ISTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

CHANGES IN ANTIOXIDANT PROFILES, METABOLITES AND ENZYMES DURING DEVELOPMENT OF TOMATO FRUIT AND TOMATO PASTE PROCESSING

Ph.D. Thesis by

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FOREWORD

The health protective effects of tomato created a great interest in the effect of processing since its industrially processed products are highly consumed worldwide. The purpose of this study was to evaluate the effects of tomato processing on its antioxidant compounds, metabolites, enzymes and to investigate the effects of development stages on the metabolites which are present in the different tissues of tomatoe. I hope this study will be enhancing the current literature on the health characteristics of a processed tomato product, the paste.

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ABBREVIATIONS

1-MCP : 1-methylcyclopropene
3GT : 3-*O*-glucosyl transferase
ABTS : 2,2- azinobis 3-ethylbenzothiazoline-6-sulfonic acid diammonium salt
AOAC : Association Official of Analytical Chemists
B : Breaker
BHT : Butylated hydroxytoluene
CL : Columella
CP : Columella & Placenta
CUPRAC : Copper Reducing Antioxidant Capacity
CV ,% : Coefficient of variation, percent
CX : Calyx
DPPH : 1,1-Diphenyl-2- picrylhydrazyl
DTPA : Diethylene triamine pentaacetic acid
DTT : Dithiothreitol
EDTA : Ethylene diamine tetracetic acid
EP : Epidermis
ESI : Electrospray Ionization
FA : Formic acid adduct
FRAP : Ferric Reducing Antioxidant Capacity
G : Green
GAE : Gallic acid equivalents
HPLC : High Performance Liquid Chromatography
JE : Jelly parenchyma
L : Lipophilic phase
LC-MS : Liquid Chromatography-Mass Spectrometry
LC-PDA-FD : Liquid Chromatography-Photodiode Array-Fluorescence Detector
LC-QTOF MS: Liquid Chromatography Quadrupole Time-of-Flight Tandem Mass Spectrometry
LDL : Low Density Lipoprotein
MMSR : Multivariate Mass Spectra Reconstruction
NMR : Nuclear Magnetic Resonance
P : Pink
PAL : Phenylalanine ammonia lyase
PBS : Phosphate Buffered Saline
PCA : Principal Component Analysis
PDA : Photodiode array
PR : Pericarp
QTOF-MS : Quadropole time of flight-Mass Spectroscopy
R : Red

SA : Specific Activity
SOTA : Self Organizing Tree Algorithm
T : Turning
TEAC : Trolox Equivalent Antioxidant Capacity
TOF : Time-of-Flight
UDP-Glu : Uridine 5'-diphosphoglucose
UPLC-MS : Ultra Performance Liquid Chromatography-Mass Spectrometry
VAR : Vascular Attachment Region

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LIST of SYMBOLS

α : alpha (in α-tocopherol)
β : beta (in β-tocopherol or β-carotene)
γ : gamma (in γ –tocopherol)
δ : delta (in δ-tocopherol)
Δ : delta (in ΔAU which defines AU_2-AU_1)
m : milli (10^{-3})
μ : micro (10^{-6})
n : nano (10^{-9})
p : piko (10^{-12})
kat : katal (SI unit of catalytic activity of enzymes)

DOMATESİN OLGUNLAŞMASI VE SALÇAYA İŞLENMESİ SIRASINDA ANTİOKSİDAN PROFİLİ, METABOLİTLERİ VE ENZİMLERDE MEYDANA GELEN DEĞİŞİMLERİN İNCELENMESİ

ÖZET

Pek çok endüstriyel ürünüyle domates, oldukça önemli ve bol tüketilen meyvelerin başında gelmektedir. Epidemiyolojik çalışmalar, domates de dahil olmak üzere düzenli sebze ve meyve tüketiminin kanseri ve kardiyovasküler rahatsızlıkları önlemede önemli bir rolü olduğunu göstermektedir. Domatesin zengin besin değeri, gıda işlemenin antioksidan aktiviteye sahip değerli bileşenlerine etkilerinin araştırılmasını önemli hale getirmiştir.

Genel olarak salça üretimi; yıkama, parçalama, ayıklama, pulper ve ince elekten geçirme, buharlaştırma, kutulama, pastörizasyon (veya sterilizasyon ve aseptik ambalajlama) ve soğutma aşamalarından oluşmaktadır. Bu çalışmada üretim sürecinin her bir aşaması, gravimetrik yöntemle nem içeriği, spektrofotometrik yöntemler ile toplam fenolik, flavonoid ve toplam antioksidan kapasitesi, HPLC ile on-line antioksidan tespiti, flavonoid ve klorojenik asit içerikleri, karotenoid içerikleri, tokoferol ve askorbik asit içerikleri ve LC-QTOF-MS yöntemiyle de tüm metabolitler (hedef dışı) analizlenerek antioksidatif bileşenlerde meydana gelen değişimleri açıklamak için ayrıntılı olarak incelenmiştir.

Çalışmadan elde edilen sonuçlar domatesin salça olarak işlenmesi esnasında %23'lük bir nem kaybına sebep olduğunu göstermiştir. İşleme basamakları sırasında nem içeriğinde meydana gelebilecek farklılıkları önlemek amacıyla örnekler sonraki analizler için dondurularak kurutulmuştur.

Spektrofotometrik analizler öncesinde aseton, metanol ve etil asetat içinden en iyi ekstraksiyon çözgeninin belirlenmesi için toplam fenolik miktarı ve ABTS ve DPPH yöntemleri kullanılarak antioksidan kapasitesi analizlenmiştir. Domates ve salça için metanol ekstratındaki toplam fenolik içerikleri aseton ekstraktlarına oranla %14 ve %11 daha fazladır. Öte yandan, etil asetat ekstraksiyonu toplam fenoliklerin ölçümünde bu çözgenin hızlı evaporasyonuna bağlı olarak oldukça yararsızdır. Antioksidan kapasite tayini için ABTS ve DPPH yöntemleri sırasıyla %67 ve %27 daha yüksek değerler sağladığından metanolün etil asetattan çok daha etkili olduğu bulunmuştur. Sonuç olarak, spektrofotometrik analizler için ekstraksiyon çözgeni olarak metanol seçilmiştir.

Domates ve salçanın toplam fenolik miktarları sırasıyla 567,5±76,2 mg GAE/100 g kuru madde ve 608,5±52,5 mg GAE/100 g kuru madde olarak bulunmuştur. Domatesin salça olarak işlenmesi esnasında toplam fenolik miktarında toplamda %6'lık bir artış gözlemlenmesine rağmen, işlemenin her bir aşamasında meydana gelen değişikliklerin hiçbiri istatistiksel olarak önemli değildir ($p<0,05$).

Domatesin toplam flavonoid miktarının 235,8±28,2 mg rutin ek./100 g kuru madde olduğu bulunmuştur. İşleme sürecinin ilk aşamasında (parçalama) toplam flavonoid içeriği meyveye oranla % 31 artmıştır. Çekirdek ve kabuk kısmının da meyveye oranla daha yüksek miktarda flavonoid içerdiği bulunmuştur. Çekirdek ve kabuğun ayrılmasının ardından önemli miktarda toplam flavonoid kaybı gözlemlenmiştir ($p<0,05$). Ancak pulp aşamasından sonra evaporatör çıkışı ile salça örneklerinde önemli bir değişiklik yoktur ($p<0,05$).

ABTS, CUPRAC, FRAP ve DPPH yöntemleri ile analiz edilen domates örneklerinin toplam antioksidan kapasitelerinin ortalama değerleri sırasıyla 4524,8, 4415,6, 2066,8 ve 1320,7 μmol TEAC/100 g kuru maddedir. Tüm kapasite tayin yöntemleri arasında, CUPRAC yönteminin işlenmiş domatesin hidrofilik ve lipofilik kısımlarında toplam antioksidan aktivitesini belirleyebildiği bulunmuştur.

Her bir bileşen ekstraktlarının toplam antioksidan aktivitesine göreceli katkısını ölçmek amacıyla on-line antioksidan tayin yöntemi kullanılmıştır. Meyvelerde yapılan analiz, kromatogramın ilk kısmının (5. dakikaya kadar) C vitamini ve glutation gibi polar antioksidanlar içeren toplam antioksidan aktivitesinin yaklaşık %85'ini (toplam pik alanı bazında) oluşturduğunu ortaya koymuştur. Salça örneklerinde ilk 5 dakikada kolondan çıkan bileşenlerin antioksidan aktivitesi toplamın %79'unu ve 5. ve 35. dakikalar arasında çıkan bileşenler ise %21'ini oluşturmaktadır.

Bileşenlerin bireysel olarak analizinden önce 2 yıllık sürede toplanan 5 tekrardan oluşan farklı partiler arasındaki varyasyonlar incelenmiştir. Bu varyasyonları anlamak için varyasyon katsayısı değerlerinin (%VK) sırasıyla hidrofilik ve lipofilik bileşenleri temsil eden rutin ve likopen için hesaplanmıştır. Yüzde VK değerleri farklı işlem basamaklarında rutin için %13-66 arasında değişirken, likopen için %11-28 arasındadır. Örnek partileri arasında önemli varyasyonlar gözlense de, işlem etkisini gösteren eğilimler birbiriyle benzerdir.

Flavonoidler arasında narincenin kalkon, rutin ve bir hidroksisinamat olan klorojenik asit sırasıyla 19,2±8,7, 19,8±11,0, 21,0±9,4 mg/100 g kuru madde miktarları ile en fazladır. Salça örneklerinin orijinal meyvedeki seviyesiyle kıyaslandığında ortalama %1 daha düşük rutin ve %25 daha düşük klorojenik asit içerdiği saptanmıştır. Rutin apiozit miktarı sabit kalsa da, narincenin kalkon salça örneklerinde tamamen kaybolmuştur. Önemli bir diğer flavonoid olan narincenin meyve örneklerinin hiçbirinde tespit edilememiş, yalnızca parçalanmış domates örneklerinde pulp esnasında küçük artış oranlarında tespit edilmiştir. Ayrıca, narincenin kalkonun düşük miktarlarda narincenine dönüşümü (narincenin kalkonun bir izomerik flavanon formu) gözlemlenmiştir.

Parçalama esnasında meyvenin flavonoid içeriğinde önemli miktarda artış gözlemlenmiştir ($p<0,05$). Rutin ve rutin apiozit meyveden, parçalanmış meyveye doğru sırasıyla 2 katı ve 1.3 katı artış göstermiştir. Parçalanmış domatesteki kuersetin olarak adlandırılan rutinin aglikon formu meyveden yaklaşık 2 kat daha yüksektir.

Flavonoid analizindeki bir diğer önemli bulgu ise, çekirdek ve kabuğun ayrılmasının ardından gözlemlenen önemli flavonoid kaybıdır (%74). Pulp örneklerinde narincenin kalkon içeriği özellikle çekirdek ve kabuğun ayrılmasına ve kısmen de narincenin izomerizasyonuna bağlı olarak, %93,6 oranında azalmıştır.

Karotenoid analizinden önce, 5 farklı yöntem arasından karotenoidler için optimum ekstraksiyon yöntemi seçilmiştir. Sonuçlar, meyveler için en yüksek likopen değerlerinin metanol ve kloroform kullanılarak ("Yöntem 5" olarak açıklanmıştır) elde edildiğini göstermiştir (yaklaşık 1,2 mg/g örnek). Likopen değerleri diğer yöntemlerle elde edilenlere göre %16-96 daha yüksektir.

Örneklerde en yüksek oranda bulunan karotenoid, domates ve salçada sırasıyla 146,0 ± 39,5 ve 98,9 ± 25,5 mg/100 g kuru madde miktarında bulunan *trans* likopendir. Domatesten salçaya doğru likopen miktarında görülen genel etki %32 oranında azalma şeklindedir. β-karoten miktarında ilk parçalama ve pulplama basamaklarında önemli bir değişiklik gözlenmemiştir. Ancak pulpun buharlaştırılmasından sonra β-karoten miktarı %34 oranında azalmıştır. İşlemenin lutein üzerindeki etkisi, β-karotene benzer şekildedir. Karotenoidlerdeki kayıplar temel olarak, kabuk ve çekirdek kısımlarının ayrılması ve ısı uygulaması içeren buharlaştırma basamakları nedeniyledir. Geriye kalan kayıplar ise işleme esnasında yer alan oksidasyon reaksiyonları sonucu olabilir.

Örneklerdeki tokoferollerin en baskın formu α-tokoferol olup 24-39 mg/100 g kuru madde aralığında değişmektedir. Yağda çözünen antioksidanlar olan α-tokoferol ve β-tokoferol endüstriyel işlemeden etkilenmemektedir. Diğer taraftan, bu bileşiklerin biyosentetik öncüleri olan γ-tokoferol ve δ-tokoferol ise sırasıyla %84 ve %69 oranında azalmıştır.

Meyvedeki C vitamini miktarı 245,7 ± 89,7 mg/100 g kuru madde olarak tespit edilmiştir. Domatesin işlenmesinden sonra C vitamininin yarısı kademeli olarak kaybolmuş ve 122,0 ± 28,7 mg/100 g kuru madde düzeyi ile sonuçlanmıştır. C vitamininde önemli kayıplara neden olan en önemli işleme basamağı, öncesinde bir ısıl işlemin uygulandığı pulplama basamağıdır.

Son ürün olan salçanın metabolit kompozisyonunu en fazla etkileyen işleme basamağını tespit etmek amacıyla LC-QTOF MS-temelli metabolomik yaklaşımı ile elde edilen sonuçları TBA (Temel Bileşen Analizi) diyagramlarına uygulanmıştır. İlk temel bileşen (veri setindeki toplam varyasyonun %36'sı) salça üretimindeki en baskın basamağa işaret etmiş ve belirgin şekilde domatesten kabuk ve çekirdek kısımlarının ayrılmasıyla ilişkili bulunmuştur. İkinci bileşen varyasyonun %20'sine karşılık gelmiş ve domatesin salçaya kademeli olarak işlenmesiyle ilişkilendirilmiştir.

Meyvede salçaya göre önemli ölçüde yüksek bulunan bileşikler arasında bir grup glikozilatlanmış alkoloidler, hidroksisinamat, flavonoidler ve saponin tomatozit A bulunmaktadır. Salçada domatese göre düşük düzeyde bulunan bileşikler, kabuk ve çekirdeğin uzaklaştırılması sırasında üretim zincirinden kaybolmuştur. Bu fraksiyon bağıl olarak yüksek miktarda tüm flavonoidlerin yanı sıra, çeşitli alkoloidleri de içermektedir. Meyve ve parçalanmış meyve arasında önemli ölçüde farklı olan bileşikler incelendiğinde, çeşitli flavonoidlerin ve glikoalkoloidlerin olduğu belirlenmiştir.

Araştırmanın ikinci kısmında, domates meyvesinin olgunluk aşamaları ve farklı dokuları nem, flavonoidler, karotenoidler, tokoferoller, C vitamini ve diğer metabolitleri açısından, tek bir tür, *Ever*, kullanılarak incelenmiştir.

Domates dokularındaki nem miktarı farklı basamaklarda %93,0-95,5 oranında değişmiştir. Her bir dokudaki nem miktarındaki değişiklikler farklı olgunlaşma aşamaları için istatistiksel olarak önemsiz bulunmuştur ($p<0.05$).

Flavonoid analizleri, tüm evrelerde rutin miktarının diğer dokularla karşılaştırıldığında en yüksek oranda epidermis dokularında bulunduğunu göstermiştir. Farklı evreler karşılaştırıldığında, yeşil evreden kırmızı evreye geçişte %11'lik artışla birlikte, rutin bileşiği en yüksek oranda kırmızı evrede (1389,6 μg/g) gözlenmiştir. Rutin apiozit içeriği de en yüksek oranda epidermis dokusunda (211,7 μg/g) bulunmuş ve yeşil evreden kırmızı evreye geçişte bu bileşiğin miktarında yaklaşık %39 oranında azalma görülmüştür. Narincenin başlıca epidermis dokusunda bulunmakla birlikte, gelişimin pembe evresinde en yüksek konsantrasyona (91,9 μg/g) ulaşmıştır. Ancak meyve gelişiminin yeşil evresinde epidermis de dahil olmak üzere hiçbir dokuda narincenin gözlenmemiştir.

Metabolomik analizleri sonucunda rutin ve narincenin kalkonun meyvedeki yoğun kütle sinyalleri gösteren en baskın flavonoidler olduğu görülmüştür. Hem rutin hem de narincenin kalkon miktarı yeşil evreden parçalanmış domates evresine geçişte artmış, kırmızı evreye dek yoğunlukları dengelenmiştir. Epidermis ve jel parenkima dokuları tüm olgunlaşma evrelerinde metabolit içeriği açısından en fazla değişimi göstermişlerdir. Flavonoidler tipik olarak domates meyvesinin epidermal dokularında bulunmuştur.

Karotenoid analizleri sonucunda en yüksek neoksantin içeriği jel parenkima dokusunun yeşil evresinde (13,57 μg/g) bulunmuş; ancak genellikle bu bileşiğe domatesin tüm dokularında düşük oranda rastlanmıştır (kuru maddede 15 μg/g'ın altında). En yüksek violaksantin miktarı dönüşüm evresinde vasküler eklenti bölgesinde (36,44 μg/g) gözlenmiş ve kırmızı evreye geçişte %37 oranında azalmıştır. En yüksek oranda β-karoten yeşil evrenin yaklaşık 11 katı olmakla birlikte kırmızı epidermiste bulunmuştur. Perikarp ve jel parenkima dokuları dışındaki tüm dokularda β-karoten miktarı yeşilden kırmızıya geçişte artmıştır. Tüm *trans*-likopen miktarı, diğer gelişim evreleri ve dokularla karşılaştırıldığında en yüksek oranda kırmızı epidermiste (kuru maddede 2786,53 μg/g) bulunmuştur. Lutein tüm gelişim evrelerinde en yüksek oranda vasküler eklenti bölgesinde, en düşük oranda ise epidermiste bulunmuştur.

Tokoferollere farklı dokularda farklı konsantrasyonlarda rastlanmıştır. Tüm dokularda δ-tokoferol seviyelerinin γ- ve α-tokoferole göre düşük olduğu bulunmuş; β-tokoferol ise saptanabilir düzeyde bulunmamıştır (kuru maddede 0,1 μg/g'ın altında). Tüm dokularda tüm gelişim evrelerinde α-tokoferolün en baskın tokoferol olduğu (kuru maddede 114,3-631,8 μg/g arasında) gözlenmiştir.

C vitamini içeriği en düşük oranda (kuru maddede146,56 μg/g) yeşil kolumela & plazenta dokularında; en yüksek oranda ise (kuru maddede 1670,74 μg/g) kırmızı epidermis dokusunda bulunmuştur. Olgunlaşma süresince tüm dokularda kırmızı evrede askorbik asit miktarı artmış ve tüm gelişim evrelerinde en yüksek değerlere epidermiste rastlanmıştır.

LC-QTOF-MS sonuçları çeşitli meyve dokusu profillerinin oldukça farklı olduğunu göstermiştir. Aynı zamanda meyvenin olgunlaşması sırasında tüm dokularda

metabolitlerde kütle sinyallerinin tamamen kaybolması veya ortaya çıkması gibi önemli değişiklikler olduğu gözlenmiştir. Domatesteki önemli bir flavonoid olan rutin, vasküler eklenti bölgesinde (alıkonma zamanı 24-25 dakika, kütle 610 g/mol), kolumela & plazenta ve epidermiste (en yüksek oranda) bulunmuş, diğer dokularda tespit edilmemiştir. Aynı zamanda likoperozit, tomatozit A, trikafeoilkuinik asit I ve narincenin kalkon miktarlarında da farklılıklar gözlenmiştir.

Domates dokularının LC-MS analizi, semi-polar metabolitlerin dokulardaki dağılımı ve bunların meyvenin olgunlaşması sırasındaki değişimleri hakkında önemli bilgiler sağlayabilir. Bu analizlerden, pek çok metabolitin meyvede eşit olarak dağılmadığı ancak bir ya da daha fazla dokuyu tercih ederek birikim gösterdiği açıkça görülmektedir.

Metabolitlerdeki temel varyasyon, temel bileşen analizi (TBA)'nin birinci ve ikinci bileşenine karşılık gelen epidermis ve jel parenkima dokuları arasında görülmüştür. Diğer taraftan, üçüncü bileşen meyve oluşumu ile ilişkilidir. Dokular arasındaki farklılıklar olgunlaşma sırasında metabolitlerin değişimine işaret ederek daha belirginleşmiştir.

Araştırmanın üçüncü bölümünde, parçalama esnasında flavonoid miktarında meydana gelen artışın nedeni ayrıntılı olarak incelenmiştir. Temel olarak, iki enzim; 3-glukozil transferaz (3GT) ve fenil alanin liyaz (FAL) analizlenmiştir.

Başlangıçta, enzim analizleme koşulları optimize edilmiştir. Sonuçlara göre, 8,3-8,4'luk optimum pH değeri Bicine pH 9,0'un EDTA, proteaz inhibitor karışımı (Serva), Triton X-100 ve DTT'nin kullanımıyla temin edilmiştir. 3GT için optimum subtrat konsantrasyonu ise 0,1 mM kuersetin olarak belirlenmiştir.

3GT aktivite ölçümleri rutin miktarındaki artışla uyumlu olarak, ilk parçalama basamağında aktivitenin iki katı arttığını göstermiştir. Bu nedenle kesmenin berelenme etkisi şeklinde ortaya çıkabileceği ve fenil propanoid yolunu teşvik ederek ve yüksek 3GT enzim aktivitesi sağlayarak yüksek rutin miktarına yol açabilir.

Farklı olgunluk evrelerindeki domateslerin etli ve kabuk kısımlarının 3GT aktivite sonuçları en yüksek 3GT aktivitesinin yeşil etli kısımda (33,4 pkat/mg) ve dönüşen renkteki kabuk kısmında (50,7 pkat/mg) olduğu görülmüştür. Ancak gelişme esnasında aktivitedeki değişimler de dokulara göre farklılık göstermiştir. Bu nedenle etli kısım ve kabuk kısmı farklı olgunlaşma evrelerinde farkı miktarda enzim içerebilmektedir.

FAL enziminin domates işleme materyalindeki aktivite ölçümleri, düşük konsantrasyon ve yöntemin düşük tespit limiti nedeniyle başarıya ulaşamamıştır. Ancak olgunlaşma basamaklarındaki örnekler için domateslerin etli kısımları en yüksek FAL aktivitesini, kırmızı etli kısımda (917,3 μkat/mg) içermekte olup, koyu kırmızı renkteki etli kısma doğru şiddetli bir azalma göstermiştir (296,4 μkat/mg). Kabuk kısmında, FAL aktivitesindeki değişimler 3GT enziminde görülen eğilime benzer şekildedir. Yüksek FAL aktivitesinin daha yüksek miktarda ürünle sonuçlanacağı ve bu ürünün fenil propanoid yolunda devam ederek, 3GT için daha fazla substrat üreteceği düşünülmektedir. Domatesin farklı dokularındaki enzim aktivitesinin metabolitlerdeki değişime paralel olarak, farklı olgunluk aşamalarında farklı olabileceği sonucuna varılmıştır.

CHANGES IN ANTIOXIDANT PROFILES, METABOLITES AND ENZYMES DURING DEVELOPMENT OF TOMATO FRUIT AND TOMATO PASTE PROCESSING

SUMMARY

Tomato is an important and highly-consumed fruit that has many different industrial products. Many epidemiological studies suggest that regular consumption of fruits and vegetables, including tomatoes, can play an important role in preventing cancer and cardiovascular problems. The nutritional value of tomato created a great interest in the effect of processing on its valuable compounds showing antioxidant activity.

In general, tomato paste production includes the basic steps of cleaning, breaking, finisher-separating the pulp from skin, and seeds, evaporating, canning, pasteurizing (or sterilizing and aseptic packaging), and final cooling. In this study, every step of processing was investigated in detail to elucidate the changes in antioxidative compounds by analyzing the moisture content by gravimetric method, total phenolic and total flavonoid contents, total antioxidant capacity by spectrophotometric methods and on-line antioxidant detection method, flavonoid and chlorogenic acid contents, carotenoid contents, tocopherol and ascorbic acid contents by HPLC and untargeted metabolomics by LC-QTOF-MS methods.

The results showed that there was a total moisture loss of 23% induced by processing tomato fruits into paste. In order to eliminate the differences in the moisture content at each processing step, samples were freeze-dried for further analysis.

Prior to the spectrophotometric analysis, the best extraction solvent out of acetone, methanol, and ethyl acetate was investigated by analyzing the amount of total phenolics and antioxidant capacities with ABTS and DPPH methods. Total phenolic contents of tomato and paste in methanol extracts were 14% and 11% higher than those of acetone extract. On the other hand, ethyl acetate extraction was totally useless for the measurement of total phenolics due to the fast evaporation of this particular solvent. For the antioxidant capacity assays, methanol was found to be much more efficient than ethyl acetate as ABTS and DPPH methods yielded 67% and 27% higher values, respectively. As a result, methanol was selected as the extraction solvent for spectrophotometric analysis.

Total amounts of phenolics were found to be 576.5 ± 76.2 mg GAE/100 g dry weight and 608.5±52.5 mg GAE/100 g dry weight for tomato and paste, respectively. Although an overall increase of 6% in total phenolic contents was observed by processing into paste, changes occurred in each step of processing were statistically insignificant ($p<0.05$).

Total flavonoid content of the fruits was found to be 235.8 ± 28.2 mg rutin eq./100 g dry weight. In the first step of processing (breaker), total flavonoid content increased

by 31% compared to the fruit. The seed and the skin parts were also found to contain high amounts of flavonoids with respect to the fruit. After the removal of seed and skin, significant amount of total flavonoid loss was observed ($p<0.05$). However, there were no significant changes after the pulping step in the samples of evaporator out and paste ($p<0.05$).

The mean values of total antioxidant capacity of tomato fruit samples analyzed by ABTS, CUPRAC, FRAP and DPPH methods were 4524.8, 4415.6, 2066.8, and 1320.7 μmol TEAC/100 g dry weight, respectively. Among all the capacity method techniques, CUPRAC method was found to be capable of both determining the antioxidant activity of hydrophilic and lipophilic phases of processed tomato.

The on-line antioxidant detection method was performed in order to assess the relative contribution of individual compounds to the total antioxidant activity of the extracts. The analysis in fruits revealed that the first part of the chromatogram (until 5 minute) comprised of approximately 85.0% of the total antioxidant activity (based on total peak area) containing polar antioxidants such as vitamin C and glutathione. In the paste samples, the antioxidant activity of the compounds eluting within the first 5 minute was 79.0% of the total, and of the compounds eluting between 5-35 minutes was 21.0%.

Prior to the analyses of individual compounds, variations between different batches composed of 5 independent replicates harvested over a 2 year period were observed. In order to understand these variations, coefficient of variation (CV %) values were calculated for rutin and lycopene representing the hydrophilic and lipophilic compounds, respectively. The CV% values ranged between 13-66% for rutin and 11-28% for lycopene at different processing steps. Although there was considerable variation between batches, the trends reflecting processing effect were similar to each other.

Among flavonoids, naringenin chalcone, and rutin as well as chlorogenic acid which is a hydroxycinnamate, were most abundant with amounts of 19.2 ± 8.7, 19.8 ± 11.0, 21.0 ± 9.4 mg/100 g dry weight, respectively. The paste samples were found to contain 1% and 25% lower rutin and chlorogenic acid in average, compared to its level in the original fruit, respectively. Although rutin apioside remained stable, the naringenin chalcone was completely lost in the paste samples. Naringenin, another important flavonoid, was undetectable in any of the fruit samples, and only small amounts were detected in the breaker samples which were increased during pulping. In addition, conversion of naringenin chalcone to lower amounts of naringenin which is an isomeric flavanone form of naringenin chalcone, was observed.

During breaking, a significant amount of increase was observed in the flavonoid content of the fruit ($p<0.05$). Rutin and rutin apioside increased by 2 and 1.3 times from fruit to breaker, respectively. The amount of aglycon form of rutin, namely quercetin, of breaked tomatoes was approximately 2 times higher than the fruit.

Another important finding in flavonoid analysis was that significant loss of flavonoids (74%) was observed after the removal of seed and skin. Naringenin chalcone content of fruit decreased by 93.6% in the pulp samples mainly because of the removal of the seed & skin fraction, and partly due to its isomerization into naringenin.

Prior to the analysis of carotenoids, optimum extraction method was selected out of 5 different methods. The results showed that the highest lycopene values for fruits were obtained by using methanol and chloroform (described as "method 5") (app. 1.2 mg/g sample). The lycopene values of tomatoes were 16-96% higher than those obtained with the rest of the methods.

The most abundant carotenoid in the samples was all *trans*-lycopene with contents of 146.0 ± 39.5 and 98.9 ± 25.5 mg/100 g dry weight in tomato and paste, respectively. The overall effect of processing from fruit to paste was a 32% decrease in lycopene content ($p<0.05$). There was no significant change in β-carotene in the first breaking and pulping steps. However, after the evaporation of the pulp, the β-carotene content decreased by 34%. The effect of processing on lutein was similar to that of β-carotene. The losses in the carotenoids were mainly caused by the removal of seed and skin and evaporation step where heat was applied. Rest of the loss could be as a result of oxidation reactions taking place during processing.

The predominant form of tocopherols in the samples was α-tocopherol ranging between 24-39 mg/100 g dry weight. The lipid-soluble antioxidants α-tocopherol and β-tocopherol were not affected by the industrial processing. On the other hand, their biosynthetic precursors, γ-tocopherol, and δ-tocopherol decreased by 84% and 69% in the paste samples, respectively.

The amount of vitamin C in fruit samples was found to be 245.7 ± 89.7 mg/100 g dry weight. After processing tomatoes, half of the vitamin C was lost gradually, ending up with 122.0 ± 28.7 mg/100 g dry weight. The main processing step which caused a significant loss in vitamin C was the pulping step, before which heat treatment was applied.

The results of LC-QTOF MS-based metabolomics approach which was performed to determine the processing steps mostly affecting the overall metabolite composition of the final paste was applied on a principal component analysis (PCA) diagram. The first principal component (36% of the total variation in the dataset) pointed to the most dominant step in the paste-production process, and it clearly corresponded to the separation of seed and skin from the rest of the tomato material. The second component explained 20% of the variation and corresponded to the step-wise processing from fruit to paste.

Among the compounds that were significantly higher in fruit versus paste were a range of glycosylated alkaloids, hydroxycinnamates, flavonoids, and the saponin tomatoside A. Compounds that were lower in paste were lost from the production chain upon removal of the seed and skin fraction. This fraction contained relatively high levels of all flavonoids, as well as several alkaloids. When the compounds that were significantly different between fruit and breaker samples were investigated, several flavonoids and glycoalkaloids were determined.

In the second part of the research, developmental stages of tomato fruit were investigated for the changes or differences in the moisture, flavonoid, carotenoid, tocopherol, and vitamin C contents and other metabolites in different tissues using a single cultivar, *Ever*.

The moisture content of tomato tissues changed between 93.0-95.5% at different stages. The differences in the moisture contents in each tissue were found to be statistically insignificant at different development stages ($p<0.05$).

Flavonoid analysis showed that rutin amount was the highest in the epidermis tissues compared to other tissues at all stages. When different stages were compared, the highest value of rutin compound was observed in the red stage (1389.6 μg/g) with an increase about 11%. Rutin apioside content was also highest in the epidermis tissue (211.7 μg/g), and decreased gradually from green to the red stage (about 39% loss). Naringenin is mainly located in the epidermis showing the highest concentration (91.9 μg/g) in the pink stage of development. However, there was no naringenin observed in the green stage of development in any tissue including the epidermis.

According to the metabolomics analysis, rutin and naringenin chalcone were the most abundant flavonoids in the fruit exhibiting intense mass signals. The content of both rutin and naringenin chalcone increased from green to the breaker stages, which was followed by stabilized intensities until the red stage. At all ripening stages, epidermis and the jelly parenchyma exhibited the most extreme differences in metabolite composition. Flavonoids were typically present in the epidermal tissue of the tomato fruit.

According to the results of carotenoid analysis, the highest neoxanthin content was observed in the green stage of jelly parenchyma (13.57 μg/g) but in general low amounts of this compound (below 15 μg/g dry weight) occurred in all tissues of tomato. The highest value of violaxanthin was observed in the vascular attachment region at the turning stage (36.44 μg/g) and it decreased by 37% during the development towards to the red stage. It was observed that the highest amount of β-carotene was found to be in the red epidermis, which is about 11 times higher than in the green stage. Except for pericarp and jelly parenchyma tissues, the content of β-carotene in all tissues increased from green to red. The all *trans*-lycopene content in red epidermis was found to be highest with respect to the other development stages and tissues (2786.53 μg/g dry weight). Lutein was found to be highest in the vascular attachment region and lowest in the epidermis at all developmental stages.

The tocopherols showed different concentrations in different tissues of tomato. The levels of δ-tocopherol were relatively low in all tissues when compared with γ- and α-tocopherols, while β-tocopherol was not detectable at all (less than 0.1 μg/g dry weight). α-Tocopherol was the most abundant tocopherol type in all tissues at all developmental stages ranging in between 114.3-631.8 μg/g dry weight.

The vitamin C content was found to be lowest in green columella & placenta (146.56 μg/g dry weight) and highest in red epidermis tissue (1670.74 μg/g dry weight). Ascorbic acid increased in the red stage of all tissues during ripening and highest values were obtained in the epidermis at all development stages.

LC-QTOF-MS results showed that different fruit tissue profiles were quite diverse. It was also visible that, in all tissues, marked changes in metabolites occurred during ripening of the fruit, such as complete disappearance as well as appearance of mass signals. Rutin, an important flavonoid in tomato, was found in vascular attachment region (retention time 24-25 minutes, mass 610 g/mol), columella & placenta and

epidermis (highest) and was not present in other tissues. There were also differences observed in the contents of lycoperoside, tomatoside A, tricaffeoylquinic acid I and naringenin chalcone.

LC-MS analysis of tomato fruit tissues can provide important information about the tissue distribution of semi-polar metabolites and their fate upon ripening. From these analyses, it became clear that most metabolites are not equally distributed over the fruit but they show preferential accumulation in one or more tissues.

Major metabolite variations were observed between epidermis and jelly parenchyma corresponding to the first and second principal component in the PCA plot, while the third component pointed out fruit development. During ripening, the differences between tissues became more pronounced suggesting ripening-dependent tissue differentiation of metabolites.

In the third part of research, the reasons causing an increase in the flavonoid content during breaking step were further investigated in detail. Basicly, the activities of two enzymes; 3-*O*-glucosyl transferase (3GT) and phenyl alanine lyase (PAL) were analyzed.

Initially, the enzyme assay conditions were optimized. According to the results a pH optimum of 8.3-8.4 was observed using Bicine pH 9.0 together with EDTA, protease inhibitor mixture (Serva), Triton X-100, and DTT. The optimum substrate concentration was also determined as 0.1 mM of quercetin for 3GT.

3GT activity measurements showed that the activity increased twice in the first breaking step being consistent with the increase in the rutin content. Therefore, it is proposed that cutting might appear as a wounding effect and may stimulate phenyl propanoid pathway and yielding higher 3GT enzyme activity which result in the formation of higher rutin content.

The results of the 3GT activities for the flesh and skin part of tomato at different development stages showed that the green flesh (33.4 pkat/mg) and the turning skin (50.7 pkat/mg) parts presented the highest 3GT values. However, the changes in the activity during development also showed variations in different tissues. Hence, the flesh and the skin part may contain different amount of enzymes at different development stages.

The activity measurement of PAL enzyme was unsuccessful for the tomato processing material probably due to its very low concentrations and low detection limit of the method. However, for the development stages samples, the flesh part of tomatoes had the highest PAL activity in the red flesh (917.3 μkat/mg) and then presented a severe decrease in the dark red flesh part (296.4 μkat/mg). In the skin part, the changes of the PAL activity showed a similar trend observed with 3GT enzyme. It is expected that higher PAL activities will result in the formation of higher amounts of products which then will go further in the phenyl propanoid pathway producing more substrates for 3GT. It is concluded that enzyme activities at different stages of development in different tomato tissues differ in parallel with the changes in the amounts of metabolites of tomatoes.

1. INTRODUCTION

Tomato is part of the *Solanum* family which contains other plant species of commercial and/or nutritional interest (potato, pepper, eggplant, tobacco and petunia). There are several quality aspects associated with the nutritional value of the tomato fruit, such as the contents of flavor volatiles, flavonoids, vitamins and carotenoids, all of which are of relevance for market consumption. Tomato fruit is widely consumed either as fresh product, or after processing into various (cooked) products. The consumption of tomatoes has been proposed to reduce the risk of several chronic diseases such as cardiovascular diseases and certain types of cancer and especially prostate cancer (Hollman *et al*., 1996; Rao and Agarwal, 1999). In addition, tomato consumption leads to decreased serum lipid levels and low density lipoprotein oxidation (Agarwal *et al*., 2001). These health protective effects have been widely attributed to the presence of key antioxidants like lipid-soluble lycopene and β-carotene, as well as water-soluble vitamin C, and compounds of intermediate hydrophobicity like quercetin-glycosides, naringenin-chalcone, and chlorogenic acid. All of these are known to contribute significantly to the antioxidant activity of tomato fruit (Rao and Agarwal, 1999; Abushita *et al*., 2000).

Besides fresh fruit, tomato paste or the more concentrated tomato puree is a significant component in the human diet. For example, in Turkey, tomato paste is a component of the majority of home-made dishes. About 115,000 tons of tomato paste was consumed in Turkey in 2006 (Erkut, 2007). From this perspective, it is important to understand the effect of industrial-scale tomato paste-making on health-associated compounds. Generally, the industrial paste-making process involves several steps including washing and selection of spoilt tomatoes, breaking (chopping), pulping, separating the seed and the skin, evaporating, pasteurizing, packaging and storage. This production process involves a number of heating steps, which may be expected to have an effect on heat-labile and oxidizable compounds. A number of studies, mainly performed on a laboratory scale, indicate that the content

of carotenoids and vitamin C in tomato products may be negatively affected by various thermal treatments such as boiling, frying, drying, and microwaving (Abushita *et al*., 2000; Takeoka *et al*., 2001). In contrast, little is known about the effects of these treatments on phenolic antioxidants including flavonoids (Re *et al*., 2002). With regard to the percentage of fruit lycopene present in the final paste, the available literature data are inconsistent with values ranging from a ca. 20% decrease to a ca. 33% increase (Takeoka *et al*., 2001; Dewanto *et al*., 2002). Clearly, these variable findings indicate that results obtained from laboratory experiments are difficult to explain the actual effects taking place during factory-scale paste making. Each processing step, to a more or lesser extent, will likely influence the composition of metabolites, including health-related antioxidants, and thus the nutritional quality of the final tomato paste. Clearly, there is a need for a better understanding of the physiological and biochemical processes that take place during the entire route from the field harvest of fresh fruit up to the pasteurized and canned tomato paste ready for retail. Besides comprehensive biochemical studies on the effect of the individual steps in factory-scale tomato processing have not yet been reported.

In addition to the nutritional value inherent to the tomato and assumed health benefits, tomato fruit is the most well studied fleshy fruit and represents a model of choice for ripening studies. During fruit development, there occur a series of physiological phenomena such as alterations in pigment biosynthesis, decreases in resistance to pathogen infection, modifications of cell wall structure, conversion of starch to sugars and increase in the levels of flavor and aromatic volatiles (Fraser *et al*., 1994; Giovannoni, 2001; Carrari *et al*., 2006). In climacteric fruits, such as the tomato fruit, ethylene plays a major role in fruit development and ripening, in addition to the plant hormones auxin and abscisic acid, as well as gibberellins and cytokinins (Srivastava, 2005). However, the dynamics and interactions within metabolic pathways, as well as the identity and concentrations of the interacting metabolites during fruit development, are mostly unknown.

The available literature proposes the changes in antioxidative compounds of tomato such as chlorogenic acid, *p*-coumaric acid, and rutin at different developmental stages of tomato (Raffo *et al*., 2002; Shahidi and Naczk, 2004a). However, there is a lack of information about the differentiation of those compounds in specific tissues.

Moreover, rutin sythesis by 3-*O*-Glucosyl transferase (3GT) from quercetin has been elucidated by researchers in buckwheat (Suzuki *et al*., 2005), onion (Latchinian-Sadek and Ibrahim, 1991), grape (Ford *et al*., 1998), red orange (Piero *et al*., 2005), maize (Futtek *et al*., 1988), strawberry (Cheng *et al*., 1994), *Arabidopsis thaliana* (Li *et al*., 2001) etc., and higher plant glucosyl transferases were reviewed by Ross *et al*. (2001) but there is no information on 3GT acitivity and rutin synthesis in tomato fruit at different ripening stages.

The first objective of this research was to investigate industrial processing of fresh fruits into tomato paste both by analyzing the fate of specific antioxidants, using dedicated analyses, and by taking a broader overview, using non-targeted metabolomics approach. The second objective was to compare fruit tissues of a tomato cultivar (*Ever*) at different ripening stages by focusing on metabolites such as carotenoids, tocopherols, ascorbic acid, phenolic acids, flavonoids, saponins and glycoalkaloids. Third objective was to investigate the activity of 3-*O*-Glucosyltransferase (3GT) and phenylalanine ammonia lyase (PAL), which is the first enzyme taking part in the phenypropanoid pathway, during the development of the tomato fruit, from green to dark red.

This research thesis is presented as literature, materials and methods, results and discussion and conclusion parts. In the literature chapter, tomatoes and tomato paste, healthy compounds of tomato and tomato products; mainly flavonoids, tocopherols, carotenoids, and ascorbic acid, were reviewed. The changes during development of tomato and effect of industrial food processing on tomato antioxidants were also presented in the literature. Materials and methods section included the detailed protocols followed for the analysis. Results and discussion part was divided into three main headlines including; effect of tomato processing on antioxidative compounds, changes in tomato fruit during developmental stages and enzyme activity measurements. In the conclusion part, new information on the changes of healthy compounds was introduced.

2. LITERATURE

2.1. Tomatoes and Tomato Paste

Tomato or *Solanum lycopersicum* (formerly *Lycopersicum esculentum*) is part of the Solanum family which contains other plant species of commercial and/or nutritional interest such as potato, pepper, eggplant, tobacco and petunia. Tomatoes were firstly originated in the Andean region of South America under extremely variable climatic conditions. Wild relatives of tomato may grow from sea level to subalpine elevations while ecotypes adapted to flooded conditions and others to extreme drought (Powell and Bennett, 2002).

Tomatoes are rich sources of vitamins, especially ascorbic acid and carotene, and antioxidants such as lycopene. A single tomato fruit is sufficient to supply about a quarter of the vitamins A and C recommended for daily consumption of humans (Powell and Bennett, 2002). There are several quality aspects associated with the nutritional value of the tomato fruit. Tomato fruit quality is assessed by the content of chemical compounds such as dry matter, Brix degree, acidity, single sugars, citric and other organic acids, and volatile compounds (Hernandez-Suarez, 2008).

Tomato fruit is a significant source of nutrition for substantial portions of the world's human population since this fruit crop is widely cultivated and consumed extensively as both fresh fruit and processed product. It has a wide variety of products including juice, paste, puree, and sauce, diced, canned or dried tomato. The stability of the concentrated processed products has made it possible to transport them widely and to prolong their shelf-life (Powell and Bennett, 2002). In Turkey, out of this wide variety of products especially tomato paste is a major component of home-made traditional dishes and has a high consumption rate (Erkut, 2007).

The tomato fruit is one of the most widely produced fruits for consumption, with more than 122 million tons produced worldwide, in 2005 (Sarısaçlı, 2005). In

Europe, and most of the developed countries, a large proportion of the tomato consumption is in form of processed tomato products, with an average of 18 kg (fresh tomato equivalent) consumed annually per capita in the European Union, with variations between 5 kg in the Czech Republic and 30 kg in Italy (Erkut, 2007).

In Turkey, tomato production accounts for approximately 38% of all vegetable production. Turkey ranks the fourth in industrial tomato production in the world. Approximately 20% of all tomato production is processed to produce products such as tomato paste, tomato juice, ketchup, tomato puree and chopped tomatoes (Table 2.1). In the past almost 95% of industrial tomato production was used to produce tomato paste but the share of different processed product varieties increased over the years. Currently, about 85% of industrial tomato production is used for tomato paste production and the rest is used for other products. The tomato paste sector is the leading sector for processed fruit and vegetable exports. Tomato paste production in Turkey is shown in Table 2.2. Traditionally, fresh tomato sale production accounts for approximately 80% of total tomato production in Turkey and the rest is utilized in the processing sector. Of the 20% of the crop that is processed, 85% is used to produce tomato paste, 10 percent is utilized for canned tomatoes and the remainder is used for ketchup, tomato juice, dried tomatoes, and other products (Sarısaçlı, 2005).

Table 2.1: Production of fresh tomatoes (million tones).

	2005	**2006**	**2007**
Fresh sale production	8,350,000	8,205,000	8,800,000
Processing production	1,700,000	1,650,000	2,000,000
Total production	10,050,000	9,855,000	10,800,000

Ecological and geographical conditions in Turkey allow producing high quality tomatoes in big quantities throughout the year all over the country. Tomatoes for industrial use are grown mainly the provinces of Balıkesir, Bursa, Manisa and Canakkale of the Aegean and Marmara regions. As a result of the high amounts of production in such regions, tomato processing facilities are also located in those regions. The Mediterranean region mostly focuses on production of tomatoes in the greenhouses for fresh consumption (Sarısaçlı, 2005; Erkut, 2007).

Table 2.2: Tomato paste production in Turkey (Sarısaçlı, 2005).

Years	Tomato Paste Production (1000 tons)
1996	308
1997	205
1998	290
1999	296
2000	260
2001	240
2002	265
2003	320
2004	260
2005	265

The tomato industry is particularly interested in planting tomato varieties with higher lycopene content, in developing industrial processes to increase lycopene content in tomato products, and in presenting lycopene content as an added value on labels of products such as tomato sauce, juice, soup, and ketchup. From this perspective, it is important to understand the effect of industrial-scale tomato paste-making on health-associated compounds (Periago *et al*., 2007).

Generally, the industrial paste-making process involves several steps, as outlined in Figure 2.1. Briefly, fruits are chopped in a "breaker" unit, after which the pulp is briefly heated and separated from seed and skin and some moisture is removed by evaporation. Finally, the product is canned and pasteurized (Anon., 2008).

Most critical tomato paste quality attributes are inherent in the fresh tomato. The tomato paste manufacturing process can only be designed and operated with the objective of preserving the natural quality of the fresh tomato. The tomatoes which will be used for paste production should have high dry matter content, high Brix value, and high lycopene content (Anon., 2008).

Assuming a given level of quality in the fresh tomatoes, shorter time and gentler methods of handling, from the growers' fields to initial processing, will result in minimum deterioration of the fresh tomato's quality. The quantity of heat units applied to the product has a specific impact on the color and viscosity of tomato paste. This is a combined function of time and temperature. Shorter holding times and lower evaporation and pasteurization/sterilization process temperatures result in higher quality tomato paste (Anon., 2008).

Fast movement of product through the process to minimize the residence time of product at elevated temperatures is achieved by high and continuous product flow rates and a minimum of product tankage. A careful blending of tomato loads from the fields and through the process is absolutely critical in the production of tomato paste. Consistent quality ingredients are required to produce consistent quality end products with minimal formulation changes, resulting in lower costs (Anon., 2008).

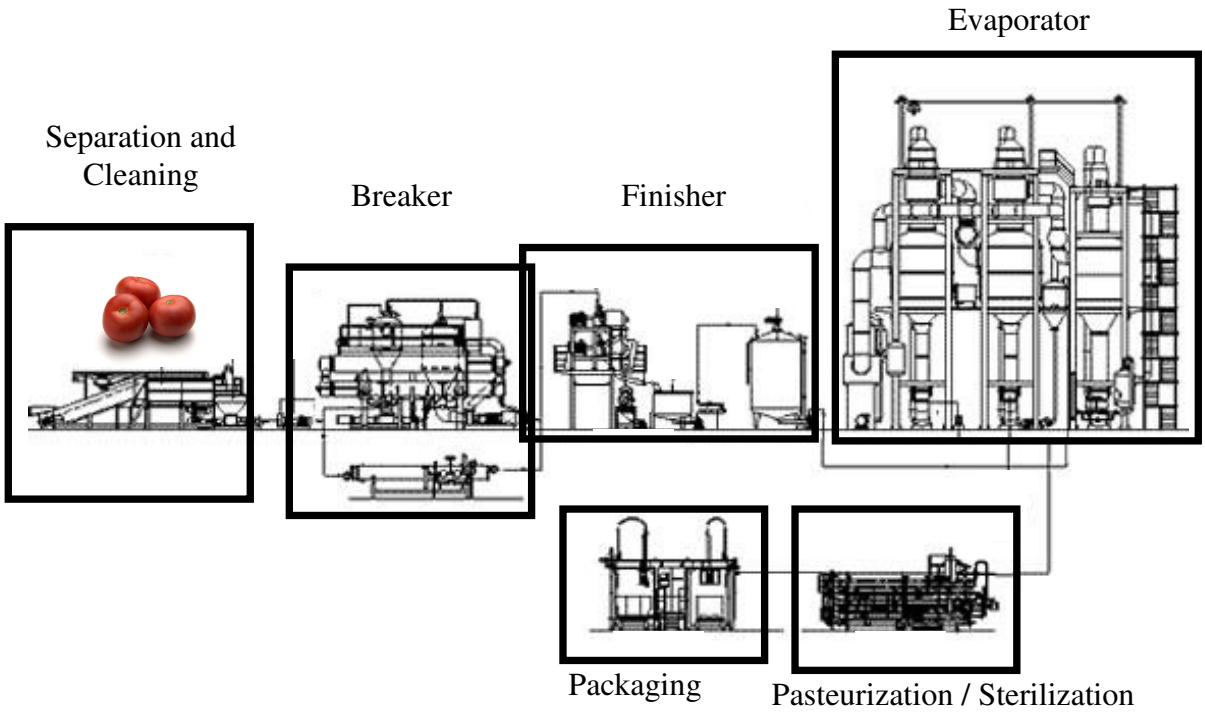


Figure 2.1: Production scheme of tomato paste.

2.2. Healthy Compounds of Tomato and Tomato Products

Since health significance of whole foods is oftenly compared to those of their corresponding individual isolated components, there is a need to explain the extent of the release of those biologically active compounds from the complex plant matrix (bioaccessibility) and their absorption metabolism (bioavailability). The concepts of both bioaccessibility and bioavailability are extremely important, since the types and quantities of biologically active components in fruits and vegetables may have insignificant health effects unless they are effectively delivered to target sites within human body (Southon and Faulks, 2002).

Unlike other micronutrients, such as water soluble vitamins, lycopene amount does not always decrease during processing. In fact, processing of tomatoes increases the lycopene content because of the concentration operations and more importantly it makes it more bioavailable. Because lycopene is insoluble in water and is very tightly bound to vegetable fiber, the bioavailability of lycopene is increased by processing. For example, cooking and crushing tomatoes and serving in oil-rich dishes greatly increases assimilation from the digestive tract into the bloodstream. Lycopene in tomato paste is four times more bioavailable than in fresh tomatoes. Thus processed tomato products such as tomato juice, soup, sauce, and ketchup contain the highest concentrations of bioavailable lycopene (Anon., 2008).

It has been reported by several studies that the *cis* lycopene stereoisomers are more bioavailable (Stahl and Sies 1992; Clinton *et al.*, 1998) than the *trans*-isomer, which is the predominant stereoisomer found in uncooked red tomato. Bioavailability depends first on the absorption of lycopene through the intestinal wall into the plasma. *Cis*-isomers of lycopene are better absorbed than the all *trans*-isomer. The characteristics of *cis* isomers that can be effective on its higher biovailability are as follows (Boileau *et al*., 1999); greater solubility in micelles, preferential incorporation into chylomicrons, less tendency to aggregate and crystallize, more efficient volatilization in lipophilic solutions, and easier transport within cells, across plasma membranes, and the tissue matrix than the all *trans*-isomers. However, little evidence is available on the absorption of specific *cis*-lycopene isomers. Cooking and processing increase the bioavailability of lycopene by softening cell walls, thereby making lycopene in tomato tissues more accessible, and by conversion of some of the *trans*-lycopene isomers to *cis*-isomers (Nguyen and Schwartz 1998; Shi and Le Maguer, 2000). Evidence also has been found indicating isomerization of all *trans* into *cis*-isomers in the gastrointestinal tract (Ishida *et al*., 2007). The increase in the bioavailability of carotenoids and lycopene in particular, is related to dissociation of protein carotenoid complexes and disruption of carotenoid crystals upon heating. Dietary fat is needed for optimum carotenoid absorption, in particular for the nonpolar carotenoids such as lycopene and β-carotene. In addition, bile salts and pancreatic enzymes are needed for absorption of these compounds. Most lycopene and other nonpolar carotenoids are transported in low-density lipoproteins. This is in contrast to polar carotenoids such as lutein and zeaxanthin, which are more

equally distributed between low-density lipoproteins and high-density lipoproteins (Russell, 2001).

Numerous research studies have demonstrated a strong association between phytochemicals from fruits and vegetables and the reduction in the risk of cancer and several other chronic diseases. These include coronary heart disease, stroke, vascular pathologies, neurodegenerative diseases, diabetes, hypertension, cataracts, macular degeneration, and osteoporosis (Wise, 2001).

There are several biological explanations why the consumption of fruits and vegetables might slow, or prevent, the onset of chronic diseases. They are a rich source of a variety of vitamins, minerals, dietary fibre and many other classes of bioactive compounds collectively called phytochemicals. Experimental dietary studies in animals, cell models, and humans demonstrate the capacity of some of these constituents to modify antioxidant pathways, detoxification enzymes, the immune system, cholesterol and steroid hormone concentrations, blood pressure, and their capacity to act as antioxidant, antiviral and antibacterial agents (Southon and Faulks, 2002).

Tomatoes are one of the main part of the Mediterranean diet which has been associated with a low mortality from cardiovascular diseases. Because tomatoes constitute the almost exclusive source of lycopene, this pigment could be one of the active agents of this protection. However, the use of only one constituent group cannot totally demonstrate the potential interaction amongst the others (Grolier *et al*., 2002). The antioxidant effect of tomato is most probably due to synergism between several compounds and it is not related to lycopene content alone as pure lycopene and several other carotenoids may act as prooxidants in a lipid environment (Yanishlieva-Maslarova and Heinonen, 2001).

The incorporation of tomatoes into diet is suggested to have a positive effect on the reduction of the risks of several chronic diseases such as cardiovascular disease and cancer (Shahidi and Wanasundara, 1992; Shahidi and Naczk, 1995; Hollman *et al*., 1996; Gerster, 1997; Clinton, 1998; Rao and Agarwal, 1999; Campbell *et al*., 2004, O'Kennedy *et al*., 2006) as well as other beneficial effects such as reducing the serum lipid and low density lipoprotein oxidation (Agarwal *et al*., 2001). Tomato

fruit is a natural source of lycopene which has been the subject of controversy due to alleged effects on prostate cancer prevention (Basu and Imrhan, 2007; Jatoi *et al*., 2007). Lycopene is the most common carotenoid in the human body and is one of the most potent carotenoid antioxidants. Lycopene is a powerful antioxidant because its multiplicity of conjugated double bonds makes it a good quencher of free radicals. It is also one of the most common carotenoids found in the blood serum. Therefore, it can be an important part of the antioxidant defense system (Burri, 2002). It is easily absorbed by the organism and is naturally present in human plasma and tissues in higher concentrations than the other carotenoids. Its level is affected by several biological and lifestyle factors. Because of its lipophilic nature, lycopene concentrates in low-density and very-low-density lipoprotein fractions of the serum. Lycopene is also found to concentrate in the adrenal, liver, testes, and prostate.

It is well-known that the positive effect on health associated with the consumption of fresh fruits and vegetables is exerted by the pool of antioxidants, with noticeable synergistic effects. Therefore, to assess the nutritional quality of fresh tomatoes, it is important to study all of the main compounds having antioxidant activity (Raffo *et al*., 2002). The protective effects of tomato and its products have been attributed to their high contents of phenolics such as quercetin glycosides, naringenin-chalcone, chlorogenic acid, vitamins C and E, lycopene, and carotene (Tavares and Rodriguez-Amaya, 1994; Bugianesi *et al*., 2004; Lenucci *et al*., 2006). In the following sections, flavonoids, tocopherols, carotenoids, and vitamin C will be evaluated.

2.2.1. Flavonoids

Plant phenols have been classiffied into major groupings distinguished by the number of constitutive carbon atoms in conjunction with the structure of the basic phenolic skeleton which are mainly simple phenols, phenolic acids, cinnamic acids, flavonoids, lignins, stilbenes, and biflavonoids (Robards *et al*., 1999).

Phenolic acids are of great interest to humans in different aspects as they contribute to the sensory and nutritional qualities of fruits and fruit products. Acylation of anthocyanins with *p*-coumaric and caffeic acids is common in fruits, and it is responsible for a better color stability in fruit products (Mazza and Miniati, 1994).

The most important group of phenolics is the flavonoids, which are highly diverse in both their chemical structure and biological functions (Robards and Antolovich, 1997). Flavonoids are diphenylpropanes with significant antioxidant and chelating properties that commonly occur in plants and cannot be synthesized by humans (Peterson and Dwyer, 1998; Shi *et al*., 2001; Heim *et al*., 2002). A single plant may contain different flavonoids, and their distribution within a plant family could be useful in the taxonomy. Flavonoids play different roles in the ecology of plants. They account for a variety of colors in flowers, berries and fruits, from yellow to red and dark purple. Flavonols, flavones, and anthocyanidins are likely to be a visual signal for pollinating insects due to their attractive colors (Heim *et al*., 2002; Rasmussen, 2004).

Structurally, flavonoids are derivatives of 1,3-diphenyl propane (Sivam, 2002) and the sub-classes vary according to their structural characteristics around the heterocyclic oxygen ring. There are many classes of flavonoids including anthocyanins, flavans, flavanones, flavones, flavonoids, and isoflavonoids (Table 2.3).

Table 2.3: The Flavonoid Classes (Peterson and Dwyer, 1998).

Class	**Color**	**Examples**	**Comments**
Anthocyanins	Blue, red, violet	Cyanidin, Delphinidin, Peonidin	They are predominant in fruit and flowers and probably the first flavonoids to be isolated.
Flavans (monoflavans, biflavans, triflavans)	Colorless	Catechin, Epicatechin, Luteoforol, Procyanidin, Theaflavin	Flavans are found in fruits and green and black teas. The astringent taste of beer, fruit juices, fruits, teas and wines is mainly due to their biflavan content.
Flavanones	Colorless to very pale yellow	Hesperidin, Naringin, Neohesperidin	They are found almost exculisively in citrus fruits. Naringin and neohesperidin have a bitter taste.
Flavones	Pale yellow	Apigenin, Diosmetin, Luteolin, Neodiosmin, Nobiletin, Tangeretin, Tricin	They are found in cereals, fruits, herbs, and vegetables. They are the yellow pigments of flowers.
Flavonols	Pale yellow	Isorhamnetin, Kaempferol, Myricetin, Quercetin	Flavonols are the most ubiquitous but are predominant in vegetables and fruits. Quercetin is the most common of all flavonoids.
Isoflavonoids	Colorless	Daidzein, Genistein	lsoflavonoids are found almost exclusively in legumes, particularly soybeans.

The flavonoids constitute a large class of polyphenols that are found ubiquitously in the plant kingdom and are thus present in fruits and vegetables regularly consumed by humans; so their amount is relatively high in our daily food (Shi *et al*., 2001; Rasmussen, 2004). In plants, the majority of the flavonoids are found as glycosides with different sugar groups linked to one or more of the hydroxyl groups. They are mainly found in the outer parts of the plants, such as leaves, flowers, and fruits. The flavonoids located in the upper surface of the leaf or in the epidermal cells have a role to play in the physiological survival of plants. Catechins and other flavanols act as feed repellents as they possess astringent characteristics, while isoflavones contribute to the disease resistance of the plant, either as constitutive antifungal agents or as phytoalexins (Naidu *et al*., 2000; Rasmussen, 2004). The flavonoids have also UV-B-protecting properties. They absorb light in the 280 ± 315 nm region, and they protect the underlying photosynthetic tissues from damage with their presence in green leaves (Stewart *et al*., 2000; Rasmussen, 2004).

Flavonoids and other polyphenols have been studied extensively and found to possess anti-tumoral, anti-allergic, anti-platelet, anti-ischemic, and anti-inflammatory activities in human beings. The role of dietary phenolic antioxidants in vivo, protecting against cancer has also been underlined by some epidemiological studies. Flavonoids and other dietary compounds have been mentioned as beneficial and protective against carcinogenesis in these studies (Shi *et al*., 2001).

In the last few decades, several epidemiological studies have shown that a dietary intake of foods rich in natural antioxidants correlates with reduced risk of coronary heart disease; particularly, a negative association between consumption of polyphenol-rich foods and cardiovascular diseases has been demonstrated (Virgili *et al*., 2003). Several clinical observations and epidemiological studies have also contributed to an emerging body of evidence for a potential role of flavonoids in the prevention of cardiovascular disease. Flavonoids have been shown to inhibit the oxidation of plasma low-density lipoprotein (LDL), decrease platelet function and to modulate cytokines and eicosanoids involved in inflammatory responses. Several epidemiological studies suggest a protection of a high flavonoid intake on the mortality of coronary heart disease (Rasmussen, 2004).

Most of these biological effects are believed to originate from their antioxidant properties. Flavonoids can exert their antioxidant activity through several mechanisms such as inhibiting the activities of enzymes including xanthine oxidase, myeloperoxidase, lipoxygenase and cyclooxygenase, by chelating metal ions, interacting with other antioxidants such as ascorbate, and most importantly, scavenging free radicals (Shi *et al*., 2001).

Tomatoes are also a good source of polyphenolic compounds, such as flavonoids and hydroxycinnamic acids (Bugianesi *et al*., 2004). In tomato fruit, kaempferol, quercetin glycosides, naringenin and naringenin chalcone are the major flavonoids (Robards and Antolovich, 1997). Besides these compounds, *p*-coumaric and ferulic acids are present both as glucosides and as glucose esters, whereas caffeic acid is only represented by caffeoylglucose (Fleuriet and Macheix, 1981; Naidu *et al*., 2000). Phenolic compounds in fresh red tomato fruit are presented in Table 2.4.

Table 2.4: Phenolic compounds in fresh red tomato fruit (Grolier *et al*., 2002).

Compounds	**Concentration (mg/kg)**	**Part of fruit**
Flavonoids		
Quercetin glycosides	3-7	Whole
Kaempferol glycosides	0.2-0.8	Whole
Naringenin	8-42 0.8 64	Whole Flesh Skin
Rutin	10-15	Whole
Phenolic acids		
Chlorogenic acid	13–38	Whole
Caffeic acid	29 56	Flesh Skin
p-Coumaric acid	16	Whole
Ferulic acid	7	Whole
Sinapic acid	2	Whole
Vanillic acid	1	Whole
Salicylic acid	1	Whole

Some recent papers reported the presence of flavonoids in tomato which also represent important antioxidative health benefits. Certain varieties of tomatoes contain high amounts of flavonols, primarily as quercetin. Flavanols and flavones are of particular interest as they are potential antioxidants and have been found to

possess antioxidative and free radical scavenging activities in foods and their consumption is associated with a reduced risk of cancer (George *et al*., 2004).

The total flavonol content of tomatoes grown in different countries ranges from 1.3 to 36.4 mg of quercetin/kg of fresh weight. Flavonols are located mostly in the tomato skin and only small quantities are found in the flesh and seeds (Shahidi and Naczk, 2004a). Distribution of flavonols in Spanish cherry (L. *esculentum* Mill.) Cv. Paloma tomatoes are shown in Table 2.5.

Table 2.5: Distribution of flavonols in Spanish cherry tomatoes μg/g fresh weight (Stewart *et al*., 2000).

Tomato	Free quercetin	Free kaempferol	Conjugated quercetin	Conjugated kaempferol	Total flavonol
Whole	0.2 ± 0.0	0.5 ± 0.1	23.4 ± 1.2	1.2 ± 0.1	25.3 ± 1.3
Skin	0.7 ± 0.0	0.4 ± 0.0	137.8 ± 5.6	4.4 ± 0.3	143.3 ± 5.8
Flesh	nd	0.1 ± 0.0	0.9 ± 0.0	0.2 ± 0.1	1.2 ± 0.1
Seed	0.1 ± 0.0	0.2 ± 0.0	1.0 ± 0.1	0.2 ± 0.0	1.5 ± 0.1

The content of flavonols in tomatoes is affected by the cultivar, harvesting season and environmental stresses such as the amount of day light and temperature, nutritional status of soil, application of plant growth regulators, and invasion with pathogens. Cherry tomatoes contain a much higher level of flavonols (17 to 203 mg/kg of fresh weight) than larger size tomato cultivars (2.2 and 11.2 mg/kg of fresh weight) (Shahidi and Naczk, 2004a).

Quercetin conjugates are the predominant form of flavonols found in tomatoes, but smaller quantities of kaempferol conjugates and traces of free aglycons have also been detected. Flavonols of tomatoes are a mixture of quercetin 3-rhamnosylglucoside (rutin), quercetin 3-rhamnosyldiglucoside, kaempferol 3-rhamnosylglucoside and kaempferol 3-rhamnosyldiglucoside. Presence of *p*-coumaric acid conjugate of rutin has also been reported. Of these, rutin is the major flavonol in tomatoes (Shahidi and Naczk, 2004a).

2.2.2. Carotenoids

Vitamin A is a generic term that refers to compounds with the biological activity of retinol. These include the provitamin A carotenoids, principally β-carotene, α-carotene, and β-cryptoxanthin, which are provided in the diet by green and yellow or

orange vegetables and some fruits and preformed vitamin A, namely retinyl esters and retinol itself, present in foods of animal origin, mainly in organ meats such as liver, other meats, eggs, and dairy products (Ross and Harrison, 2006).

Vitamin A is an essential dietary factor for normal embryogenesis, cell growth and differentiation, reproduction, maintenance of the immune system, and vision (Ball, 2006a). Most carotenoids act as macular pigments (lutein and zeaxanthin) and they have antioxidant and biochemical properties other than pro-vitamin A activity (Southon and Faulks, 2003). Although some 600 carotenoids have been isolated from natural sources, only about one-tenth of them are present in human diets, and only about 20 have been detected in blood and tissues. β-Carotene, α-carotene, lycopene, lutein, and β-cryptoxanthin are the five most prominent carotenoids in the human body (Ross and Harrison, 2006).

Recent chromatographic methods have led to identify in tomatoes at least 17 different carotenoids of which the coloring and antioxidant properties may vary. It has been reported that tomatoes contain mainly lycopene and also β-carotene, ζ-carotene, phytofluene and phytoene and traces of lutein, α-carotene, and etc. The composition of carotenoids greatly depends on the variety. Some red varieties, like Flavourtop and Beefsteak, contain up to 50 mg/kg of lycopene, of which 87% in the former and 56% in the latter of *trans*-isomers and very little β-carotene. By contrast, Tigerella variety contains similar quantities of the 3 pigments. In the yellow varieties β-carotene is the major pigment. Finally, it should be noted that the wild varieties can contain up to twice the lycopene and vitamin C quantity of the cultivated varieties (Grolier *et al*., 2002).

Tomatoes contain moderate amounts of α- and β-carotene and lutein. β-Carotene is known for its provitamin A activity and lutein for reduced risk of lung cancer. Consumption of tomato and tomato products is thus being considered as a nutritional indicator of good dietary habits and healthy life styles (George *et al*., 2004). The carotenoid composition of raw tomatoes and several tomato products including ketchup, tomato juice, tomato sauce and tomato soup are shown in Table 2.6.

Table 2.6: Carotenoid content of tomatoes and its products, µg/100 g (Campbell *et al*., 2004).

Carotenoid	Raw tomatoes	Ketchup	Tomato juice	Tomato sauce	Tomato soup
β-carotene	449	560	270	290	75
α-carotene	101	0	0	0	0
Lycopene	2573	17007	9037	15152	5084
Lutein + zeaxanthin	123	0	60	0	1
Phytoene	1860	3390	1900	2950	1720
Phytofluene	820	1540	830	1270	720

The most representative antioxidant compounds in tomato fruits are β-carotene and lycopene, the contents of which are affected by ripening stage. Indeed, β-carotene represents the precursor of vitamin A, which is essential to the diet of animals as an antioxidant, whereas lycopene is not present in all vegetables. Tomato and its byproducts represent the most important source of this antioxidant compound in the human diet (Guintini *et al*., 2005).

Lycopene is a long symmetrical hydrocarbon chain with eleven conjugated double bonds that lacks the characteristic ring structures found in most carotenoids. There are many isomers of lycopene; an all *trans*-isomer and many varieties of *cis* isomer. All *trans*-lycopene is the most common form of lycopene found in foods. In the common red tomato, about 90% of total lycopene is in the form of all *trans*-isomer, and isomerization has been suggested to derive from thermal treatment in food processing (Riso and Porrini, 2001; Burri, 2002; Grolier *et al*., 2002).

At least 85% of dietary lycopene comes from tomato fruit and tomato-based products as can be seen in Table 2.7 (Bramley, 2000). Lycopene is a powerful antioxidant because of its multiplicity of conjugated double bonds makes it capable of fighting the reactive oxygen species and of avoiding cell injury. Lycopene is also usually one of the most common carotenoids in the blood serum. Therefore, it can be an important part of the antioxidant defense system (Burri, 2002).

Table 2.7: Lycopene contents of tomato, tomato products and some other foods (Bramley, 2000).

Food	Lycopene Content (mg/g wet weight)
Fresh tomato	8.8-42.0
Watermelon	23.0-72.0
Pink guava	54.0
Pink grapefruit	33.6
Papaya	20.0-53.0
Tomato sauce	62.0
Tomato paste	54.0-1500.0
Tomato juice	50.0-116.0
Tomato ketchup	99.0-134.4
Pizza sauce	127.1

Many *in vitro* studies demonstrated that lycopene has the best singlet oxygen quenching properties and radical scavenging activity among the carotenoids (Riso and Porrini, 2001). Singlet oxygen is quenched by lycopene at a rate of almost twice β-carotene. In addition to its antioxidant properties, lycopene has also been shown to induce cell to cell communication and modulate hormonal, immune system, and other metabolic pathways, which may also be responsible for the beneficial effects (Guintini *et al*., 2005).

2.2.3. Tocopherols

Tocopherols are monophenolic antioxidants that help to stabilize most vegetable oils and they occur widely in nature. Tocopherols are composed of eight different compounds belonging to two families, namely, tocols and tocotrienols, referred to as α, β, γ, or δ, depending on the number and position of methyl groups attached to the chromane rings. Tocopherols also possess vitamin E activity. With regard to vitamin E activity, α-tocopherol is the most potent member of this family (Shahidi and Naczk, 2004b).

Vegetable foods contain considerable amounts of different tocopherols and tocotrienols in their lipid fractions. Cereals and cereal products, oilseeds, nuts and vegetables are rich sources of tocopherols. Tocopherols are important biological antioxidants. α-Tocopherol, or vitamin E, prevents oxidation of body lipids including polyunsaturated fatty acids and lipid components of cells and organelle membranes.

Tocopherols are produced commercially and used as food antioxidants. The antioxidant activity of tocopherol is based mainly on the tocopherol–tocopheryl quinone redox system (Shahidi and Naczk, 2004b).

It has been reported that, in non-peeled tomato fruit, the mean vitamin E content was 60 mg/kg dry matter or 3 mg/kg fresh matter, with 35-100 mg/kg (dry matter basis) as 95% confidence limits. From various sources of data in the 70's, it has been indicated the following values of vitamin E concentration: 0.3, 2.7, 3.0, 12 mg/kg raw edible part of tomato fruit. The tocopherol content of tomato fruit was found to be moderately high 6.6 mg/kg fresh matter at 95.4 % moisture. Citing the U.S. Department of Agriculture, a vitamin E content of tomatoes of 3.2 mg/kg fresh matter was reported (Grolier *et al*., 2002). Vitamin E contents of tomatoes and tomato products are shown in Table 2.8.

Table 2.8: Vitamin E contents of tomato and tomato products (Heinonen and Meyer, 2002; Anon., 2007).

Product	**Vitamin E (mg/kg fresh weight)**
Tomatoes	7
Tomato juice	7
Ketchup	23
Sundried tomatoes	0.1
Canned tomato sauce	14.2
Tomato puree	19.7
Tomato paste	43.0

2.2.4. Ascorbic acid

Ascorbic acid (vitamin C) plays a role as a redox cofactor and catalyst in a broad array of biochemical reactions and processes. Ascorbic acid is synthesized in plants from D-glucose and other sugars. It functions in many mono- and dioxygenases to maintain metals in a reduced state. The functions of ascorbic acid are based primarily on its properties as a reversible biological antioxidant (Ball, 2006b).

Ascorbic acid readily scavenges reactive oxygen and nitrogen species, such as superoxide and hydroperoxyl radicals, aqueous peroxyl radicals, singlet oxygen, ozone, peroxynitrite, nitrogen dioxide, nitroxide radicals, and hypochlorous acid. Excesses of such products have been associated with lipid, DNA, and protein oxidation (Johnston *et al*., 2006).

Ascorbic acid, in collaboration with α-tocopherol and β-carotene, plays an important role in the defense against cellular damage by oxidants. In this role, ascorbic acid scavenges many types of free radical and also regenerates the reduced form of α-tocopherol (Ball, 2006b).

Fresh fruits and vegetables constitute rich sources of vitamin C; but the level depends on many factors such as genetic variation, maturity, climate, sunlight, method of harvesting, and storage (Ball, 2006b). Apart from flavonoids, tocopherols and carotenoids; tomato is also a source of vitamin C, providing a significant contribution to dietary intake. Ascorbic acid is very susceptible to chemical and enzymatic oxidation during the processing, storage, and cooking of food (Ball, 2006b). Raw tomato contains more vitamin C than processed tomato, and there is a higher loss of the vitamin during the production of tomato concentrates than in tomato juice or whole canned tomatoes (Riso and Porrini, 2001). Vitamin C contents of tomato and its products are shown in Table 2.9.

Table 2.9: Vitamin C contents of tomato and tomato products (Canene-Adams *et al*., 2004; Anon., 2007).

Product	**Vitamin C (mg/100 g)**
Raw tomatoes	12.7
Ketchup	15.1
Tomato juice	18.3
Tomato sauce	7.0
Tomato soup	27.3
Tomato paste	21.9
Tomato puree	10.6
Sun dried tomatoes	39.2

2.3. Changes in Antioxidants During Development of Tomato Fruit

Climacteric fruits such as tomato are distinguished from those that are non-climacteric by their increased respiration and ethylene biosynthesis rates during ripening. Tomato fruit development is marked by significant changes in the cell wall components, and a handful of polysaccharides degrading enzymes have received much attention over the last fifteen years. The activity of these enzymes is directly linked to the shelf-life of the fruits, one of the crucial characteristics in tomato market. Fleshy fruits as tomatoes are predominantly composed by parenchyma cells walled by an unlignified layer of cellulose microfibrils suspended in a matrix of

glycoproteins, water and pectic and hemicellulose polysaccharides. The latter accounts for 90% of the cell wall, with cell wall polysachharides largely derived from sugars and sugar phosphates (Burg and Burg, 1965; Carrari *et al*., 2007).

The growth of tomato fruits goes through different phases. The early phase of fruit development is characterized by high metabolic activity and a rapid cell division of the tissue, whereas at a later developmental phase, the cells expand. Fruit ripening begins when seeds are completely formed and the fruit reaches its final size. This ripening process involves a series of coordinated events including changes at the physiological and biochemical levels. There are few reports about the enzymes involved in amino acid biosynthesis in the fruit (Boggio *et al*., 2000).

A series of quantitative and qualitative changes of the chemical composition take place during tomato fruit ripening. Organic acids, soluble sugars, amino acids, pigments and over 400 aroma compounds contribute to the taste, flavor and aroma volatile profiles of the tomatoes. The ripening of tomatoes is characterized by the softening of the fruit, the degradation of chlorophylls and an increase in the respiration rate, ethylene production, as well as the synthesis of acids, sugars and lycopene (Hernandez-Suarez *et al*., 2008).

The nitrogen metabolism in tomato fruit is also important in ripening and is closely related to the central carbon metabolism. Interesting pattern of amino acid contents in tomato fruit has been observed during several stage of ripening. At earlier stages of development glutamine, alanine, asparagine, arginine, valine and proline were found as predominant amino acids and their concentrations decrease at later stages of development. In contrast glutamate, cysteine, aspartate, tryptophan, methionine and putrescine increase at the later stage of fruit development and ripening (Boggio *et al*. 2000; Carrari *et al*. 2006).

In case of antioxidants, immature green tomato fruits contain a high level of chlorogenic acid in the pericarp and pulp. The level of chlorogenic acid rapidly declines as the color of the fruit changes from green to pink and then to red. The content of rutin also reaches a maximum in green tomato and then decreases during successive stages of fruit development. On the other hand, *p*-coumaric acid glucoside has been found only in the pulp and reaches a maximum level at the breaker stage (i.e., when the fruit color changes from green to pink). However, no rapid decline in

p-coumaric acid glucoside content has been noted at successive stages of fruit development. Changes in chlorogenic acid and rutin are similar to those observed for auxin (indole-3-acetic acid) metabolism. Therefore, rutin and chlorogenic acid have been suggested as regulators of auxin metabolism during ripening of tomato fruit (Shahidi and Naczk, 2004a).

During fruit development tissue specialization takes place which is a complex biological process where transformations at the physical, chemical and biological level are taking place simultaneously. These transformations result in a series of dynamic modifications in the whole metabolic pathway network leading to cell division, cell expansion and ripening. The transformation of the ovary into the mature fruit has been target of intensive studies, in practically all aspects of fruit development. In particular at the early stage of fruit set, the hormonal regulation and the interaction of different hormones have been studied extensively (Gillaspy *et al.*, 1993; Giovannoni, 2001). The assignment of genes involved in fruit formation and ripening functions, the analysis of transcripts and proteins as well as registering alterations of a limited set of known metabolites during development have been previously documented (Gillaspy *et al*., 1993; Buta and Spaulding, 1997; Srivastava and Handa, 2005). Recently, a comprehensive study on the primary metabolism in tomato fruit has been performed at both metabolite and transcript levels (Carrari *et al*., 2006). In this latter study, a GC-TOF MS based metabolomics approach was used to detect changes in mainly sugars, organic acids and amino acids upon fruit development. So far, tissue differentiation during fruit formation and maturation has been monitored mostly at the morphological level and much less at the biochemical level.

During ripening, tomato fruit undergoes a wide range of biosynthetic as well as degradative reactions that dramatically affect the final fruit composition. These changes are highly coordinated, taking place in every subcellular compartment, and are regulated by plant hormones and modified by genetic and environmental factors. At the breaker stage no more than 10% of surface color is pink or red; at the firm red stage >90% of surface color shows red. The production of the red color of the ripe fruit is due to the degradation of chlorophyll and the high accumulation of carotenoids as lycopene and β-carotene as the chloroplasts are transformed to

chromoplasts. In addition to the genetic back ground of the plants, growing conditions also affect the antioxidant content of the tomato fruit. For example, light plays a fundamental role in determining the final content of carotenoids (Guintini *et al*., 2005). The factors affecting the antioxidant content of fresh tomatoes can be listed as the influence of cultivar, cultural practices, ripening stage at harvest, and storage conditions (Raffo *et al*., 2002). Leonardi *et al*. (2000) found that carotenoid content as well as lipophilic antioxidant activity of tomato was more affected by ripening stage than by cultivar, which nevertheless determined significant effects; cherry tomatoes showed a relatively high level of carotenoids and higher lipophilic and hydrophilic antioxidative abilities than other varities of tomatoes commonly used for fresh consumption in Italy.

The color of red tomatoes is determined primarily by their lycopene content. Johjima and Matsuzoe (1995) have shown that the fruit color values (a/b) were highly correlated with lycopene content, including *cis* and *trans* forms. At lycopene concentrations between 32 and 43 mg/kg fresh matter, fruit color turns from orange to red and satisfactory red coloration required a total carotene concentration in excess of 55 mg/kg fresh matter of which lycopene accounted for 90% of the carotenes. Giovanelli *et al*.(1999) measured the color changes at seven stages during wine and post-harvest ripening on two genotypes (Normal Red and Crimson) of the tomato cv. *Moneymaker* grown in a greenhouse. It was found that ripening conditions affected the final antioxidant content, especially lycopene which mainly accumulated in the very last period of ripening as also followed by the color changes.

The rate of softening after processing depends on many factors related to the product and to the processing and the storage conditions. The stage of ripening of the fruit at harvest and at cutting is of particular importance, since both affect post-cutting quality and shelf-life. Equally important is the temperature at which the processed product is stored, since most of the reactions in the product are biochemical and hence temperature dependent. Although storage at low temperature is a common practice to retard softening of fruit and vegetables, accelerated softening of tomato can occur at low temperature due to chilling injury (Lana *et al*., 2005).

Ethylene is one of several plant growth regulators that affect growth and developmental processes including ripening and senescence and can profoundly

influence the quality of harvested products (Watkins, 2006). Living tissues show physiological response to minimal processing procedures as well to post-processing treatments and to the package environment in which they are enclosed. Wounding of plant tissues has long been known to induce ethylene production, and the time line for the initiation of this response can range anywhere from a few minutes to an hour after wounding. Large increases in ethylene production, as a consequence of cutting, have been shown in tomato (*Lycopersicon esculentum* Mill.) by several studies. Ethylene production is localized to tissue in surrounding area of the wound or cutting injury. Storage temperature also has an effect on wound-induced ethylene production. It has been shown that storage of the cut fruits and vegetables at 0–2.5°C will almost completely suppress wound-induced ethylene as compared to higher storage temperatures (Toivonen and DeEll, 2002).

The use of technologies that minimize the accumulation of ethylene in the storage environment or inhibit ethylene action would extend the time to ripen of green tomatoes. 1-Methylcyclopropene (1-MCP) is proposed to interact with ethylene receptors and thereby prevent ethylene-dependent responses (Watkins, 2006). In a study by Wills and Ku (2002), 1-MCP has been used to inhibit the action of ethylene and thereby extend the storage life by incresing ripening period of green tomatoes and postharvest life of riped tomatoes.

The observed loss of rigidity during ripening involves physicochemical changes occurring in the fruit cell walls derived from modifications in the cell wall polymers, mainly, due to enzymatic hydrolysis of pectin (Carrari *et al*., 2006; Tomassen *et al*., 2007). The alteration of fruit size during development is almost irrelevant after the green stage, as the fruit size appears stable (Giovannoni, 2001).

2.4. Effect of Industrial Food Processing on Tomato Antioxidants

The nutritional value of tomato created a great interest in the effect of food processing and storage on its nutritive compounds showing antioxidant activity. Although most of the nutritional components in tomato fruit are stabilized by acidic pH of the fruit tissue and many of nutrients are conserved during relatively short and mild processing steps of tomato products, the antioxidant capacity of the products may change depending on processing type and conditions (Powell and Bennett, 2002). Intact fruits and vegetables are prone to deleterious changes induced by

respiratory, metabolic and enzymatic activities, as well as by transpiration, pest and microbial spoilage, and temperature-induced injury. Many of those changes may affect adversely the antioxidant status of tomato products (Lindley, 1998). However, there are also recent studies in which the compounds having antioxidative effects, such as lycopene or β-carotene or the total antioxidant capacity of end products increased as a result of food processing.

The starting point for the retaining tomato's nutritional properties during processing into different products is the raw material. Hence, great attention should also be given to avoiding or minimizing the detrimental effects of technological processing methods and conditions of processed products. Although the effect of processing on the antioxidant level of tomatoes has been reviewed moderately, the exact level of losses or even gains of antioxidants differ widely according to the type and conditions of the process applied or the variety/origin of the fruits used in the experiments. However, understanding the mechanisms taking place in the tissue during processing will definitely lead to several developments in the food industry for controlling the technical and environmental parameters. Indeed, most of the tomato products (such as tomato paste, diced tomatoes, puree) are exposed to secondary heat treatments during home processing. Therefore, elucidating the extent of biochemical changes during industrial processing is of critical importance. The effects of thermal and non-thermal treatments also need more investigation for interpreting the changes under different conditions.

The demand for tomato processing usually arises from needs to prolong the shelf-life; to make it available out of season; to produce practical products especially for home-consumption; to convert into new products with alternative flavor and texture; to provide better nutritional characteristics; and to add value for extra income. Processing of tomato might be simple or complicated depending on the end product to be produced. For example, fresh-cut tomato production includes the steps of washing, selection of tomatoes, cutting and packaging whereas tomato paste production includes several steps like washing, selection of tomatoes, breaking (cutting), separating the seed and the skin, evaporating, pasteurizing, canning and storage. Those kinds of productions with many steps, including heat treatments, have higher potentials of affecting the nutritional status of the end product.

Most of the industrial processing steps include heat treatments and the present studies are mainly focused on the investigation of the effects of these heat treatments on the nutritional or quality characteristics of tomatoes. Frying, boiling or microwaving removes 35 to 78% of quercetin conjugates originally present in tomatoes (Crozier *et al*., 1997). These losses may be due to the degradation or extraction of flavonols from tomato by water. Tomato juice and puree are rich sources of flavonols. Processing tomatoes to juice and puree increases the content of free quercetin by up to 30%, an increase that may be brought about by enzymatic hydrolysis of quercetin conjugates. Tomato juice and puree contain 15.2 to 16.9 mg/L and 16.6 to 72.2 mg/kg of fresh weight of flavonols, respectively (Stewart *et al*., 2000). On the other hand, canned tomatoes are a poor source of flavonols (Shahidi and Naczk, 2004a). These various findings on the industrial treatments are classified as thermal and non-thermal treatments in this section.

2.4.1. Thermal treatments

For most cases, industrial processing of tomatoes into different products includes several steps of heat treatments such as drying, heating, pasteurizing, etc. The aim of these treatments might be to inhibit / inactivate microorganisms or enzymes, to decrease the moisture content and concentrate the product, or to soften the tissue in order to separate the skin easily. During these treatments, several changes might occur in the composition or the nutritional value beside changes in sensory properties such as color, texture, and flavor of the product. The effect of thermal treatments on individual functional components of tomato has been reviewed below.

Lycopene and carotenes: Lycopene, the most special component of tomato have been studied particularly for its health effect, bioavailability, and changes that occur during processing or their developmental stages. The primary sources of lycopene in the diet are tomatoes and tomato products, although other foods such as apricots, pink grapefruit, watermelon, guava, and papaya are also accepted as dietary sources (Russell, 2001). Besides lycopene, tomatoes also contain α-, β-, γ-, δ-carotene and lutein and also neurosporene, phytoene, and phytofluene (Riso and Porrini, 2001). Of all the carotenoid pigments, lycopene is the most efficient singlet oxygen quencher. The quenching constant of lycopene was found to be more than twice β-carotene (Di Mascio *et al.*, 1989).

Lycopene in tomatoes seems to be more stable compared to other carotenoids to changes during peeling and juicing of vegetables. Among commercial juices tested, tomato juice has a higher oxygen radical absorbance capacity than orange juice and apple juice. According to Anese *et al.* (1999), antioxidant activity of tomato juice decreased after an initial 2–5 hours of heating but was restored after prolonged heating. Gazzani *et al.* (1998) reported that while boiled vegetable juices were generally found to exert antioxidant activity, tomato juice was pro-oxidant. These contradictory findings may be explained by differences in the amounts of the antioxidant compounds in the tomato juices because Gazzani *et al.* (1998) used a filtration method resulting in loss of most of the juice coloration. Apart from lycopene, another interesting antioxidant compound, naringenin chalcone, is present in tomato skin (64 mg/kg) and may also be present in juice, paste and ketchup. In processing tomato into ketchup, naringenin chalcone is transformed to naringenin (Heinonen and Meyer, 2002).

The effect of thermal treatments on lycopene in tomato products have attracted many researchers (Sharma and Le Maguer, 1996; Zanoni *et al.*, 1999; Shi and Le Maguer, 2000; Takeoka *et al.*, 2001; Graziani *et al.*, 2003; Sahlin *et al.*, 2004; Goula and Adamopoulos, 2005; Goula *et al.*, 2006; Toor and Savage, 2006). Lycopene appears to be a relatively stable compound during food processing but Sharma and Le Maguer, (1996) reported that heating of tomato pulp to produce paste, ketchup, juice, and other products may cause degradation of lycopene and other carotenoids. For example, heating tomato juice for 7 min. at 90 °C and 100 °C resulted in a 1.1 and 1.7% decrease in lycopene content, respectively (Shi and Le Maguer, 2000). Drying tomato halves at 110 °C for app. 4 hours caused a lycopene loss of 12% (Zanoni *et al.*, 1999). Also, semi-drying of tomato quarters at 42 °C for 18 hours resulted in 10.5-20.5% loss for three different cultivars (Toor and Savage, 2006). In another study, a statistically significant loss in lycopene at levels of 9-28% was observed as the tomatoes were processed into paste. According to Takeoka *et al.* (2001), significant losses in lycopene occurred during processing into paste and overall losses were less than 30% and much lower compared to those observed for pure lycopene in model systems under similar conditions.

Antioxidants naturally present in tomato such as vitamin C, vitamin E, flavonoids or carotenoids in tomato products may play a role in preventing the degradation of lycopene (Takeoka *et al*., 2001; Sanchez-Moreno *et al*., 2006). Shi *et al*. (2004) evaluated the synergistic effect of lycopene and other tomato carotenoids. Tomato juice which was enriched in vitamin C according to the label description showed the highest lycopene content among the juices tested. However, possible interactions of antioxidants with the other compounds in tomatoes, has not been sufficiently investigated (Riso and Porrini, 2001).

The loss of antioxidants as a result of oxidative damage especially during drying was also evaluated in several articles (Zanoni *et al*., 1999; Lavelli *et al*., 2000; Giovanelli *et al*., 2002; Goula *et al*., 2005). These studies proposed that oxidative damage can be avoided by optimizing operating conditions for both drying and storage of dried tomato products. For example, low temperature application for short times, reduction of tomato thickness, partial removal of water, or osmotic and vacuum drying techniques can be used to obtain dried tomatoes with higher antioxidant activities (Zanoni *et al*., 1999).

It has been reported that there is an increase in the bioavailability of some of the carotenoids due to heating and distruption of cell walls (Gartner *et al*., 1997; Russell, 2001). Böhm and Bitsch (1999) supported this view by demonstrating better absorption of lycopene in tomato juice (processed tomatoes) from the intestine than absorption in raw tomatoes.

Recently, researchers reported that antioxidant activity and nutritional properties of tomato products may also be improved by several heat treatments as well as increased bioavailability. A higher antioxidant activity was obtained through thermal treatments such as steaming, microwaving, frying, and drying of the tomato fruits (Chen *et al*., 2000; Chang *et al*., 2006). Graziani *et al*. (2003) showed that lycopene content significantly increased during heating of tomatoes in an oil bath at 100 °C for 2 h (Graziani *et al*., 2003). Re *et al*. (2002) also reported that processing tomato pulp under different temperatures applied to produce paste resulted in higher contents of lycopene and higher antioxidant activity. According to the results of Gahler *et al*. (2003), homogenization and thermal treatments during tomato juice production provided an increase in both of the hydrophilic and lipophilic antioxidant activity.

Shi and Le Maguer (2000) suggested that during processing heat induces isomerization of all *trans* to *cis*-forms, and Dewanto *et al*. (2002) found that total *trans* and *cis*-lycopene content in the tomatoes increased with increased heating time. Abushita *et al*. (2000) proposed that *trans* to *cis* isomerization of β-carotene and lycopene occurred during thermal processing of tomato particularly during dehydration step to produce the paste. Besides isomerization reactions thermal processing is reported to increase carotenoid concentration probably due to greater extractability, enzymatic degradation and unaccounted losses of moisture and soluble solids (Heinonen and Meyer, 2002).

Dewanto *et al*. (2002) and Chang *et al*. (2006) suggested that thermal processes might break down cell walls and weaken the bonding forces between lycopene and tissue matrix. Such distruptions in the cell walls may increase the release of phytochemicals from the matrix and make lycopene more accessible rendering an increase in nutritional quality and antioxidant activity of product. On the other hand, Abushita *et al*. (2000) reported that all *trans*-lycopene and the total carotenoid content increased by 37% and 29%, respectively, in the dry matter of tomato paste, most likely due to removal of seeds and peels and loss of soluble volatile compounds during evaporation steps. The increase in carotenoids by thermal processing was also attributed to enzymatic degradation which causes weakening in protein-carotenoid aggregates (Stahl and Sies, 1992; Sahlin *et al*., 2004). Similarly, for some other vegetables and fruits the increase in the amounts of some phenolic substances having antioxidant activity was associated to two concomitant events: the thermally induced extraction of antioxidant molecules previously complexed or polymerized and the retention of active compounds caused by the inactivation of the enzymes involved in their catabolism (Scalzo *et al*., 2004).

In the view of available literature it is apparent that heat treatment might produce changes in the extractability of phenolics due to the disruption of the plant cell wall and, thus, result in more easily release of bound polyphenolic and flavonoid compounds (Peleg *et al*., 1991). Heat treatment can deactivate endogenous oxidative enzymes. Therefore, another reason for the increased amount of antioxidants could also be explained by the prevention of enzymatic oxidation reactions which cause losses of such compounds in the raw plant materials (Dewanto *et al*., 2002; Choi *et al*., 2006). According to Gahler *et al*. (2003) another reason for the increase of the

hydrophilic antioxidant capacity is the formation of Maillard products. Maillard products are antioxidant active substances that are formed at high temperatures and are suggested to balance the loss of vitamin C or even to lead to an increase in the hydrophilic antioxidant capacity.

However, there are also conflicting results on the effect of thermal treatments in the current literature. Seybold *et al*. (2004), observed an increase in the lycopene content of tomato sauce from Spanish tomatoes whereas they have found a decrease in sauces prepared from Holland tomatoes.

In conclusion, the different behavior of processed tomatoes which has been observed as an increase or decrease in lycopene or other components might be due to the type or variety of the tomatoes used, agricultural treatments, conditions such as temperature, time, presence of oxygen or light and methods of processing, or it might be simply a matter of insufficient extraction of compounds to be analyzed. It was also reported that even the extractability of carotenoids may vary according to the ripening stage, firmness, and genotype of the fruit (Seybold *et al*., 2004). Therefore, it is somewhat difficult to compare all the data available on phenolics and antioxidant capacities of tomato and its products.

Vitamin C and tocopherols: The vitamin C content of tomatoes depends on variety and cultivation conditions (Abushita, 1996). During processing, vitamin C is destroyed mainly due to oxidation reactions and heat applied in the presence of air (Leoni, 2002). Particularly, industrial processing of tomatoes decreases the moisture content less than 15% in some products utilizing heat treatments (at about 60-110°C for 2-10 hours). Such high temperature applications cause oxidative stress in addition to the effect of oxygen. The decrease in vitamin C content of tomatoes by several heat applications and processes has been reported extensively in the literature (Zanoni *et al*., 1999; Giovanelli *et al*., 2002; Gahler *et al*., 2003; Sahlin *et al*., 2004). The degree of losses in ascorbic acid is highly related to the drying temperature used for the production of the end product. For example, drying at 80°C, resulted with 10% of ascorbic acid whereas all the ascorbic acid was lost upon drying at 110 °C. Abushita *et al*. (2000) also reported that during hot-break extraction, tomato lost about 38% of its original ascorbic acid, and further processing to produce tomato paste by vacuum evaporation caused the product to lose more than 16% of the

ascorbic acid content. In that study, 45% of the initial content of ascorbic acid was retained in the final tomato paste which can play an important role in prevention of tomato paste against oxidative degradation during storage or cooking (Abushita *et al*., 2000).

Vitamin E in tomatoes is predominantly represented by α-tocopherol. Like lycopene, vitamin E belongs to the lipophilic fraction of the tomato fruit and proved to provide positive effects on human health. Investigations on the effects of tomato processing on the content of vitamin E were limited similar to flavonoids. In a study conducted by Abushita *et al*. (2000) α-tocopherol lost 20.3% of its content during thermal processing of tomato paste, while α-tocopherol quinone and γ-tocopherol lost 46.5 and 32.7% of their original content, respectively. According to Seybold *et al*. (2004), homogenization and sterilization of tomatoes during production of tomato juice resulted in significant losses of contents of α-tocopherol both on wet and dry weight bases. In contrast, short-term heating of tomato sauce, tomato soup, and baked tomato slices led to a significant rise (51-73%) of α-tocopherol contents on wet as well as dry weight bases.

Total phenolics, flavonoids and antioxidant activity: Similar to the findings of lycopene, there are conflicting results for total phenolics, flavonoids or the total antioxidant acitivities of processed tomato samples in the current literature. Some studies indicate that a considerable loss of antioxidants is caused by the technological process. For example, Crozier *et al*. (1997) studied the effect of cooking on the quercetin content of onions and tomatoes. With both vegetables, boiling reduced the quercetin content by 80%, microwave cooking by 65%, and frying by 30%.

In contrary to those claims, those properties were found to be increased as a result of processing in other studies. For example, in a study conducted by Chang *et al*. (2006), two tomato varieties (Sheng-Neu (SN), and I-Tien-Hung (ITH)) were air dried (AD) at 80°C for 2 hours and then 60 °C for 6 hours, and, subsequently, total phenolic and total flavonoid contents were analyzed. Total flavonoid contents in AD-SN and AD-ITH were increased by 89% and 50% and the total phenolic contents increased by 13% and 50% compared to the fresh ones, respectively (Chang *et al*., 2006).

According to Re *et al*. (2002), the overall antioxidant activity of the hydrophilic extract of tomato paste was improved after processing. In this study, two samples were collected during tomato paste production: before and after the evaporation. The breaking process was applied using three different techniques: hot-break, cold-break and super cold-break. The results showed that total antioxidant activities of products obtained from hot break process were significantly higher than those of obtained from cold-break and super cold-break processes. In addition, TEAC value for the whole fruit was significantly lower than that of samples before evaporation and products of all three types of breaking techniques (Re *et al*., 2002).

Dewanto *et al*. (2002) also proposed that heat-treated samples had slightly higher contents of total phenolics and flavonoids but there were no significant differences between raw and processed samples at 88 °C for 2, 15, 30 minutes. However, the increase in the total antioxidant activity between the raw and heat processed tomatoes was found to be statistically significant. The increase in total antioxidant activity of the heat-processed tomatoes was explained by the increased amount of lycopene and other bound phytochemicals released from the matrix with thermal processing or a possible synergistic effect of other phytochemicals such as phenolics and flavonoids (Dewanto *et al*., 2002).

The total phenolics and carotenoids of tomato have been reported to be quite stable during processing under high temperature conditions, and thermal processing has been reported to release more bound phenolics due to the breakdown of cellular constituents (Dewanto *et al*., 2002). The increase of the hydrophilic antioxidant capacity was proposed to be depending on the increase of phenolics according to Gahler *et al*. (2003).

Changes in the antioxidant activity of tomato products are complex and highly depend on the specific compounds being studied. Initial results suggest that losses in antioxidant activity associated with decreases in lycopene concentration during processing may be accompanied by increases in the antioxidant activity of other components, particularly polyphenolics. Therefore, further studies characterizing the changes in polyphenol content and antioxidant activity during thermal processing should be performed to fully understand the role of processed foods in preventing human diseases (Takeoka *et al*., 2001).

Graziani *et al*. (2003) applied heat treatments in samples produced under both laboratory and industrial scale conditions to observe changes in carotenoids and antioxidant activity. However, since the processing methods and conditions were different for industrial and lab-scale production, the results were not comparable. Abushita *et al*. (2000) analyzed samples taken from three steps of paste processing (raw tomato, crushed sieved puree, and pasteurized paste) which was obtained from a canning factory in Hungary. The results showed that the contents of ascorbic acid and tocopherols decreased during processing while carotenoids unchanged or increased as a result of processing and *trans* to *cis* isomerization of β-carotene was observed. In another study, where samples were supplied from a real-life factory, Takeoka *et al*. (2001) observed a statistically significant decrease (9-28%) in lycopene and vitamin C as the tomatoes were processed into paste. Although both studies evaluated samples taken from a factory instead of lab-scale production, their findings are limited as evaluating of all the steps of processing would have given a better and a broader perspective to fully understand the effect of processing on antioxidants.

2.4.2. Non-thermal treatments

There is also a lack of information on the effect of non-thermal treatments such as cutting, homogenization, peeling, etc. on the antioxidant components of tomatoes. Lana and Tsijkens (2005) investigated the antioxidative capacity of fresh cut tomatoes and proposed that fresh cut tissues are primarily exposed to oxidative stress, presumably causing membrane damage and altering the composition and content of antioxidant compounds.

Gahler *et al*. (2003) investigated home-preparation methods such as peeling, tomato soup preparation, etc. and three different steps of tomato juice production including sieving, homogenization, sterilization, filling, and pasteurization. Those researchers proposed that homogenization increased the hydrophilic antioxidant capacity of different tomato products. However, the exact mechanism still remains unclear.

During direct consumption of tomatoes, consumers may prefer to remove some parts of tomatoes such as skin, calyx (top part), and sometimes seeds. However, it has been reported that tomato skin has higher levels of antioxidants comparing to the

pulp (Toor and Savage, 2005). This finding was explained by the fact that DNA-damaging UV light induces the accumulation of UV light-absorbing flavonoids and other phenolics, predominantly in the epidermis tissues of the plant body (Toor *et al*., 2006) and 98% of the total flavonols were found in the tomato skin as conjugated forms of quercetin and kaempferol phenolics (Canene-Adams *et al*., 2004). The majority of studies investigating the antioxidant composition of different fractions of tomatoes fractionated the tomato to skin and flesh portions while few studies investigated the seeds. In a recent study, other parts of tomatoes including calyx, columella, and jelly parenchyma as well as epidermis and pericarp were investigated with respect to their metabolite profile. The tissues which are generally removed during home-preparation such as calyx or epidermis were found to contain high contents of carotenoids, flavonoids or other compounds (Moco *et al*., 2007). Besides home processing, separation of certain parts of tomatoes may also cause a loss in antioxidants during industrial processing. For example, approximately one-third of the total weight of tomatoes in the form of skin and seeds is discarded during processing of tomatoes into paste (Al-Wandawi *et al*., 1985). Capanoglu *et al*. (2008) claimed that the losses in lycopene, β-carotene, and lutein caused by the removal of seed and skin during the production of tomato paste comprised the ratios of 4.6, 8.6, and 93.3%, respectively. Rest of the loss is presumably due to the oxidation reactions taking place during processing (Capanoglu *et al*., 2008). Another observation in this study is the increase in the rutin content and total flavonoids of tomatoes during the breaking step where the tomatoes are chopped into small particles and homogenized. This increase in the breaking step has been explained by the continuing synthesis of flavonoids as a response to wounding (Capanoglu *et al*., 2008). Similar observations were reported for apples (Abdallah *et al*., 1997), lettuce leaves (Kang and Saltveit, 2002), and potatoes (Tudela *et al*., 2002). On the other hand, Lana and Tjiskens (2006) found that fresh cut tomatoes have a lower antioxidant activity with respect to the intact fruit. However, they observed an increase in the hydrophilic antioxidant activity at the end of the storage period and reported that it might be associated to some repair or recycling mechanism.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals

The standards of chlorogenic acid, β-carotene, lutein, all *trans*-lycopene (from tomato, 90-95%), chlorophyll a and b from spinach, α-, δ- and γ-tocopherol were purchased from Sigma (St. Louis, USA), naringenin from ICN (Ohio, USA), rutin from Acros (New Jersey, USA), naringenin chalcone from Apin Chemicals (Abingdon, UK), ascorbic acid from Merck (Darmstadt, Germany), neoxanthin and violaxanthin from CaroteNature (Lupsingen, Switzerland), and lutein from Extrasynthese (Genay, France). The solvents of acetonitrile, methanol and chloroform were of HPLC supra gradient quality and were obtained from Biosolve (Valkenswaard, The Netherlands) and ethyl acetate (for HPLC) from Acros (New Jersey, USA). Tris (hydroxymethyl) methylamine (Tris) was purchased from Invitrogen (Carlsbad, USA). Meta-phosphoric acid (HPO_3), sodium chloride (NaCl), diethylene triamine pentaacetic acid (DTPA), butylated hydroxytoluene (BHT), and leucine enkaphaline were obtained from Sigma (St. Louis, USA). Formic acid (98-100%) for synthesis was purchased from Merck-Schuchardt (Hohenbrunn, Germany), monopotassium phosphate tetrahydrate ($KH_2PO_4.4H_2O$) pro analysis from Merck (Darmstadt, Germany) and dipotassium phosphate tetrahydrate ($K_2HPO_4.4H_2O$) 98% from Sigma (St. Louis, USA). MES buffer (2-Morpholinoethanesulfonic acid monohydrate), Bicine (N,N-Bis(2-hydroxyethyl)glycine), CAPS (N-cyclohexyl-3-aminopropanesulfonic acid) were obtained from Sigma (USA). Ultra pure water was produced on an Elga Maxima purification unit (Bucks, UK).

For the enzyme assay analyses Uridine 5'-Diphosphoglucose (UDP-Glu) Disodium from *Saccharomyces cerevisiae* was obtained from Sigma-Aldrich (Steinheim,

Germany) and NAP-5 desalting columns were purchased from Amersham Biosciences (Freiburg, Germany). Microcon Centrifugal Filter Devices were bought from Amicon Bioseparations, Millipore (Germany).

3.1.2. Tomatoes and tomato products

During the processing of the same batch starting from fresh tomato, intermediate processing materials yielded from the different steps, and the final tomato paste products were collected at an established tomato paste factory (Tamek Co.) in Bursa, Turkey. In total, 30 samples from 5 different processing events using independent tomato batches (biological replicates) were collected: two times in 2005 and three times in 2006 in order to create seasonal replicates with about 4 weeks intervals between each sampling time. All samples were snap-frozen in liquid nitrogen, transported to The Netherlands in dry ice, and subsequently ground to a fine powder using a precooled grinder (IKA Model A10). All samples were individually freeze-dried to compensate for the differences in water content, and stored at -80 ºC until analysis.

The scheme of factory-scale tomato paste production is shown in Figure 3.1. The first sample ("Fruit") was taken from tomatoes as they arrived at the factory. The second sample ("Breaker") was taken from the breaker unit where tomatoes had been separated, washed in a water bath, and transported by means of water tunnels. In this unit tomatoes were chopped with knives in a few seconds and collected in the tanks ready to be sent to the finisher unit. The third and fourth samples were taken after the material had been heated to 60-80 °C for 2.0-2.5 minutes in the Finisher unit, which was followed by the removal of seed and skin parts (sample "Seed & Skin") from the remaining pulp by means of filters (sample "Pulp"). The amount of seed and skin removed at this step represented about 3.5% of the original whole fruit wet-weight. The fifth sample ("Evaporator out") was taken after the pulp had been treated in a three-effect evaporator unit, where water was removed, by heating up to 80°C until a Brix value of 28-30° was reached. The sixth sample ("Paste") was collected after the final paste was canned and pasteurized (5-10 min at 93 °C). For each sample, five biological replications (from starting fruit material and all processing steps) were collected at five different time points in consecutive two years.

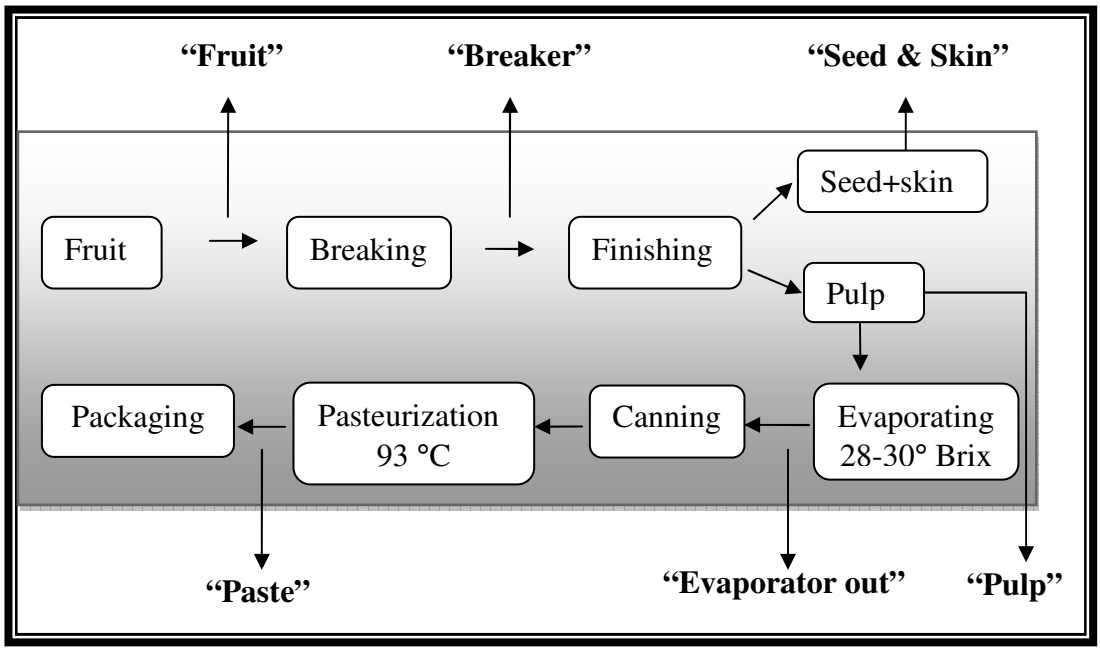


Figure 3.1: Production scheme of tomato paste samples (Arrows point the samples collected during processing).

3.1.3. Tissues of tomato at different developmental stages

For a single tomato cultivar, *Ever*, fruits were harvested at different stages of development, i.e. green, breaker, turning, pink, and red (Figure 3.2A-E) from plants grown in an environmentally controlled greenhouse located at Wageningen University. Tissues were collected from: the vascular attachment region (VAR) or calyx (CX), epidermis (EP), pericarp (PR), columella & placenta (CP), and jelly parenchyma (JE) (including the seeds) (Figure 3.2F), using 10 fruits for each developmental stage. Following collection of tissues all samples were immediately frozen in liquid nitrogen. After grinding the frozen tomato material, different tissue samples were freeze-dried. These samples were analyzed and quantified firstly for the occurrence of specific metabolites (isoprenoid derivatives and ascorbic acid) using Liquid Chromatography-Photodiode Array-Fluorescence Detector (LC-PDA-FD) and were then also profiled for semi-polar metabolites using Liquid Chromatography-Mass Spectrometry (LC-MS). Seeds from variety *Ever* were kindly provided by Seminis Seed Company, Inc. (Wageningen, The Netherlands).

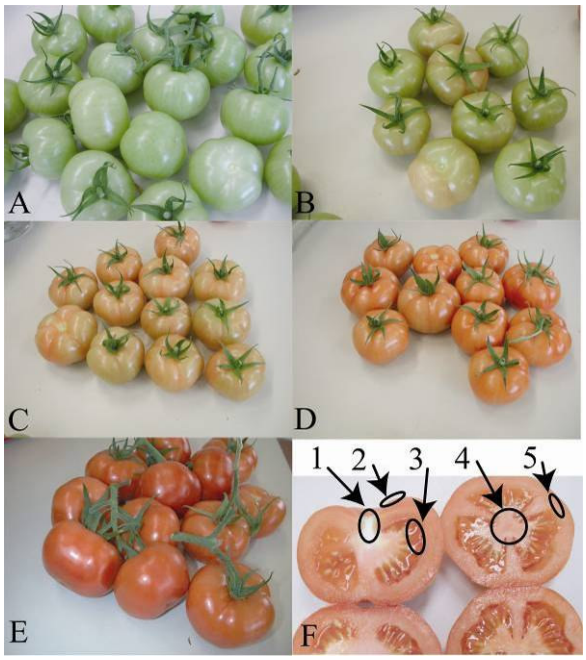


Figure 3.2: Fruit ripening stages of the tomato cultivar *Ever*: green (A), breaker (B), turning (C), pink (D) and red (E) and different tissues within the fruit: VAR (1), epidermis (2), jelly parenchyma (3), CP (4) and pericarp (5).

3.1.4. Tomato samples at different developmental stages for measurement of enzyme activity

Samples from the processing steps of tomato paste production as defined in Figure 3.1 were transported in dry ice from Wageningen, The Netherlands to Gatersleben, Germany for the analysis of the 3-*O*-glucosyl transferase (3GT) and phenylalanine ammonia lyase (PAL) enzymatic activities.

In the second part of the study, tomatoes of *Moneymaker* variety at 6 different developmental stages (green, breaker, turning, pink, red, dark red) were grown in green house of Gatersleben, IPK (Light: 16 hours, Temperature: Day 20 °C, Night 18 °C) as shown in Figure 3.3. All samples were collected and snap-frozen in liquid nitrogen, and subsequently ground to a fine powder using a precooled grinder (IKA, Model A11). All samples were stored at -86 ºC in deep-freezers (Sanyo VIP Series) until analysis.

The tomatoes were divided into flesh and skin parts and analyzed to observe the enzymatic changes taking place during the development of tomato.

Figure 3.3: Developmental stages of tomato.

3.2. Methods

The chemical analyses applied on collected samples are summarized in Table 3.1.

Table 3.1: Summary of all analyses carried out in tomato and tissue samples.

Analyses	**Samples**		
	Tomato, intermediate processing materials, and Paste	Tissues of different developmental stages of tomato cult. *Ever*	Flesh and skin of different developmental stages of tomato cult. *Moneymaker*
Moisture content	√	√	
Total phenolics	√		
Total flavonoids	√		
Antioxidant capacity assays	√		
On-line antioxidant detection	√		
Flavonoid analysis	√	√	√
Carotenoid analysis	√	√	
Metabolomics studies	√	√	
3GT activity measurement	√		√
PAL activity measurement			√

3.2.1. Moisture analysis

Moisture contents of the samples were analyzed according to "Association Official of Analytical Chemists" (AOAC, 1990) method 925.10. Approximately 2 g of each sample was taken and exactly weighed before and after air-drying at 130 °C until the equilibrium is reached (app. 2-3 hours) in oven.

3.2.2. Determinations of optimum solvent for the analysis of phenolic content and antioxidant capacity

The most effective extraction solvent system for the analysis of total phenolics and antioxidant capacity was investigated. Three different solvents included 80% methanol, 80% acetone, and ethyl acetate. Three mL of each solvent was added to 0.2 g ± 0.01 g freeze-dried samples. After shaking for 4 hours at 4-7 °C, the solutions were centrifuged at 2500 rpm for 10 minutes and the supernatants were analyzed for their total phenolic content and antioxidant capacities using ABTS and DPPH methods (Sahlin *et al*., 2004). Based on those results and available information in the literature printing out the higher extraction yields of phenolic compounds obtained by an increase in polarity of the solvent (Moure *et al*., 2000; Cheung *et al*., 2003), methanol was selected for the extraction of hydrophilic phenolic compounds. As lipophilic phenolics may also be present in tomatoes (Abushita *et al*., 2000) the residue was re-extracted with chloroform and dissolved in isopropanol in order to extract the remaining lipophylic residues if available.

The procedure applied for the preparation of hydrophilic and lipophilic extracts were as follows: Three mL 75% methanol was added to 0.2 ± 0.01 g freeze-dried sample and sonicated for 15 min. After centrifugation at 2500 rpm for 10 min, the supernatant was collected, and another 2 mL 75% methanol was added to the pellet. This extraction procedure was repeated twice. Both supernatants were combined and this solution was used as the hydrophilic extract. Following the extraction of lipophilic compounds from the pellet based on the procedure for carotenoid extraction as described at 3.2.8 the metabolites were dissolved in 5 mL of isopropanol. This solution was used as the lipophilic extract.

3.2.3. Analysis of total phenolics

The level of total phenolics was estimated using Folin- Ciocalteau method (Singleton and Rossi, 1965; Spanos and Wroldstad, 1990; Toor and Savage, 2006) by mixing 100 µL sample extract, 900 µL pure water and 5 mL Folin-Ciocalteau reagent. Then, 4 mL saturated Na_2CO_3 was added and 2 hours later, the absorbance was measured at 765 nm. For the preparation of a standard curve, 0.10-0.50 mg/mL gallic acid was used and data were expressed in mg gallic acid equivalents (GAE) per 100 g dry weight. The calibration curve is shown in Appendix, Figure A.1.

3.2.4. Analysis of total flavonoids

0.25 mL sample extract or standart was mixed with 1.25 mL of ultra pure water. After adding 75 µL of 5% $NaNO_2$ solution and, by allowing to stand for 6 minutes, 150 µL 10% $AlCl_3.6H_2O$ was added. After 5 minutes 0.5 mL of 1 M NaOH was added and the total volume was adjusted to 2.5 mL with ultra pure water. Samples were vortexed for 10 seconds and measurements was done at 510 nm against a reagent blank with a spectrophotometer (Perkin Elmer, 2000). Rutin with concentrations of 0.05-0.50 mg/mL were used as standart (Dewanto *et al*., 2002). The calibration curve is shown in Appendix, Figure A.2.

3.2.5. Total antioxidant capacity assays

Four different *in vitro* tests were applied to estimate differences in total antioxidant levels between samples using Perkin Elmer UV-VIS (2000) model Spectrophotometer. The calibration curves obtained by each method are shown in the Appendix, Figures A.3-A.10.

The ABTS (2,2- azinobis 3-ethylbenzothiazoline-6-sulfonic acid diammonium salt) method was based on the method of Miller and Rice Evans (1997) with slight modifications. For the hydrophilic extracts the ABTS stock solution was diluted in 50 mM potassium phosphate buffer pH 8.0 instead of 5 mM Phosphate Buffered Saline (PBS). Then, 100 µL sample extract or reference was mixed with 1 mL ABTS-working solution (final pH of the reaction mixture was about 7.4). After exactly 40 seconds the remaining ABTS radicals were measured at 734 nm. Trolox was used as a reference compound and results were expressed in terms of µmol Trolox Equivalent Antioxidant Capacity (TEAC) per 100 g dry weight.

The DPPH (1,1-diphenyl-2- picrylhydrazyl) method (Kumaran and Karunakaran, 2002; Rai *et al*., 2006) was performed by mixing 100 µL sample extract with 2 mL 0.1 mM DPPH in methanol (not buffered). After incubating at room temperature for 30 min in the dark, the absorbance of the mixture was measured at 517 nm. The results were expressed as µmol TEAC/100 g dry weight.

The FRAP (Ferric Reducing Antioxidant Power) method (Benzie and Strain, 1996) was performed using 100 µL sample extract or Trolox which was pipetted into 1.5 mL reaction tubes, followed by the addition of 900 µL FRAP reagent (pH 3.6). This mixture was then quickly vortexed for 20 sec. Absorbance was measured at 593 nm against a reagent blank exactly 4 min after the addition of sample to the FRAP reagent. The results were expressed as µmol TEAC/100 g dry weight.

The CUPRAC (Copper Reducing Antioxidant Capacity) method was performed according to the method previously described by (Apak *et al*., 2004; Apak *et al*., 2006; Bektaşoğlu, 2006). The method involved the preparation of 10^{-2} M Copper (II) chloride solution, ammonium acetate (NH_4Ac) buffer (pH=7), and $7.5x10^{-3}$ M Neocuproin (Nc) solutions. To a test tube 1 mL each of Cu (II), Nc, and NH_4Ac buffer solutions were added. Sample extract or standard solution (100 mL) and H_2O (1 mL) were added to the initial mixture so as to make the final volume 4.1 mL. The tubes were stoppered, and after 1 h, the absorbance at 450 nm was recorded against a reagent blank. The results were expressed in terms of µmol TEAC/100 g dry weight.

3.2.6. On-line antioxidant capacity

An HPLC-PDA system (Figure 3.4) coupled to post-column antioxidant detection, in which ABTS cation radicals are mixed on-line with the separated compounds was used to determine which antioxidant species are present in the aqueous-methanol tomato extracts. The on-line ABTS-antioxidant reaction lasted 30 sec, at 40°C and pH 7.4 (Koleva *et al*., 2001; Beekwilder *et al*., 2005).

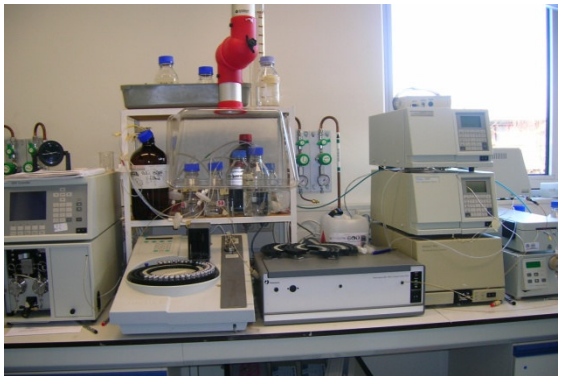


Figure 3.4: On-line antioxidant detection system.

The HPLC system comprised a Waters 600 control unit, a Waters 996 photodiode array (PDA) detector, and a column incubator at 40 °C. Columns used for flavonoid and carotenoid analyses were LUNA 3 µ C18 (2) 150x4.60 mm and YMC C30, 250 x 4.6 mm S-5 µm column, respectively. Solvent systems were A (Ultra pure water with 0.05% formic acid) and B (acetonitrile with 0.05% formic acid) for flavonoids and A (ultra pure water:methanol, 20:80 v/v, 0.2% amonium acetate in ultra pure water), B (*t*-butyl methyl ether), and C (methanol) for carotenoids with a flow rate of 1 mL/min. Separation of compounds in the extracts was conducted in a 60 min run. Compounds eluting from the column passed first through a PDA detector (set at an absorbance range of 240-600 nm) and were then allowed to react for 30 s with a buffered solution of 2,2-azinobis, 3-ethylbenzothiazoline-6-sulfonic acid (ABTS; Roche) cation radicals online, before passing through a second detector, which monitored ABTS reduction. The ABTS radical solution was prepared by dissolving 55 g of ABTS in 50 mL of water, followed by the addition of potassium permanganate (19 mg potassium permanganate was dissolved in 1 mL water and 0.5 mL of this solution was added). After 16 h of incubation in the dark, the radical solution was diluted further with 0.1 M pH 8.0 potassium phosphate buffer until the absorbance of 1.2 was reached for flavonoids, and for carotenoids, the dilution of ABTS was done by methanol instead of the buffer. The postcolumn reaction loop was a 3 m stainless steel tube (internal diameter 0.508 mm) at 40 °C. The decreased absorption by ABTS by reaction with antioxidants was monitored as it passed through a dual-wavelength UV-vis detector (Waters) at 412 and 650 nm. The absorption at 412 nm was the maximum absorption of the ABTS cation, while the 650 nm absorption was used when interference of compounds at 412 nm was observed.

3.2.7. Flavonoid composition

Non-hydrolyzed phenolic extracts were obtained by extracting 25 (± 0.05) mg dry weight with 2.0 mL 75% methanol in ultrapure water. After 15 min. sonofication, the samples were centrifuged at 2500 rpm for 10 min., and filtered from 0.2 µm filters (Bino *et al*., 2005). The filtrates were injected to the online-antioxidant detection HPLC system as described in section 3.2.6. Calibration curves for some of the flavonoids are shown in Appendix, Figure A.11-A.16.

Hydrolysis of flavonoids was performed as described by Muir *et al*. (2001), by weighing 30 ± 0.5 mg freeze-dried sample and adding 0.57 mL water, 1 mL methanol containing 0.5% (w/v) TBHQ, and 0.4 mL 6M HCl, followed by 1 h hydrolysis at 90°C.

3.2.8. Carotenoids

3.2.8.1. Selection of extraction method

For the analysis of carotenoids, 5 different extraction methods were tested for their efficiency in extracting lycopene from both "Fruit" and "Paste" samples. The first method was developed by Sahlin *et al*. (2004) using 80% acetone ("method 1"). The mixture of 5 mL of chloroform, 3 mL of acetone, and 15 mL hexane was used according to the method of Dewanto *et al*. (2002) representing the second method ("method 2"). In the third method, 35 mL ethanol and hexane (4:3, v/v) was used according to the procedure of Taungbodhitham *et al*. (1998) ("method 3"). The fourth method included the extraction of carotenoids using 40 mL tetrahydrofuran and methanol (1:1, v/v) mixture according to the method of Jaarsveld *et al*. (2006) ("method 4"). The method yielding the highest recovery in both type of samples was named as fifth method ("method 5"), which was used in further experiments (Bino *et al*., 2005). Briefly, freeze-dried samples (25 ± 0.05 mg) were extracted with 4.5 mL of methanol:chloroform solvent mixture (2.5:2.0 v/v), shaked with mini-shaker, and waited for 5 min. Afterwards, 2.5 mL of Tris buffer (pH = 7.5, with 1 M NaCl) was added and shaked again. After 5 min. the samples were centrifuged at 2500 rpm for 11 min., and the lower chloroform phase was separated. The upper phase was washed with 1 mL chloroform three times and all the chloroform phases were collected. After the complete evaporation of chloroform phase under nitrogen, 1 mL of ethyl acetate was added, and sonicated for 10 min. The samples were used for further HPLC analysis for carotenoids.

3.2.8.2. Carotenoid and chlorophyll composition

For chromatographic separation of carotenoids the extracts (10 µL) were injected into a LC-PDA-FD system composed of a W600 pump system (Waters Chromatography, Milford, MA, USA) equipped with a YMC-Pack reverse-phase

C30 column (250 x 4.6 mm, particle size 5 µm), maintained at 40 °C. Eluting compounds were detected using a Waters 996 PDA detector over the UV/Vis range of 240 to 750 nm coupled online to a Waters 2475 fluorescence detector. Data were analyzed using "Empower Pro" software (2002, Waters). Measurements for neoxanthin and violaxanthin were made at 440 nm, chlorophyll b at 470 nm, β-carotene, lutein and lycopene at 478 nm, and chlorophyll a at 665 nm. The quantification of carotenoids was based on calibration curves constructed from injecting known amounts of the respective standard compounds.

3.2.9. Tocopherol composition

The same extraction procedure as carotenoids was applied for tocopherol analysis. HPLC system defined in 3.2.8.2 for carotenoids was used to analyze tocopherol content of samples. α-, δ- and γ-Tocopherols were detected using Waters 996 PDA detector over the UV/Vis range of 240 to 750 nm coupled online to a Waters 2475 fluorescence detector with excitation at 296 and emission at 340 nm.

3.2.10. Analysis of vitamin C

For the analysis of ascorbic acid, a 5% (m/v) metaphosphoric acid with 1 mM aqueous diethylene triamine pentaacetic acid (DTPA) was prepared as extraction solution (continuous stirring and sonication was needed to obtain a homogeneous solution). This solution was stored at 4 ºC before analysis. To 25 (± 0.05) mg freeze-dried tomato tissue, 0.475 mL water was added, immediately followed by 2 mL ice-cold extraction solution. The extracts were stirred and left on ice before 15 min sonication. After centrifugation at 2500 rpm for 10 min, the supernatants were filtered through 0.2 µm polytetrafluoroethylene filters and taken for LC-PDA analysis. The same LC-PDA system was used as for the analysis of isoprenoids. Separation was performed at 30°C on a YMC-Pack Pro C18 (150 x 4.6 mm, 5 µm particle size) column using 50 mM phosphate buffer (pH 4.4) as mobile phase. After 15 minutes separation, the column was washed with acetonitrile and reconditioned for the injection of the next sample (Helsper *et al*., 2003). The detection and quantification of ascorbic acid was made at 262 nm by means of a calibration curve using ascorbic acid as standard which is shown in the Appendix, Figure A.17.

3.2.11. High Performance Liquid Chromatography Quadrupole Time-of-Flight Tandem Mass Spectrometry based metabolomics

For the analysis of the fruit tissues from the cultivar *Ever*, the extraction procedure described in section 3.2.7 was applied for non-hydrolyzed aqueous methanol extracts using 25 ± (0.05) mg freeze-dried material and 2 mL 75% methanol (in 3 replicates). The seeds of *Ever* and *Money Maker* were also extracted with this procedure using 50 ± (0.05) mg in 2 mL methanol. The extracts obtained were taken for High performance liquid chromatography quadrupole time-of-flight tandem mass spectrometry (LC-QTOF MS) analysis in ESI negative mode, as previously described (Moco *et al*., 2006, De Vos *et al*., 2007). In brief, a Waters Alliance 2795 HT system equipped with a Luna C18 (2) pre-column (2.0 x 4 mm) and analytical column (2.0 × 150 mm, 100 Å, particle size 3 µm) from Phenomenex (Torrance, CA, USA) were used for chromatographic separation. The HPLC system was connected online to a Waters 2996 PDA detector and subsequently to a QTOF Ultima V4.00.00 mass spectrometer (Waters-Corporation, MS technologies, Manchester, UK). For LC-MS measurements 5 µL of sample (methanolic extract) was injected into the system and for LC-MS/MS 10 µL. The MS/MS measurements were made with increasing collision energies according to the following program: 10, 15, 25, 35 and 50 eV. Leucine enkaphalin ($[M-H]^-$ = 554.2620) was injected through a separate inlet and used as 'lock mass'.

3.2.11.1. Data analysis and alignment

Acquisition, visualization and manual processing of LC-PDA-MS/MS data were performed under MassLynx™ 4.0 (Waters). Mass data were automatically processed by metAlign version 1.0 (Anon., 2006a). Baseline and noise calculations were performed from scan number 70 to 2,400, corresponding to retention times 1.4 min to 48.6 min. The maximum amplitude was set to 35,000 and peaks below two times the local noise were discarded (de Vos *et al*., 2007). The accurate masses from the metAlign-extracted peaks were recalculated from the 3 top scans of each MassLynx-signal.

3.2.11.2. Annotation of metabolites

The obtained datasets were analyzed as [retention time x accurate mass x peak intensity] matrixes for metabolite identification. The matrix was reduced by discarding all signals below a signal intensity of 100 (ion counts/scan at the centre of the peak) and those eluting within the first 4.0 min of chromatography. This dataset was then checked for the presence of known tomato fruit metabolites using the MoTo Database (DB) (Anon, 2006b), after manually calculating the accurate masses by taking into account an mass signal intensity ratio of analyte versus lock mass of 0.25-2.0 (Moco *et al*., 2006). For mass signals lower than 0.25 x lock mass intensity it was impossible to calculate a correct accurate mass. To annotate compounds, the tolerance for mass deviation was set at 5 ppm, taking into account the correct analyte/lock mass ratio. For an observed accurate mass, a list of possible molecular formulas was obtained, selected for the presence of C, H, O, and N, S or P. In addition, raw datasets were checked manually in MassLynx software for retention time, UV/VIS spectra and QTOF-MS/MS-fragmentation patterns for chromatographically separated peaks not present in the MoTo DB, to complement the accurate mass-based elemental formulas.

3.2.11.3. Multivariate analyses of LC-MS data

For the comparison and visualisation of the main tendencies of the LC-MS data acquired for the tissues of *Ever* at different ripening stages, the data matrix obtained from MetAlign was loaded into Genemaths software (Applied Maths, Belgium). Principal component analysis was performed after logarithmic (of base 2) transformation and standardization of mass signals over the samples (by subtraction of the average).

The LC-MS derived dataset after processing by Metalign initially consisted of about 20,000 mass peaks aligned across all samples analyzed. Low intensity mass peak patterns were discarded (as described above), thereby reducing the data set to 10,388 mass peaks. Most compounds were usually represented by a number of ions (isotopes and unintended fragments and adducts) that makes the entire LC-MS data highly redundant. This redundancy was removed by clustering of mass peak patterns using an approach called Multivariate Mass Spectra Reconstruction (MMSR) (Tikunov *et*

al., 2005). This resulted in 504 mass peak clusters, each of which was represented by a single mass signal in further analyses. A small data set containing the quantified levels of carotenoids, tocopherols, chlorophylls and ascorbic acid, analyzed by LC-PDA-Fl, was appended to the LC-MS data resulting in a final (variable) data set comprising 528 components. Each variable was normalized across the samples using range scaling (Smilde *et al*., 2005). The normalized data were subjected to an unsupervised clustering using Self Organizing Tree Algorithm (SOTA) (Herrero *et al*., 2001). Fourteen clusters with significant internal variability ($p < 0.001$) were derived using this procedure.

3.2.12. Enzyme activity measurements

3.2.12.1. 3-*O*-Glucosyl transferase assay

Optimization of the extraction and assay conditions: The optimal pH of 3-*O*-glucosyl transferase (3GT) (EC 2.4.1.91) was determined under standard assay conditions at pH values of 5.0-6.0 (50 mM MES buffer), 7.0 (50 mM MES, and Tris-HCl buffer), 8.0 (50 mM Tris–HCl, and Bicine buffer), 9.0 (50 mM Bicine buffer), and 10.0 (CAPS buffer).

Saturation curve for the substrate was performed from 0.01 M to 1.0 M of quercetin. Reaction time curve was also prepared from 10 to 60 minutes within 10 minute intervals.

Extaction from the material: Samples were ground into a fine powder using a precooled grinder (IKA A1000). 500 µL of extraction buffer was added on 100 mg of fresh weight sample and homogenized on ice. Extraction buffer was composed of 50 mM Bicine buffer (pH 9.0), 1 mM Ethylene diamine tetracetic acid (EDTA), 10 µL protease inhibitor mixture (Serva), 0.1% (v/v) Triton X-100, and 10 mM Dithiothreitol (DTT). Samples were then centrifuged at 16,000 rpm for 10 minutes at 4°C and supernatants were filtered from NAP-5 column (Suzuki *et al*., 2005). The filtered samples were used for enzyme assay and protein content analysis by Bradford method.

Enzyme assay: The standard assay for 3GT activity was performed using HPLC to measure the initial rate of increase in the isoquercitrin concentration in the reaction mixture. The standard assay mixture consisted of 50 mM Bicine buffer (pH 9.0), 1 mM EDTA, 50 mM NaCl, 0.1% (v/v) Triton X-100, 0.1 mM quercetin and 10 mM UDP-Glucose in a final volume of 100 µL. 55 µL of the crude extract was mixed with 25 µL of Bicine assay buffer, 10 µL of 1 mM quercetin, 10 µL of 10 mM UDP-Glucose. The reaction was performed at 30 °C and then stopped by the addition of 100 µL methanol. One unit of the 3GT activity corresponded to the amount of enzyme activity that produces 1 pmol of isoquercitrin per minute under standard assay conditions (Suzuki *et al*., 2005). The specific enzyme activity was calculated with the formula: (pmole of enzyme/time of reaction in seconds*protein content in the extract).

The isoquercitrin concentration was detected using HPLC coupled with W2690 Controller unit (Waters) and Waters 996 PDA dedector. Separation was performed on C18, Phenomenex Aqua column, 125 A°, 250 x 4.60 mm, 5 µ (Figure 3.5). The calibration curves prepared for isoquercitrin, quercetin, quercitrin, and rutin are presented in Appendix, Figures A.13-A.16.

The enzyme assay measurement was repeated using Ultra Performance Liquid Chromatography-Mass Spectrometry (UPLC-MS) system for tomato processing samples because of the conflicting results obtained by HPLC. The LC-MS analyses of fractions were elucidated on an UPLC™ (Acquity UPLC™, Waters, Eschborn, Germany) with a photodiode array detector (2996, Waters) coupled on-line with a mass detector. LC-separation was performed by reverse-phase chromatography on an Acquity UPLC™ BEH C18, 1.7µm, 4.1x50 mm column (Waters), maintained at 25°C. The mobile phase was composed of 98% water, 2% formic acid solution (= 5% ammonium formate in formic acid) (solvent A) and 100% acetonitrile (solvent B). At a flow rate of 0.2 mL/min, the following gradient was applied: initial 0% B; 1.25 min, 0% B; 10 min, 40% B; 11 min, 100% B; 12 min, 100% B; 12.2 min, 0% B, 13 min, 0% B.

10.000 absorbance spectra were recorded every 1 s, between 210 and 600 nm, with a bandwidth of 1.2 nm, and chromatograms were extracted from the PDA data at 280 and 535 nm. Data were analyzed using Waters MassLynx software.

The LCT Premier™ time-of-flight (TOF) mass spectrometer was equipped with an electrospray ionization (ESI) and modular LockSpray™ interface. The capillary voltage was 2.200 V, and the source temperature was 100°C. Spectra were recorded in positive-ion W-mode between mass-charge ratios of 100 and 1.000. Mass calibration was performed in the range of 100-1000 *m/z* with phosphoric acid. LockSpray™ interface was used to allow automated accurate mass measurements of the plant metabolites. Leucine enkephalin, 2 ng/µL in 1/1 (v/v) acetonitrile/water, was employed during Lockspray operation as internal mass reference.

(A) **(B)**

Figure 3.5: (A) HPLC and (B) UPLC-MS systems used for the measurement of the substrate and product concentrations.

3.2.12.2. Protein determination

The total soluble protein concentration was measured according to the method reported by Bradford (Bradford, 1976) using bovine serum albumin as a standard. Calibration curve is presented in the Appendix, Figure A.18. The measurements were performed using a Molecular Devices Spectra Max Plus spectrophotometer coupled with SOFTMAX PRO®.

3.2.12.3. Phenylalanine ammonia lyase assay

Fine powdered plant material was mixed with 100 mM Sodium tetraborat (Borax) buffer (pH 8.8) including 5 mM mercaptoethanol and protease inhibitor mix with a ratio of 1:1 (mg sample: µL buffer). A small spoon of unsoluble Polyclar AT is added and homogenized on ice. After incubation on ice for 30 min, sonification was

done for 30 seconds for 2 times. Samples were centrifuged for 5 min. at 10000 g. This procedure was repeated untill the total volume of 500 µL was obtained. Collected supernatants were centrifuged and filtered through NAP5 desalting column (Amersham Biosciences). The samples were concentrated using an Amicon concentrator (Millipore, Germany). The protein content of the concentrated extract was measured by Bradford method and the enzyme assay was performed.

For the assay, 180 µL protein extract was mixed with 20 µL of 100 mM L-Phenylalanin which has been incubated at 37 °C. The kinetic reaction was performed using microplate at 290 nm for 2 hours within intervals of 3 minutes (Figure 3.6). Assay without sample, without phenylalanine and a heating control were also carried out as controls. The curves obtained after the kinetic reaction of PAL (EC 4.3.1.5) was used for the calculation of the enzyme activity. Then the specific enzyme activity was calculated according to the calibration curve of *t*-cinnamic acid, which is the product of the reaction (Appendix, Figure A.19).

According to the calibration curve, 0.1 AU (absorbance unit) is equal to 4.464 nmol (4464 µmol) *t*-cinnamic acid. Specific activity (SA) is calculated in terms of (µkat.mg-1) using the formula (3.1):

$SA = (\Delta OD/\Delta t).\ (1000/\text{ protein content } (\mu g).\ (4464\ \mu mol/0.1\ AU)$ **(3.1)**

$\Delta OD\ (\Delta AU) = (AU2 - AU1)$ obtained from the kinetic reaction diagram.

$\Delta t = (t_2 - t_1)$ where t is the time in seconds that corresponds to the AU_1 and AU_2 values. Protein content is the total amount of protein determined by the Bradford method as explained above.

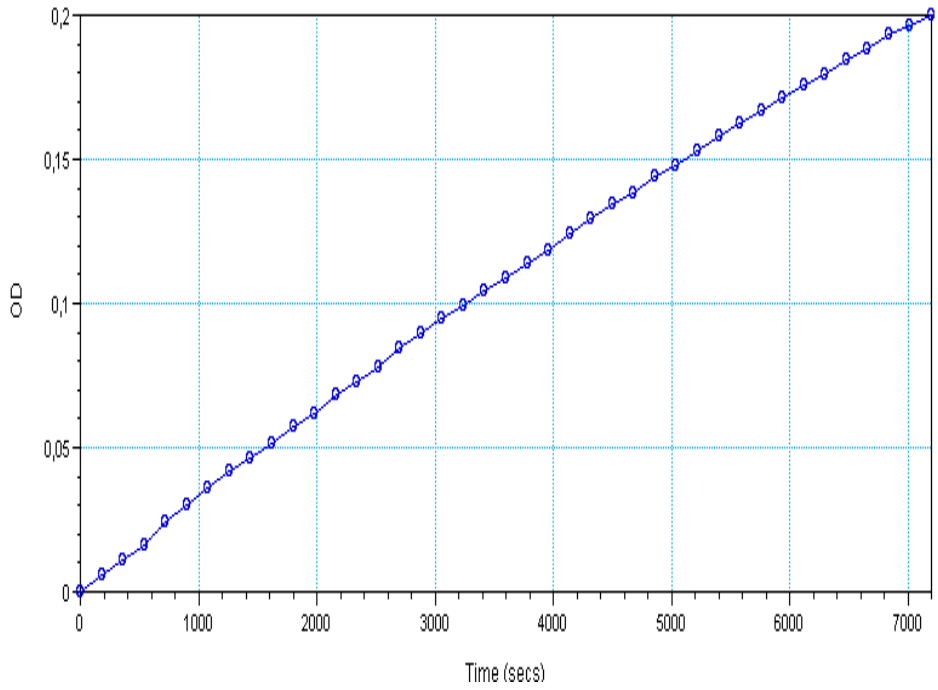


Figure 3.6: Kinetic reaction diagram for PAL assay.

3.2.13. Statistical analysis

All analyses were performed in triplicate. Data were subjected to statistical analysis using SPSS software (version 11.5 for Windows XP, SPSS Inc.) for the Analysis of Variance (ANOVA). Duncan's New Multiple Range Test was used to analyze differences between treatments, and Student t-test was performed to analyze the differences of the data obtained from LC-QTOF-MS analyses. For multivariate analysis (Cluster Analysis and Principal Component Analysis-PCA), the LC-MS data were read into GeneMaths software (Applied Maths, Belgium) after 2 log-transformation of mass signal intensities. Mass signals (variables) were normalized by dividing by the mean of each variable (as described in detail in section 3.2.11.2 and 3.2.11.3). Statistical analysis tables are given in the Appendix Table B.1.

4. RESULTS AND DISCUSSION

4.1. Effect of Tomato Processing on Antioxidative Compounds

In this study samples representing a number of different well-defined processing steps were taken from a Turkish tomato paste producer. In order to decrease batch-to-batch variations, each process step was independently sampled five times using different batches processed during two successive years. Each sample was analyzed for the contents of moisture, total phenolics, total flavonoids, antioxidant capacity, carotenoid and flavonoid profiles, tocopherols, vitamin C, and metabolomics.

4.1.1. Moisture contents

Moisture contents of each step of processing are shown in Table 4.1. The dry-matter content from fruit to paste increased from 5% to 30%.

Table 4.1: Moisture contents of paste processing steps[1].

Processing Steps	Moisture content, %
Fruit	94.6 ± 0.9 a
Breaker	94.2 ± 0.7 a
Finisher pulp	94.5 ± 1.4 a
Seed & Skin	78.7 ± 3.1 b
Evaporator out	71.6 ± 5.1 c
Paste	71.9 ± 2.7 c

[1]Data represent average quantities ± standard deviation of 5 independent processing events. Different letters in the columns represent statistically significant differences ($p<0.05$).

The total moisture loss was found to be about 23% by processing fruit into the paste. A difference of 19% moisture from tomato to paste was reported by Periago *et al*. (2007). The moisture contents observed at the first breaking step and the pulp obtained after the separation of the seed and the skin were insignificant from that of the fruit. However, the moisture content of seed and skin fraction was significantly lower than those of the fruit and the pulp. In the evaporator out fraction which is obtained after the evaporation of the pulp, the moisture content decreased

significantly as expected ($p<0.05$). There was no significant difference between moisture contents of the evaporator out and the paste samples. Since the moisture contents changed in each step, samples were freeze-dried for reporting the results of further analysis. It is known that freeze-drying process has no significant effect on antioxidant activity or total phenolics (Toor *et al*., 2006).

4.1.2. Solvent choice for spectrophotometric methods

The results of three different solvent systems (80% methanol, 80% acetone, and 100% ethyl acetate) are given in Table 4.2.

Table 4.2: Comparison of three solvent systems to determine total phenolics and antioxidant capacities in tomato and paste samples[1].

	Extraction solvent	Tomato	Paste
Total Phenolics (mg GAE/100 g dry weight)	Methanol	315.1 ± 0.0	333.4 ± 1.5
	Acetone	271.1 ± 3.9	298.4 ± 7.7
	Ethyl acetate	-	-
ABTS method (µmol TEAC/100 g dry weight)	Methanol	2,385.8 ± 10.7	2,174.7 ± 21.3
	Acetone	1,813.5 ± 28.2	2,000.8 ± 0.0
	Ethyl acetate	1,075.9 ± 47.5	719.1 ± 103.7
DPPH method (µmol TEAC/100 g dry weight)	Methanol	1,619.5 ± 8.9	1,093.3 ± 99.6
	Acetone	1,529.1 ± 43.7	1,075.8 ± 82.0
	Ethyl acetate	1,040.2 ± 0.20	799.3 ± 6.1

[1]Data represent average quantities ± standard deviation of 3 replicates.

According to the results, total phenolic contents of tomato and paste in methanol extract were 14% and 11% higher than that of acetone extract. No measurement could be done with ethyl acetate due to the fast evaporation of ethyl acetate. ABTS and DPPH results of tomato sample showed that methanol extract yielded with 24% and 6% higher values than acetone extractions, respectively. Similarly, yields obtained with methanol extractions were much higher compared to the ethyl acetate extracts using both antioxidant assays (55% in ABTS method and 36% in DPPH method).

In addition, paste samples also presented higher ABTS and DPPH results when methanol was the choice of solvent for extraction. The antioxidant capacities were 8% and 2% higher with methanol extraction compared to those of acetone, respectively, with both antioxidant capacity assays. Moreover, methanol was

particularly much more efficient than ethyl acetate as ABTS and DPPH methods yielded 67% and 27% higher values, respectively.

It is known that higher extraction yields of phenolic compounds can be obtained by increasing polarity of the extraction solvent (Cheung *et al*., 2003). Moure *et al*. (2000) also showed that total phenolic content in the methanol or ethanol extracts of seed hulls was higher than that in the acetone extract. In the light of current literature and the results obtained in this study methanol was selected for the extraction of hydrophilic phenolics. These hydrophilic extracts were used for the analysis of total phenolics, total flavonoids and antioxidant capacity tests.

After the extraction of hydrophilic antioxidants, there might be still remaining hydrophobic phenolic compounds. Those lipophilic structures deserve attention since they might also possess antioxidant activity (Abushita *et al*., 2000). In the view of current literature the antioxidant capacity tests were performed on both hydrophilic and lipophilic extracts. The results of the assays are given in section 4.1.5 in detail. The extraction procedure of lipophilic compounds applied for antioxidant capacity tests was based on the carotenoid extraction method in order to obtain comparable results with HPLC and spectrophotometric methods. The only difference in two extraction methods was the solvent used at the final stage to dissolve the antioxidants. Isopropanol was used for spectrophotometric methods instead of chloroform due to its high immiscibility in water, which is an important characteristic for antioxidant assays.

4.1.3. Total phenolics

Total phenolic contents of the samples are presented in Table 4.3 and Figure 4.1. In tomato samples, total amount of phenolics was found to be 576.5 ± 76.2 mg GAE/100 g dry weight. Those findings were similar to the results of Lavelli *et al*. (2000) who reported values changing between 429.0 ± 5.0 and 464.0 ± 2.0 mg GAE/100 g dry weight of tomatoes and slightly lower than results provided by Marinova *et al*. (2005) reporting 76.9 mg GAE/100 g fresh weight phenolics for Bulgarian tomatoes.

Processing tomato fruit into paste resulted in an overall increase of 6% in phenolic content (Figure 4.1). However, the changes occurred in each step were not

statistically different ($p<0.05$), similar to the findings of Dewanto *et al*. (2002) and Gahler *et al*. (2003).

Table 4.3: The contents of total phenolics and total flavonoids of tomato processing samples[1].

Processing Steps	Total phenolics (mg GAE/100 g)	Total flavonoids (mg rutin/100 g)
Fruit	576.5±76.2 a	235.8±28.2 bc
Breaker	592.0±21.8 a	307.7±37.3 b
Finisher pulp	565.2±47.5 a	206.3±42.8 c
Seed & Skin	543.5±83.8 a	525.6±112.8 a
Evaporator out	596.3±54.0 a	221.9±29.4 c
Paste	608.5±52.5 a	232.7±35.6 bc

[1]Data represent average quantities ± standard deviation of 5 independent processing events. All contents are expressed per 100 g dry weight. Different letters in the columns represent statistically significant differences ($p<0.05$).

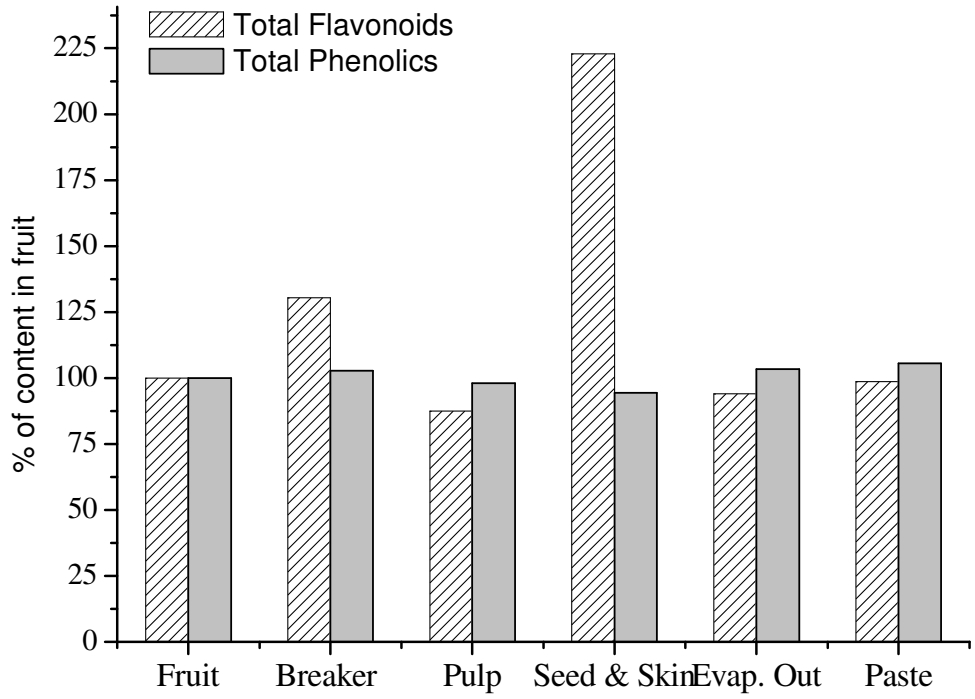


Figure 4.1: Total phenolic and total flavonoid contents of samples collected from each processing step with normalized values (The percent values were calculated by accepting the initial content of each component in the fruit as 100 unit).

Based on the results of total phenolics, all fractions contained more or less the same amount of phenolic compounds at each processing step (Figure 4.1). In contrast, both the dedicated quantitative HPLC analyses (Table 4.7, 4.10 and 4.11) and the untargeted metabolic profiling approach (Table 4.12, 4.13 and 4.14) pointed out significant differences in phenolics at specific processing steps. Those differences will be discussed further in sections 4.1.8 to 4.1.12. It should be noticed that the Folin-Ciocalteau assay is not specific for phenolic compounds, as other reducing compounds present in crude extracts may also interfere the results (George *et al*., 2004).

4.1.4. Total flavonoids

Total flavonoid content of the fruits was found to be 235.8 ± 28.2 mg rutin eq./100 g dry weight (Table 4.3). Toor *et al*. (2006) also reported total flavonoid contents of 197-211 mg rutin eq./100 g dry matter for tomatoes. A 31% increase was observed in the flavonoid content of samples collected from breaking step (Figure 4.1). Total flavonoid assay results were also in accordance with HPLC flavonoid analysis where in both cases an increase in the first breaking step was observed (Table 4.7). However, following the separation of seed and the skin, about 33% decrease was observed in the finisher pulp compared to the breaker samples.

The seed and the skin part doubled the amount of total flavonoids with respect to the fruit (Figure 4.1). The high amount of flavonoids in the seed and the skin is probably associated to the fact that DNA-damaging UV light induces the accumulation of UV light-absorbing flavonoids and other phenolics, predominantly in the dermal tissues of the plant body (Toor *et al*., 2006). In addition, about 98% of the total flavonols were found in the tomato skin as the conjugated forms of quercetin and kaempferol (Canene-Adams *et al*., 2004). As a result, by the effect of seed and skin removal, significant amount of total flavonoid loss was observed ($p<0.05$). Therefore, recovery of flavonoids and other compounds from the seed and skin fraction might be considered to serve as a source of functional ingredients for the food industry (Peschel *et al*., 2006) and as a high quality plant protein source (Sogi *et al*., 2005). However, there were no significant change after the pulping step, in the samples of evaporator out and paste ($p<0.05$).

4.1.5. Antioxidant capacity

The antioxidant capacity of samples using 4 different methods including ABTS, DPPH, FRAP, and CUPRAC are represented in Table 4.4.

The mean values of total antioxidant capacity of tomato fruit samples analyzed by ABTS, CUPRAC, FRAP and DPPH methods were 4524.8, 4415.6, 2066.8, and 1320.7 μmol TEAC/100 g dry weight, respectively. As presented in Table 4.4 the FRAP method gave about 53% and 54% lower total antioxidant capacity values compared to the ABTS and CUPRAC methods. However, much lower (about 70 % and 71 %) values were observed with DPPH method in total antioxidant capacities than both previous methods. Inspite of the differences in these methods, the observed values are consistent with the findings of Raffo *et al.* (2006) who reported approximately 1990 – 4540 μmol TEAC/100 g dry weight in tomato. However, the results of ABTS and CUPRAC methods were higher than the results of Toor *et al.* (2006) who reported values of 2540 ± 56 – 2579 ± 137 μmol TEAC/100 g dry weight tomato.

The differences in total antioxidant capacities observed in each processing step were found to be statistically insignificant based on the data obtained with ABTS test ($p<0.05$). ABTS method yielded a 9% decrease in the total antioxidant capacity of the fruit when processed into the paste (Table 4.4). Similarly, this decrease was also insignificant for both hydrophilic and lipophilic extracts in paste ($p<0.05$) Although lipophilic extract of seed and skin fraction showed a slight decrease in antioxidant potential, ABTS method did not reveal any significant effect of other processing steps on the total antioxidant capacity (Table 4.4).

CUPRAC method showed a single significant increase in total antioxidant capacity of the seed and skin fraction but there was no difference in the rest of all processing steps (Table 4.4). The amount of hydrophilic antioxidants remained fairly stable but lipophilic antioxidants gradually decreased during processing (about 73% decrease from fruit to paste). This significant decrease was mostly associated to the removal of the seed and the skin fraction but, obviously, it was not apparent in total

antioxidant potential since hydrophilic antioxidants were at high levels in all process samples.

In contrast to these findings, both FRAP and DPPH methods represented significant decreases in lipophilic and hydrophilic antioxidant activities during processing from fruit to the paste ($p<0.05$) (Table 4.4). FRAP method yielded decreases of 38%, 78%, and 77% for hydrophilic, lipophilic, and total antioxidant capacity values in paste samples, respectively. Moreover, the decrease in the hydrophilic antioxidant capacity was highest in samples collected at breaking step (about 18% compared to the values observed in fruits), and the samples of finisher pulp stage also decreased about 12% compared to the previous step. Whereas the most significant decrease by about 68% in lipophilic antioxidant capacity values was observed in the finisher pulp step with respect to its previous step.

DPPH method showed that total antioxidant capacity of tomato fruit was decreased 41% when processed into paste (Table 4.4). Similarly, both hydrophilic and lipophilic antioxidant capacities presented consistent decreasing trends towards to the paste step. The major decrease in hydrophilic antioxidant capacity was observed in breaking step (about 19%) compared to the original fruit. The lipophilic antioxidant activity was mostly decreased at evaporator out processing step which is probably due to an increase in seed and skin removal step (Table 4.4).

The antioxidant capacity of seed and skin fractions measured by all the methods were observed to have high levels of antioxidants, and even those values were higher than those of the final paste. The general effect observed during tomato processing from fresh fruit towards paste was a modest decrease both in hydrophilic and lipophilic antioxidant capacities. However, there were inconsistencies between the results of methods.

In addition to the above findings, it was observed that CUPRAC method yielded consistent data or, in some cases, higher values with respect to the results of ABTS method. The antioxidant capacity values obtained by DPPH method were found to be the lowest amongst all other methods. Only two tests yielded sufficiently high TEAC values in the analyzed samples of various tomato processing steps; namely the ABTS and CUPRAC methods. The chromogens of both methods are soluble in both

aqueous and organic solvents (Apak *et al*., 2007) which was the reason of high TEAC values for both hydrophilic and lipophilic extracts, while FRAP oxidations are probably incomplete for some phenolics during the standard time period of the protocol (Berker *et al*., 2007). The relative losses in carotenoids including lycopene, β-carotene, and lutein, during paste making process are best reflected in the lipophilic phase (L) antioxidant activity findings obtained with the CUPRAC and DPPH assays (Table 4.4). However, the DPPH test gave very low results for the hydrophilic fractions in all samples. In other words, CUPRAC method was capable of both determining the total antioxidant activity of hydrophilic and lipophilic phases of processed tomato.

Antioxidant capacity tests are widely used together with other analysis to characterize different plant materials or their products (Arnao, 2000). However, the current antioxidant tests are not capable of analyzing all the antioxidants available in the tissues, and methods may represent several advantages and/or disadvantages over each other. The principles of the methods vary greatly depending on the radical that is generated or the time of reaction. Even the methods having the same principle such as ABTS and DPPH can show differences in their responses to different types of antioxidants. The reactions for the radical formations or the solubility of radicals in different solvent systems also differ in each method (Arnao, 2000). The measurement of antioxidant activities, especially in case of multifunctional or complex multiphase systems, cannot be evaluated satisfactorily by a simple antioxidant test due to the many variables influencing the results. Therefore, it has been recommended to apply several test procedures to evaluate antioxidant activities of plant tissues (Antolovich *et al*., 2002).

Table 4.4: The antioxidant capacities of tomato processing samples[1].

Methods		Fruit	Breaker	Finisher pulp	Seed & Skin	Evaporator out	Paste
ABTS (µmol TEAC/100 g)	H[2]	4385.7± 514.3 a	4409.4±82.7 a	4056.3±338.3 a	4235.1± 710.9 a	4155.6± 355.1 a	3988.0± 385.0 a
	L[3]	139.1± 22.1 a	143.8± 11.7 a	140.1± 9.4 a	103.7± 54.1 b	123.8± 4.4 ab	118.5± 10.4 ab
	T[4]	4524.8±529.4 a	4553.2±86.7 a	4196.4±341.8 a	4338.8±753.2 a	4279.4±353.2 a	4106.5±391.6 a
CUPRAC (µmol TEAC/100 g)	H	3819.4± 452.1 ab	3744.1± 286.3 ab	3347.0± 309.1 b	4098.6± 801.6 ab	3985.2 ±803.8 ab	4311.3 ±807.9 a
	L	595.2 ±167.2 a	544.3± 102.1 a	324.5 ±45.4 b	492.2±24.9 a	282.1 ±173.7 bc	162.6 ±80.6 c
	T	4415.6±588.0 b	4288.4±219.7 b	3671.5±270.2 b	4590.8±769.6 a	4267.3±820.9 b	4473.9±877.1 b
FRAP (µmol TEAC/100 g)	H	1709.2 ±139.4 a	1402.1±54.0 b	1234.8±260.7 bc	1131.1±232.1 c	1178.7±104.6 bc	1054.1±90.3 c
	L	357.6±125.6 a	298.7±95.2 ab	143.2±44.1 c	240.7±15.8 b	101.7±5.2 c	79.9±29.0 c
	T	2066.8±132.6 a	1700.8±90.7 b	1378.0±221.5 c	1371.8±218.8 c	1280.4±100.7 c	1134.0±73.3 c
DPPH (µmol TEAC/100 g)	H	1211.4±217.5 a	980.4±83.4 b	872.3±241.1 bc	879.4±184.6 bc	849.4±47.5 bc	706.7±33.5 c
	L	109.3±21.4 b	108.1±17.4 b	103.6±10.9 bc	132.6±10.2 a	84.0±5.9 cd	78.1±20.7 d
	T	1320.7±217.9 a	1088.5±74.7 b	975.9±231.6 bc	1012.0±189.9 b	933.4±48.5 bc	784.8±45.3 c

[1]Data represent average quantities ± standard deviation of 5 independent processing events. All contents are expressed per 100 g dry weight. Different letters in the columns represent statistically significant differences ($p<0.05$); [2]H= Hydrophilic extract; [3]L = Lipophilic extract; [4]T = Total.

4.1.6. On-line antioxidant detection

The percent areas of the negative peaks obtained by on-line antioxidant detection for flavonoids in fruit and paste are presented in Table 4.5 and the chromatograms are shown in Figure 4.2 and 4.3.

The aim of on-line antioxidant detection is to assess the relative contribution of individual compounds to the total antioxidant activity of the extracts, and to observe the potential chemical basis of any changes (Beekwilder *et al*., 2005). This was done by HPLC analysis with a specific on-line antioxidant detection system based on post-column reaction with ABTS-cation radicals. As shown in Figure 4.2 (A, B and C, top panels), in fruit samples the first part of the chromatogram (until 5 minute) comprised approximately 85.0% of the total antioxidant activity (based on total peak area). This region contains polar antioxidants including vitamin C and glutathione. Between 5 and 35 minutes after sample injection, phenolic compounds like chlorogenic acid, rutin and rutin apioside representing 12.5% of total antioxidant activity were eluted. Naringenin chalcone, eluting at a retention time of 42 min, showed an activity equivalent to about 2.5% of the total antioxidant activity. In the paste samples (Figure 4.2, bottom panels), the antioxidant activity of the compounds eluting within the first 5 minute was 79.0% of the total, and of the compounds eluting between 5-35 minutes was 21.0%. Since naringenin chalcone was completely lost upon tomato paste production, and the content of its isomeric form naringenin was relatively low (as discussed in section 4.1.8), no other compound with significant antioxidant activity was observed in the paste after 35 minutes of chromatography.

Table 4.5: Percent (%) of total antioxidant capacity based on total peak area obtained from on-line antioxidant detection.

	% of Total Antioxidant Capacity	
Retention time	**Fruit**	**Paste**
≤5 min.	85.0	79.0
5-35 min.	12.5	21.0
≥35 min.	2.5	-

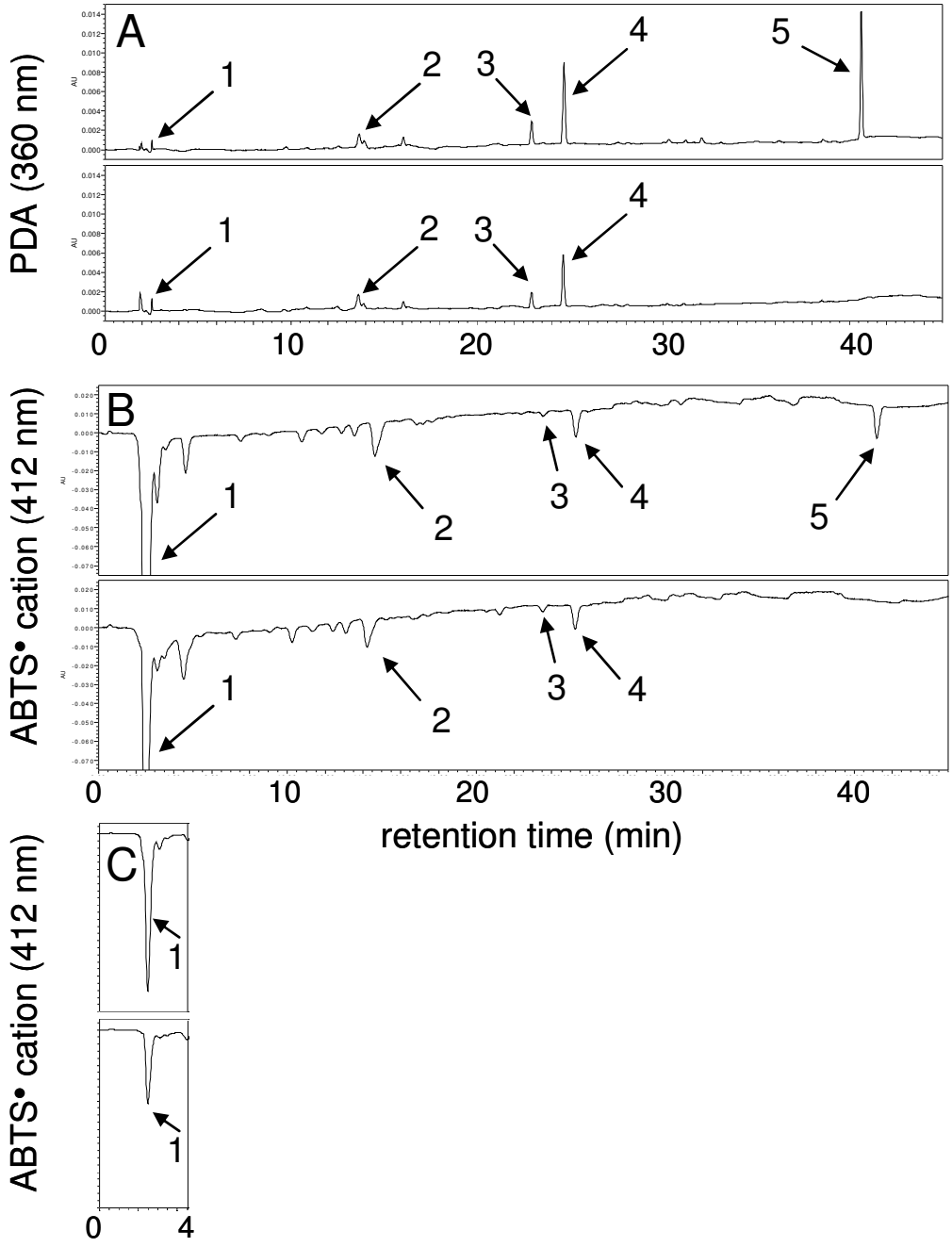


Figure 4.2: Profiling antioxidants in aqueous-methanol extracts using HPLC-PDA with on-line antioxidant detection. A: Typical chromatograms (PDA, recorded at 360 nm) of "Fruit" (upper panel) and "Paste" (lower panel). B: Antioxidants in "Fruit" (upper panel) and "Paste" (lower panel), as determined from reaction with ABTS$^{\bullet}$-cation radicals. C: Same as B, comparing the antioxidant activity in the first 4 minutes of the chromatogram. Y-axes in upper and lower panels are directly comparable. Numbers refer to the main antioxidants identified: (1) Ascorbic acid, (2) Chlorogenic acid, (3) Rutin-apioside, (4) Rutin, (5) Naringenin chalcone.

The results of on-line antioxidant detection system also showed that the key antioxidant in hydrophilic extracts both in the original fruit and in the final tomato paste was found to be vitamin C (Figure 4.2). Next to vitamin C, phenolic antioxidants including rutin, rutin-apioside, chlorogenic acid and naringenin chalcone (only in original fruit) were also identified as major antioxidants in these hydrophilic extracts.

In the lipophilic (chloroform) extracts, on-line carotenoid analysis showed a similar peak as flavonoid assay in the first 5 minute, showing the effect of vitamin C and other polar compounds (Figure 4.3). After 5 minutes, lutein, β-carotene, phytofluene, and lycopene became the responsible compounds for the small negative peaks. However, the negative antioxidant peaks for lipophilic extracts were not as clear as hydrophilic extracts because of their lower concentrations as also shown in Table 4.4.

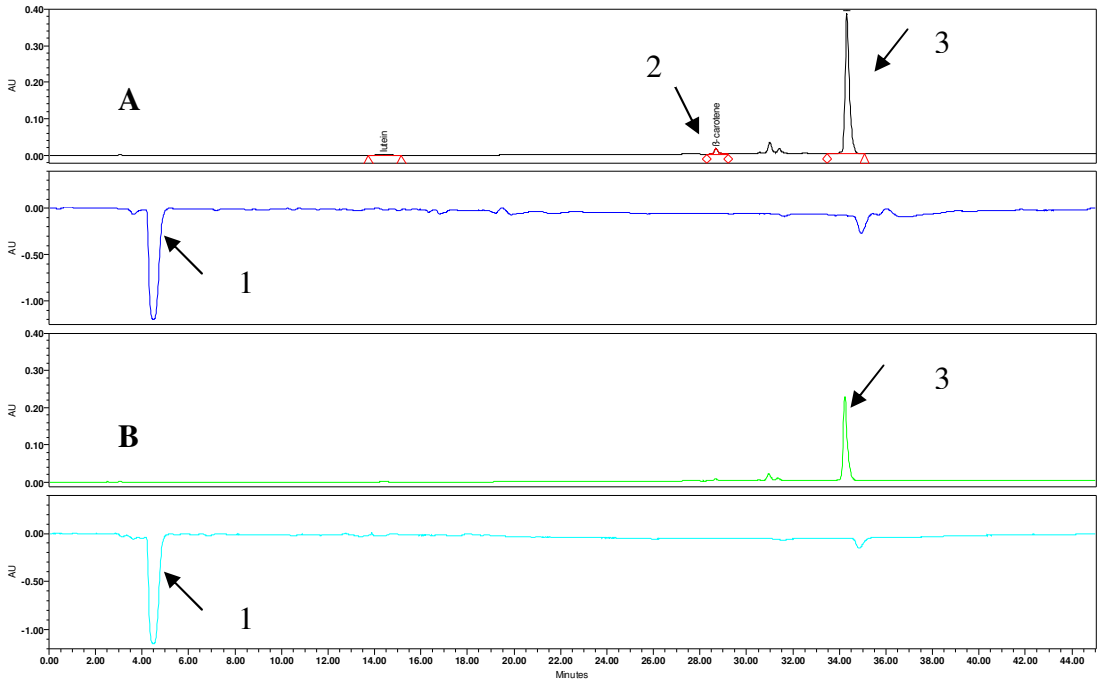


Figure 4.3: Profiling antioxidants in chloroform extracts using HPLC-PDA with on-line antioxidant detection. A: Typical chromatograms (PDA, recorded at 360nm) (upper panel) and antioxidants (lower panel) of "Fruit". B: Typical chromatograms (PDA, recorded at 360nm) (upper panel) and antioxidants (lower panel) of "Paste". Y-axes in upper and lower panels are directly comparable. Numbers refer to the main antioxidants identified: (1) Ascorbic acid, (2) β-Carotene, (3) Lycopene.

In the lipophilic extracts, all *trans*-lycopene was by far the most abundant compound detected. Lycopene is a good antioxidant, with *in vitro* activities higher than those of vitamin C and vitamin E (Re *et al*., 2002). The decrease in the amount of those compounds from fruit to paste can be observed from the chromatogram (Figure 4.3). This decrease was found to be statistically significant ($p<0.05$), and it will be discussed further in section 4.1.9, in detail.

4.1.7. Variation between batches

All the analyses were applied on 5 independent replicates harvested over a 2 year period and variations between these different batches were observed. In order to evaluate these variations, coefficient of variation (CV %) values were calculated for rutin and lycopene as representatives of the hydrophilic and lipophilic compounds, respectively (Table 4.6). The changes in these values ranged between 13%-66% for rutin and 11-28% for lycopene at different processing steps. These variations might be as a result of sampling, or changes in the variety or type of tomatoes used. It is known that the variety/cultivar (Abushita *et al*., 1997; George *et al*., 2004), climate (Bradfield and Stamp, 2004; Toor *et al*., 2006), size of the fruit (Sahlin *et al*., 2004; Toor *et al.*, 2006), growing conditions (Guintini *et al*., 2005), ripening stage (Abushita *et al*., 2000; Leonardi *et al*., 2000) may be effective on the content of compounds such as lycopene, β-carotene, or vitamin C. In this study, mean values were used to report the values of each analysis.

Table 4.6: CV% values of rutin and lycopene for tomato processing samples[1].

Processing Steps	Rutin	CV%	Lycopene	CV%
Fruit	19.8±11.8	59.6	146.0±39.5	27.1
Breaker	43.7±16.7	38.2	130.8±28.9	22.1
Finisher pulp	12.5±8.2	65.6	114.1±31.8	27.9
Seed & Skin	167.3±22.2	13.3	61.9±14.5	23.4
Evaporator out	16.5±5.4	32.7	89.2±10.0	11.2
Paste	16.9±6.1	36.1	98.9±25.5	25.8

[1]Data represent average quantities ± standard deviation of 5 independent processing events. Contents of rutin and lycopene are expressed per 100 g dry weight. Different letters in the columns represent statistically significant differences ($p<0.05$).

Although there was considerable variation between batches, as explained above, the trends reflecting the effect of processing were similar as can be seen in Figure 4.4 and 4.5 for rutin and lycopene, respectively. The rutin content in the breaker was

found to be 0.7 to 3.3 times higher than fruit for 5 batches followed by a decrease from breaker to finisher pulp steps (about 15-91%). After the evaporation step, the rutin content decreased about 0.1 to 1.9 times in all batches except for the 5th batch where a 5% increase was observed. Similarly, slight increases (8%) or decreases (6%) were detected in samples collected from evaporator out to paste stage. Such fluctuations could be related to sampling from different batches.

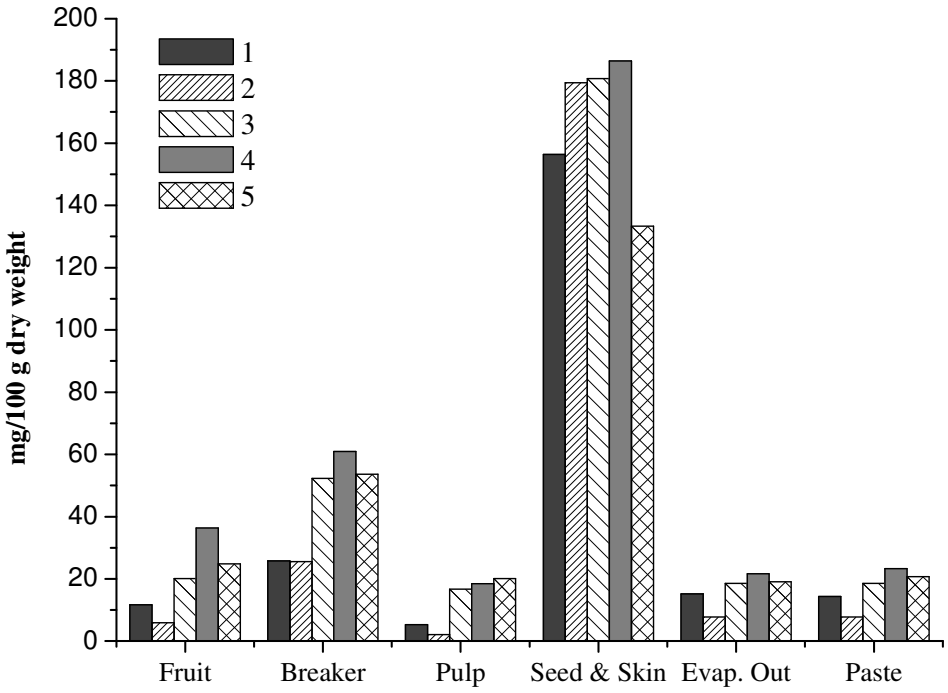


Figure 4.4: Changes in rutin during processing of tomato using 5 independent replicates harvested over a 2 year period. The numbers in the legend (1 to 5) represent the batch numbers.

Lycopene contents, on the other hand, for the original values of fruit and paste and normalized values of 5 replicates are shown in Figure 4.5. For all the batches, the lycopene content decreased from fruit to paste by about 19% to 42%. In the middle steps of processing, namely from fruit to breaker, and from breaker to finisher pulp, increases about 15% or decreases about 17% were observed, respectively. After evaporation, the evaporator out samples showed 18% increase or 47% decrease when compared with pulp samples in various batches. The lycopene content in the paste was found to decrease by 16% or increase by 59% compared to the evaporator out samples depending on the batch.

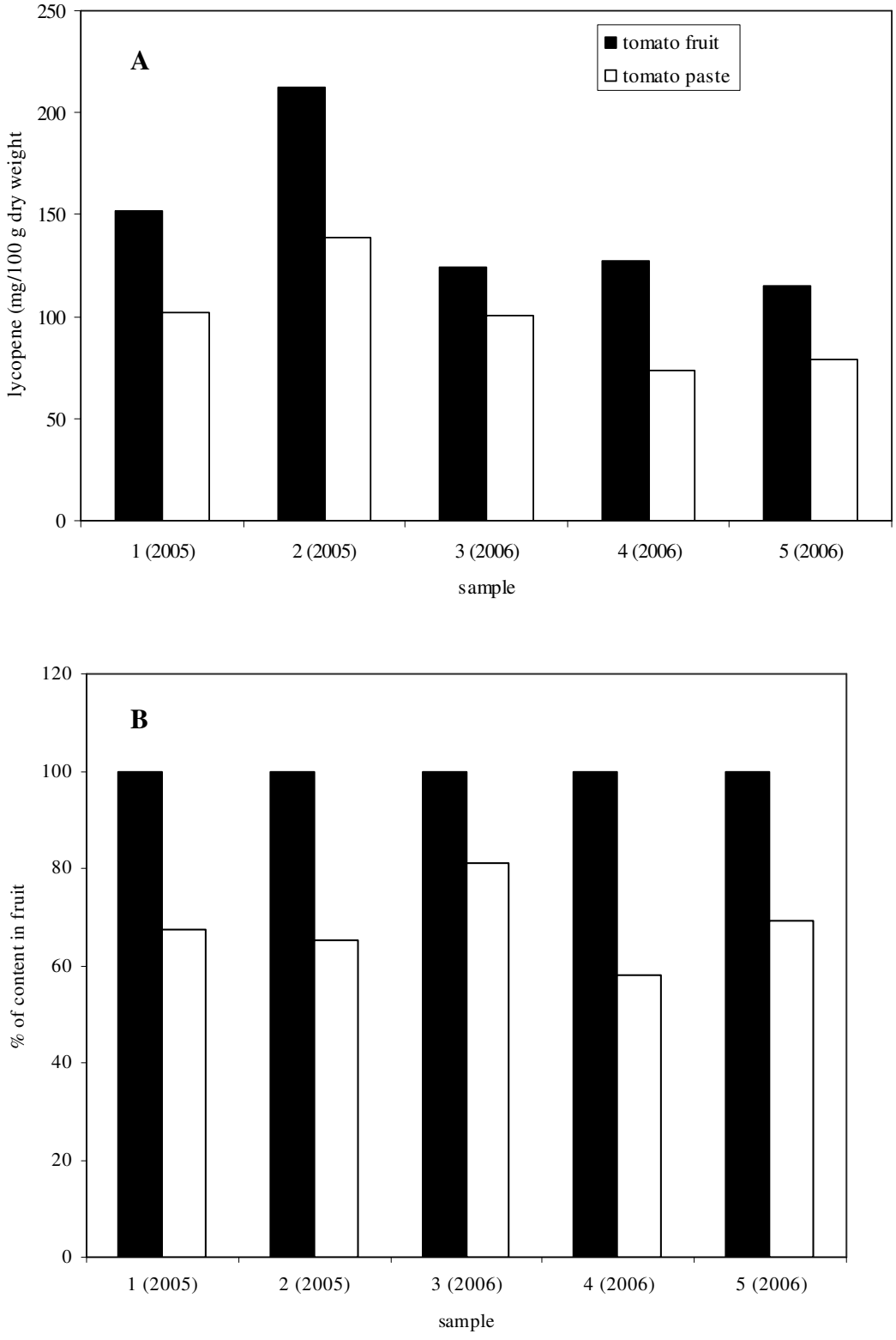


Figure 4.5: Changes in A: lycopene B: lycopene with normalized values (The percent values were calculated by accepting the initial content of each component in the fruit as 100 unit) during processing of tomato using 5 independent replicates harvested over a 2 year period. Y axes (1 to 5) represent the batch numbers with sampling years.

4.1.8. Flavonoid profile and chlorogenic acid

The flavonoid and chlorogenic acid contents of processing samples are shown in Table 4.7. Among flavonoids, naringeninchalcone, and rutin as well as chlorogenic acid which is a hydroxycinnamate, were most abundant with amounts of 19.2 ± 8.7, 19.8 ± 11.0, 21.0 ± 9.4 mg/100 g dry weight, respectively. According to the values obtained by Re *et al.* (2002), chlorogenic acid was reported to be much lower at 3.7 ± 0.2 mg/100 g dry weight levels. However, the rutin (31.93 ± 4.8 mg/100 g dry weight) and naringenin (22.5 ± 5.9 mg/100 g dry weight) contents published by the same research group were found to be more comparable with the presented data in this study.

Table 4.7: The contents of flavonoids and chlorogenic acid of tomato processing samples[1].

Processing Steps	Rutin	Rutin apioside	Naringenin	Naringenin chalcone	Chlorogenic acid
Fruit	19.8±11.8 c	4.8±1.9 c	0.0±0.0 d	19.2±8.7 bc	21.0±9.4 a
Breaker	43.7±16.7 b	10.6±2.3 b	1.1±0.2 c	43.8±8.1 b	15.1±7.2 a
Finisher pulp	12.5±8.2 c	4.7±1.8 c	2.5±0.6 b	2.8±2.1 c	18.4±14.0 a
Seed & Skin	167.3±22.2 a	35.1±7.0 a	3.6±1.2 a	166.2±50.0 a	13.3±4.2 a
Evaporator out	16.5±5.4 c	5.1±1.2 c	2.0±0.8 b	0.0±0.0 c	16.2±7.6 a
Paste	16.9±6.1c	4.9±1.3 c	2.2±0.9 b	0.0±0.0 c	16.7±8.0 a

[1]Data represent average quantities ± standard deviation of 5 independent processing events. All contents are expressed per 100 g dry weight. Different letters in the columns represent statistically significant differences ($p<0.05$).

Rutin content increased consistently during breaking in both tomato batches collected in 2 years (Figure 4.4). However, this increase was lost by the removal of the seed and the skin part resulting in 74% lower amounts in the finisher pulp samples. About 96% increase was then observed from pulp to evaporator out steps. The paste samples were found to contain 1% lower rutin in average than its level in the original fruit samples. Similarly, rutin apioside showed the same trend with an increase of 1.3 times from fruit to breaker which was followed by a decrease of 56% from breaker to finisher pulp. The rutin apioside content increased by 16% when it was subjected to evaporation and a decrease (6%) was observed at the last stage of processing into paste. The seed and skin fractions for all batches were found to contain high levels of both rutin and rutin apioside. The amount of seed and skin removed during processing is 3.5% of the whole fruit, and this should also be taken

into consideration while calculating the loss of compounds during the separation of seed and skin.

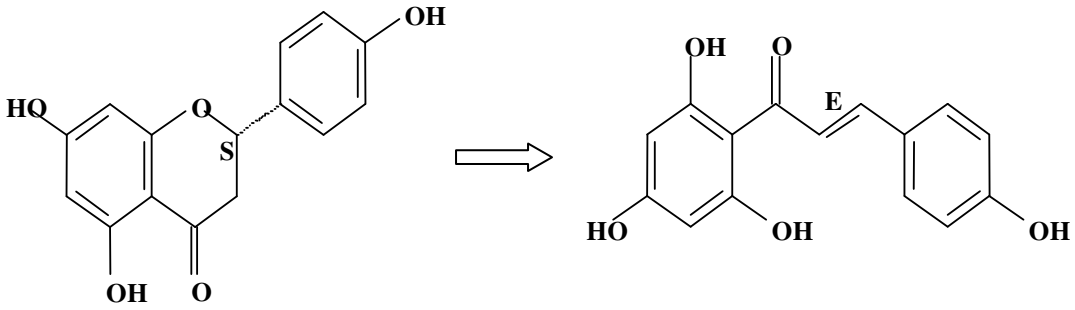
The increase observed in the breaking step is a new finding in the available literature. This increase can be explained by the continuing synthesis of flavonoids as a response to wounding as similar observations were reported for apples (Abdallah *et al*., 1997), lettuce leaves (Kang and Saltveit, 2002), and potatoes (Tudela *et al*., 2002). To assess whether this was due to an increased extractability, or a partial de-glycosylation or de-esterification of more complexed flavonoid species, acid hydrolysis of the samples followed by HPLC-PDA analyses of the resulting the formation of flavonoid aglycons was performed. The aglycon form of rutin, namely quercetin, was investigated for all the processing steps. The areas of quercetin for each step are shown in Table 4.8. Results showed that quercetin content of breaked tomatoes were approximately 2 times higher than fruit itself. This ratio between fruit and breaker was similar to the findings obtained with non-hydrolyzed extracts confirming that the increase in these compounds was the result of synthesis, rather than release from more complex forms. In the later stages of the paste-making process, the increase in flavonoids which was observed during the breaking step was followed by a decrease upon removal of the seed and skin fraction resulting in levels in the paste that were similar to those in the original fruit.

Table 4.8: Areas of quercetin in the hydrolyzed samples.

Processing Steps	**Area**
Fruit	35655
Breaker	102520
Finisher pulp	32480
Seed & Skin	350457
Evaporator out	33525
Paste	41199

Naringenin, another important flavonoid, was undetectable in any of the fruit samples, and small amounts were already detected in the breaker samples and increased during pulping. The seed and skin fraction contained the highest amount of naringenin. During evaporation and pasteurization, 17% decrease and %8 increases were observed, respectively. Re *et al*. (2002) also reported a significant loss of naringenin upon processing.

In addition, the data presented here showed conversion of naringenin chalcone to lower amounts of naringenin, which is the isomeric flavanone form of naringenin chalcone (Figure 4.6) (Dey, 1993). The chalcone form was highest (43.8±8.1 mg/100 g dry weight) after breaking and was mainly lost on separation of the seed and skin fraction. At the end of the total paste making procedure, including the evaporator out and paste samples, the chalcone was undetectable while the flavanone naringenin represented 11% of total naringenin/naringenin chalcone present in fruit samples.



Naringenin chalcone Naringenin

Figure 4.6: Conversion of naringenin chalcone into naringenin.

The chlorogenic acid contents of the samples for 5 batches are shown in Figure 4.7. The loss of chlorogenic acid from fruit to paste was 21% in total. However, there was no significant change during processing steps. In contrast to the flavonoids, chlorogenic acid, the main hydroxycinnamate compound in tomato, was not increased in the breaker samples with respect to the fruit samples.

The chlorogenic acid values of the 2nd batch were found to be different from the rest of the series. When the vitamin C values of this batch were compared with the values of other batches, it was observed that 2nd batch had the lowest values. This effect might be due to the use of overripe tomatoes for processing, which could result in low amounts of vitamin C and other metabolites as well (Abushita *et al*., 1997; Guintini *et al*., 2005). It is also known that the carotenoid content, antioxidant activity or levels of other tomato compounds vary considerably according to the genetic variety, ripening stage and growing conditions of the fruit (Leonardi *et al*., 2000; Raffo *et al*., 2002; Spencer *et al*., 2005).

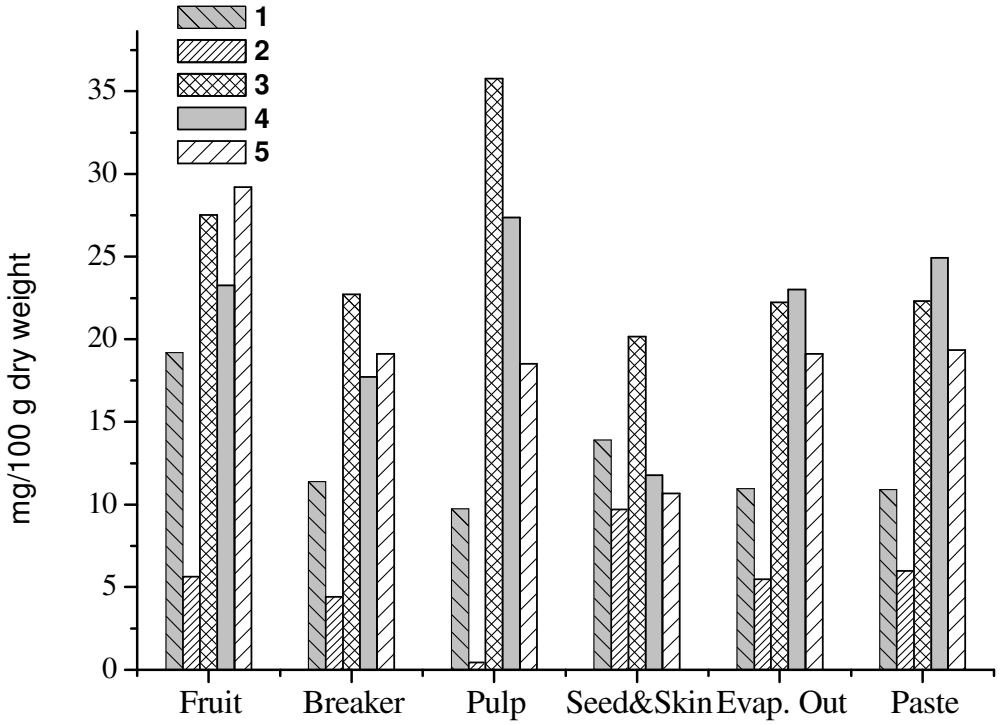


Figure 4.7: Chlorogenic acid content of tomato processing samples.

4.1.9. Carotenoids

4.1.9.1. Optimum extraction method

Lycopene content of fruit and paste samples obtained with different carotenoid extraction methods are presented in Figure 4.8. The results showed that the highest lycopene values for fruits were obtained by using the solvents of methanol and chloroform ("method 5") (app. 1.2 mg/g sample). The lycopene values were 16-96% higher than those obtained with the rest of the methods. Similarly, the lycopene contents of paste samples obtained by the "method 5" were found to be 22-95% higher compared to methods 3, 2, and 1, except for the 4th method which showed no difference. Therefore, it could be concluded that the "method 5" provided the highest extractability for both fruit and the paste. Also during the extraction, the color of the residue (after extraction) was found to be completely colorless with this particular method. In addition, high extraction solvent volumes were needed with other methods and decoloration of the analyzed sample was incomplete in some cases.

Hence, the “method 5” was selected for the extraction of carotenoids followed by further profiling analysis by HPLC.

In the view of current literature some researches presented increases in lycopene or β-carotene as a result of processing. Especially the effect of heat treatments have been explained by higher extractability of compounds from the samples as a result of break down in cell walls and bonding forces (Dewanto *et al*., 2002; Chang *et al*., 2006; Choi *et al*., 2006). Therefore, choice of the best extraction method is important from this point of view as well.

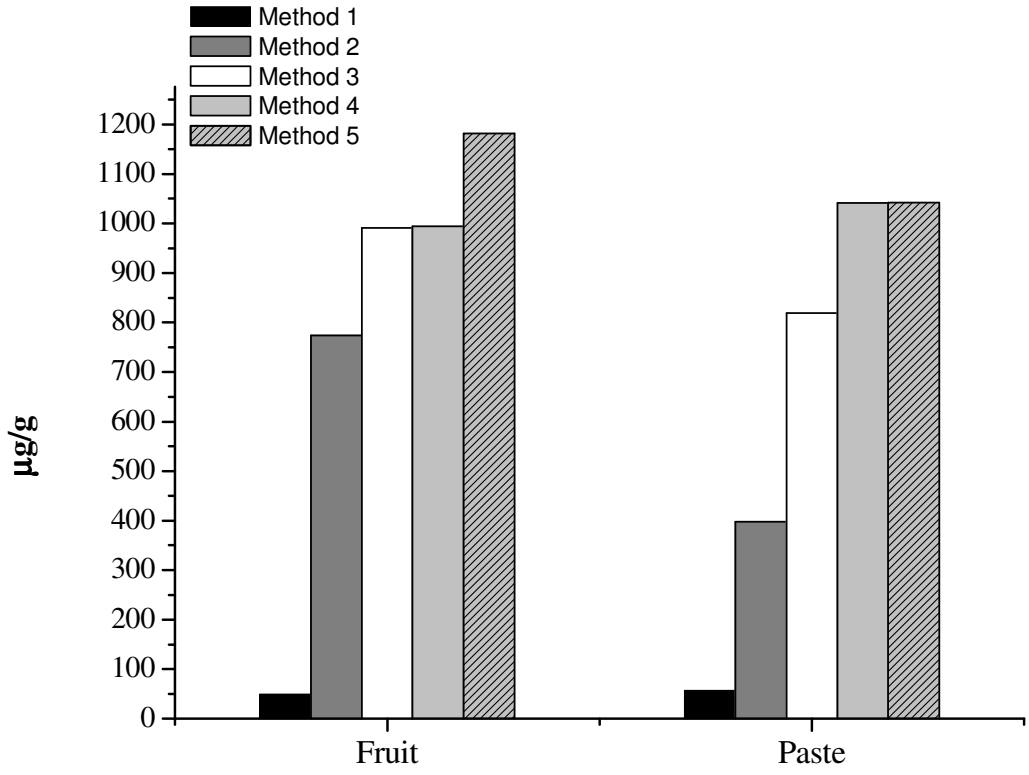


Figure 4.8: Lycopene content of fruit and paste obtained with different extraction methods.

4.1.9.2. Carotenoid profile

The carotenoid contents of processing samples are shown in Table 4.9. The all *trans*-lycopene contents of tomatoes and paste were found to be 146.0 ± 39.5 and 98.9 ± 25.5 mg/100 g dry weight, respectively. Lycopene contents of about 101.2-304.5 mg/100 g dry weight for tomato and its products reported by Tavares and Rodriguez-

Amaya (1994) are consistent with our data. The differences in lycopene contents of tomatoes might be due to many pre- and postharvest factors such as agronomic and cultural practices, ripening stage at harvest, or storage conditions (Lenucci *et al*., 2006).

Table 4.9: The contents of carotenoids of tomato processing samples[1].

Processing Steps	All *trans*-lycopene	β-Carotene	Lutein
Fruit	146.0±39.5 a	4.7±1.4 a	2.0±0.4 a
Breaker	130.8±28.9 ab	4.8±1.0 a	2.1±0.4 a
Finisher pulp	114.1±31.8 abc	4.2±0.9 a	1.9±0.4 a
Seed & Skin	61.9±14.5 d	4.2±0.6 a	1.4±0.2 b
Evaporator out	89.2±10.0 cd	2.8±0.4 b	0.9±0.2 c
Paste	98.9±25.5 bc	3.0±0.5 b	0.5±0.1c

[1]Data represent average quantities ± standard deviation of 5 independent processing events. All contents are expressed per 100 g dry weight. Different letters in the columns represent statistically significant differences ($p<0.05$).

All *trans*-lycopene content decreased by 10% when fruit was processed in the breaker unit. This decrease continued further by 13% and 22% during pulping and evaporation steps, respectively. At the last step, pasteurization did not cause a significant change with respect to its previous evaporator out stage. However, when the overall effect was investigated from fruit to paste, a 32% decrease in lycopene content was found to be statistically significant ($p<0.05$).

There was no significant change in β-carotene in the first breaking and pulping steps. However, after the evaporation of the pulp the β-carotene content decreased significantly by 34% ($p<0.05$) but there was no significant difference between evaporator out and paste samples. In overall processing from fruit to paste there was about 36% loss of β-carotene ($p<0.05$).

The effect of processing on lutein was similar to that of β-carotene. Breaking and removal of seed and the skin did not have a significant effect but evaporation caused a significant loss in β-carotene by about 53 % ($p<0.05$). Losses in β-carotene were more emphasized (75%) when fruit was processed into paste.

The losses in lycopene, β-carotene or lutein were mainly caused by the evaporation step where heat was applied, and removal of seed and skin. Rest of the loss was probably due to the oxidation reactions taking place during processing. For lycopene, many researchers have reported significant losses during different treatments, such as

drying, heating, and cooking of tomato and its products (Sharma and Le Maguer, 1996; Zanoni *et al*., 1999; Takeoka *et al*., 2001; Sahlin *et al*., 2004; Goula *et al*., 2005; Goula *et al*., 2006; Toor *et al*., 2006). In contrast, higher lycopene contents through several thermal treatments and by homogenization and juice or paste production have been reported as well (Dewanto *et al*., 2002). Seybold *et al*. (2004) observed an increase in the lycopene content of tomato sauce from Spanish tomatoes whereas they have found a decrease in sauces prepared from Holland tomatoes. In conclusion, the differences observed in processed tomatoes might be due to the type or variety of the tomatoes used, conditions such as temperature, time, presence of oxygen or light and methods of processing, or it might be a matter of insufficient extraction of compounds to be analyzed (Seybold *et al*., 2004; Lenucci *et al*., 2006). It was also reported that even the extractability of carotenoids may vary according to the ripening stage, firmness, and genotype of the fruit (Seybold *et al*., 2004).

These increases in lycopene might be associated to the increased extractability of these compounds during fruit processing. The results of present study indicate a gradual and significant decrease in carotenoids (total loss of 32-75% depending on carotenoid species) upon industrial processing from fruit to tomato paste. It is possible that this result was caused by differential extractability in carotenoids between fractions. However, the efficiency of carotenoid extraction by the method used in the present study was highest among five common methods tested, and no residual color was observed in the material after extraction. Therefore, it can be concluded that the observed carotenoid losses are most likely resulted from oxidation reactions taking place during factory-scale tomato processing.

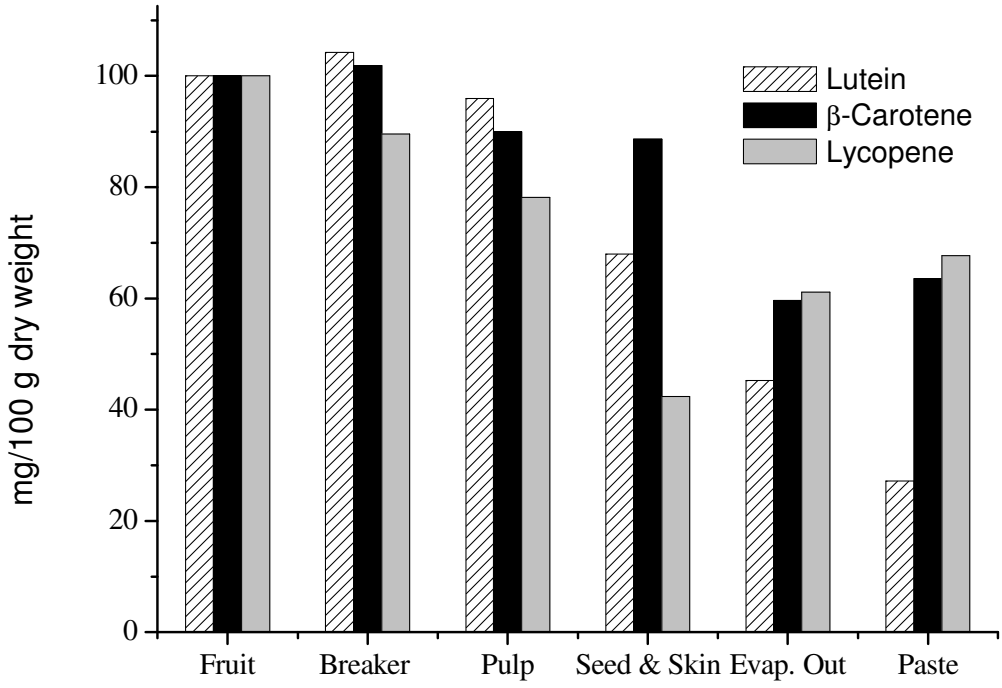


Figure 4.9: Normalized values (The percent values were calculated by accepting the initial content of each component in the fruit as 100 unit) of lutein, β-carotene, and lycopene contents of tomato processing samples.

4.1.10. Tocopherols

Tocopherol content of processing samples are presented in Table 4.10. Notably, the lipid-soluble antioxidant α-tocopherol (vitamin E) was not affected by the industrial processing. There was no significant difference in the samples of each processing step ($p>0.05$). Although its biosynthetic precursor γ-tocopherol did not change during breaking, there was a significant loss by about 72% after the removal of seed and the skin. The seed and skin fraction contained the highest amount of γ-tocopherol. On the other hand, evaporation and pasteurization steps had no significant effects ($p<0.05$). The γ-tocopherol was significantly lower (84%) in paste compared to fruit sample. The relatively high concentration of γ-tocopherol in the seed and skin fraction explains its lower content in the final paste. But α-tocopherol concentration remained fairly constant in all fractions. The β-tocopherol content did not change significantly by processing fruit into paste ($p<0.05$). However, its biosynthetic precursor, δ-tocopherol, was found to be decreased significantly (69%) in the paste samples.

Table 4.10: The contents of tocopherols of tomato processing samples[1].

Processing Steps	α-Tocopherol	γ-Tocopherol	β-Tocopherol	δ-Tocopherol
Fruit	31.62±8.84 a	8.85±5.38 b	1.08±1.49 ab	0.35±0.19 b
Breaker	32.99±15.01 a	7.75±2.24 b	0.0±0.0 b	0.28±0.11 bc
Finisher pulp	24.39±12.67 a	2.17±1.12 c	2.07±0.60 a	0.18±0.09 bcd
Seed & Skin	31.36±12.41 a	23.18±6.32 a	1.38±1.33 ab	0.71±0.17 a
Evaporator out	23.95±5.98 a	1.20±0.31 c	1.48±0.40 ab	0.07±0.04 d
Paste	38.67±2.29 a	1.45±0.25 c	2.46±0.17 a	0.11±0.05 cd

[1]Data represent average quantities ± standard deviation of 5 independent processing events. All contents are expressed per 100 g dry weight. Different letters in the columns represent statistically significant differences ($p<0.05$).

4.1.11. Vitamin C

The vitamin C content of each sample is presented in Table 4.11. The amount of vitamin C in fruit samples was found to be 245.7 ± 89.7 mg/100 g dry weight in average. These findings are consistent with the values in the literature reported as 14.7-44.6 mg/100 g fresh matter in tomatoes having moisture content of 90-95% (Gahler *et al*., 2003), or 330.0 ± 10.0 mg/100 g dry weight in fresh tomatoes (Lavelli *et al*., 1999).

Table 4.11: The contents of vitamin C of tomato processing samples[1].

Processing Steps	Vitamin C
Fruit	245.7±89.7 a
Breaker	174.3±34.9 ab
Finisher pulp	124.8±51.6 b
Seed & Skin	10.0±11.8 c
Evaporator out	184.9±6.1 ab
Paste	122.0±28.7 b

[1]Data represent average quantities ± standard deviation of 5 independent processing events. All contents are expressed per 100 g dry weight. Different letters in the columns represent statistically significant differences ($p<0.05$).

By the effect of processing, half of the vitamin C was lost gradually, ending up with 122.0 ± 28.7 mg/100 g dry weight of vitamin C (Figure 4.10). The breaking step did not cause any significant change in the vitamin C content. The main processing step which caused a significant loss in vitamin C was the pulping step at which heat treatment was applied previously (2.0-2.5 min at 60-80°C). Abushita *et al.* (2000) also reported that during hot-break pulping tomato lost about 38% of its original ascorbic acid, and further processing to produce tomato paste by vacuum evaporation resulted in losses more than 16% of the ascorbic acid content (Abushita *et al*., 2000).

The next heating treatment during the evaporation stage (up to 80°C untill the desired Brix-value was reached) did not further affect the vitamin C content, while the subsequent pasteurization step (5-10 min at 93°C) towards the final paste reduced it by 34% (compared to the evaporator out sample). The decrease in vitamin C content by several heat applications and processes has been reported extensively in the literature (Zanoni *et al*., 1999; Shi and Le Maguer, 2000; Giovanelli *et al*., 2002; Gahler *et al*., 2003). In contrast to the flavonoids, no increase in vitamin C was observed as a result of the breaking step, and a relatively low content was detected in the seed and skin fraction as compared to the pulp and final paste.

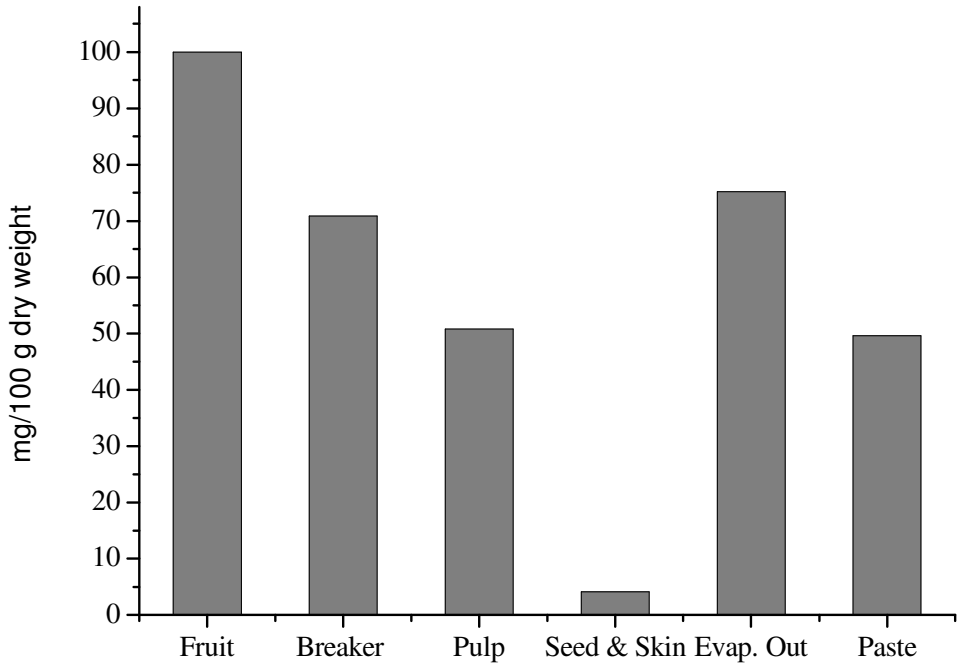


Figure 4.10: Normalized values (the percent values were calculated by accepting the initial content of each component in the fruit as 100 unit) of vitamin C contents of tomato processing samples.

4.1.12. Untargeted metabolomics analysis

A representative LC-MS profile and the score plot of the Principal Component Analysis (PCA) diagram is shown in Figure 4.11 and 4.12. In order to determine which of the processing steps mostly affects the overall metabolite composition of the final paste, all tomato samples were extracted in aqueous-methanol and analyzed using an untargeted LC-QTOF MS - based metabolomics approach. The mass

profiles thus obtained (Figure 4.11) were processed and aligned across all samples using Metalign™ software. After filtering out mass signals that were < 10 times the local noise in all 30 samples, the resulting data matrix contained intensity values (calculated as peak heights) of 3,177 mass signals aligned across all samples. The mass intensity data were 2 log-transformed and then subjected to multivariate analysis using GeneMaths software.

Metabolomics allows the diagnostics of plant status, with direct relationship to the exhibited visual characteristics (phenotype). Using metabolomics technologies, a comprehensive description of naturally occurring metabolites (primary and secondary metabolites) in a biological system, such as tomato fruit, is feasible. The expansion of metabolomic technologies resulted in the usage of a diverse range and configuration of instruments and analytical methods. Mostly MS (Mass Spectrometry) (Schauer *et al*., 2005; Tikunov *et al*., 2005; van der Werf *et al*., 2005; Moco *et al*., 2006; Fraser *et al*., 2007) and NMR (Nuclear Magnetic Resonance) (Keun *et al*., 2002; Le Gall *et al*., 2003; Ward *et al*., 2003; Kochhar *et al*., 2006; Griffin and Kauppinen, 2007) technologies are used, but also other techniques such as LC-photo diode array (PDA) (Porter *et al*., 2006), infrared and Raman spectroscopy (Ellis and Goodacre, 2006) have been applied in plant metabolomics. Among a wide variety of applications (Hall, 2006; Schauer and Fernie, 2006), plant metabolomics approaches provide insight into the biochemical composition of the plant system, allowing the establishment of links to possible metabolite functions.

The representative LC-MS profile shows the changes when the fruit is processed into paste. The changes in flavonoids such as rutin, rutin apioside or naringenin chalcone have been already discussed in the previous sections. However, changes in glycosylated alkaloids such as lycoperoside or tomatin which is a saponin, were also observed and discussed further in this section.

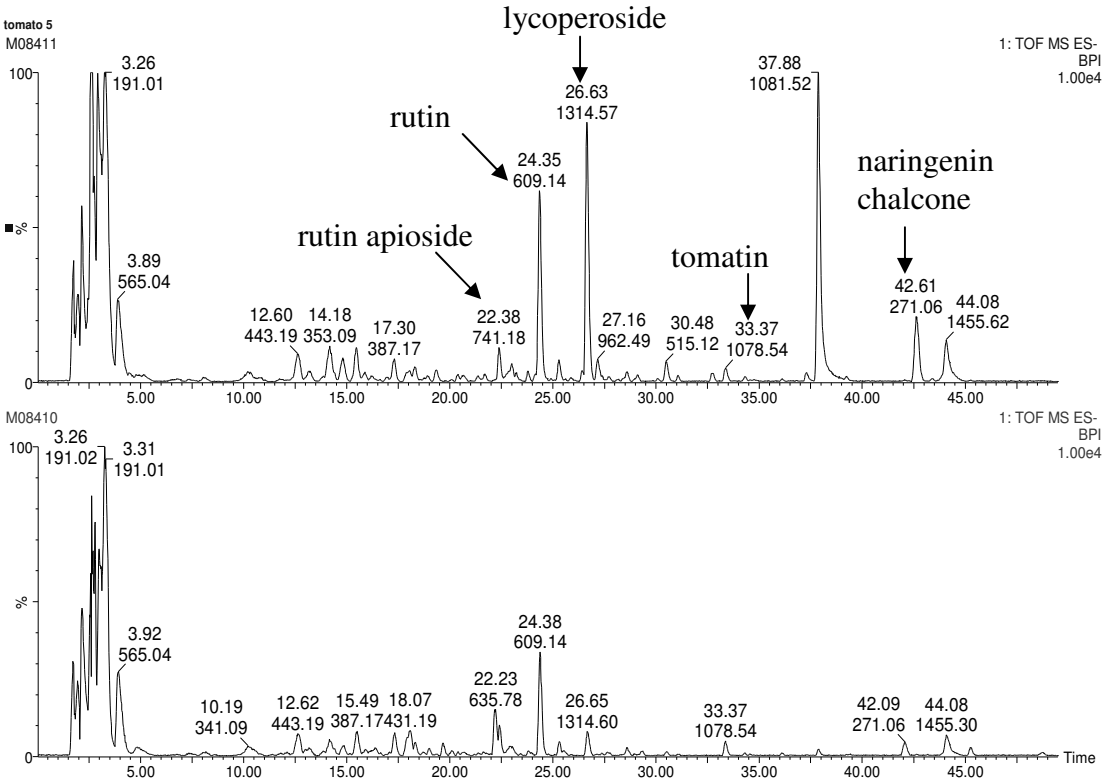


Figure 4.11: Representative LC-MS profiles of original "Fruit" samples (upper panel) and final "Paste" samples (lower panel). Numbers above peaks indicate retention time (in minutes: upper) and accurate m/z (ESI neg mode: lower). Chromatograms are on the same scale.

To visualize the effect of each industrial tomato processing step, PCA of the dataset, after normalization of each mass signal towards the mean of all samples, was performed using GeneMaths software. As shown in the score plot (Figure 4.12), the 5 biological replicates of samples clustered together according to each processing step. This indicates that the mass signals (metabolites) were mainly influenced by various processing steps rather than by possible variation between individual tomato fruit batches and processing events.

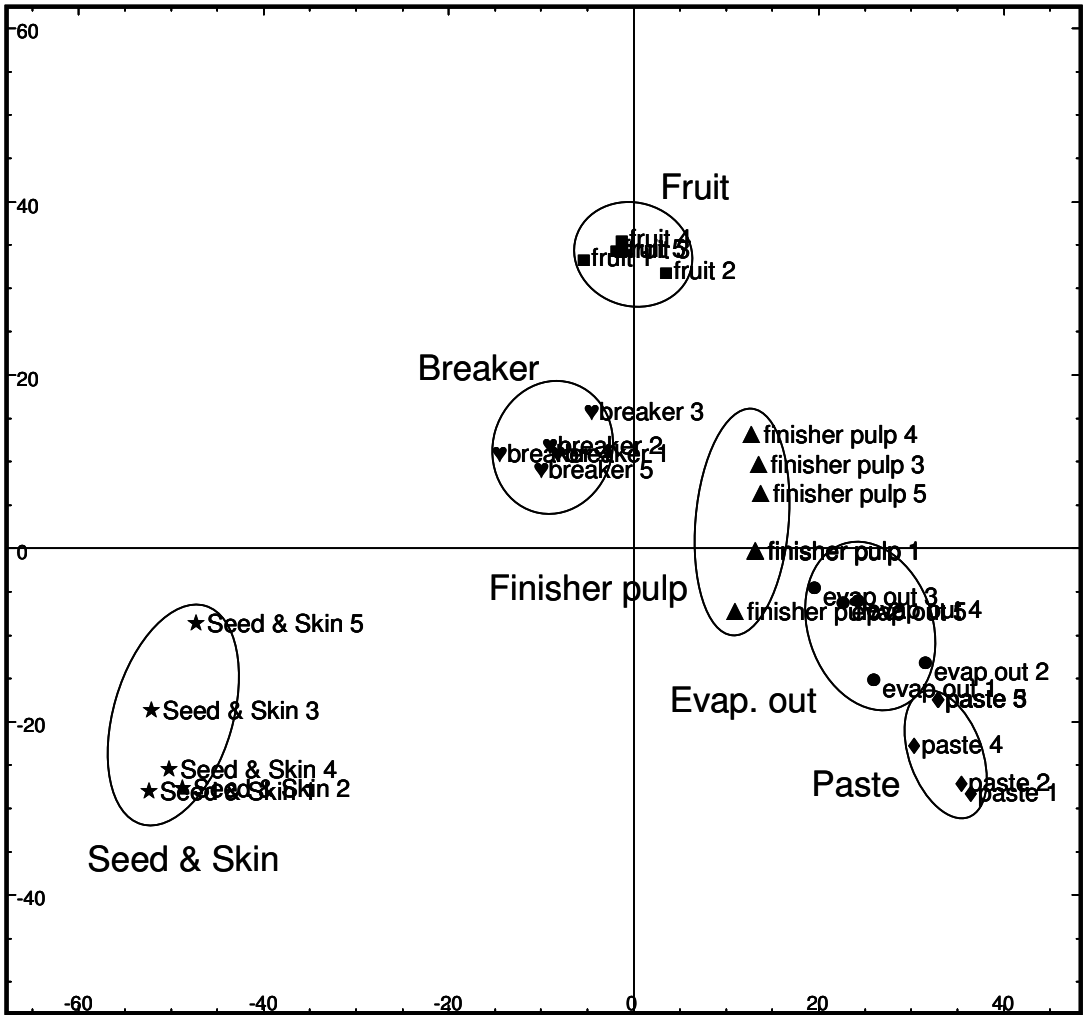


Figure 4.12: Principal component analysis of untargeted LC-MS based metabolomics data. LC-MS chromatograms of all samples were processed and aligned in an unbiased manner using Metalign^{TM} software. Mass signal intensities were subjected to multivariate analyses after 2log-transformed and normalization through dividing by the mean value across all samples. Independent replicate samples per processing step have been numbered 1 to 5.

The first principal component (X-axis) explained 36% of the total variation in the dataset. This component points to the most dominant step in the paste-production process and clearly corresponded to the separation of seed and skin from the rest of the tomato material, with the largest difference in metabolite profiles between seed and skin and paste. The second component (Y-axis) explained 20% of the variation and corresponded to the step-wise processing from fruit to paste. The largest overall variation between the 5 biological replicates at each processing step, calculated over

all 3,177 mass signals detected, was observed within the pulp samples (with a CV% of 44%), where this value was 32% in all the other samples.

The compounds that are affected by the various processing steps were deduced from the differential mass signals, by annotating them by their observed accurate masses and retention times, using a recently published LC-MS based database of tomato fruit metabolites (Moco *et al*., 2006). The most important differences in those metabolites were observed in between fruit and paste, fruit and seed and the skin, and fruit and breaker. In general, nearly 43% (1356 signals) of the total number of mass signals differed significantly ($p<0.05$) between fruit and paste samples. Likewise, about 60% of the signals were statistically different between fruit and seed and skin, and about 25% between fruit and breaker as can be seen in Table 4.12, 4.13, and 4.14.

Among the compounds that were significantly higher in fruit versus paste (Table 4.12) were a range of glycosylated alkaloids (lycoperoside- and esculeoside-isomers), hydroxycinnamates (mono-, di- and tricaffeoyl derivatives), flavonoids (specifically naringenin chalcone and two of its glycosides), and the saponin tomatoside A. The ratio for the compounds higher in the fruit compared to the paste, changed from 1.4 to 62.3 as in pantothenic acid-hexose and tomatoside A, respectively. In contrast, only the alkaloid lycoperoside H and the flavonoid naringenin were, respectively, 2-fold and 6-fold higher in paste compared to original fruit.

Clearly, most compounds that were lower in paste were lost from the production chain upon removal of the seed and skin fraction (Table 4.13). This fraction, therefore, contained relatively high levels of all flavonoids, as well as several alkaloids. On the other hand, compounds that were relatively low in the seed and skin fraction were several hydroxycinnamates (chlorogenic acid and 3 caffeic acid hexosides), citric acid, a glycoside of pantothenic acid (vitamin B5), and UDP-Glucose which is 22-fold higher than in the seed and skin fraction. For the rest of the compounds listed in Table 4.13 the seed and skin fraction contained 3 to 14-fold higher amounts compared to the fruit as in coumaric acid-hexose, and α-tomatin, respectively.

When the compounds that were significantly different between fruit and breaker samples were investigated, again several flavonoids and glycoalkaloids were determined (Table 4.14). The relative levels of compounds including, naringenin, naringenin chalcone, rutin, rutin apioside, kaempferol-rutinoside, lycoperoside H, increased with ratios of 1.3 to 4.3-fold after the breaking treatment as compared to the intact fruits. On the other hand, only pantothenic acid-hexose, UDP-Glucose, ferulic acid-hexose decreased by 25% to 50% when fruit was processed into breaker.

The untargeted LC-MS analysis gave a more comprehensive view on the biochemical changes that occur during pastemaking, beyond carotenoids and vitamin C. When all the metabolites were evaluated, it can be observed that more than 40% of a total of 3177 metabolite-related mass signals were significantly changed during the entire process from fruit to paste.

Table 4.12: Identified metabolites detected by LC-QTOF MS that were significantly different between original "Fruit" and final "Paste".

RT (min)[1]	Metabolite	Probability[2]	Ratio
			Fruit : Paste
42.10	Naringenin	0.006200	0.16
26.57	Lycoperoside H	0.012900	0.54
7.29	Pantothenic acid-hexose	0.044900	1.36
13.17	Caffeic acid hexose	0.049100	1.46
14.81	Chlorogenic acid	0.029700	1.79
23.85	Lycoperoside G or F, or esculoside A	0.023100	2.26
31.06	Quercetin-hexose-deoxyhexose-pentose-p-coumaric acid	0.039300	2.36
39.27	Tricaffeoylquinic acid	0.017100	2.87
20.45	Esculeoside B	0.019900	3.04
30.52	Dicaffeoylquinic acid	0.004460	3.14
33.39	Lycoperoside A, B, or C	0.023600	3.39
33.42	Naringenin chalcone-hexose	0.025000	6.70
32.75	Naringenin chalcone-hexose	0.015800	8.64
25.87	Lycoperoside G or F, or esculoside A	0.000589	14.53
26.64	Lycoperoside G or F, or esculoside A	0.003730	16.86
42.67	Naringenin chalcone	0.008980	22.82
37.90	Tomatoside A	0.019700	62.33

[1]RT: chromatographic retention (in minutes). Indicated for each compound is the significance of the difference between "Fruit" and "Paste" samples, and the calculated ratio of the concentration of the compound in both samples.

[2] Significance level is 0.05 (n=5).

Table 4.13: Identified metabolites detected by LC-QTOF MS that were significantly different between "Fruit" and "Seed & Skin"samples.

RT (min)[1]	Metabolite	Probability[2]	Ratio
			Fruit : Seed&Skin
33.39	α-Tomatine	0.011700	0.07
42.10	Naringenin	0.008600	0.09
22.47	Rutin apioside	0.006240	0.12
33.39	Lycoperoside A, B, or C	0.002820	0.14
42.67	Naringenin chalcone	0.000253	0.16
24.40	Rutin	0.000596	0.19
15.51	Quercetin-dihexose-deoxyhexose	0.001540	0.19
27.41	Kaempferol-rutinoside	0.002690	0.19
15.82	Naringenin-dihexose	0.003910	0.23
31.06	Quercetin-hexose-deoxyhexose-pentose-p-coumaric acid	0.000869	0.26
37.90	Tomatoside A	0.000395	0.26
39.27	Tricaffeoylquinic acid	0.003660	0.31
32.75	Naringenin chalcone-hexose	0.013000	0.34
26.57	Lycoperoside H	0.001820	0.39
13.85	Coumaric acid-hexose	0.029000	0.39
3.26	Citric acid	0.000239	1.92
13.17	Caffeic acid hexose	0.001170	2.29
14.81	Chlorogenic acid	0.033200	2.50
11.73	Caffeic acid hexose	0.006830	2.63
4.83	Phenylalanine	0.001190	2.68
15.90	Benzyl alcohol-hexose-pentose	0.049000	3.15
7.29	Pantothenic acid-hexose	0.000613	3.72
10.19	Caffeic acid hexose	0.014400	4.51
3.92	UDP-Glucose	0.002020	21.53

[1]RT: chromatographic retention (in minutes). Indicated for each compound is the significance of the difference between "Fruit" and "Seed & Skin" samples, and the calculated ratio of the concentration of the compound in both samples.

[2] Significance level is 0.05 (n=5).

Table 4.14: Identified metabolites detected by LC-QTOF MS that were significantly different between original "Fruit" and "Breaker" samples.

RT (min)[1]	Metabolite	Probability[2]	Ratio
			Fruit : Breaker
42.10	Naringenin	0.00185	0.23
33.39	Lycoperoside A, B, or C	0.02220	0.35
24.40	Rutin	0.00584	0.41
42.67	Naringenin chalcone	0.01550	0.47
22.47	Rutin apioside	0.01220	0.49
15.82	Naringenin-dihexose	0.03800	0.56
27.41	Kaempferol-rutinoside	0.02410	0.59
26.57	Lycoperoside H	0.03810	0.66
15.51	Quercetin-dihexose-deoxyhexose	0.01330	0.67
20.45	Esculeoside B	0.01320	0.79
7.29	Pantothenic acid-hexose	0.03140	1.34
3.92	UDP-Glucose	0.04670	1.80
12.90	Ferulic acid-hexose	0.02010	1.98

[1]RT: chromatographic retention (in minutes). Indicated for each compound is the significance of the difference between "Fruit" and "Breaker" samples, and the calculated ratio of the concentration of the compound in both samples.

[2] Significance level is 0.05 (n=5).

It appears that two steps (removal of the seed and the skin and breaking) are specifically important in changing the metabolic profiles during the paste making as shown in the PCA plot (Figure 4.12, and Table 4.13, Table 4.14).

Firstly, the removal of seed and skin in the finisher step caused a major change in the overall metabolic profile. This change include a strong reduction in the level of a number of flavonoids and alkaloids relative to the original fruit samples (Table 4.13). For some of the flavonoids, for which standards were commercially available, these results were confirmed in the targeted HPLC-PDA (Table 4.7). These flavonoids and alkaloids mostly end up in the seed and skin fraction, which is therefore relatively rich in these compounds (on basis of the same weight). Both the flavonoids and the identified alkaloids have been mainly reported to occur in the epidermal tissue of tomato fruits (Bovy *et al*., 2002; Moco *et al*., 2006; Moco *et al*., 2007). It is, therefore, likely that the loss of flavonoids and alkaloids during the pasting process resulted from incomplete extraction of these compounds from the epidermal tissue during processing. An exception in this respect is tomatoside A, which is a seed-specific compound (Moco *et al*., 2007), and is relatively abundant in the seed and skin fraction. As a result, the removal of seed and skin material thus seems to have a strong effect on the metabolite composition of tomatoes.

Secondly, the transition from fruit to breaker was an important step (Table 4.14). After this step, most flavonoids and some alkaloids were reproducibly increased by a factor of 2 to 3. During this transition, the fruits are washed, transported and chopped. It is yet unclear which of these activities contribute to the observed enrichment in flavonoids and alkaloids. One explanation could be that the breaking of the fruit triggers a wound response in the fruit tissue. Increases in flavonoids have been frequently reported upon cutting (wounding) of plant tissues (Tudela *et al*., 2002; Vina and Chaves, 2006; Vina and Chaves, 2007) as also explained in section 4.1.8. Moreover, it is known that wounding of tomato fruit induces production of enzymes such as ascorbate free-radical reductase, which are involved in the regeneration of antioxidant compounds (Grantz *et al*., 1995).

4.2. Changes in Tomato Fruit During Developmental Stages

In order to evaluate the changes in different tissues of a tomato fruit, which occur during the development of the tomato fruit, a single cultivar, *Ever*, was investigated. For further analysis the fruit was divided into five different tissues starting from the outside to the centre of the fruit. The following fruit parts were separated and analyzed separately: the fruit tissue where the sepals were directly connected (vascular attachment region or calyx), the external epidermal tissue layer (exocarp or epidermis), the fleshy tissue layer below the epidermis (pericarp), the gelatinous locular tissue of the fruit including the seeds (jelly parenchyma), and the central inner fleshy tissue of fruit (columella), as can be seen in Figure 3.2.

4.2.1. Moisture contents

The moisture contents of each tissue collected at each development stage are given in Table 4.15. The moisture content of tomato tissues changed between 93.0-95.5% at different stages. The moisture contents of green and red tomato have been reported to be approximately 93.0 and 94.5%, respectively (Anon., 2007). In a study where tomatoes were divided into three parts as skin, pulp and seed, the moisture contents of each portion were found to be 94.2-94.5%, 95.3-95.9%, and 92.9-94.8%, respectively, in 3 different cultivars (Toor and Savage, 2005). The differences in the moisture contents in our study were found to be statistically insignificant in each tissue at different development stages ($p>0.05$).

Table 4.15: Moisture content in the fruit tissues during development (%)[1].

	Green	**Breaker**	**Turning**	**Pink**	**Red**
Calyx	93.7 ± 0.1 a	93.4 ± 0.2 a	93.9 ± 0.3 a	94.0 ± 0.4 a	93.6 ± 0.3 a
Columella & Placenta	93.6 ± 0.3 a	93.7 ± 0.4 a	94.6 ± 0.5 a	94.1 ± 0.4 a	94.8 ± 0.6 a
Epidermis	93.9 ± 0.3 a	93.9 ± 0.2 a	94.1 ± 0.3 a	94.2 ± 0.2 a	93.4 ± 0.5 a
Pericarp	95.0 ± 0.6 a	94.9 ± 0.3 a	95.4 ± 0.2 a	95.5 ± 0.4 a	95.3 ± 0.2 a
Jelly paranchyma	93.8 ± 0.4 a	93.2 ± 0.1 a	93.6 ± 0.3 a	93.0 ± 0.3 a	94.3 ± 0.4 a

[1]Data represent average quantities (n=2). Different letters in the rows represent statistically significant differences ($p<0.05$).

4.2.2. Flavonoids

The flavonoid contents of all samples are presented in Figure 4.13, 4.14., and 4.15. Rutin amount was found to be highest in the epidermis tissues (Figure 4.13) compared to its amount in other tissues at all stages. When different stages were compared, the highest value of rutin compound was observed in the red stage (1389.6 µg/g) with an increase about 11% from green to red stage. Second highest values were obtained in the vascular attachment region which was found to be highest in the breaker (398.0 µg/g) and lowest in the red stage (92.2 µg/g) of development. Columella & Placenta also included low amounts of rutin changing in between 37.4-143.5 µg/g. Moreover, pericarp and jelly parenchyma tissues had very low values, including the pink jelly parenchyma, being the lowest (2.1 µg/g).

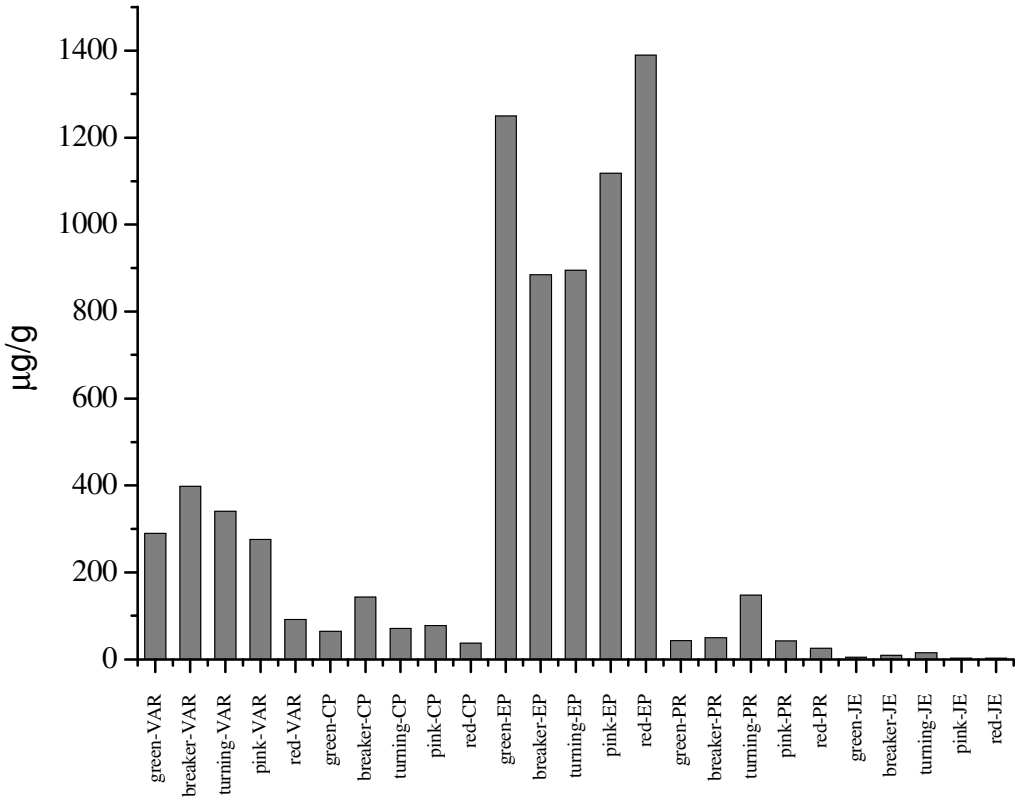


Figure 4.13: Rutin content of tomato tissues at different development stages.

The levels of rutin apioside were much lower than levels of rutin (0-211.7 µg/g) (Figure 4.14). Rutin apioside, being again highest in the epidermis, decreased gradually from green to the red stage (about 39%). In the vascular attachment region, the rutin apioside decreased by 62% from green to red stage. The values of rutin apisode in the pericarp tissue were found to be very low between concentrations 0-4 µg/g. On the other hand, there was no rutin apioside in the columella & placenta and jelly parenchyma tissues.

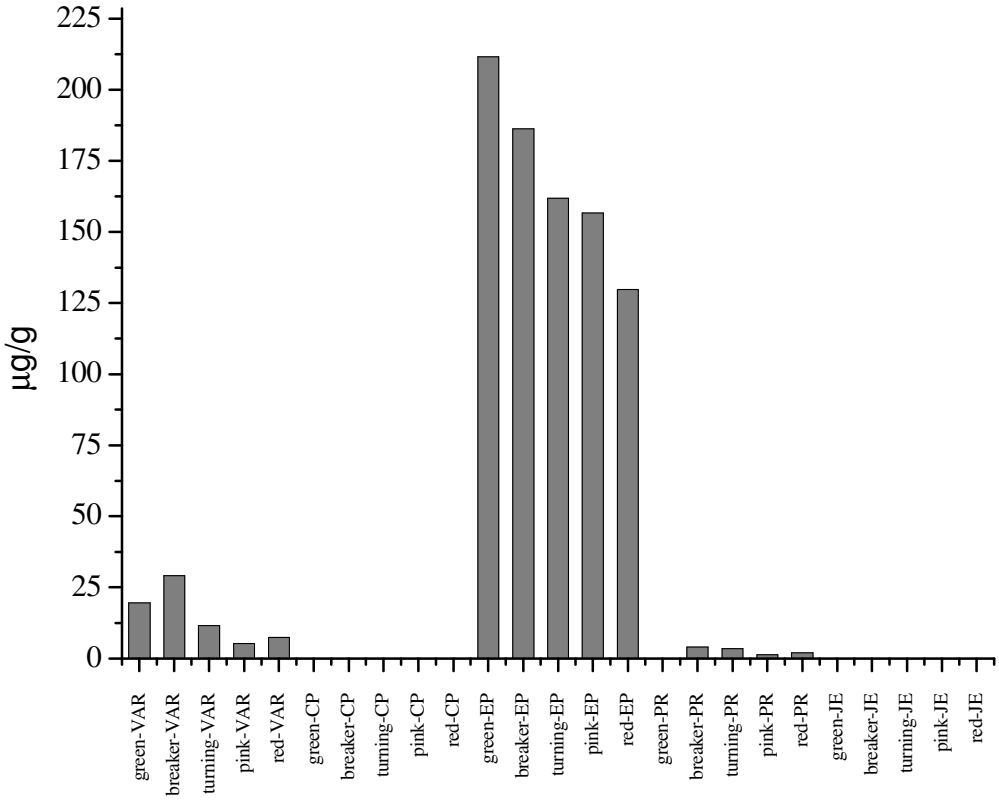


Figure 4.14: Rutin apioside content of tomato tissues at different development stages.

The contents of naringenin in different tissues and development stages are shown in Figure 4.15. Naringenin is mainly located in the epidermis showing the highest concentration (91.9 µg/g) in the pink stage of development. However, there was no naringenin observed in the green stage of development in any tissue including the epidermis. In the vascular attachment region naringenin reached to its highest value during breaker stage (23.7 µg/g). The pericarp and the jelly parenchyma contained

very low values between 0 and 4.3 µg/g. None of the development stages of columella & placenta contained naringenin.

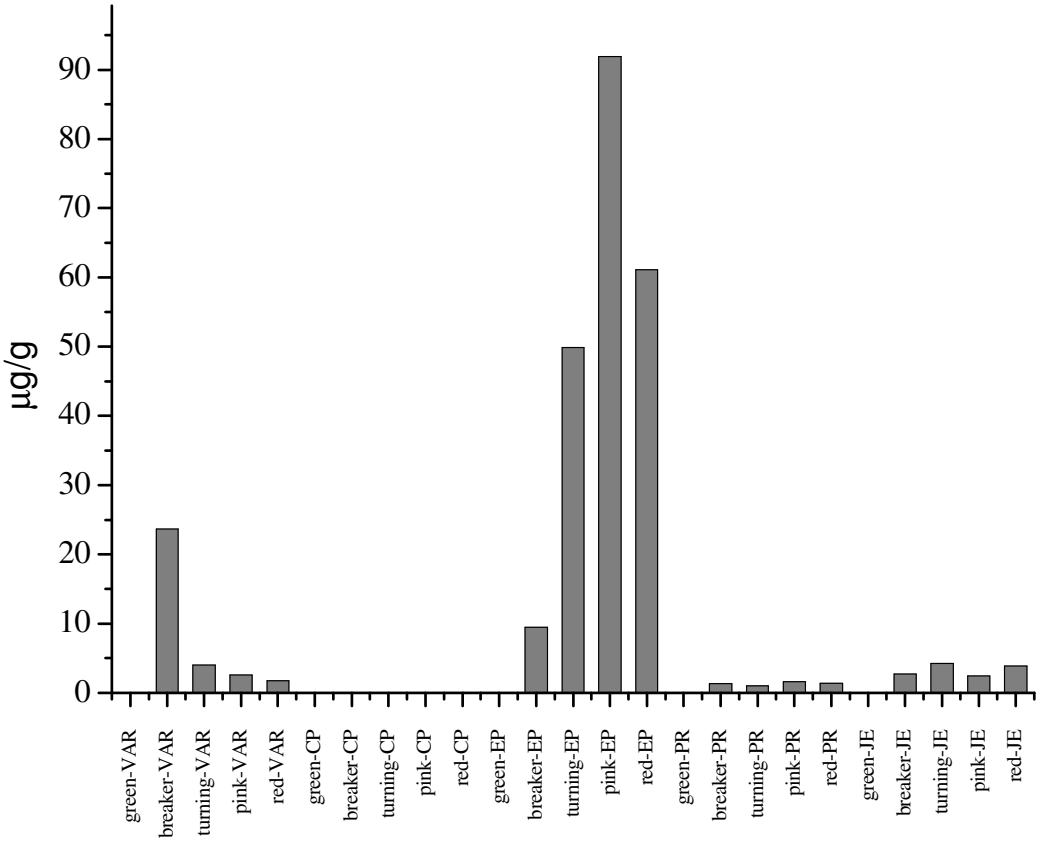


Figure 4.15: Naringenin content of tomato tissues at different development stages.

Naringenin chalcone amount was found to be highest in the epidemis tissues at the pink stage (10451.0 µg/g) followed by a 44% of decrease from pink to red stage (Figure 4.16). In the vascular attachment region, naringenin chalcone content increased from 0 (green) to 728.2 µg/g (turning) and then decreased by 62% in the red stage of development. Similarly, the content in the pericarp increased from 0.9 µg/g (green) to 24.8 µg/g (turning), which was followed by a decrease about 85% until reaching to the red stage. The columella & placenta and the jelly parenchyma tissues contained very low amounts in few development stages but there was no naringenin chalcone observed in the pink and the red stage in both of these tissues.

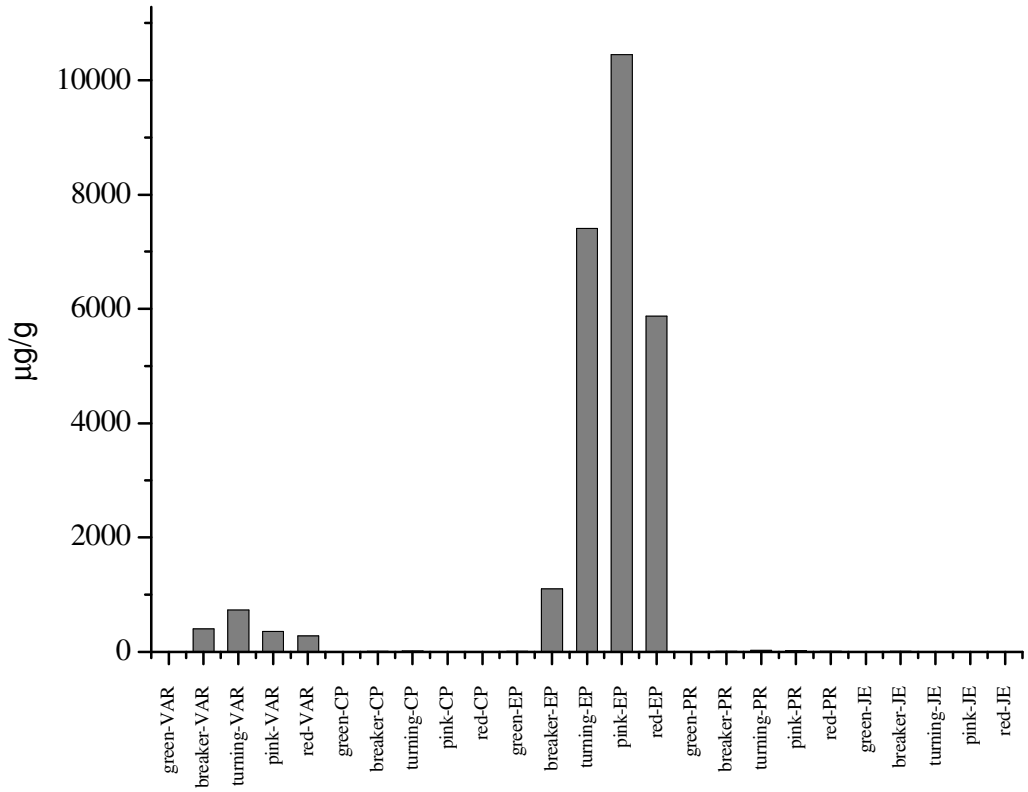


Figure 4.16: Naringenin chalcone content of tomato tissues at different development stages.

According to the metabolomics analysis, flavonoids were typically present in the epidermal tissue of the tomato fruit as also observed by the HPLC analysis (Figure 4.19). Quercetin, kaempferol and naringenin (chalcone) derivatives were found mostly in the epidermis and some, such as naringenin chalcone, naringenin and the trisaccharides of kaempferol and quercetin, also in the calyx tissues. Quercetin-dihexose-deoxyhexose, quercetin-hexose-deoxyhexose-pentose-coumaric acid, kaempferol-dihexose-deoxyhexose, naringenin, naringenin chalcone-hexose and naringenin-dihexose increased during development, while quercetin-hexose-deoxyhexose-pentose decreased. Rutin and naringenin chalcone were the most abundant flavonoids in the fruit, exhibiting intense mass signals. In the present study an increase in the rutin and naringenin chalcone was only observed from the green to the breaker stages, followed by stabilized intensities until the red stage. However, in the literature it is assumed that these flavonoids increase during all development stages (Bovy *et al*., 2002). Two glycosylated derivatives of kaempferol, namely

kaempferol-rutinoside and kaempferol-hexose-deoxyhexose-pentose, exhibited non-linear patterns during development. There was an increase in the first derivative from breaker to red stage, which was followed by a decrease from the green to the breaker stage. The second derivative was decreased at the turning stage, increased at the pink stage, and returned to lower intensities at the red stage of the fruits.

The epidermis and the jelly parenchyma, at all ripening stages, exhibited the most extreme differences with regard to metabolite composition. Glycosylated flavonoids, including rutin, kampferol rutinoside and a quercetin trisaccharide, are specifically abundant in the epidermis, either present at similar levels in all developmental stages or accumulating upon ripening. Especially, deglycosylated flavonoids are known for their capacity of electron transfer, as antioxidants but also as prooxidants (Awad *et al*., 2001; Lemanska *et al*., 2001). As such, flavonoids can participate as plant protective elements in both biotic and abiotic phenomena: defense against pathogens and environmental stress (drought, UV radiation, wounding) (Pourcel *et al*., 2007). In addition, flavonoids have been associated with auxin transport in the plant, acting as endogenous mediators of auxin flow (Besseau *et al*., 2007). During fruit development, high levels of naringenin chalcone are being formed in the epidermis. The concentration of glycosylated flavonoids (dihexose-deoxyhexoses of kampferol and quercetin) increased during development possibly associated to the increase in the formation of sugars with higher conjugation possibilities. The presence of flavonoid derivatives in the outer layers of the tomato fruit is in accordance with the increased transcription of phenylalanine ammonia-lyase, flavanone 3-hydroxylase, flavonol synthase and sugar transporters in the exocarp tissues compared to the inner locular tissue (Lemaire-Chamley *et al*., 2005).

4.2.3. Carotenoids and chlorophylls

The carotenoid contents of samples are given in Table 4.16. According to the results, low amounts of neoxanthin occurred in all tissues of tomato (below 15 μg/g dry weight). The highest neoxanthin content was observed in the green stage of jelly parenchyma (13.57 μg/g). However, its concentration decreased gradually by about 86% from green to red stage of development.

Table 4.16: Mass contents of carotenoids and chlorophylls in the tissues of tomato fruit (calyx, columella, epidermis, pericarp and jelly parenchyma), at different development stages (in µg/g dry weight)[1].

Development Stages	Neoxanthin	Violaxanthin	β-Carotene	All *trans*-lycopene	Lutein	Chlorophyll a	Chlorophyll b
Vascular attachment region							
G	11.81 ± 1.31	20.93 ± 0.47	10.32 ± 1.70	1.91 ± 0.48	34.12 ± 4.27	254.56 ± 23.51	96.32 ± 10.70
B	10.68 ± 0.81	27.01 ± 0.35	20.07 ± 3.70	4.96 ± 0.80	34.02 ± 0.89	214.12 ± 19.65	69.37 ± 6.63
T	10.97 ± 0.69	36.44 ± 0.10	38.12 ± 5.39	48.86 ± 8.25	36.63 ± 1.18	166.13 ± 11.84	53.40 ± 3.59
P	8.46 ± 0.42	32.68 ± 0.19	52.25 ± 5.48	110.15 ± 18.02	30.06 ± 0.44	108.02 ± 5.01	30.60 ± 2.38
R	5.51 ± 0.27	22.87 ± 0.67	74.03 ± 3.89	400.83 ± 35.19	30.77 ± 0.67	49.13 ± 2.02	14.29 ± 1.29
Columella							
G	6.25 ± 0.40	12.19 ± 0.24	4.17 ± 0.56	nd	16.28 ± 0.99	98.06 ± 6.85	37.47 ± 2.09
B	4.91 ± 0.19	20.29 ± 0.42	21.81 ± 1.77	20.22 ± 1.09	18.24 ± 1.28	51.40 ± 3.37	18.32 ± 1.59
T	5.73 ± 0.56	22.8 ± 0.40	28.15 ± 3.82	35.68 ± 4.51	15.85 ± 0.60	45.95 ± 7.42	15.99 ± 1.24
P	4.04 ± 0.45	21.43 ± 0.35	43.84 ± 2.47	110.26 ± 7.53	11.42 ± 0.24	15.73 ± 1.46	7.52 ± 0.92
R	3.62 ± 0.44	23.95 ± 0.33	60.98 ± 3.10	253.98 ± 16.29	12.24 ± 0.61	nd	2.23 ± 0.50
Epidermis							
G	2.01 ± 0.46	8.55 ± 0.45	7.64 ± 0.12	nd	9.15 ± 1.27	102.56 ± 7.22	26.07 ± 0.67
B	3.13 ± 0.56	15.71 ± 0.87	23.61 ± 1.53	40.18 ± 3.24	11.35 ± 1.20	81.02 ± 5.15	20.56 ± 1.74
T	1.7 ± 0.16	13.23 ± 0.94	39.54 ± 0.46	214.69 ± 2.32	8.95 ± 0.56	24.4 ± 1.49	3.75 ± 0.45
P	1.8 ± 0.21	6.47 ± 0.49	64.89 ± 1.78	874.25 ± 23.38	8.58 ± 0.13	5.03 ± 0.51	1.28 ± 0.08
R	nd	9.92 ± 0.14	84.64 ± 4.54	2786.53 ± 86.83	9.04 ± 0.38	nd	nd
Pericarp							
G	6.34 ± 0.56	11.39 ± 0.08	8.29 ± 0.23	0.48 ± 0.04	18.08 ± 0.54	121.83 ± 13.37	43.41 ± 4.03
B	6.73 ± 0.62	16.84 ± 0.31	29.15 ± 0.33	41.58 ± 1.22	25.36 ± 0.50	73.10 ± 6.25	23.54 ± 3.33
T	2.91 ± 0.61	11.96 ± 1.18	44.66 ± 6.89	78.44 ± 33.75	21.37 ± 0.60	27.57 ± 2.74	8.49 ± 0.70
P	3.09 ± 0.20	11.00 ± 1.32	50.03 ± 5.68	301.50 ± 9.98	20.90 ± 0.78	6.65 ± 0.26	3.34 ± 0.05
R	nd	5.48 ± 0.09	49.9 ± 1.83	845.68 ± 17.21	14.16 ± 0.45	nd	nd
Jelly parenchyma							
G	13.57 ± 1.57	25.09 ± 0.77	15.21 ± 0.59	nd	39.73 ± 1.44	281.95 ± 8.1	119.37 ± 9.09
B	4.62 ± 0.12	17.83 ± 1.61	64.90 ± 4.35	115.02 ± 7.44	25.75 ± 1.01	21.78 ± 3.34	5.48 ± 0.82
T	2.90 ± 0.38	13.29 ± 0.59	73.99 ± 1.59	213.18 ± 5.47	20.23 ± 0.21	2.39 ± 1.43	0.86 ± 0.43
P	2.63 ± 0.20	12.88 ± 0.77	73.74 ± 4.11	366.83 ± 16.84	21.16 ± 0.96	0.96 ± 0.96	nd
R	1.93 ± 0.32	8.34 ± 0.40	69.27 ± 2.41	542.63 ± 21.10	21.46 ± 1.09	0.32 ± 0.32	nd

[1]Data represent average quantities (n=3) ± standard error of the means; nd = not detectable; Dev = developmental stages: G = green, B = breaker, T = turning, P = pink, R = red.

Similarly, in the vascular attachment region and columella & placenta, the highest values were observed in the green stage (11.81 μg/g and 6.25 μg/g, respectively) and then decreased by 53% and 42% until reaching to the red stage. On the other hand, in the epidermis and pericarp, its content was relatively low in the green stage and was, even, not detectable in the red stage (Table 4.16).

The levels of violaxanthin were relatively stable during development of all tissues. The highest value was observed in the vascular attachment region at the turning stage (36.44 μg/g). Then its concentration decreased about 37% during the development towards to the red stage. In the jelly parenchyma, the green fruit contained the highest violaxanthin amount (25.09 μg/g) and decreased gradually to 8.34 μg/g (a 67% decrease). Pericarp tissue contained lower amounts of violaxanthin changing between 5.48-16.84 μg/g. From green to red stage there was a 52% decrease in the pericarp tissue. Similarly, Fraser *et al*. (1994) observed a decrease in the the characteristic carotenoids found in green fruit, i.e. lutein, neoxanthin, and violaxanthin as the ripening process progressed. However, in the epidermis and columella & placenta tissues increases about 14% and 49%, respectively, were observed from green to red stage (Table 4.16).

It was observed that the highest amount of β-carotene was found to be in the red epidermis, which was about 11 times higher than in the green stage (Table 4.16). Except for pericarp and jelly parenchyma tissues, the content of β-carotene in all tissues increased from green to red. In those tissues the highest values of β-carotene were obtained in the pink and turning stage (50.03 μg/g and 73.99 μg/g, respectively). Particularly, in the vascular attachment region, there was about 86% increase in β-carotene levels from green to red stage. Similar to these findings, in the columella and parenchyma tissue about 15 times higher β-carotene values were observed in the red tissue when compared to the green phase (Table 4.16).

The all *trans*-lycopene content in red epidermis was found to be highest with respect to the other development stages and tissues (2786.53 μg/g dry weight) (Table 4.16). These findings were higher than the results of Toor and Savage (2005) who observed a lycopene content of 1526 μg/g dry weight in the tomato skin. In the green fruit lycopene was not detectable as can also be observed from the color of the fruit in

Figure 3.2. However, lycopene content increased about 69 times from breaker to red stage. Moreover, red pericarp tissue contained even higher amounts of lycopene (845.68 μg/g dry weight) with an increase of about 1750 times compared to the green stage. The lycopene level was undetectable in tissues at green stage except for the pericarp and vascular attachment region, in which very small amounts were observed (0.48 and 1.91 μg/g dry weight, respectively). In the vascular attachment region, the lycopene content increased 210 times with respect to the green fruit (400.83 μg/g dry weight). Columella & Placenta in the red fruit contained 253.98 μg/g dry weight lycopene which was 13 times higher than that of the breaker stage. Similarly, lycopene in the jelly parenchyma increased 5 times from breaker to red stage of development and reached to a content of 542.63 μg/g dry weight (Table 4.16).

In the epidermis, lutein showed the lowest amount and the highest amount in the vascular attachment region at all developmental stages. In the vascular attachment region from green to red stage a 10% decrease was observed. This slight decrease was observed consistently at all tissues during development stages. In the epidermis this decrease was about 1%, on the other hand for columella & placenta it was about 25%. From green pericarp to breaker pericarp an increase by 40% was detected initially but this increase was then followed by a gradual decrease reaching to 14.16 μg/g dry weight. On the other hand, lutein in the jelly parenchyma decreased gradually by 46% during development from green to red tissue (Table 4.16).

The transformation of chloroplasts into chromoplasts during fruit ripening is paired with the degradation of chlorophylls and production of carotenoids, in particular lycopene. Lycopene is the pigment conferring the red color of ripe tomato fruits and is accumulated in fruit-localized phytochromes. These organelles regulate the color development in tomato by controlling the amount of accumulated lycopene (Alba *et al*., 2000). Lycopene is strongly present in the epidermis, but also, to a lesser extent, in all other fruit tissues similar to our findings. Carotenoids, in general, attract seed dispersals and, therefore, can influence the propagation of the species. The profiles given by the concentration levels of carotenoids in tomato fruit through development seem to be comparable to previous studies, despite the differences in cultivar and sample preparation (Fraser *et al*., 1994). The main carotenoids detected (neoxanthin,

violaxanthin, β-carotene, lycopene and lutein) are present in all tissues of the fruit, including the inner parts of the fruit such as the jelly parenchyma.

Chlorophyll a was higher than chlorophyll b with a ratio of about 3 in all tissues. Chlorophyll b was more abundant in the calyx tissue and less abundant in the jelly parenchyma tissue, while these were the two tissues where chlorophyll a was the richest while being the poorest in the columella and epidermis. The highest chlorophyll a and b was in the green jelly parenchyma (281.95 µg/g dry weight) but decreased sharply starting from the breaker and ended with 0.32 µg/g dry weight in the red tissue. In the red stage of columella & placenta, epidermis, and pericarp no chlorophyll a was observed. On the other hand, no chlorophyll b was observed in the epidermis, pericarp of the red fruit. In the jelly parenchyma chlorophyll b content was 119.37 µg/g dry weight at the red stage but decreased sharply to 5.48 µg/g dry weight in the breaker and was not detectable at the pink and red stages.

The green color of the tissues in the early stages of development is associated to the presence of chlorophylls. In tomato, two chlorophyll molecules are detected: chlorophyll a and b, differing in an aldehyde group (in chlorophyll b) instead of a methyl group (in chlorophyll a) (Rüdiger, 2002; Daun, 2005; Larkum and Kühl, 2005). These two related metabolites complement each other in their photoreception capabilities, enlarging the light absorbing spectrum. At each ripening stage the level of total chlorophylls was the highest in the calyx and the lowest in the epidermis. In fact, there is evidence that the chlorophylls are photosynthetically active including the calyx and inner tissues as the jelly parenchyma (Smillie *et al*., 1999). It has been further suggested that the photosynthetic abilities of the jelly parenchyma might be of importance in the development and maturation of the seeds. The presence of chlorophylls in the inner fruit layers of the tomato fruit is in accordance with the transcriptional patterns of chlorophyll a/b related proteins, which were found preferentially expressed in the locular fruit tissue (Lemaire-Chamley *et al*., 2005). Similarly, the highest chlorophyll a and b values were obtained in the inner fruit layers, specifically at the green stage of jelly parenchyma tissues.

In summary, the tendencies observed during development were similar for all tissues: there was an increase in lycopene during fruit development and a decrease in chlorophylls (a and b), which was also obvious from the fruit color (from green to a

red colored-fruit). β-carotene increased during fruit development, neoxanthin slightly decreased, and lutein was almost constant during development. Violaxanthin showed a slightly different tendency from other xanthophylls as firstly increased until the breaker/pink stage and then decreased in the red stage.

4.2.4. Tocopherols

The tocopherol content in the fruit tissues during development are presented in Table 4.17. In general, the α-, γ- and δ-tocopherols increased during development in all tissues except for the jelly parenchyma where all tocopherols decreased.

Table 4.17: Mass contents of tocopherol and vitamin C in the tissues of tomato fruit at different development stages (µg/g dry weight)[1].

Development Stages	δ-tocopherol	α-tocopherol	γ-tocopherol	Vitamin C
Vascular attachment region				
G	0.22 ± 0.02	406.29 ± 9.39	10.19 ± 0.16	274.09 ± 2.83
B	1.08 ± 0.00	483.93 ± 15.6	15.42 ± 0.20	796.03 ± 2.31
T	2.60 ± 0.07	554.89 ± 7.49	32.09 ± 0.47	412.44 ± 6.73
P	2.84 ± 0.04	631.76 ± 9.76	37.91 ± 0.81	415.6 ± 3.47
R	3.23 ± 0.09	537.59 ± 3.40	62.73 ± 1.13	1286.85 ± 5.84
Columella				
G	0.03 ± 0.02	209.03 ± 1.36	3.11 ± 0.11	146.56 ± 2.51
B	0.44 ± 0.01	210.75 ± 2.79	7.34 ± 0.06	549.76 ± 3.2
T	0.82 ± 0.01	278.47 ± 2.66	10.05 ± 0.14	701.97 ± 3.37
P	0.83 ± 0.01	279.53 ± 2.47	9.99 ± 0.12	869.48 ± 6.38
R	1.06 ± 0.00	316.95 ± 1.13	15.34 ± 0.34	1302.36 ± 5.08
Epidermis				
G	1.76 ± 0.03	181.19 ± 5.54	33.67 ± 0.31	1176.59 ± 7.24
B	3.27 ± 0.06	193.69 ± 7.94	41.55 ± 1.54	1609.36 ± 10.28
T	5.86 ± 0.04	208.25 ± 5.31	62.11 ± 0.34	1553.45 ± 5.96
P	7.38 ± 0.05	214.31 ± 1.89	77.38 ± 0.73	1616.4 ± 3.27
R	7.57 ± 0.11	193.69 ± 3.26	69.11 ± 1.21	1670.74 ± 7.61
Pericarp				
G	2.72 ± 0.57	155.4 ± 11.52	77.32 ± 25.33	703.75 ± 2.48
B	6.16 ± 0.22	157.54 ± 5.72	151.07 ± 5.64	1174.49 ± 8.25
T	5.19 ± 0.16	165.18 ± 1.71	128.69 ± 4.63	1404.24 ± 10.97
P	4.66 ± 0.16	195.60 ± 3.80	85.58 ± 2.81	1141.82 ± 7.67
R	4.33 ± 0.13	216.62 ± 7.06	129.74 ± 6.14	1517.8 ± 6.08
Jelly parenchyma				
G	3.07 ± 0.51	133.02 ± 8.58	102.11 ± 22.16	830.34 ± 12.8
B	1.02 ± 0.00	121.36 ± 1.60	13.37 ± 0.21	1040.81 ± 11.57
T	1.37 ± 0.00	132.45 ± 2.41	21.61 ± 1.19	1108.41 ± 8.82
P	1.42 ± 0.00	136.40 ± 1.80	25.28 ± 0.96	1039 ± 7.24
R	1.52 ± 0.01	114.29 ± 0.54	22.22 ± 0.31	1141.9 ± 8.36

[1]Data represent average quantities (n=3) ± standard error of the means; Dev = developmental stages: G = green, B = breaker, T = turning, P = pink, R = red.

The tocopherols showed different concentrations in diverse parts of tomato fruit. The levels of δ-tocopherol were relatively low in all tissues when compared with γ- and α-tocopherols, while β-tocopherol was not detectable at all (less than 0.1 µg/g dry weight). The highest δ-tocopherol was in the red epidermis which increased 4 times

when compared to that of green tissue. Similarly, δ-tocopherol content in the vascular attachment region, columella & placenta, and pericarp increased 15, 35, 2 times following transition from green to red stage, respectively. On the other hand, a decrease by 50% was observed in the jelly parenchyma during fruit development.

α-Tocopherol (vitamin E) was the most abundant tocopherol type in all tissues at all developmental stages. The vascular attachment region was the richest tissue for this tocopherol, while the jelly parenchyma was the poorest. Except for jelly parenchyma, α-tocopherol content increased in all tissues from green to red stage. The percent increases were 32%, 52%, 7%, and 39% for vascular attachment region, columella and placenta, epidermis, and pericarp, respectively. In the jelly parenchyma, 14% decrease was observed by transition from green to red tissue stage.

γ-Tocopherol, which is the biosynthetic precursor of α-tocopherol, was highest in the jelly parenchyma of the tomato fruit. Similar to the other tocopherols, its content in the jelly parenchyma decreased 5 times during fruit development. However, in other tissues an increase between 2 (in pericarp) to 6 (in vascular attachment region) times was observed. The ratio α- versus γ-tocopherol clearly differed between tissues, which suggest tissue-dependent differences in the activity of the corresponding γ-tocopherol methyltransferase catalyzing the methylation of γ-tocopherol to yield α-tocopherol. γ-Tocopherol methyltransferase is believed to be involved in regulating the relative amounts of the various tocopherols present in photosynthetic organisms (Backash *et al*., 2005).

4.2.5. Vitamin C

The vitamin C contents (Table 4.17) of tissues at different development stages varied in the ranges between 146.56 μg/g dry weight (lowest in green columella & placenta) and 1670.74 μg/g dry weight (highest in red epidermis). The lowest vitamin C contents were observed in columella & placenta and vascular attachment region tissues in which an increase by about 9 and 5 times was observed from green to red stage development, respectively.

In the view of current literature it is known that ripening stage is very effective on the content of vitamin C (Abushita *et al*., 2000; Leonardi *et al*., 2000). The level of

vitamin C (ascorbic acid) was increased during ripening, in all tissues, although its increase was generally largest between green and breaker or turning development stages (Table 4.17). In the vascular attachment region, vitamin C displayed a rather specific pattern upon fruit ripening: a nearly 3-fold increase from green to breaker stage, followed by a 2-fold decrease from breaker to turning stage and again a 3-fold increase from pink to red stage. When red fruit is compared to green fruit, ascorbic acid increased nearly 10 fold in the columella and less than 2 fold in the epidermis. At all ripening stages, the highest levels of this antioxidant were detected in the epidermis of the fruits.

The antioxidant ascorbic acid increases in the fruit during ripening, and is particularly high in the epidermis (Toor and Savage, 2005). By acting as a major electron transfer element in numerous reactions, ascorbic acid is likely to be involved in vital plant processes such as hormone biosynthesis (e.g. abscisic acid), detoxification of reactive oxygen species, regeneration of isoprenoid derivatives (e.g. α-tocopherol, zeaxanthin) and consequently, photosynthetic activity and plant growth (Chen and Gallie, 2006). The transcript level of guanidine diphosphate-mannose pyrophosphorylase, an enzyme involved in ascorbic acid biosynthesis, is higher in the exocarp than in the locular tissue explaining the high amounts of ascorbic acid in epidermis tissue (Lemaire-Chamley *et al*., 2005).

4.2.6. Metabolomics approach in the fruit tissues during development

The LC-QTOF-MS analyses of metabolites are presented in Figures 4.17 to 4.19. Metabolite analysis allowed the detection of mostly glycosylated derivatives of phenolic acids, alkaloids and other small molecules. The different fruit tissue profiles were quite diverse, as can be seen from the obtained mass chromatograms in Figure 4.17. It was also visible that, in all tissues, marked changes in metabolites occurred during ripening of the fruit, such as the complete disappearance as well as the appearance of mass signals. The most specific examples were 3-Caffeoylquinic acid (retention time 14 min., mass 354) and ferulic acid-hexose I (retention time 12-13 min., mass 356 g/mol) which were only found in vascular attachment region. Similarly, quercetin-dihexose-deoxyhexose was only observed in epidermis at 15-16 minutes (mass 771 g/mol). Rutin, which is an important flavonoid in tomato was found in vascular attachment region (retention time 24-25 minutes, mass 610 g/mol),

columella & placenta and epidermis (highest) and was not clearly observed in other tissues. Similarly, based on the HPLC analysis results, the other tissues were found to contain very low amounts of rutin. There were also differences observed in the content of lycoperoside which eluted at 26-27 minutes with a molecular weight of 1315 g/mol (high in the epidermis and jelly parenchyma tissues and low in the pericarp). Tomatoside A in jelly parenchyma tissue (retention time of 37.9 minutes, mass of 1082 g/mol) was not detected in other tissues. On the other hand, tricaffeoylquinic acid I (retention time 39.3 min., mass 678 g/mol) and naringeninchalcone (retention time 42.7 min., mass 272 g/mol) were only observed in epidermis.

Principle component analyses of the LC-MS profiles are shown in Figure 4.18. PCA plot over selected metabolites are shown in A and PCA plot over the samples are shown in B section. The explained variance over the x axis (PC1) was found to be 33.6%, y axis (PC2) 22.2 % and z axis (PC3) of 13.2%.

Accoring to the PCA plot, it appeared that differences between tissues are more pronounced than differences between ripening stages. Major metabolite variations were observed between the tissues epidermis and jelly parenchyma, which corresponded to the first and second principal component in the PCA plot, while the third component corresponds to fruit development. During ripening the differences between tissues become more pronounced, suggesting ripening-dependent tissue differentiation of metabolites.

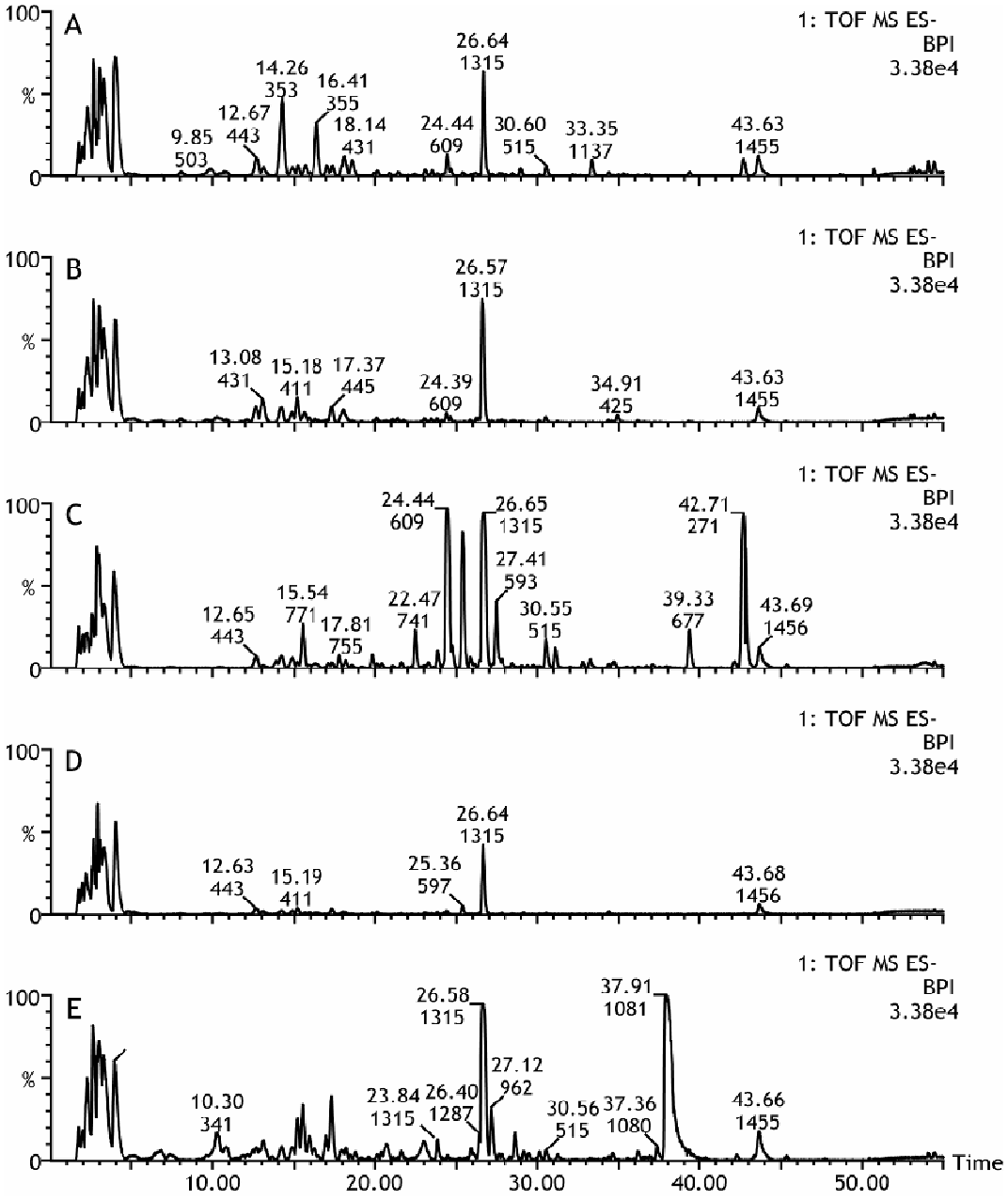


Figure 4.17: LC-MS chromatograms of the tissues vascular attachment region (A), columella & placenta (B), epidermis (C), pericarp (D) and jelly parenchyma (E) at the red stage of the tomato fruit *Ever*.

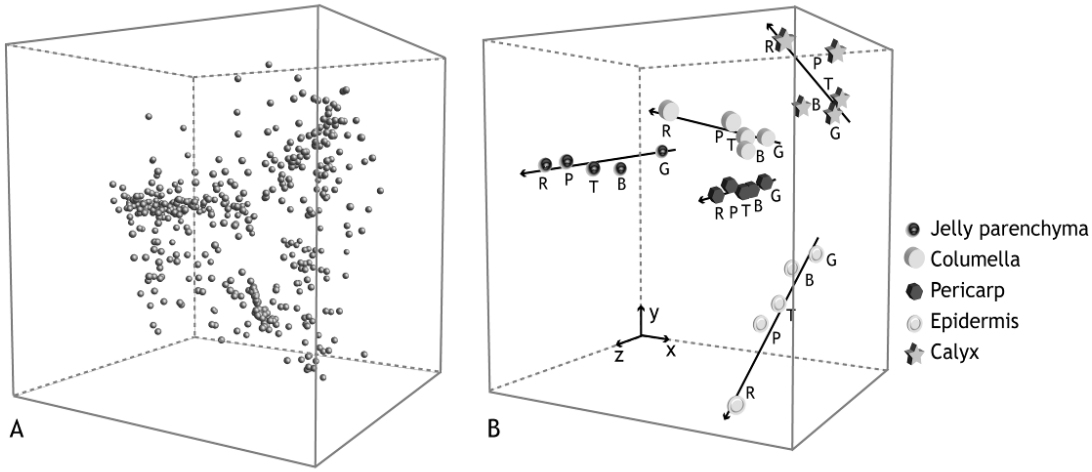


Figure 4.18: Principal component analysis of LC-MS data from tomato fruit *Ever* over different developmental stages (G = green, B = breaker, T = turning, P = pink and R = red) and different tissues within the fruit.

Quantification of compounds could not be performed in these analyses, as most of the naturally occurring compounds are not commercially available as standards. Compounds identified using LC-PDA-MS/MS in the analyzed tissues (calyx, columella, epidermis, pericarp and jelly parenchyma) are listed in Table 4.18. In the tables, Ret (min) corresponds to averaged retention time, in minutes. Mass corresponds to averaged accurate mass, in D, obtained from signals with an intensity ratio 0.25 < analyte / lock mass < 2.0 - in italic, from signals with analyte / lock mass > 0.25. Δmass (ppm) is the deviation between the averages of found accurate mass and real accurate mass, in ppm, obtained manually, by calculation from mass signal, when intensity of analyte ~ lock mass. UV/Vis is the absorbance maximums in the UV/Vis range (not detectable absorbances are represented by "-"). MS/MS are the fragments obtained through increased collision energy on indicated parent mass. Metabolite name is the common name of putatively identified metabolite. Mol Form corresponds to molecular formula of the metabolite. MM is the molecular mass of the metabolite. (FA) is the formic acid adduct, and I, II, III, IV corresponds to different isomers of the same compound.

The performance of the LC-MS system and the results obtained in this study are in accordance with previous findings (Moco *et al.*, 2006). Some of the compounds reported have been detected before in tomato peel and are present in the MoTo DB database. The analysis of different fruit tissues enables a complementation of the

putative identifications with additional or improved experimental data, in addition to other newly found compounds.

LC-MS analysis of tomato fruit tissues can provide important information about the tissue distribution of semi-polar metabolites and their fate upon ripening of the fruit. From these analyses, it became clear that most metabolites are not equally distributed over the fruit, but show preferential accumulation in one or more tissues.

Table 4.18: Metabolites putatively identified by LC-PDA-ESI-QTOF-MS/MS in tissues of tomato fruit (continuing).

Ret (min)	Max intensity	Mass	Δmass (ppm)	UV/Vis	MS/MS	Metabolite Name	Mol Form	MM
4.82	797	164.0725	-4.7		146, 103	Phenylalanine	$C_9H_{11}NO_2$	165.0790
7.38	1071	380.1561	3.8	-	308, 263, 218, 200, 174, 161, 146, 134	Zeatin hexose	$C_{16}H_{23}N_5O_6$	381.1648
10.27	8816	341.0880	-0.6			Caffeic acid-hexose I	$C_{15}H_{18}O_9$	342.0951
10.47	2864	325.0930	-0.3			Coumaric acid-hexose I	$C_{15}H_{18}O_8$	326.1002
10.88	913	341.0884	-1.6			Caffeic acid-hexose II		
12.67	1081	443.1918	1.0	-	381, 307, 281, 237, 219, 201, 189, 179, 161, 153, 143, 119, 113, 101, 89	Dehydrophaseic acid-hexose	$C_{21}H_{31}O_{10}$	444.1995
12.91	2077	355.1035	0.0			Ferulic acid-hexose I	$C_{16}H_{20}O_9$	356.1107
13.19	2340	341.0879	-0.3			Caffeic acid-hexose III	$C_{15}H_{18}O_9$	342.0951
13.90	1630	325.0929	-0.1		163, 119, 93	Coumaric acid-hexose II	$C_{15}H_{18}O_8$	326.1002
14.23	14901	353.0873	1.3		191	3-Caffeoylquinic acid	$C_{16}H_{18}O_9$	354.0951
14.26	1510	325.0935	-2.0	-		Coumaric acid-hexose III	$C_{15}H_{18}O_8$	326.1002
14.86	2800	353.0878	0.1			5-Caffeoylquinic acid	$C_{16}H_{18}O_9$	354.0951
15.20	7603	411.1872	0.0	-	249, 161, 101	(iso)pentyl dihexose	$C_{17}H_{32}O_{11}$	412.1945
15.39	286	325.0936	-2.1	-	265, 235, 205, 163, 145, 117	Coumaric acid-hexose IV	$C_{15}H_{18}O_8$	326.1002
15.55	7842	771.1989	0.1			Quercetin-dihexose-deoxyhexose	$C_{33}H_{40}O_{21}$	772.2062
15.89	631	595.1660	1.4		549, 475, 433, 415, 385, 355, 313, 271, 263	Naringenin dihexose	$C_{27}H_{32}O_{15}$	596.1741
16.38	9995	355.1038	-1.0			Ferulic acid-hexose II	$C_{16}H_{20}O_9$	356.1107
17.18	2280	353.0876	0.6			4-Caffeoylquinic acid	$C_{16}H_{18}O_9$	354.0951
17.29	1312	355.1031	0.9	297sh, 329	193, 175, 160, 132	Ferulic acid-hexose III	$C_{16}H_{20}O_9$	356.1107
17.83	2177	755.2036	0.6	264, 349	593, 447, 285	Kaempferol-dihexose-deoxyhexose	$C_{33}H_{40}O_{20}$	756.2113
20.40	1396	1272.5891	-2.0			(Esculeoside B) FA	$C_{57}H_{95}NO_{30}$	1273.5939
22.47	16993	741.1946	-8.4			Quercetin-hexose-deoxyhexose-pentose	$C_{32}H_{38}O_{20}$	742.1956
23.85	3619	1314.5978	-0.5			(Lycoperoside F) FA or (Lycoperoside G) FA or (Esculeoside A) FA I	$C_{59}H_{97}NO_{31}$	1315.6045
24.14	232	1094.5459	-6.4	-	1049, 917, 887, 754, 736, 718, 700, 688, 609, 592, 395, 305, 143, 89	(Lycoperoside H) FA I	$C_{51}H_{85}NO_{24}$	1095.5462
24.44	33024	609.1459	0.4			Rutin	$C_{27}H_{30}O_{16}$	610.1534
24.76	7396	725.1936	-0.2	264, 345	593, 575, 285, 255	Kaempferol-hexose-deoxyhexose-pentose	$C_{32}H_{38}O_{19}$	726.2007
25.50	737	1094.5419	-2.8	-	1049, 917, 887, 754, 688, 592, 455, 305, 143	(Lycoperoside H) FA II	$C_{51}H_{85}NO_{24}$	1095.5462

Table 4.18: Metabolites putatively identified by LC-PDA-ESI-QTOF-MS/MS in tissues of tomato fruit.

Ret (min)	Max intensity	Mass	Δmass (ppm)	UV/Vis	MS/MS	Metabolite Name	Mol Form	MM
25.70	353	425.1821	-1.0	-	263, 153	Abscisic acid-hexose	$C_{21}H_{30}O_9$	426.1890
25.83	980	1314.5954	1.4			(Lycoperoside F) FA or (Lycoperosyde G) FA or (Esculeoside A) FA II	$C_{59}H_{97}NO_{31}$	1315.6045
25.87	2053	1312.5817	-0.2			(Dehydrolycoperoside F) FA or (Dehydrolycoperoside G) FA or (Dehydroesculeoside A) FA I	$C_{59}H_{95}NO_{30}$	1313.5888
26.54	2044	1094.5397	-0.7	-	1049, 917, 887, 754, 688, 592, 179, 143, 125	(Lycoperoside H) FA III	$C_{51}H_{85}NO_{24}$	1095.5462
26.62	695	1312.5881	-5.0	-	1267, 1137, 1107, 975, 944, 812, 746, 650, 275, 143	(Dehydrolycoperoside F) FA or (Dehydrolycoperoside G) FA or (Dehydroesculeoside A) FA II	$C_{59}H_{95}NO_{30}$	1313.5888
26.62	32144	1314.6003	-2.4			(Lycoperoside F) FA or (Lycoperoside G) FA or (Esculeoside A) FA III	$C_{59}H_{97}NO_{31}$	1315.6045
27.31	537	1094.5421	-3.0			(Lycoperoside H) FA IV	$C_{51}H_{85}NO_{24}$	1095.5462
27.45	16802	593.1514	-0.3			Kaempferol-3-O-rutinoside	$C_{27}H_{30}O_{15}$	594.1585
27.60	1810	1312.5843	-2.1	-	1267	(Dehydrolycoperoside F) FA or (Dehydrolycoperoside G) FA or (Dehydroesculeoside A) FA III	$C_{59}H_{95}NO_{30}$	1313.5888
27.62		1314.5920	-1.8	-		(Lycoperoside F) FA or (Lycoperosyde G) FA or (Esculeoside A) FA IV	$C_{59}H_{97}NO_{31}$	1315.6045
27.82	1662	515.1199	-0.8			Dicaffeoylquinic acid I	$C_{25}H_{24}O_{12}$	516.1268
28.47	868	515.1199	-0.7			Dicaffeoylquinic acid II	$C_{25}H_{24}O_{12}$	516.1268
30.58	4972	515.1193	0.5			Dicaffeoylquinic acid III	$C_{25}H_{24}O_{12}$	516.1268
31.13	3614	887.2255	-0.4	258, 321		Quercetin-hexose-deoxyhexose-pentose-*p*-coumaric acid	$C_{41}H_{44}O_{22}$	888.2324
32.19	3517	1076.5283	0.0	-	1031, 899, 868, 736, 670, 574, 305, 143, 119, 113	(α-Dehydrotomatin)FA I	$C_{51}H_{83}NO_{23}$	1077.5356
32.75	380	1136.5520	-2.2	-		(Lycoperoside A) FA or (Lycoperoside B) FA or (Lycoperoside C) FA I	$C_{53}H_{87}NO_{25}$	1137.5567
32.82	1081	433.1141	-0.2			Naringenin chalcone-hexose	$C_{21}H_{22}O_{10}$	434.1213
32.84	1960	1078.5451	-1.1	-		(α-Tomatin)FA I	$C_{51}H_{85}NO_{23}$	1079.5512
33.31	686	1076.5309	-2.4	-		(α-Dehydrotomatin) FA II	$C_{51}H_{83}NO_{23}$	1077.5356
33.33	33734	1078.5438	0.1		1033, 901, 870, 738, 672, 576, 305, 143, 119, 113	(α-Tomatin) FA II	$C_{51}H_{85}NO_{23}$	1079.5512
33.35	23219	1136.5489	0.4		1092, 959, 929, 796, 731, 634	(Lycoperoside A) FA or (Lycoperoside B) FA or (Lycoperoside C) FA II	$C_{53}H_{87}NO_{25}$	1137.5567
33.46	2614	433.1143	-0.6			Naringenin chalcone-hexose II	$C_{21}H_{22}O_{10}$	434.1213
34.09	918	1136.5505	-0.9	-		(Lycoperoside A) FA or (Lycoperoside B) FA or (Lycoperoside C) FA III	$C_{53}H_{87}NO_{25}$	1137.5567
37.94	33859	1081.5448	-1.1	-	1037, 919, 903, 757, 740, 595, 161	Tomatoside A	$C_{51}H_{86}O_{24}$	1082.5509
39.32	7103	677.1519	-1.0			Tricaffeoylquinic acid I	$C_{34}H_{30}O_{15}$	678.1585
40.63	*145*	*677.1533*	*1.1*			Tricaffeoylquinic acid II	$C_{34}H_{30}O_{15}$	678.1585
41.35	*112*	*677.1533*	*4.5*	-	515, 353	Tricaffeoylquinic acid III	$C_{34}H_{30}O_{15}$	678.1585
42.17	5885	271.0621	-3.4			Naringenin	$C_{15}H_{12}O_5$	272.0685
42.73	34406	271.0622	-3.6			Naringenin chalcone	$C_{15}H_{12}O_5$	272.0685

The metabolites which were detected and quantified by LC-PDA system were flavonoids, carotenoids, chlorophyll, tocopherols, and vitamin C. However, during the analysis of untargeted metabolomics by LC-QTOF-MS many other metabolites such as phenolic acids, glycoalkaloids, saponins, glycosylated hormonal metabolites, etc. were detected. These metabolites are described below as phenolic acids, glycoalkaloids, and other metabolites.

4.2.6.1. Phenolic acids

Conjugated phenolic acids of caffeic acid, coumaric acid (most likely *p*-coumaric acid) and ferulic acid are abundant in all tissues of tomato fruit (Figure 4.17, Tables 4.19 and 4.20). Three isomers of caffeic acid-hexose, eluting at 10.27, 10.88 and 13.19 min, were present in all fruit tissues. These compounds were more abundant in the jelly parenchyma, in particular the first eluting isomer, being in the turning and pink stages where their mass signals showed to be the highest. The second isomer (10.88 min) was almost uniquely present in the calyx. Four isomers of coumaric acid-hexose were found in tomato fruit, eluting at 10.47, 13.90, 14.26, and 15.39 min. The first and fourth isomers were more abundant in the jelly parenchyma, the second in the epidermis and the third in the calyx. Three isomers of ferulic acid-hexose, 12.91, 16.38 and 17.29 min, appeared mostly in the jelly parenchyma (first eluting isomer) and calyx (last two eluting isomers).

Four isomers of caffeoylquinic acid were present through all the different tomato tissues, appearing in the chromatograms at 14.23, 14.86 and 17.18 min. In addition, there were three isomers of dicaffeoylquinic acids (eluting at 27.82, 28.47 and 30.58 min), as well as three isomers of tricaffeoylquinic acids (appearing at 39.32, 40.63 and 41.35 min in the chromatograms) which were present in all tomato tissues, in particular in the epidermis, and increased in amount upon development.

4.2.6.2. Glycoalkaloids

A variety of glycoalkaloids, detected as their formic acid adducts (Moco *et al*., 2006), were present in different tissues and specific developmental stages (Figure 4.17, Tables 4.19 and 4.20). Esculeoside B was the first eluting alkaloid, at a retention time of 20.40 min. This compound mainly occurred in the jelly parenchyma

and its signal intensity slightly decreased during fruit development (1.4 fold from green to red stage). Four isomers of lycoperoside H were found in tomato fruit, at retention times of 24.14, 25.50, 26.54 and 27.31 min, essentially in the epidermis and showing higher intensities at the early stages of ripening (green and breaker). The alkaloids lycoperoside F and G and esculeoside A have the same molecular mass and appeared four times in the mass signal chromatogram, suggesting four different isomers (at 23.85, 25.83, 26.62 and 27.62 min). These compounds were present in all tissues, in particular in the epidermis and jelly parenchyma, and hardly occurred in the green fruit. In the red stage, the third isomer appeared as one of the highest signals in the mass chromatograms. With a 2 Dalton difference, three derivatives of lycoperosides F and G or esculeoside A, recently suggested to be named as dehydrolycoperosides F and G or dehydroesculeoside A (Moco *et al*., 2006), occurred in all tomato fruit tissues. These metabolites had lower signal intensities than the lycoperosides F and G or esculeoside A isomers, but displayed analogous behavior through development and tissues. The last isomer, eluting at 27.60 min, was only present in the epidermis and increased more than 1500 fold from the green (below the detection limit) to the red stage. The most known tomato alkaloids, α-tomatin and dehydrotomatin, occurred as two different isomers in all tissues at the green stage, in particular in the epidermis and jelly, decreasing up to levels below the detection limit at the red stage of development. In both cases, one isomer was highly abundant at the green stage: the first eluting isomer of dehydrotomatin, retention time 32.19 min (more than 110 fold decrease) and the second eluting isomer of α-tomatin, retention time 33.33 min (more than 65 fold decrease). Three isomers of the equal mass metabolites lycoperoside A, B and C were found in all tissues. Analogous to α-tomatin, these alkaloids preferably accumulated in the epidermis and jelly parenchyma, and in the green stage of the fruits. The second isomer, at retention time 33.35 min, was the most abundant one.

A class of compounds that exhibited marked developmental features is the glycoalkaloids. Glycoalkaloids such as tomatine are proposed to be formed through the cholesterol pathway, in which a series of modifications takes place in the steroid moiety. Only at the last step, the steroid tomatidine is glycosylated into α-tomatine (Friedman, 2002). The green stage-specific-metabolites α-tomatine and dehydrotomatine could be detected in all fruit tissues, being specifically abundant in

the epidermis, and both alkaloids strongly decreased at the first signs of fruit ripening (Figure 4.19). In contrast, several other alkaloids, also preferentially accumulating in the epidermis, markedly increased upon ripening: lycoperosides F, G and esculeoside A and their related dehydro forms: dehydrolycoperosides F, G and dehydroesculeoside A (Figure 4.19). Esculeoside A was postulated to be formed from α-tomatine, through a ring rearrangement in the steroid moiety (Fujiwara *et al*., 2004). The functions of these high molecular mass metabolites in the plant are still not fully understood. Glycoalkaloids are known to have antimicrobial and anti-insect properties, and participate in plant defense mechanisms (Friedman, 2002). The antifungal activity of both α-tomatine and its aglycon tomatidine has been studied recently (Simons *et al.*, 2006). α-Tomatine is easily permeabilized through the cell membrane, however it has lower antifungal activity compared to tomatidine, suggesting that the sugar moiety is important for cell penetration while the steroid moiety confers toxicity to the fungus. In fact, upon internalization, α-tomatine is recognized by enzymes produced by the fungal pathogens that can hydrolyze one sugar unit or even the complete sugar moiety yielding tomatidine. Based on fungal gene expression data, tomatidine (and not α-tomatine) inhibits ergosterol biosynthesis which is key target for chemical control of fungal pathogens of plants and animals (Simons *et al*., 2006).

4.2.6.3. Other metabolites

A large number of metabolites present in the extract appear in the chromatogram before 4 minutes of retention time, as a large and mostly asymmetrical peak (Figure 4.17, Tables 4.19 and 4.20). These are (very) polar metabolites that do not interact with the stationary phase. Amino acids, nucleosides, mono-, di-, and tri-phosphate nucleotides, sugars and organic acids are present in this part of the chromatogram. Phenylalanine is the only amino acid actually separated by the instrumental setup (retention time 4.8 min), as it interacts with the reverse-phase column and appears as a separate mass signal. This amino acid occurs through all different tomato fruit tissues, slightly increasing during development (about 2-fold). The tissues where this amino acid was the most abundant were the pericarp and columella.

The metabolite assigned as the saponin tomatoside A seemed to be a jelly parenchyma specific metabolite, displaying extremely high intensity values through all stages of development within this tissue.

Glycosylated hormonal metabolites were also found in the tomato tissues, in particular in the jelly parenchyma: zeatin hexose and abscisic acid hexose. The levels of these metabolites increased during development (up to 8 fold). The metabolite dehydrophaseic acid-hexose, belonging to the abscisic acid pathway, was found in all tissues and developmental stages of the fruit, being highest in the red calyx.

The jelly parenchyma material, which in this study included the seeds, is relatively rich in specific semi-polar metabolites. The locular tissue surrounding the seeds is of importance to the maturation of seeds during fruit development. The presence of hormonal compounds in this tissue suggests that the cellular environment surrounding the seeds is subjected to hormonal regulation processes (Gillaspy *et al*., 1993). The specific accumulation of glycosylated forms of several plant hormones in the jelly parenchyma material (Figure 4.19), may therefore suggest storage of these signalling molecules as glycosides in close proximity to the place of need. Alternatively, the origin of these hormonal compounds might be in the seeds themselves and not in the tissue surrounding these. Abscisic acid is involved in seed dormancy phenomena which can explain the presence of abscisic acid pathway related metabolites such as dehydrophaseic acid-hexose and absisic acid-hexose in the jelly parenchyma material (Bewley, 1997). A glycosylated cytokinin, zeatin-hexose, is also specifically present in this material, increasing in concentration during fruit ripening (Figure 4.19). This is in agreement with the preferential expression of the zeatin-*O*-glucosyltransferase gene in the locular tissue (Lemaire-Chamley *et al*., 2005). The increase of glycosylated hormonal metabolites during fruit development is in accordance with the discontinued need of hormonal (non-glycosylated) triggering molecules, such as abscisic acid and zeatin, after the cell expansion of the fruit (prior to the mature green formation) (Gillaspy *et al*., 1993). In addition to these plant hormones, the saponin tomatoside A specifically accumulated in the jelly parenchyma tissue. However, the biological function of this compound is still completely unknown.

4.2.7. Metabolite pattern classification

The data obtained from LC-PDA-FI and LC-PDA-MS of the analyses of tissues and ripening stages from the tomato variety *Ever* was joined to be processed together. For this reason, the Multivariate Mass Spectra Reconstruction (MMSR) method previously developed for the data analysis of Gas Chromatography-MS data (Tikunov *et al*., 2005) was used. According to this method, the combined dataset was classified into 14 different clusters (Figure 4.19). In this way, the classification of previously (putatively) assigned metabolites was feasible. These metabolites fitted into 9 of the 14 clusters. The clusters A, B and C indicate the presence of metabolites that are abundant in the epidermis, either constantly present (A), decreasing (B) or increasing (C) during fruit ripening. In these clusters, flavonoids, phenolic acids and alkaloids are present. In detail, the metabolites constantly present in epidermis were lycoperoside H (FA) II and IV, coumaric acid-hexose II and IV, kaemferol-3-rutinose, kaemferol-hexose-deoxyhexose-pentose, quercetin, hexose-deoxyhexose-pentose, and rutin. As shown in B, the metabolites which found to decrease during fruit development were lycoperoside A/B/C (FA) III, lycoperoside H (FA) I, α-dehydrotomatin (FA) I, α-tomatin (FA) I and II, and caffeoylquinic acid III. On the other hand, the metabolites which increased during ripening were mainly lycopene, β-carotene, ascorbic acid, naringenin, naringenin chalcone, quercetin-dihexose-deoxyhexose, esculeoside B (FA), etc. The clusters D and E group the metabolites which were abundant in the jelly parenchyma (Table 4.17, Figure 4.17). These were; caffeic acid-hexose I, tomatoside A, zeatin hexose, γ-tocopherol, dehydro (lycoperoside F/G Esculeoside A) (FA) II, (iso) pentyl dihexose, and caffeoylquinic acid II. Metabolites abundant in the calyx were grouped in the clusters F, G, and I. Caffeic acid-hexose II, dehydrophaseic acid-hexose, ferulic acid-hexose II and III are shown in F. Caffeoylquinic acid I, α-tocopherol, lutein, and violaxhantin are shown in G. Chlorophyll b and neoxhantin are shown in I. Phenylalanine which is the abundant metabolite in the pericarp is shown in the cluster H. As shown in Figure 4.19, the chemical class of metabolites was found to be independent from the cluster, as in each cluster chemically diverse metabolites (biosynthetically produced from different pathways) are grouped. This analysis led to a biological classification, according to tissue and ripening stage.

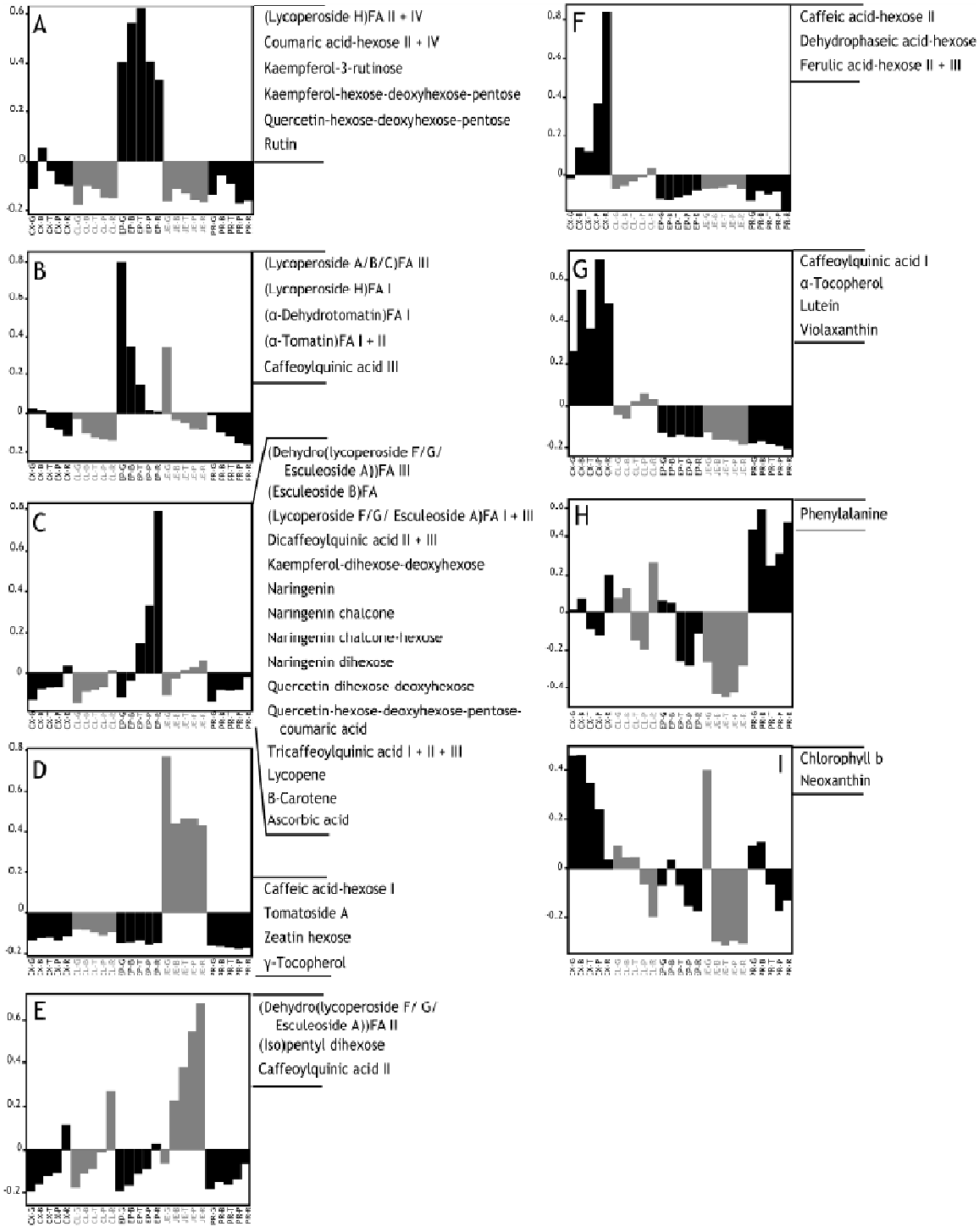


Figure 4.19: Classification of the assigned metabolites according to their abundance in the tissues of tomato fruit through ripening stages into behavioral pattern groups (in terms of normalized LC-MS intensity values): A to I. The patterns display, from left to right, the different fruit tissues, calyx (CX in black), columella (CL in grey), epidermis (EP in black), jelly parenchyma (JE in grey) and pericarp (PR in black); within each tissue, the ripening stages are displayed: green (G), breaker (B), turning (T), pink (P) and red (R).

4.3. Enzyme Activity Measurements

According to the previous findings presented in section 4.1, an increase in the flavonoid content (especially in rutin) as a result of breaking step was observed. On the basis of this observation, it was concluded that this situation could be as a result of wounding effect where the activity of enzymes were stimulated. Hence, the activities of two enzymes: 3-*O*-Glucosyltransferase (3GT), and phenylalanine ammonia lyase (PAL) were investigated in tomato processing samples from fruit to paste. 3GT is the first enzyme responsible for the rutin biosynthesis, and it catalyzes the conversion of quercetin into isoquercitrin in the presence of UDP-Glucose, which is then converted into rutin by the effect of rhamnosyl transferase (Figure 4.20) (Barber, 1963; Barber, 1991). Glycosyltransferases are a large family of enzymes capable of transferring sugar to wide ranges of acceptor molecules (Campbell *et al*., 1997; Coutinho *et al*., 2003) and catalyzes the conversion of quercetin into isoquercitrin in the presence of UDP-Glucose which is then converted into rutin by the effect of rhamnosyl transferase.

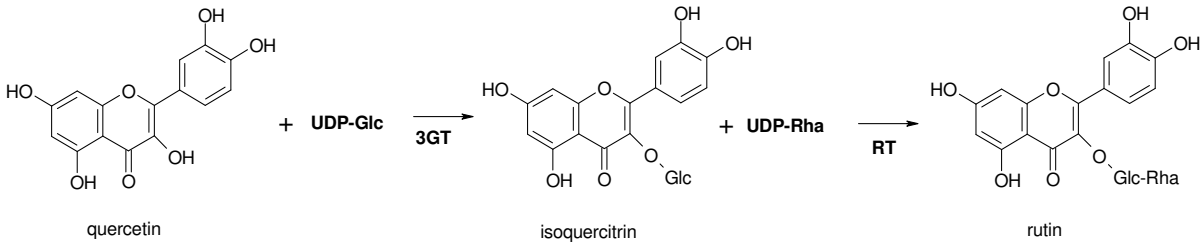


Figure 4.20: Reactions catalyzed by glucosyl and rhamnosyl transferase.

Following the activity measurement of the 3GT enzyme which uses quercetin as a substrate, a previously acting enzyme activity, PAL enzyme, in the phenyl propanoid pathway was investigated to observe the changes leading to higher quercetin concentrations (Blount *et al*., 2000).

Besides the investigation of these two enzymes in the processing materials, the changes in the enzyme activities of fruit tissues (flesh and skin part) during the development of the tomato fruit, from green to dark red, were also followed.

Prior to the enzyme activity measurements, the assay conditions and extraction procedure of enzyme from the plant tissues were optimized for obtaining best results.

4.3.1. 3-*O*-Glucosyl transferase activity

4.3.1.1. Optimization of the extraction and assay conditions

In order to optimize the extraction and assay conditions, pH, buffer composition and quercetin saturation concentrations were determined. According to the pH experiment, the optimum pH for the enzyme was found to be 8.3-8.4 using Bicine pH 9.0 together with EDTA, protease inhibitor mixture (Serva), Triton X-100, and DTT (Figure 4.21). The obtained pH was found to be higher with respect to the pH of 3GT extracted from buckwheat or grape (around pH 7.0) (Ford *et al*., 1998; Suzuki *et al*., 2005). Glucosyl transferases (GT) have been identified in several plants including buckwheat (Suzuki *et al*., 2005), onion (Latchinian-Sadek and Ibrahim, 1991), grape (Ford *et al*., 1998), red orange (Piero *et al*., 2005), maize (Futtek *et al*., 1988), strawberry (Cheng *et al*., 1994), *Arabidopsis thaliana* (Li *et al*., 2001), and higher plant glucosyl transferases were reviewed by Ross *et al*. (2001). It was reported that most of the glucosyl transferases exhibit pH optima in the range of 7.5-8.5, although there could be exceptions such as 9.0 to 9.5 (Ibrahim and Varin, 1993).

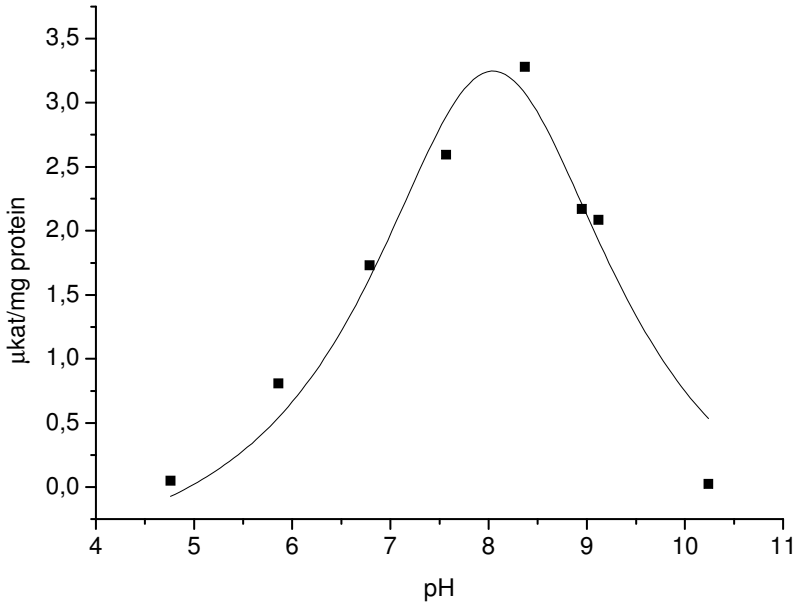


Figure 4.21: pH curve for 3GT.

The concentration of quercetin, which is the substrate of this reaction (Figure 4.20), is another key factor for the optimization of the reaction and the assay. In order to determine the optimum quercetin concentration its saturation curve was prepared (Figure 4.21). The saturation curve showed that the reaction was equilibrated starting from 0.07 mM quercetin concentration, and the product concentration became stable as can be observed by the stabilized line. The end concentration of 0.1 mM quercetin was used during the assay (Brazier *et al*., 2003; Suzuki *et al*., 2005; Ko *et al*., 2007).

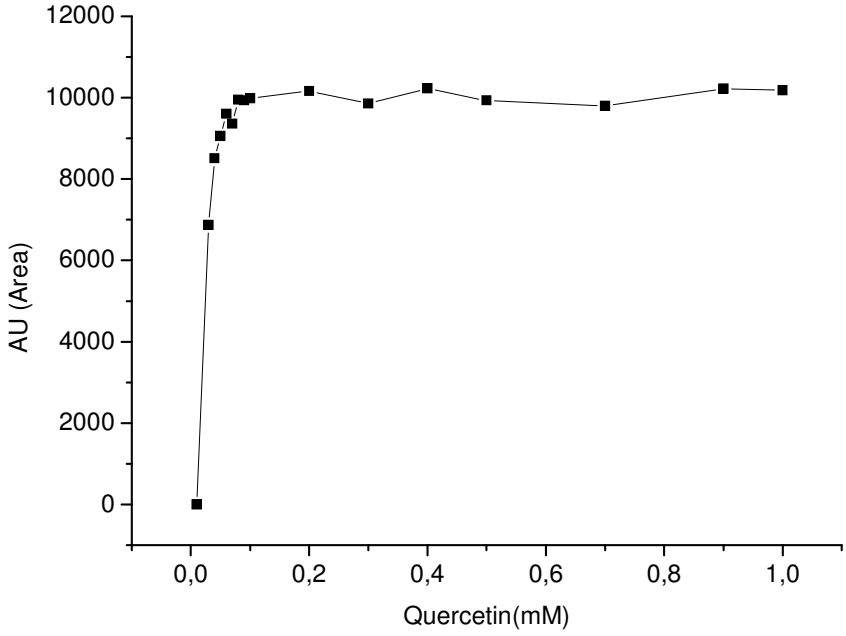


Figure 4.22: Quercetin saturation curve for 3GT.

After the optimization of assay conditions, four control samples were prepared; (1) without enzyme (3GT), (2) without substrate (quercetin), (3) without UDP-Glucose (which is necessary for the reaction), and (4) with denatured enzyme (Figure 4.23 and Appendix, Figure C.1-Figure C.3). These chromatograms were compared with a full assay including all the required reagents (substrate, UDP-Glucose) under standart assay conditions (Figure 4.24). It was observed that the production of isoquercitrin, which is the product of the reaction, was absent in the control samples. On the other hand, when the optimum conditions were provided for the assay, isoquercitrin production was clearly observed at the 27^{th} minute (Figure 4.24).

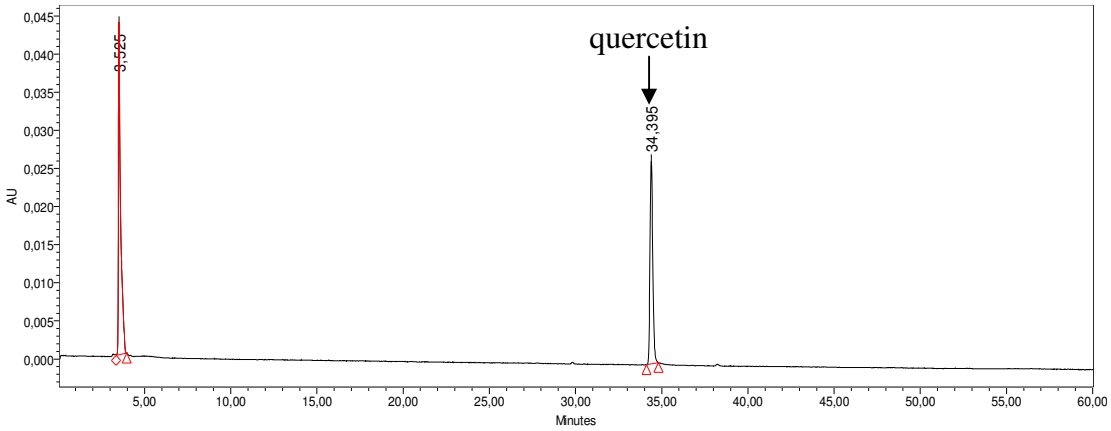


Figure 4.23: Enzyme assay performed without UDP-Glucose.

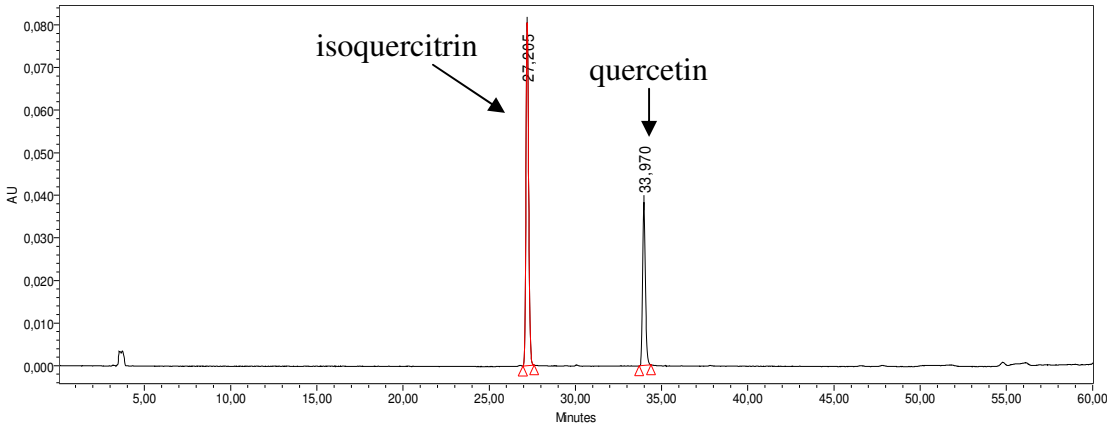


Figure 4.24: Enzyme assay performed including all the reagents.

4.3.1.2. 3-*O*-Glucosyl transferase activity of tomato processing samples

The protein contents of all steps of paste processing materials obtained are shown in Table 4.19. The protein amounts were used for the calculation of specific enzyme activity.

Table 4.19: Protein content of paste processing material.

Processing Step	Protein content (mg)
Fruit	0.61
Breaker	0.67
Finisher pulp	0.29
Seed & Skin	0.56
Evaporator out	0.18
Paste	0.18

The specific 3GT activity of the samples are presented in Figure 4.25-A. The results showed an increase in the 3GT enzyme activity during the breaking process of the tomatoes. This increase in the first step resembles the previous findings presented in section 4.1.8 showing an increase in the rutin content (Figure 4.25-B).

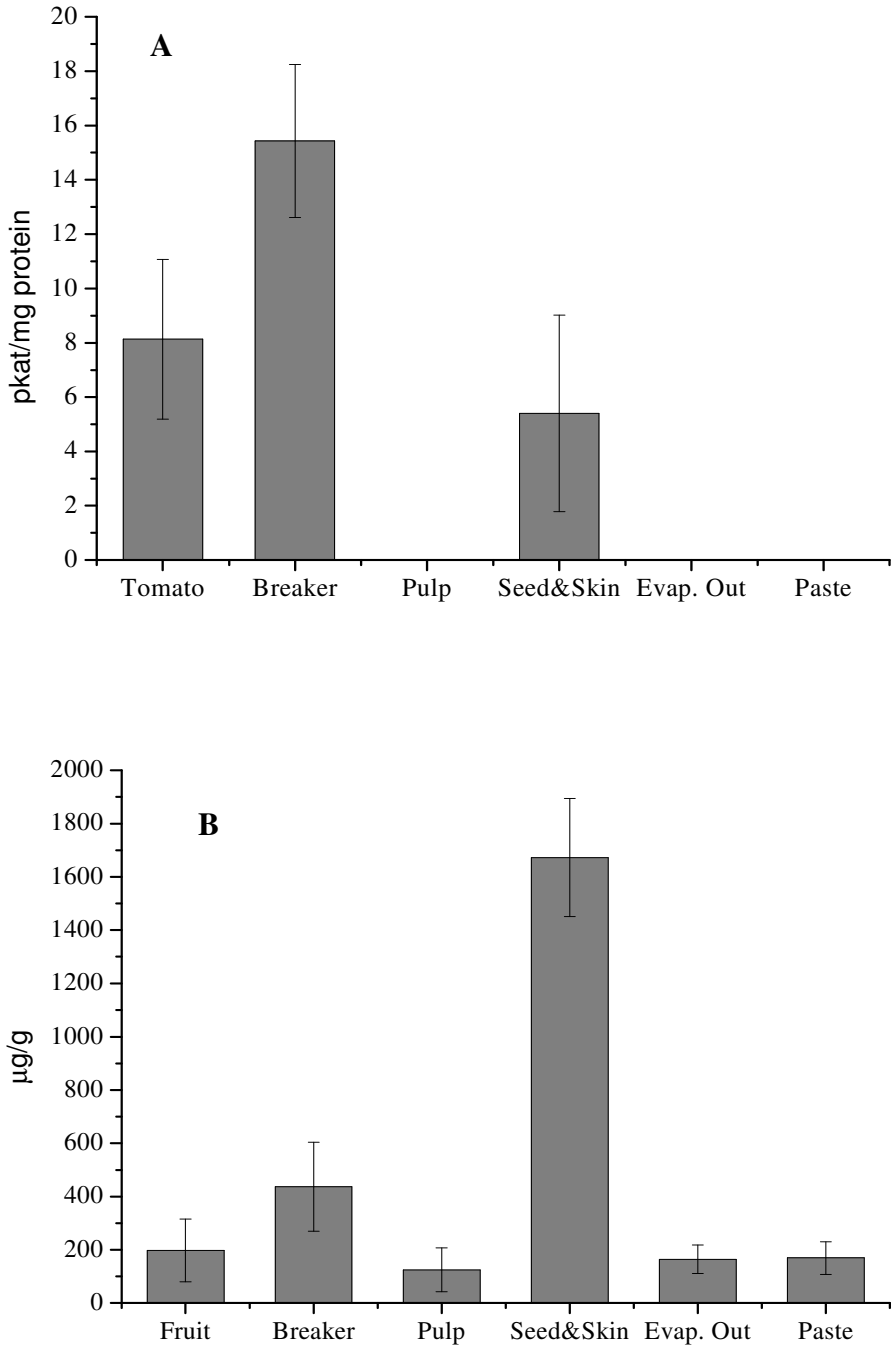


Figure 4.25: Changes during tomato processing A: 3GT activity and B: rutin content.

The increase in the rutin content was consistent with the increase in 3GT activity during rutin biosynthesis. This finding supports the hypothesis that cutting might appear as a wounding effect and might result in an increase in the enzyme activity leading to higher rutin biosynthesis. Under stress conditions such as temperature or water stress, rutin content found to increase in a medicinal herb *Hypericum brasiliense* (De Abreu and Mazzafera, 2005). Truesdale *et al*. (1996) also identified a novel wound-responsive gene of tomato which shared sequence homology with the family of glucosyl transferases involved in the conjugation of small molecular weight substrates such as phenolics and flavonoids.

It has been reported that classes of secondary metabolites have a variety of biological functions, such as elements involved in pollination, photoprotection, seed dispersal, adaptation to abiotic conditions and defense, as well as being involved in other non-ecological phenomena such as auxin transport (Tracewell *et al*., 2001; Friedman, 2002; Taylor and Grotewold, 2005; Kunz *et al*., 2006; Simons *et al*., 2006).

Among flavonoids, rutin found to contribute to the greatest extent to the antioxidant capacity of tomatoes and it was suggested that this flavonoid may be a useful target for up-regulation in tomatoes in order to improve their antioxidant status (Spencer *et al.*, 2005).

The finisher seed & skin samples collected from the pulper stage also had enzyme activity and contained high amounts of flavonoids as already presented in section 4.1.8. In the samples taken from the evaporator out and paste stage, where a heat treatment was applied, no activity was observed. This was assumed to be due to the denaturation of the enzyme at such high temperature (80 ºC).

The chromatograms of the assay for tomato and breaker samples at 360 nm are shown in Figure 4.26. Another significant finding of the assay was that some additional compounds, besides the substrate, quercetin, and the product, isoquercitrin, were observed during the reaction, which were absent in the control. These additional compounds were found to be higher in tomato when compared to the breaker.

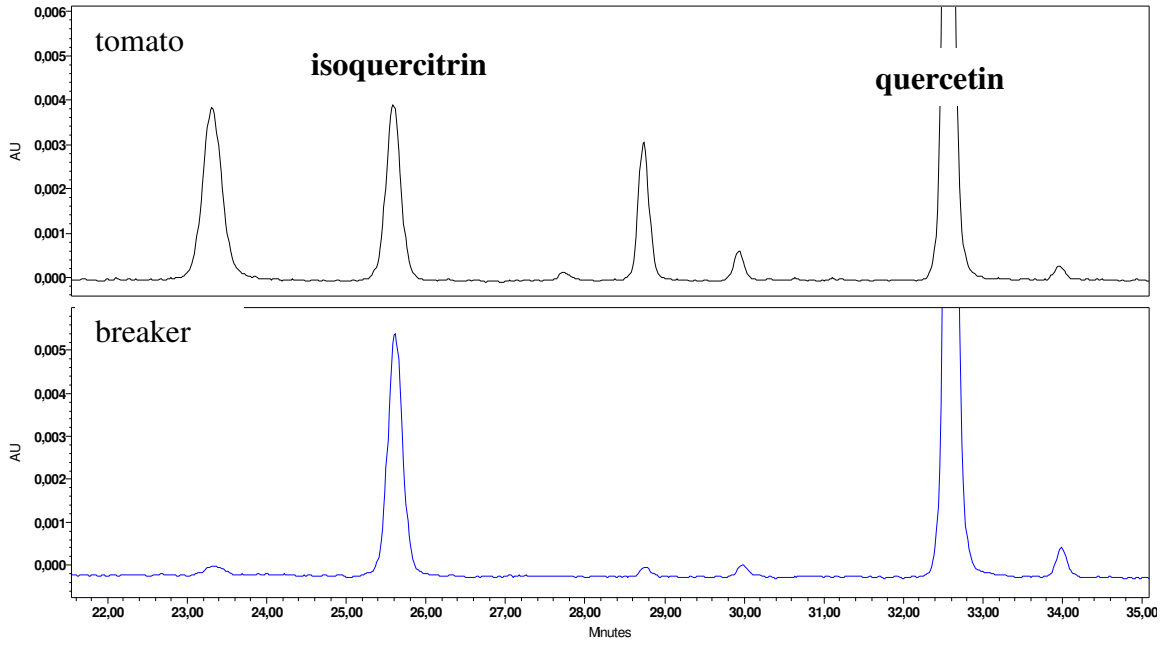


Figure 4.26: HPLC chromatogram at 360 nm of tomato and breaker samples.

In order to find out the nature of additional peaks, samples which had 3GT activity were re-analyzed using UPLC-MS (

Figure **4.27**). In figure 4.27 peak "2" was estimated to be isoquercitrin and peak "4" to be quercetin. Peaks "1" and "3" were unknown compounds. In order to define these compounds, their UV spectra were compared with the spectra of isoquercitrin and quercetin (Figure 4.28). The results showed that the spectra of unknown compounds were very similar to that of isoquercitrin resembling a flavonol structure.

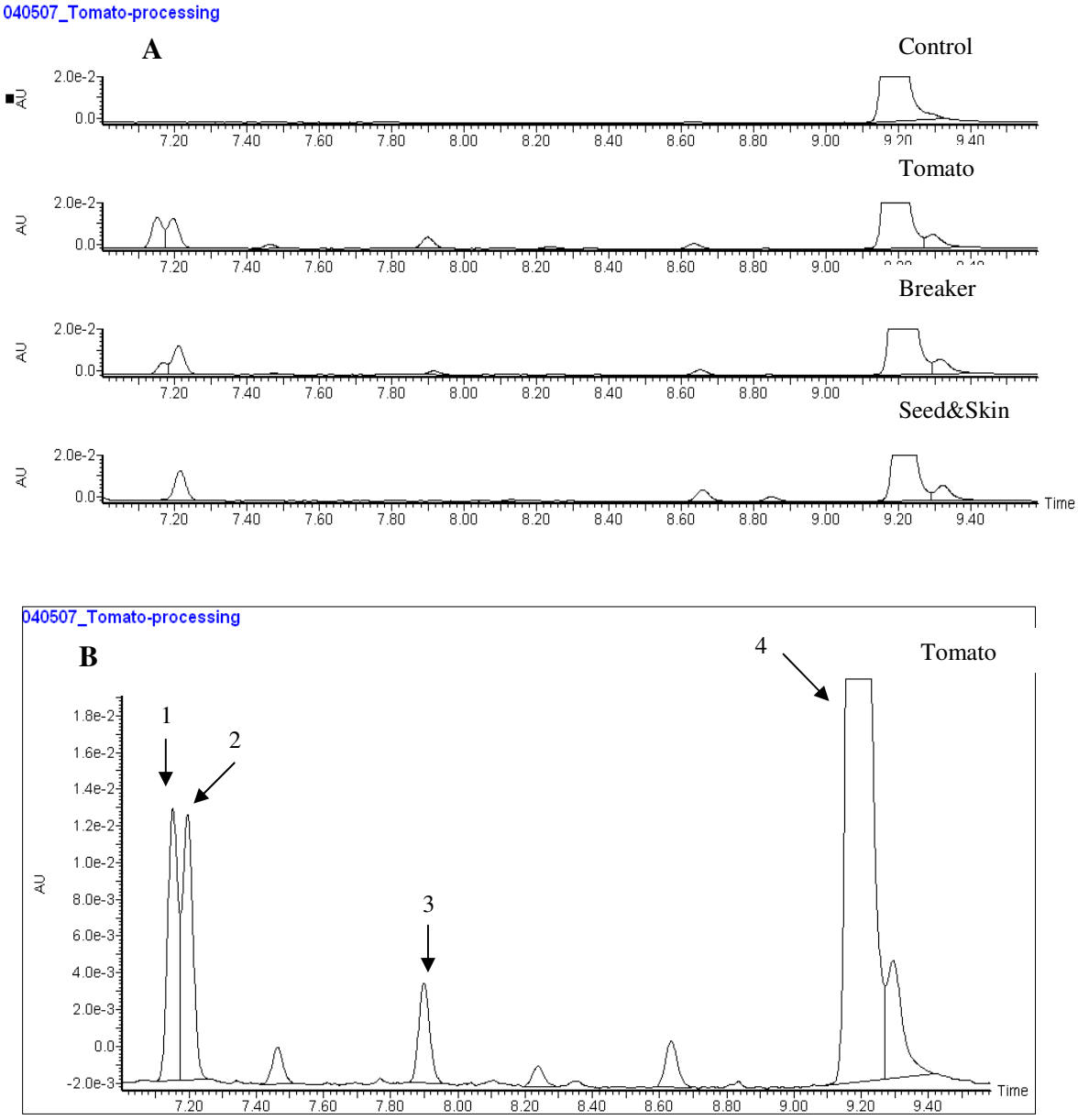


Figure 4.27: UPLC-MS chromatograms, A: Control, tomato, breaker, seed&skin fractions; B: enlarged chromatogram of tomato sample.

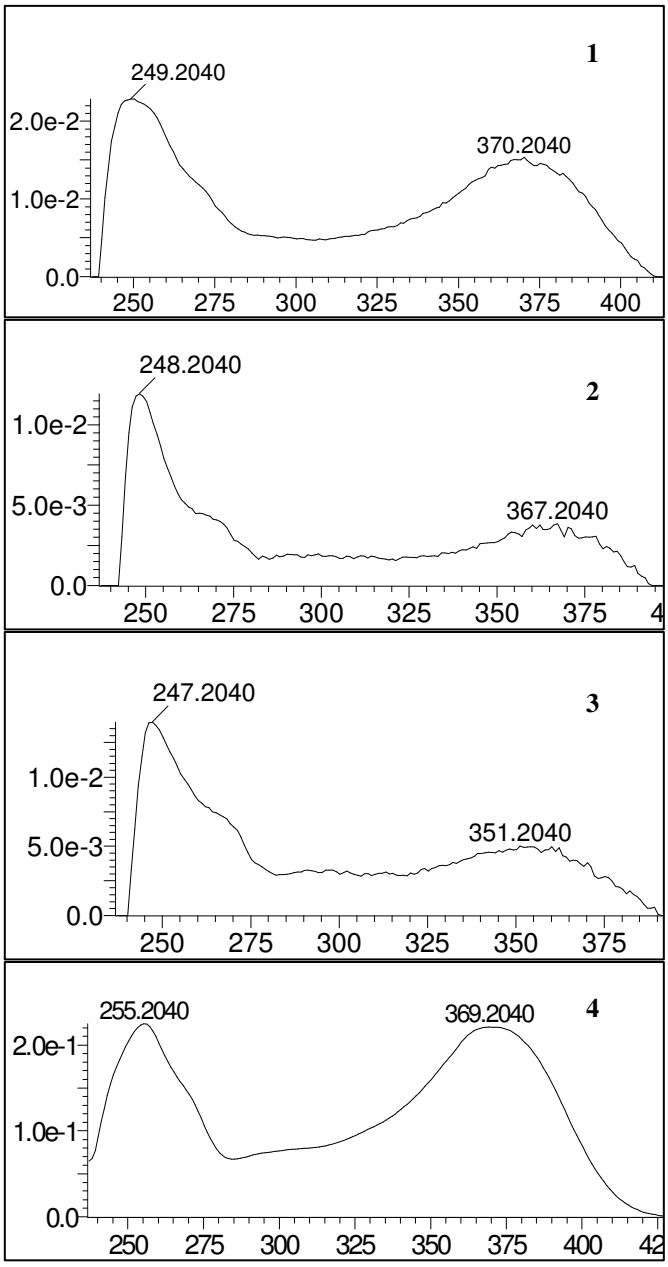


Figure 4.28: UV spectra of the peaks, (1) unknown compound, (2) isoquercitrin, (3) unknown compound, (4) quercetin.

In the MS data, there were two major m/z values of 301 and 463 g/mol. The accurate mass of 301 g/mol corresponds to the aglycon, quercetin and the mass of 463 g/mol corresponds to aglycon plus the glucoside. The glycosides are known to have increased solubility, stability, and bioavailability when compared with the quercetin aglycone (Hollman *et al*., 1999). In the view of current literature, however, detailed analysis of individual glycosides of quercetin are not available. Different plants

contain different spectra of glycosides depending on both the sugars attached to the flavonol and to the regioselectivity of glycosylation (Harborne and Baxter, 1999).

Due to the complexity of additional secondary metabolites in the extracts, purification of significant amounts of each quercetin glycoside was found to be difficult. Based on the available literature, the flavonol aglycone has five potential glucosylation sites and, to synthesize any single monoglucoside, four other hydroxyl groups must be protected. However, it could be difficult in some cases since some additional untargeted compounds can be synthesized as similarly observed in this study (Lim *et al*., 2004). Glucose is attached to the 3rd binding site in the "*O*" ring of isoquercetin (quercetin-3-*O*-glucoside) (Figure 4.29-A). Considering the retention times (Lim *et al*., 2004), the additional peaks were predicted to be quercetin-7-*O*-glucoside and quercetin-4'-*O*-glucoside (Figure 4.29-B). The formation of these additional peaks could be disadvantageous in the reactions where regioselectivity is expected (Lim *et al*., 2004).

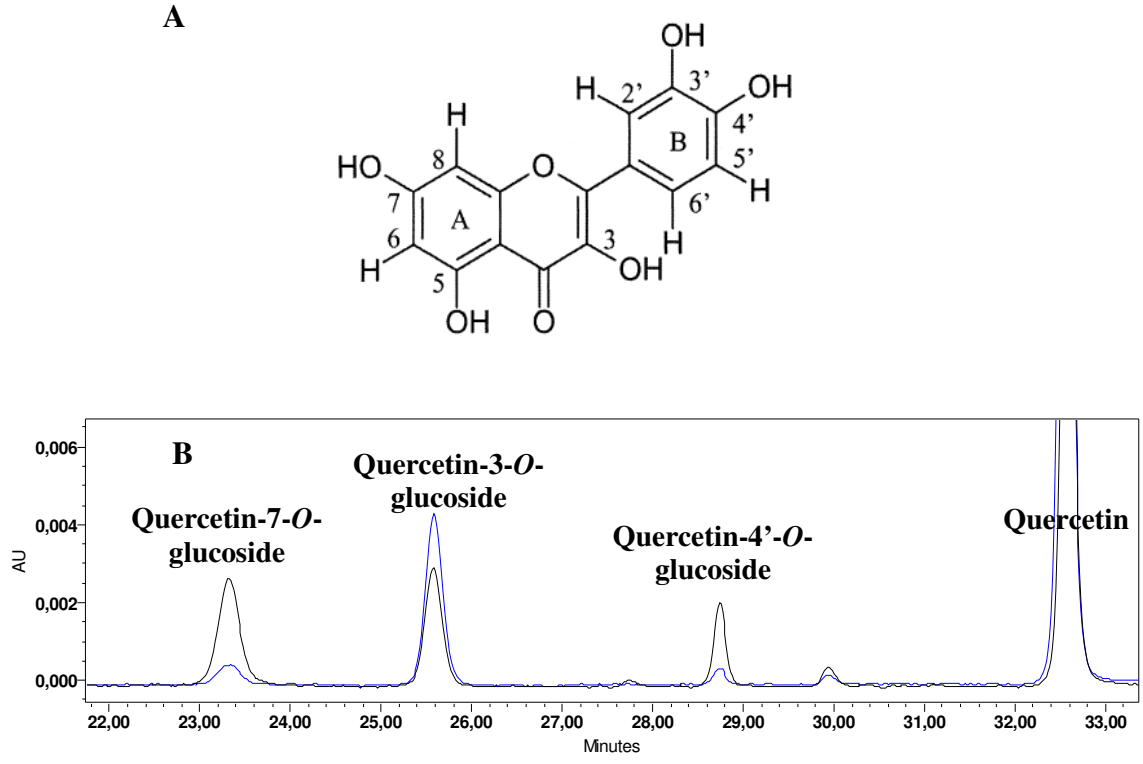


Figure 4.29: Quercetin and its derivatives (A) Binding sites of quercetin, (B) Presence of isoforms.

4.3.1.3. 3-*O*-Glucosyl transferase activity of tomatoes from different developmental stages

In addition to the tomato processing material, the flesh and skin parts of different developmental stages of tomatoes were investigated for their 3GT activity (Figure 4.30 and Figure 4.31). The results showed that green flesh had the highest 3GT values (33.4 pkat/mg), which were followed by a decrease immediately after the breaker stage. After the breaker step, 3GT activity was found to be maintained approximately at the same level (Figure 4.30).

Similarly, for the skin part, first an increase and then again a decrease was observed (Figure 4.31). The highest 3GT activity in the skin part was obtained at the turning stage (50.7 pkat/mg). On the other hand, the lowest activity was observed at the dark red stage (20.5 pkat/mg) which gradually decreased from the turning stage. The rest of the steps showed similar activities changing in between 30.0-34.3 pkat/mg.

These results showed that the flesh and the skin part can contain different amounts of enzymes at different development stages. The changes in all of the metabolites during development were explained in detail in section 4.2. Skin part contained higher enzyme activities which might be related to their higher substrate concentration. The studies investigating the flavonoid composition of different fractions of tomatoes reported that tomato skin has higher levels of flavonoids compared to the pulp (Toor and Savage, 2005) and that the main flavonols found in the tomato skin are the conjugated forms of quercetin and kaempferol (Canene-Adams *et al*., 2004).

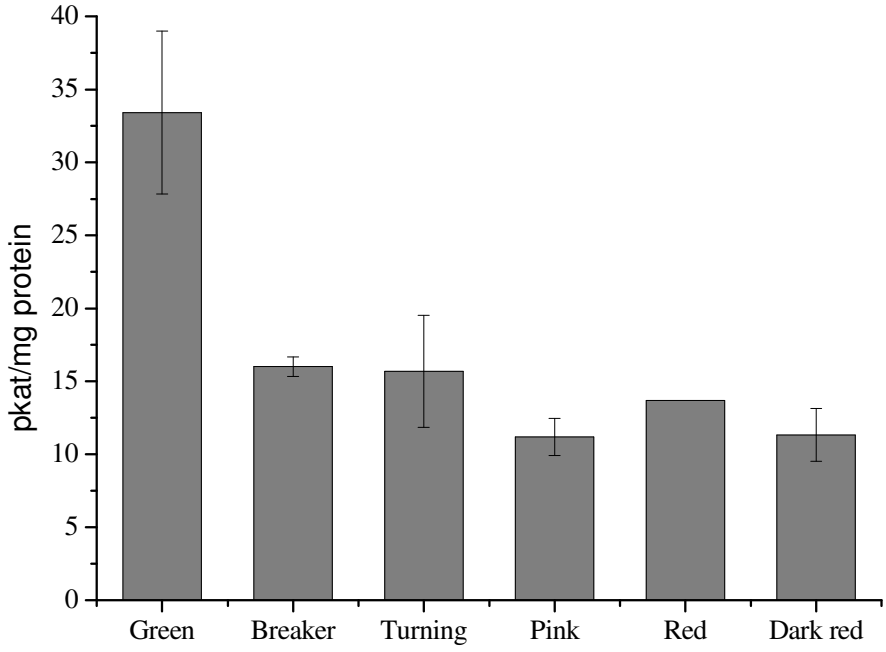


Figure 4.30: 3GT activity of the flesh part of developmental stages of tomato.

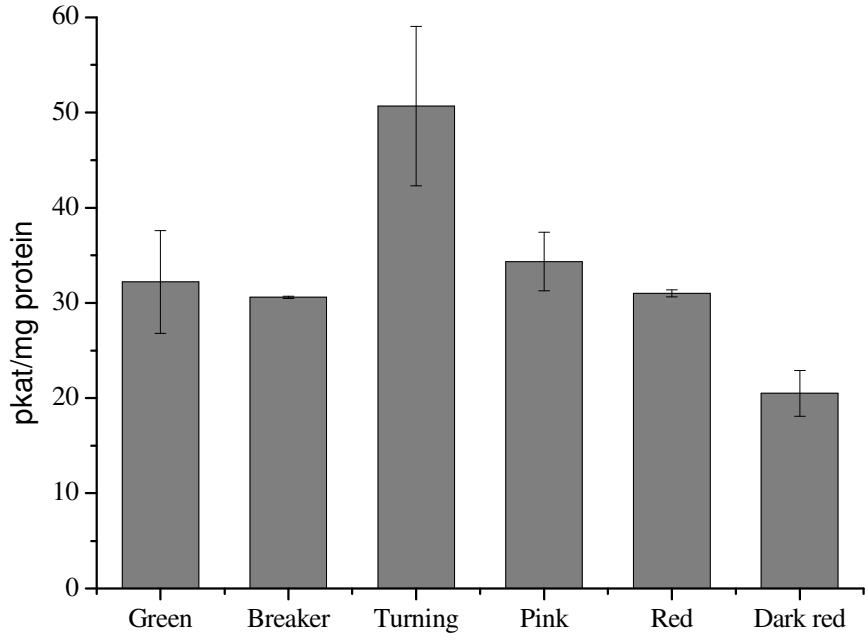


Figure 4.31: 3GT activity of the skin part of developmental stages of tomato.

4.3.2. Phenyl alanine ammonia lyase activity

The studies concerning biosynthesis of flavonoids have shown that in several systems, biosynthesis of flavonoids is stimulated by light, and the photo-induced accumulation of flavonoids is preceded by photo-induction of the enzyme, phenylalanine ammonia lyase (Sarma and Sharma, 1999). Hence, following the measurement of the 3GT activity, a previously acting enzyme in the phenyl propanoid pathway was measured to observe the changes in the substrate amount. The reaction of PAL, the first enzyme in this pathway, is shown in Figure 4.32.

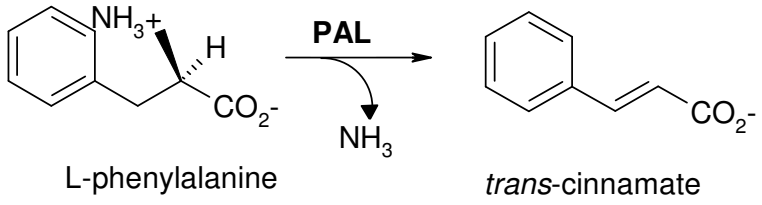


Figure 4.32: PAL reaction.

4.3.2.1. Phenyl alanine ammonia lyase activity of tomato processing samples

The activity measurement of PAL enzyme was unsuccessful probably due to its very low concentrations and low detection limit of the method in tomato processing samples.

4.3.2.2. Phenyl alanine ammonia lyase activity of tomatoes from different developmental stages

The protein contents of samples determined by Bradford method are shown in Table 4.20. The protein amounts were used for the calculation of specific enzyme activity.

Table 4.20: Protein content of development stages material.

Development Stages	Protein content (mg)	
	Flesh part	Skin part
Green	1.7	2.6
Breaker	1.8	4.0
Turning	2.2	1.9
Pink	2.1	3.8
Red	1.3	2.5
Dark red	1.6	4.0

The PAL activity in the flesh and skin part of tomatoes at different developmental stages is shown in Figure 4.33.

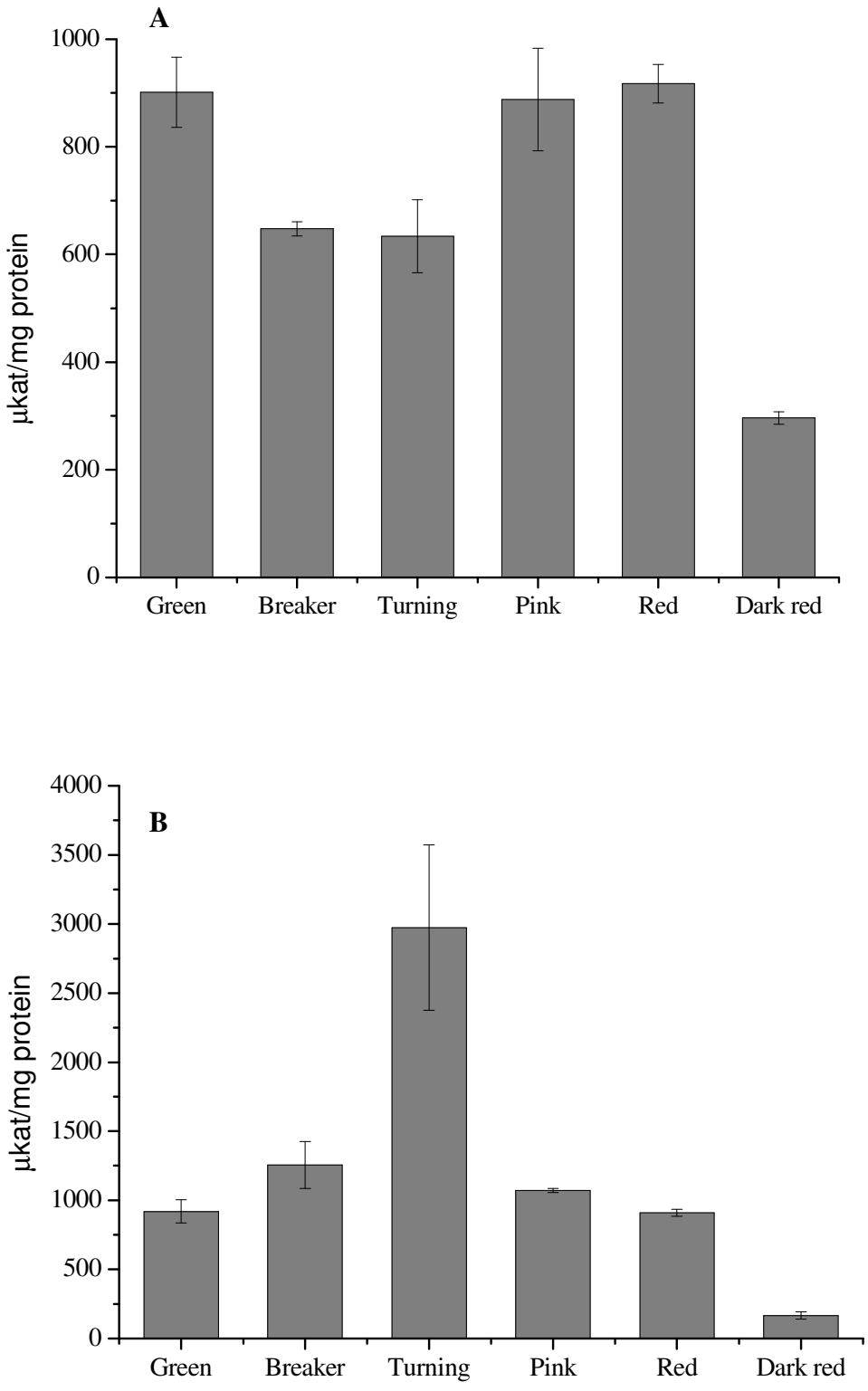


Figure 4.33: Specific PAL activity for the (A) flesh part and (B) skin part.

In the flesh part, the highest PAL activity was in the red flesh (917.3 μkat/mg), and, then, a severe decrease yielding the lowest activity (296.4 μkat/mg) in the dark red flesh part was observed. The rest of the steps contained PAL activity of 633.8-888.0 μkat/mg. In the skin part, the same trend of the 3GT activity was observed with the highest activity at the turning stage (2974.6 μkat/mg). The lowest activity, on the other hand, was again observed in the dark red stage, similar to the flesh part (166.0 μkat/mg).

In general, the skin part showed higher PAL activities similar to the observation in the 3GT enzyme which could be associated to the higher substrate concentrations. It is known that the accumulation of UV absorbing compounds such as flavones, flavonols, isoflavonoids, and anthocyanins in the vacuoles of the epidermal cell layers seem to provide a selective way against the UV light. A role for these compounds in UV protection is apparent from the studies in *Arabidopsis,* where mutations block the synthesis of specific group of flavonoids and significantly reduce the UV tolerance of these mutants (Li *et al*., 1993; Sarma and Sharma, 1999).

In order to investigate the relation between two enzyme activities, the correlation factors between 3GT and PAL for the flesh and skin parts were calculated (Figure 4.34). The coefficient of correlation of the flesh part of development stages gave very low values (r=0.14). On the other hand, correlation between 3GT and PAL activities was found to be higher for the skin parts (r=0.88).

It is expected that higher activities of PAL will result in higher concentrations of *trans*-cinnamate which then will take place in the rest of the phenyl propanoid pathway and, therefore, will provide more substrate for the rutin synthesis. This hypothesis is in accordance with those findings for the skin part where the activity of both enzymes was highly correlated.

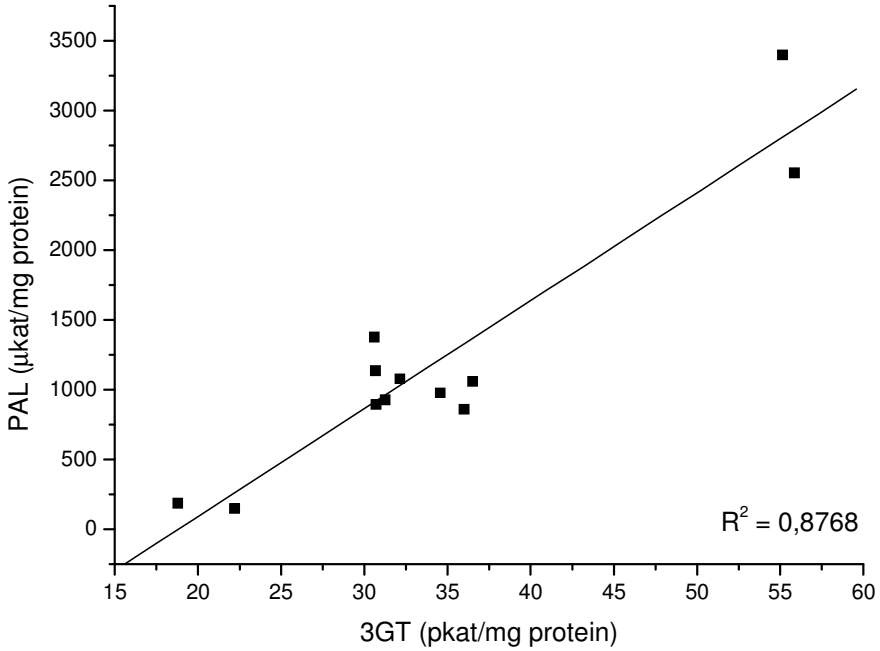


Figure 4.34: Correlation between 3GT and PAL activities for the skin part of tomato.

5. CONCLUSION

Total phenolic assay is insufficient to reflect the changes in total phenolics during processing and, therefore, is not recommended for monitoring changes in phenolic compounds during tomato fruit processing as a single method. Due to the lack of specificity of this method for phenolic compounds, the presence of other reducing compounds reduces the accuracy of the assay. On the other hand, total flavonoid assay yields more accurate results in accordance with individual analysis of flavonoids performed by HPLC.

The results showed that relative losses in carotenoids including lycopene, β-carotene, and lutein, during paste making process are best reflected in the lipophilic phase antioxidant activity findings obtained with the CUPRAC and DPPH assay. However, the DPPH test is incapable of measuring the hydrophilic fractions efficiently. In other words, it is important to measure both hydrophilic and lipophilic antioxidant capacity of samples to be able to evaluate the total capacity correctly. It is expected that a single method can not determine all the antioxidant compounds available, and it is apparent that all the methods may represent several advantages and disadvantages over each other. Even the methods based on the same principle such as ABTS and DPPH can show several important differences in their response to antioxidants. Therefore, it is highly recommended to apply several test procedures to evaluate antioxidant activities.

Vitamin C is the major antioxidant compound comprising of approximately 85% of the total antioxidant activity. On the other hand, rutin, rutin apioside, chlorogenic acid, naringenin chalcone, lycopene, lutein and β-carotene also contribute to the total antioxidant capacity of the samples. The effect of processing on vitamin C is the key factor affecting the resulting antioxidant capacity of tomato paste because of its high contribution to the total antioxidant capacity.

Naringenin chalcone, naringenin, rutin and rutin-apioside are the most abundant flavonoids in tomato samples. Notably, the abundance of all these flavonoids significantly increases more than 2-fold as a result of the breaking step. Another change that occured in flavonoids during processing is the conversion of naringeninchalcone into naringenin in between breaking and evaporation steps. Therefore, it is concluded that the removal of seed and the skin has a significant effect on the loss of flavonoids in the processed tomatoes. As a result, the most critical events in tomato processing are the breaking step, which causes a significant increase in a range of flavonoids and alkaloids, and the pulping step, after which the seed and skin are removed. Recovery of flavonoids and other compounds from the seed & skin fraction can be taken into consideration to add value to those by-products during tomato processing.

In all lipophilic extracts, all *trans*-lycopene, β-carotene and lutein are identified as the main carotenoids. It is apperant that losses in lycopene, β-carotene or lutein are mainly caused by the removal of seed and skin and by evaporation step. Rest of the losses is probably due to the oxidation reactions taking place during processing. The major step causing a decrease in the carotenoid content is the evaporation step indicating that thermal treatments are resulted in degradation of carotenoids.

The lipid-soluble antioxidant α-tocopherol (vitamin E) and β-tocopherol are not affected by the industrial processing. On the other hand, their biosynthetic precursors, γ-tocopherol, and δ-tocopherol decrease significantly in the paste through processing. The main step giving significant losses of γ-tocopherol, and δ-tocopherol is the removal of seed and the skin from the pulp.

By the effect of processing, half of the vitamin C was lost as a result of the breaking and pulping steps, where the tomatoes are chopped and a heat treatment was applied. In contrast to the flavonoids, no increase in vitamin C was observed as a result of the breaking step, and a relatively low content was detected in the seed & skin fraction as compared to the pulp and final paste. It is obvious that vitamin C has the highest antioxidant activity in tomato fruit. Major losses particullarly occur in the breaker and pulper steps of paste processing. However, still about 50% of the vitamin C is preserved in the paste.

According to the overall metabolite composition of the final paste, analyzed by an untargeted metabolomics approach, the metabolite profiles are mainly influenced by the various processing steps, rather than by variation between individual tomato fruit batches, season or processing event.

Due to their high antioxidant contents tomato processing wastes reserve a particular attention as they are functional ingredients for the food industry as a source of high quality plant proteins. In addition, processing wastes can have a potential to be used as raw materials for producing alternative fuels. Another way of getting use of the residues is that compounds present in the seed & skin fraction may be re-extracted and then (partly) returned to the paste. In addition, as the breaker step results in increased amounts of flavonoids, further research into the biochemical processes underlying this aspect and adjustment of the breaker step towards optimized flavonoid levels may help to enhance the amount of these health-related compounds in the final paste.

It has been clearly demonstrated that the physical state and processing history of a food item have a very marked effect on the availability of these compounds for absorption by human body. This indicates that disruption of the food matrix and thermal treatments during the processing could be major factors affecting the bioavailability of the antioxidative compounds. Therefore, in addition to *in vitro* studies, clinical studies investigating the bioavailability of those compounds would provide valuable data for elucidating the effect of food processing on human health.

Tomato fruit ripening involves a complex set of unique biological processes with the regulation of metabolic pathways taking place within specific tissues of the fruit. In the present study, an extensive biochemical characterization of different fruit tissues, at different ripening stages, revealed that rutin and naringenin chalcone are the most abundant flavonoids in the fruit, exhibiting intense mass signals. Flavonoids are typically present in the epidermal tissue of the tomato fruit. Quercetin, kaempferol and naringenin (chalcone) derivatives are located mostly in the epidermis and some, such as naringenin chalcone, naringenin and the trisaccharides of kaempferol and quercetin, also in the calyx tissues. The changes in the flavonoids during development differed widely according to the metabolite. In addition, the tendencies observed during development are similar for all tissues for carotenoids and

chlorophylls. The tocopherols have different concentrations in diverse parts of the tomato fruit. Ascorbic acid level is relatively high in the epidermis increasing in the fruit during ripening. All the tissues found to contain different amount and type of antioxidants which were changing during development showing that any tomato sample collected at a different stage may result with a completely different antioxidant profile.

Metabolomics study showed that different fruit tissue profiles are quite diverse. Many of the metabolites are not uniformly distributed over the tomato fruit upon fruit ripening, but rather, preferentially accumulate in specific fruit tissues as well as at specific ripening stages. This information provides important information about the tissue distribution of semi-polar metabolites and their fate upon ripening of the fruit. Additionally, ripening-dependent changes in metabolites are the second source of metabolite profile segregation. Additionally, biochemical studies on fruits, including tomato, generate knowledge that potentially can have a direct consumer impact as it provides insight into nutritional and quality aspects. Future studies, by using fruits from other genotypes and natural or induced mutants, might also be performed to confirm the observed tissue-preference of metabolites and to give more insight into the tissue-specific regulation of metabolic pathways and the biological function of secondary metabolites.

The increase in the flavonoid content during breaking step is related to the enzyme activities of 3GT and PAL. The increase in 3GT activity in the first breaking step is consistent with the increase in the rutin content. Cutting may result in the stimulation of phenyl propanoid pathway and therefore stimulating higher 3GT enzyme activies and higher rutin contents. Moreover, low regioselectivity of glycosylation for quercetin yields different additional compounds such as quercetin-7-O-glucoside and quercetin-4'-O-glucoside.

The differences in the 3GT activities at different development stages indicate that the flesh and the skin part can contain different amount of enzymes at different development stages. The existence of higher enzyme activities might be related to their higher substrate concentration.

The highest PAL activity in the flesh and skin part of tomatoes differs according to the developmental stages reaching lowest level in the dark red flesh part. Such activity variations could be as a result of changes in the antioxidants and metabolites which are taking place as substrates in the enzymatic reactions. It is concluded that different enzymes in the phenyl propanoid pathway may show different activities during development in the fruit.

There is still a need for information to fully understand the mechanism behind the flavonoid regulation and synthesis pathways. Understanding the structure-activity relationships for the enzymes taking place in these pathways will also provide useful information in order to clarify the flavonoid synthesis mechanisms and the interactions of different enzymes taking place in the pathways.

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APPENDIX

APPENDIX A. CALIBRATION CURVES

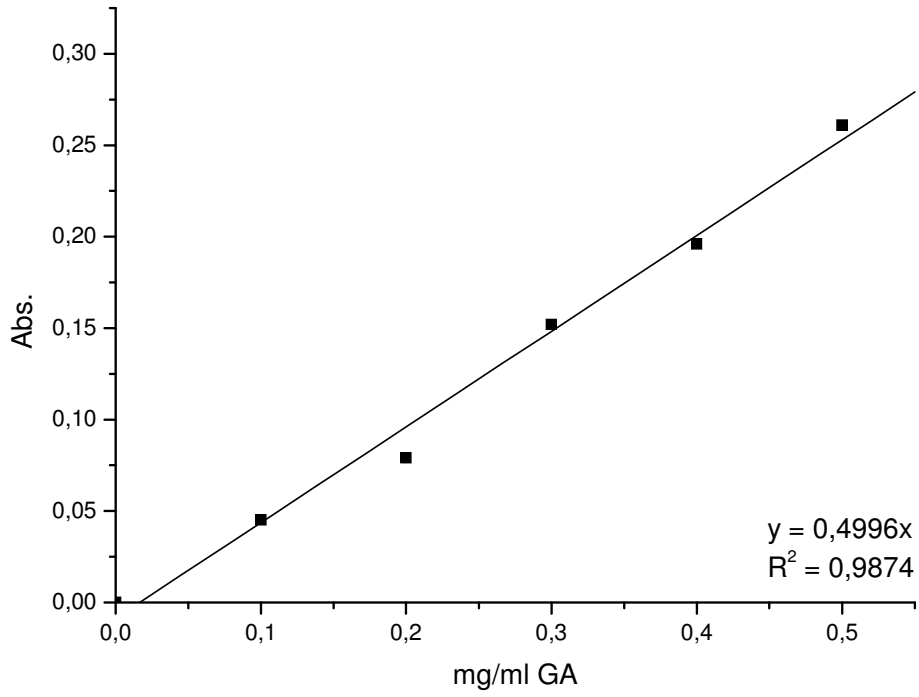

Figure A.1: Calibration curve for total phenolics.

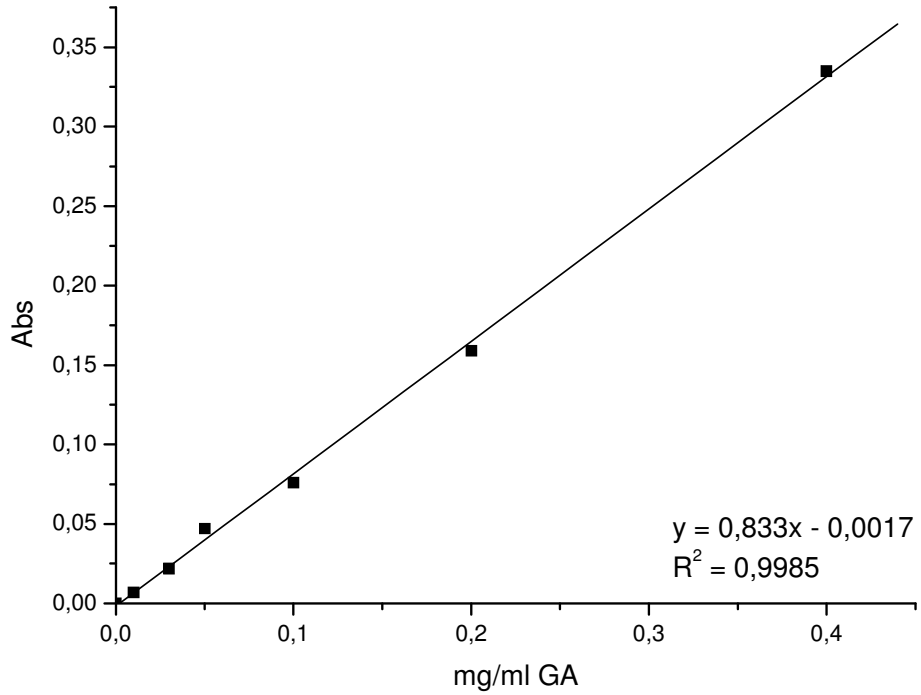

Figure A.2: Calibration curve for total flavonoids.

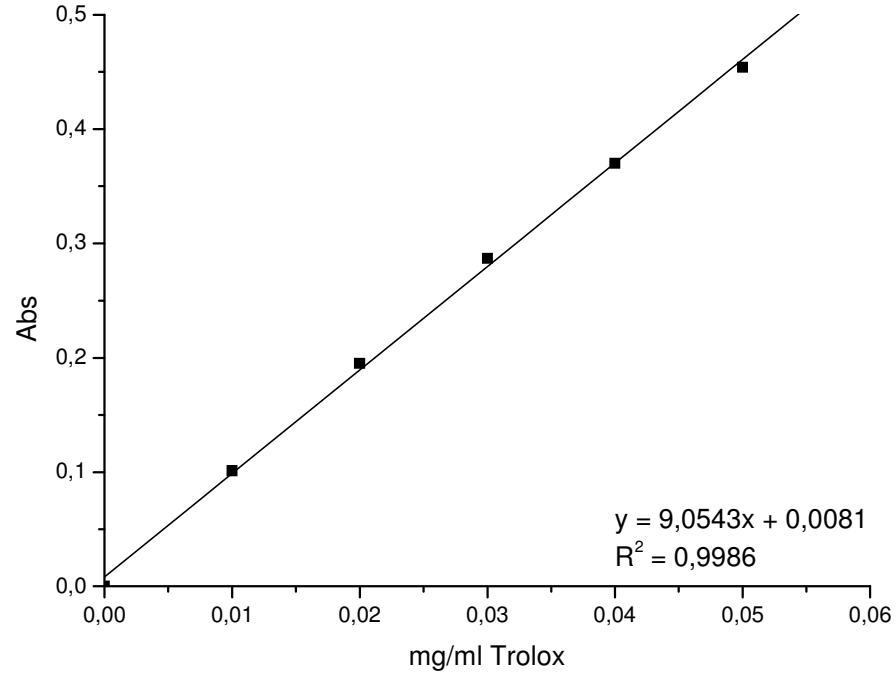

Figure A.3: Calibration curve for ABTS method in methanol.

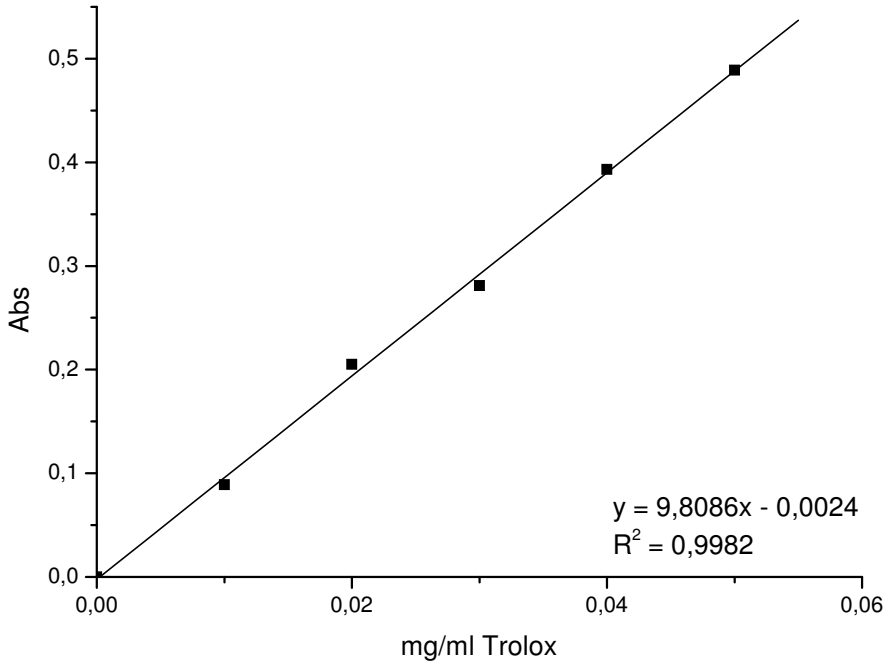

Figure A.4: Calibration curve for ABTS method in acetone.

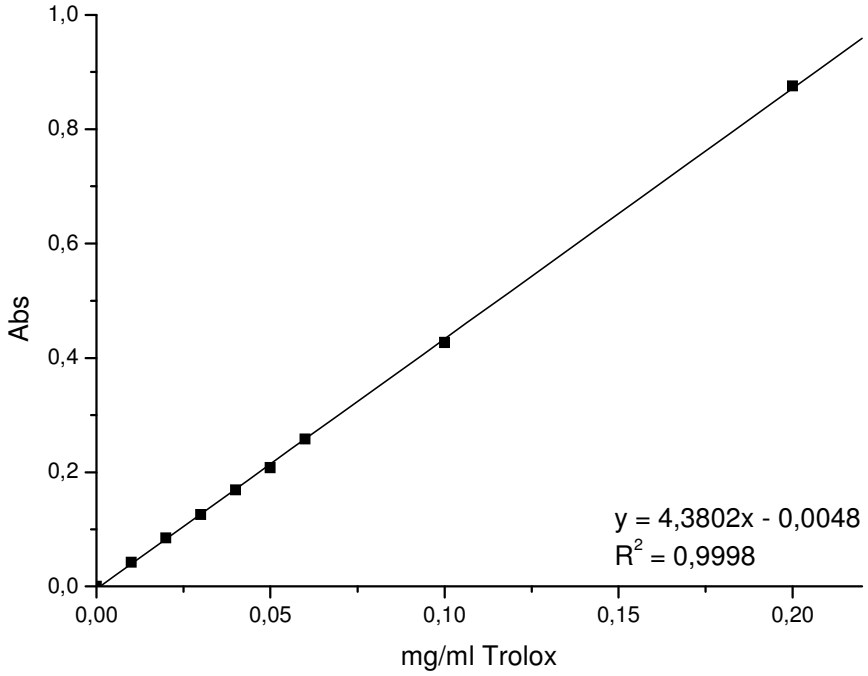

Figure A.5: Calibration curve for DPPH method in methanol.

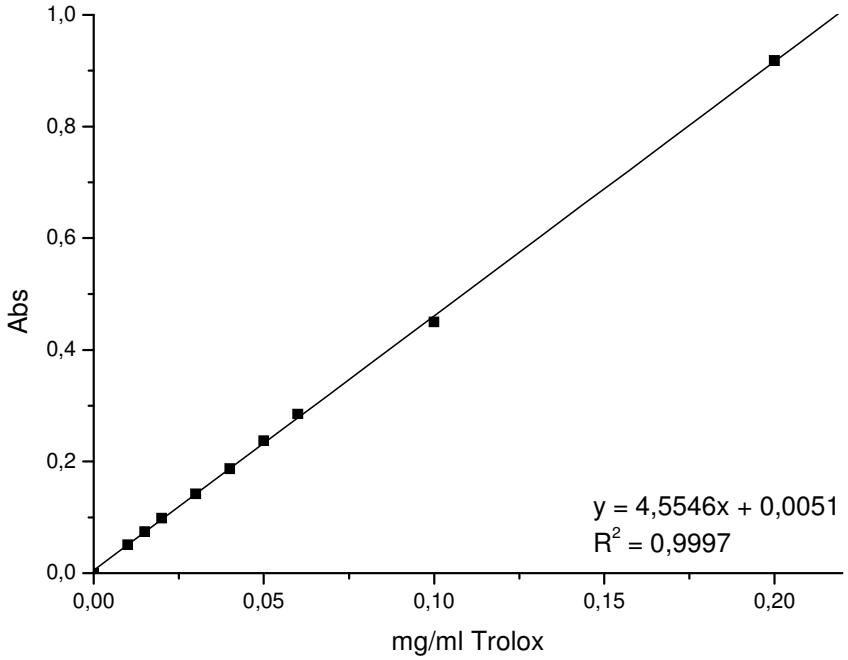

Figure A.6: Calibration curve for DPPH method in acetone.

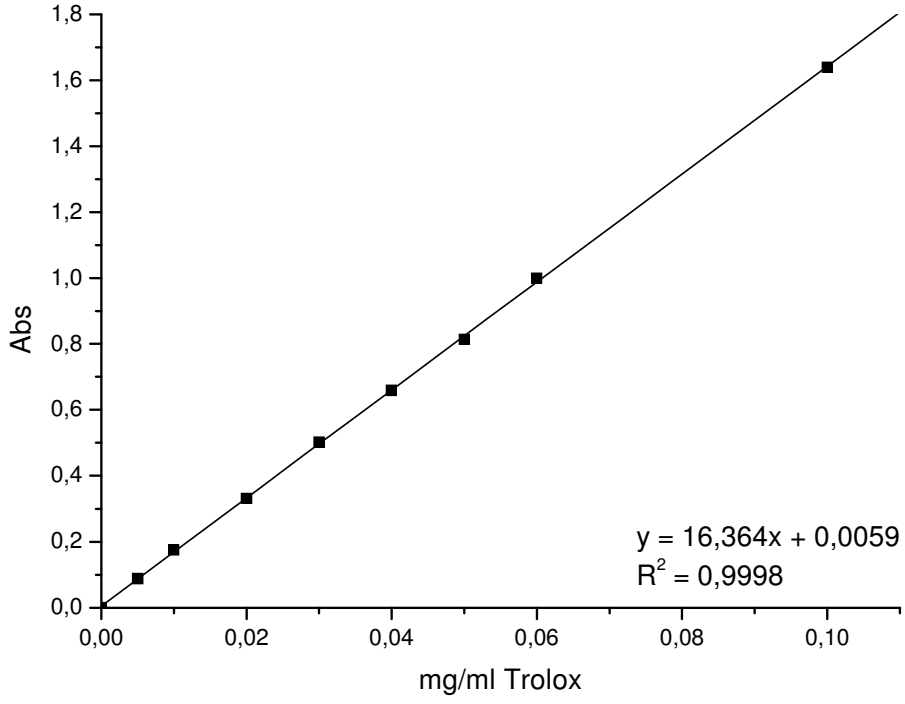

Figure A.7: Calibration curve for FRAP method in methanol.

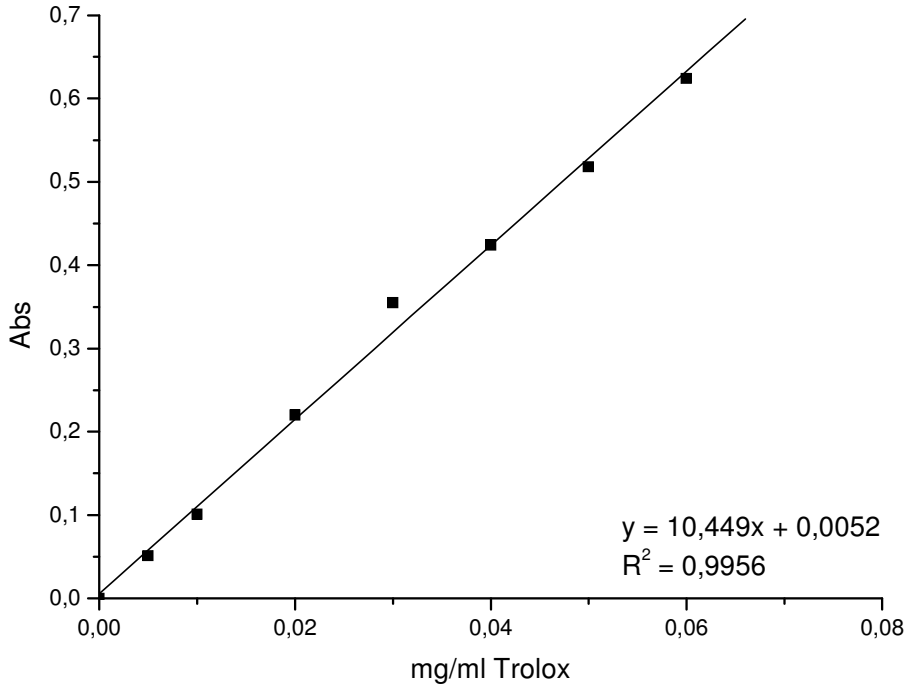

Figure A.8: Calibration curve for FRAP method in acetone.

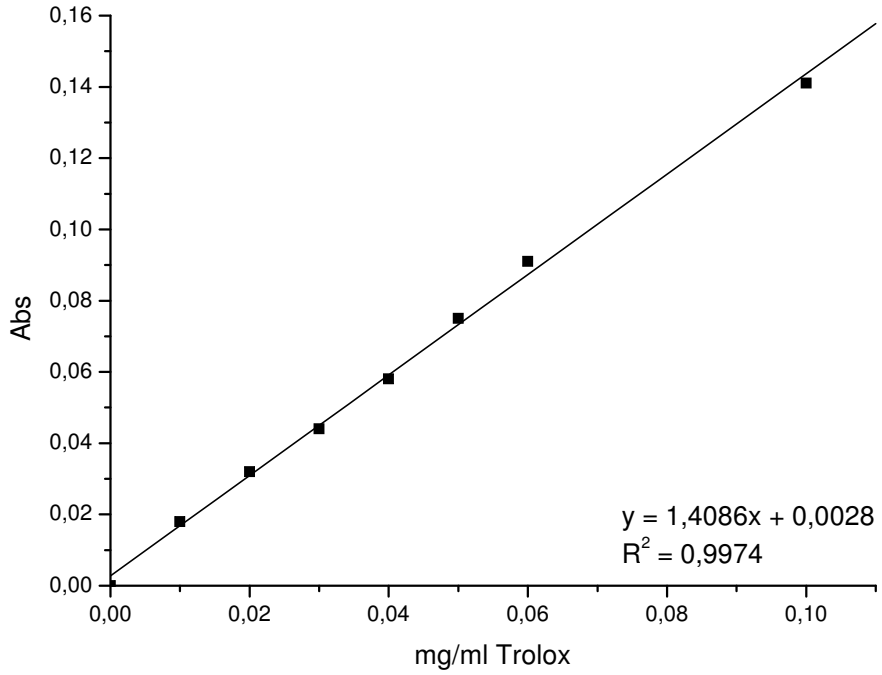

Figure A.9: Calibration curve for CUPRAC method in methanol.

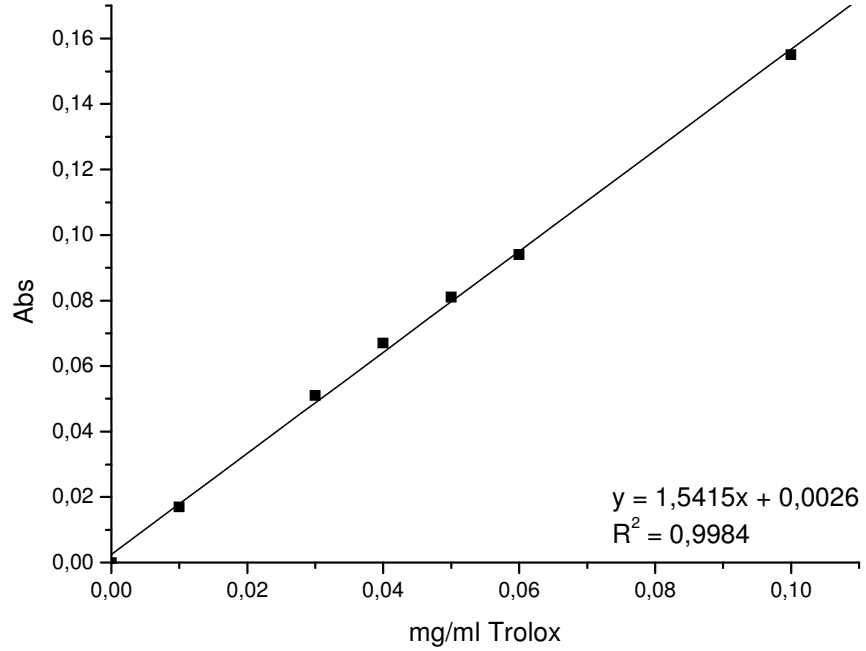

Figure A.10: Calibration curve for CUPRAC method in acetone.

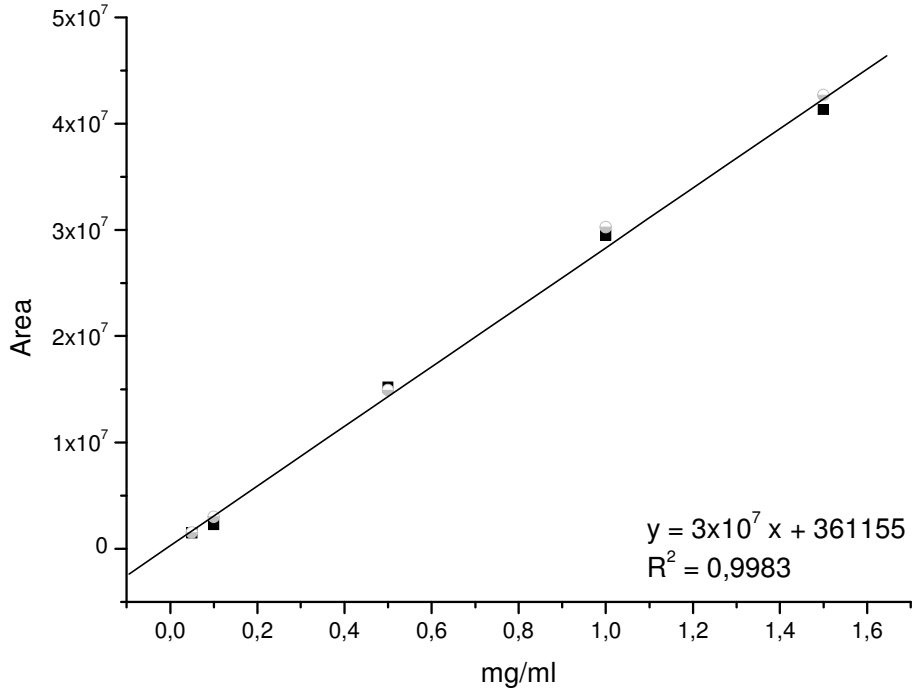

Figure A.11: Calibration curve for naringenin.

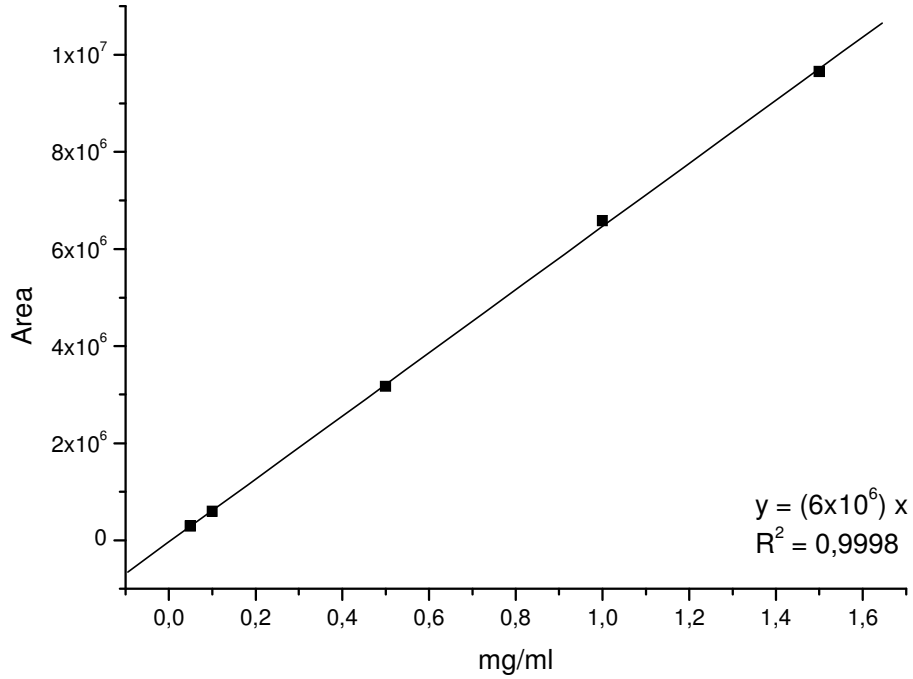

Figure A.12: Calibration curve for naringenin chalcone.

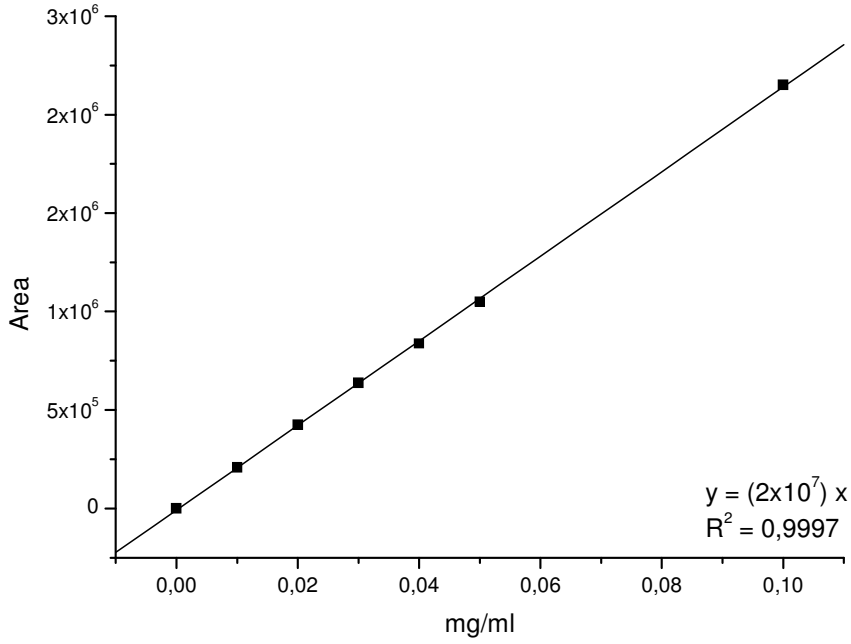

Figure A.13: Calibration curve for isoquercetin.

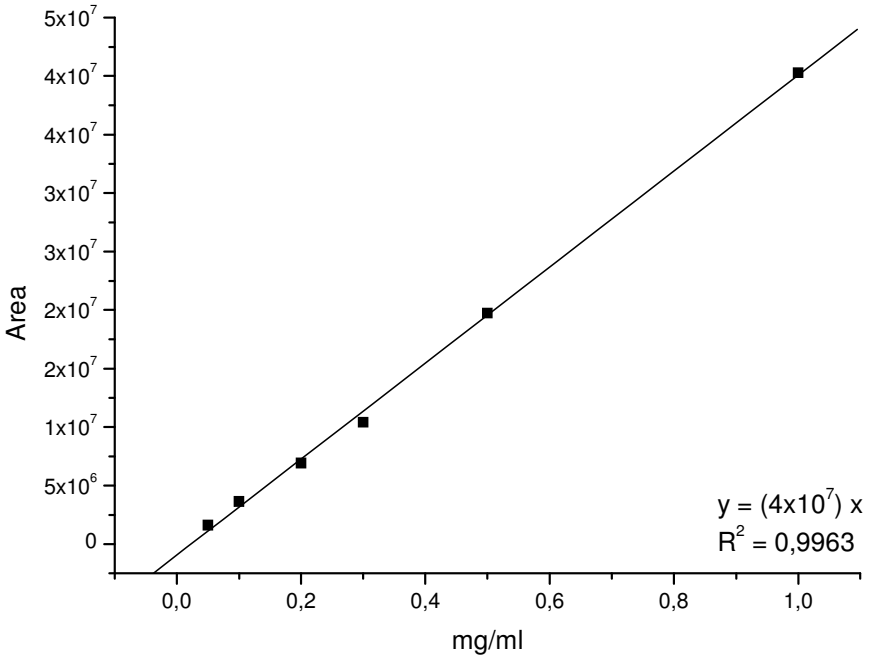

Figure A.14: Calibration curve for quercetin.

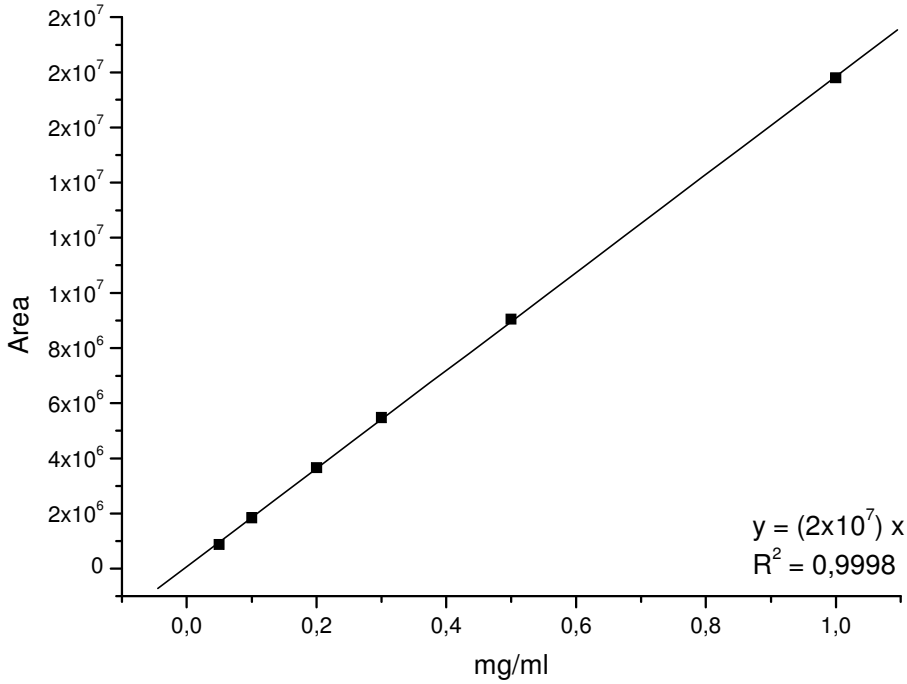

Figure A.15: Calibration curve for quercitrin.

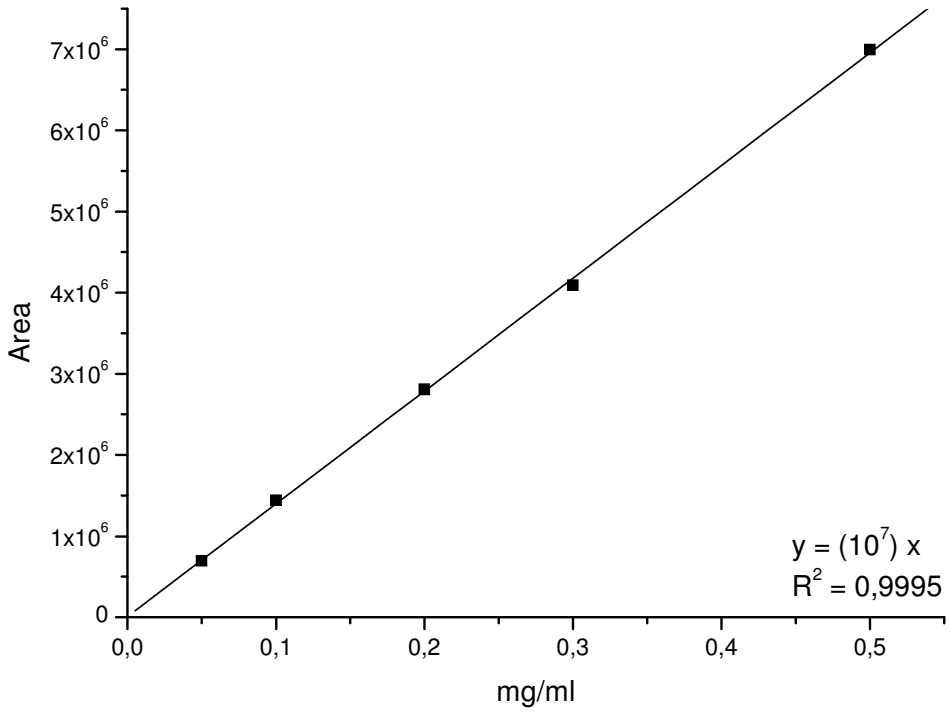

Figure A.16: Calibration curve for rutin.

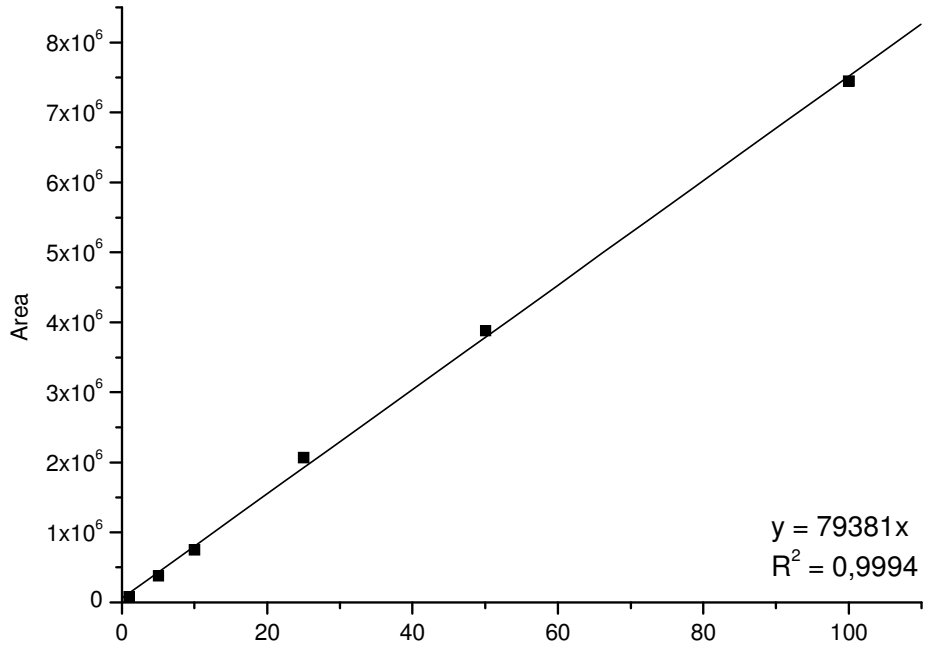

Figure A.17: Calibration curve for vitamin C.

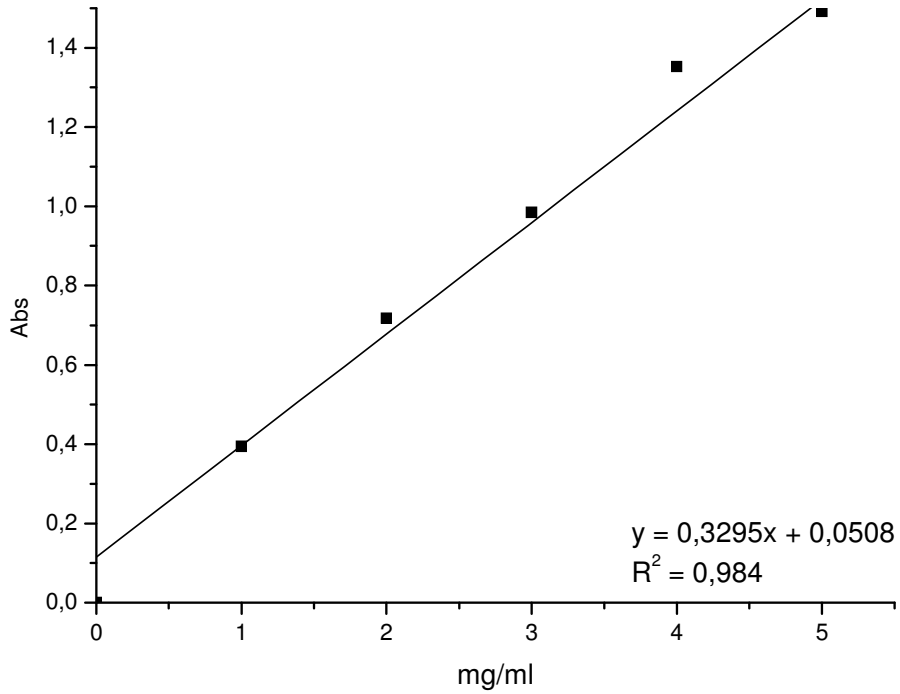

Figure A.18: Calibration curve for protein assay.

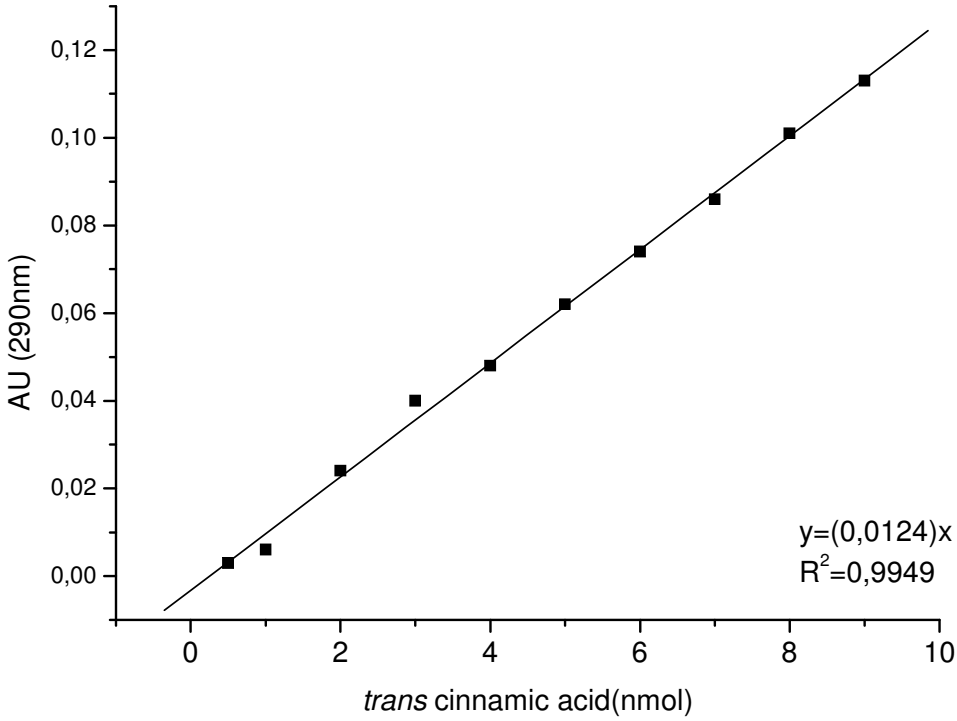

Figure A.19: Calibration curve for PAL assay.

APPENDIX B. ANOVA TABLES

Table B.1: Statistical analysis results of different tomato processing samples (continuing).

		Sum of Squares	df	Mean Square	F	P
Moisture	Between Groups	3399,4	5	679,9	88,8	0,000
	Within Groups	183,7	24	7,7		
	Total	3583,0	29			
Vitamin C	Between Groups	158718,0	5	31743,6	9,7	0,000
	Within Groups	78710,3	24	3279,6		
	Total	237428,3	29			
β-Carotene	Between Groups	18,1	5	3,6	4,7	0,004
	Within Groups	18,3	24	0,8		
	Total	36,5	29			
Lycopene	Between Groups	22764,9	5	4553,0	6,2	0,001
	Within Groups	17468,6	24	727,9		
	Total	40233,6	29			
Lutein	Between Groups	10,2	5	2,0	25,4	0,000
	Within Groups	1,9	24	0,0		
	Total	12,2	29			
Naringenin	Between Groups	38,1	5	7,6	14,3	0,000
	Within Groups	12,8	24	0,5		
	Total	50,9	29			
Rutin apioside	Between Groups	3645,3	5	729,1	67,5	0,000
	Within Groups	259,1	24	10,8		
	Total	3904,4	29			
Rutin	Between Groups	91177,6	5	18235,5	104,9	0,000
	Within Groups	4173,0	24	173,9		
	Total	95350,5	29			
Naringenin chalcone	Between Groups	104758,0	5	20951,6	47,4	0,000
	Within Groups	10601,0	24	441,7		
	Total	115359,0	29			
Chlorogenic acid	Between Groups	179,4	5	35,9	0,5	0,807
	Within Groups	1899,7	24	79,2		
	Total	2079,1	29			
ABTS	Between Groups	790476,0	5	158095,2	0,8	0,588
	Within Groups	5000374,2	24	208348,9		
	Total	5790850,2	29			
DPPH	Between Groups	799468,3	5	159893,7	6,5	0,001
	Within Groups	588530,2	24	24522,1		
	Total	1387998,5	29			
CUPRAC	Between Groups	2613628,4	5	522725,7	1,2	0,316
	Within Groups	10009418,6	24	417059,1		
	Total	12623047,0	29			

Table B.1: Statistical analysis results of different tomato processing samples (continuing).

		Sum of Squares	df	Mean Square	F	P
FRAP	Between Groups	2871563,5	5	574312,7	24,9	0,000
	Within Groups	552924,1	24	23038,5		
	Total	3424487,6	29			
Total Flavonid	Between Groups	368381,6	5	73676,3	23,4	0,000
	Within Groups	75486,8	24	3145,3		
	Total	443868,5	29			
Total Phenolics	Between Groups	13905,9	5	2781,2	0,8	0,570
	Within Groups	84931,4	24	3538,8		
	Total	98837,3	29			
δ-Tocopherol	Between Groups	1,4	5	0,3	16,8	0,000
	Within Groups	0,4	24	0,0		
	Total	1,7	29			
γ-Tocopherol	Between Groups	1762,7	5	352,5	28,0	0,000
	Within Groups	302,6	24	12,6		
	Total	2065,2	29			
β-Tocopherol	Between Groups	18,3	5	3,7	2,7	0,042
	Within Groups	31,9	24	1,3		
	Total	50,2	29			
α-Tocopehrol	Between Groups	775,7	5	155,1	0,6	0,686
	Within Groups	6006,6	24	250,3		
	Total	6782,3	29			
ABTS-hydrophilic	Between Groups	734713,6	5	146942,7	0,8	0,590
	Within Groups	4661927,8	24	194247,0		
	Total	5396641,4	29			
ABTS-lipophilic	Between Groups	6101,6	5	1220,3	1,9	0,124
	Within Groups	15078,6	24	628,3		
	Total	21180,2	29			
CUPRAC-hydrophilic	Between Groups	2755367,5	5	551073,5	1,4	0,252
	Within Groups	9293221,2	24	387217,5		
	Total	12048588,7	29			
CUPRAC-lipophilic	Between Groups	716867,6	5	143373,5	11,2	0,000
	Within Groups	308435,6	24	12851,5		
	Total	1025303,1	29			

Table B.1: Statistical analysis results of different tomato processing samples (continuing).

		Sum of Squares	**df**	**Mean Square**	**F**	**P**
FRAP-hydrophilic	Between Groups	4648959,4	5	929791,9	12,5	0,000
	Within Groups	1786311,3	24	74429,6		
	Total	6435270,7	29			
FRAP-lipophilic	Between Groups	317149,7	5	63429,9	13,8	0,000
	Within Groups	110707,7	24	4612,8		
	Total	427857,3	29			
DPPH-hydrophilic	Between Groups	714480,0	5	142896,0	5,7	0,001
	Within Groups	599363,9	24	24973,5		
	Total	1313843,9	29			
DPPH-lipophilic	Between Groups	9605,8	5	1921,2	8,0	0,000
	Within Groups	5775,4	24	240,6		
	Total	15381,2	29			

APPENDIX C. CONTROL SAMPLES FOR ENZYME ASSAYS

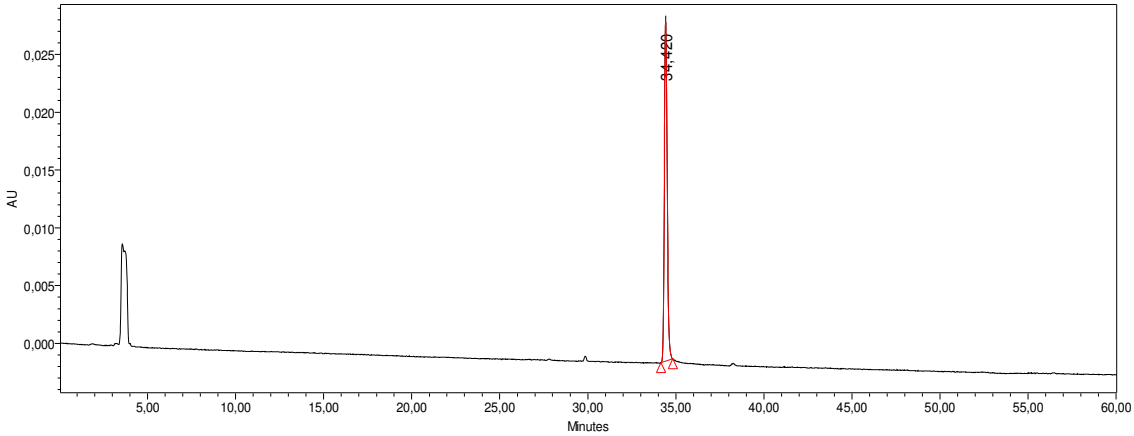

Figure C.1: Enzyme assay control without enzyme.

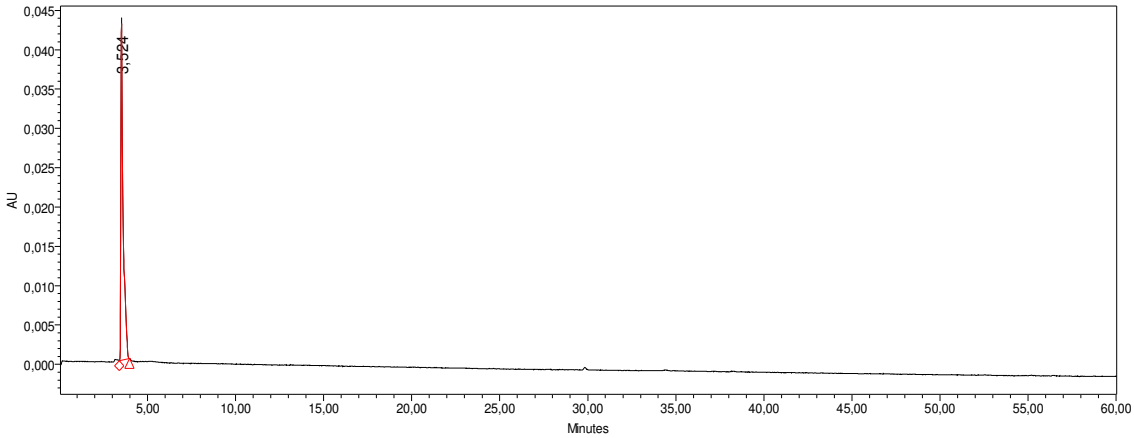

Figure C.2: Enzyme assay control without quercetin.

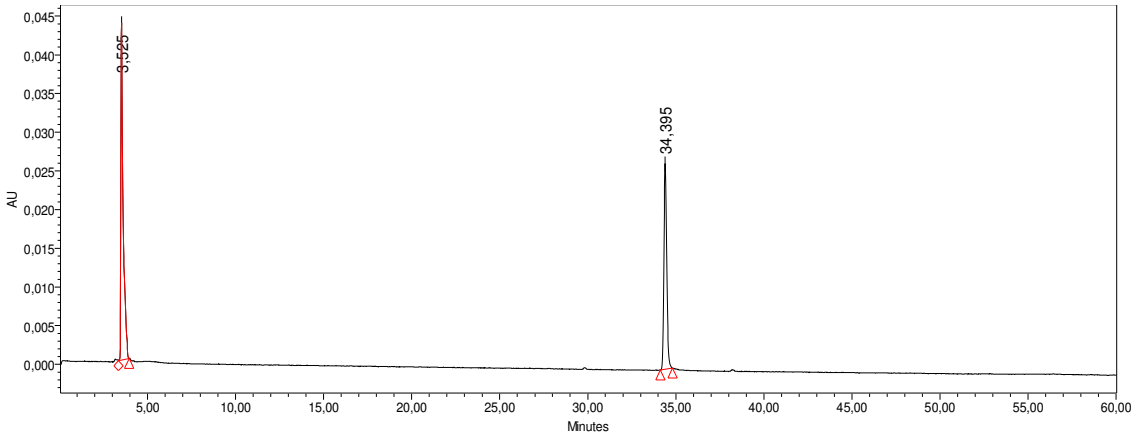

Figure C.3: Enzyme assay control with denatured enzyme.

BACKGROUND

Esra Çapanoğlu was born in 1978 in Erciş, Turkey. She graduated from ISTEK Uluğ Bey High School in 1995 and enrolled to Istanbul Technical University (ITU) Food Engineering Department in the same year. She graduated in 1999 having the first rank of the class and registered to ITU Food Engineering Department Graduate Programme and was awarded with a M.Sc. degree in 2002. She has started her PhD in the same programme in the same year.

During her Ph.D study, she has worked as a visiting scientist for 6 months in PRI (Plant Research International), Wageningen, The Netherlands in 2006. Then, she worked in the laboratories of IPK (The Leibniz Institute of Plant Genetics and Crop Plant Research), Germany for 6 months in 2007.

She has joined several international and national conferences and has 4 full articles published in the international journals. She also has 24 international conference papers and 8 national articles and conference papers. She has a second place award from IFT 2003, in Chicago, USA from Food Product Development Section and a third place award from CFFN Functional Food Congress, 2006 in Istanbul, Turkey. She was awarded with Tinçel Proficient and DAAD (German Academic Exchange Programme) Scholarships. She also had scholarships from Wageningen, PRI in 2006, and from EU 6th Framework FLORA project in 2007.

She has been working as a research assistant in Food Engineering Department since June, 2000. She has assisted several courses in Food Engineering Department, including Food Quality Control Laboratory, Sensory Analysis, Mass Transfer.