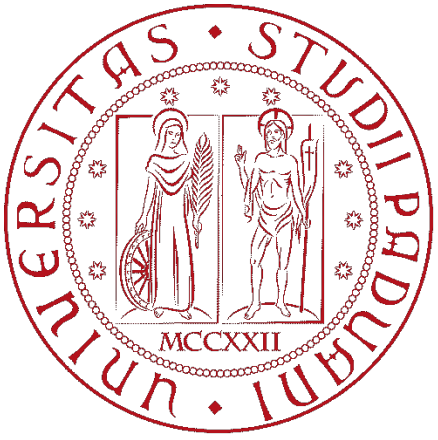


UNIVERSITÀ DEGLI STUDI DI PADOVA

Department of Comparative Biomedicine and Food Science

Second Cycle Degree (MSc)

In Biotechnologies for Food Science

Characteristics Of Polyphenolic Compounds In fruits Using UV/Vis Spectrometry And HPLC Technique

Supervisor: Prof. dr. sc. Lidija Jakobek Barron

Co-Supervisors: Prof. Dr. Antonio Masi

Submitted By: Abdul Wahab

Student No. 2005506

Academic Year: 2022

Index

Abbreviations and Acronyms

FRAP	Ferric reducing antioxidant power (FRAP)
HPLC	High-Performance Liquid Chromatography
MS	Mass Spectrometer
RPLC	Reverse Phase Liquid Chromatography
PDA	Photodiode-Array Detection
Uv/Vis	Ultraviolet-visible spectroscopy
Cons	Concentration
SD	Standard Deviation
rT	Retention Time
LOQ	Limit of Quantification
CV%	Correlation Coefficient
LOD	Limit of Detection

Acknowledgments

Thanks to Almighty Allah for blessing me with great achievements throughout my life. Thanks to Erasmus+ Programs which gave me this prestigious opportunity to do my thesis research work at Josip Juraj Strossmayer University of Osijek (Croatia).

I would to thank Prof. dr. sc. Lidija Jakobek Barron for supervising me during my research work and helping me out every day by teaching me new skills without her supportive behavior and encouragement I would not be able to finish my research work on time.

A big thanks to my co-supervisor prof doctor Antonio Masi for being an amazing mentor and always available for any queries and help. I am very thankful to Professor Mauro Dacasto for guiding me and helping me out during my Master's program at Unipd.

All the lab workers and specially Jozo Istuk, Petra Matic, and Evana for being there during my experimental work.

Last but not least special thanks to my family and my friend Muhammad Ahmad Bajwa for being supportive during my whole education carrier and for their kind prayers. Without their support and prayers, I will never be able to study at the best universities in the world and progress in my life.

Summary

Polyphenols extraction from the peels and flesh of apples and oranges was obtained using different processes of extraction. A variable amount of solvents were used in many different concentrations to get the best extraction of polyphenols from samples. Different concentrations of water and methanol were used or methanol was acidified with HCL (0.1%).

An ultrasonic bath was used to perform an extraction with different time periods ranging from five to 15 minutes and after that centrifugation helped to get better results from extracts of samples. The total polyphenols and total flavonoid content of apples and oranges peels and flesh were analyzed using Folin-Ciocalteau and spectrophotometer methods.

RP-HPLC Reversed-phase high-performance liquid chromatography assisted with a PDA detector was used to analyze the total amount of individual polyphenols in three different apple varieties (Pink Lady, Red Delicious, and Braeburn) and one variety of sweet oranges (Navelina).

Statistical analysis was used with the help of t-tests and regression line using Microsoft Excel to validate the amount of polyphenol present in samples of apples and oranges.

In apple flesh samples, extraction with 80% methanol in water was used to analyze hydroxycinnamic acids, flavan-3-ols, and dihydrochalcones while in the case of apple peel samples 80 percent methanol in water was used for an efficient extraction of Flavonols, dihydrochalcones, flavan-3-ols, and anthocyanins. Acidified methanol could also be useful for the extraction of anthocyanins and flavonols from the peel. Also in the case of the peel and flesh of oranges, 80% methanol with water was used to analyze Flavanones naringin, naringenin, and hesperidin.

Introduction

1.2 Phenolic compounds

Polyphenols are considered among the most widespread antioxidants found in the body.One g/d is considered the highest dietary intake of polyphenols and it is ten times more than vitamin C daily intake and a hundred times more than Vitamin E and carotenoids intake in the body. General sources of polyphenols in regular diet are tea, cereals, vegetables, red wine, and cocoa (Manach, Scalbert et al. 2004).

Despite their widespread distribution in plants, nutritionists have only lately become aware of many different beneficial health effects of dietary polyphenols. Minerals, vitamins, carotenoids, and minerals were the most frequently researched antioxidants until the mid-1990s. After 1995, researchers began studying polyphenols and flavonoids, their antioxidant qualities, and their involvement in the prevention of disease.

'Polyphenol' is not a strict chemical term. Phenolic acids, tannins, and flavonoids, and also their polymerized or chemically modified counterparts, are included under the same term of polyphenol. Phenolic acids, Flavonols, anthocyanins, Dihydrochalcones, and Flavan-3-ols, are the main classes of polyphenols.

1.2.1 Phenolic acids

In polyphenols, phenolic acids rank as the second major group in apples Compared to the peel of apples phenolic acids are found more in the flesh portion (Wojdyło, Oszmiański et al. 2008, Purushotham, Tian et al. 2009). Phenolic acids play a great role in antioxidant activity in the apple's flesh (Klinder, Shen et al. 2016).

Chlorogenic acid, gallic acids, p-Coumaric acid and Caffeic acid are some examples of phenolic acids discussed in this research. In many apple cultivars, chlorogenic acid is the most abundant phenolic acid present in flesh of apple (Tsao, Yang et al. 2003) and it can be absorbed in the intestine without any structural changes.

In vegetables, fruits, herbs, and mushrooms the most commonly found phenolic acid is caffeic acid. It has a major role in many medicinal activities in bodies including anti-diabetic, anti-inflammatory antioxidant, and antimicrobial (Andrikopoulos, Kaliora et al. 2002) (Pearson, Tan et al. 1999).

.

p-Coumaric acid is commonly present in cereals, mushrooms, vegetables and fruits. It is the precursor of the synthesis of other phenolic acids and it also has medicinal benefits in the body including anti-tumor, anti-oxidant, antiplatelet aggregation, and anti-microbial properties (Burcelin, Mrejen et al. 1998, Guo, Xia et al. 2012).

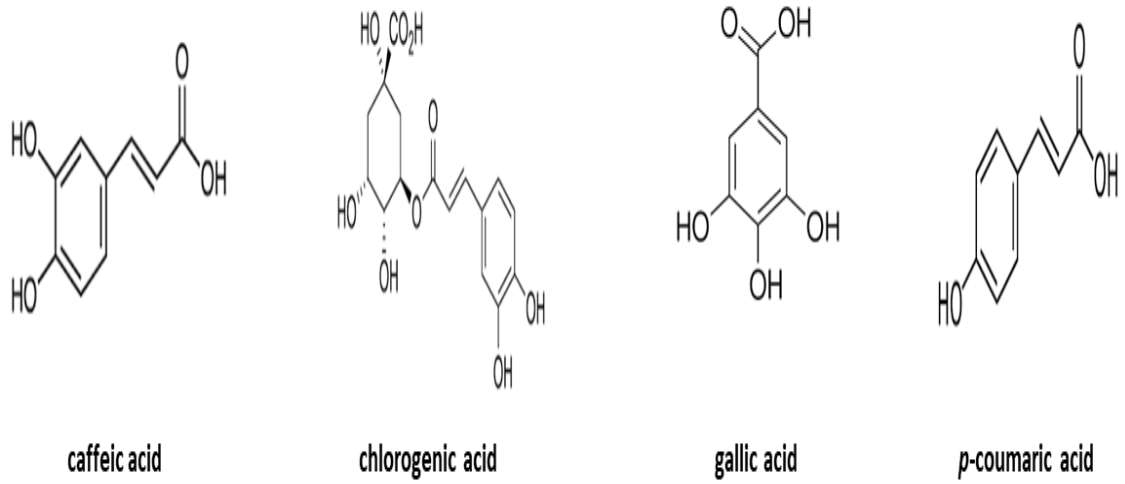


Figure 1: molecular structure of Phenolic acids (Caffeic Acid, Chlorogenic acid, Gallic Acid, *p*-coumaric acid)

1.2.2 Flavan-3-ols

Catechin and epicatechin (Figure 2) fall under the category of flavan-3-ols. These monomeric substances concentration is higher in the apple peel compared to apple flesh (Burcelin, Mrejen et al. 1998). Epicatechin concentration is higher in immature apples compared to catechin (Burcelin, Mrejen et al. 1998).

The antioxidant activity of epicatechin was checked by ferric reducing antioxidant power (FRAP) and it is higher than catechin. In apples, it is considered the most important polyphenol (Klinder, Shen et al. 2016). Both monomeric favan-3-ols catechin and epicatechin can be absorbed in the small intestine's epithelial cells. Catechin undergoes sulfation in a metabolic pathway and, most

of the catechin in plasma and urine is present in the form of glucuronide conjugates or as methylated (Gerhauser 2008).

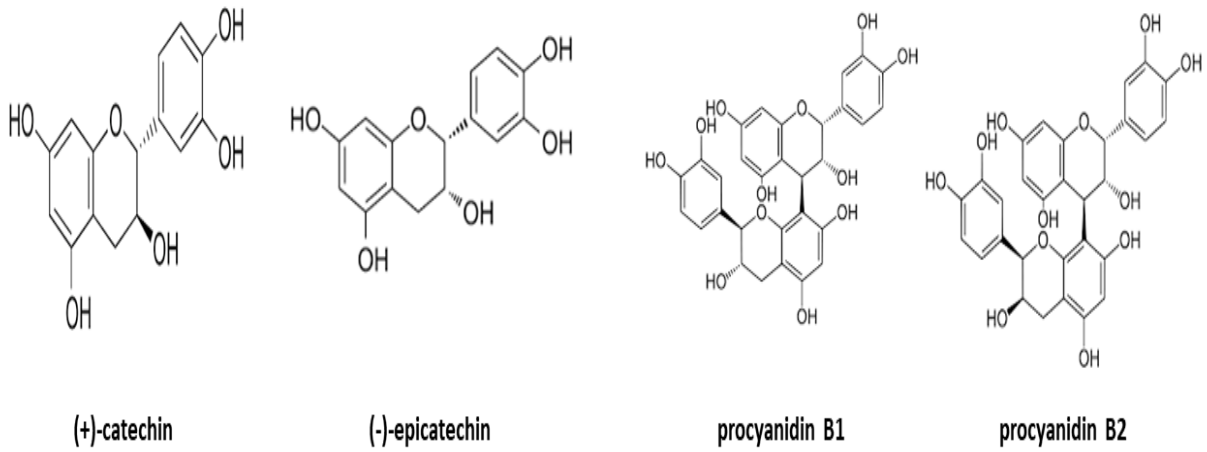


Figure 2: molecular structure of Flavan-3-ols (+) catechin, (-) epicatechin, procyanidin B1, procyanidin B2

1.2.3 Flavonols

Figure 3 shows Flavonols. Quercetin flavanols and are found in the peels of apples (Manach, Scalbert et al. 2004) (Wojdyło, Oszmiański et al. 2008). As they are found in a very low concentration, they are called as minor groups of polyphenols in apples. (Wojdyło, Oszmiański et al. 2008).

Quercetin is found in a high concentration in apples where they have a major role and show very strong bioactivity. Among all polyphenols, quercetin is about 1 to 11 percent (Burcelin, Mrejen et al. 1998). Quercetin is usually present in apples in six different conjugated forms; free quercetin is present in apples but in trace amounts (Burcelin, Mrejen et al. 1998). For transition metal ions quercetin acts as a radical chelator and it is also known as a free radical scavenger.

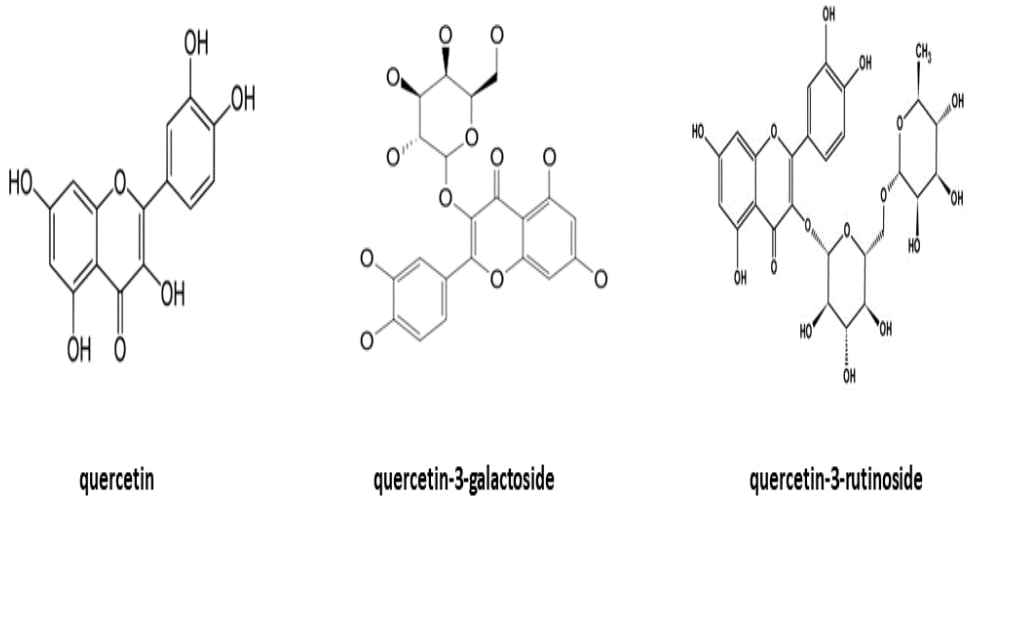
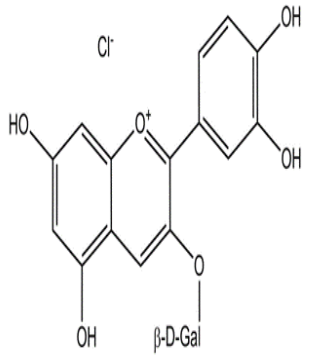


Figure 3: molecular structure of flavonols (quercetin, quercetin 3-galactoside, quercetin 3-rutinoside

1.2.4 Anthocyanins

Anthocyanins are present in the peel of apples and they give the red color to the peel of apples. Anthocyanins are a mixture of more common cyanidin-3-O-galactoside and less common cyanidin 3-O-glucoside (Wojdyło, Oszmiański et al. 2008). In the case of red cultivars, 3-arabinoside and 3-xyloside are present in trace amounts (Burcelin, Mrejen et al. 1998). In crab apple species, cyanidin-3-O-galactoside is found in high concentration.

As the concentration of anthocyanins is just less than 1 percent of apple polyphenols, their concentration does not correlate with the antioxidant activity (Klinder, Shen et al. 2016).



cyanidin-3-galactoside

Figure 4: molecular structure of an Anthocyanin (cyanidin 3-galactoside)

1.2.5 Dihydrochalcones

A total of 4 to 6 percent dihydrochalcones are of total polyphenols in apples (Burcelin, Mrejen et al. 1998) and they are mainly composed of glucose and xyloglucose (Klinder, Shen et al. 2016). The main compound under this group are mainly phloridzin (phloretin-2'-glucoside) and phloretin-2'-xyloglucoside in peel and flesh of apples (Tsao, Yang et al. 2003, Klinder, Shen et al. 2016). Most of the dihydrochalcones are found in the seed and peel of apples (Tsao, Yang et al. 2003).

Apart from being in very low concentration in apples, these polyphenols are used to identify different fruits as well different other types of apple cultivars(Tsao, Yang et al. 2003).

1.3 Bioavailability of polyphenols

Polyphenols' biological characteristics are mostly determined by their bioavailability. In the intestine, the rate of absorption of polyphenols is determined by the chemical structure of polyphenols (Srinivasan 2005).

In the intestines, the content of plasma steadily declines when a high number of flavonoids are absorbed. There is a high affinity for quercetin in the plasma albumin so it has a longer elimination half-life. Glycosylation is common in flavones, isoflavones, flavonols, and anthocyanins(Zhang, Wang et al. 2006). Chemically, glucose and rhamnose are connected with xylose, glucuronic acid, and galactose. Usually, there is a single sugar in polyphenols, but they can have two or three and

a variety of sugar substitutes. Sugars can also be used to replace the malonic acid group. Glycosylation impacts the biological, physical, and chemical properties of polyphenols, which explains the hydrophilic property difference between quercetin-3-O-rhamnoglucoside and quercetin.

Microflora-rhamnosidases are the only source because glucosidases in the human body cannot break the polyphenol that is linked to rhamnose. Epicatechin are acylated primarily with acids such as gallic acid. Galloyl substitution of flavanols has no effect on the partition coefficient and does not affect the bioavailability, unlike glycosylation. While passing through cellular membranes the flavanols do not undergo hydrolysis (Zhang, Wang et al. 2006). To esterify sugars, organic acids, and lipids, caffeic acid is used. It is present in high amounts in coffee and it is produced when caffeic acid and quinic acid are esterified. Esterases found in human cells cannot produce chlorogenic acid, and chlorogenic acid cannot release caffeic acid.

Furthermore, polyphenols move through the small intestine without being absorbed, therefore influencing the intestinal bacteria. Because of the passing polyphenols, two-dimensional reactions occur (Kim, Vance et al. 2016). Polyphenols are first physiologically converted into their more bioavailable metabolites. Second, polyphenols alter the gut microbial community's composition, most likely by inhibiting harmful bacteria while stimulating good bacteria. These may serve as a prebiotic metabolite in the latter, enriching the beneficial bacteria. As a result, dietary polyphenols and gut microbial interactions may have an influence on human host health (Klinder, Shen et al. 2016).

1.4 Phenolic compounds in fruits

1.4.1 Polyphenols from apples and their benefits:

Figure 5 shows the different varieties of apples used during experiments. Apples are the most popular fruit among Western nations, In the United States apples account for 22 percent of the total consumed per year (He and Liu 2008). Extracts of apples and their component chemicals are one of the most researched fruit bio-actives so far. Apples have more bioactive compounds than many other tree fruits, such as peach and pear (Leontowicz, Leontowicz et al. 2007). Flavonoids and phenolic acids are the most common among them. Because apple peel contains three to six times the amount of flavonoids as apple flesh, compared to the flesh extracts peel extracts have higher antioxidant activity (Barbosa, Pinto et al. 2010).

Phenolic compounds of several fruits have been demonstrated to have antioxidant and numerous physiological actions, which lead to cardioprotective characteristics in vitro (Chen, Cai et al. 2006) (Andrikopoulos, Kaliora et al. 2002) and in experimentation on animals (Ellingsen, Hjerkinn et al. 2008) (Aprikian, Busserolles et al. 2002).

Research shows that apple polyphenols may aid to prevent type 2 diabetes through a variety of mechanisms, that includes inhibition of starch digesting enzymes such as amylase and glucosidase (Hyson 2011). In experimental animals, anthocyanins, phenolic acids, flavonols, and

dihydrochalcones have also been linked to increased sensitivity of insulin and also lower the glucose level of the blood (Burcelin, Mrejen et al. 1998, Gerhauser 2008, Hyson 2011, Guo, Xia et al. 2012).

Polyphenols appear to have numerous mechanisms of action, including antioxidant capabilities that remove or trap mutagens, increases xenobiotic metabolism that detoxifies procarcinogens, activation of apoptosis, and antiproliferative effects.

Figure 5: different varieties of apples (Pink Lady, Red Delicious, Braeburn)

1.4.2 Polyphenols from Oranges and their benefits

Interest in citrus fruits is increasing in recent years due to their relation in lowering the risk of colorectal (Levi, Pasche et al. 1999), stomach (McCullough, Robertson et al. 2001), gastric (Palli, Russo et al. 2001) and esophageal (Levi, Pasche et al. 2000, Chen, Ward et al. 2002) cancer.

Apart from role in preventing cancer, citrus fruits also appear to be in correlation with blood lipid profiles improvements (Kurowska, Spence et al. 2000) and a better survival rate in elderly people (Fortes, Forastiere et al. 2000).

There are several types of oranges under the genus Citrus which includes sour sweet oranges as well as tangelos, tangerines and tangros. In sour oranges, neohesperidin and naringin are the main flavanone glycosides. The sugar neohesperidose play an important role in giving a bitter or tangy

taste to sour oranges. In sweet oranges the main flavanone glycosides are narirutin and hesperidin. In sweet oranges, sugar rutinose imparts the neutral taste.

Figure 6: a variety of orange used in experiment

1.5 Determination of Polyphenols

1.5.1 Liquid Chromatography

Liquid chromatography endures a wide range of mobile phase properties and the choices of numerous types of stationary phases and a variety of detectors used in the process.

1.5.2 HPLC

In High-Performance Liquid Chromatography (HPLC), components in a liquid mixture are separated by forcing a high pressure liquid or solvent through a porous material column that contains a stationary phase on its surface. With the help of a high-pressure pump, the solvent moves through the column. Depending upon different components present in the sample these components have different affinities for the mobile phase and stationary phase used(He and Liu 2008).

1.5.3 Reversed-Phase Liquid Chromatography

Depending upon the interaction occurring between the stationary phase and the solutes in the eluent,there are different types of liquid chromatography: Normal phase, ion–exchange gel filtration, and reversed-phase.

In reversed-phase chromatography, the mobile phase is more polar compared to the stationary phase. Mostly two types of stationary phases are used in one type the nonpolar groups are bonded on the silica (18 carbon chain or commonly known as the Octadecyl group are most common in it.) The second type of stationary phase used is organic polymer beads.

Thus, the mobile phases enable the passage of the polar liquid through the stationary non-polar phase. In the pore space, analytes are separated, and as a stationary phase adhered to the surface of a silica particle, they are adsorbed onto a hydrophobic surface or hydrophobic functional groups. Because of this, sample compounds are divided into groups based on how hydrophobic they are, with highly polar analytes having different selectivity and eluting quickly.

1.6 Main Components Of HPLC

1.6.1 Reservoir

In RP-HPLC there are two types of solvent, organic and aqueous and these solvents are in the reservoir of the HPLC. Solvents are placed in different bottles named A and B. Usually, A contains the aqueous solvent while B contains the organic solvent.

1.6.2 Pump

Solvent moves from the reservoir to the column with the help of the Pump. The pump has great importance in maintaining this flow with the appropriate provided pressure. A certain pressure is adjusted depending on the type of solvent used.

1.6.3 Injector

Sample injection can be manual as well as automatic. Precision accuracy is the most important factor in sample injection in HPLC. During the loading, position injection is filled with the given amount of sample and in the injection position, it injects into the high-pressure column.

1.6.4 Column

Reversed-phase HPLC column is widely used and has the capability to handle a large variety of analytes. For varied analytical purposes, a large variety of HPLC columns are currently available. The following are a handful of the most frequent categories, organized by separation mechanism. Nonpolar packing is found in reversed-phase HPLC columns. They are employed with organic solvent mobile phases that are aqueous or water-miscible. Tetrahydrofuran, Acetonitrile, and methanol are the most commonly used solvents. The pH of the mobile phase and also a lot of factors influence selectivity and retention times.

1.6.5 PDA Detector:

Detectors in HPLC have the ability to convert a physical or chemical signal which can be measured in terms of concentration or any other type of identity (Scott,1996). Preferred detectors have high sensitivity as well as good reproducibility. There should not be an effect of the mobile phase on the result of the detector. It should have a fast response and be easy and reliable to use. Analytical methods such as Photodiode-Array Detection (PDA) can be used to assess the purity of an analyte or associated impurity peak eluting during an HPLC separation.

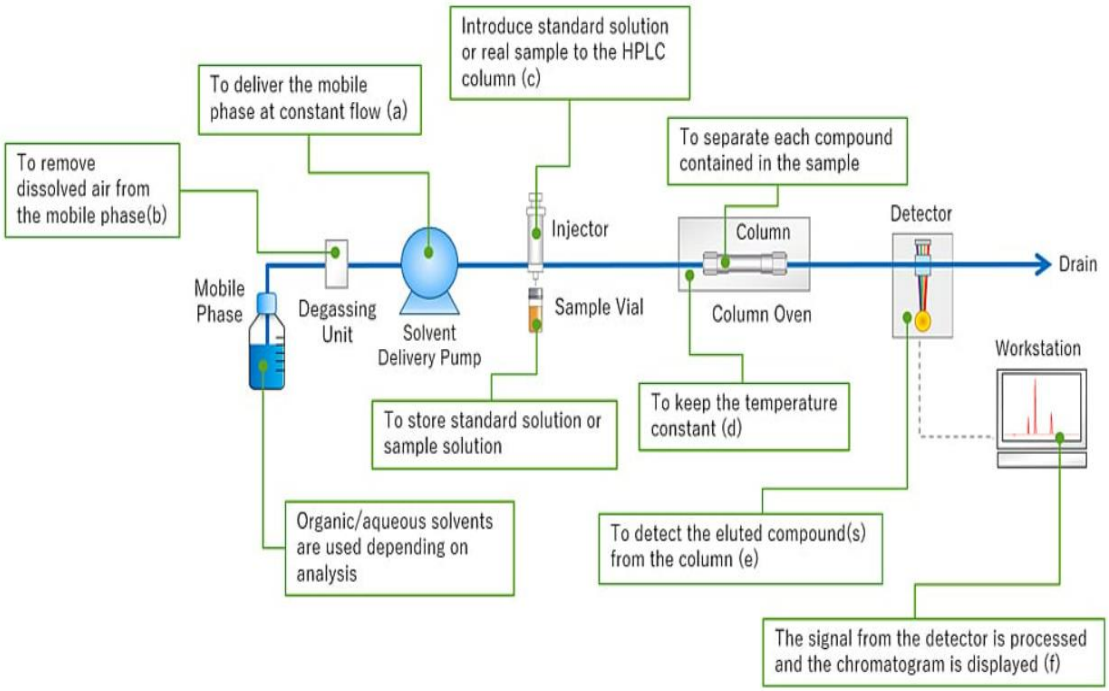


Figure 7: flow diagram of HPLC

2. Materials and Methods

This section contains materials and methods used for this research.

2.1. Chemicals and materials

Phloretin-2′-glucoside (1361 S), procyanidin B1 (0983), quercetin-3-galactoside (1027 S), procyanidin B2 (0984), cyanidin-3-galactoside chloride (0923 S) and phloretin (1360 S) were purchased from Extrasynthese (Genay, France). Methanol (HPLC grade) was obtained from J.T. Baker (Gliwice, Poland), and orto-phosphoric acid (85 % HPLC-grade) was purchased from Fluka (Buchs, Switzerland). Na_2CO_3 and $NaNO_2$ was bought from Gram mol (Zagreb, Croatia).

Pellets of sodium hydroxide (NaOH), were bought from Carlo Erba (Chaussée du Vexin, France). Reagent Folin–Ciocalteu was obtained from Kemika (Zagreb, Croatia). (+)-catechin hydrate (C1251), (-)-epicatechin (E1753), caffeic acid (C0625), quercetin dihydrate (Q0125), *p*-coumaric acid (C9008), gallic acid monohydrate (398225), rutin hydrate (quercetin-3-rutinoside hydrate; R5143), anhydrous $AlCl_3$, chlorogenic acid (C3878), (+)-naringenin (N5893), and hesperidin (H5254) were bought from Sigma-Aldrich (St. Louis, MO). Naringin hydrate (206910250) was bought from Acros Organics (ThermoFisher Scientific, Waltham, MA, USA).

2.2. Preparation of solutions

2.2.1. Preparation of polyphenol standards

Stock solutions of polyphenol standards for HPLC analyses were prepared in methanol and then used to prepare four different dilutions according to Table…. γ_1 is the mass concentration of stock solution, V_1 is the volume of stock solution, γ_2 is the mass concentration of the dilution, and V_2 is the volume of prepared dilution. calibration curves were created using dilutions.

Table 1 Preparation of polyphenol standard dilutions from stock solutions.

	First dilution				Second dilution			Third dilution			Fourth dilution		
Standards	γ_1 mgl^{-1}	V_1 µl	γ_2 mgl^{-1}	V_2 µl	V_1 µl	γ_2 mgl^{-1}	V_2 µl	V_1 µl	γ_2 mgl^{-1}	V_2 µl	V_1 µl	γ_2 mgl^{-1}	V_2 µl
Flavan-3-ols													
(+)-catechin	904.3	1	0.9	1000	10	9.0	1000	25	22.6	1000	100	90.4	1000
(-)-epicatechin	1160.0	1	1.2	1000	10	11.6	1000	25	29.0	1000	100	116.0	1000
procyanidin B1	450.0	1	0.5	1000	10	4.5	1000	25	11.3	1000	100	45.0	1000
procyanidin B2	450.0	1	0.5	1000	10	4.5	1000	25	11.3	1000	100	45.0	1000
Phenolic acids													
gallic acid	1205.3	1	1.2	1000	10	12.1	1000	25	30.1	1000	100	120.5	1000
p-coumaric acid	940.0	1	0.9	1000	10	9.6	1000	25	24.0	1000	100	96.0	1000
caffeic acid	910.8	1	0.9	1000	10	9.1	1000	25	22.8	1000	100	91.1	1000
chlorogenic acid	893.0	1	0.9	1000	10	8.9	1000	25	22.3	1000	100	89.3	1000
Dihydrochalcones													
phloretin	940.0	1	0.9	1000	10	9.4	1000	25	23.5	1000	100	64.0	1000
phloretin-2'-glucoside	940.0	1	0.9	1000	10	9.4	1000	25	23.5	1000	100	94.0	1000
Flavonols													
quercetin	910.7	1	0.9	1000	10	9.1	1000	25	22.8	1000	100	91.1	1000
quercetin-3-rutinoside	1134.5	1	1.1	1000	10	11.3	1000	25	28.4	1000	100	113.4	1000
quercetin-3-galactoside	163.3	1	0.2	1000	10	1.63	1000	25	4.1	1000	100	16.33	1000
Anthocyanins													
cyanidin-3-galactoside	161.7	1	0.2	1000	10	1.61	1000						
Flavanones													
naringin	922.0	1	0.9	1000	10	9.2	1000	25	23.5	1000	100	92.2	1000
naringenin	912.0	1	0.9	1000	10	9.1	1000	25	22.8	1000	100	91.2	1000
hesperidin	1760.0	1	1.8	1000	10	17.6	1000	25	44	1000	100	176	1000

2.2.2. Preparation of solutions for spectrophotometric methods

Reaction solutions were prepared as $NaNO_2$ solution (5%), $AlCl_3$ solution (10%), NaOH solution (1 M), Na_2CO_3 (200 g l^{-1}).

For determination of total polyphenols Gallic acid is used as standard, and prepared in 1, 10, 25, 50, 75 and 100 mg l^{-1}. (+)-catechin was used as a standard for total flavonoid determination, and prepared in 1, 10, 25, 50, 75 and 100 mg l^{-1}.

2.3. Apple and orange samples

Apples (Pink Lady, Red Delicious, and Braeburn cultivars), and sweet oranges (Navelina) were bought from the local supermarket. Around 1 kg of apples was peeled, and the core was removed. A coffee grinder was used to homogenize the peel. After homogenization, it to stored in plastic bag at − 18 ◦C. The flesh of apples was homogenized with the help of a stick blender, transferred to plastic bags, and stored at − 18 ◦C. Around 1 kg of oranges was peeled, the peel and flesh were separately homogenized with the stick blender, and stored likes samples of apple. Extraction of polyphenols from the samples was done on the second day of storage.

2.4. Preparation of extracts

2.4.1 Preparation of extracts of apples

For the preparation of extracts of each apple flesh and apple peel, around 0.2 g of homogenized samples were put in the plastic cuvettes, 1200 µl of methanol and 300 µl of distilled H_2O were added (80 % methanol), and the cuvettes were vortexed (Grant Bio, Cambridgeshire, England) and for 15 minutes put in an ultrasonic bath.

After the ultrasonic bath, cuvettes were centrifuged at10,000 rpm for 10 minutes (Eppendorf, Hamburg, Germany). Extracts were removed from residues by pipetting, and transferred into new cuvettes. The whole procedure was repeated with the remaining residue one more time using 400 µl of methanol and 100 µl H_2O (80 % methanol in water). These extracts were combined and used to measure total polyphenols and total flavonoids with spectrophotometric methods, and individual polyphenols with the HPLC method

2.4.2. Preparation of extracts of oranges

Extracts of orange peel and pulp were prepared by weighing homogenized samples in plastic cuvettes (around 0.1g of orange peel and 0.3 g of orange pulp), adding 800 µl of methanol and 200

µl of water (80 % methanol in water), and by extracting samples with the help of an ultrasonic bath (15 minutes). All the samples were then centrifuged for 5 minutes at 10,000 rpm. Extracts were transferred into new cuvettes. These extracts were used to measure total polyphenols and total flavonoids with spectrophotometric methods, and individual polyphenols with the HPLC method.

2.5. Spectrophotometric methods

2.5.1. Spectrophotometric method for total polyphenols

To prepare the reaction solution for total polyphenol determination, 2370 µl of H_2O, 30 µl of extract, 150 µl of Folin-Ciocalteu reagents, and 450 µl of Na_2CO_3 (200 g/L) were added to the glass cuvette. Reaction solution was mixed in a vortex and incubated (IN 30, Memmert, Buechenbach, Germany) for 30 minutes at 40 °C. Absorbance at 765 nm was measured on a UV/Vis spectrophotometer (UV 1280, Shimadzu, Kyoto, Japan) against a blank solution (instead of a prepared solution, blank contained 30 µL of distilled water). The same procedure was used for the measurement of different concentrations of gallic acid. These results were used to create the calibration curve of gallic acid. The results of total polyphenols were expressed in mg l^{-1} of gallic acid equivalent.

2.5.2. Spectrophotometric method for total flavonoids:

1000 µl of distilled water and 250 µl of a sample were added to the glass cuvette. To this solution at specific times, reagents were added (at time t = 0 min 75 µl $NaNO_2$ (5%), at time t = 5 min 75 µl $AlCl_3$ (10%), at the time t = 6 min 500 µl NaOH (1 M), and 600 µl of distilled water). The reaction solution developed pink color. At 510 nm absorbance was measured with spectrophotometer against a blank (distilled water was used in blank instead of sample solution). The same procedure was used for the measurement of different concentrations of (+)-catechin. These results were used to create the calibration curve of (+)-catechin, which was used to express total flavonoids of samples in mg l^{-1} of (+)-catechin equivalent.

2.6. Reversed-phase high-performance liquid chromatography

Samples from apples and oranges were analyzed with the help of the HPLC system (1260 Infinity II, assisted with a sampler, PDA detector and a quaternary pump,) (Agilent technology, Santa Clara, USA). Polyphenol characterization was done using a Poroshell 120 EC C-18 column (4.6 × 100 mm, 2.7 µm), and a Poroshell 120 EC-C18 4.6 mm guard column. During the procedure, two mobile phases named A (0.1 % H_3PO_4) and B (100 % methanol) were used.

Table 02

Time (min)	Methanol %
0	5
5	25
14	34
25	37
30	40
34	49
35	50
54	50.8
56	80
58	80
59	5
60	5

The gradient in HPLC for apples was set for a total of 60 minutes and different percentages of mobile phases. 10 µl of samples were injected and flow was set at 0.8 ml min^{-1}.

Table 03

Time (min)	Methanol(%)
0	5
3	22
33	65
35	65
37	80
39	5

The gradient in HPLC for oranges was set for a total of 37 minutes and different percentages of mobile phases.10 µl of samples were injected and flow was set at 0.8 ml min^{-1}.

Identification of polyphenols was done by spiking extracts with authentic standards bought from different companies, and by comparing UV/Vis spectra and retention times of peaks with those of authentic standards (200 to 600 nm). Calibration curves were prepared. Some polyphenols were identified tentatively (quercetin derivatives 1 and 2, quercetin-3-glucoside, quercetin-3-xyloside and quercetin-3-rhamnoside), and quantified by using calibrations curve of quercetin.

2.7. Method Validation

2.7.1. Linearity

The least-square method was used for calculating the regression line to evaluate the Linearity and sensitivity. R^2 and a linear equation were obtained.

In the linear equation y= ax+ b, output y is the absorbance, input x is the concentration (mg l^{-1}), b is the intercept, and a is the slope of the curve (sensitivity of the method).

2.7.2. Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD was calculated by using the standard deviation of the intercept (SD) and the mean slope of a calibration curve (S) according to equation (1). It was expressed in milligrams per liter.

$$LOD = 3.3\ SD\ /S \qquad (1)$$

The Limit of quantification was calculated similarly, by using the standard deviation of the intercept (SD), and the mean slope of a calibration curve (S) according to equation (2). LOQ was also expressed in milligrams per liter.

$$LOQ = 10\ SD\ /S \qquad (2)$$

2.7.3. Precision

Precision was determined by measuring different concentrations of standards two times and calculating the coefficient of variation (CV in %). CV is expressed as:

$$CV = SD/S \times 100 \qquad (3)$$

Where SD is a standard deviation of amounts (mg l^{-1}), S is a sample mean (mg l^{-1}).

2.7.4. Accuracy

Accuracy was determined by regression analysis, with confidence interval 95 %.

2.8. Statistical Analysis

Polyphenol standards were prepared in four different dilutions, each measured two times with HPLC methods (n=2). Standards used for spectrophotometric methods were prepared in six different dilutions, each measured three times (n=3). For HPLC analyses, extracts of apples (flesh and peel) were prepared in two parallels, each measured once (n=2), while extracts of oranges (flesh and peel) were prepared in three parallels, each measured two times (n=6). For spectrophotometric analyses, one extract of apples and oranges was prepared, each measured two times (n=2). Results were expressed as means ± standard deviation. Microsoft Excel was used to analyze the available data for preparing calibration curves, and for regression analysis.

3. Results

Validation of spectrophotometric methods for the determination of total polyphenols and total flavonoids

Table 04 Linearity, limit of detection (LOD) and limit of quantification (LOQ) of the method for the determination of total polyphenols and the method for total flavonoids

Compound	Equation	Linearity R^2	LOD (mg l^{-1})	LOQ (mg l^{-1})
Total polyphenols				
gallic acid	y = 0.0011x - 0.0037	0.9743	3.7	11.1
Total flavonoids				
(+)-catechin	y = 0.0021x + 0.0524	0.9868	1.9	5.9

Each concentration measured three times (n=3)

Table 05 Precision of the method for the determination of total polyphenols and the method for the determination of total flavonoids

Compound	Precision (%)	Mean precision (%)
Total polyphenols		
gallic acid (mg l^{-1})		
10	27.3	
25	2.4	
50	2.5	
75	13.6	
100	12.0	11.5
Total flavonoids		
(+)-catechin		
10	17.2	
25	11.9	
50	1.4	
75	3.8	
100	0	6.8

Each concentration measured three times (n=3)

Table 06 Analysis of the accuracy of the method for the determination of total polyphenols and the method for the determination of total flavonoids

Polyphenol	Slope	95 % confidence interval		Intercept	95 % confidence interval	
		Lower	Upper		Lower	Upper
Total polyphenols						
gallic acid	1.0415	0.9485	1.1345	0.0069	-5.2378	5.2515
Total flavonoids						
(+) catechin	0.9801	0.9121	1.0481	0.0201	-3.6386	3.6789

Determination of total polyphenols and total flavonoids in apples and oranges

Table 07 The amount of total polyphenols and total flavonoids in apple flesh and peel and in orange pulp and peel

Fruit	Total polyphenols (mg kg^{-1})	Total flavonoids (mg kg^{-1})
Apple flesh		
Pink Lady	217.6 ± 0.0	83.1 ± 45.7
Red Delicious	438.1 ± 38.2	144.9 ± 38.4
Braeburn	253.6 ± 11.7	21.7 ± 2.1
Apple peel		
Pink Lady	2258.4 ± 12.1	1118.4 ± 79.2
Red Delicious	2547.8 ± 263.9	1120.7 ± 122.6
Braeburn	2528.9 ± 36.6	1045.9 ± 102.3
Orange pulp		
	1302.9 ± 6.6	126.6 ± 6.4
Orange peel		
	1412.9 ± 7.2	819.5 ± 0.0

Extracts were prepared once and measured two times (n=2)

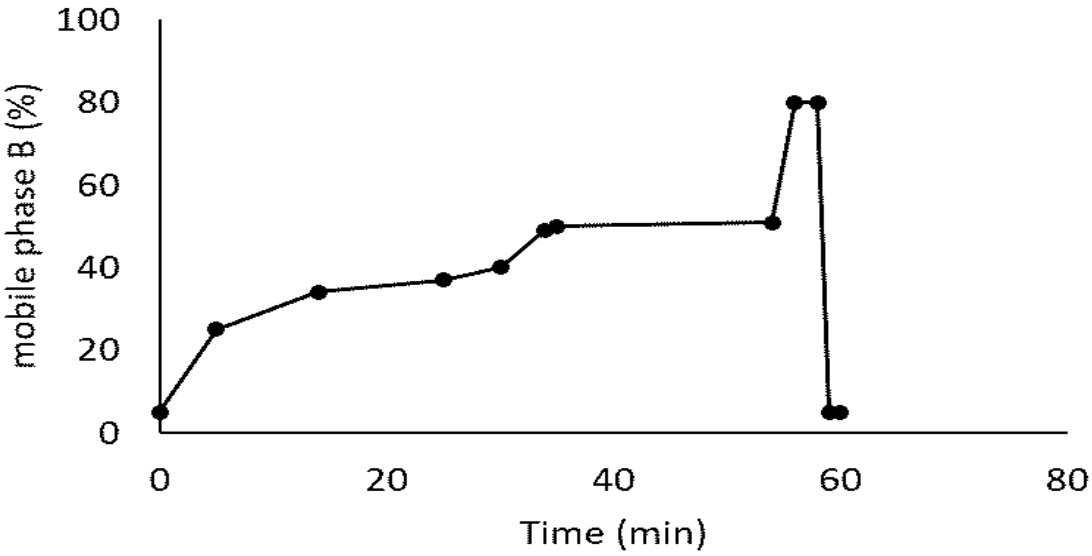
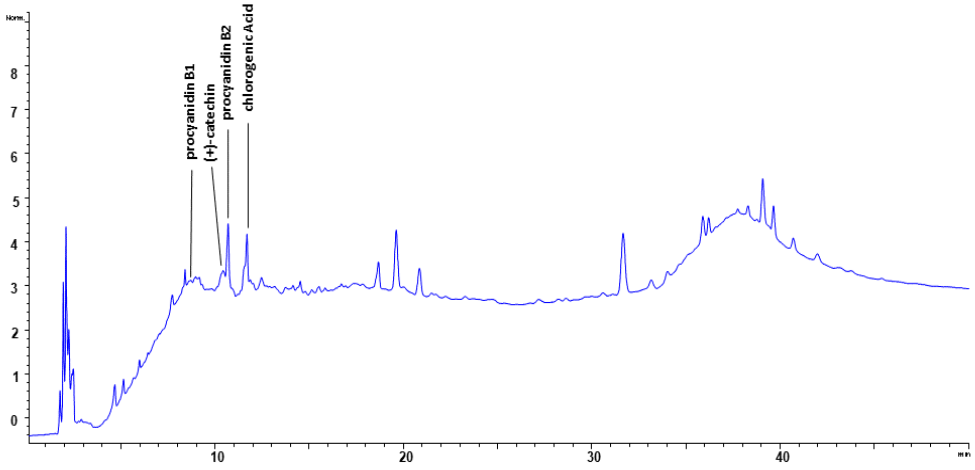
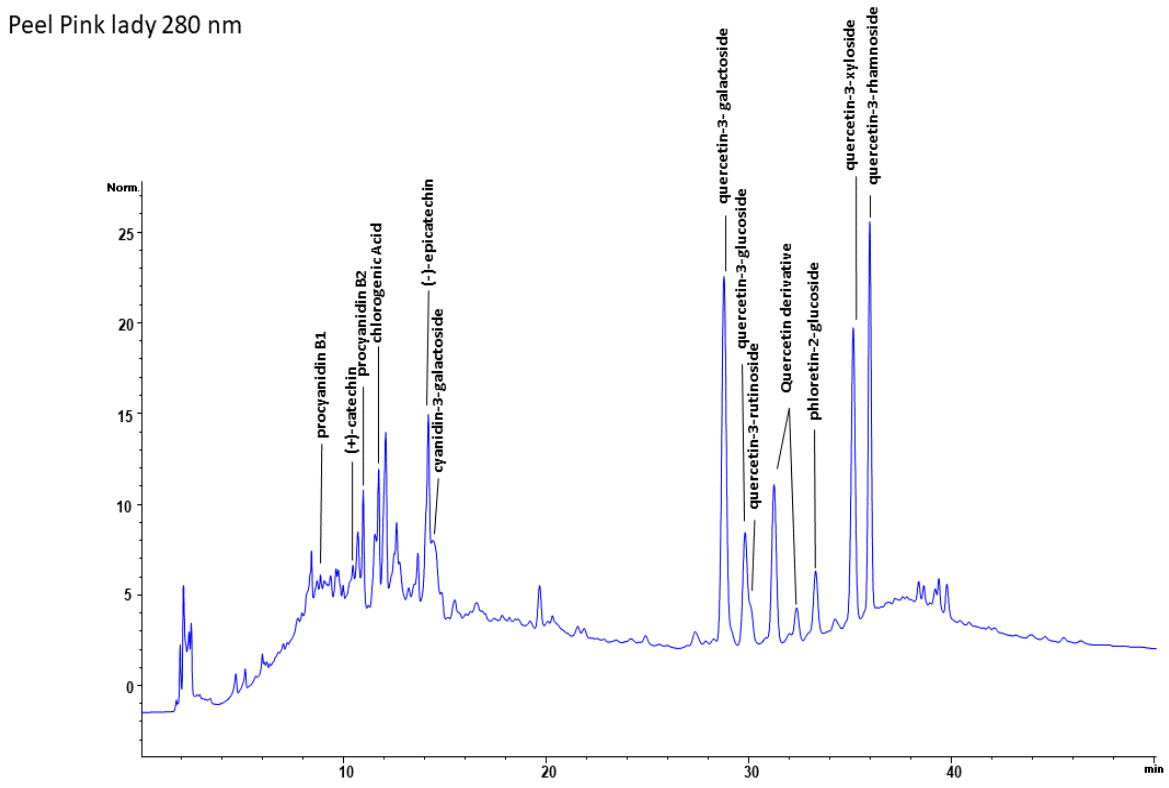


Figure 07 The relationship between time and the percentage of mobile phase B (100 % methanol) for the RP-HPLC method for the determination of polyphenols in apples



A



B

Figure 08 Chromatogram of the extract of A) apple flesh and B) apple peel scanned at 280 nm

Table Linearity, limit of detection (LOD) and limit of quantification (LOQ) of the method for the determination of polyphenols in apples

Compound	Equation	Linearity R^2	LOD (mg l^{-1})	LOQ (mg l^{-1})
Flavan-3-ols				
(+)-catechin	y = 9.543x - 2.875	0.9995	0.90	2.72
(-)-epicatechin	y = 9.4034x - 3.712	0.9997	0.16	0.50
procyanidin B1	y = 5.0977x - 0.2178	0.9992	0.11	0.32
procyanidin B2	y = 6.7554x + 1.4187	0.9995	0.06	0.19
Phenolic acids				
gallic acid	y = 51.702x + 206.5	0.9966	0.09	0.29
p-coumaric acid	y = 114.22x - 119.59	0.9984	1.17	3.56
caffeic acid	y = 143.22x - 413.28	0.9891	0.14	0.42

chlorogenic acid	y = 58.842x - 62.323	0.9998	0.17	0.51
Dihydrochalcones				
phloretin	y = 99.785x - 324.78	0.9877	0.05	0.15
phloretin-2'-glucoside	y = 36.755x - 14.645	0.9989	0.58	1.76
Flavonols				
quercetin	y = 73.049x + 171.41	0.9996	0.31	0.93
quercetin-3-rutinoside	y = 30.323x + 92.097	0.9969	0.63	1.92
quercetin-3-galactoside	y = 87.257x - 22.681	0.9999	0.01	0.02
Anthocyanins				
cyanidin-3-galactoside	y=227.14x-579.81	0.9996	0.67	2.25

Standards were prepared in four different concentrations, each measured two times (n=2)

Table Precision of RP-HPLC method for determination of polyphenols from apples

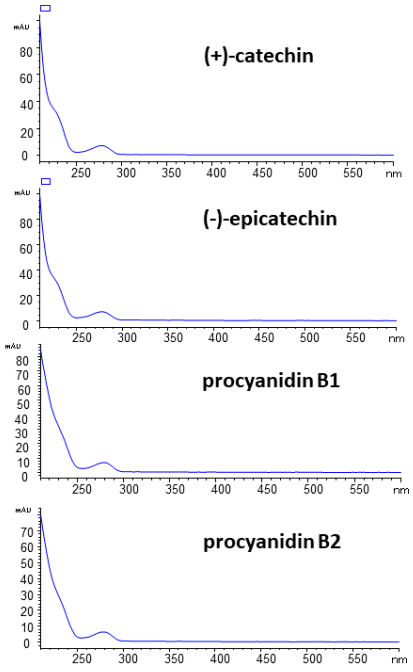
Compound	Concentration (mg l^{-1})	Precision (%)	Mean precision (%)
Flavan-3-ols			
(+)-catechin	1	3.9	
	9	0.3	
	23	4.5	
	90	0.1	2.2
(-)-epicatechin	1	4	
	12	2.9	
	29	1.6	
	116	1.5	2.5
procyanidin B1	0.5	2.2	
	5	2.2	
	11	2.7	
	45	0.3	1.9
procyanidin B2	0.5	16.8	
	5	1.1	
	11	1.1	
	45	0.4	4.9
Phenolic acids			
gallic acid	1	1.5	
	12	1.3	
	30	0.02	
	121	0.1	0.7
p-coumaric acid	1	0.4	
	10	1	
	24	0.02	
	96	3.4	1.2

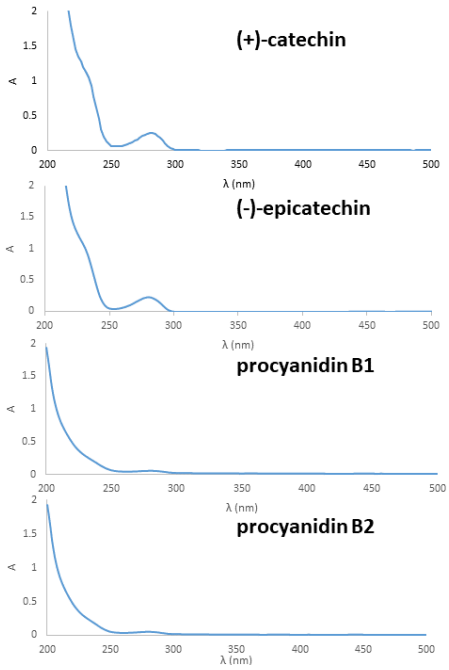
caffeic acid	1	0.3	
	9	0.6	
	23	0.01	
	91	0.6	0.4
chlorogenic acid	1	0.8	
	9	1	
	22	0.4	
	89	0.03	0.6
Dihydrochalcones			
phloretin	1	0.2	
	9	0.009	
	24	0.6	
	64	0.2	0.3
phloretin-2-glucoside	1	1.8	
	9	0.6	
	24	0.09	
	94	1.4	1
Flavonols			
quercetin	1	3.6	
	9	1.7	
	23	0.9	
	91	0.1	1.6
quercetin-3-rutinoside	1	16.7	
	11	4.2	
	28	1.7	
	113	0.01	5.7
quercetin-3-galctoside	0.3	0.6	
	2	0.6	
	4	0.5	
	16	0.6	0.6
Anthocyanins			
cyanidin-3-galactoside	24	0.3	
	48	1.8	1.1

Standards were prepared in four different concentrations, each measured two times (n=2)

Table Analysis of the accuracy of RP-HPLC method for the determination of polyphenols from apples

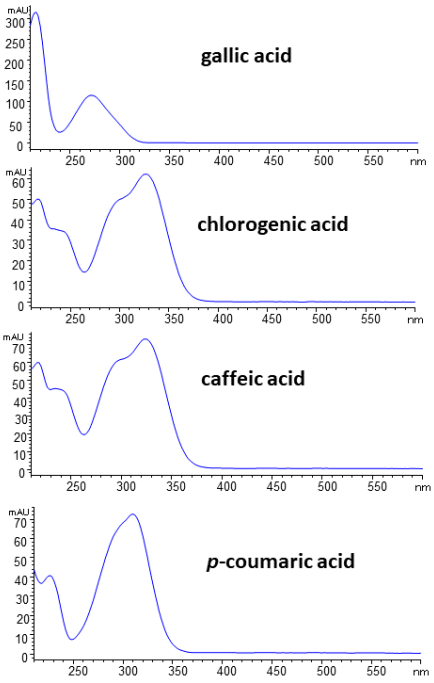
Polyphenol	Slope	95 % confidence interval		Intercept	95 % confidence interval	
		Lower	Upper		Lower	Upper
Flavan-3-ols						
(+)-catechin	1.0000	0.9767	1.0232	2.44E-06	-1.0895	1.0895
(-)-epicatechin	1.0000	0.9825	1.0174	-5.2E-06	-1.0485	1.0485
procyanidin B1	0.9999	0.9724	1.0274	-3.9E-06	-0.6408	0.6408
procyanidin B2	0.9999	0.9766	1.0233	1.78E-06	-0.5445	0.5445
Phenolic acids						
gallic acid	1.0000	0.9419	1.0580	6.36E-05	-3.6234	3.6235
p-coumaric acid	0.9999	0.9594	1.0404	-1.5E-05	-2.0125	2.0124
caffeic acid	0.9999	0.8948	1.1050	-1.5E-05	-4.9584	4.9584
chlorogenic acid	0.9999	0.9873	1.0126	-4.9E-06	-0.5859	0.5858
Dihydrochalcones						
phloretin	0.9999	0.8886	1.1113	-1.8E-05	-3.8324	3.8323
phloretin-2-glucoside	0.9999	0.9673	1.0326	6.74E-06	-1.5889	1.5889
Flavonols						
quercetin	0.9999	0.9803	1.0196	-5.5E-05	-0.9277	0.9276
quercetin-3-rutinoside	1.0000	0.9443	1.0557	7.18E-06	-3.2714	3.2714
quercetin-3-galactoside	1.0000	0.9885	1.0114	3.54E-06	-0.0964	0.0964
Anthocyanins						
cyanidin-3-galactoside	0.9660	0.9159	1.0161	1.2984	-0.0664	2.6632

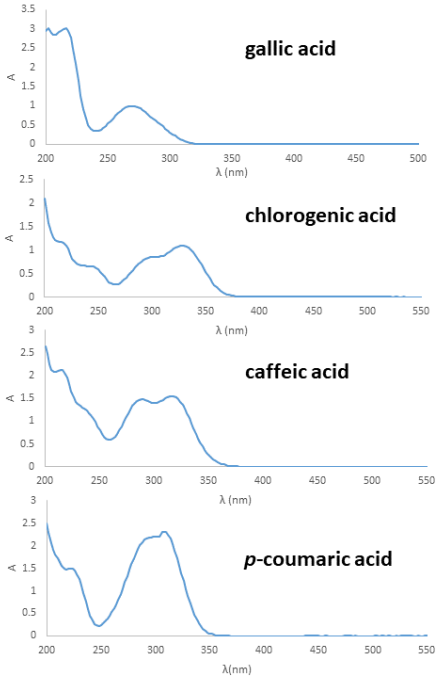




A) B)

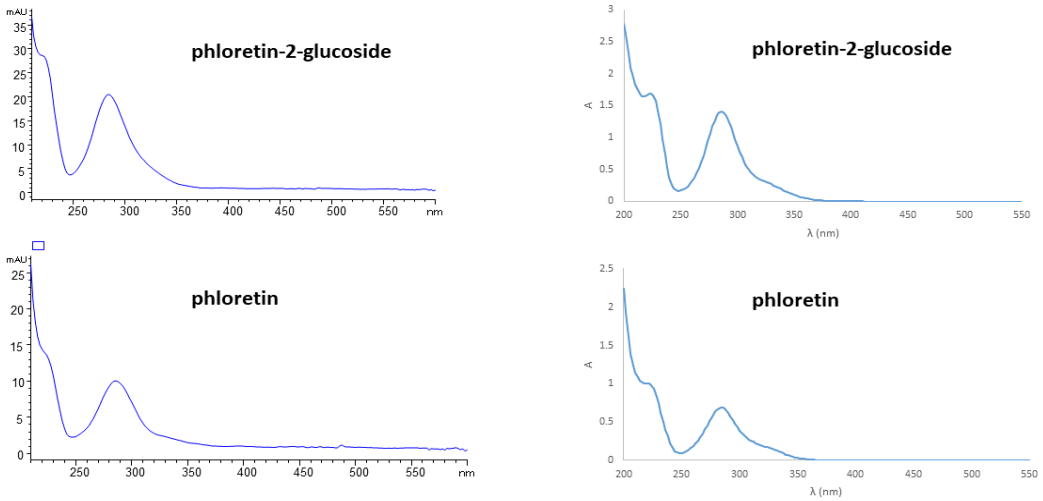
Figure 09 Spectra of flavan-3-ols scanned from 200 to 600 nm, obtained on A) HPLC with PDA detector and B) with UV/Vis spectrophotometer





A) B)

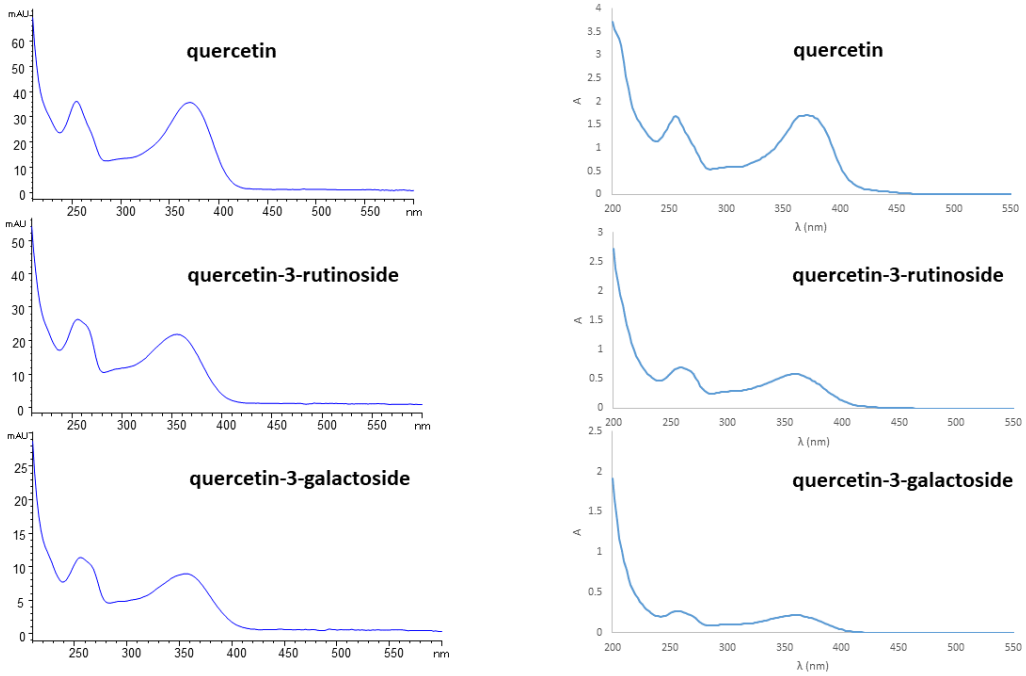
Figure 10 Spectra of phenolic acids scanned from 200 to 600 nm obtained on A) HPLC with PDA detector and B) with UV/Vis spectrophotometer



A)

B)

Figure 11 Spectra of dihydrochalcones scanned from 200 to 600 nm obtained on HPLC with PDA detector and with UV/Vis spectrophotometer



A)

B)

Figure 12 Spectra of flavonols scanned from 200 to 600 nm obtained on A) HPLC with PDA detector and B) with UV/Vis spectrophotometer

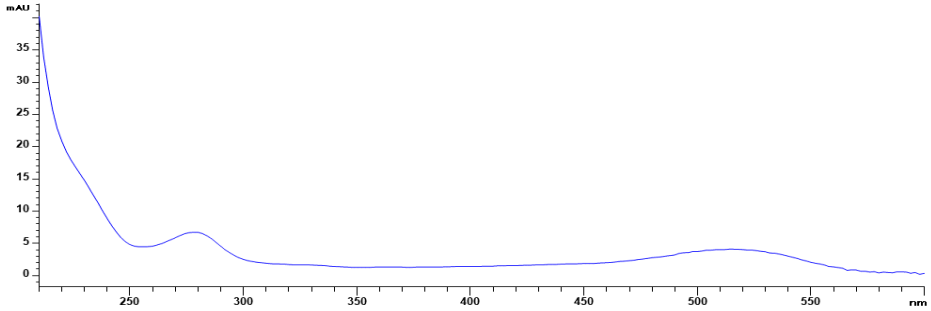


Figure 13 Spectra of anthocyanins scanned from 200 to 600 nm obtained on HPLC with PDA detector

Identification and quantification of polyphenols in apples

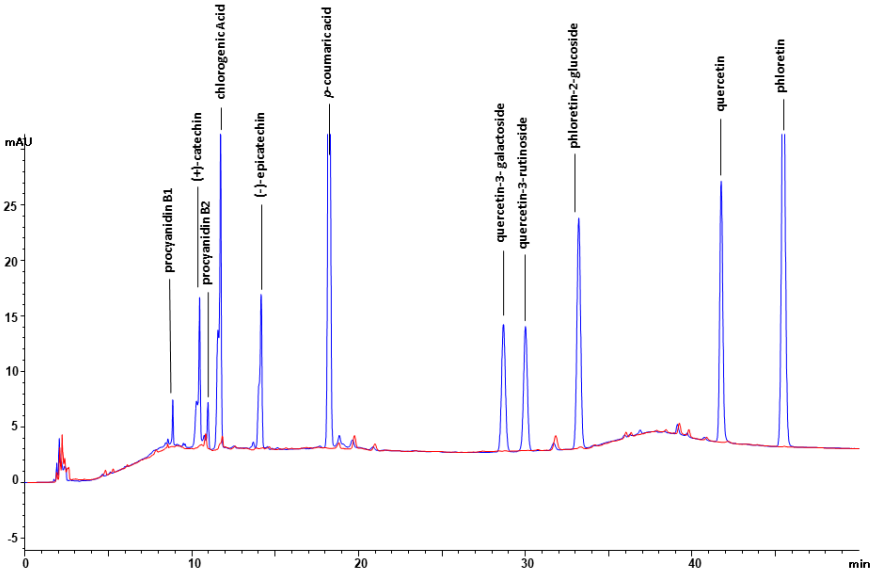


Figure 14 Chromatogram of apple flesh overlayed with a chromatogram of apple flesh spiked with polyphenol standards, both scanned at 280 nm

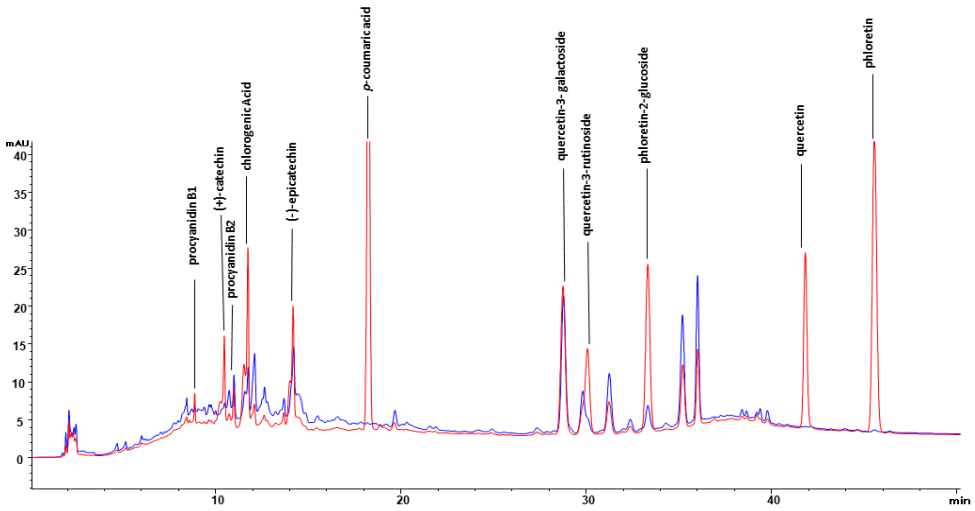


Figure 15 Chromatogram of apple peel overlayed with a chromatogram of apple peel spiked with polyphenol standards, both scanned at 280 nm

Table Amount of polyphenols in apple peel and flesh (mg kg^{-1} flesh weight)

	Pink lady		**Red delicious**		**Braeburn**	
Compound	**Flesh (mg kg^{-1})**	**Peel (mg kg^{-1})**	**Flesh (mg kg^{-1})**	**Peel (mg kg^{-1})**	**Flesh (mg kg^{-1})**	**Peel (mg kg^{-1})**
Flavan-3-ols						
procyanidin B1		5.1 ±0.7		7.9±0.7		7.6±0.5
(+)-catechin	10.3 ± 2.2	20.8 ±0.3	9.9±0.2	32.4±0.6	5.8±0.1	4.8±0.1
procyanidin B2	18.8 ± 0.7	38.0 ± 2.8	7.5±0.5	10.8±2.6	3.2±0.0	59.5±6.5
(-)-epicatechin	4.8 ± 0.2	119.4 ± 0.4	10.6±0.3	31.4±3.2	5.8±0.3	51.2±0.4
Phenolic acids						
chlorogenic acid	14.1 ± 0.1	23.5 ±0.9	22.7±0.7	26.2±1.1	15.7±0.5	17.5±0.6
Dihydrochalcones						
phloretin-2-glucoside		17.7 ±0.7		11.9±2.6		20.0±1.7
Flavonols						

quercetin-3- galactoside		63.5 ±1.2		49.6±2.4		49.9±12.6
quercetin-3-glucoside		22.8 ± 0.3		14.4±0.5		11.4±1.5
quercetin-3-rutinoside		14.3 ± 0.3				
quercetin derivative 1		31.8 ± 0.4		29.2±2.0		17.4±2.3
quercetin derivative 2		6.6 ± 0.1		4.4±0.2		2.8±0.7
quercetin-3-xyloside		49.1 ±0.6		58.4±4.3		31.8±4.9
quercetin-3-rhamnoside		46.7 ±0.6		19.4±1.0		23.9±2.0
Anthocyanins						
cyanidin-3-galactoside		24.1 ±0.5		17.2±3.2		15.2±3.3
TOTAL	48.0 ± 3.2	483.4 ±9.8	50.8±1.7	313.4±24.4	30.4±0.9	312.9±37.1

Two parallel extracts were prepared, each measured once (n=2)

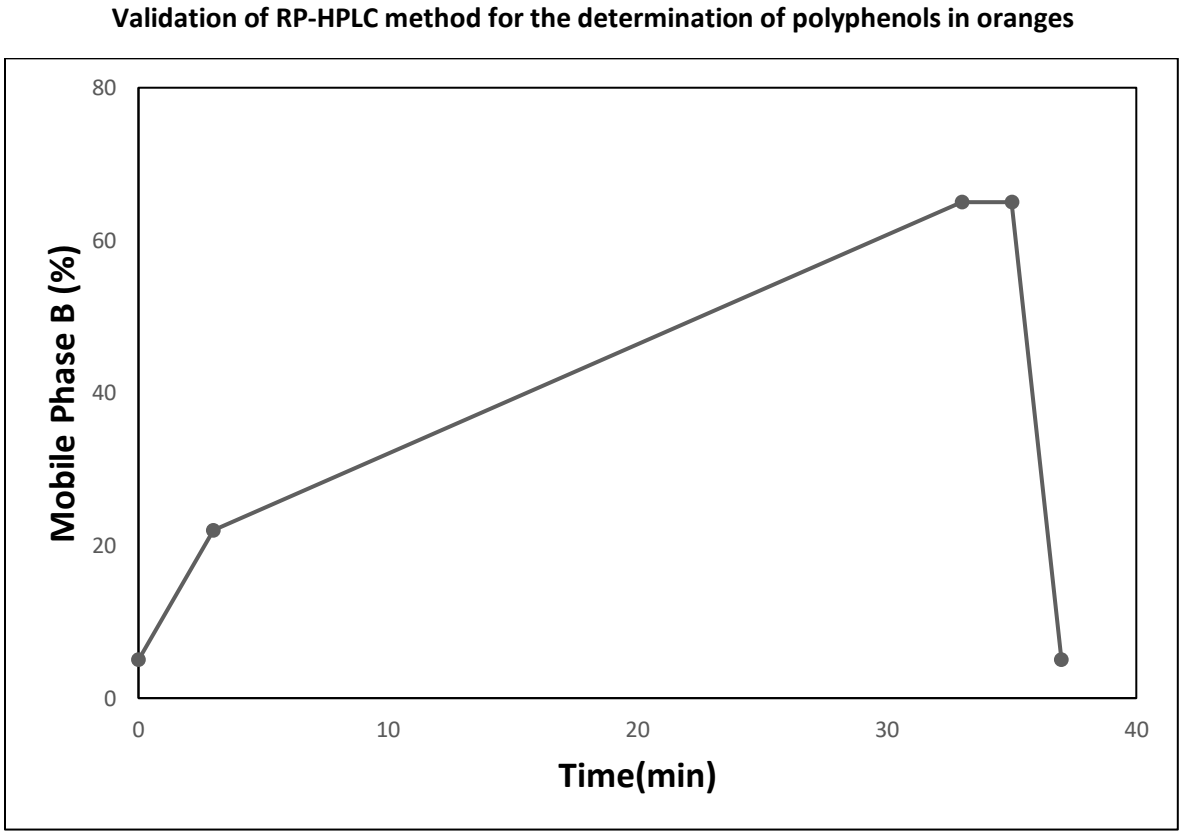
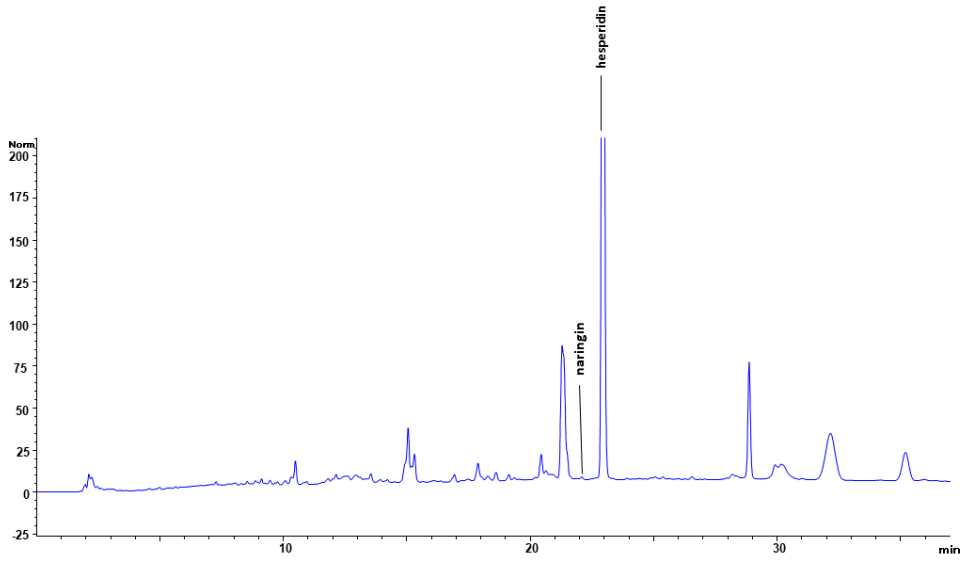
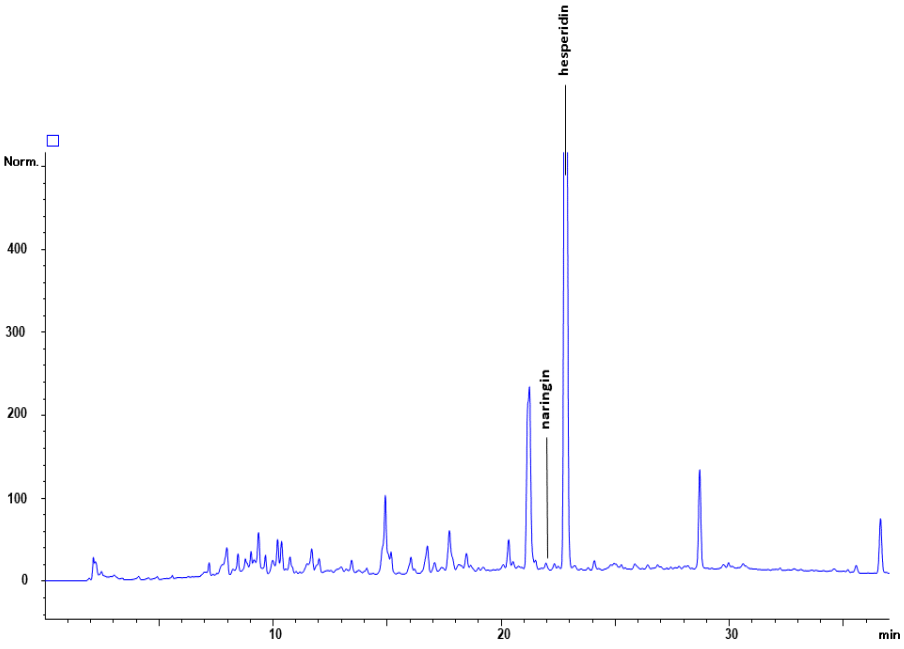


Figure 16 The relationship between time and the percentage of mobile phase B (100 % methanol) for the RP-HPLC method for the determination of polyphenols in oranges



A



B

Figure 17 Chromatogram of the extract of A) orange pulp and B) orange peel scanned at 280 nm

Table Linearity, limit of detection (LOD) and limit of quantification of the method for the determination of polyphenols in oranges

Compound	Equation	Linearity R^2	LOD (mg l^{-1})	LOQ (mg l^{-1})
Flavanones				
naringin	y =29.723x– 40.289	0.9991	0.0008	0.0025
naringenin	y = 45.681x – 47.365	0.9991	0.0002	0.0009
hesperidin	y = 22.614x – 44.056	0.9989	9.34E-05	0.0002

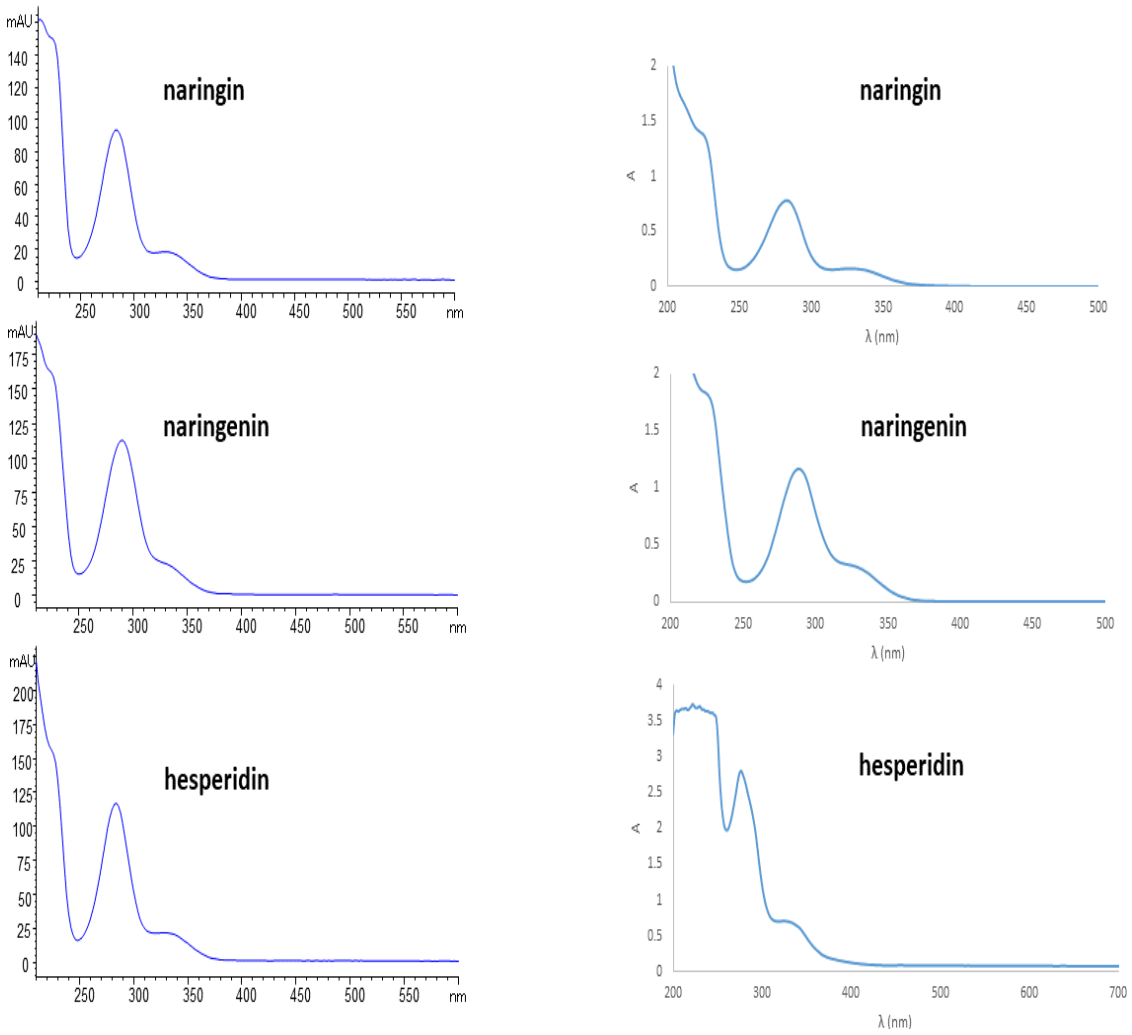
Standards were prepared in four different concentrations, each measured two times (n=2)

Table Precision and accuracy of the RP-HPLC method for the determination of polyphenols from oranges

Compound	Concentration (mg l^{-1})	Precision (%)	Mean precision (%)
Flavanones			
naringin	1	0	
	10	0.7	
	25	0	
	100	0.9	0.4
naringenin	1	8.8	
	10	1.2	
	25	0	
	100	0.8	2.7
hesperidin	1	0.3	
	10	1.2	
	25	0.9	
	100	0.01	0.6

Standards were prepared in four different concentrations, each measured two times (n=2)

Polyphenol	Slope	95 % confidence interval		Intercept	95 % confidence interval	
		Lower	Upper		Lower	Upper
Total Flavanones						
naringin	1.0001	0.9697	1.030	1.75E-05	-1.5723	1.5723
naringenin	1.0000	0.9707	1.0293	-0.0001	-1.5179	1.5176
hesperidin	1.0001	0.9674	1.0328	0.0001	-1.6931	1.6935



A) B)

Figure 18 Spectra of flavanones scanned from 200 to 600 nm, obtained on A) HPLC with PDA detector and B) with UV/Vis spectrophotometer

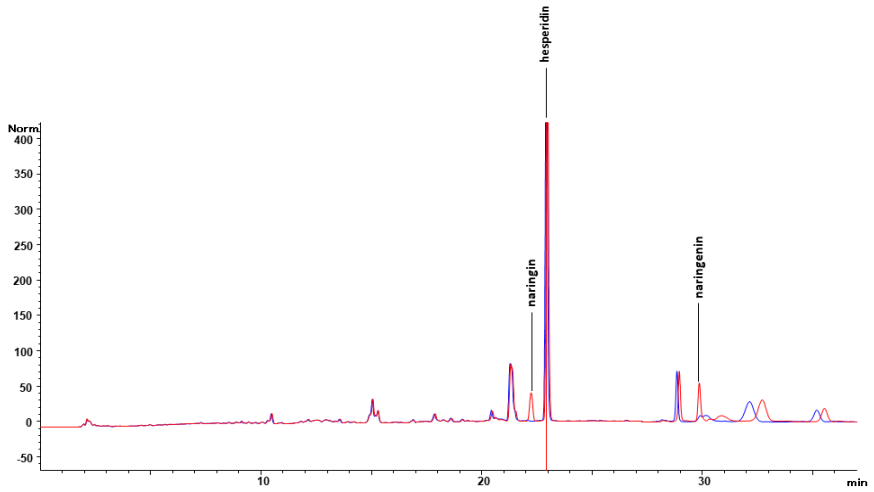


Figure 19 Chromatogram of orange pulp overlayed with a chromatogram of orange pulp spiked with polyphenol standards, both scanned at 280 nm

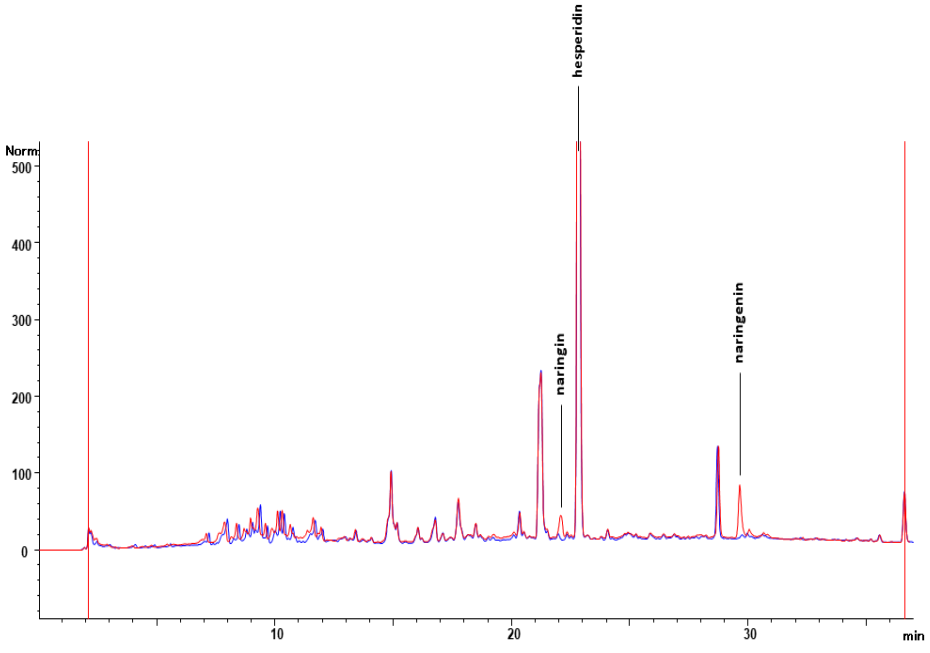


Figure 20 Chromatogram of orange peel overlayed with a chromatogram of orange peel spiked with polyphenol standards, both scanned at 280 nm

Table Amount of polyphenols in orange pulp and peel (mg kg^{-1} flesh weight)

Compound	Flesh ($mg\ kg^{-1}$)	Peel ($mg\ kg^{-1}$)
Flavanones		
naringin	9.1 ± 0.1	30.8 ± 0.6
naringenin		
hesperidin	2179.0 ± 390.4	6441.8 ± 604.9

Three parallel extracts were prepared, each measured two times (n=6)

Discussion:

The Influence of the Extraction Solvent

Different percentages of methanol with water were used as a solvent in ultrasonic bath in for 15 minutes for polyphenol extraction from apples and orange's peel and flesh samples. Total polyphenols and total flavonoids were measured using Folin-Ciocalteau method and spectrophotometric method. With increasing the extraction time the total polyphenols and total flavonoids increases. Different extraction times were tested but a total 15 minutes are enough for extraction using ultrasonic bath.

When methanolic solvents were examined for the peel (Table 07), larger amounts of total polyphenols in (Pink Lady 2258.4 **mg kg^{-1}** , Red Delicious 2547.8 **mg kg^{-1}** , Braeburn 2528.9**mg kg^{-1}** and in orange peel 1412.9 **mg kg^{-1}**) obtained compared to the amount of polyphenols in Flesh portion of apples and oranges as (Pink Lady 217.6 **mg kg^{-1}** , Red Delicious 438.1 **mg kg^{-1}** , Braeburn 253.6 **mg kg^{-1}** and in orange pulp 1302.9 **mg kg^{-1}**).

In case of total flavonoids, the same trend can be seen as larger amount of total flavonoids obtained (Pink Lady 1118.4 **mg kg^{-1}** , Red Delicious 1120.7 **mg kg^{-1}** , Braeburn 2528.9 **mg kg^{-1}** and in orange peel 819.5 **mg kg^{-1}**) compared to total flavonoids in flesh portion of apples and oranges (Pink Lady 83.1 **mg kg^{-1}** , Red Delicious 144.9 **mg kg^{-1}** , Braeburn 21.7 **mg kg^{-1}** and in orange pulp 126.6.9 **mg kg^{-1}**). Higher concentrations of methanol, such as 80 or 100%, were demonstrated to be superior to solvents containing a higher proportion of water, such as 40% methanol. This is because methanol reduces the activity of the enzyme polyphenol oxidase.

Fruit Weight to Solvent Ratio

The ratio is crucial since it determines how much polyphenolic content will be extracted. In other words, too much solvent may isolate the bulk of polyphenolic compounds, but it may also dilute the extract, necessitating an additional concentration step and extra time. An inadequate amount of solvent might result in insufficient extraction. 0.2g of peel and flesh of apple sample and 0.1g of orange peel and 0.3g of orange flesh sample were extracted for 15 minutes in an ultrasonic bath with 5 ml of 80% methanol. Total polyphenols and total flavonoids in extracts were determined using the Folin-Ciocalteau technique and Spectrophotometric method.

The findings demonstrated that when the peel/solvent ratio increased, total polyphenols and total flavanoids dropped. Due to a reduced surface area of contact between the extracted material and solvent, it is typical to predict a lesser number of extracted compounds with a larger amount of apple material per milliliter of solvent. The majority of polyphenols were removed during the first extraction step.

At least two extraction processes must be carried out in order to obtain all polyphenols. Furthermore, because the fresh material's weight, which yielded the greatest quantity of polyphenols, it could be advised to pick a heavier weight (0.2 g) than a lighter weight (0.1 g) and repeat the extraction process two times. The kind of apple and the quantity of polyphenols both

influence how many extraction processes are necessary. Finally, extracting polyphenols from apple material for 15 minutes in an ultrasonic bath would be the ideal circumstances for doing so.

For the flesh, 60 to 80% methanol is a good extraction solvent to employ, and for the peel, 80% methanol. To complete the polyphenol extraction, the residue should be extracted twice for a total of 15 minutes in an ultrasonic bath. The analysis of polyphenols should employ a combination of extracts. A third extraction of the residue can be advised, depending on the apple type. The use of methanol/water combinations as extraction solvents is supported by the literature data. In prior research, various methanol to water ratios were shown to be the ideal solvent (Arts and Hollman 1998, Escarpa and Gonzalez 1998, Tsao, Yang et al. 2003, Veberic, Trobec et al. 2005, Khanizadeh, Tsao et al. 2008)

Additionally, methanol can lower the activity of the enzyme polyphenol oxidase, which helps to accelerate the conversion of polyphenols into brown pigments through oxidation and polymerization (Arts and Hollman 1998) Methanol can shield polyphenols and improve extraction by lowering polyphenol oxidase activity.

Analysis of Extracts with RP-HPLC-PDA

For the identification of specific polyphenols, the RP-HPLC-PDA technique was created and validated. Utilizing spectral data and the retention durations of real standards, compounds were discovered. Additionally, recognized standards were added to extracts to validate the identity. Using information from the literature, several of them have been provisionally identified, including quercetin-3-xyloside (Jakobek, García-Villalba et al. 2013)phloretin-2′-xyloglucoside (Tsao, Yang et al. 2003, Balázs, Tóth et al. 2012). In (Fig.15), identified chemicals are displayed.

In chromatograms of apples peel and flesh there can be seen peaks of chlorogenic acids,(−)-epicatechin, (+)-catechin, cyanidin-3-galactoside, flavan-3-ols, phloridzin, procyanidin B1 procyanidin B2 and Quercetin derivatives. Incase of orange peel and flesh samples peaks of naringin and hesperidin can be seen. All of these compounds were found in apple peel in earlier studies ((Lamperi, Chiuminatto et al. 2008, Balázs, Tóth et al. 2012, Jakobek, García-Villalba et al. 2013)

The components that make up the peel's content include flavonoids and anthocyanins. Earlier investigations have revealed several (iso)rhamnetin and kaempferol derivatives (Alonso-Salces, Ndjoko et al. 2004) as well as galactoside, glucoside, xyloside, arabonoside, and rhamnoside derivatives of quercetin (van der Sluis, Dekker et al. 2001, Khanizadeh, Tsao et al. 2008) derivatives of cyanidinand Galactoside of cyanidin (van der Sluis, Dekker et al. 2001, Khanizadeh, Tsao et al. 2008) are mentioned as anthocyanins in the peel . Phloridzin and phloretin-2′-xyloglucoside, which are dihydrochalchones, chlorogenic acid, and pcoumaroylquinic acid, which are hydrocinnamic acids, (+)-catechin, ()-epicatechin.

Compounds identified during this study in previous studies of recent years. (Napolitano, Cascone et al. 2004, Iacopini, Camangi et al. 2010, Jakobek, García-Villalba et al. 2013). In past studies the amount of flavonols in apples flesh is reported to be low (Lamperi, Chiuminatto et al. 2008,

Iacopini, Camangi et al. 2010, Jakobek, García-Villalba et al. 2013) although quercetin-3-galactoside, quercetin-3-xyloside and quercetin-3-glucoside, quercetin-3-arabinoside, were also found(Lamperi, Chiuminatto et al. 2008) as can be seen in this study.

The Folin Ciocalteau method can not only detect polyphenols but also some other compounds including different amino acids and sugars. While RP-HPLC gives far better results when we need to detect individual polyphenols, in the case of Folin Ciocalteau method in order to get better results, we need to remove all interferences and clean the extracts before measuring. As during this study extracts were not clear as they should be to get better results, the amount of polyphenols is overestimated as it includes other (interfering) compounds also. For this reason,in case of HPLC as mentioned in results the amount of polyphenols results to be lower.

Conclusion:

For extraction of polyphenols from apples and orange peel and flesh samples a different number of methods used with the help of ultra-sonic bath different percentages of mixture of methanol and water used to get the best extractions. In addition to this, acidified methanol was used as a solvent for extraction of polyphenols.

Folin-Ciocalteau and spectrophotometry methods were used to check total polyphenols and total flavonoids. Results obtaine from the Folin-Ciocalteau method are higher than individual polyphenols results in RP-HPLC-PDA because apart from polyphenols Folin-Ciocalteau detects other sugars and amino acids.

Upon using different concentration of methanol with water, 80% methanol gave the best value for extraction of dihydrochalcones, flavan-3-ols and anthocyanins, from samples of apple peel. Obtained results show that acidified methanol can also be more helpful because anthocyanins are more stable in acidified environment and with high diversity of Flavonols. A solution of 80% methanol with water was used as solvent to get the best possible extractions of flavan-3-ols , dihydrochalcones and hydroxycinnamic acid. Also in the case of flavanones extraction from orange peel and orange flesh sample, 80%methanol can be used as extraction solvent for analyzing naringin, naringenin and hesperidin.

Spectra from the spectrophotometer were obtained to analyze the polyphenols presence in apple and oranges sample. Individual polyphenols from apples and oranges samples were analyzed by spiking with authentic standards in RP-HPLC-PDA. Several compounds were identified as in previous studies but the amount of polyphenols was low, suggesting that apple and orange varieties used in this study contain little amounts of these compounds.

References:

Alonso-Salces, R. M., et al. (2004). "On-line characterisation of apple polyphenols by liquid chromatography coupled with mass spectrometry and ultraviolet absorbance detection." Journal of chromatography A **1046**(1-2): 89-100.

Andrikopoulos, N. K., et al. (2002). "Inhibitory activity of minor polyphenolic and nonpolyphenolic constituents of olive oil against in vitro low-density lipoprotein oxidation." Journal of medicinal food **5**(1): 1-7.

Aprikian, O., et al. (2002). "Lyophilized apple counteracts the development of hypercholesterolemia, oxidative stress, and renal dysfunction in obese Zucker rats." The Journal of nutrition **132**(7): 1969-1976.

Arts, I. C. and P. C. Hollman (1998). "Optimization of a quantitative method for the determination of catechins in fruits and legumes." Journal of Agricultural and Food Chemistry **46**(12): 5156-5162.

Balázs, A., et al. (2012). "Investigation of dietary important components in selected red fleshed apples by GC–MS and LC–MS." Fitoterapia **83**(8): 1356-1363.

Barbosa, A. C. L., et al. (2010). "Varietal influences on antihyperglycemia properties of freshly harvested apples using in vitro assay models." Journal of medicinal food **13**(6): 1313-1323.

Burcelin, R., et al. (1998). "In vivo and in vitro regulation of hepatic glucagon receptor mRNA concentration by glucose metabolism." Journal of Biological Chemistry **273**(14): 8088-8093.

Chen, D.-M., et al. (2006). "Inhibitory effects of procyanidin B2 dimer on lipid-laden macrophage formation." Journal of cardiovascular Pharmacology **48**(2): 54-70.

Chen, H., et al. (2002). "Dietary patterns and adenocarcinoma of the esophagus and distal stomach." The American journal of clinical nutrition **75**(1): 137-144.

Ellingsen, I., et al. (2008). "Consumption of fruit and berries is inversely associated with carotid atherosclerosis in elderly men." British Journal of Nutrition **99**(3): 674-681.

Escarpa, A. and M. Gonzalez (1998). "High-performance liquid chromatography with diode-array detection for the determination of phenolic compounds in peel and pulp from different apple varieties." Journal of chromatography A **823**(1-2): 331-337.

Fortes, C., et al. (2000). "Diet and overall survival in a cohort of very elderly people." Epidemiology: 440-445.

Gerhauser, C. (2008). "Cancer chemopreventive potential of apples, apple juice, and apple components." Planta medica **74**(13): 1608-1624.

Guo, H., et al. (2012). "Cyanidin 3-glucoside attenuates obesity-associated insulin resistance and hepatic steatosis in high-fat diet-fed and db/db mice via the transcription factor FoxO1." The Journal of nutritional biochemistry **23**(4): 349-360.

He, X. and R. H. Liu (2008). "Phytochemicals of apple peels: isolation, structure elucidation, and their antiproliferative and antioxidant activities." Journal of Agricultural and Food Chemistry **56**(21): 9905-9910.

Hyson, D. A. (2011). "A comprehensive review of apples and apple components and their relationship to human health." Advances in nutrition **2**(5): 408-420.

Iacopini, P., et al. (2010). "Antiradical potential of ancient Italian apple varieties of Malus× domestica Borkh. in a peroxynitrite-induced oxidative process." Journal of Food Composition and analysis **23**(6): 518-524.

Jakobek, L., et al. (2013). "Polyphenolic characterisation of old local apple varieties from Southeastern European region." Journal of Food Composition and analysis **31**(2): 199-211.

Khanizadeh, S., et al. (2008). "Polyphenol composition and total antioxidant capacity of selected apple genotypes for processing." Journal of Food Composition and analysis **21**(5): 396-401.

Kim, K., et al. (2016). "Greater flavonoid intake is associated with improved CVD risk factors in US adults." British Journal of Nutrition **115**(8): 1481-1488.

Klinder, A., et al. (2016). "Impact of increasing fruit and vegetables and flavonoid intake on the human gut microbiota." Food & function **7**(4): 1788-1796.

Kurowska, E. M., et al. (2000). "HDL-cholesterol-raising effect of orange juice in subjects with hypercholesterolemia." The American journal of clinical nutrition **72**(5): 1095-1100.

Lamperi, L., et al. (2008). "Polyphenol levels and free radical scavenging activities of four apple cultivars from integrated and organic farming in different Italian areas." Journal of Agricultural and Food Chemistry **56**(15): 6536-6546.

Leontowicz, H., et al. (2007). "Apple peels and pulp as a source of bioactive compounds and their influence digestibility and lipid profile in normal and atherogenic rats-in English." Medycyna weterynaryjna **63**: 1434.

Levi, F., et al. (1999). "Food groups and colorectal cancer risk." British journal of cancer **79**(7): 1283-1287.

Levi, F., et al. (2000). "Food groups and oesophageal cancer risk in Vaud, Switzerland." European Journal of Cancer Prevention: 257-263.

Manach, C., et al. (2004). "Polyphenols: food sources and bioavailability." The American journal of clinical nutrition **79**(5): 727-747.

McCullough, M. L., et al. (2001). "A prospective study of diet and stomach cancer mortality in United States men and women." Cancer Epidemiology Biomarkers & Prevention **10**(11): 1201-1205.

Napolitano, A., et al. (2004). "Influence of variety and storage on the polyphenol composition of apple flesh." Journal of Agricultural and Food Chemistry **52**(21): 6526-6531.

Palli, D., et al. (2001). "Red meat, family history, and increased risk of gastric cancer with microsatellite instability." Cancer research **61**(14): 5415-5419.

Pearson, D. A., et al. (1999). "Apple juice inhibits human low density lipoprotein oxidation." Life Sciences **64**(21): 1913-1920.

Purushotham, A., et al. (2009). "The citrus fruit flavonoid naringenin suppresses hepatic glucose production from Fao hepatoma cells." Molecular nutrition & food research **53**(2): 300-307.

Srinivasan, V. S. (2005). Bioavailability of Ingredients in Dietary Supplements: A Practical Approach to the In Vitro Demonstration of the Availability of Ingredients in Dietary Supplements. Pharmaceutical Dissolution Testing, CRC Press**:** 425-438.

Tsao, R., et al. (2003). "Polyphenolic profiles in eight apple cultivars using high-performance liquid chromatography (HPLC)." Journal of Agricultural and Food Chemistry **51**(21): 6347-6353.

van der Sluis, A. A., et al. (2001). "Activity and concentration of polyphenolic antioxidants in apple: effect of cultivar, harvest year, and storage conditions." Journal of Agricultural and Food Chemistry **49**(8): 3606-3613.

Veberic, R., et al. (2005). "Phenolic compounds in some apple (Malus domestica Borkh) cultivars of organic and integrated production." Journal of the Science of Food and Agriculture **85**(10): 1687-1694.

Wojdyło, A., et al. (2008). "Polyphenolic compounds and antioxidant activity of new and old apple varieties." Journal of Agricultural and Food Chemistry **56**(15): 6520-6530.

Zhang, H., et al. (2006). "New insight into the structures and formation of anthocyanic vacuolar inclusions in flower petals." BMC Plant Biology **6**(1): 1-14.