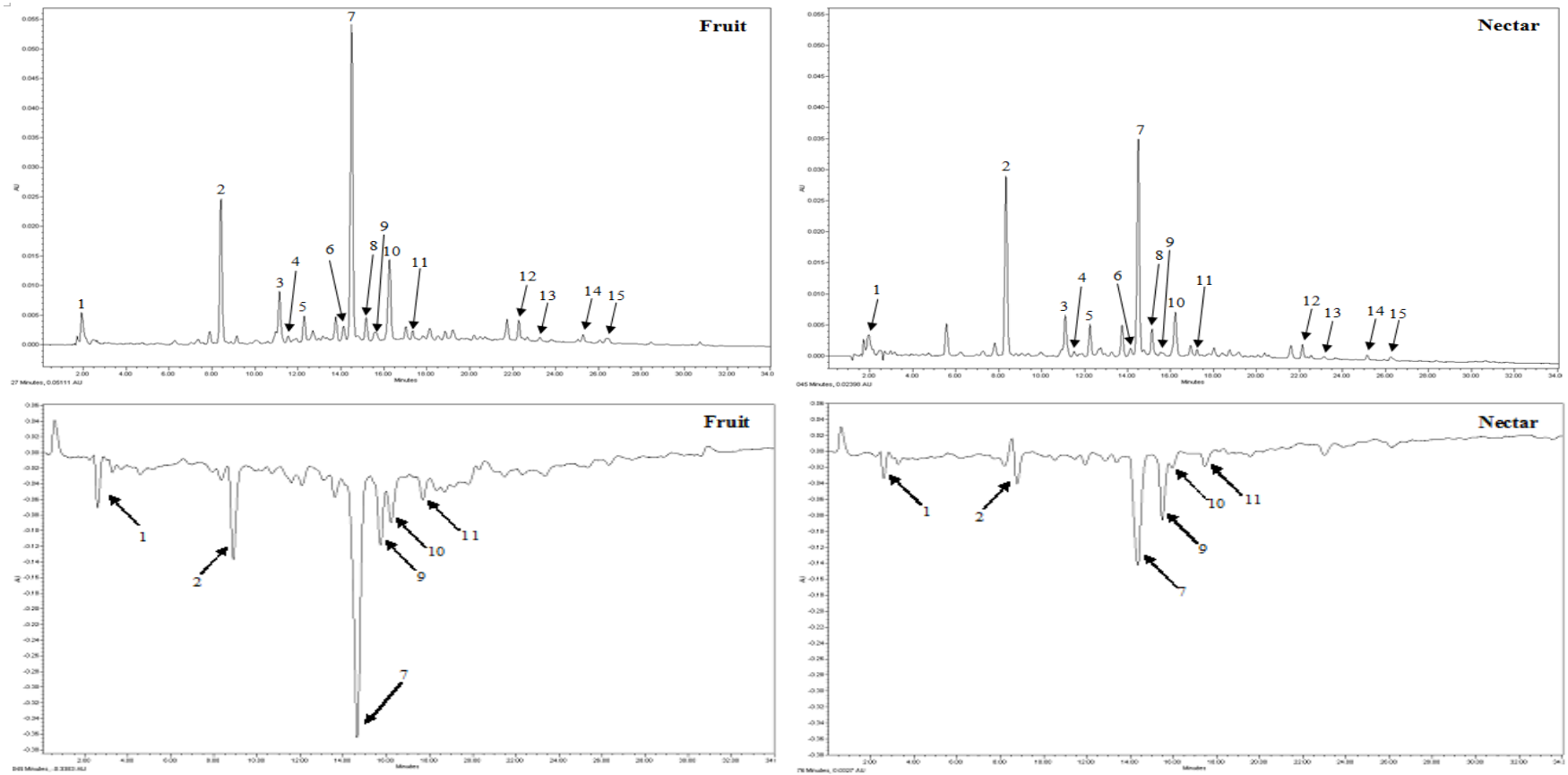

Figure 4.7 : (**Upper panels**) Representative HPLC chromatograms of sour cherry original fruit (FF-St) (left panel) and final nectar (PN) (right panel) extracts recorded by PDA, 280 nm. Chromatograms are on the same scale. (**Lower panels**) Representative chromatograms of antioxidant peaks in (FF-St) (left panel) and PN (right panel) determined on-line by a post-column reaction with ABTS cation radicals on HPLC. Numbers refer to the main peaks identified: 1, putative ascorbic acid derivative (Capanoglu *et al.*, 2008); 2, neochlorogenic acid (Bonerz *et al.*, 2007); 3, 3-coumaroylquinic acid (Bonerz *et al.*, 2007); 4, (+)-catechin (standard); 5, chlorogenic acid (standard); 6, cyanidin-3-sophoroside (Bonerz *et al.*, 2007); 7, cyanidin-3-(2^G-glucosylrutinoside) (Bonerz *et al.*, 2007; Chandra *et al.*, 2001); 8, (-)-epicatechin (standard); 9, cyanidin-3-glucoside (standard); 10, cyanidin-3-rutinoside (Bonerz *et al.*, 2007; Chandra *et al.*, 2001); 11, quercetin-rutinoside-glucoside (Bonerz *et al.*, 2007); 12, rutin (quercetin-3-rutinoside) (standard); 13, quercetin-3-glucoside (standard); 14, kaempferol-3-rutinoside (standard); 15, rosmarinic acid (standard).

Table 4.8 : Percent contributions of sour cherry phenolics to the total antioxidant capacity as determined using on-line HPLC antioxidant detection in each nectar processing step.

Sample	cyanidin-3-(2^{G}-glucosylrutinoside) (%)	cyanidin-3-glucoside (%)	cyanidin-3-rutinoside (%)	total anthocyanins (%)	total quercetin derivatives (%)	total phenolic acids (%)	ascorbic acid derivative (%)	total antioxidant area (%)[a]
FF	40.9	5.2	6.6	52.7	8.9	13.4	4.2	79.3
FF-St	48.5	7.0	5.0	60.6	5.6	16.4	5.8	88.3
St	3.1	4.1	0.0	7.2	16.9	6.9	2.8	33.8
MH	43.3	11.2	4.6	59.2	10.4	10.7	2.7	83.0
MP	44.8	12.2	4.3	61.3	8.0	10.7	3.2	83.3
MPEX-I	40.8	11.4	4.8	57.0	10.2	10.7	3.5	81.4
MPEX-II	33.3	12.2	3.7	49.2	11.8	13.3	2.0	76.3
MPEX-III	24.5	13.1	2.9	40.5	13.4	12.8	1.4	68.1
PrJ	43.1	11.3	4.5	58.9	8.3	13.9	2.8	83.8
PC	9.2	0.9	0.9	10.9	10.7	1.5	4.0	27.1
Sd	0.0	0.0	0.0	0.0	8.3	1.8	9.6	19.8
PsJ	44.3	12.5	4.7	61.5	8.8	12.7	2.7	85.7
EnJ	41.0	12.8	4.1	57.9	11.7	11.1	2.6	83.3
ClJ	43.1	11.9	4.2	59.2	9.2	12.4	3.1	83.9
FtJ	40.4	12.3	3.5	56.3	8.0	14.8	4.2	83.2
FR	37.1	12.2	5.0	54.3	11.5	12.6	3.0	81.4
CJ	42.2	13.3	3.4	58.9	8.6	16.8	3.0	87.3
CJ-BPF	41.8	12.3	3.5	57.5	8.8	13.0	3.9	83.2
CJ-APF	41.6	12.4	3.4	57.4	8.6	13.9	3.3	83.2
N	40.9	12.6	2.5	56.0	8.2	14.8	4.0	83.0
SSN	39.8	14.8	1.8	56.4	7.4	12.1	5.1	80.9
PN	40.3	14.7	2.0	57.0	8.0	14.1	5.0	84.1

[a] Total antioxidant area is the sum of total anthocyanins, total quercetin derivatives, total phenolic acids, and ascorbic acid derivative.

4.6 Identification of Sour Cherry Compounds Using LC-QTOF-MS Based Metabolomics Approach

Metabolomics has been defined as the technology designed to give us the broadest, essentially non-targeted, insight for the comprehensive analysis of the composition of complex biochemical mixtures, such as plant extracts (Hall, 2006). Metabolomics approach provides a better understanding of a biological system through enabling the data for a number of participating metabolites with known identities (Moco *et al.*, 2007). Moreover, obtaining high-quality data suitable for metabolite identification is only a step that further needs to be supported with the use of bio-computational tools for automotion of data analysis (Moco *et al.*, 2007). This includes processing the data by dedicated bioinformatics and biostatistics software tools in order to identify biochemical differences and key differential metabolite markers for specific treatments. These analyses are of importance for specific reasons, including those they are initially untargeted, no need for any previous knowledge on the biochemistry, and are providing valuable information on the composition of complex biochemical mixtures, including food materials, and on the effects of internal (i.e., genetic) and external (i.e., processing) factors influencing this composition (Hall, 2006; Hall *et al.*, 2008). Moreover, this technology also enables the analysis of the complex data in a readily understandable way using dedicated visualization strategies those simplify and more easily comprehend the multidimensional complexity of the growing sets of data that we are generating (Hall, 2006; Kell, 2002).

Untargeted LC-QTOF-MS based metabolomics analysis was performed on 22 samples from each of the 5 production replicates. Representative LC-MS profiles of original fruit (FF-St) and final nectar (PN) samples are shown in Figure 4.8. After processing the LC-MS data, 193 mass clusters (representing individual compounds) were interpreted for compound identification. Based on the MS data, elemental composition could be determined and putative identities were established for 38 compounds, by comparing the elemental composition to public and commercial databases (Table 4.9). For 26 of these compounds, all fragmentation, UV absorption and retention time data corresponded to those for the compounds described in the

literature for *Prunus* spp. or other fruit species (Dictionary of Natural Products and KnapSAcK databases, and Bonerz *et al.*, 2007; Iijima *et al.*, 2008; Moco *et al.*, 2006; Tikunov *et al.*, 2010; van der Hooft *et al.*, 2012; Wagner *et al.*, 1969). For 12 of these, their identity was confirmed by comparison to authentic standards (Table 4.9). Two additional compounds, cyanidin-3-glucoside (Figure 4.7, peak 9) and rosmarinic acid (Figure 4.7, peak 15) were not identified in the MS analysis, but were identified using HPLC analysis and comparing retention times and absorption spectra with those of original standards. The metabolites identified included four anthocyanins, five flavan-3-ols, eight phenylpropanoids, eight flavonols, four flavanones, four organic acids, and two carbohydrates.

The LC-MS system is not very optimal for detecting anthocyanins. In liquid chromatography analyses, the pH of the elution system is kept below pH 2 by the inclusion of small amounts of formic, acetic, or trifluoroacetic acid. Anthocyanins are known to exist in two interconvertible forms below pH 3.2, which are the red flavylium cation form and the blue quinoidal species (Strack and Wray, 1989). On the other hand, 96% of an anthocyanin is in the red flavylium form at pH 1.5, whereas only 67% is in that form at pH 2.5 (Wulf and Nagel, 1978). The pH values higher than pH 2 give rise to severe peak broadening due to the slow interconversions between different structures (Hale *et al.*, 1986), resulting in poor resolution and reduced detection limits (Da Costa *et al.*, 2000). Since formic acid was used to acidify the eluents in the LC-MS system used in this study, it could result in such detection problems as a weaker acid compared to the trifluoroacetic acid (Poll and Harding, 1989). Therefore an HPLC-DAD system using a more acidic eluents (with trifluoroacetic acid) could much successfully detect all anthocyanins present in sour cherry samples.

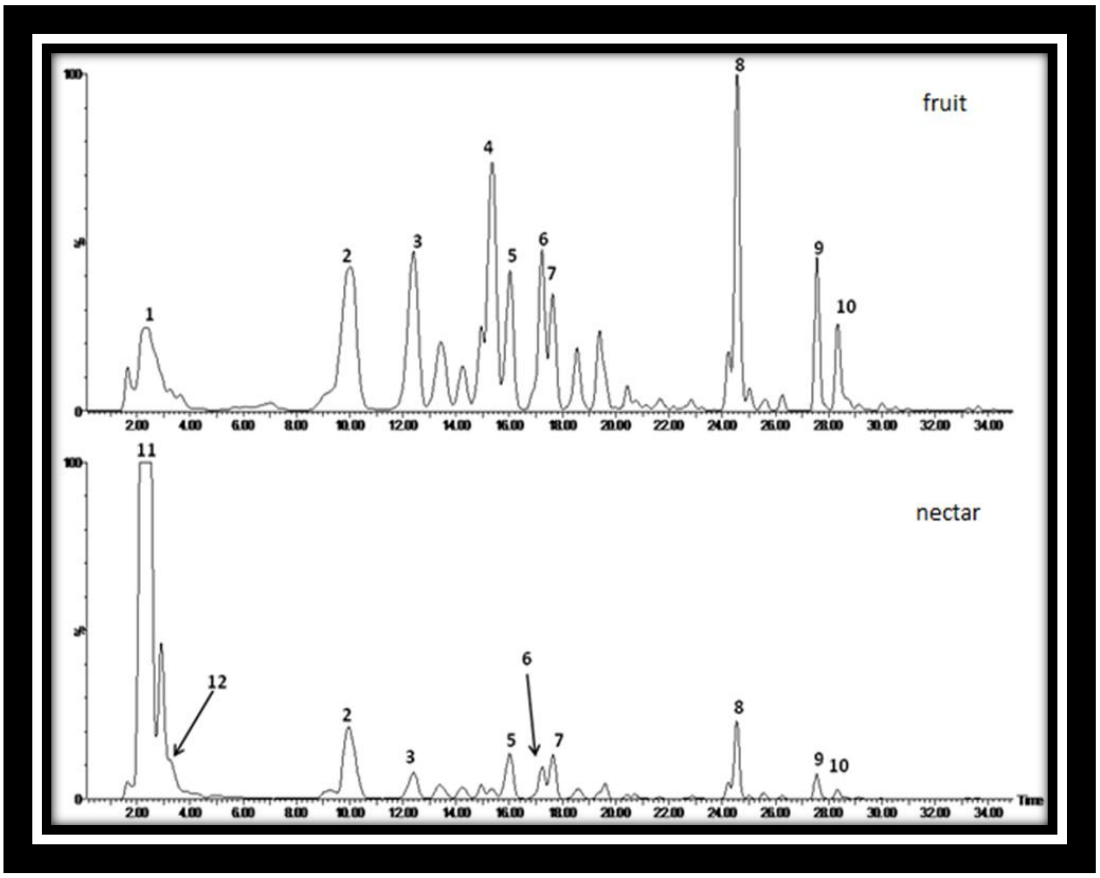

Figure 4.8 : Representative LC-MS chromatograms of methanolic extracts of the original fruit (FF-St) (upper panel) and final nectar (PN) (lower panel) samples. The chromatograms have the same scale: 100% indicates 6000 mass counts. Numbers refer to the main peaks identified: 1, quinic acid; 2, neochlorogenic acid; 3, cyanidin-3-(2^{G}-glucosylrutinoside); 4, amygdalin; 5, procyanidin B2; 6, benzyl alcohol-hexose-pentose isomer; 7, epicatechin; 8, quercetin-3-O-rutinoside; 9, kaempferol-3-O-rutinoside; 10, isorhamnetin-3-rutinoside; 11, sucrose; 12, citric acid.

To visualize the changes in metabolic composition in different steps during sour cherry nectar processing, a multivariate analysis was carried out on the LC-MS data. Comparison and visualization of the main features of the LC-MS data were performed by loading the data matrix into GeneMaths XT 1.6 software (www.applied-maths.com). Principal Components Analysis (PCA) was performed for unsupervised (not any structural classification, instead the classification emerges from the data) comparison of samples and metabolites (Figure 4.9). To allow a fair comparison, data for nectar samples including nectar (N), sucrose syrup added nectar (SSN), and pasteurized nectar (PN) were re-calculated by compensating for the additional sucrose in dry-weight composition of these samples.

Table 4.9 : Annotated sour cherry metabolites detected using LC-QTOF-MS.

RT (min)	λ_{max}	Observed mass (Da)	Calculated mass (Da)	Fragments	m/z error ppm	Elemental formula	(Putative) ID	Ref	Identificati-on level[a]	Compound group
2.15		181.0692	181.0712	-	14.07	C6H14O6	Sugar alcohol	KNapSAcK	3	carbohydrate
2.22		341.1120	341.1084	-	9.25	C12H22O11	Sucrose		1	carbohydrate
2.40		191.0539	191.0556	-	11.29	C7H12O6	Quinic acid		1	organic acid
2.40		295.0687	295.0665	-	5.71	C10H16O10	Malic acid		1	organic acid
2.67		337.0792	337.0771	114.9985 133.0104	4.71	C12H18O11	Ascorbic acid-glucoside		1	organic acid
3.13		191.0218	191.0192	-	10.80	C6H8O7	Citric acid		1	organic acid
9.89	324	353.0881	353.0873	179.0389 191.0587	0.93	C16H18O9	Neochlorogenic acid (5-caffeoylquinic acid)	Bonerz *et al.*, 2007	2	phenylpropanoid
11.23	272, 515	609.1512	609.1456		8.43	C27H30O16	Cyanidin-3-sophoroside	Bonerz *et al.*, 2007	2	anthocyanin
11.68	273, 326	577.1393	577.1346	425.0946	7.27	C30H26O12	Procyanidin B1	Bonerz *et al.*, 2007	2	flavan-3-ol
12.37	277, 516	755.1976	755.2035	-	8.54	C33H40O20	Cyanidin-3-(2^{G}-glucosylrutinoside)	Bonerz *et al.*, 2007	2	anthocyanin
13.32	312	337.0941	337.0923	163.0435 191.0594	3.73	C16H18O8	Coumaroylquinic acid isomer	Moco *et al.*, 2006	2	phenylpropanoid
13.32	281, 515	593.1510	593.1506	-	0.32	C27H30O15	Cyanidin-3-rutinoside	Bonerz *et al.*, 2007	2	anthocyanin
13.43	282	431.1615	431.1553	-	13.02	C19H28O11	Benzyl alcohol-dihexose	Moco *et al.*, 2006	2	phenylpropanoid
13.54	277	289.0753	289.0712	-	12.20	C15H14O6	Catechin		1	flavan-3-ol
14.21	277, 324	353.0902	353.0873	191.0579	6.89	C16H18O9	Chlorogenic acid (3-caffeoylquinic acid)		1	phenylpropanoid
14.91	280, 330	773.2079	773.2082	-	0.99	C40H38O16	catechin diacyl-glucoside	KNapSAcK	3	flavan-3-ol
15.31	263	456.1526	456.1506	323.1033	3.32	C20H27NO11	Amygdalin	KNapSAcK	2	cyanogenic glycoside

Table 4.9 (continued) : Annotated sour cherry metabolites detected using LC-QTOF-MS.

RT (min)	λ_{max}	Observed mass (Da)	Calculated mass (Da)	Fragments	m/z error ppm	Elemental formula	(Putative) ID	Ref	Identification level[a]	Compound group
15.98	272	577.1337	577.1346	289.0770 425.0920	2.46	C30H26O12	Procyanidin B2	Bonerz *et al.*, 2007	2	flavan-3-ol
16.21	274	417.1449	417.1397	121.0327 295.1087	11.10	C18H26O11	Guaiacol-hexose-pentose	Tikunov *et al.*, 2010	2	phenylpropanoid
17.20	277, 312	401.1477	401.1448	269.1077	6.02	C18H26O10	Benzyl alcohol-hexose-pentose isomer	DNP	3	phenylpropanoid
17.60	272	289.0724	289.0712	-	2.06	C15H14O6	Epicatechin		1	flavan-3-ol
18.52	281	327.1101	327.1080	165.0591	4.88	C15H20O8	Hydroxycinnamic acid-hexose	DNP	3	phenylpropanoid
19.59	267, 342	771.1918	771.1984	-	9.20	C33H40O21	Quercetin-rutinoside-glucoside	KNapSAcK	3	flavonol
20.71	267, 325	357.1218	357.1186	195.0702	7.62	C16H22O9	Monoterpene-hexose	KNapSAcK	3	terpene
21.54	267	509.2283	509.2234	415.1676 191.0594 337.0989	8.46	C22H38O13	Octenoic acid-dihexose I	DNP	3	fatty acid
22.42	277	465.1097	465.1033	303.0563	12.54	C21H22O12	Pentahydroxyflavanone-hexose	KNapSAcK	3	flavanone
24.23	225, 282	433.1157	433.1135	271.0666	3.79	C21H22O10	Naringenin-7-*O*-beta-D-glucoside (Prunin)	Van der Hooft *et al.*, 2012	2	flavanone
24.53	258, 353	609.1442	609.1456	-	3.19	C27H30O16	Quercetin-3-*O*-rutinoside		1	flavonol
24.86	277, 331	449.1139	449.1084	287.0616 151.0078	11.04	C21H22O11	Tetrahydroxyflavanone-hexose	Iijima *et al.*, 2008	2	flavonol
25.56	353	463.0880	463.0877	301.0410	7.90	C21H20O12	Quercetin-3-*O*-glucoside		1	flavonol

Table 4.9 (continued) : Annotated sour cherry metabolites detected using LC-QTOF-MS.

RT (min)	λ_{max}	Observed mass (Da)	Calculated mass (Da)	Fragments	m/z error ppm	Elemental formula	(Putative) ID	Ref	Identification level[a]	Compound group
27.55	348	593.1503	593.1506	-	1.56	C27H30O15	Kaempferol-3-*O*-rutinoside	Bonerz *et al.*, 2007	1	flavonol
28.34	353	623.1604	623.1612	-	2.18	C28H32O16	Isorhamnetin-3-rutinoside	Bonerz *et al.*, 2007	2	flavonol
28.67	339	447.0981	447.0927	-	10.80	C21H20O11	Kaempferol-7-glucoside	KNapSAcK	3	flavonol
29.40	277, 332	355.1091	355.1034	193.0552	15.9	C16H20O9	Ferulic acid-hexose isomer	DNP	3	phenylpropanoid
30.43	268	447.1342	447.1291	285.0805	10.24	C22H24O10	Dihydrowogonin-hexose isomer I	Wagner *et al.*, 1969	2	flavanone
31.08	277	447.1361	447.1291	285.0821	14.40	C22H24O10	Dihydrowogonin-hexose isomer II	Wagner *et al.*, 1969	2	flavanone
34.19	267	493.2347	493.2285	447.2281	11.35	C21H36O10 (+FA)	Octenoic acid-dihexose II	DNP	3	fatty acid
37.82	370	301.0396	301.0348	-	14.17	C15H10O7	Quercetin		1	flavonol

[a] Numbers used for the representation of identification level refer to: (1) Identity confirmed by comparison to chemical reference standards. (2) Putatively annotated compounds based on fragmentation, UV absorption and retention time corresponding to compounds described in the literature for *Prunus* spp. or other fruit species. (3): Putatively characterized compound classes assigned by comparison of the elemental composition to public and commercial databases. FA, Formic Acid.

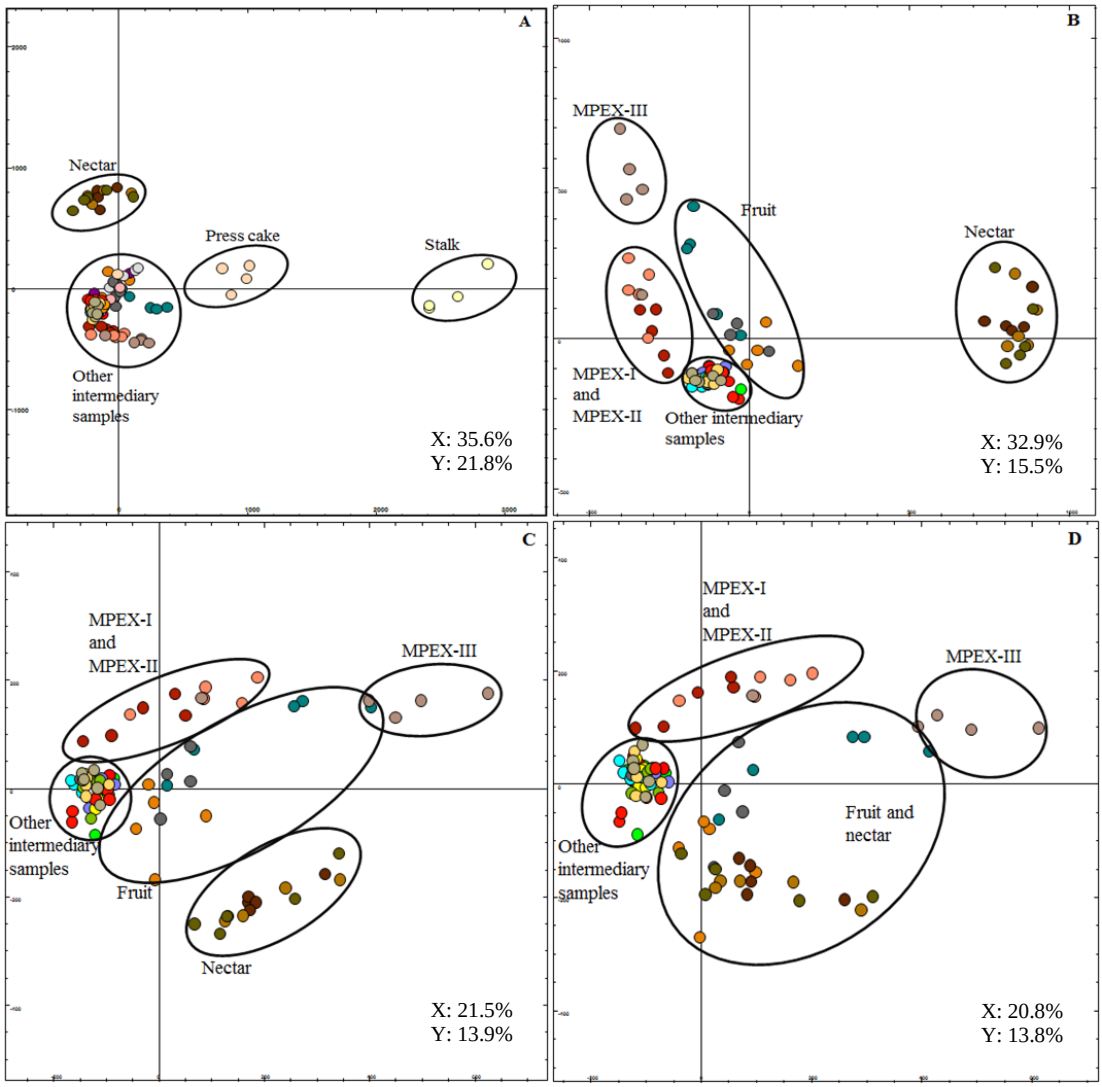

Figure 4.9 : Principal Components Analysis of untargeted LC-QTOF-MS based metabolomics data of all biological replicates of sour cherry juice processing samples (**A**) all samples, including waste samples. (**B**) excluding waste samples and stalk metabolites. (**C**) excluding sucrose and citric acid. (**D**) excluding other nectar metabolites.

4.6.1 Waste materials

The PCA grouped different replicates from the same processing steps together (Figure 4.9). This indicated consistent effects of the treatments on metabolite composition. Stalk and press cake waste materials differ substantially from the other samples, as can be seen in Figure 4.9.A, where all samples collected from five different batches of nectar production were included in the analyses. In Figure 4.9.A, the first principal component (*X-axis*), explaining 36% of total variation in the data set, separates stalk and press cake samples from the rest of the samples. Compounds that were significantly over-represented in stalk material (n=5; $p<0.05$) were

identified by t-test analysis (Table 4.10). These stalk specific metabolites were considered as irrelevant for the fruit, and were not taken into account in the further analysis. In the press cake, 24 metabolites were over-represented (Table 4.11), and 26 under-represented, relative to the fruit (Table 4.11). Compounds over-represented in the press cake relative to the fruit included catechin and a number of flavonols, while anthocyanins and phenolic esters such as neochlorogenic acid were under-represented, meaning that they were retained in the juice fraction and not lost in the waste material. In the repeated washes from the press cake (MPEX-I, II and III), a large number of phenolic compounds, in particularly flavonols, showed higher concentrations on dry-weight basis, relative to the fruit (Table 4.12). Apparently, the MPEX press washes add to the recovery of these compounds in the juice. Amygdalin, a typical seed compound (Krafft *et al.,* 2012), was the only compound found to be over-represented in the fruit, relative to the pressed juices (Table 4.12). This compound is likely to have been lost due to removal of the cherry stones.

Table 4.10 : Metabolites observed using LC-QTOF-MS analysis which were shown to differ significantly (Student's *t* Test, $p < 0.05$) between original fruit (FF-St) and stalk (St) samples.

Retention time (min)	Mass (Da)	Putative ID	Fruit:Stalk Ratio[a]
12.37	755.1976	cyanidin-3-(2^G-glucosylrutinoside)	143.27
14.92	773.2079	catechin diacyl-glucoside	87.98
13.29	611.1630	unidentified	17.41
13.33	593.1510	cyanidin-3-rutinoside	15.43
2.43	755.2072	unidentified	11.53
15.31	456.1526	amygdalin	7.80
2.41	295.0688	malic acid	5.02
22.89	441.1817	unidentified	3.66
2.95	333.0614	unidentified	2.91
3.62	293.0547	unidentified	2.70
20.40	771.1937	unidentified	2.58
9.12	515.1428	unidentified	2.43
9.90	353.0881	neochlorogenic acid (5-caffeoylquinic acid)	2.12
2.41	191.0540	quinic acid	0.49
3.13	191.0218	citric acid	0.43
33.63	451.1084	unidentified	0.38
18.53	327.1101	hydroxycinnamic acid-hexose	0.32
13.22	341.0933	unidentified	0.30
28.34	623.1604	isorhamnetin-3-rutinoside	0.27
2.39	195.0515	unidentified	0.26
28.78	515.1249	unidentified	0.26
19.59	771.1918	quercetin-rutinoside-glucoside	0.23
26.27	449.2077	unidentified	0.17
22.82	611.2585	unidentified	0.17
18.15	449.1145	unidentified	0.17
15.51	367.1086	unidentified	0.16
24.54	609.1442	quercetin-3-*O*-rutinoside	0.15
11.69	577.1393	procyanidin B1	0.12
20.71	357.1218	monoterpene hexose	0.11
20.40	772.2017	unidentified	0.10
35.75	417.2556	unidentified	0.10
18.35	431.1964	unidentified	0.10
17.79	489.1669	unidentified	0.09
25.13	567.2133	unidentified	0.09
13.54	289.0753	catechin	0.08
14.14	325.0986	unidentified	0.08
16.54	489.1667	unidentified	0.08
17.14	325.1006	unidentified	0.08
28.87	431.1045	unidentified	0.08
29.92	575.1209	unidentified	0.08

Table 4.10 (continued) : Metabolites observed using LC-QTOF-MS analysis which were shown to differ significantly (Student's *t* Test, $p < 0.05$) between original fruit (FF-St) and stalk (St) samples.

Retention time (min)	Mass (Da)	Putative ID	Fruit:Stalk Ratio[a]
30.29	449.1150	unidentified	0.07
24.23	433.1157	naringenin-7-*O*-beta-D-glucoside (prunin)	0.07
14.86	465.1079	unidentified	0.07
23.89	473.1693	unidentified	0.07
23.00	447.0975	unidentified	0.06
17.37	355.1087	unidentified	0.06
45.91	327.2229	unidentified	0.06
39.77	447.1355	unidentified	0.05
20.10	325.0976	unidentified	0.05
30.04	417.2197	unidentified	0.05
27.55	593.1503	kaempferol-3-*O*-rutinoside	0.05
37.44	433.1184	unidentified	0.05
23.20	433.1183	unidentified	0.05
30.44	447.1342	dihydrowogonin-hexose isomer I	0.04
21.54	509.2283	octenoic acid-dihexose I	0.04
23.62	521.2087	unidentified	0.04
40.53	463.1316	unidentified	0.04
21.78	581.2141	unidentified	0.04
29.54	477.1108	unidentified	0.04
29.14	271.0661	unidentified	0.04
15.82	459.1570	unidentified	0.04
38.89	593.1363	unidentified	0.04
20.84	755.2044	unidentified	0.03
20.28	449.1144	unidentified	0.03
33.27	433.1191	unidentified	0.03
31.54	555.1553	unidentified	0.03
16.78	449.1141	unidentified	0.03
27.84	431.1035	unidentified	0.03
25.57	463.0919	quercetin-3-*O*-glucoside	0.03
22.43	465.1097	pentahydroxyflavanone-hexose	0.02
42.66	271.0664	unidentified	0.02
37.35	493.2355	unidentified	0.02
36.14	447.1359	unidentified	0.02
33.49	283.0663	unidentified	0.02
32.26	445.1202	unidentified	0.02
24.86	449.1139	tetrahydroxyflavanone-hexose	0.02
37.08	415.1090	unidentified	0.02
37.98	417.1249	unidentified	0.02
7.48	315.0764	unidentified	0.02
40.75	447.1361	unidentified	0.02

Table 4.10 (continued) : Metabolites observed using LC-QTOF-MS analysis which were shown to differ significantly (Student's *t* Test, $p < 0.05$) between original fruit (FF-St) and stalk (St) samples.

Retention time (min)	Mass (Da)	Putative ID	Fruit:Stalk Ratio[a]
18.69	449.1118	unidentified	0.02
33.53	449.1118	unidentified	0.02
28.67	417.1256	kaempferol-7-glucoside	0.02
16.22	447.0981	guaiacol-hexose-pentose	0.02
26.49	431.1035	unidentified	0.02
31.09	447.1361	dihydrowogonin-hexose isomer II	0.01
34.19	493.2347	octenoic acid-dihexose II	0.01
29.41	711.2170	ferulic acid-hexose isomer	0.01

[a] ratio of the mass ion counts in fruit and in stalk materials for this compound: compounds with fruit:stalk ratios between 0.5 and 2 were not considered, and when maximal mass signals did not exceed 10x the noise, these compounds were also excluded.

Table 4.11 : Metabolites observed using LC-QTOF-MS analysis which differed significantly (Student's *t* Test, $p < 0.05$) between original fruit (FF-St) and press cake (PC).

Retention time(min)	Mass(Da)	Putative ID	Fruit:Press cake Ratio [a]
12.37	755.1976	cyanidin-3-(2G-glucosylrutinoside)	16.52
19.59	771.1918	quercetin-rutinoside-glucoside	15.33
14.92	773.2079	catechin diacyl-glucoside	15.26
3.62	293.0547	unidentified	14.85
22.89	441.1817	unidentified	12.56
9.12	515.1428	unidentified	12.50
21.65	403.1655	unidentified	12.43
13.29	611.1630	unidentified	9.43
17.21	401.1477	benzyl alcohol-hexose-pentose isomer	6.15
13.33	593.1510	cyanidin-3-rutinoside	6.13
22.82	611.2585	unidentified	5.89
2.41	295.0688	malic acid	5.45
2.95	333.0614	unidentified	5.24
18.53	327.1101	hydroxycinnamic acid-hexose	3.99
26.27	449.2077	unidentified	3.41
17.37	355.1087	unidentified	3.41
9.90	353.0881	neochlorogenic acid (5-caffeoylquinic acid)	3.08
13.33	337.0941	coumaroylquinic acid isomer	2.87
2.88	292.9268	unidentified	2.87
2.68	337.0792	ascorbic acid-glucoside	2.68
2.08	343.1254	unidentified	2.46
1.85	128.9606	unidentified	2.29
1.63	272.9607	unidentified	2.14
1.76	242.9428	unidentified	2.08
45.91	327.2229	unidentified	0.46
20.28	449.1144	unidentified	0.37
34.19	493.2347	octenoic acid-dihexose II	0.33
28.78	515.1249	unidentified	0.32
23.20	433.1183	unidentified	0.32
29.14	271.0661	unidentified	0.31
24.23	433.1157	naringenin-7-*O*-beta-D-glucoside (Prunin)	0.28
13.54	289.0753	Catechin	0.28
22.43	465.1097	pentahydroxyflavanone-hexose	0.27
33.53	417.1256	unidentified	0.26
18.69	449.1118	unidentified	0.25
2.08	146.0496	unidentified	0.25
27.84	431.1035	unidentified	0.24
37.98	417.1249	unidentified	0.24
40.75	447.1361	unidentified	0.22

Table 4.11 (continued) : Metabolites observed using LC-QTOF-MS analysis which differed significantly (Student's *t* Test, $p < 0.05$) between original fruit (FF-St) and press cake (PC).

Retention time(min)	Mass(Da)	Putative ID	Fruit:Press-cake Ratio [a]
25.57	463.0919	quercetin-3-*O*-glucoside	0.22
24.86	449.1139	tetrahydroxyflavanone-hexose	0.22
33.27	433.1191	unidentified	0.19
28.67	447.0981	kaempferol-7-glucoside	0.18
31.54	555.1553	unidentified	0.17
33.49	283.0663	unidentified	0.16
31.09	447.1361	dihydrowogonin-hexose isomer II	0.12
2.23	341.1121	sucrose	0.08

[a] ratio of the mass ion counts in fruit and in press cake for this compound: compounds with fruit : press cake ratios between 0.5 and 2 were not considered, and when maximal mass signals did not exceed 10x the noise, these compounds were also excluded.

Table 4.12 : Metabolites observed using LC-QTOF-MS analysis that differed significantly (Student's *t* Test, $p < 0.05$) between original fruit (FF-St) and MPEX steps.

Retention time (min)	Mass (Da)	Putative ID	Fruit:MPEX Ratio [a]		
			III	II	I
15.31	456.1526	amygdalin	3.00	3.14	3.23
15.98	611.1644	unidentified	0.50	0.49	0.49
22.82	611.2585	unidentified	0.49	0.54	0.56
9.90	353.0881	neochlorogenic acid (5-caffeoylquinic acid)	0.47	0.52	0.55
14.92	773.2079	catechin diacyl-glucoside	0.46	0.48	0.44
13.29	611.1630	unidentified	0.43	0.44	0.45
13.33	593.1510	cyanidin-3-rutinoside	0.43	0.44	0.45
26.27	449.2077	unidentified	0.42	0.49	0.54
17.61	289.0724	epicatechin	0.40	0.45	0.50
25.13	567.2133	unidentified	0.38		
15.51	367.1086	unidentified	0.35	0.46	0.55
18.15	449.1145	unidentified	0.35		
18.35	431.1964	unidentified	0.35	0.41	0.43
20.71	357.1218	monoterpene-hexose	0.34	0.38	0.41
15.98	577.1337	procyanidin B2	0.32	0.37	0.41
21.54	509.2283	octenoic acid-dihexose I	0.29	0.36	0.36
28.34	623.1604	isorhamnetin-3-rutinoside	0.28	0.31	0.36
22.14	785.2166	unidentified	0.28	0.31	0.34
14.21	353.0902	chlorogenic acid (3-caffeoylquinic acid)	0.27	0.33	0.39
39.05	417.11258	unidentified	0.26		
25.02	577.1365	unidentified	0.25	0.34	0.44
17.37	355.1087	unidentified	0.24	0.28	0.31
19.95	401.0941	unidentified		0.27	0.27
29.14	271.0661	unidentified	0.24	0.35	0.47
23.20	433.1183	unidentified	0.24	0.35	0.47
22.43	465.1097	pentahydroxyflavanone-hexose	0.23	0.36	0.47
15.24	353.0927	unidentified	0.23	0.29	0.35
44.41	285.0450	unidentified	0.23		
13.54	289.0753	catechin	0.22	0.33	0.45
20.28	449.1144	unidentified	0.21	0.34	0.43
24.23	433.1157	naringenin-7-*O*-beta-D-glucoside (Prunin)	0.21	0.32	0.41
37.98	417.1249	unidentified	0.21	0.36	
20.40	772.2017	unidentified	0.21	0.24	0.25
27.84	431.1035	unidentified	0.21	0.33	0.44
21.49	720.1579	unidentified	0.20	0.29	
24.54	609.1442	quercetin-3-*O*-rutinoside	0.19	0.20	0.22
40.75	447.1361	unidentified	0.19	0.31	

Table 4.12 (continued) : Metabolites observed using LC-QTOF-MS analysis that differed significantly (Student's *t* Test, $p < 0.05$) between original fruit (FF-St) and MPEX steps.

Retention time (min)	Mass (Da)	Putative ID	Fruit:MPEX Ratio [a]		
			III	II	I
31.54	555.1553	unidentified	0.18		
30.04	417.2197	unidentified	0.18	0.22	
20.40	771.1937	unidentified	0.18	0.21	
33.49	283.0663	unidentified	0.17		
21.13	865.1960	unidentified	0.17	0.24	0.32
33.27	433.1191	unidentified	0.16	0.26	0.36
11.69	577.1393	procyanidin B1	0.16	0.24	0.29
18.69	449.1118	unidentified	0.15	0.24	0.32
28.78	515.1249	unidentified	0.15	0.25	0.38
33.63	451.1084	unidentified	0.15	0.22	0.30
29.54	477.1108	unidentified	0.14	0.21	
24.86	449.1139	terahydroxyflavanone-hexose	0.14	0.22	0.29
16.22	417.1449	guaiacol-hexose-pentose	0.14		
24.72	451.1090	unidentified	0.13	0.19	
31.09	447.1361	dihydrowogonin-hexose isomer II	0.13	0.22	0.32
27.55	593.1503	kaempferol-3-*O*-rutinoside	0.12	0.16	0.19
20.84	755.2044	unidentified	0.12	0.15	0.17
21.78	319.0512	unidentified	0.12		
25.57	463.0919	quercetin-3-*O*-glucoside	0.11	0.17	
7.48	315.0764	unidentified	0.11		
28.67	447.0981	kaempferol-7-glucoside	0.10	0.15	0.20
34.19	493.2347	octenoic acid-dihexose II	0.08	0.12	0.19
20.93	709.2016	unidentified	0.06	0.08	0.09
29.41	711.2170	ferulic acid-hexose isomer	0.05	0.07	0.09

[a] ratio of the mass counts in fruit and in mash press washes (MPEX) for this compound: compounds with fruit:MPEX ratios between 0.5 and 2 were not considered, and when maximal mass signals did not exceed 10x the noise, these compounds were also excluded.

4.6.2 Nectar samples

To explore further the differences between original fruit and final nectar, the waste samples (stalks, press cake, filtration residue) and the metabolites that occur only in the stalks were excluded from the data set, after which a PCA analysis was again performed (Figure 4.9.B). This leads to the observation of a clear separation of the nectar samples from the others (*X-axis* –representing the first component of variance-explains 33% of total variation). Metabolites that strongly distinguished nectar from the original fruit were dominated by polar compounds (Table 4.13), like sucrose and citric acid, that had been added as a supplement during the last production steps. Consequently, another PCA was performed using the same dataset, now omitting the sucrose and citric acid signals (Figure 4.9.C). This PCA showed that the nectar samples were still different, although the degree of variation (*X-axis* 21.5%) became smaller. This separation of nectar from the other samples could be ascribed to nine compounds (Table 4.13), apart from the added sucrose and citric acid, including neochlorogenic acid, which were higher in nectar on dry-weight basis. Remarkably, only 11 out of the total of 193 detected compounds observed in the original cherry fruit had levels which were significantly different (decreased or increased) in the final nectar (Table 4.13). Noteworthy, anthocyanins were absent in this list of significantly different metabolites between fruit and nectar, suggesting that this class of compounds were recovered well. The majority of the anthocyanins is extracted during nectar production and protected in the final product. When the additional nine different compounds were also removed from the dataset, the fruit and nectar samples were no longer consistently separated from each other in the PCA (Figure 4.9.D).

Table 4.13 : Metabolites observed using LC-QTOF-MS analysis that differed significantly (Student's *t* Test, $p < 0.05$) between original fruit (FF-St) and nectar.

Retention time(min)	Mass (Da)	Putative ID	Fruit: Nectar Ratio[a]
15.31	456.1526	Amygdalin	4.08
9.90	353.0881	Neochlorogenic acid (5-caffeoylquinic acid)	0.49
24.54	609.1442	Quercetin-3-*O*-rutinoside	0.45
2.68	115.0000	Ascorbic acid-glucoside	0.44
15.24	353.0927	unidentified	0.42
2.41	191.0540	Quinic acid	0.35
2.39	179.0576	unidentified	0.23
3.13	191.0218	Citric acid	0.11
2.68	663.2047	unidentified	0.05
2.23	341.1121	Sucrose	0.02
2.84	439.0911	unidentified	0.01

[a] ratio of the mass ion counts in fruit and in nectar for this compound: compounds with a fruit:nectar ratio between 0.75 and 2 were not included, and when maximal mass signals did not exceed 10x the noise, these compounds were excluded from the analysis.

4.6.3 Ascorbic acid glucoside

Our HPLC results of vitamin C contents revealed that original fruit (FF-St) samples contained only the oxidized form of ascorbic acid, DHAA (8.3-16.0 mg/100 g dry-weight), whereas there was no AA or DHAA detected in the final nectar (PN) sample. The level of AA in our original cherry sample appears relatively low as compared to those reported for sour-cherry fruit (10 mg/100 g fresh weight fruit) (NutritionData, 2012) and juices in literature (43 – 177 mg / L) (Bonerz *et al.*, 2007), which may be due to differences in cherry varieties, juice processing and/or analysis methods (HPLC-PDA in this study versus potentiometric analysis in Bonerz *et al.*, 2007).

Nevertheless, using HPLC with online antioxidant detection (Figure 4.7 and Table 4.8), we noted that there was a polar antioxidant in the extracts. We therefore applied LC-QTOF-MS analysis to identify other compounds that might be responsible for this polar antioxidant activity. Based on the observed accurate mass ($[M-H]^-$ = 337.0792) and retention time (2.71 min) both corresponding with the authentic standard, we putatively identified a conjugated form of AA, i.e. ascorbic acid-glucoside (AAG; $C_{12}H_{18}O_{11}$, calculated $[M-H]^-$ = 337.0776) (Figure 4.10), in sour cherry samples. By subsequent analysis of a standard calibration series, ascorbic acid-glucoside concentrations in sour cherry could be calculated: 2.2 mg/100 g dry-

weight and 1.3 mg/100 g dry-weight in FF-St and PN samples, respectively (Table 4.14).

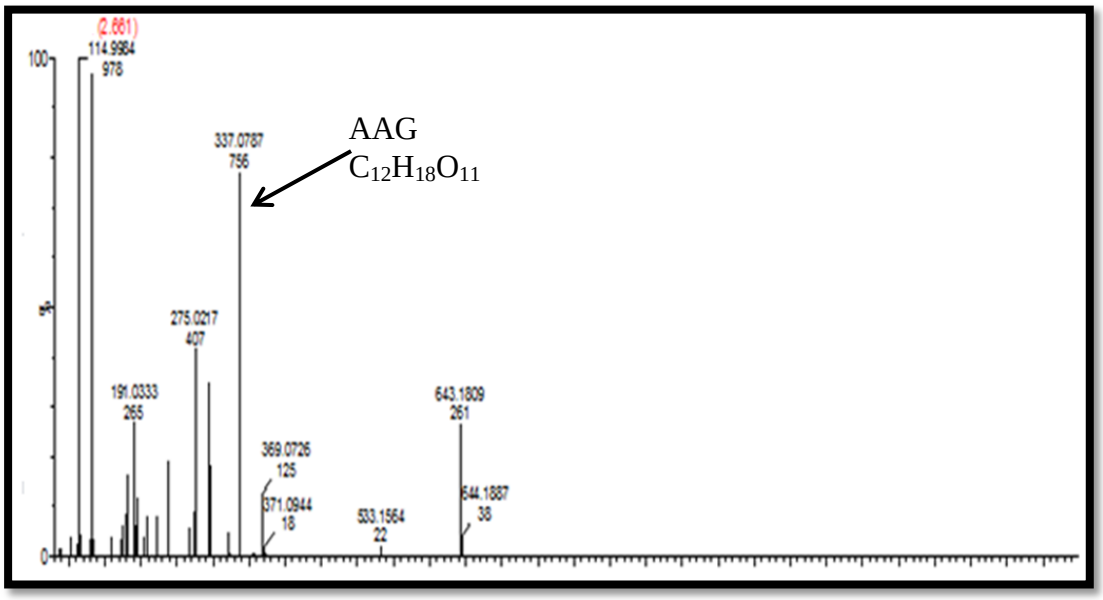

Figure 4.10 : Detection of AAG in sour cherry fruit; LC-MS spectra of the parent peak obtained with searching for $[M-H]^{-}$ = 337 (*m/z*).

Ascorbic acid-glucoside has so far been reported in only three plant species, which are *Goji* berry (*Lycium barbarum* L.) (5 mg per 100 g dry-weight) (Toyada-Ono *et al.*, 2004), zucchini (*Cucurbita pepo* L.) (ranging between 0.001 mg to 0.098 mg / g fresh weight in different plant parts) (Hancock *et al.*, 2008), and baechu kimchi (napa cabbage) (Jun *et al.*, 1998). Stable forms of ascorbic acid that are glycosylated at the 2- or 3-hydroxyl groups are already reported. For instance, 2-*O*-(*α*-D-glucopyranosyl)ascorbic acid was produced by glucosyltransferase (Yamamoto *et al.*, 1990), 2-*O*-(*β*-D-galactopyranosyl)ascorbic acid was obtained by galactosidase (Shimono *et al.*, 1994), and 3-*O*-(*β*-D-glucopyranosyl)ascorbic acid was obtained by chemical synthesis (Sato and Miyakada, 1983). Among these three, 2-*O*-(*α*-D-glucopyranosyl)ascorbic acid has been pointed out to be most investigated as a highly stable α-glucoside form of AA to acidic and various oxidative conditions. This AA analogue with provitamin C activity has been indicated to be a good alternative to traditional AA in fruit products (Toyada-Ono *et al.*, 2004). After its oral administration, α-glucosidases present in the digestive organs (i.e., saliva, intestinal digestive juices, and the small intestinal tract) hydrolyze this compound, generating active AA (Yamamoto *et al.*, 1990). AA2G is not detected in blood on oral administration, therefore it is suggested to be completely hydrolyzed in the

digestive organs (in which α-glucosidase is widely distributed) to exist in blood as ascorbic acid (Toyado-ono *et al.*, 2004; Yamamoto *et al.*, 1990). It is also suggested that hydrolysis and activation by β-glucosidase, which is less widely distributed in the body, instead of activation and hydrolysis by α-glucosidase, which is widely distributed, would be more advantageous in terms of the transport of this stable form of AA into tissues and long lasting action (Yamamoto *et al.*, 1990). As well as, 2-*O*-(*β*-D-glucopyranosyl)-L-ascorbic acid, which is present in *Goji* berry (*Lycium barbarum* L.) naturally, has been indicated to possess multiple pharmalogical functions, such as antioxidant, antiaging, immune promoting, and antitumorigenic activities (Amagase *et al.*, 2009; Potterat, 2010; Reeve *et al.*, 2010). However, it should be emphasized that the absolute levels of this antioxidant compound in the sour cherry variety analyzed were low compared to the other existing fruit antioxidants (Figure 4.7 and Table 4.14).

Table 4.14 : Change in AAG concentrations between processing steps.

Sample	**AAG concentration (mg / 100 g dw)** [a]	**% of FF-St**
FF	2.3 ± 0.6 abc	
FF-St	2.2 ± 0.7 a	100
St	1.2 ± 0.4 cd	54
MH	2.2 ± 0.3 abc	103
MP	2.7 ± 0.2 a	123
MPEX-I	2.6 ± 0.2 a	119
MPEX-II	2.5 ± 0.2 a	116
MPEX-III	2.4 ± 0.1 ab	109
PrJ	2.7 ± 0.2 a	126
PC	0.3 ± 0.3 d	14
PsJ	2.7 ± 0.3 a	122
EnJ	2.6 ± 0.2 a	120
ClJ	2.6 ± 0.2 a	118
FtJ	2.5 ± 0.2 a	117
FR	1.1 ± 0.7 bcd	52
CJ	2.5 ± 0.3 ab	114
CJ-BPF	2.5 ± 0.3 a	116
CJ-APF	2.4 ± 0.3 ab	111
N	1.3 ± 0.5 d	62 (142)[b]
SSN	1.4 ± 0.7 d	64 (186)[c]
PN	1.3 ± 0.6 d	61 (178)[c]

[a] Data represent average quantities ± standard deviation of five biological replications for each sample. Different letters show statistically significant differences between the concentrations ($p<0.05$).

[b] The original percent is multiplied by factor 2.3 to compensate for the additional sucrose in dry-weight composition of the initial nectar (N) sample.

[c] The original percent is multiplied by factor 2.9 to compensate for the additional sucrose in dry-weight composition of the sucrose syrup added nectar (SSN) and final pasteurized nectar (PN) samples.

4.7 Bioavailability Assays

4.7.1 Simulated *in vitro* gastrointestinal (GI) digestion

The impact of *in vitro* GI digestion on health-related antioxidant compounds of sour cherry was monitored during *in vitro* gastric and intestinal digestion conditions applied to the fruit and its processed nectar. Samples were taken prior to digestion (initial), after gastric digestion (post gastric, PG), and after intestinal digestion. The latter was separated in serum-available material (IN) and non-serum available material (OUT). The changes in the levels of TP, TF, TA contents and TAC during gastric and intestinal digestion were determined with spectrophotometric methods. The recoveries were calculated relative to the values of the initial samples for both fruit and nectar material (Figure 4.11 and Figure 4.12).

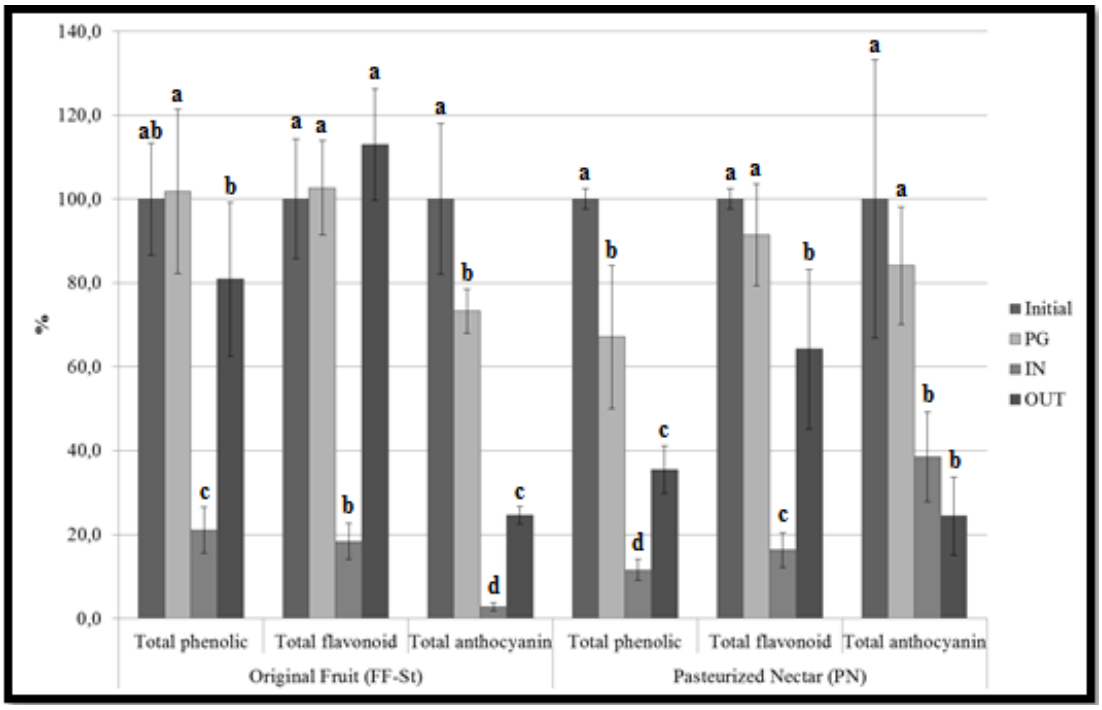

Figure 4.11 : Percent recoveries of TP, TF and TA in original fruit (FF-St) and pasteurized nectar (PN) after *in vitro* gastrointestinal digestion. The terms represent; Initial = initially determined values in fruit and nectar matrixes (100% in percent); PG, phenolics remaining after gastric digestion; IN, dialyzed fraction after intestinal digestion; OUT, non-dialyzed fraction after intestinal digestion. Data represent average values ± standard deviation of five independent samples.

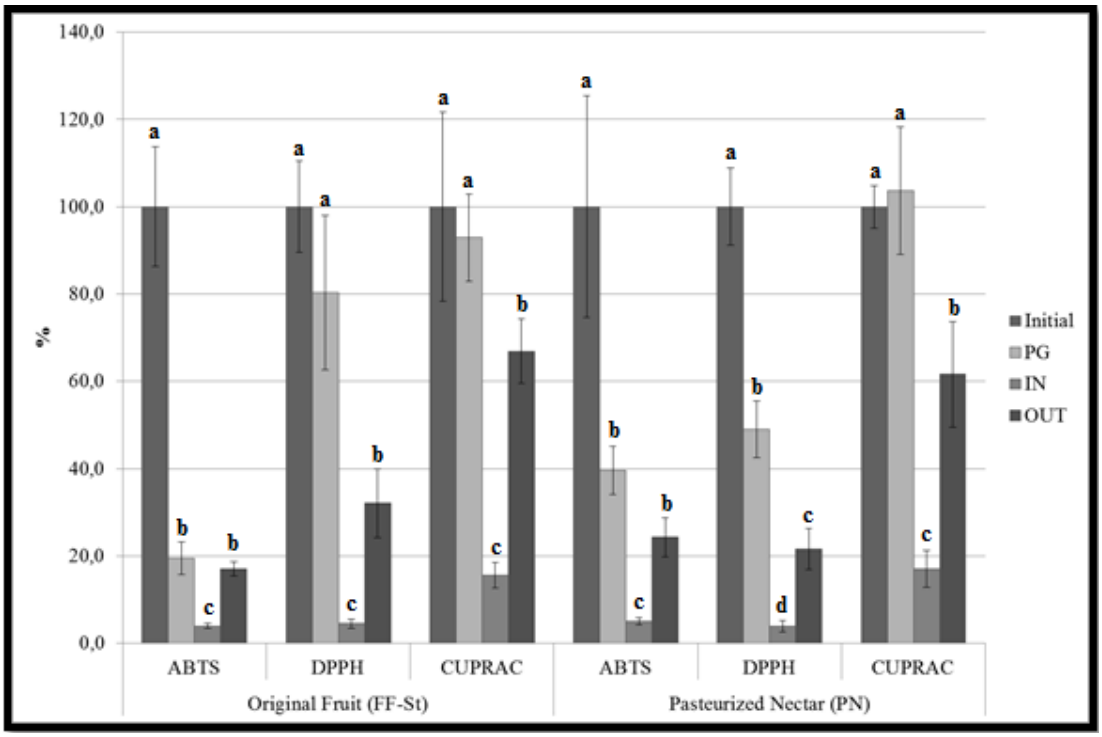

Figure 4.12 : Percent recoveries of TAC (ABTS, DPPH and CUPRAC) in original fruit (FF-St) and pasteurized nectar (PN) after *in vitro* gastrointestinal digestion. The terms represent; Initial, initially determined values in fruit and nectar matrixes (100% in percent); PG, phenolics remaining after gastric digestion; IN, dialyzed fraction after intestinal digestion; OUT, non-dialyzed fraction after intestinal digestion. Data represent average values ± standard deviation of five independent samples.

As shown in Figure 4.11, with regard to TP values, the gastric digestion did not have a profound effect: 100% of the cherry fruit and 70% of the cherry nectar TP values were recovered. In consistence with these results, TF values did not show significant changes in both fruit and nectar after gastric digestion (103 % and 92% of the inital values were available in PG samples of fruit and nectar, respectively), on the other hand, most TF was not serum available (i.e. it occurred in the OUT fraction). The values for TA showed a remarkable trend: significant amounts (39% of the initial value) of TA were found in the IN fraction from the nectar. This was highly different from the cherry fruit, where TA recovery in the IN fraction was only 3%. Thus, it seems that fruit processing leads to enhanced serum availability of anthocyanins. The various antioxidant capacity assay methods showed large differences in recovery values, in particular in post-gastric samples. On the other hand, all methods indicated that the recovery of TAC in the serum-available fraction (the IN values) was the same for both fruit and nectar (Figure 4.12).

The major finding from this work was that the total anthocyanin recovery obtained for dialyzable fraction (IN) of nectar sample (39%) was 13-fold higher than the recovery obtained for the same fraction of fruit sample (3%) (Figure 4.11). This may indicate a higher *in vitro* anthocyanin bioavailability in the nectar sample. This could be correlated to a higher stability of anthocyanins in the nectar matrix. This stability has previously been reported to be affected by a combination of several factors, including structure and concentration, pH, temperature, light, metallic ions, enzymes, oxygen, ascorbic acid, and sugars (Francis, 1989). The industrially-processed sour cherry nectar samples used in this study contained more than 50% sucrose added to their dry-matter, a key differentiator of the nectar and the fruit. It has been reported previously that high concentrations of sugar in fruit, preserves and stabilizes the anthocyanins (Wrolstad *et al.*, 1990). This stabilizing effect of sugar addition has been investigated by several studies and explained as added sugar has led to reduced water activity (a_w) and low changes of sugar concentration and a_w have been reported to have an effect on pigment stability. Rubinskiene *et al.* (2005) examined the impact of 10-40% sucrose inclusion to water solutions of spray-dried anthocyanin pigments. A positive effect on the stability of pigments was found when sucrose concentration increased > 20%. Moreover, Tsai *et al.* (2004) revealed that sucrose was a good anthocyanin protector, specifically at high concentrations. They assessed the kinetics of anthocyanin degradation and water mobility (after heating) on their model system of roselle (*Hibiscus sabdariffa* L.) anthocyanins with different sugar concentrations (20, 40, 60%). Their results indicated that anthocyanin stability in sucrose was significantly higher at concentrations ≥ 40%, perhaps because increased ability to bind water molecules favored the stability of anthocyanins in sucrose solutions. Similarly, in our case, the sucrose added to the nectar could have had a stabilizing effect on anthocyanins explaining the higher levels of these components available in nectar after simulated digestion. However, since anthocyanin stability in a food matrix may depend on a combination of numerous factors, future research may be directed to unravel the effect of nectar constituents on anthocyanin stability and transportation during gastrointestinal digestion.

4.7.2 Assays with *in vitro* Caco-2 cell model

4.7.2.1 Optimum cell medium for transport experiments

The first parameter investigated in order to select the optimum cell medium for use in transport experiments was the stability of sour cherry anthocyanins when fruit and nectar samples were diluted in selected mediums including HBSS, HBSS with 100 mM HEPES, DMEM, and DMEM with 9.1% FBS.

In the human body it can be expected that pH in the intestine lumen will end up near neutral. The sample used in transport experiments therefore should also be held in medium at neutral pH. HBSS is a common medium for this kind of transport experiments but does not have pH buffering capacity, that is why both HBSS and HBSS with HEPES were included. The standard culturing medium for Caco-2 cells is DMEM with FBS which contains HEPES for buffering at neutral pH. On the other hand, the proteins in FBS might cause binding of phenolic compounds (Bartolome *et al.*, 2000) and thereby could disturb analytical analysis. Therefore, DMEM without FBS was also tried as having the potential of being a better alternative medium to the cell culture medium (DMEM with 9.1% FBS).

Fruit and nectar samples were diluted in the four different media, incubated for 6 h at 37ºC and 5% CO_2 in air and anthocyanin levels determined by HPLC-DAD. The percentages of change in cyanidin-3-(2^G-glucosylrutinoside) (C3GR) concentrations were monitored as the representative anthocyanin compound, since it was determined to be the major anthocyanin in sour cherry samples. As shown in Figure 4.13 the stability of C3GR in HBSS medium was found to be significantly ($p < 0.05$) higher than in other media tested. Although, DMEM (no FBS) medium and DMEM with 9.1% FBS medium were found to have significantly lower stability in some cases, the stability (%) values recorded among the three media (other than HBSS) were still comparable (Figure 4.13). The higher stabilities obtained with HBSS medium is very likely linked to the lower pH values measured for samples diluted in this medium. While the pH values obtained for fruit and nectar in HBSS were around pH 4; both samples in the other three media gave pH values of around pH 7. Although the recovery of anthocyanins throughout 6 h incubation in HBSS was higher, the low pH of samples in HBSS was not appropriate to mimic the human intestinal conditions.

The lower pH values obtained with HBSS medium could be linked to the medium properties which was not buffered with buffering agents. So, when the sour cherry fruit and its nectar, having pH values of around pH 3-3.5, were diluted in HBSS, they were able to lower the pH of this medium. On the other hand, higher pH values (pH ≈ 7) were obtained for both samples in HBSS medium buffered with HEPES.

The second parameter investigated for selecting the optimum medium for transport analysis was the integrity of the Caco-2 cell monolayers which should not be affected negatively as a result of exposure to the medium tried out during a 6 h incubation, at 37ºC and 5% CO_2. For the decision of the optimum medium, the tight junctions of the cell monolayers in different mediums were evaluated with TEER measurements which were performed and recorded: first, in the culture medium (initial TEER) before it was changed; second, after 1 h of pre-incubation with the replaced medium (TEER at 0 h); and third, 6 h of incubation after the previous (0 h) measurements (TEER at 6 h). The initial TEER values recorded in the cell culture medium, and % changes obtained with 1 h of pre-incubation after replacement, and with 6 h of incubation after 0 h TEER measurements were given in Table 4.15.

Results indicate that the TEER was negatively affected by HBSS media, either non-buffered or buffered with HEPES, resulting in significant decreases in TEER values, even below 200 $\Omega.cm^2$ which were not acceptable for a proper confluent Caco-2 layer representing small intestine. Although, the decrease in TEER with exposures to both DMEM media were found to be significant, the values were still > 200 $\Omega.cm^2$ at the end of incubation. Therefore, these media were thought to be appropriate media for transport experiments (Table 4.15).

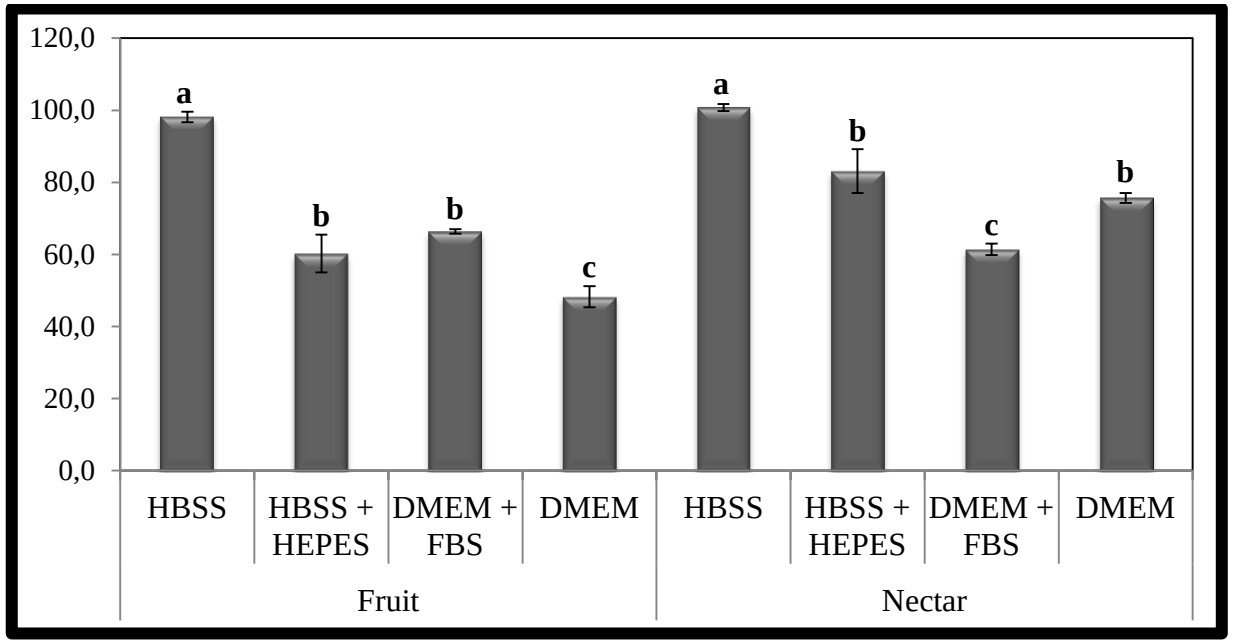

Figure 4.13 : Percentage changes in cyanidin-3-(2^{G}-glucosylrutinoside) concentrations in fruit (left panel) and nectar (right panel) during 6 h of incubation in different media (without cell monolayers). Values represent the averages of duplicates ± standard deviations. Different letters on the bars represent statistically significant differences ($p < 0.05$) between samples. Statistical analysis for fruit and nectar was performed separately.

Table 4.15 : TEER measurements performed in cell culture medium and during incubation in four different cell media.

Medium	initial TEER value in culture medium	TEER at 0 h as % of initial value[a]	TEER at 6 h as % of 0 h[a]
HBSS	277 ± 21.2 a	57 ± 4.1 (158±0.7) b	70 ± 3.9 (110±5.7) b
HBSS with 100 mM HEPES	288 ± 0.7 a	55 ± 9.5 (157±26.9) b	77 ± 5.9 (120±12.7) b
DMEM with 9.1% FBS	273 ± 7.1 a	91 ± 1.1 (248±3.5) ab	91 ± 8.2 (226±17.0) b
DMEM (no FBS)	285 ± 9.2 a	86 ± 5.0 (244±6.4) b	95 ± 0.5 (232±4.9) b

[a]Numbers in brackets represent the average TEER values measured duplicate at respective times. Different letters in the rows figure out statistically significant differences between the measurements ($p < 0.05$).

When the results obtained for anthocyanin stability and cell monolayer integrity experiments, as well as the pH value measurements for fruit and nectar in four different mediums, were evaluated, DMEM mediums, supplemented with FBS or non-supplemented, were determined to be acceptable for further transport experiments. Because of the fact that the proteins present in DMEM supplemented with 9.1% FBS could potentially have interactions with sour cherry phenolic compounds which could further lead to precipitations (Hagerman and Butler, 1981; Siebert *et al.*, 1996) in samples on cell layers, as a conclusion, DMEM with no FBS was decided to be used as the transport medium for further transport experiments.

4.7.2.2 Transport experiments with whole fruit and whole nectar samples

Transport experiments were performed with whole sour cherry fruit (WF) and its processed nectar (WN) which were dissolved in transport medium, DMEM (no FBS), in concentrations of 35 mg dry-weight / mL medium, and 55 mg dry-weight / mL medium, respectively. In order to assess the possible effects of the nectar ingredients, also WF supplemented with sucrose and / or citric acid were prepared. These samples, with sample volume of 2 mL, were applied to the apical side of the Caco-2 cell monolayers, cultured on 6-well tissue culture plate inserts, and incubated for 6 h at 37ºC and 5% CO_2. Transport of anthocyanins across the cell monolayers was studied in the apical to basolateral direction. The apical and basolateral sides were collected at the end of 6 h incubation and were analysed and quantified by HPLC. Since cyanidin-3-(2^G-glucosylrutinoside) (C3GR) (peak 1 in Figure 4.14) comprised around 75% of the total anthocyanins in both WF and WN samples, the recovery of anthocyanins on cell monolayers and the transport of anthocyanins

through the cell monolayers were monitored for this major compound (Figure 4.14). Although in nectar samples, cyanidin-3-rutinoside (peak 2, in Figure 4.14) was also detected at the basolateral side, the calculations were based on only C3GR anthocyanin compound. Anthocyanin recovery percentages on the apical side and anthocyanin transport efficiency percentages to the basolateral side were calculated for each sample (Figure 4.15 and Figure 4.16; Table 4.16). Calculation for anthocyanin recovery was based on the formula: (C3GR concentrations at the apical side after 6 h of incubation) / (C3GR concentrations at the apical side at 0 h) x 100%; and calculation for anthocyanin transport efficiency was based on the formula (C3GR concentrations at the basolateral side after 6 h of incubation) / (C3GR concentrations at the apical side at 0 h) x 100%. Figure 4.15 shows the recovery percentages of C3GR on the apical side of cells and Figure 4.16 shows the transport efficiency percentages of C3GR from the apical to the basolateral side of cell monolayers. Table 4.16 shows both the data for stability and transport efficiency percentages, as well as the statistical grouping ($p < 0.05$) of the samples on the basis of these terms.

The recovery percentages (Table 4.16 and Figure 4.15) obtained for WF and WF with sucrose-added (WF + SUC) samples were significantly lower with approximately 55% disappearance of C3GR in 6 h of incubation with Caco-2 monolayers. The highest recovery was obtained for the WF sample with citric acid-and-sucrose-added (WF + CA + SUC) with 82% recovery. On the other hand, recovery percentages obtained for WN sample and WF with citric acid-added (WF + CA) sample were not significantly different with 77% and 78% recoveries, respectively. Although the difference between these samples and WF + CA + SUC sample was found to be statistically significant ($p < 0.05$), their recoveries were still comparable. The higher recovery values of anthocyanins in the apical solutions could be linked to the lower pH values (pH = 4) obtained for the citric acid including samples. The WF and WF + SUC samples had a higher pH value (pH = 6), and lower anthocyanin recovery.

The anthocyanin transport efficiency percentages (expressed in terms of C3GR) determined in the basolateral side extracts, and collected after 6 h incubation with Caco-2 cells, is shown in Figure 4.16 and also given in Table 4.16. In general, samples that provided higher recovery of anthocyanins on the apical side of cell

monolayers, were also found to have higher transport efficiencies to the basolateral side of cell monolayers in 6 h incubation, except for the WF + SUC sample. This sample gave the lowest recovery of anthocyanins on the apical side of cells, but was not significantly different from the WF + CA + SUC sample that was determined to have the highest transport efficiency (%) to the basal side. The transport efficiencies (%) of anthocyanins to the basolateral side were determined to have the highest values for the samples including both citric acid and sucrose added to their contents, which were WF + CA + SUC and WN, with 3.9% and 2.6% of transport efficiency percentages, respectively. Following these, WF + SUC and WF + CA samples gave comparable transport efficiencies of 2.4% and 2.3%, respectively. The WF sample which had the lowest recovery on the apical side was also found to have the lowest transport (0.52%) to the basal side. Both citric acid and/or sucrose inclusion in the content of the samples resulted in an enhanced transport of anthocyanins. Citric acid inclusion in the content of the samples could be related to the higher anthocyanin concentrations in the apical solutions over incubation time (as a result of lower pH), thus enhancing the transport efficiency (%) as well. On the other hand, sucrose inclusion had no effect on recovery (%) values, but contributed more significantly to the transport (Table 4.16 and Figure 4.16).

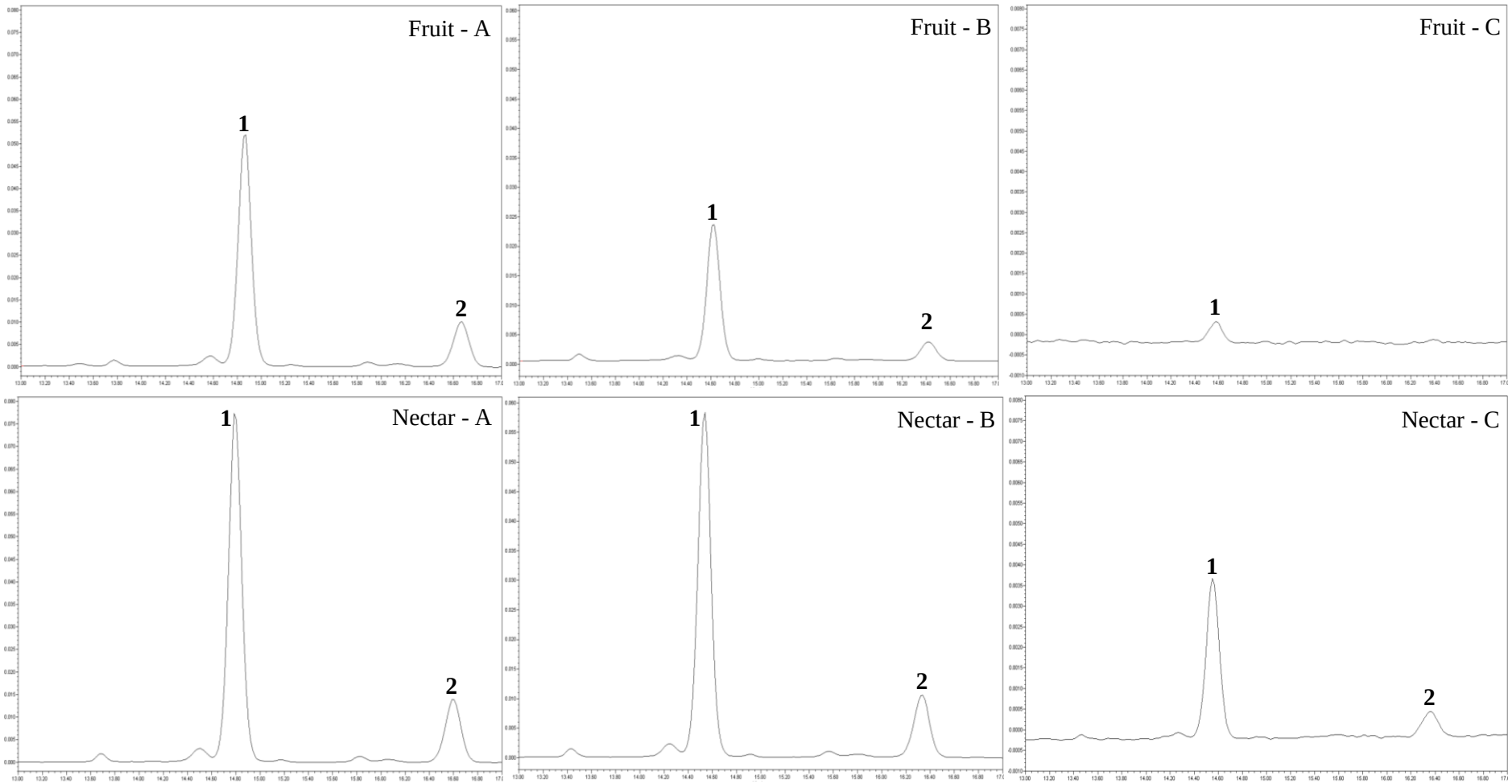

Figure 4.14 : Representative HPLC chromatograms of sour cherry fruit (upper panel) and nectar (lower panel) anthocyanins in the apical side extracts at 0 h (A); in the apical side extracts after 6 h incubation (B); and in the basolateral side extracts after 6 h incubation (C). Numbers refer to the anthocyanins peaks: (1) cyanidin-3-(2^{G}-glucosylrutinoside), (2) cyanidin-3-rutinoside. The chromatograms for apical side at 0 h, apical side at 6 h and basolateral side at 6 h are seperately on the same scale for fruit and nectar samples.

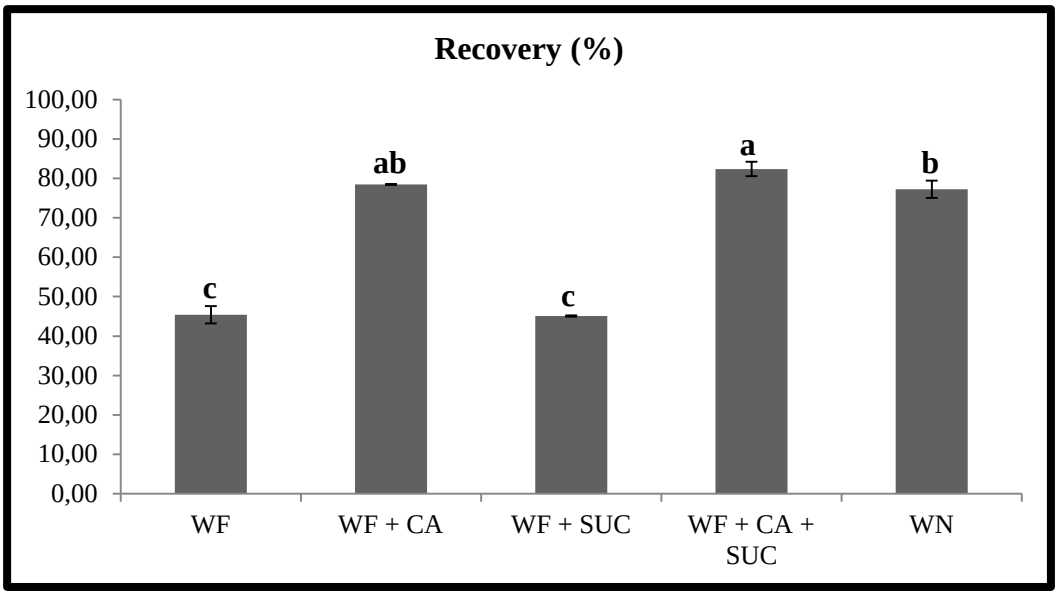

Figure 4.15 : Anthocyanin recovery percentages (for whole samples) at the apical side of cell monolayers during 6 h of incubation calculated as (C3GR concentrations at the apical side after 6 h of incubation) / (C3GR concentrations at the apical side at 0 h) x 100%. Values are the means ± SD (n = 3). Differences between samples those do not share a letter given on the bars are statistically significant ($p < 0.05$). The terms represent: WF = whole fruit (pH = 6); WF + CA = whole fruit with citric acid (pH = 4); WF + SUC = whole fruit with sucrose (pH = 6); WF + CA + SUC = whole fruit with citric acid and sucrose (pH = 4); WN = whole nectar (pH = 4).

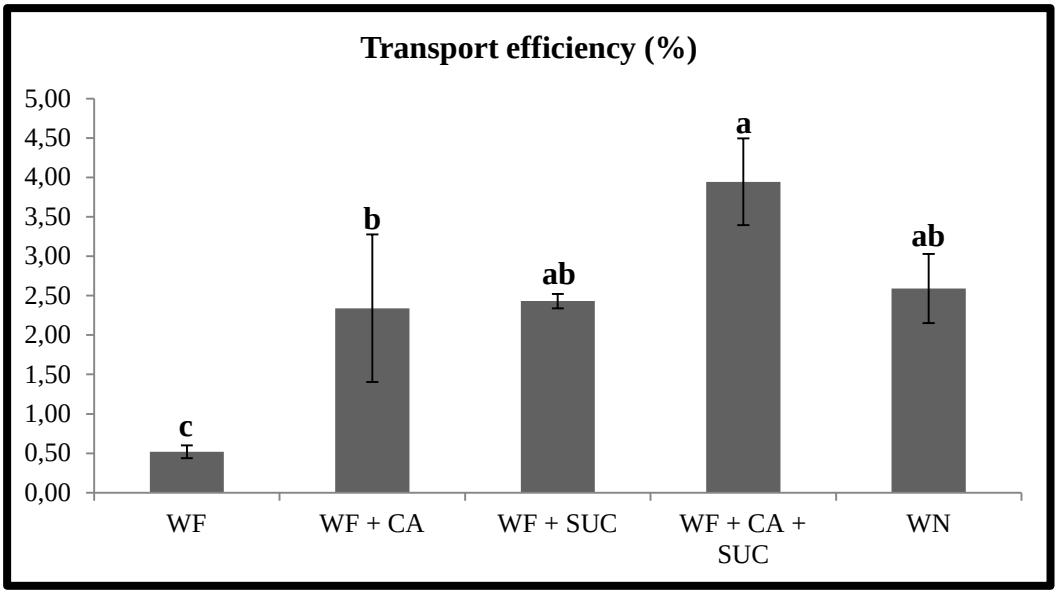

Figure 4.16 : Anthocyanin transport efficiency percentages (for whole samples) to the basal side of cell monolayers during 6 h of incubation, calculated as (C3GR concentrations at the basolateral side after 6 h of incubation) / (C3GR concentrations at the apical side at 0 h) x 100%. Values are the means ± SD (n = 3). Differences between samples those do not share a letter given on the bars are statistically significant ($p < 0.05$). The terms represent: WF = whole fruit (pH = 6); WF + CA = whole fruit with citric acid (pH = 4); WF + SUC = whole fruit with sucrose (pH = 6); WF + CA + SUC = whole fruit with citric acid and sucrose (pH = 4); WN = whole nectar (pH = 4).

Table 4.16 : Stability (%) on apical side of Caco-2 cell monolayer and transport efficiency (%) of anthocyanins from apical to basolateral side of Caco-2 cell monolayer in whole fruit and whole nectar samples.

Sample & pH	Recovery (%)[a]	Transport efficiency (%)[b]
WF (pH 6)	45.4 ± 2.4 c	0.52 ± 0.1 c
WF + CA (pH 4)	78.4 ± 0.1 ab	2.34 ± 0.9 b
WF + SUC (pH 6)	45.1 ± 0.2 c	2.43 ± 0.1 ab
WF + CA + SUC (pH 4)	82.4 ± 1.8 a	3.94 ± 0.6 a
WN (pH 4)	77.2 ± 2.2 b	2.59 ± 0.4 ab

[a] Recovery percentages were calculated based on (C3GR concentrations at the apical side after 6 h of incubation) / (C3GR concentrations at the apical side at 0 h) x 100%. Values represent the averages of triplicates ± standard deviation. Differences between samples those do not share a letter given in the columns are statistically significant ($p < 0.05$).

[b] Transport efficiency percentages were calculated based on (C3GR concentrations at the basolateral side after 6 h of incubation) / (C3GR concentrations at the apical side at 0 h) x 100%. Values represent the averages of triplicates ± standard deviation. Differences between samples those do not share a letter given in the columns are statistically significant ($p < 0.05$).

4.7.2.3 Transport experiments with fruit and nectar samples treated with solid phase extraction procedure

Sour cherry fruit and nectar samples were subjected to solid phase extraction (SPE) procedure in order to purify anthocyanins and other flavonoids and to eliminate the possible effects of the other food matrix ingredients. These purified fruit-SPE and nectar-SPE pellets were dissolved in transport medium, in certain concentrations, in order to obtain the anthocyanin concentrations in their non-SPE counterparts, WF and WN samples, when they were diluted in transport medium in concentrations of 35 mg dry-weight / mL medium, and 55 mg dry-weight / mL medium, respectively. Subsequently, they were applied to Caco-2 cells grown in 6-well inserts, following the same procedure used for WF and WN samples. After 6 h incubation at 37ºC and 5% CO_2, the apical and basolateral side samples were collected and analysed on HPLC in order to monitor the recovery and transport of anthocyanins (investigated for C3GR), respectively. The effects of sucrose and/or citric acid inclusion on transport were also investigated on SPE samples.

The recovery percentages of C3GR on the apical side of cells and the transport efficiency percentages of C3GR from the apical to the basolateral side of cell monolayers are shown in Figure 4.17 and Figure 4.18, respectively, for fruit-SPE and nectar-SPE samples, as well as for their citric acid-added forms. In addition, Table 4.17 shows both the data for recovery and transport efficiency percentages, as well as

the statistical grouping ($p < 0.05$) of the SPE samples on the basis of these terms. Sucrose-added SPE samples showed a different and inconsistent trend in transport of anthocyanins than the trend observed for sucrose-added WF sample (data not shown).

The recovery percentages of C3GR anthocyanin compound for fruit-SPE and nectar-SPE samples were not statistically different ($p < 0.05$) (Figure 4.17). Citric acid-added fruit-SPE and citric acid-added nectar-SPE samples showed higher anthocyanin recoveries than their non-citric acid-added counterparts. This could be linked to the lower pH (pH = 4) they had than the non-citric acid-added SPE samples (pH = 8). This observation was similar to the results obtained for WF with citric acid-added sample. On the other hand, while citric acid addition to the fruit-SPE sample resulted in a statistically significant ($p < 0.05$) increase in recovery, the difference in recovery percentages of nectar-SPE and citric acid-added nectar-SPE samples were found to be not statistically significant.

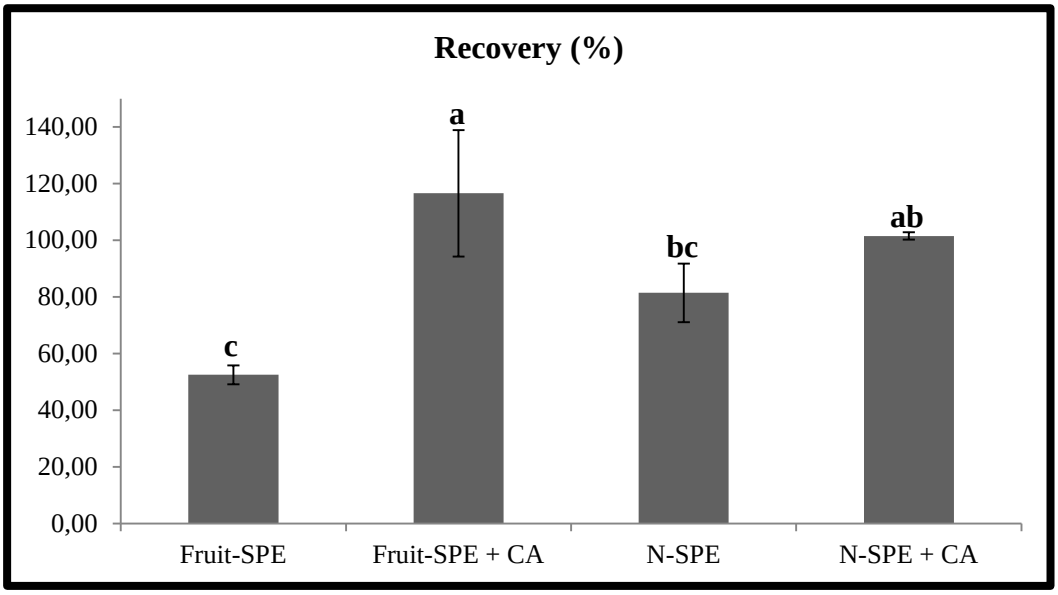

Figure 4.17 : Anthocyanin recovery percentages (for SPE samples) at the apical side of cell monolayers during 6 h of incubation calculated as (C3GR concentrations at the apical side after 6 h of incubation) / (C3GR concentrations at the apical side at 0 h) x 100%. Values are the means ± SD (n = 3). Differences between samples those do not share a letter given on the bars are statistically significant ($p < 0.05$). The terms represent: Fruit-SPE = solid phase extracted fruit sample (pH = 8); Fruit-SPE + CA = solid phase extracted fruit with citric acid (pH = 4); N-SPE = solid phase extracted nectar (pH = 8); N-SPE + CA = solid phase extracted nectar with citric acid (pH = 4).

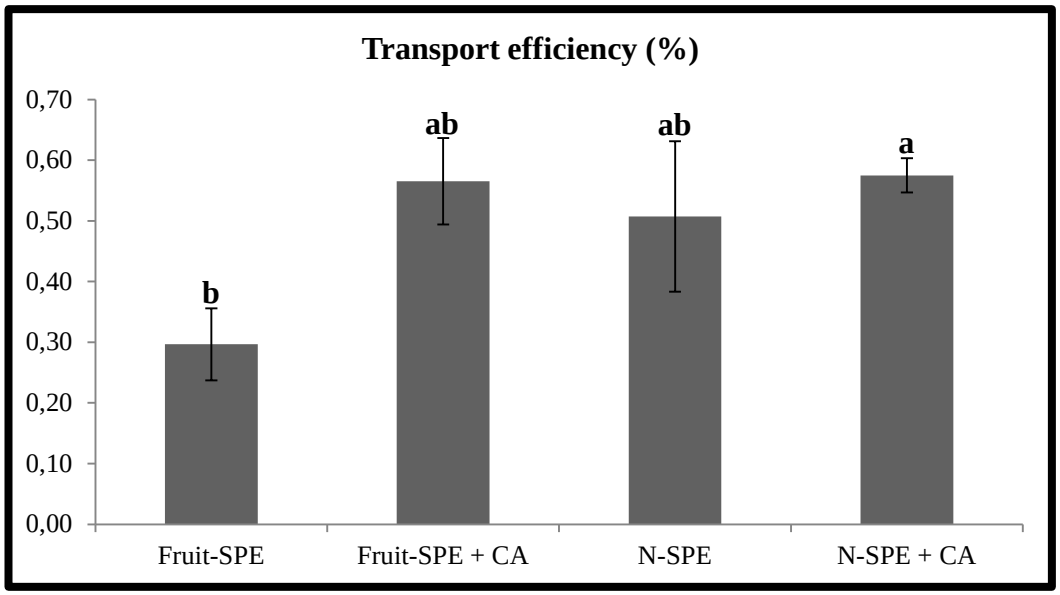

Figure 4.18 : Anthocyanin transport efficiency percentages (for SPE samples) to the basal side of cell monolayers during 6 h of incubation, calculated as (C3GR concentrations at the basolateral side after 6 h of incubation) / (C3GR concentrations at the apical side at 0 h) x 100%. Values are the means ± SD (n = 3). Differences between samples those do not share a letter given on the bars are statistically significant ($p < 0.05$). The terms represent: Fruit-SPE = solid phase extracted fruit sample (pH = 8); Fruit-SPE + CA = solid phase extracted fruit with citric acid (pH = 4); N-SPE = solid phase extracted nectar (pH = 8); N-SPE + CA = solid phase extracted nectar with citric acid (pH = 4).

Table 4.17 : Recovery (%) on apical side of Caco-2 cell monolayer and transport efficiency (%) of anthocyanins from apical to basolateral side of Caco-2 cell monolayer in fruit-SPE and nectar-SPE samples.

Sample & pH	Recovery (%)[a]	Transport efficiency (%)[b]
Fruit-SPE (pH 8)	52.5 ± 3.4 c	0.30 ± 0.06 b
Fruit-SPE + CA (pH 4)	116.6 ± 22.4 a	0.57 ± 0.07 ab
Nectar-SPE (pH 8)	81.4 ± 10.4 bc	0.51 ± 0.12 ab
Nectar-SPE + CA (pH 4)	101.5 ± 1.3 ab	0.58 ± 0.03 a

[a] Recovery percentages were calculated based on (C3GR concentrations at the apical side after 6 h of incubation) / (C3GR concentrations at the apical side at 0 h) x 100%. Values represent the averages of triplicates ± standard deviation. Differences between samples those do not share a letter given in the columns are statistically significant ($p < 0.05$).

[b] Transport efficiency percentages were calculated based on (C3GR concentrations at the basolateral side after 6 h of incubation) / (C3GR concentrations at the apical side at 0 h) x 100%. Values represent the averages of triplicates ± standard deviation. Differences between samples those do not share a letter given in the columns are statistically significant ($p < 0.05$).

The trend of change in transport efficiency percentages of the SPE samples analysed were determined to be consistent with the trend of change observed for recovery percentages of the same samples (Figure 4.18). The higher recovery on the apical side of cells during incubation provided a higher transport of anthocyanins through cell monolayers to the basal side. Fruit-SPE sample was found to have the lowest transport efficiency (%) of anthocyanins, whereas citric acid-added nectar-SPE sample had the highest transport efficiency (%) values (Table 4.17). The transport efficiencies of anthocyanins in fruit-SPE and nectar-SPE samples were not

statistically different ($p < 0.05$). Although citric acid addition to the fruit-SPE sample resulted in an approximately 2-fold increase in transport, the transport efficiency (%) values obtained for citric acid-added fruit-SPE was not statistically different in comparison to its non-citric acid-added counterpart. The citric acid inclusion in the nectar-SPE sample did also result in non-significant changes in transport. Citric acid inclusion in SPE samples did not lead to substantial increases in transport, which was a distinct observation when compared to the results obtained for inclusion of citric acid into WF sample.

The anthocyanins in fruit-SPE and nectar-SPE samples were found to have lower efficiencies in transport through the Caco-2 cell monolayers in comparison to the transport efficiencies obtained for the whole fruit and whole nectar samples (≈ 2-fold and ≈ 5-fold lower, respectively). While citric acid addition to the WF resulted in an approximately 5-fold increase in transport efficiency (%), citric acid-added fruit-SPE sample had an approximately 2-fold higher transport efficiency (%) than the fruit-SPE sample.

The small intestine is responsible for several functions including uptake of food components, and the production and modification of the bioactive compounds. On the other hand, it has also been reported that intestinal functions can be regulated by food components and the response can be mimicked with *in vitro* cell lines (Shimizu, 2010). To analyse food-intestine interactions at the cellular and molecular levels, cell lines derived from human intestinal tissues, including colon cancer tissues, can be introduced to various cell-based assays. Caco-2 cell lines from colon cancer, when cultured for 2-3 weeks on semipermeable membranes, spontaneously differentiates and expresses many small intestinal functions, e.g. tight-junctions between the cells, microvillus structures on the apical cell surface, expression of several brush-border digestive enzymes , transporters, and receptors (Hashimoto and Shimizu, 1993; Shimizu, 2010). In this present Caco-2 cell assays, we have studied the transport of sour cherry anthocyanins through intestinal epithelium, simulated using an *in vitro* Caco-2 cell model. The transport experiments were carried out with different forms of sour cherry material, including whole fruit (WF), whole nectar (WN) samples, and purified flavonoids (including anthocyanins) from both of these fruit and nectar samples (fruit-SPE and nectar-SPE).

Our results have revealed the transport of the major sour cherry anthocyanin, cyanidin-3-(2^G-glucosylrutinoside) (C3GR), through the Caco-2 cell monolayers in its intact glycosidic form, agreeing with the results of McGhie *et al.* (2003). They already reported the unmetabolized excretion of this specific anthocyanin compound, extracted from boysenberry fruit, by both rats and humans after ingestion. There are also several other *in vivo* and *in vitro* studies those have readily determined the intact absorption/transport of different individual anthocyanin compounds from different plant sources (Bub *et al.*, 2001; Cao and Prior, 1999; Cao *et al.*, 2001; Matsumoto *et al.*, 2001; Miyazawa *et al.*, 1999; Tsuda *et al.*, 1999; Wu *et al.*, 2002; Yi *et al.*, 2006). On the other hand, more recent *in vivo* studies on anthocyanin absorption and metabolism reported the occurrence of glucuronide and methylated conjugates of anthocyanins, besides the original glycosidic form, in plasma and urine, as the two major types of metabolites (Ichiyanagi *et al.*, 2004; Ichiyanagi *et al.*, 2005a; Ichiyanagi *et al.*, 2005b; Wu *et al.*, 2004).

The transport efficiency of sour cherry anthocyanins (represented in terms of cyanidin-3-(2^G-glucosylrutinoside) (C3GR)) were determined to range between 0.5 – 4% for whole materials; and 0.3 – 0.6% for their purified SPE samples. Yi *et al.* (2006), who studied the transport of seven monoglycoside anthocyanins (delphinidin-3-*O*-*β*-glucopyranoside (Dp-glc), cyanidin-3-*O*-*β*-galactopyranoside (Cy-gal), cyanidin-3-*O*-*β*-glucopyranoside (Cy-glc), petunidin-3-*O*-*β*-glucopyranoside (Pt-glc), peonidin-3-*O*-*β*-galactopyranoside (Pn-gal), peonidin-3-*O*-*β*-glucopyranoside (Pn-glc), malvidin-3-*O*-*β*-glucopyranoside (Mv-glc)) extracted from blueberries using Caco-2 cell monolayers, reported the transport efficiency of anthocyanins to be averaged between 3 – 4% (except for delphinidin-3-*O*-*β*-glucopyranoside, less than 1%). The values obtained for C3GR in sour cherry fruit and nectar samples subjected to solid phase extraction were much lower (0.3 – 0.6%) compared to these values. On the other hand, for whole sour cherry samples, the transport efficiency values (2 – 4%) were comparable to the results of *Yi et al.* (2006), except for WF (0.5%). In another *in vitro* Caco-2 cell model study, Faria *et al.* (2009) tested the transport of anthocyanins, extracted from red grape skins (rich in malvidin-3-glucoside), through Caco-2 cell monolayers and reported the transport efficiency (%) to be ranged between 0.14 – 1.77% over an incubation time of 15 min to 120 min. Distinctively, Steinert *et al.* (2008) couldn't observe any anthocyanin

transport to the basolateral side of Caco-2 cell monolayers in their study with black currant anthocyanin extracts. These considerable differences recorded for the bioavailability of different individual anthocyanins derived from various fruits can be affected by several factors such as the nature of the phenolic aglycone and the sugar conjugates attached (McGhie *et al.*, 2003; Wu *et al.*, 2004; Wu *et al.*, 2005). Free hydroxyl groups in the aglycone structure has been reported to have the potential of hindering transport in Caco-2 cell monolayers (Tammela *et al.*, 2004) which can be the result of the formation of hydrogen-bonds with these groups and the polar groups of lipid molecules in membranes. This was linked to longer retention delays of polyhydroxylated flavonoids in membranes (Ollila *et al.*, 2002) and lower transport efficiencies through cell monolayers (Yi *et al.*, 2006). Wu *et al.* (2004) observed a much higher total urinary excretion of pelargonidin-based anthocyanins (having four hydroxyl groups) than the cyanidin-based ones (having five hydroxyl groups), while McGhie *et al.* (2003) reported lower relative concentrations of delphinidin-based anthocyanins (having six hydroxyl groups) than those of malvidin-based anthocyanins (having four hydroxyl groups) in the urine of rats and humans. In agreement with McGhie *et al.* (2003), Yi *et al.* (2006) also showed a lower transport/absorption efficiency of Dp-glc, through Caco-2 cell monolayers, than that of Mv-glc. In addition, Dp has no hydrophobic methoxyl (-OCH_3) group, while Mv has two, resulting in a greater hydrophobic nature, facilitating increased partitioning into cells and tissue. These data provided further evidence that hydrophilic and hydrophobic groups in the aglycone structure are important for the bioavailability of anthocyanins (Yi *et al.*, 2006). Besides the aglycone structure, sugar moieties those attached to the anthocyanidin aglycone have also been pointed out to play a substantial role in terms of absorption/transport of these compounds (Cooney *et al.*, 2004; Ichiyanagi *et al.*, 2006; McGhie *et al.*, 2003; Wu *et al.*, 2005). McGhie *et al.* (2003) investigated the effect the sugar conjugate included in the anthocyanin structure, on absorption and excretion, with both animal and human experiments. Their results, those were observed for boysenberry anthocyanins, revealed that cyanidin-3-*O*-2^G-glucosylrutinoside (C3GR), cyanidin-3-*O*-rutinoside, and cyanidin-3-*O*-sophoroside all behaved similarly with respect to absorption and excretion; while, in contrast to this observation, the relative concentration of cyanidin 3-*O*-glucoside was less in the urine as compared with the original concentrations in the berry extract. Additionally, for black currant anthocyanins, the bioavailability of

delphinidin-3-*O*-glucoside and cyanidin-3-*O*-glucoside was determined to be apparently lower as compared to those of delphinidin-3-*O*-rutinoside and cyanidin-3-*O*-rutinoside. Furthermore, Ichiyinagi *et al.* (2006) carried out an *in vivo* (using rats) experiment on anthocyanins from bilberry extracts, in which they compared the bioavailability values between the different types of sugars attached to the same aglycon, and showed that galactosides had higher values than glucosides. Some possible explanations for this phenomena are highlighted as: a higher antioxidant capacity of cyanidin-3-*O*-glucoside may lead to a higher oxidation rate *in vivo* (McGhie *et al.*, 2003), and/or specific transport mechanisms of various cells for glucose-containing compounds (*e.g.* sodium dependent glucose transporter SGLT1) may provide a more rapid transport of this type of anthocyanins than others, across the apical membrane of enterocytes (Gee *et al.*, 1998; Walgren *et al.*, 2000). However, in contrast with the results of McGhie *et al.* (2003) and Ichiyinagi *et al.* (2006), Yi *et al.* (2006) reported a significantly higher transport efficiency of cyanidin-3-*O*-*β*-glucopyranoside (Cy-glc) and peonidin-3-*O*-*β*-glucopyranoside (Pn-glc) than cyanidin-3-*O*-*β*-galactopyranoside (Cy-gal) and peonidin-3-*O*-*β*-galactopyranoside (Pn-gal), respectively. These data can be a clear evidence of that the nature of the sugar conjugate may significantly influence the differences in bioavailability between anthocyanins, although the mechanisms are yet far from clear.

The transport efficiencies (%) of anthocyanins obtained for all sour cherry samples tested in this study, as well as in other similar studies, performed using *in vitro* Caco-2 cell model for assessing the bioavailability of other fruit anthocyanins (Faria *et al.*, 2009; Steinert *et al.*, 2008; Yi *et al.*, 2006), were relatively low comparing to the results obtained for some other flavonoids (Spencer, 2003; Sesink *et al.*, 2001). Additionally, the results of various *in vivo* animal and human studies also showed the low bioavailability of anthocyanins, determined to be ranging between 0.005 – 0.190% urinary excretion in animals, and 0.003 – 5.10% urinary excretion in humans, respectively (reviewed in McGhie and Walton, 2007). Although anthocyanins are a group in flavonoids, their biochemistry has been reported to be more complex than other flavonoid groups (McGhie and Walton, 2007). The relative low bioavailability (reported mostly to be less than 0.1% of the ingested dose recovered in urine) of anthocyanins compared to other flavonoid groups can be

influenced by various factors, such as poor lipophilic properties of anthocyanins (Yi *et al.*, 2006). Anthocyanins are highly reactive and rapidly lost in the presence of oxygen, light, various enzymes, and as a result of high temperature. In addition, pH has been known to have a significant effect on anthocyanin stability (Jackman *et al.*, 1987). Changes in pH can lead to reversible transformations in anthocyanin structure. Anthocyanins generally exist in the form of flavylium cation in foods, as the most abundant molecular form at low pH values. However, as anthocyanins are exposed to different pH values (high pH) during passage through the gastrointestinal tract (GIT), the other forms (*e.g.* quinonoidal bases, hemiketals, and chalcones) will dominate (McGhie and Walton, 2007). More importantly, anthocyanins are unstable at pH 7.0 and reported to degrade into chalcone and benzoic acid derivatives at pH 7.0 and 37ºC (Kang *et al.*, 2003). Yi *et al.* (2006) evaluated, in their study, whether the change in concentration of an anthocyanin compound in apical side during incubation under cell culture conditions (neutral pH and 37ºC) (determined in the absence of cells) could be related to the change in concentration of this compound in basal side. Due to their results, they concluded that a higher loss of a compound in the apical solution could explain to some degree its remarkable lower transport efficiency to the basolateral compartment. This result is in good agreement with our results. In this present study, sour cherry samples that provided higher recovery of anthocyanins on the apical side of cell monolayers, were also found to have higher transport efficiencies to the basolateral side of cell monolayers in 6 h incubation (except for the WF + SUC sample). Due to these similar observations in related studies, one conclusion in our work could be that the transport efficiency of anthocyanins through the cell monolayers could partly be related to the concentration in the apical solution during incubation. In relation to this conclusion, citric acid could be pointed out as an important driving force in transport of anthocyanins with providing samples with lower pH values (pH $\approx$ 4) (thus, a higher stability of anthocyanins) and higher recovery percentages on apical side, and so higher transport efficiency percentages to the basal side. The necessity of using pH-gradient condition was already suggested by Yi *et al.* (2006). This was supported by the fact that even though the cellular interstice and blood has the pH value of around pH 7.4, the pH ranges from 5.0 to 6.5 in the upper gastrointestinal tract under fasting conditions, and additionally, the pH of the acidic microclimate just above the epithelial cell layer has been reported to be between 5.8 and 6.3 (Lucas, 1983).

A distinctive result observed in this study was that the WF + SUC sample revealed an exceptional behaviour with the lowest concentration of anthocyanins in the apical side over 6 h incubation time (which was linked to the high pH compared to the citric acid including samples; pH ≈ 6), but significantly higher transport efficiency (%) value than the WF sample which also had the same recovery (%) and the same pH value. Therefore, the second conclusion could be that the sucrose inclusion had a significant positive contribution to the transport through Caco-2 cell monolayers. However, Mulleder *et al.* (2002), who investigated and compared the urinary excretion of cyanidin-3-glucoside in humans after consumption of elderberry anthocyanins with and without simultaneous ingestion of sucrose, found out that the ingestion of sucrose led to a reduced excretion of cyanidin-3-glucoside. In addition, quercetin-glucoside uptake in Caco-2 cells was reported to be inhibited by adding glucose (Walgren *et al.*, 2000). Quercetin glycosides were already shown to be absorbed in the small intestine by interaction with the intestinal glucose transporter (SGLT-1) (Gee *et al.*, 1998; Hollman *et al.*, 1995). In addition, quercetin and anthocyanin aglycones share a similar basic flavonoid structure. These data were in accordance with the hypothesis indicating the involvement of the glucose transport receptors (*e.g.* SGLT-1) in anthocyanin absorption. Since anthocyanins include a sugar conjugate attached, especially a glucose residue, glucose transporters were pointed out as the candidates for anthocyanin absorption/transport (Milbury *et al.*, 2002). In contrast to this hypothesis, a recent study which investigated the transport of various flavonoid glycosides (quercetin, luteolin, apigenin, naringenin, pelargonidin, daidzein, and genistein), revealed that the compounds, those were tried out, were not transported by human SGLT-1 when expressed in *Xenupus laevis* oocytes (Kottra and Daniel, 2007). However, the same study also pointed out that not only glycosylated flavonoids but also a few nonglycosylated counterparts showed a structure-related tendency for effective inhibiton of SGLT-1. Walton *et al.* (2006) also could not find any relation between the presence of glucose and the absorption of cyanidin-3-glucoside by jejunum tissue,suggesting that their mechanism of absorption do not follow the same pathway, but in contrast, they determined that cyanidin-3-glucoside absorption was significantly reduced in the presence of quercetin-3-glucoside. On the other hand, Walgren *et al.* (2000) determined in their study, using the Caco-2 cell model, that although quercetin glycosides were absorbed across the apical membrane via SGLT-1, these compounds only accumulated within

the cell, which was linked to the no further translocation across the basolateral membrane. In relation to the latter finding, in our case, the contradictory results obtained for WF + SUC sample, with the low recovery on the apical side but the high transport efficiency to the basal side, may reveal the fact that anthocyanin disappearance from the apical side for this sample was not due to the high degradation caused by experimental conditions but could be rather related to the physiological actions of the cells (defined as "absorption efficiency"). A similar observation of anthocyanin disappearance in transport chambers of Caco-2 cells was also explained by Steinart *et al.* (2008) with this cell absorption phenomena. Moreover, sucrose could have had a preserving and stabilizing effect on anthocyanins (Wrolstad *et al.*, 1990) and could have provided both high transport through the cell monolayers, in addition to cell accumulation.

Our results also demonstrated that anthocyanins in WN sample were transported more efficiently than anthocyanins in WF, but the transport of fruit anthocyanins was "up-graded" to the similar values as in nectar, by adding citric acid. This higher transport obtained by the citric acid inclusion could be related to the higher recovery of anthocyanins over incubation time with cell monolayers. On the other hand, the transport efficiency values of anthocyanins in fruit-SPE and nectar-SPE samples were determined to be much lower than the values obtained for WF and WN samples (≈ 2-fold and ≈ 5-fold lower, respectively). This could be explained by the fact that the fruit matrix helped to the transport of anthocyanins. In addition, while this matrix effect was conserved in nectar processing, it was not conserved during SPE procedure. McDougall *et al.* (2005a) stated the positive effect of food matrix on anthocyanin bioavailability. In their study, they evaluated the bioavailability of raspberry anthocyanins, in the form of extracts, using *in vitro* digestion system, and reported that *in vitro* exposure of anthocyanins to differences in pH, oxygen, and temperature significantly reduced the anthocyanin availability, but codigestion with common foodstuffs (*e.g.* bread, ice cream, breakfast cereal) provided the protection of the labile anthocyanins and the level of serum bioavailability did not markedly decrease. Felgines *et al.* (2003) investigated the bioavailability of strawberry anthocyanins, consumed in the form of whole fruits, and suggested that anthocyanin absorption was probably affected by the food matrix. These results may indicate the possible binding of polyphenols to food matrices during digestion, and that can

protect the more labile anthocyanins from degradation. Tthe fruit matrix could contain factors (e.g. proteins, or salts) which help transport, and which are were removed by SPE. An alternative explanation for reduced transport over Caco-2 cells could be that the SPE process itself introduces factors such as soluble resin contaminants which may bind phenolics and thus hinder proper transport.However, the matrix components involved and the factors laying behind this process require further attention.

5. CONCLUSIONS

The majority of the health-associated phenolic compounds present in sour cherry fruit is conserved in nectar, as revealed by an extensive phytochemical analyses during the entire industrial-scale nectar processing. In particular, anthocyanins are well conserved in sour cherry nectar, compared to the other fruit juices in which these compounds often end up in the waste fractions. During processing sour cherry fruit into nectar, the repeated press-cake extraction steps (MPEX-I, MPEX-II, and MPEX-III) are considered to be the most significant steps facilitating high recovery of fruit antioxidants into the juice fraction. Other factors favoring the greater levels of anthocyanins in the processed nectar can be listed as the relatively higher stability of sour cherry anthocyanins (composed of cyanidin derivatives) than those present in other anthocyanin-rich fruits (i.e., delphinidins) and the localization of these pigments in the fruit tissue (with relatively higher contents in the flesh). In addition, the removal of processing wastes (stalks, seeds, and press cake), which are poor in anthocyanins, do not lead to anthocyanin losses during processing. On the other hand, the reductions (2 to 4-fold) obtained in the amounts of sour cherry phytochemicals and the antioxidant capacity (on dry-weight basis) during processing of concentrated juice to nectar are related with the sucrose syrup addition, that makes more than 50% of the dry-matter of nectar comprised from sucrose.

Sour cherry is an anthocyanin-rich fruit, having cyanidin-3-(2^{G}-glucosylrutinoside) as the major anthocyanin component, which comprises around 75% of total anthocyanins. The other anthocyanin compounds are cyanidin-3-rutinoside, cyanidin-3-sophoroside, and cyanidin-3-glucoside. The sour cherry fruit is also a substantial source of short-chain procyanidins (DP values <3), predominantly epicatechin. Differentiating from anthocyanins, procyanidins are in higher amounts in waste materials of nectar processing, including the press-cake. This can most likely be linked to the strong affinity of these compounds to the cell-wall polysaccharides. Loss of some procyanidins with processing wastes results in lower DP values in final

nectar, showing that the wastes contain longer-chain procyanidins. This may also provide higher procyanidin bioavailability in nectar than in fruit, since low-molecular-weight oligomers (polymerization degree up to 3) are known to be absorbed to a greater extent than polymeric procyanidins.

Untargeted metabolomics approach, using LC-QTOF-MS, enables monitoring the sour cherry metabolite profile throughout the nectar processing. There are 38 compounds identified in sour cherry samples based on the MS data, including four anthocyanins, five flavan-3-ols, eight phenylpropanoids, eight flavonols, four flavanones, four organic acids, and two carbohydrates. The stalk and the press-cake waste materials differentiate significantly from the other samples in principal component analysis (PCA) which is performed for the comparison and the visualization of the LC-MS data. Anthocyanins and phenolic esters such as neochlorogenic acid are under-represented in these processing wastes, relative to the fruit, meaning that they are retained in the juice fraction and not lost in the waste material. Remarkably, only 11 out of the total 193 detected compounds observed in the sour cherry fruit have levels which are significantly different (decreased or increased) in the final nectar. These are dominated by polar compounds, including the nectar ingredients, sucrose and citric acid, as the predominant ones. Noteworthy, anthocyanins are not included in this list of key differentiators between fruit and nectar, indicating the well recovery of this class of compounds into the final nectar.

Sour cherry variety (“Kütahya” local variety) investigated in this study is poor in vitamin C content (contains only DHAA). On the other hand, there is a polar antioxidant in the extracts that contributes to the on-line antioxidant activity. This antioxidant compound is identified as a stable conjugated form of ascorbic acid, ascorbic acid-glucoside, through further LC-QTOF-MS analysis. This is the first report, to our knowledge, to reveal the existence of this (rarely existing) metabolite in a sour cherry variety.

In conclusion, the most remarkable result of those chemical assays is that the sour cherry juice is differentiating from other fruit juices by preservation or high recovery of antioxidants, predominantly anthocyanins, in the juice fraction. The repeated press-cake extractions applied during processing are most likely the major factors contributing to the high recovery values. In the other fruit juice processes, in which this additional press-cake extraction procedure is not included, substantial amounts

of fruit phytochemicals are removed with the waste. In addition, a broader overview of sour cherry metabolite profile is achieved by the metabolomics analysis, through which the key differentiator metabolites between the starting fruit material and the final nectar product are elucidated in a detailed way, as well as new metabolites are identified. This will strongly contribute to the limited data on the bioactive components present in sour cherry and its products.

The cell-based *in vitro* bioavailability assays points out that sour cherry anthocyanins are transported through Caco-2 cell monolayers in their intact glycosidic form. Citric acid inclusion in food matrix during nectar processing seems to be an important factor, which increases the transport of anthocyanins to the basolateral side of the cell monolayers. This is linked to the higher concentrations observed at the apical side during incubation for citric acid including samples, due to the lower pH, resulting in more constant transport efficiencies. Additionally, sucrose inclusion also contributes positively to the *in vitro* bioavailability of sour cherry anthocyanins, providing higher transport efficiencies to the basolateral compartment. This latter data needs further research on the role of glucose transport receptors (*e.g.* SGLT-1) in anthocyanin absorption. Additionally, the lower transport efficiencies obtained in the solid phase extracts of fruit and nectar samples indicates the importance of food matrix in enhanced bioavailability.

For the cell-based assays, it should be considered that these results are all obtained based on *in vitro* studies. Using a Caco-2 cell model, we are able to show that sour cherry anthocyanins can be transported across cell monolayers. However, it is still necessary to further investigate the presence of other transport mechanisms and the influence of various factors, such as food matrix, processing, etc., in bioavailability studies. Moreover, it should be taken into consideration that the anthocyanin absorption and bioavailability may depend on the models used (e.g. cell monolayers, animal models, human trials). Thus, the extrapolation of the observed *in vitro* bioavailability results to the complex *in vivo* conditions in the human gut is needed. On the other hand, anthocyanin bioavailability and absorption is mostly evaluated with the detection methods based on the measurements of the red flavylium cationic form by HPLC analysis. More selective and sensitive methods should be developed to further identify the alternative molecular forms of anthocyanins (quinoniodal bases, hemiketals, and chalcones). It is suggested that further additional approaches

are of importance in order to confirm our results and to elucidate the missing details about anthocyanin bioavailability issue.

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APPENDICES

APPENDIX A: Calibration curves

APPENDIX B: ANOVA tables

APPENDIX A : CALIBRATION CURVES

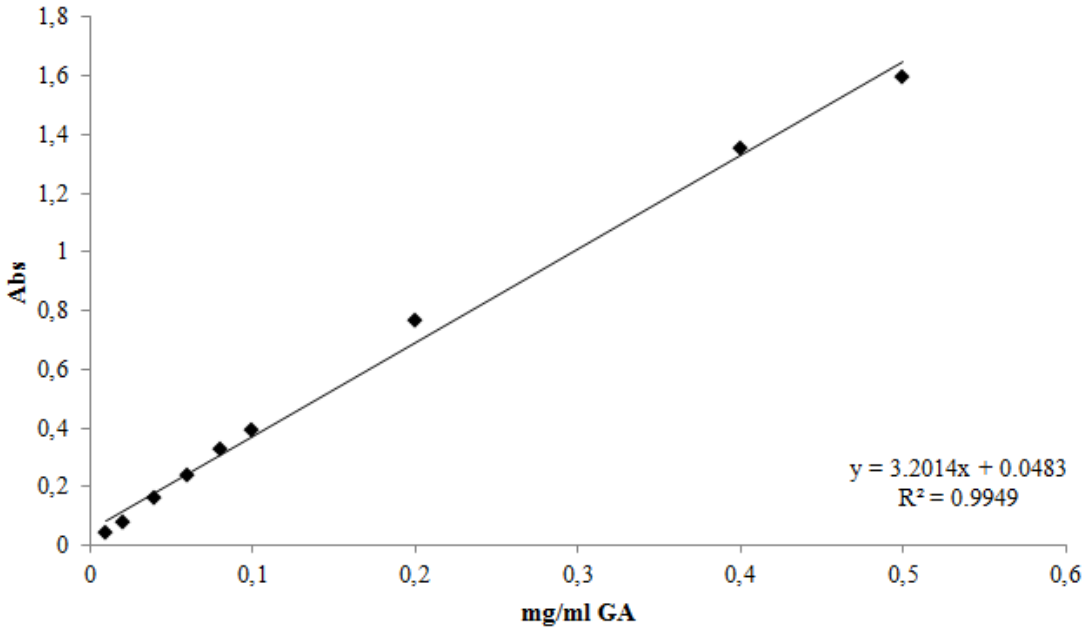

Figure A.1 : Calibration curve for total phenolics (with 900 µl water dilution).

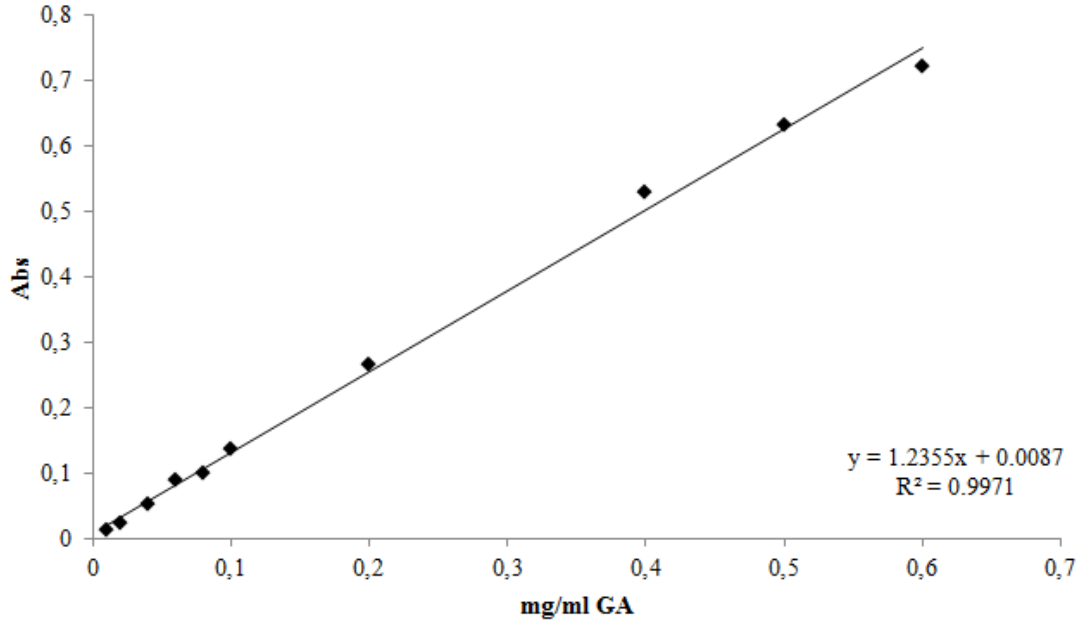

Figure A.2 : Calibration curve for total phenolics (with 5000 µl water dilution).

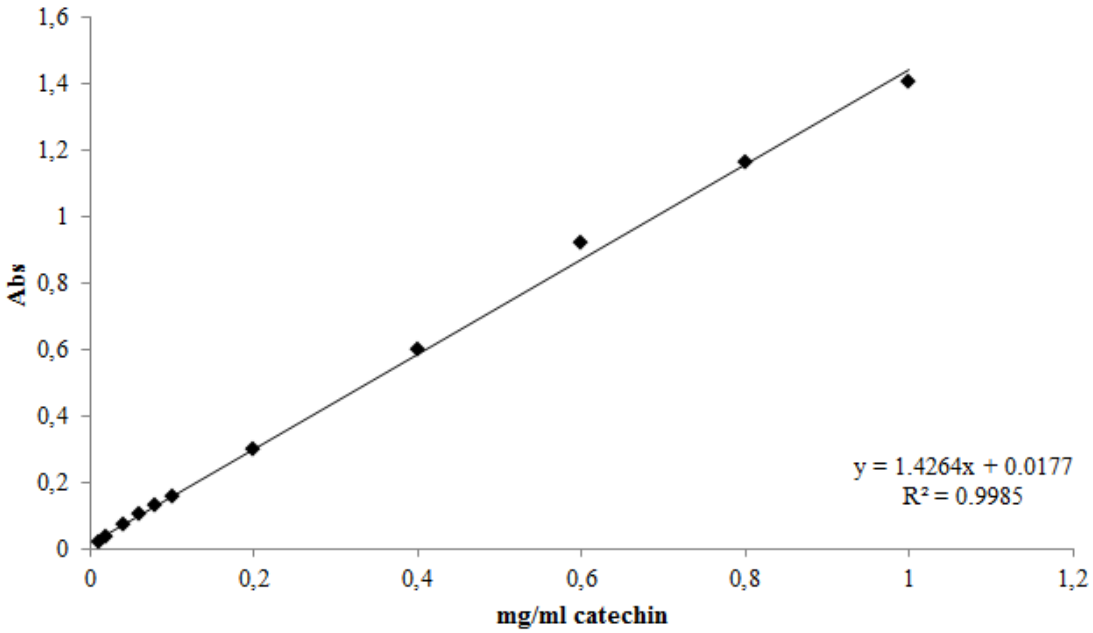

Figure A.3 : Calibration curve for total flavonoids.

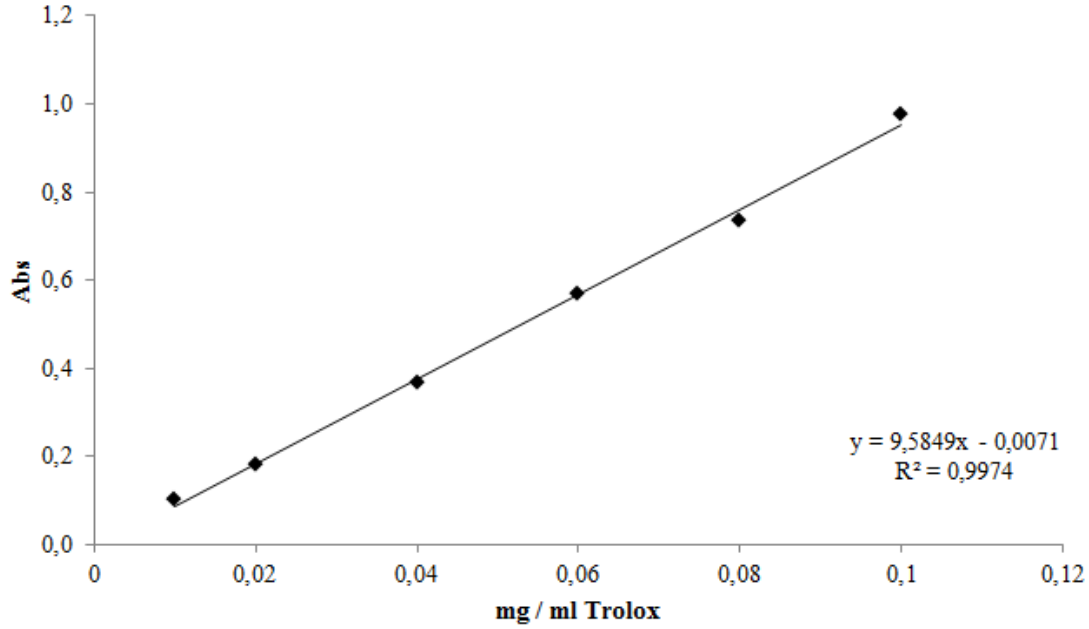

Figure A.4 : Calibration curve for ABTS method.

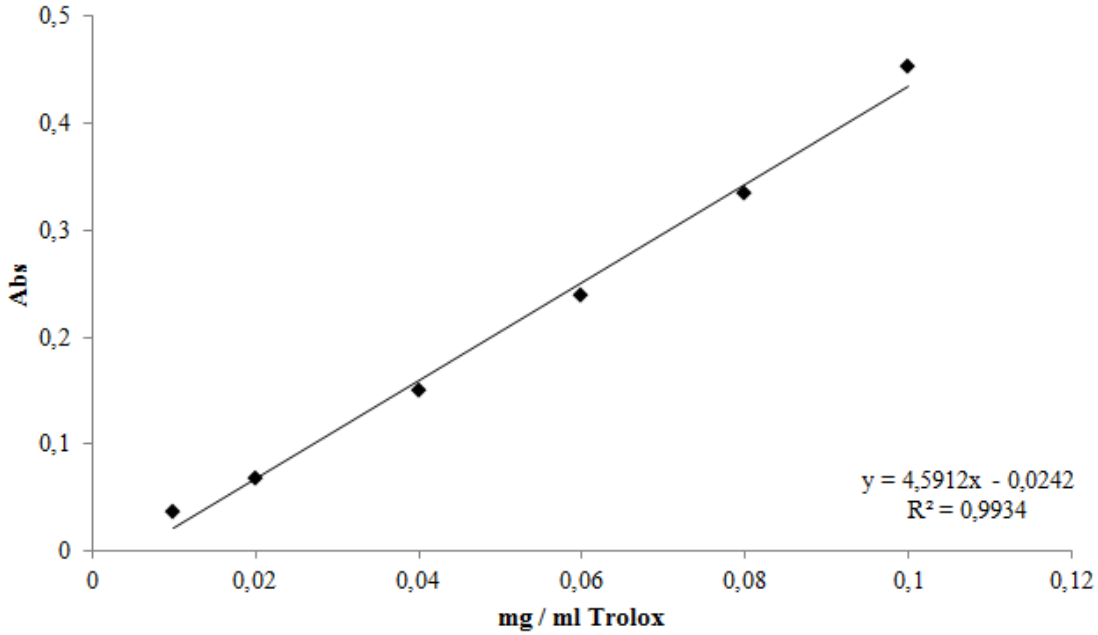

Figure A.5 : Calibration curve for DPPH method.

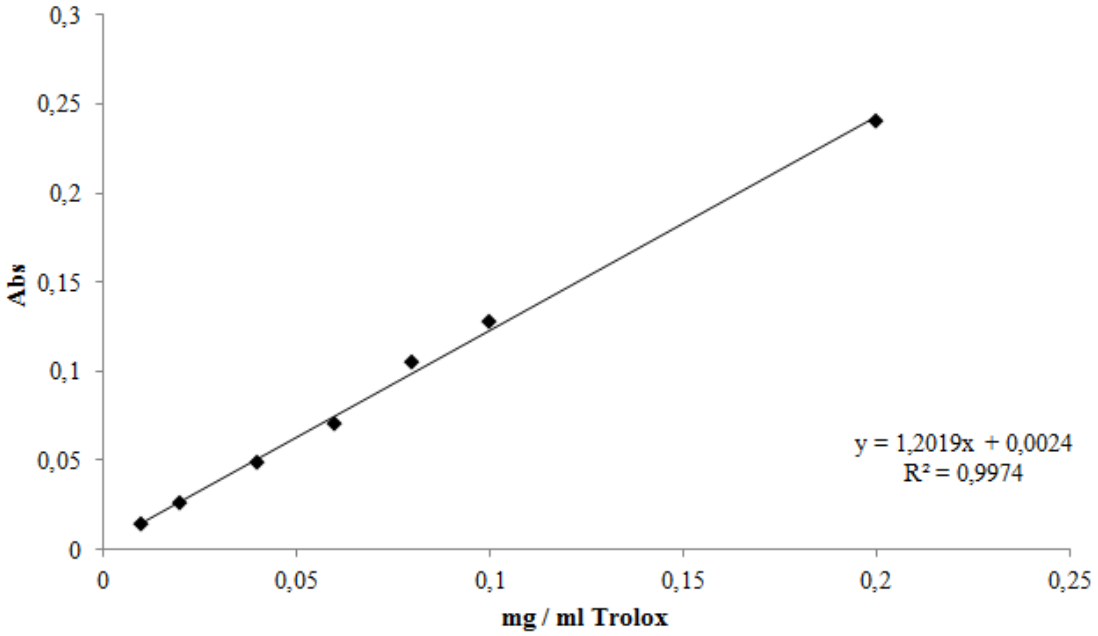

Figure A.6 : Calibration curve for CUPRAC method.

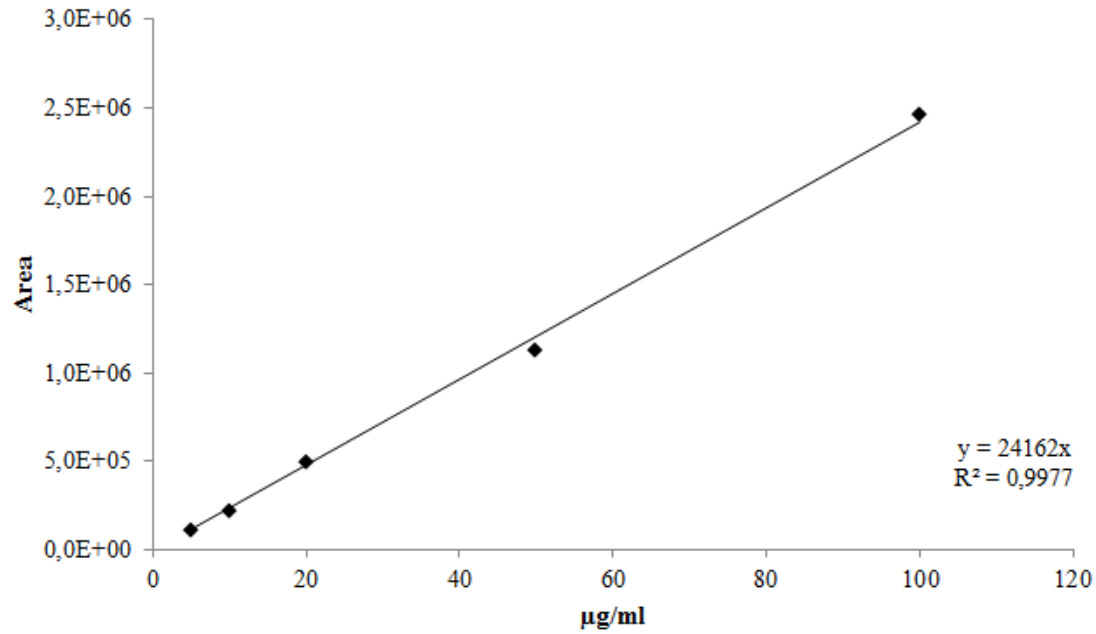

Figure A.7 : Calibration curve for cyanidin-3-glucoside.

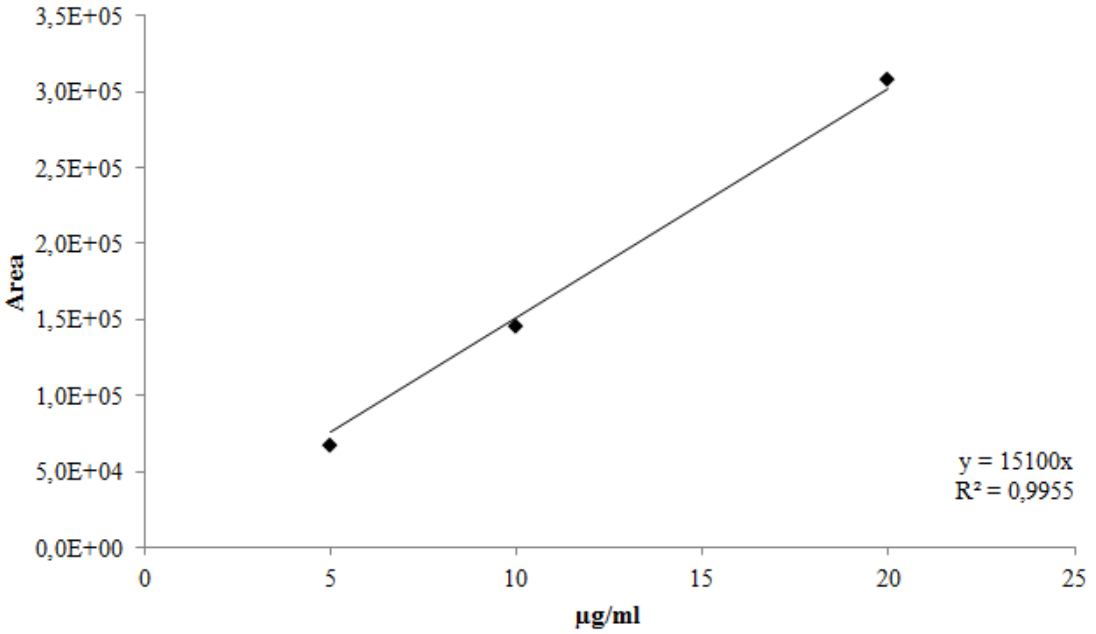

Figure A.8 : Calibration curve for chlorogenic acid.

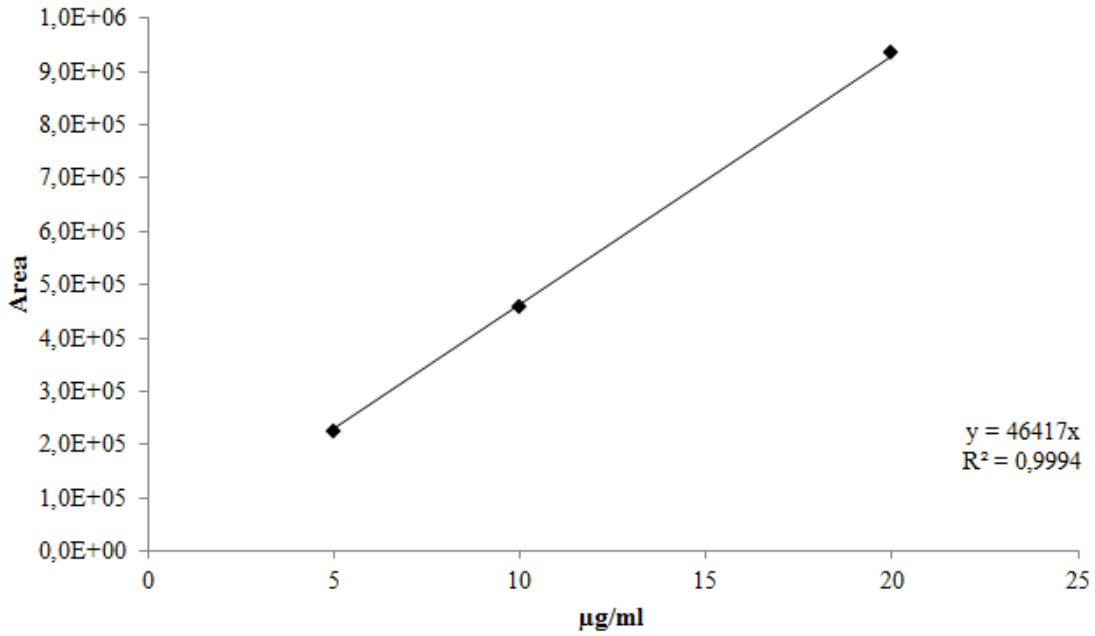

Figure A.9 : Calibration curve for quercetin-3-glucoside.

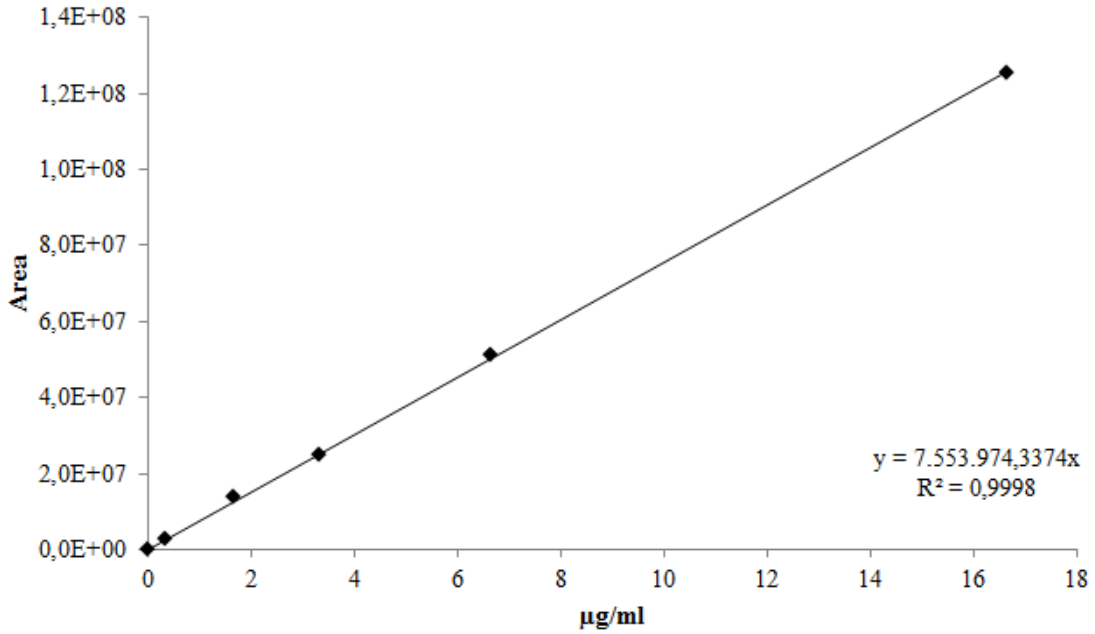

Figure A.10 : Calibration curve for (+)-catechin.

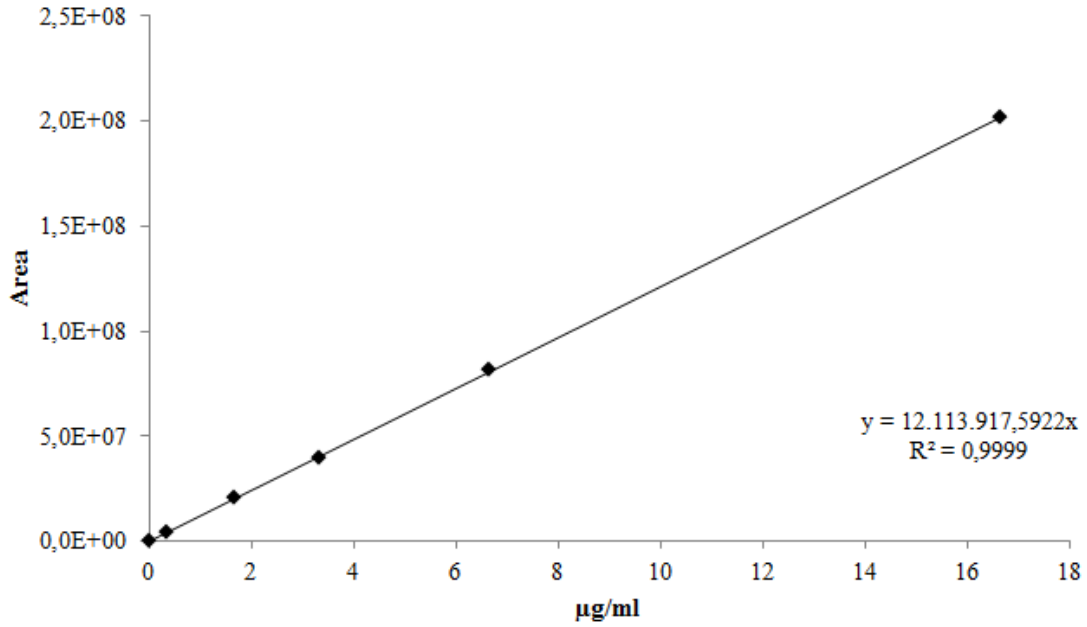

Figure A.11 : Calibration curve for (-)-epicatechin.

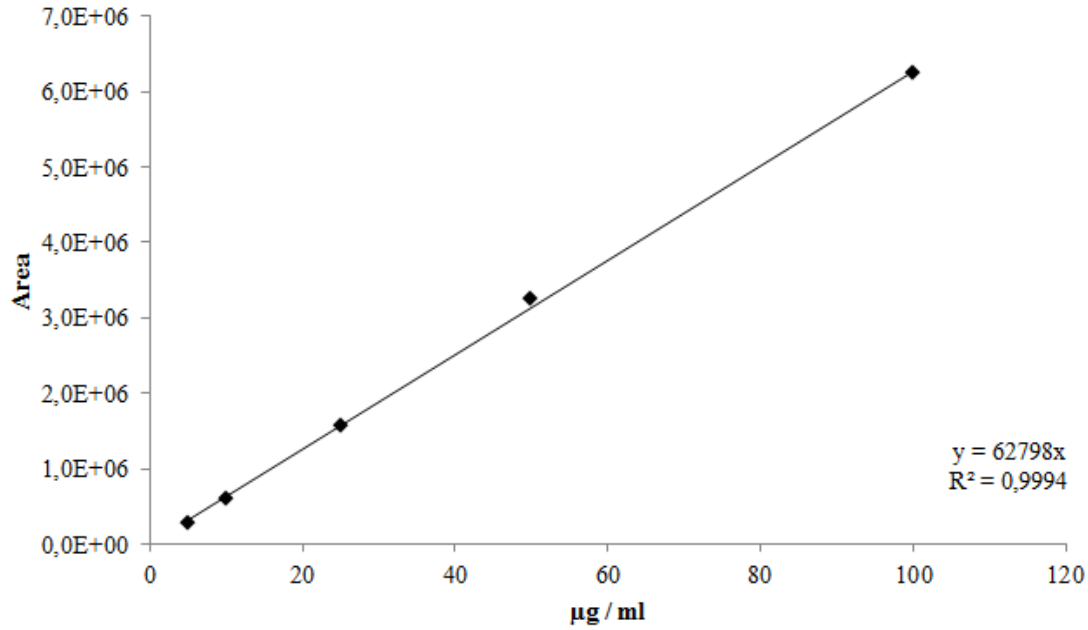

Figure A.12 : Calibration curve for ascorbic acid.

APPENDIX B : ANOVA TABLES

Table B.1 : Statistical analysis results for the methods applied to the sour cherry nectar processing samples.

		Sum of Squares	**df**	**Mean Square**	**F**	**P**
Moisture	Between Groups	31673.6	21	1508.3	112.6	0.000
	Within Groups	1138.4	85	13.4		
	Total	32811.9	106			
Total Phenolics	Between Groups	17514330	21	834016	17.4	0.000
	Within Groups	1584012	33	48000		
	Total	19098342	54			
Total Flavonoids	Between Groups	587700902	21	27985757	25.0	0.000
	Within Groups	38067313	34	1119627		
	Total	625768216	55			
Total Anthocyanins	Between Groups	748904	21	35662	17.8	0.000
	Within Groups	71968	36	1999		
	Total	820871	57			
ABTS	Between Groups	115326606	21	5491743	13.4	0.000
	Within Groups	15176639	37	410179		
	Total	130503245	58			
CUPRAC	Between Groups	395487043	21	18832716	14.3	0.000
	Within Groups	47586612	36	1321850		
	Total	443073655	57			
DPPH	Between Groups	60279502	21	2870452	14.5	0.000
	Within Groups	7111864	36	197552		
	Total	67391366	57			
Cyanidin-3-(2^G-glucosylrutinoside) (C3GR)	Between Groups	1898982	21	90428	50.1	0.000
	Within Groups	151768	84	1807		
	Total	2050751	105			
Cyanidin-3-rutinoside	Between Groups	404075	21	19242	25.7	0.000
	Within Groups	63679	85	749		
	Total	467754	106			
Cyanidin-3-sophoroside	Between Groups	4864.4	21	231.6	32.5	0.000
	Within Groups	599.2	84	7.1		
	Total	5463.6	105			
Cyanidin-3-glucoside	Between Groups	18087.4	21	861.3	24.7	0.000
	Within Groups	3004.6	86	34.9		
	Total	21092.0	107			
Total catechin	Between Groups	235813	21	11229	33.9	0.000
	Within Groups	27813	84	331		
	Total	263626	105			
Total epicatechin	Between Groups	8620554	21	410503	26.3	0.000
	Within Groups	1312612	84	15626		
	Total	9933166	105			
Degree of polymerization	Between Groups	10.5	21	0.5	28.4	0.000
	Within Groups	1.5	84	0.02		
	Total	12.0	105			
Neochlorogenic acid	Between Groups	2670710	21	127177	9.5	0.000
	Within Groups	1123788	84	13378		
	Total	3794499	105			
p-Coumaroylquinic acid	Between Groups	277630	21	13220	15.7	0.000
	Within Groups	70678	84	841		
	Total	348308	105			

Table B.1 (continued) : Statistical analysis results for the methods applied to the sour cherry nectar processing samples.

		Sum of Squares	df	Mean Square	F	P
Chlorogenic acid	Between Groups	142701.9	21	6795.3	18.2	0.000
	Within Groups	31340.1	84	373.1		
	Total	174042.1	105			
Rosmarinic acid	Between Groups	1398.2	21	66.6	34.5	0.000
	Within Groups	164.1	85	1.9		
	Total	1562.3	106			
Rutin	Between Groups	54519.3	21	2596.2	20.3	0.000
	Within Groups	10370.3	81	128.0		
	Total	64889.6	102			
Quercetin-3-glucoside	Between Groups	1629.1	21	77.6	68.9	0.000
	Within Groups	94.6	84	1.1		
	Total	1723.2	105			
Kaempferol-3-rutinoside	Between Groups	16064.7	21	765.0	59.5	0.000
	Within Groups	1055.0	82	12.9		
	Total	17119.7	103			
Ascorbic acid-glucoside	Between Groups	44.9	20	2.2	14.9	0.000
	Within Groups	12.2	81	0.2		
	Total	57.1	101			
Total Phenolics (*in vitro* GI digestion; fruit)	Between Groups	1122475	3	374158	67.3	0.000
	Within Groups	77896	14	5564		
	Total	1200370	17			
Total Flavonoids (*in vitro* GI digestion; fruit)	Between Groups	26161020	3	8720340	163.5	0.000
	Within Groups	746620	14	53330		
	Total	26907640	17			
Total Anthocyanins (*in vitro* GI digestion; fruit)	Between Groups	51074	3	17025	144.6	0.000
	Within Groups	1649	14	118		
	Total	52723	17			
ABTS (*in vitro* GI digestion; fruit)	Between Groups	133735188	3	44578396	205.6	0.000
	Within Groups	3036250	14	216875		
	Total	136771438	17			
DPPH (*in vitro* GI digestion; fruit)	Between Groups	1616135	3	538712	72.7	0.000
	Within Groups	103706	14	7408		
	Total	1719841	17			
CUPRAC (*in vitro* GI digestion; fruit)	Between Groups	31240434	3	10413478	58.3	0.000
	Within Groups	2498767	14	178483		
	Total	33739201	17			
Total Phenolics (*in vitro* GI digestion; nectar)	Between Groups	395895	3	131965	54.7	0.000
	Within Groups	33766	14	2412		
	Total	429661	17			
Total Flavonoids (*in vitro* GI digestion; nectar)	Between Groups	5290569	3	1763523	241.0	0.000
	Within Groups	102439	14	7317		
	Total	5393008	17			
Total Anthocyanins (*in vitro* GI digestion; nectar)	Between Groups	7762.3	3	2587.4	34.6	0.000
	Within Groups	1048.0	14	74.9		
	Total	8810.3	17			
ABTS (*in vitro* GI digestion; nectar)	Between Groups	13250966	3	4416989	56.8	0.000
	Within Groups	1088362	14	77740		
	Total	14339328	17			
DPPH (*in vitro* GI digestion; nectar)	Between Groups	1022066	3	340689	278.5	0.000
	Within Groups	17124	14	1223		
	Total	1039190	17			

Table B.1 (continued) : Statistical analysis results for the methods applied to the sour cherry nectar processing samples.

		Sum of Squares	df	Mean Square	F	P
CUPRAC (*in vitro* GI digestion; nectar)	Between Groups	7037897	3	2345966	71.9	0.000
	Within Groups	456390	14	32599		
	Total	7494287	17			
Cell; optimum medium; stability C3GR; fruit	Between Groups	4088.2	3	1362.7	143.0	0.000
	Within Groups	76.2	8	9.5		
	Total	4164.4	11			
Cell; optimum medium; stability C3GR; nectar	Between Groups	2410.1	3	803.4	75.6	0.000
	Within Groups	85.0	8	10.6		
	Total	2495.1	11			
HBSS; TEER	Between Groups	29617	2	14809	92.1	0.002
	Within Groups	483	3	161		
	Total	30100	5			
HBSSwithHEPES; TEER	Between Groups	30970	2	15485	52.5	0.005
	Within Groups	885	3	295		
	Total	31885	5			
DMEM; TEER	Between Groups	3089.3	2	1544.7	31.0	0.010
	Within Groups	149.5	3	49.8		
	Total	3238.8	5			
DMEMwithFBS; TEER	Between Groups	2214.3	2	1107.2	9.5	0.051
	Within Groups	350.5	3	116.8		
	Total	2564.8	5			
Whole samples; recovery	Between Groups	0.3868	4	0.0966	317.1	0.000
	Within Groups	0.0030	10	0.0003		
	Total	0.3898	14			
Whole samples; transport	Between Groups	0.0018	4	0.0004	15.6	0.000
	Within Groups	0.0003	10	0.0000		
	Total	0.0021	14			
SPE samples; recovery	Between Groups	0.5731	3	0.1910	15.5	0.003
	Within Groups	0.0738	6	0.0123		
	Total	0.6469	9			
SPE sample; transport	Between Groups	0.0000	3	0.0000	6.2	0.029
	Within Groups	0.0000	6	0.0000		
	Total	0.0000	9			

CURRICULUM VITAE

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Work Experience and Awards:

- 2007 October – Present: Research Assistant, Istanbul Technical University
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- 2011 November – 2012 September: PhD research in Wageningen University, Food and Biobased Research, Wageningen-The Netherlands. Working with Dr. Jurriaan Mes on in vitro bioavailability of sour cherry anthocyanins and other phenolics by using Caco-2 cell model, in the framework of the EU 7th Framework Project, ATHENA.
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Publications:

- **Toydemir, G.**, Capanoglu, E., Gomez Roldan, M.V., De Vos, R.C.H., Boyacioglu, D., Hall, R.D., and Beekwilder, J. 2013. Industrial processing effects on phenolics compounds in sour cherry (*Prunus cerasus* L.) fruit. *Food Research International,* 53: 218-225.
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- **Toydemir Otkun, G.**, Boyacioglu, D., Demir, N. 2010. "Starch Retrogradation Behavior of Different Rice Varieties Used in Canned Stuffed Vine Leaves During Storage". *IFT 2010 Annual Meeting & Food Expo*, July 2010, Chicago, IL, USA.
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