Hochschule Neubrandenburg
University of Applied Sciences

In Cooperation with the Swedish University of Agricultural Sciences (Alnarp)

# Bachelor Thesis

# The Fate and Behaviour of Cinnamon Acid Derivatives in Yeast based Food Processing


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**Abstract**

A method for identifying and quantifying cinnamic acid and its derivatives in four commercial fruit juices (blueberry juice, cranberry juice, apple juice, sea buckthorn berry juice) was developed. Not only the pure fruit juices were analyzed, but also the effect of fermentation by *Saccharomyces cerevisiae* on the content of hydroxycinnamic aids. Seven organic acids (cinnamic acid, coumaric acid, chlorogenic acid, sinapic acid, rosmarinic acid, ferulic acid, caffeic acid) were determined. Therefore unconcentrated and concentrated samples were used. The analysis of the phenolic compounds was carried out by reversed-phase high-performance liquid chromatography (RP-HPLC) coupled to an diode array detector (DAD). The separation was performed on a C18 column within 30 min using a gradient system with water (pH3) and methanol as eluting solvents. Using the flow rate at 1 ml/min, the column temperature at 35 °C and the detection wavelength at 280 nm, the HPLC analyzing was performed. The quatification was performed by comparison of peak areas with external standards. Chlorogenic acid and rosmarinic acid were the most abundant phenolic compounds, especially found in blueberry juice.

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## 1 Introduction

An increased scientific interest in health promoting nutrition can be observed during the last 10 years (KROON AND WILLIAMSON 2005). In addition to low sugar content, presence of unsaturated fatty acids and dietary fibers (DEUTSCHE GESELLSCHAFT FÜR ERNÄHRUNG 2010), less known substances are nowadays considered to improve food quality by being capable to prevent age-related diseases including cancer and cardiovascular diseases (KROON AND WILLIAMSON 2005).

First and foremost, more attention is paid to the value of secondary plant metabolites, especially polyphenols. The effect of these phenolic compounds to prevent diseases or affect peoples´ physical conditions in a positive way, is documented in several studies. As antioxidants, they help to protect us against environmental stresses. Since we have to deal with smoke, oxygen, toxins and also sunlight, polyphenols are a essential part of our life (KROON AND WILLIAMSON 2005).

According to HASNA (2009) these features make polyphenols a potentially interesting material for the development of functional foods or possible therapy for the prevention of some diseases.

In foods, phenolic compounds can predominantly be found in fruits and beverages like tea, red wine and coffee, but also vegetables and cereals are good sources (HASNA 2009). In plants, these aromatic compounds contribute for growth, pigmentation, reproduction and protection against pathogens (ZERN AND FERNANDEZ 2005). But especially for fruits, phenolic compounds are of importance for the taste, color and nutritional properties, in food quality (HASNA 2009).

In food processing phenolics are often subjected to fermentation processes, i.e. wine made of juices, sauerkraut made of cabbage and also wheat or barley used for beer. A common food process is the fermentation by yeasts. Especially in the sector of the beverage industry yeast-fermentation is of particular importance. In this combination it could be of significance to know, if the content of phenolic compounds is changing in foodstuffs during yeast-fermentation. Therefore the focus of this study is to contemplate phenolic acids and derivatives as precursors of polyphenolics. Instead of hydrxoybenzoic acids, the hydroxycinnamic acids, which were chosen for this work, are more common (HASNA 2009). Dependent on environmental and technologic factors the qualities and concentrations of food compounds can change (HASNA 2009, SWIEGERS 2005). Under certain conditions it might be possible that the cinnamic acid derivatives can be modified in a way o improve food quality.

Here, we are following the presence of selected polyphenolic compounds in a synthetic mineral medium and fruit juices during the process of yeast fermentation.

The impact of fermentation on cinnamic acid and derivatives was analysed for *Saccharomyces cerevisia*, the baker´s yeast.

## 2 Literature Overview

In this part, the reader can find a detailed demonstration to the subject areas of polyphenols, fruit juices and the level of science. Next to the classification and synthesis of polyphenols, topics like yeast fermentation and the constitutional effect of phenolic compounds are specified in this point, as well.

### 2.1 Polyphenols

In the following, characteristics and behavior of phenolic compounds are listed. Not only the main structures of the cinnamic acid and derivatives, but also the enhancements to polyphenols and lignin are shown. Further, also the significance for the human health is discussed.

#### 2.1.1 Classification

Polyphenols are secondary metabolites that can be found in every plant species (ZERN AND FERNANDEZ 2005). The structure consists of an aromatic ring, carrying one or more hydroxyl groups.

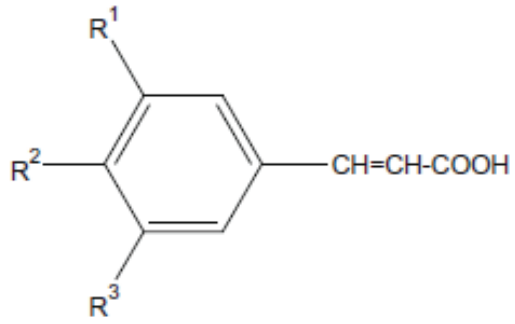



**Figure 1: Chemical structure of cinnamic acid derivatives (BARROS et al. 2009)**

Phenolic compounds can be classified in numerous categories. Some of them are the simple phenols, the phenolic carboxylic acids, the phenylpropanoids and flavan_derivatives (HEß 1999).

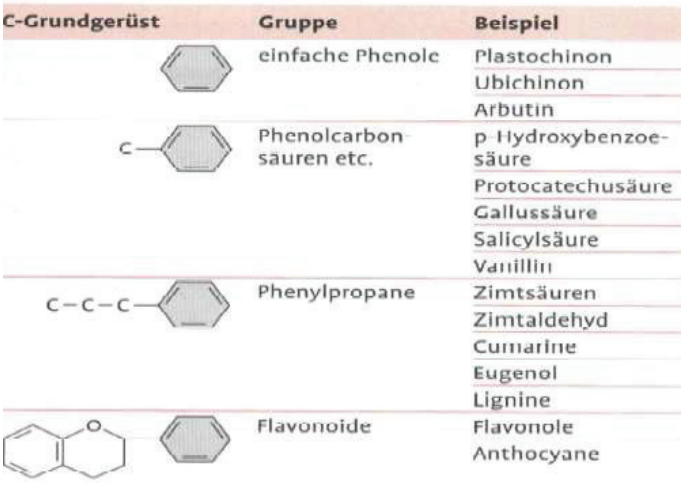



**Figure 2: Classification of some phenolic groups with german captions (HEß 2008)**

Simple phenols composed of one aromatic ring, holding one or more hydroxyl groups and furthermore methyl groups, as well. Phenolic carboxylic acids are arranged like simple phenols, with a carboxyl group as substituent. Phenylpropanoids show a side chain of 3 carbon atoms on the aromatic ring. Cinnamic acid, cinnamic alcohols, coumarins, cinnamic aldehyds and also lignins are parts of this section. The flavan derivatives consist of 2 aromatic rings, having an oxygen hetero-cycle. Depending on the oxidation status of this cycle, it is distinguished between flavanons, anthocyanidinen or flavonols (HEß 1999).

In plants, phenolic compounds guard against herbivores and pathogenic microbes. Alongside, phenols make sure that plants are structurally tightened or embody gorgeous colours (anthocyanidine). Absorption of ultraviolet radiation, or inhibition of the growth of other competing plants, are furthermore functions of the phenolic compounds (TAIZ AND ZEIGER 1998). They are essential for the growth and reproduction of plants, as well (SHAHIDI AND NACZK 1995).

In foods, phenols can commonly be found as flavonoides, phenolic acids, stilbens, coumarins, lignanes and tannins (SHAHIDI AND NACZK 1995; HASNA 2009).

### 2.1.2 Synthesis

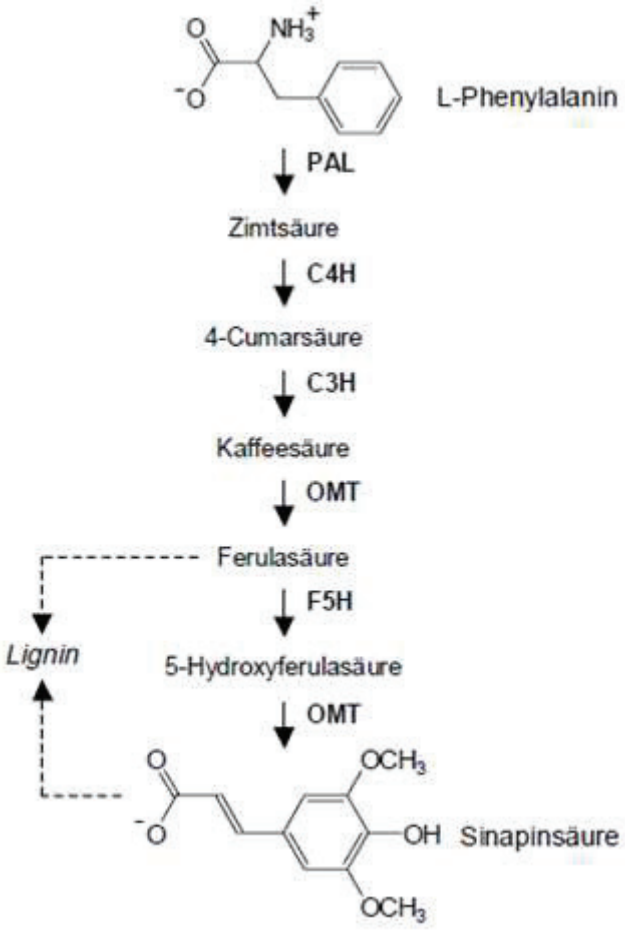
Polyphenols are mostly made by 3 different ways. These are the shikimic pathway, the acetate-malonate pathway and the acetate-mevalonate pathway. The latter do not play any role for higher plants. On the acetate-malonate pathway, the importance is due to the supply of the aromatic ring for the acculmulation of flavonoides, but not for the biosynthesis of the aromatic compounds (HEß 1999).

Here, the most important way is the shikimic pathway, which got their name from the intermediate level, the shikimic acid. Next to the delivery of polyphenols, the skikimic pathway is very significant for the allocation of the amino acids tyrosine, tryptophan and phenylalanine (HEß 1999).

The shikimic pathway begins with the substances phosphoenolpyruvat and d-erythrose-4-phosphate, which transform into 5-dehydrochina acid. The following synthesis way is followed 5-dehydroshikimic acid to shikimic acid and further to 5-phosphoreshikimi acid. Amplify one unit of phosphoenolpyruvat, chorismic acid is formed. At this point the path splits in 2 ways (greek:chorizo = to split) (HEß 1999).

One branch changes from anthranilate-synthases to anthralinic acid and leads to the aromatic amino acid tryptophan.

The second way of the chorismic acid goes over chorismate mutase to prephenic acid, which reacts with one unit of phenylpyruvat to the amino acid phenylalanine. In addition, also tyrosin was formed with p-hydroxy-phenylpyruvate (HEß 1999).




Both, phenylalanine and tyrosine are the mother substances for the formation of phenol derivatives. Because of secession of ammonia of phenylalanine, cinnamic acid is formed. Tyrosine is transformed in p-coumar acid (LUCKNER 1969).

The cinnamic acid can be seen as the mother substance for herbal phenols, because it describes the synthesis of almost all of them (HEß 1999).

**Figure 3: Synthesis of different cinnamic acids based on phenylalanine with german captions (www.bibliothek.uni-halle.de)**

With the help of hydroxylation and methylisation of the p-coumaric acid and the cinnamic acid, all other cinnamic acid derivatives are formed, like caffeic acid, sinapic acid or ferulic acid (LUCKNER 1969).

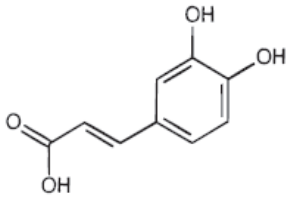



**Figure 4: Chemical structure of caffeic acid (FRIEDMAN & JÜRGENS 2000)**

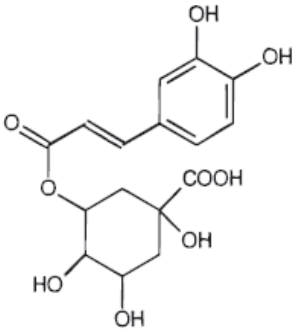



**Figure 5: Chemical structure of chlorogenic acid (FRIEDMAN & JÜRGENS 2000)**

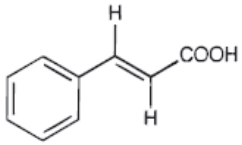



**Figure 6: Chemical structure of cinnamic acid (FRIEDMAN & JÜRGENS 2000)**

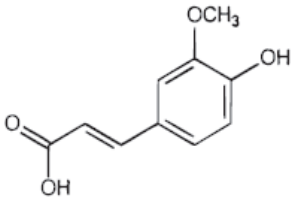



**Figure 7: Chemical structure of ferulic acid (FRIEDMAN & JÜRGENS 2000)**

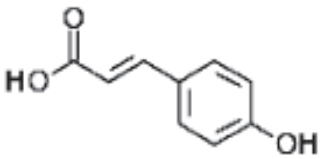



**Figure 8: Chemical structure of coumaric acid (LAFAY & GIL-IZQUIERDO 2008)**

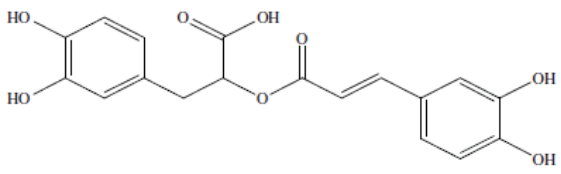



**Figure 9:Chemical structure of rosmarinic acid (WANG et al. 2004)**

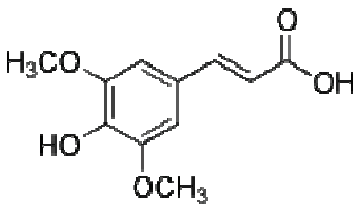



**Figure 10: Chemical structure of sinapic acid (www.wikipedia.org)**

Both together, cinnamic acid and chlorogenic acid are forming caffeic acid. Two units of caffeic acids are becoming rosmarinic acid. All these acids named above are precursors of polyphenols. The other three dehydroxycinnamics, coumaric acid, ferulic acid and sinapic acids are essential for the constitution of lignins.
Lignin is formed of 3 different phenylpropanoid alcohols - coumaryl, coniferyl and sinapyl. The alcohols are dehydrogenized to organic radicals and get polymerized to lignin (TAIZ AND ZEIGER 1998). With usage of adenosintriphosphate (ATP), the cinnamic acids are reduced to cinnamic aldehydes. Further, by another reduction formed the cinnamic alcohols (HEß 1999).
The details of the biosynthesis of flavonoids, simple phenols or phenol carbonic acid are for this work not of particular importance.

### 2.1.3 Constitutional Effect

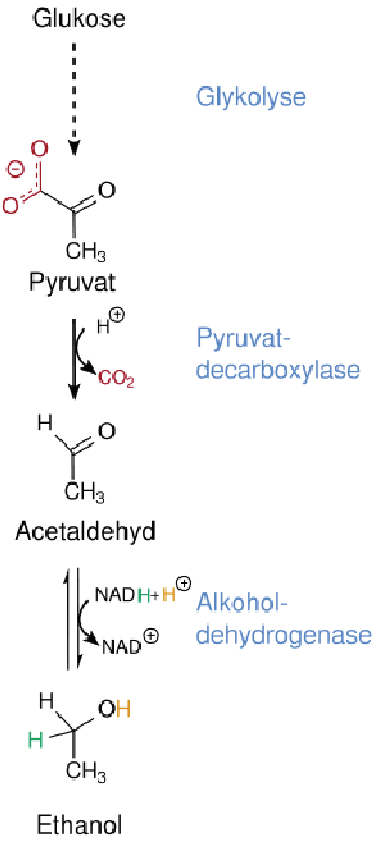
According to RODRÌGUEZ-MEDINA et al. 2009, health is the principal concern of modern society and the food habits are part of a good health.
10 years ago, little was known about bioavailability, metabolism and fate of polyphenols in humans (KROON AND WILLIAMSON 2005).
The phenolic compounds showed that they have multiple functions. They cause not only antioxidative activities, but also the ability to bind proteins, which even influences gene expression and cell signaling (KROON AND WILLIAMSON 2005; HASNA 2005).
An established example for the properties of polyphenols is the “French Paradox”. Despite intake of lots of unsaturated fatty acids, the rate of getting sicken with coronary heart diseases in France, is much lower than in other countries. (STOCLET et al. 2004; SUN 2001; ZERN AND FERNANDEZ 2005; DE LANGE 2006).
The reason considered for this low incidences is the regularly consumption of red wine. Including compounds, like resveratol. Quercetin, catechin and proanthocyanidins already got inhibitors against platelet aggregation. Still, these compounds have a protective effect against low-density lipoproteins (LDL), as well. Thereby the neuronal cell death gets attenuated, which causes of oxidized LDL (SUN 2001).
Another characteristic of polyphenols is their antioxidant activity, due to an interaction of superoxide and other reactive oxygen species, like hydroxyl and peroxy radicals (STOCLET 2004).

A last advice, which became known in the last years, is that phenolic compounds can react beneficial on inflammations, because of the significant alteration of adhesion molecules and monocyte adhesion to endothelial cells (ZERN UND FERNANDEZ 2005).

## 2.2 Yeast and Fermentation

According to FLEET 2008, grape juice underwent a natural or a spontaneous alcoholic fermentation that, almost invariable, was dominated by strains of yeast, *Saccharomyces cerevisiae*. Because of that, pure cultures of this baker´s yeast were isolated and developed as starter cultures for wine fermentations.

*S.cerevisiae* is a budding yeast, using almost simply sugars for its metabolism. Baker´s yeast is facultative anaerobic and releases carbon dioxide and ethanol during fermentation. The simple schema on the left (figure…) shows the alcoholic fermentation which was used for this work. The optimal fermentation temperature for *s.cerevisiae* is around 30 °C. Temperatures over 45 °C causes for yeasts die out.




No other yeast is of particular significance as *S.cerevisiae*. This yeast is more resisted to ethanol than non-*Saccharomyces* species which used dying off earlier, because of their sensitiveness to ethanol. There are a lot of *S.cerevisiae* strains, which are available as commercial preparations for fermentation. These strains have been selected on the basis of the following criteria. The yeasts have to be fast, vigorous and must be able to ferment the grape juice sugars to high ethanol concentrations. Further they should produce only minimal foam and sediment quickly from the wine at the end of the fermentation. Another very important criterion is that the yeast does not give sluggish, slow or stuck fermentations (FLEET 2008).

**Figure 11: Alcoholic fermentation with german captions (www.bdbe.de)**

## 2.3 Fruits and Juices

The juices are the main objects of investigation in this work. Because of that, the different species must be characterized more precise. Differences between cultivation and the content of phenolic acids are described continuative.

According to RECHNER 2000, the composition of phenolic substances in all juices is always depending on the compounds finding in the whole fruits. Also the aging of polyphenols during storing fruit juices has influences of its compounds. Because of oxidation, polymerization and condensation reactions, phenolic compounds can be catabolized (RECHNER 2000).

*Blueberry and Cranberry*

Berry fruits are characterized by a high content of polyphenols, including phenolic acids like benzoic and cinnamic acid derivatives (SZAJDEK & BOROWSKA 2008, GIOVANELLI & BURATTI 2009). The contents of all phenolic compounds are determined by several conditions, such as variety, region, cultivation, species, ripeness, weather conditions and also storage time. Berry plants, which grew in cold northern climate with a short vegetation season and without pesticides and fertilizers, showed higher content of phenolic compounds than the same ones grew in a milder climate. In that case it would be god to know the cultivars and were the berries were grown (BORGES 2010). Blueberry marked a phenolic content of 1.811 – 4.730 mg/kg. Cranberries offered values from 1.200 to 1.765 mg/kg. Phenolic acids in berries are mainly founded in bound forms as glycosides or esters. For hydroxycinamic acid derivatives, caffeic, ferulic and coumaric acid were presented. Especially caffeic acid can be found in blueberries (CLIFFORD 2000). Also high concentrations of chlorogenic acid were determined in berries, which due to the tart taste of fruit and its products. Bilberry wine was reported to contain 50 mg/l of chlorogenic acid. For cranberry and blueberry, large amounts of ferulic acid were determined (SZAJDEK 2008). GIOVANELLI & BURATTI 2009, suggested that wild blueberries contain higher levels of total phenolics (approximately 6.000 mg/kg) than cultivated ones. The cultivated blueberry “Bluecrop” offered a total phenolic amount of 2.990 mg/kg.

CLIFFORD 2000 analyzed that a long maturation of berry wine can offer up to 3 or 4 mg/l of caffeic and coumaric acid by hydrolysis.

*Apple*

According to RECHNER 2000, six categories of polyphenols can be find in apples. Amongst others, also hydroxycinnamic acid and its derivatives. Chlorogenic acid presents the highest concentration of cinnamic acid derivatives, with concentrations between 62 – 385 mg/kg (average 139 mg/kg) fresh fruit. Also ESCARPA & GONZÀLEZ 1999, reported the importance of chlorogenic acid for apples and pears. LEE 2003, described concentrations of clorogenic acid between 44,0 – 142,8 mg/kg. According to KAHLE et al. 2005, chlorogenic concentrations of 54,0 mg/l, were detected for juice made of Granny Smith apples. For Golden Delicious apples, 37,6 mg/l were achieved. Red Dilicious and Fuji apples showed amounts of 32,7 mg/l and 54,1 mg/l, respectively. Cider apples reached concentrations around 200 to 450 mg/l. The other compounds, like caffeic acid and coumaric acid, are of secondary importance. In contrast CLIFFORD 2000, recorded that apples are typical sources for caffeic acid. Also KAHLE et al. 2005, recorded 3,8 mg/l, 4,8 mg/l, 6,1 mg/l and 2,5 mg/l for Granny Smith, Golden Delicious, Red Delicious and Fuji, in each case. The total hydroxycinnamic acids amounts varied from 56,8 mg/l to 67,7 mg/l for dessert apples.

The polyphenol configuration of apple juice is different to them of the fresh fruits. The contents of phenolic acids are fluctuating. For chlorogenic acid, concentration between 2,3 – 557,4 mg/l can be reached (RECHNER 2000). The data for caffeic acid and coumaric acid showed 0,7 – 13,9 mg/l and 0,3 – 6,2 mg/l, respectively. The style of mashing the fresh fruits and the procedure of straining can abet or degrade the crossover of the polyphenols from the fruit into juice. The content of polyphenols is also contigent on the sort of apples (RECHNER 2000). CLIFFORD 1999, summarized that the heat processing of apple juice reduces the content of chlorogenic acid, but the fate of cinnamic acids was not debated.

*Sea Buckthorn Berry*

Sea buckthorn is a shrub or a tree, which extends in the temperate zones of Asia and Europe and all over the subtropical zones (HEINAAHO et al. 2009).

The berries are a good source of bioactive substances. It has high contents of fatty acids, amino acids, minerals, carotenoids and vitamins, but also amounts of phenolic compounds (HEINAAHO et al. 2009; ZADERNOWSKI et al. 2005).

ZADERNOWSKI et al. 2005 found out, that the total phenolic acids (benzoic and hydroxycinnamic acids) in the berries range from around 3570 to 4400 mg/kg. In detail, caffeic acid reached values of 6,3 -15,8 mg/kg. Depending on the species of sea buckthorn berries, 90,3 – 290,8 mg/kg for coumaric acid and 5,1 -17,8 mg/kg for ferulic acid were

achieved. According to ARIMBOOR et al. 2007, sea buckthorn berry extracts offer 10,1 – 166,8 µg/ml for cinnamic acid, 6,6 – 208,5 µg/ml for caffeic acid, 5,0 – 220,0 µg/ml for ferulic acid and 10,4 – 240,8 µg/ml for coumaric acid. Depending on the part of the berry (pulp, coat, leaves), different concentrations for these four acids can be found. In total, cinnamic acid was presented with 238 mg/kg. Coumaric acid showed 1 mg/kg, ferulic acid 175 mg/kg and caffeic acid 18 mg/kg.

## 2.4 Analysing Methods

According to KELEBEK et al., 2008, the most important phenolic acid in orange juice is hydroxycinnamic acid and its derivatives: ferulic, p-coumaric, sinapic, caffeic and chlorogenic acids. These compounds can not only be found in orange juice, but also in other juices and wine (GRUZ et al., 2008).
Fruit juices are a perfect source of phenolic compounds, easily available and thus a good possibility for studying its phenolic ingredients (RODRÌGUEZ-MEDINA et al., 2009).
The procedures for analyzing phenolic compounds can be very different.

*Solvents*
Some taken solvents of all these numerous analysis were formic acid in water and in acetonitrile (RODRÌGUEZ-MEDINA et al. 2009, KAHLE et al. 2005), orthophosphoric acid in water and in methanol (WANG et al., 2004) or acetic acid in water and in methanol (MOUSAVINEJYD et al., 2009; DE SIMÒN et al., 1992). Usually, standard peaks offer the phenomenon of tailing, because of the ability of ionizing of the phenolic hydroxyl group. According to FANG et al., 2007, adding phosphoric acid to the water for the mobile phase and keep it on pH 3, helps to separate the standard peaks more successfully. To get a good ionization of the phenolic compounds, formic acid should be added to the solvents (RODRÌGUEZ-MEDINA et al., 2009).

*Detection wavelengths*
For determination rosmarinic acid and caffeic acid in aromatic herbs, a detection wavelength at 330 nm was used (WANG et al., 2004). 320 nm were chosen by KAHLE et al. 2005, to analyze typical polyphenol profiles of apple juices.
280 nm is the wavelength used for benzenoid derivatives (CAO et al. 2009) and also for other phenolic compounds (ESCARPA & GONZÀLEZ 1999).

For determination red wine flavonoids, 360 nm were chosen (FANG et al., 2007). GRUZ et al., 2008, detected phenolic acids in beverages at 230 nm. The detection of nonflavonoid phenolic compounds in commercial juices and nectars was performes simultaneously at 280 and 340 nm (DE SIMÒN et al., 1992).

*Calibration*

For calibrating the external standards, different dilutions must be prepared and analyzed. Therefore TOLONEN & UUSITALO, 2004, took eight levels (0.04, 0.1, 0.2, 0.4, 1.0, 2.0, 4.0 and 10 µg/ml) of calibration solutions.

The calibration curve of FANG et al., 2007, was established by taking five different standard substances of flavonoids.

*Methods*

The mostly taken flow rate for analyzing phenolic compounds is 1,0 ml/min, but also 0,5 ml/min were used for analysing the phenolic fraction in organic commercial juices (RODRÌGUEZ-MEDINA et al., 2009). Therefore a small injection volume of 5 µl was established, flowing through the separating column set at 35 °C.

| Time in min | 0 | 5 | 5,5 | 6 |
|---|---|---|---|---|
| Water/formic acid (99,9:0,1) | 99 | 0 | 99 | 9 |
| Acetonitrile/formic acid (99,9:0,1) | 1 | 100 | 1 | 1 |

**Table 1: Gradient for identification of phenolic compounds (RODRÌGUEZ-MEDINA et al., 2009)**

The decuple, even 50 µl of the sample mixture was injected for identification and quantification of phenolic compounds (MOUSAVINEJYD et al., 2009). The separation was carried out on a RP-C18 column (125 x 5,0 mm x 5,0 µm) with a flow rate of 1,0 ml/min and a gradient seen in table… .

| Time in min | 0 | 5 | 15 | 45 | 55 |
|---|---|---|---|---|---|
| Water/acetic acid (97,5:2,5) | 100 | 100 | 90 | 50 | 100 |
| Methanol/acetic acid (97,5:2,) | 0 | 0 | 10 | 50 | 0 |

**Table 2: Gradient for characterization of phenolic fraction in commercial juices (MOUSAVINEJYD et al., 2009)**

GRUZ et al., 2008, developed a rapid analysis of phenolic acids in beverages (white wine, grapefruit juice and green tea) by using UPLC-MS/MS (ultra performance liquid chromatography coupled with tandem mass spectrometry). With that method, 17 phenolic acids, including chlorogenic acid, caffeic acid, coumaric acid, sinapic acid and ferulic acid, were analyzed qualitative and quantitative. With the following gradient (table…) and a flow rate of 0,25 ml/min, signals were detected from a DAD. A RP column (BEH $C_8$, 150 x 2,1 mm, 1,7 µm), which was maintained at 30 °C, was used to identify the phenolic acids.

| Time in min | 0 | 0,8 | 1,2 | 1,9 | 2,4 | 3,7 | 4,0 | 5,2 | 5,7 | 8,0 | 9,0 | 9,5 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Water 7,5 mM HCOOH in % | 95 | 90 | 90 | 85 | 85 | 79 | 79 | 73 | 50 | 0 | 0 | 95 |
| Acetonitrile in % | 5 | 10 | 10 | 15 | 15 | 21 | 21 | 27 | 50 | 100 | 100 | 5 |

**Table 3: Gradient for rapid analysis of phenolic acids in beverages (GRUZ et al., 2008)**

SEERAM et al. 2006, studied phenol fingerprint profiles of six different berry extracts (blackberry, black raspberry, blueberry, cranberry, red raspberry, strawberry) using HPLC. With a C18 column (250 x 4, mm, 5µm) and a DAD, scanning from 250 – 600 nm, the extracts were separated in 70 min. The column remained at 25 °C, the flow rate was 1 ml/min.

For a fast separation of phenolic compounds from apples and pears, ESCARPA & GONZÀLEZ 1999, used the HPLC with DAD. 20 µl of sample mixtures were injected and eluted in the column, which remained at room temperature, to separate cinnamic acids, like clorogenic, caffeic and coumaric acid.

40 µl injection volume and a constant flow rate of 1,0 ml/min were occurred to analyse red wine flavonoids on a 100RP-18e column (250 x 4,0 mm, 5 µm) guarded with a RP-18 column (10 x 4,0 mm) placed in a column oven set at 20 °C. The used proportions of the solvents, used for HPLC analyzing, are shown in table… (FANG et al., 2007).

| Time in min | 0 | 15 | 28 | 40 | 44 | 45 | 52 |
|---|---|---|---|---|---|---|---|
| Water (pH3)/19%acetonitrile/5%methanol/1%THF | 100 | 98 | 72 | 64 | 64 | 20 | 20 |
| Water (pH3)/55%acetonitrile/15%methanol | 0 | 2 | 28 | 36 | 36 | 80 | 80 |

**Table 4: Gradient for determination of red wine flavonoids by HPLC (FANG et al., 2007)**

For identifying rosmarinic acid and caffeic acid in aromatic herbs by HPLC, the solvents were pumped through a C18 column (150 x 4,6 mm), maintained at 30 °C (WANG et al., 2004). A 25 min lasting gradient (see table…) and a flow rate of 1,0 ml/min were used for analyzing a sample injection volume of 10 µl. The contents of both acids in the numerous samples can be seen in table… .

| Time in min | 0 | 10 | 15 | 25 |
|---|---|---|---|---|
| Water/o-phosphoric acid (99,9:0,1) | 60 | 50 | 40 | 40 |
| Methanol /o-phos. acid (99,9:0,1) | 40 | 50 | 60 | 60 |

**Table 5: Gradient for determination of rosmarinic acid and caffeic acid in aromatic herbs by HPLC (WANG et al., 2004)**

| Samples | RA | CA |
|---|---|---|
| Rosemary 1 | 10.0 | 0.1 |
| Rosemary 2 | 10.0 | 0.1 |
| Rosemary 3 | 11.0 | 0.2 |
| Sage 1 | 8.7 | 0.3 |
| Sage 2 | 14.1 | 0.3 |
| Sage 3 | 8.5 | 0.4 |
| Thyme 1 | 8.7 | 0.3 |
| Thyme 2 | 4.5 | 0.1 |
| Spearmint 1 | 14.3 | 0.3 |
| Spearmint 2 | 7.1 | 0.2 |
| Balm | 27.4 | 0.3 |
| Self-heal | 21.7 | 1.8 |
| Lavender | 2.0 | nd |

RA – rosmarinic acid; CA – caffeic acid; nd – not detectable.

**Table 6: Results of the determination of rosmarinic acid and caffeic acid in aromatic herbs (WANG et al., 2004)**

The determination of organic acids in orange juice and orange wine was analysed on a HPX-87H column (300 x 7,8 mm) with a set temperature at 55 °C and a flow of 0,3 m/min (KELEBEK et al., 2008). For detecting phenolic compound in the same samples, the mobile phase was flowing with 1 ml/min through a Ultrasphere ODS column (25 x 4,6 mm, 5µm), which was equipped with a precolumn (10 x 4,6 mm). Both were kept at 25 °C. The used gradient can be find in table… . Caffeic acid (≈ 5,66 mg/l), chlorogenic acid (≈ 8,49 mg/l), coumaric acid (≈ 3,52 mg/l), ferulic acid (≈24,06 mg/l) and sinapic acid (≈ 18,65 mg/l) were detected with last mentioned method.

| Time in min | 0 | 10 | 40 | 58 | 72 | 103 | 106 |
|---|---|---|---|---|---|---|---|
| Water/formic acid (95:5) | 100 | 100 | 95 | 85 | 75 | 50 | 0 |
| Acetonitrile/solvent A (60:40) | 0 | 0 | 5 | 15 | 25 | 50 | 100 |

**Table 7: Gradient for determination organic acids, sugars and phenolic compositions in orange juice and orange wine (KELEBEK et al., 2008)**

KAHLE et al. 2005, used a C18 column (100 x 4,6 mm, 3 µm), aconstant flow rate of 1 ml/min and an injection volume of 20 µl to determine polyphenol profiles of apple juices.

CAO et al., 2009, separated and identified polyphenols in apple pomace by HPLC, while using a SB-C18 separating column (250 x 4,6 mm, 5 µm) and absorbance spectra at 280 and 380 nm. The result showed, that they detected mainly flavonols and their glycosides.

For analysing concentrations of phenolic compounds in several commercial juices and nectars, DE SIMÒN et al., 1992, used to take a C18 Nova-Pak column. Figure… shows the gradient and the flow rate, which changes over analysing. The measured concentrations of the phenolic compounds can be find in figure… .

| time, min | flow, mL/min | % A | % B |
|---|---|---|---|
| 0 | 0.6 | 100 | 0 |
| 2 | 0.6 | 100 | 0 |
| 10 | 0.6 | 60 | 40 |
| 15 | 0.6 | 50 | 50 |
| 20 | 0.5 | 40 | 60 |
| 30 | 0.4 | 30 | 70 |
| 40 | 0.4 | 25 | 75 |
| 45 | 0.3 | 15 | 85 |
| 50 | 0.7 | 15 | 85 |
| 60 | 0.8 | 15 | 85 |
| 65 | 0.6 | 100 | 0 |

**Figure 12: Gradient For analysing concentrations of phenolic compounds in several commercial juices and nectars (DE SIMÒN et al., 1992)**

| | peach | peach + grape | apricot | apple | apple + peach | pear | pear + grape | grape | orange | pineapple |
|---|---|---|---|---|---|---|---|---|---|---|
| 1. *p*-hydroxybenzoic ac | 0.01–0.16 | 0.98–3.42 | 0.06–0.39 | t | 0.09 | | t | 0.81–2.60 | | 0.17–0.72 |
| 2. protocatechuic ac | | 0.32–0.69 | | | | | | | | |
| 3. gallic ac | | 0.38–0.84 | | | | | 0.01 | 0.86–2.01 | | |
| 4. *p*-hydroxybenzoic ald | 0.02–0.06 | | | 0.02–0.12 | | | | | t | 0.02–0.11 |
| 5. vanillic ald | 0.01–0.08 | 0.01–0.04 | 0.01–0.16 | t | 0.01 | 0.02–0.12 | 0.03 | | t | t |
| 6. syringic ald | 0.01–0.04 | t | 0.01–0.07 | t | | 0.03–0.11 | 0.03 | | 0.10–0.25 | 0.10–0.38 |
| 7. 3,4-dihydroxybenzoic ald | 0.03–0.18 | 0.10–0.20 | 0.04–0.08 | 0.05–0.26 | 0.10 | 0.02–0.06 | 0.04 | 0.03–0.18 | | t |
| 8. *p*-coumaric ac | 0.06–0.15 | 0.11–0.22 | 0.07–0.11 | 0.56–0.77 | 0.06 | 0.08–0.12 | 0.39 | 0.26–1.91 | 0.10–0.30 | 0.11–0.44 |
| 9. ferulic ac | nd–0.05 | 0.02–0.07 | 0.05–0.18 | t | 0.19 | t | t | 0.10–0.12 | 0.23–0.54 | 0.11–1.77 |
| 10. caffeic ac | 0.02–0.31 | 0.20–0.37 | 0.09–0.27 | 0.86–1.05 | 0.18 | 0.01–0.07 | 0.04 | 0.38–0.81 | | 0.08–0.16 |
| 11. sinapic ac | | | | | | | | | 0.36–1.11 | 0.22–0.64 |
| 12. scopoletin | | | 0.03–0.07 | | | | | | t | |
| 13. aesculetin | t | | 0.02–0.05 | 0.20–0.45 | | | | | | |
| 14. chlorogenic ac | 0.22–7.12 | 1.18–1.48 | 0.65–3.09 | 2.49–9.14 | 3.52 | 1.14–7.88 | 11.56 | | 0.13–2.04 | |
| 15. *p*-coumaroylquinic ac | | 0.27–0.48[b] | | 0.81–2.58 | 0.52 | 0.17–0.43 | 0.46 | | | 0.25–0.39 |
| 16. feruloylglucose | | | | 0.02–0.14 | | nd–0.30 | 0.03 | | 0.08–0.19 | 0.09–0.14 |
| 17. caffeoyl ester | 0.11–1.68[b] | 0.14–0.37[c] | 0.36–1.06[b] | | 0.99[b] | | | 0.10–1.78[c] | | |
| 18. feruloyl ester | | 0.07–0.16[c] | | | | | | 0.74–1.01[c] | | |
| 19. *p*-coumaroyl ester | | 0.05–0.07[c] | | | | | | 0.78–1.61[c] | | |

[a] ac, acid; ald, aldehyde; nd, not detected; t, traces (only detected by TLC). [b] Possible 3′-caffeoylquinic acid. [c] Tartaric ester.

**Figure 13: Concentrations (in mg/l) of certain nonflavonoid phenolic compounds in commercial juices and nectars (DE SIMÒN et al., 1992)**

*Sample preparation*

For preparing juice samples for the HPLC analyses, KELEBEK et al., 2009, were using a centrifuge at 4.000 rpm for 20 min. Then, the supernatant were filtered through 0.45 µm pore size membrane and kept at a temperature of -18 °C until analysis.

MOUSAVINEJAD et al., 2009, used to centrifuge their pomegranate juice samples, as well. Therefore they set 5.000 rpm for 4 min and filtered the supernatant through a 0.45 µm filter.

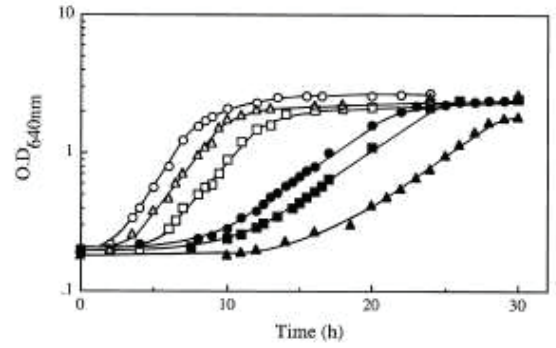
For preparing the samples GRUZ et al., 2008, used to centrifuge the wine, juice and tea at 3.500 rpm in 5 min. After that, the samples were filtered through a 0,2 µm micro filter.

*Reproducibility*

The reproducibility of the HPLC analysis is mostly performed by comparing retention times and peak areas or absorption spectra of unknown peaks with external standards and those reported in the literature (INBARAJ et al., 2010; FANG et al., 2007). Therefore the coefficient of variation will be determined. FANG et al., 2010, used to inject substances five times and could reach a CV less than 5% for standard mixtures.

*Yeast based studies*

CHAMBEL et al., 1999, inquired on the effect of cinnamic acid on the growth and on plasma membrane $H^+$-ATPase activity of *Saccharomyces cerevisiae*. Beause of the presence of cinnamic acid and derivatives in plants and fruits, it could be, that they also inhibit wine or fruit juices fermentations by *Saccharomyces cerevisiae*. Therefore defined concentrations of cinnamic acid were given to a synthetic produced medium containing *S.cerevisiae.* The results were detected with an optical density and shown in figure… .




Effect of increasing concentrations of cinnamic acid on the growth curve of *S. cerevisiae* YPH499, at pH 4 and 30°C. Total cinnamic acid concentrations were: 0 (○), 10 (△), 15 (□), 20 (●), 25 (■) and 35 (▲) mg/l. Results are representative of the many growth experiments carried out.

**Figure 14: Effect of cinnamic acid on the growth of S.cerevisiae (CHAMBEL et al., 1999)**

Determination of phenolic compounds from the fermentation of cultivars Cabernet Sauvignon and Merlot, was studied by KLENAR et al., 2004. The totalphenols were measured by a spectrophotometer at 765 nm. The results showed more phenolic substances in Cabernet Sauvignon. The mass concentration reached from 4109 to 4240 mg/l. The following figure shows the increasing of the phenolic compounds during the fermentation of Merlot. The second figure offers the increasing in Cabernet Sauvignon.

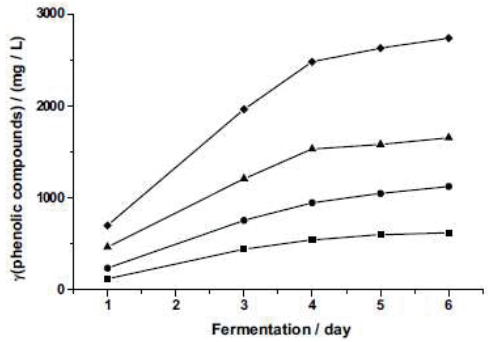



Fig. 2. Increase of phenolic compounds during fermentation of Merlot. Microlocation Škocjan; the average sugar grade at harvest was 22.5 °Brix.
◆ Phenols; ■ Anthocyanins; ▲ Nontannins; ● Tannins

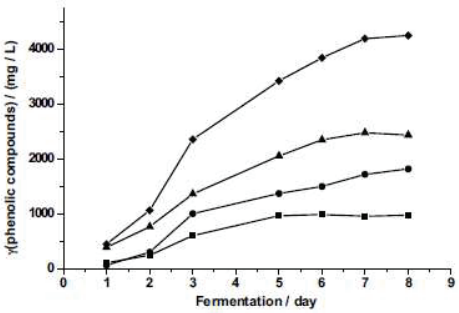



Fig. 6. Increase of phenolic compounds during fermentation of Cabernet Sauvignon. Microlocation Labor; the average sugar grade at harvest was 22.0 °Brix.
◆ Phenols; ■ Anthocyanins; ▲ Nontannins; ● Tannins

**Figure 15: Increase of phenolic compounds during fermentation of Merlot (KLENAR et al., 2004)**

**Figure 16: Increase of phenolic compounds during fermentation of Cabernet Sauvigon (KLENAR et al., 2004)**

# 3 Materials and Methods

In this chapter, the operation for the HPLC analyzing is described detailed. Next to it, the reagents and materials used for research are listed and declared for the way of sample preparation. Moreover the functions of all used technical machines and the fermentation procedure by yeasts are given below.

## 3.1 Materials

In the following points, the standard substances, used for the calibration, but also the mediums, taken for the HPLC evaluation, are listed.

### 3.1.1 Standard Substances and Yeast

For HPLC analysing the standard substances cinnamic acid, ferulic acid, chlorogenic acid, caffeic acid andcoumaric acid from ´Sigma-Aldrich Chemie GmbH Germany` and rosmarinic acid and sinapic acid from ´Carl Roth GmbH Germany`, were used.

Baker´s yeast, *Saccharomyces cerevisiae* was used for fermentation of fruit juices and synthetic mineral medium. Preliminary experiments (data not shown) were performed using the yeasts *Kluyveromyces lactis* and *Dekkera bruxellensis* obtained from a culture collection at Lund University.

### 3.1.2 Minimal Medium and Natural Medium

A mineral medium, based on MERICO et al. 2007 was used for fermentation of baker´s yeast in synthetic medium.

As natural mediums four different Fruit Juices (Blueberry Juice, Cranberry Juice, Apple Juice and Sallow Thorn Juice) were used.
Therefore the juices were taken from ´Kivik´s Musteri` in Sweden. All juices but the apple juice had a fruit content of 40 %. Except the ecological apple juice, which had a fruit content of 100 %, in other juices were mixed with grape juice. In the sallow thorn juice there was also

orange juice added. The content of blueberry in the juice was only 11 %, of cranberry 16 % and of the sallow thorn 12 %. All juices were free of pulp.

### 3.2 Technical Equipment

In the following, the HPLC plays the primary role. The construction, the operation methods and the application for this machine are elaborated in the part below. Next to the signal collection of the DAD, also the evaluation of the column-capacity with the help of HETP, are summarized in the following points. Furthermore, the procedural method for the sample preparation with the centrifuge and the concentrator are shown below, as well.

#### 3.2.1 HPLC

HPLC is the abbreviation for High Performance Liquid Chromatography. It is an analytical process for detaching substances of a liquid mixture. The visualization of the separated components occurs by peaks, which let establishing a chromatogram.
In this chromatographic separation process, the dissolved mixture, which is going to be analysed, is given to a flux material, also called mobile phase or eluent. The mobile phase can be polar, unpolar or a mixture of both solvents. The eluent, including the sample, is pumped through a separating column. After passing the column, compounds are delivered to a detector, which sends a signal to an evaluation unit.

*Diode Array Detector (DAD)*
In the detector a ray of lights of four different wavelengths encounters on the eluted substance and gets dispersed in the particular spectral colours by a prism. These hit the light-sensitive diodes and generate a characteristically spectral-curve. All collected signals are transmitted to the computer-assisted software.

*Column*
The column contains the stationary phase, which incurs interactions with the sample mixture. Polar or unpolar stationary phases are common in HPLC. The most commonly used method is the application of a column with an unpolar stationary phase. It is called the reversed phase chromatography (RP). In that case the elution power degrades with higher polarity of the

substances. For the mobile phase water, methanol or acetonitrile are the most ordinary solvents. A sample mixture can be separated with a gradient or in an isocratic way.

Based on the idea, that the sample substances crosses over from the mobile phase to the stationary phase and then back to the mobile phase, the number of the changeovers can be used to describe the performance of the column. These transitions are described as zones alternatively as theoretical plates. The theoretical plates are depending on the length of the column, the sample substances and the constitution of the stationary phase, as well. The calculation of the separation stairs should be supplemented to check the height of the plate (HETP – Height Equivalent to a Theoretical Plate).
Summarized, a column has high-capacity, if there is a high number of theoretical plates while these should offer the lower HETP possible.

*Calibration*
For identifying and quantifying the substances, the methods with an external or an internal standard can be used.
The former is given into the HPLC in different concentrations. The chromatogram will show a peak which offers a characteristic retention time. For quantifying this component, the peak-area and the concentration of standard is needed. With the help of these values the concentration of a sample mixture can be calculated.
Using the method of the internal standard, a standard substance is given to the sample mixture, which should be similar to the components in the sample. With the peak area and the peak height of the standard substance, the concentrations of the sample-peaks can be assessed.

### 3.2.2 Centrifuge

For centrifuge the samples, a Galaxy 16DH Microcentrifuges of VWR was used. The settings of machine can be adjusted individually by the user. One can choose the revolutions per minute (1.000 – 13.000 rpm) and the running time in minutes. For centrifuging the samples, we set 4.000 rpm at 20 min.
The principle is based on utilisation of the mass inertia. Because of the centripetal acceleration, which occurs due to an equably circular motion, particles with higher density get transported external.

### 3.2.3 Concentrator

Samples were concentrated using an Eppendorf Concentrator 5301.
With that apparatus it is possible to run fourty-eight Eppendorf tubes. Under vacuum and rotation, water of the samples will be evaporated, controlled by temperature and running time.
A temperature-range let you chose the values 30 °C, 45 °C and 60 °C. Depending on desired end-concentration and chosen temperature, the operating time can be set up to hours.
At 45 °C, the samples were evaporated triplicate, except the unfermented juices, which were concentrated duplicate.

## 3.3 Methods

In point 3.3, the reader will find out all of the operation ways, using for furnishing samples for the HPLC analyzing. From fermentation, to sample preparation up to extraction, the sample mixtures must follow all of these steps, as per particulars given below.

### 3.3.1 HPLC Analysis

For analysing cinnamic acid and derivatives, a HPLC of the company "Merck Hitachi" was used. The machine consists of a solvent-storage container, a degasser, a high-pressure pump (L-7100), an auto sampler with the injection-syringe (L-7200), a heatable column oven (L-7360) and a diode array detector (DAD, L-7455) with an affiliated electronic data processing system. The later is operating with the software "Chromatography Data Station Software" (Version 4.1) of Merck Hitachi.
First a separation column of Thermo Electron Corporation (Hy Purity C18; 100 x 4,6 mm, 5 µm particle size; TEC) was used. Because of problems with that column, a Chromolith Performance column (RP 18e; 100 x 4,6 mm; CPC) was taken for further analysis. The columns consist of an unpolar stationary phase.
With changing the column, a new lamp was put in the DAD, as well.
For the gradient-system, solvent A, methanol and solvent B, deionized water made by a purification system (Simplicity 185, Millipore, Simpak) were used. For microbial stability, the water was regulated at pH 3 with orthophosphoric acid. Before passing the column, the solvents were pumped through the degasser at a controlled flow of 1 ml/min.
All used mixtures were filtered through a 0,45 µm membrane (17 mm, Sun Sri Titan, USA).

### 3.3.2 Gradient

For appointing an optimal gradient for the polyphenol-analysis, a pre-existing gradient (table 8) was used. In previous analysis, the gradient was taken to analyse cinnamic acid. With the help of this gradient, an optimization of the separation was achieved.
The aim was to detect all substances as separated peaks in the shortest run-time.
At first all needed standard-substances were dissolved in methanol and measured with the given gradient. Because of overlapping peaks, a stepwise optimization of the solvent-concentrations was arranged. The gradient was optimized until improved results could be achieved.
Because of some frictions, the column needed to be changed within the analysis. Therefore the previous developed gradient was adopted for the new column.

| time in min | H2O in % | MeOH in % |
|---|---|---|
| 0 | 80 | 20 |
| 1 | 80 | 20 |
| 4 | 60 | 40 |
| 12 | 50 | 50 |
| 16 | 20 | 80 |
| 18 | 0 | 100 |
| 20 | 0 | 100 |
| 22 | 80 | 20 |

**Table 8: Pre-existing gradient**

### 3.3.3 Yeast Fermentation

For juice fermentation, the bakers-yeast *Saccharomyces cerevisiae* (Kron Jäst, Jästbolaget AB, Sweden) was used.
For 100 ml juice, 2 % of freeze-dried yeast was applied. The mixture was filled in 200 ml Erlenmeyer-flasks and closed with a plug or aluminum foil. The flasks were put on a shaker for 24 h at 25°C. The shaker rotated with 150 revolutions per minute (rpm).
After fermentation, the flasks were taken out of the shaker and prepared for the HPLC-analysis (see 3.3.4.)

### 3.3.4 Standard and Sample Preparation

*Standard Preparation*
Initially the standard-substances were prepared for the first HPLC-analyses. Therefore 1,5 ml Eppendorf-tubes were used.
1 mg of the standard-substance was given into the Eppendorf-tube under the fume hood. After that, 1 ml methanol was given to the substance. It was shaken per hand for 1 to 2 min. The next step was to transfer this solution in a 10 ml volumetric flask and to fill it up to the 10 ml

mark. After repeated shaking and degassing in an ultrasonic bath, the steps were accomplished until 5 dilution-levels were generated.
Then, the sample were filtered and filled with a syringe (Becton Dickinson, Plastipak, 1 ml) into vials.

*Sample Preparation*

For the preparation of the fermented and unfermented juice-samples, the following procedure was used.
The unfermented samples were directly filtered and given into the vials for analysing.
Another step was to concentrate the juice by using the Eppendorf Concentrator (see 3.2.3). The concentration process was regularly checked and ended before the liquid samples gelled. This was up to a 2- or 3-fold concentration, depending on the juice.
The procedure with the fermented juices was similar to the unfermented samples.
After the fermentation process (see 3.3.3), the solutions were filled in several Eppendorf-tubes and centrifuged. The residue, containing the yeast cells, stayed in the tubes. The supernatant was transferred in new tubes and concentrated like the unfermented juice.
The residue was extracted with water (pH3) for 4 h. Then, the extracted supernatant was removed from the biomass and concentrated, as well. Afterwards the biomass was concentrated. Later, the balance weights from the insoluble fractions were compared with the concentration of the phenolic compounds in the juice-samples.
Finally, all samples were given in the HPLC for analyzing.
Before and after measuring, all samples were always kept cooled. The standard-substances were stored in a fridge at 4°C. For longer storage, the fermented and unfermented juices were kept in a freezer at -22°C.

### 3.3.5 Extraction

The extraction was performed to determine phenolic compounds in the unsoluble biomass.
Therefore 200 µl of water (pH 3) were given to the containing biomass-tubes. With a reagent-shaker the tubes were shaken for a few seconds. Thereupon the tubes were stored for 4 h at room-temperature. Chromatograms of 4 h-stored and 24 h-stored samples were used to determine an adequate extraction time.
Finally the liquid supernatant was removed for concentrating.
The residue was evaporated as long as the pure biomass was presented.

## 4 Results

In this chapter all results are presented. The main focus lies on the presentation of the chromatograms, including the different hydroxycinnamic acid and its derivatives in fermented and unfermented fruit juices. Both the analyses for the standard substances performed on TEC and CPC are shown here while the chromatograms which were formed by duplication with the CPC, are not shown in this work.

### 4.1 Gradient

With the pre-existing gradient, the standard mixtures were analyzed. With the help of the generated chromatograms, the concentrations of the solvents were adapted. If two peak of two different standard substances held nearly the same retention time, it was an attempt made to lengthen the separation time, or to change the concentration configuration of both solvents. In that way, the gradient, showed in table 10, was developed for the TEC. The same gradient was also used for the CPC.

**Table 9: Pre-existing gradient**
**Table 10: Developed gradient**

| time in min | H2O in % | MeOH in % |
|---|---|---|
| 0 | 80 | 20 |
| 1 | 80 | 20 |
| 4 | 60 | 40 |
| 12 | 50 | 50 |
| 16 | 20 | 80 |
| 18 | 0 | 100 |
| 20 | 0 | 100 |
| 22 | 80 | 20 |

| time in min | H2O in % | MeOH in % |
|---|---|---|
| 0 | 80 | 20 |
| 2 | 90 | 10 |
| 4 | 95 | 5 |
| 6 | 85 | 15 |
| 8 | 80 | 20 |
| 12 | 75 | 25 |
| 15 | 70 | 30 |
| 18 | 60 | 40 |
| 22 | 45 | 55 |
| 25 | 30 | 70 |
| 30 | 80 | 20 |

### 4.2 Standard Substances, Minimal Medium and Biomass

For evaluating the standard substances for the CPC and TEC, the same mixtures and minimal mediums were used for both columns.

*Standard substances*

Before analyzing and quantifying the concentrations of the hydroxycinnamic acids, we need to detect the acid at a characteristic retention time and establish a straight calibration line of cinnamic acid and its derivatives. Furthermore, it was decided to detect all standards at the same wavelength, so that all results could be compared with each other. The chosen wavelength was 280 nm.

*Cinnamic Acid*

For cinnamic acid, the following standard concentrations were used:

| | |
|---|---|
| Blank | 0 mg cinnamic acid per l |
| Standard 1 | 10 mg cinnamic acid per l |
| Standard 2 | 100 mg cinnamic acid per l |
| Standard 3 | 1.000 mg cinnamic acid per l |

For quantifying the acids, the peak areas were used. Therefore, the data at 280 nm were taken. A correlation coefficient r = 0,988 for cinnamic acid could be reached by detecting this standard substance with the CPC and the new installed DAD lamp. A similarly high correlation coefficient of cinnamic acid was reached with the TEC (data for correlation coefficients are not shown).

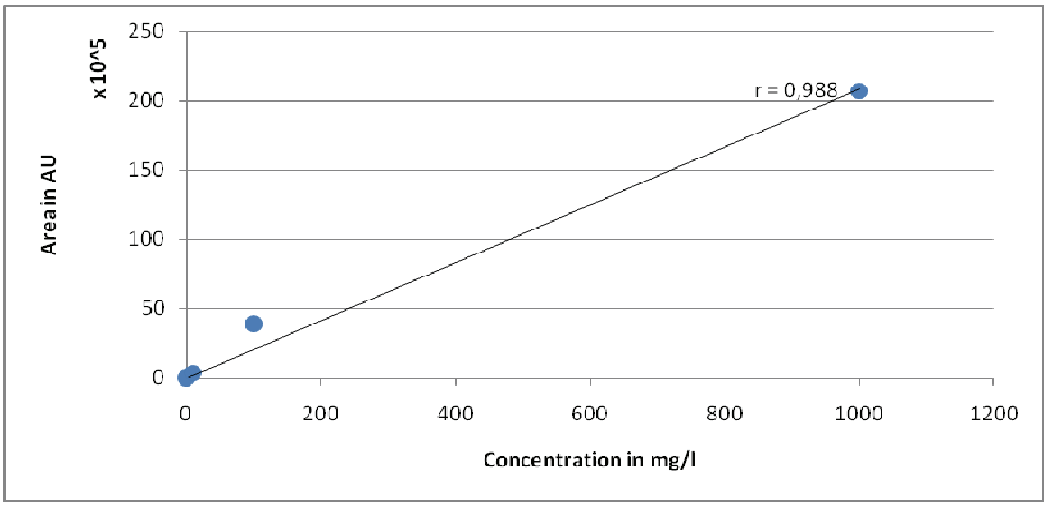



**Figure 17: Straight calibration line for cinnamic acid (CPC)**

The tailing cinnamic acid was detected at a retention time at around 25,72 min (see the following figures). The figur 20 show the cinnamic acid peak at 5 different wavelenghs. For detecting the hydroxycinnamic acids, 280 nm was used. At this wavelengh, the biggest area could be determined for cinnamic acid.

For the TEC, the cinnamic peak was found at RT 22,01, detected at 280 nm, as well (figure 19). The peak, which eluted at RT 24,19 min (± 0,2 min), was also found in all other chromatograms for the TEC. It seems that there had been a contamination in the mixtures of all standards.

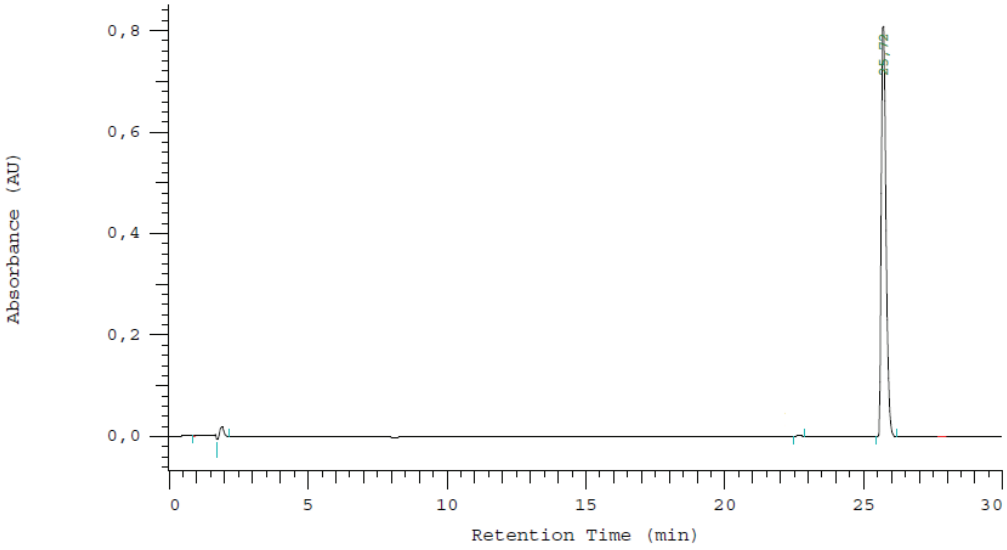



**Figure 18: Chromatogram of cinnamic acid (100 mg/l) at 280 nm (CPC)**

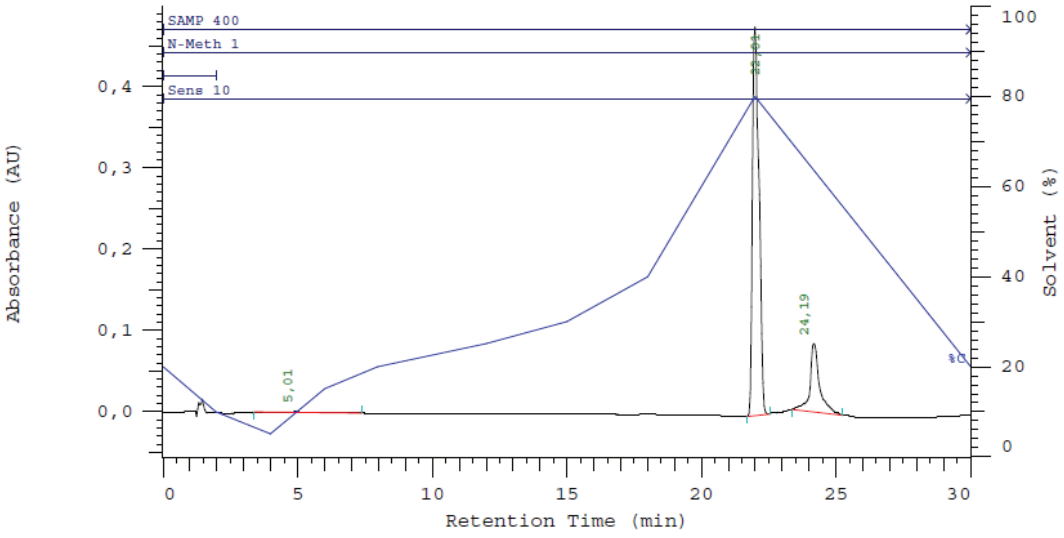



**Figure 19: Chromatogram of cinnamic acid (100 mg/l) at 280 nm (TEC)**

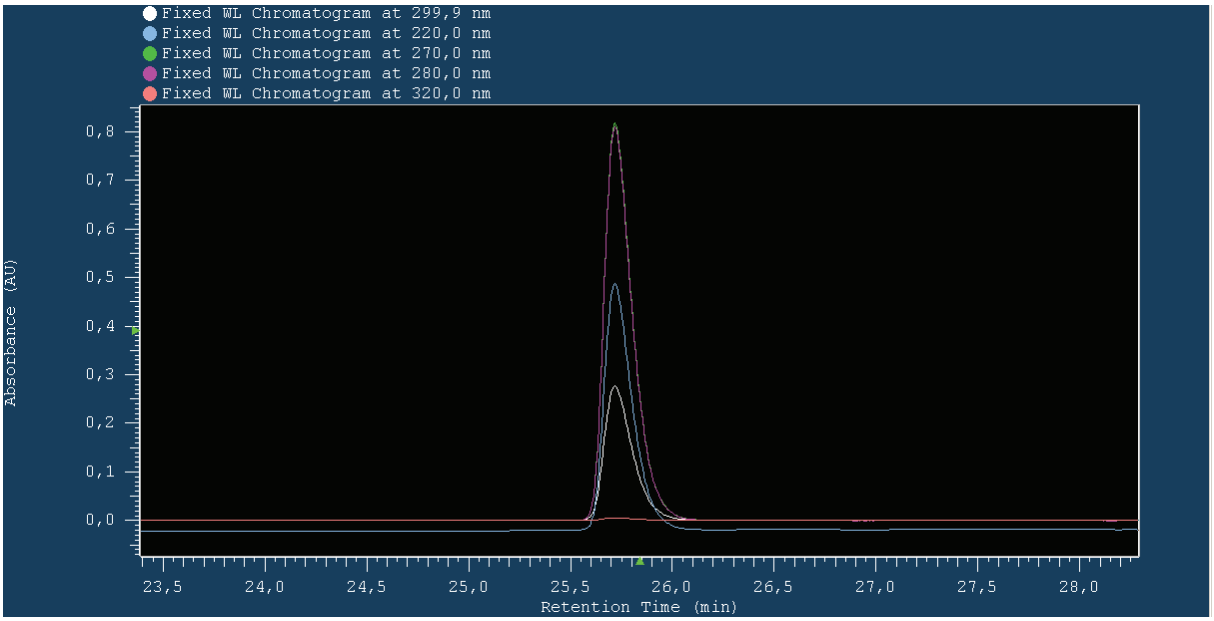



**Figure 20: Peak of cinnamic acid at 220, 270, 280, 300 and 320 nm (CPC)**

In the figures mentioned below, the characteristical peak spectrum of cinnamic acid, analysed with the CPC, is shown. The TEC could detect a similar spectrum for this standard substance (figure 92, appendix).

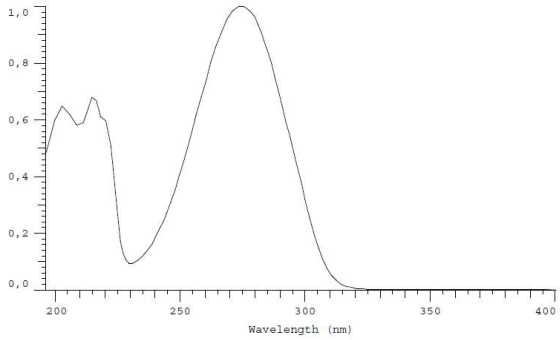



**Figure 21: Spectrum of cinnamic acid at RT 25,72 min (CPC)**

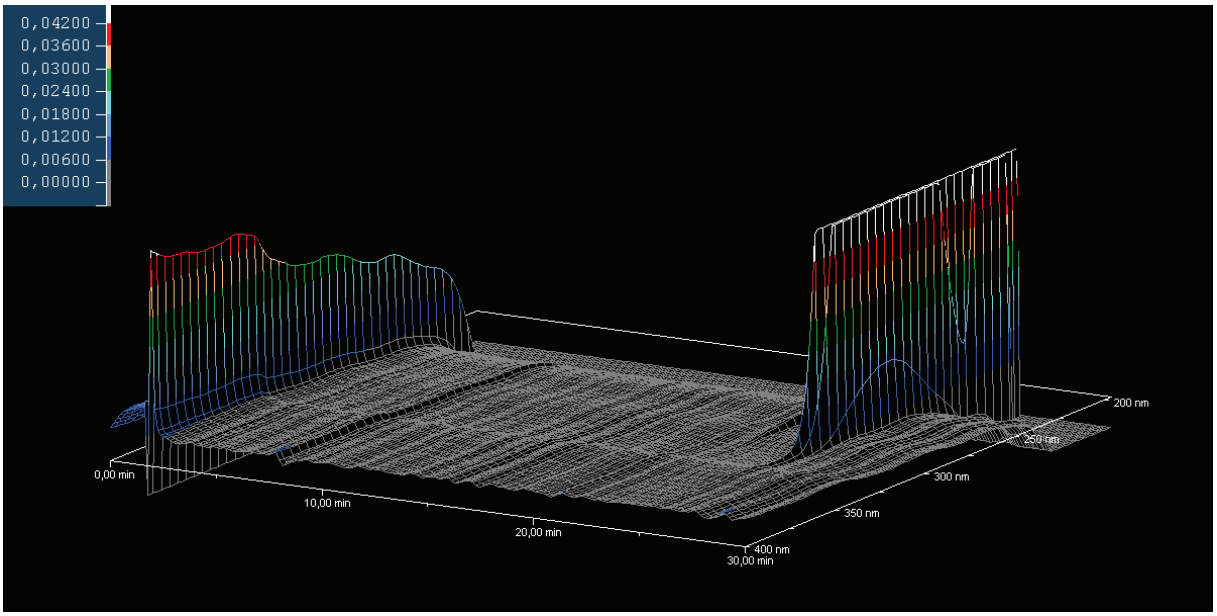



**Figure 22: 3-D spectrum of cinnamic acid (CPC)**

In figure 22 the 3-D spectrum of cinnamic acid can be seen. The colors represent the different wavelengths. The first spectrum, on left side of the figure, is the injection peak, but not relevant for this work.

*Caffeic Acid*

Like cinnamic acid, caffeic acid was also detected at 280 nm. The caffeic peak was detected at around 25,72 min. The standard concentrations for the calibration line were the same as those selected for cinnamic acid. A correlation coefficient at r = 0,999 was determined. With the TEC, caffeic acid was detected after 3,72 min (figure 25). Consequently, the peaks of the different analysis have a range of about 22 min. The correlation coefficients were as good as the ones which were achieved by CPC.

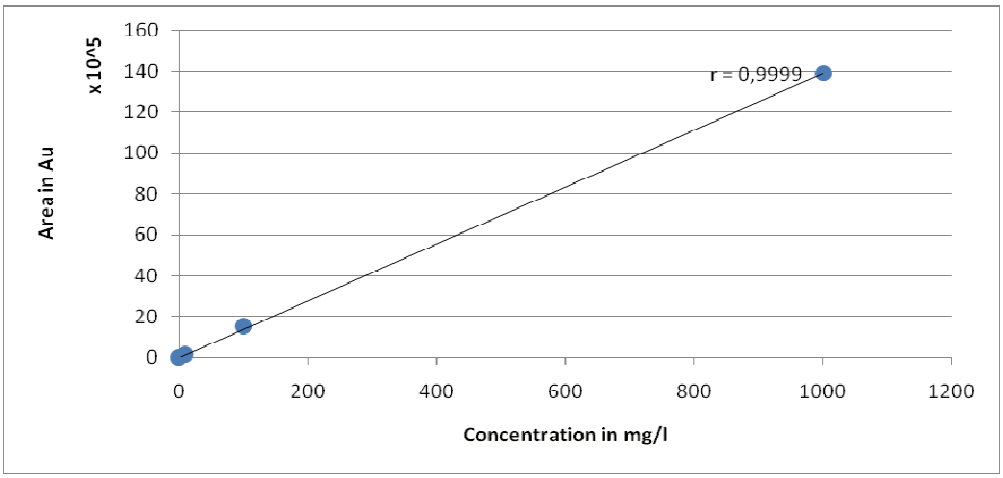


**Figure 23: Straight calibration line for caffeic acid (CPC)**

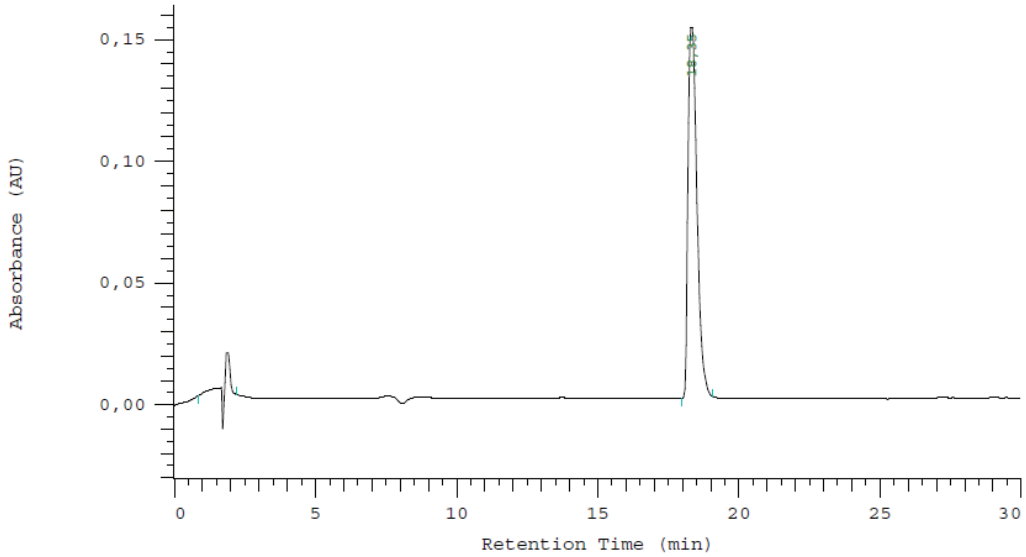



**Figure 24: Chromatogram of caffeic acid (100 mg/l) at 280 nm (CPC)**

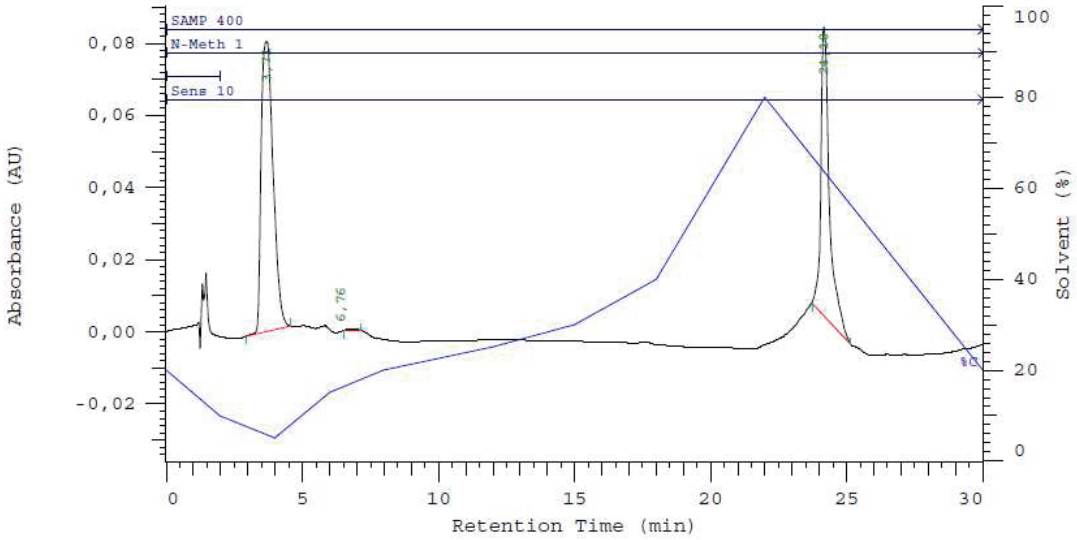



**Figure 25: Chromatogram of caffeic acid (100 mg/l) at 280 nm (TEC)**

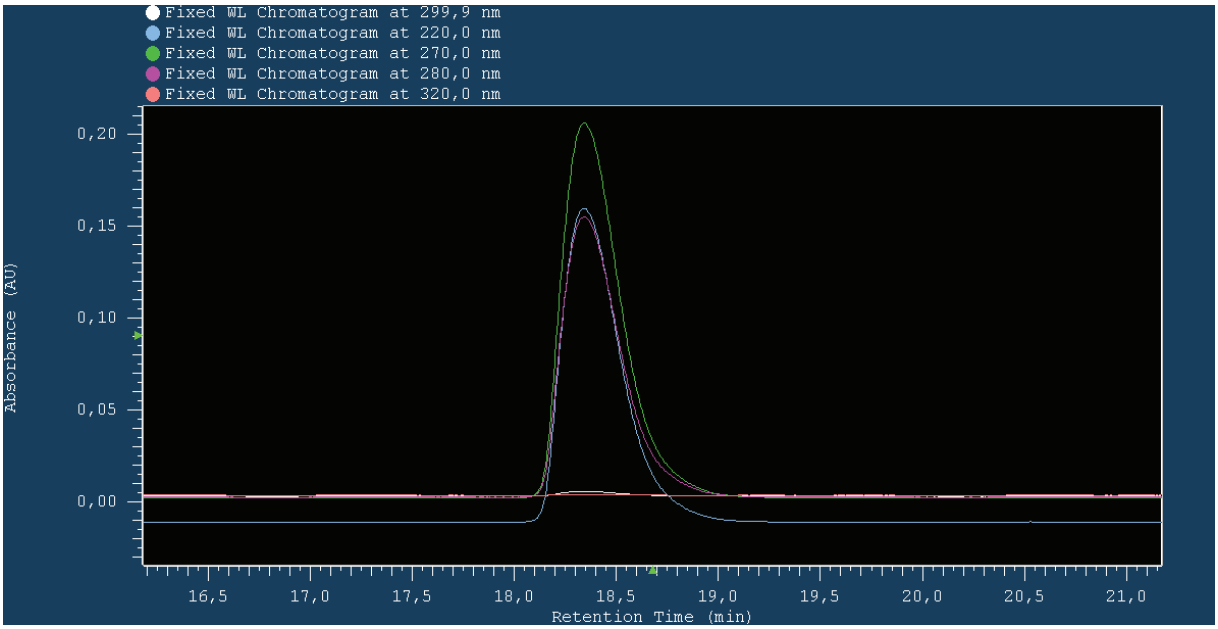



**Figure 26: Peak of caffeic acid at 220, 270, 280, 300 and 320 nm (CPC)**

Especially in figure 26 it can be seen, that the peak of caffeic acid shows a tailing shape. It is also observed that the absorbance at 270 nm was much higher than at 280 nm.

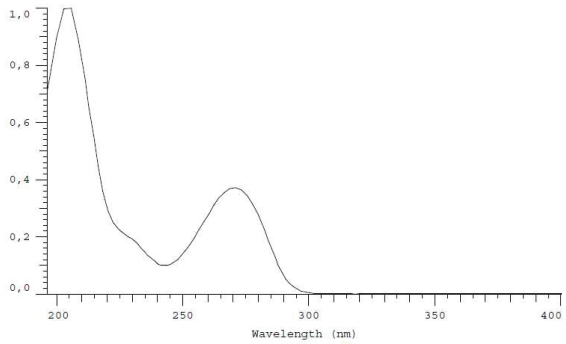



**Figure 27: Spectrum of caffeic acid at RT 18,35 min (CPC)**

The shape of the spectrum shown in figure 27 can be easily found in the 3-D spectrum. The analyses of caffeic acid, with the TEC, showed a similar shape (figure 93, appendix).

Because of similarity of the RTs of caffeic acid with another acid, caffeic acid cannot be identified precisely in the juice samples. Therefore, the concentrations were generally described as “phenolic compounds”.

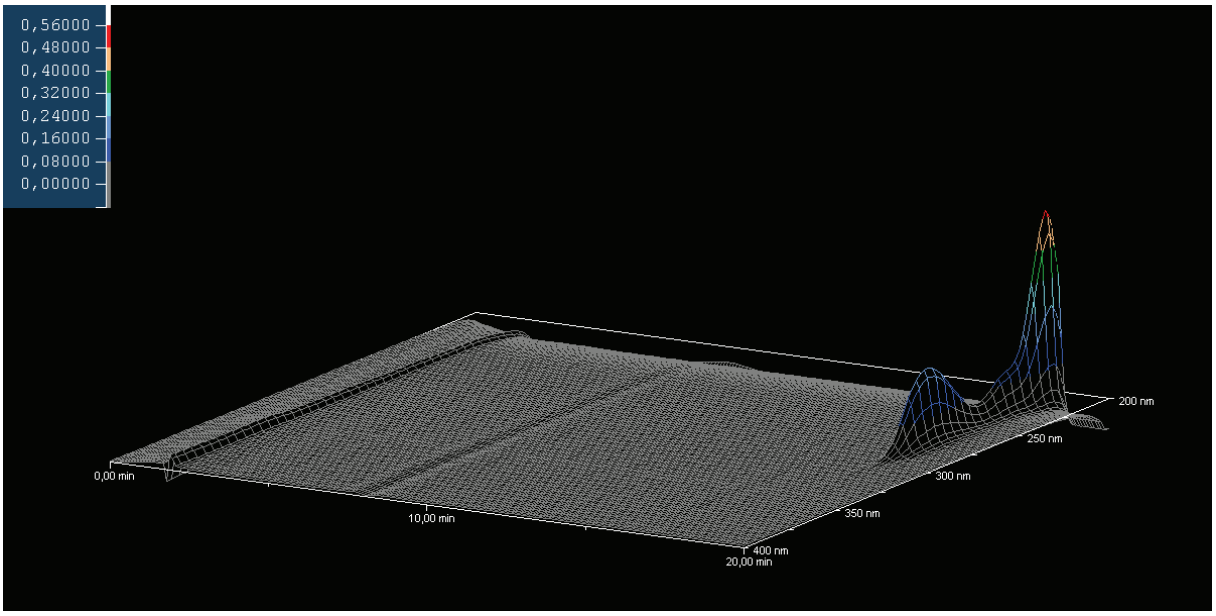



**Figure 28: 3-D spectrum of caffeic acid (CPC)**

*Coumaric Acid*

By detecting coumaric acid, two separate peaks were recorded with the CPC at 18,06 and 20,23 min.

The detection was carried out at 280 nm, as with all substances. The concentration dilutions were the same as the two phenolic acids described previously. With increasing levels of standard substance, both peaks became higher, whereupon the peak with the earlier retention time was always smaller than the other one. The calibration lines could reach correlation coefficients of r = 0,998 for peak 1, and r = 0,999 for peak 2. Both peaks are characterized with a tailing shape.

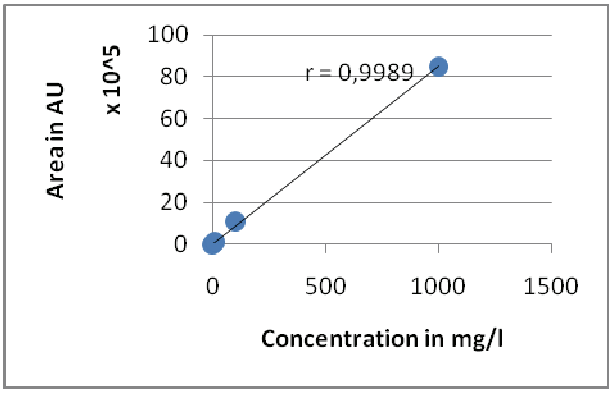



**Figure 29: Straight calibration line for coumaric acid peak 1 (CPC)**

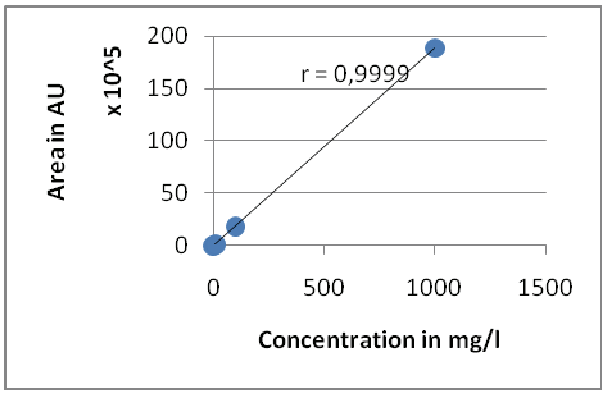



**Figure 30: Straight calibration line for coumaric acid peak 2 (CPC)**

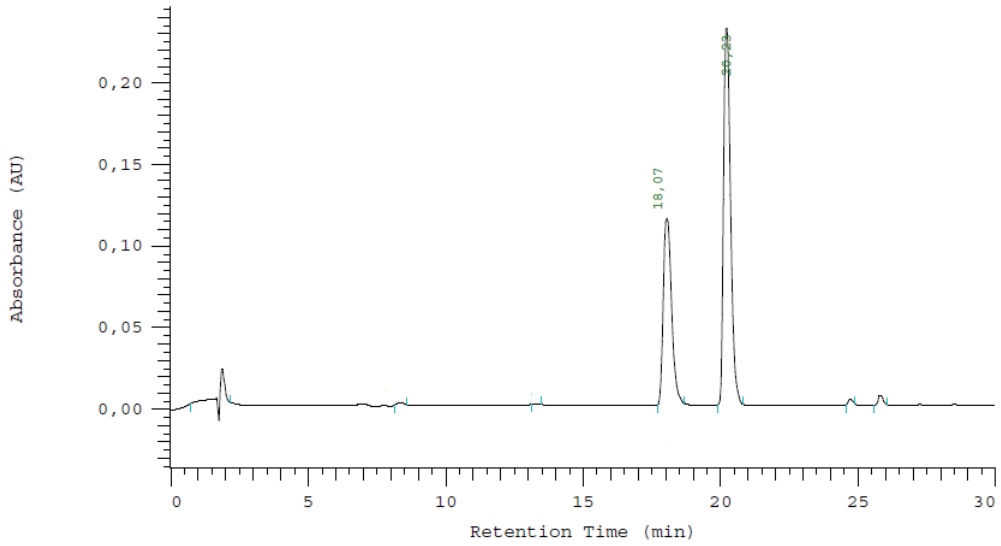



**Figure 31: Chromatogram of coumaric acid (100 mg/l) at 280 nm (CPC)**

Peak 2 of coumaric acid and a peak 1 of another standard substance showed overlapping RTs. Regarding the spectra of these two peaks, it is seen that both spectra are similar. Therefore it is not possible to appoint a peak one of these acids. Due to this, peaks found in juice samples at that RT cannot be identified, but described as phenolic compounds as well. The highly similar RTs of caffeic acid and coumaric peak 1 resulted in inclusion of the first peak of coumaric acid to the category of phenolic compounds.

A double peak was identified by detecting coumaric acid with the TEC (figure 32). But the double peak was treated like two peaks. The RT´s were 11,34 min for peak 1, and 12,18 min for peak 2.

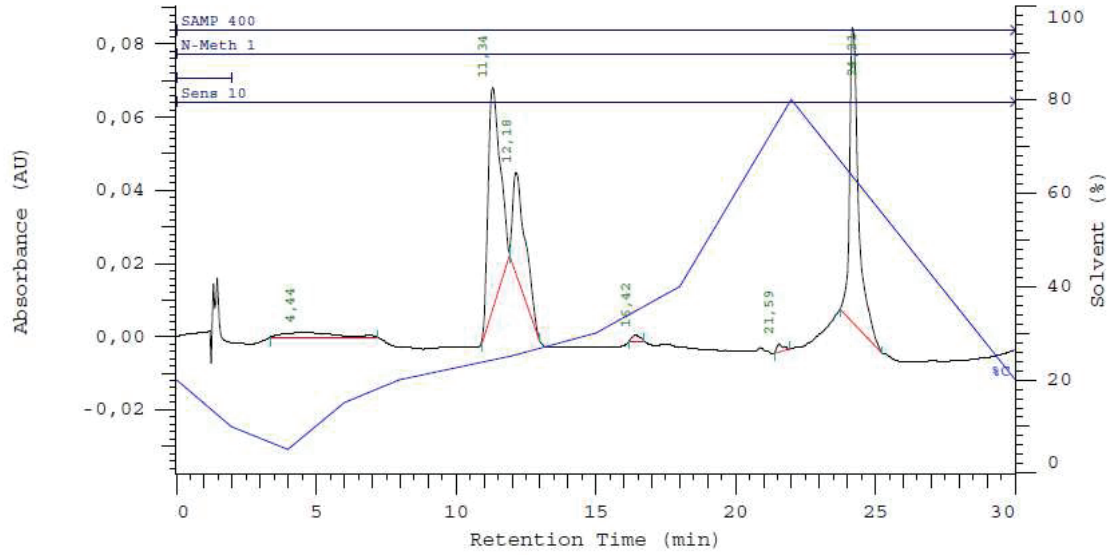



**Figure 32: Chromatogram of coumaric acid (100mg/l) at 280 nm (TEC)**

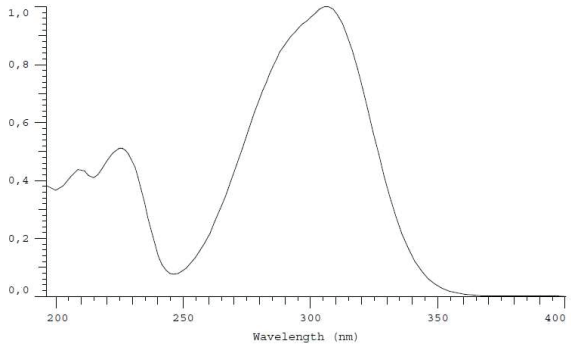



**Figure 33: Spectrum of coumaric acid at RT 18,07 min (CPC)**

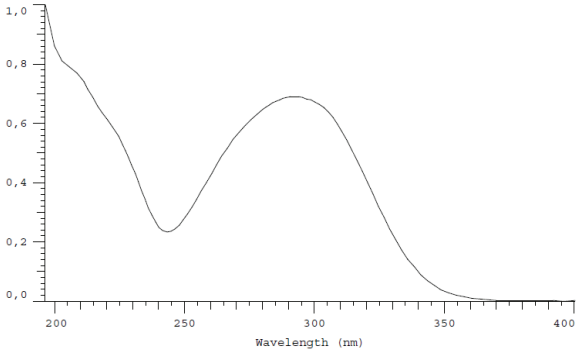



**Figure 34: Spectrum of coumaric acid at RT 20,23 min (CPC)**

The discrepancy of the spectra of the coumaric acid peaks shows that both peaks are two dissimilar substances. The following spectra of coumaric acid, analyzed with TEC, showed completely different shapes than the figures 33 & 34. Both shapes are very similar.

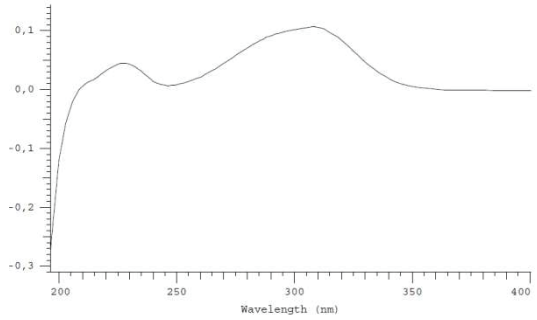



**Figure 35: Spectrum of coumaric acid at RT 18,07 min (TEC)**

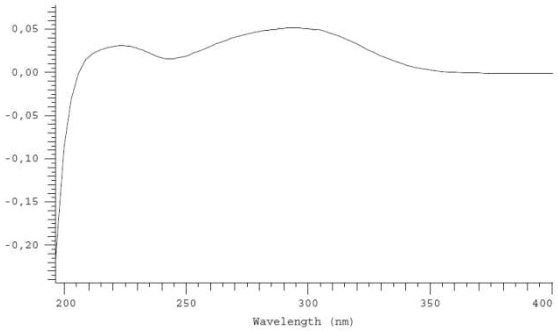



**Figure 36: Spectrum of coumaric acid at RT 21,63 min (TEC)**

*Ferulic Acid*

With the concentrations 0, 10, 100 and 1.000 mg/l, two peaks were detected at 20,57 min (Peak 1) and 21,63 min (Peak 2), using the CPC. The straight calibration lines of both peaks showing very good correlations, but they are barely separated from the base line (seen in figure 39).

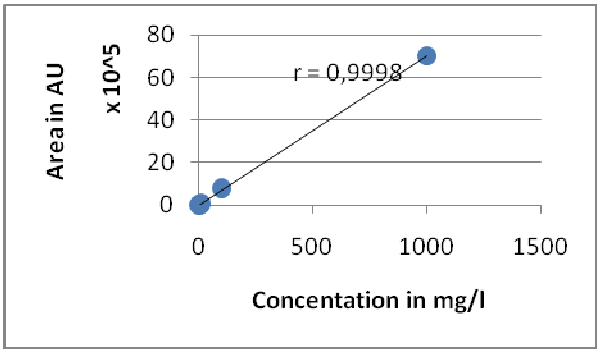



**Figure 37: Straight calibration line for ferulic acid peak 1 (CPC)**

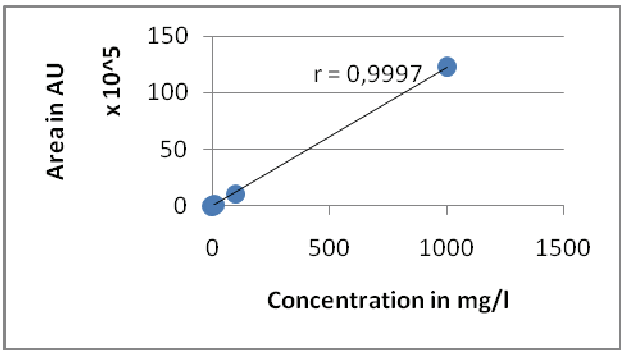



**Figure 38: Straight calibration line for ferulic acid peak 2 (CPC)**

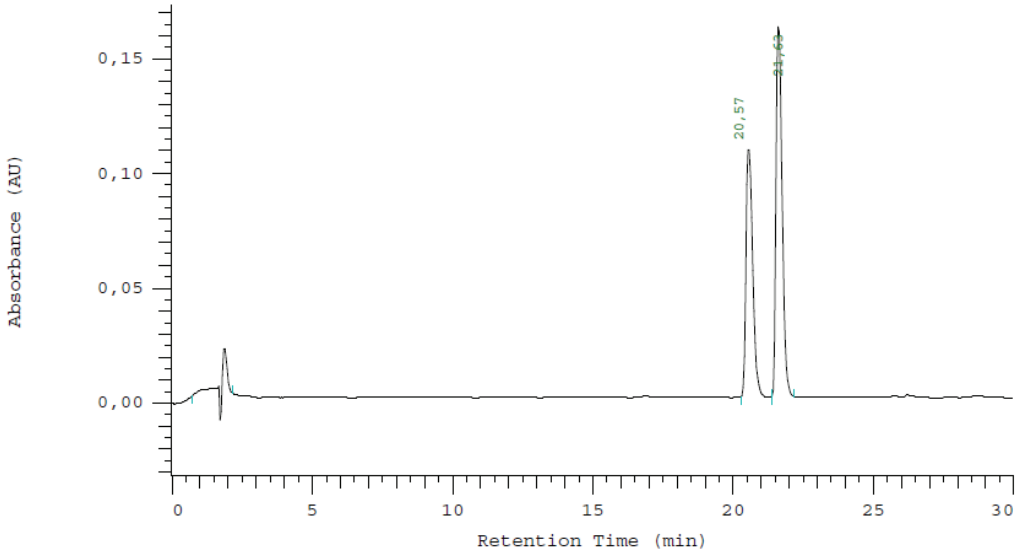



**Figure 39: Chromatogram of ferulic acid (100 mg/l) at 280 nm (CPC)**

Regarding the peaks of ferulic acid, measured with the TEC, it is seen that two peaks are also present. However, the shapes of the peaks are not as filigree as those produced with CPC, but also having good correlation coefficients. The RT´s (13, 46 min and 14, 73 min) are much earlier than the ones, performed with CPC. Another difference between these peaks, analyzed with CPC, is that the first peak is higher than the second one. In the chromatogram above, the first peak displays less height than the second one.

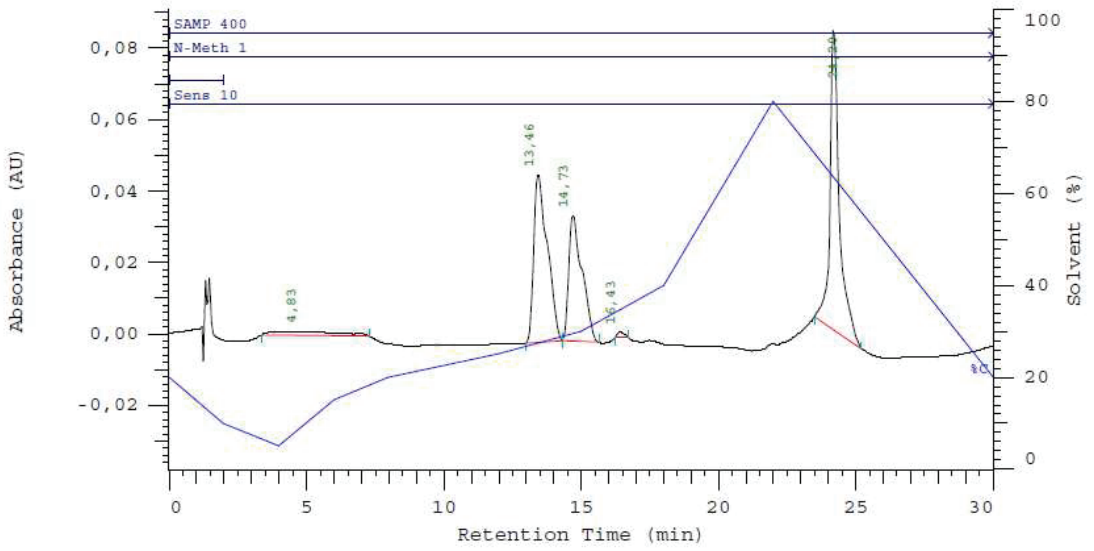



**Figure 40: Chromatogram of ferulic acid (100 mg/l) at 280 nm (TEC)**

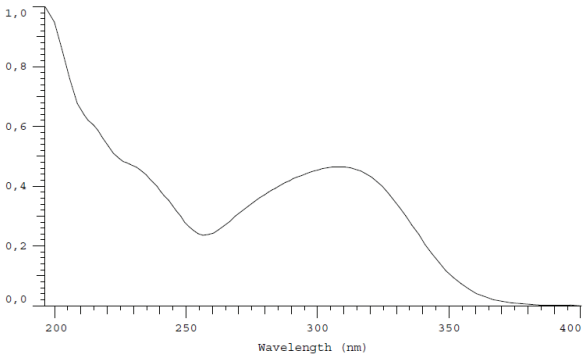



**Figure 41: Spectrum of ferulic acid at RT 20,57 min (CPC)**

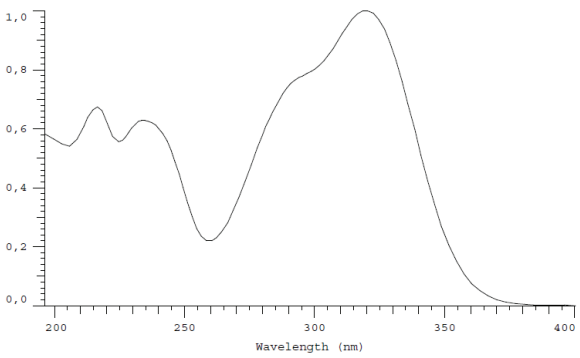



**Figure 42: Spectrum of ferulic acid at RT 21,63 min (CPC)**

Like coumaric acid, the spectra of ferulic acid showed different curves for both peaks. Because of the similarity of the RT of the first ferulic peak, compared with the second peak of coumaric acid, the concentrations of both acids cannot be evaluated precisely. By having only the second peak of ferulic acid left for determination of the concentrations, the results would have been adulterated. Therefore, also ferulic acid is strictly interpreted as a phenolic compound. Additionally, the second peak of ferulic acid has the same spectrum as the first peak of sinapic 1. In that case, a flawless analyzing of the concentrations would have been not possible.

*Rosmarinic Acid*

Because of used lower concentration factors than in cinnamic, caffeic, coumaric and chlorogenic acid, no peaks could be detected for the first standard sample. It seems that the 10 µl injection volume was not enough for detecting this substance. For the other concentrations, rosmarinic acid showed two peaks, as well (CPC)

The following standard concentrations were used:

| | |
|---|---|
| Blank | 0 mg rosmarinic acid per l |
| Standard 1 | 1 mg rosmarinic acid per l |
| Standard 2 | 10 mg rosmarinic acid per l |
| Standard 3 | 100 mg rosmarinic acid per l |

Anyhow, the straight calibration lines of both peaks reached correlation coefficients at r = 0,999 (peak 1) and r = 0,998 (peak 2).

For analyzing rosmarinic acid with the TEC, the concentrations had been the same. The calibration line was very good, as well.

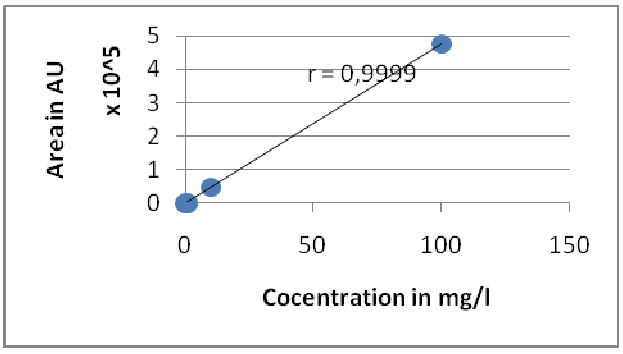



**Figure 43: Straight calibration line for rosmarinic acid peak 1 (CPC)**

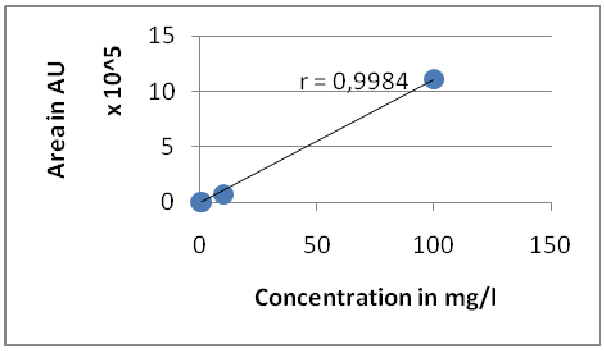



**Figure 44: Straight calibration line for rosmarinic acid peak 2 (CPC)**

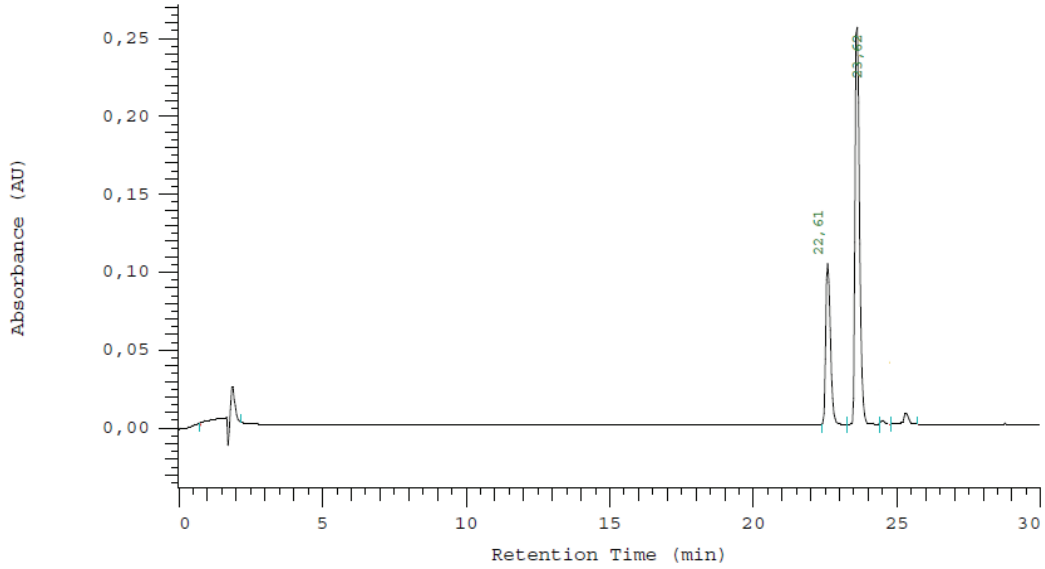



**Figure 45: Chromatogram of rosmarinic acid (100 mg/l) at 280 nm (CPC)**

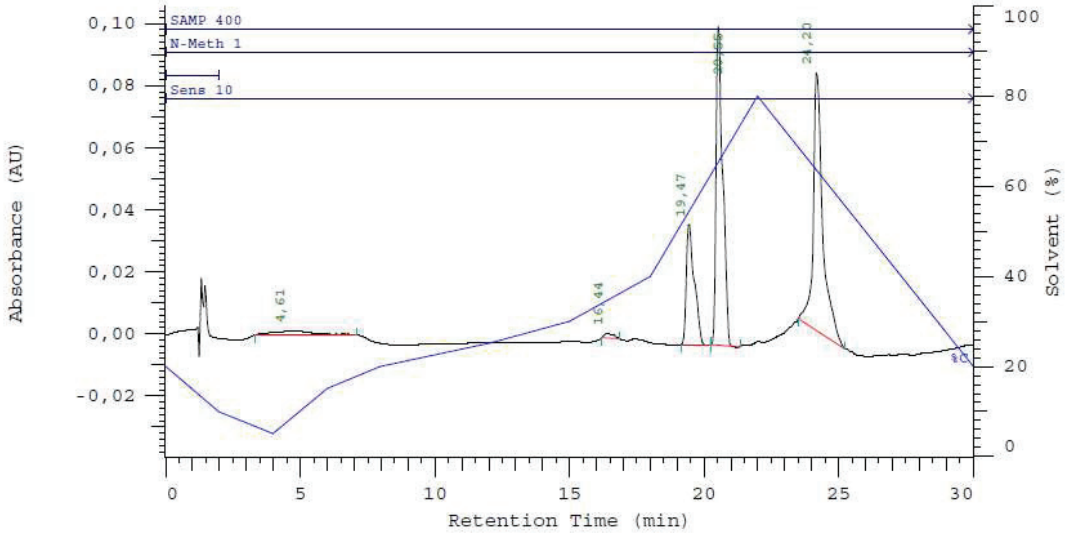



**Figure 46: Chromatogram of rosmarinic acid (100 mg/l) at 280 nm (TEC)**

The phenomenon of filigree peaks by analysing ferulic acid with the CPC is the same as found with dissecting rosmarinic acid. With the TEC the standard mixture was eluted at 19,47 min and 20,55 min, respectively. Using the CPC rosmarinic was detected at 2,61 min and 23,62 min.

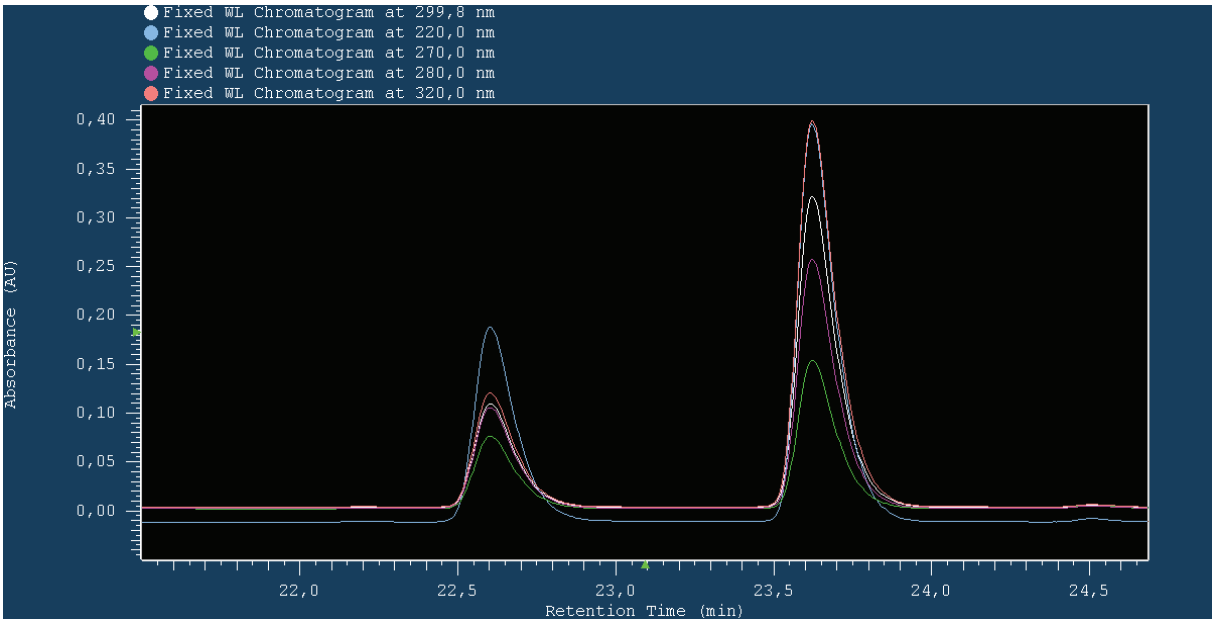



**Figure 47: Peaks of rosmarinic acid at 220, 270, 280, 300 and 320 nm (CPC)**

Figure 47 shows that the absorbance at 280 nm is located in the medium region. In using 320 nm for evaluating both peaks, a higher concentration level could have been obtained. As with ferulic acid, both rosmarinic acid peaks are close together. The differences of their peak optima are only around 1 min. The first peak is around half the height of the second one. But as shown in figure 48 & 49, the spectra of the peaks show a specific correlation. For evaluation of the concentrations in the juice samples, both peaks areas for rosmarinic acid were added. The same conditions were applied for determining the concentrations of the juice samples, analyzed with the TEC. The spectra curves were identical to the ones in figure… and … .

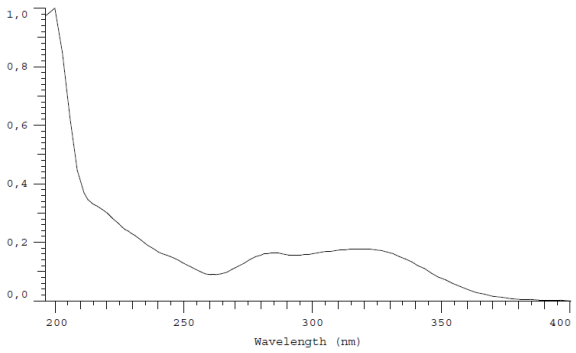



**Figure 48: Spectrum of rosmarinic acid at RT 22,61 min (CPC)**

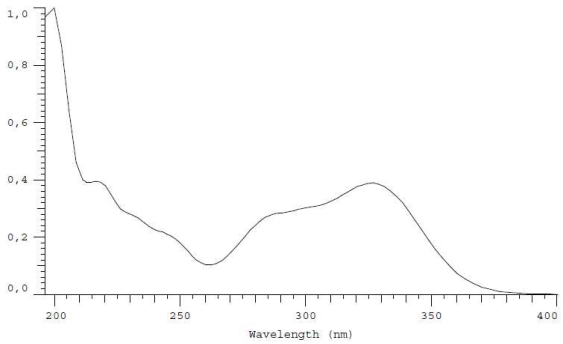



**Figure 49: Spectrum of rosmarinic acid at RT 23,62 min (CPC)**

*Sinapic Acid*

For the detection of sinapic acid, the same standard concentrations were used as those used to identify rosmarinic acid. At 1 mg/l, only one peak was detected around RT 23,62 min. At the concentrations 10 and 100 mg /l, two peaks (RT 21,50 and 22,19 min) were observed (figure 52). Both correlation coefficients reached very high values, as seen in the figures below.

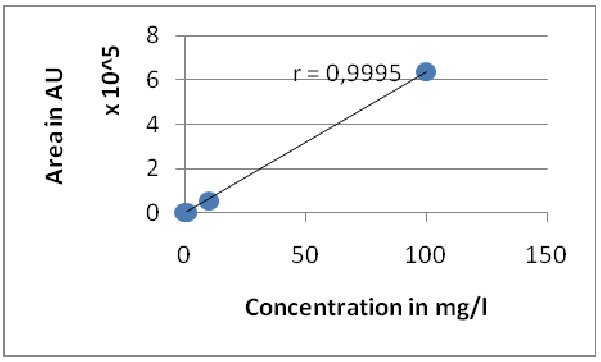



**Figure 50: Straight calibration line for sinapic acid peak 1 (CPC)**

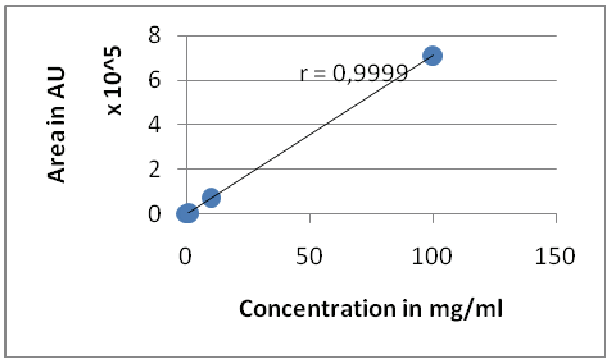



**Figure 51: Straight calibration line for sinapic acid peak 2 (CPC)**

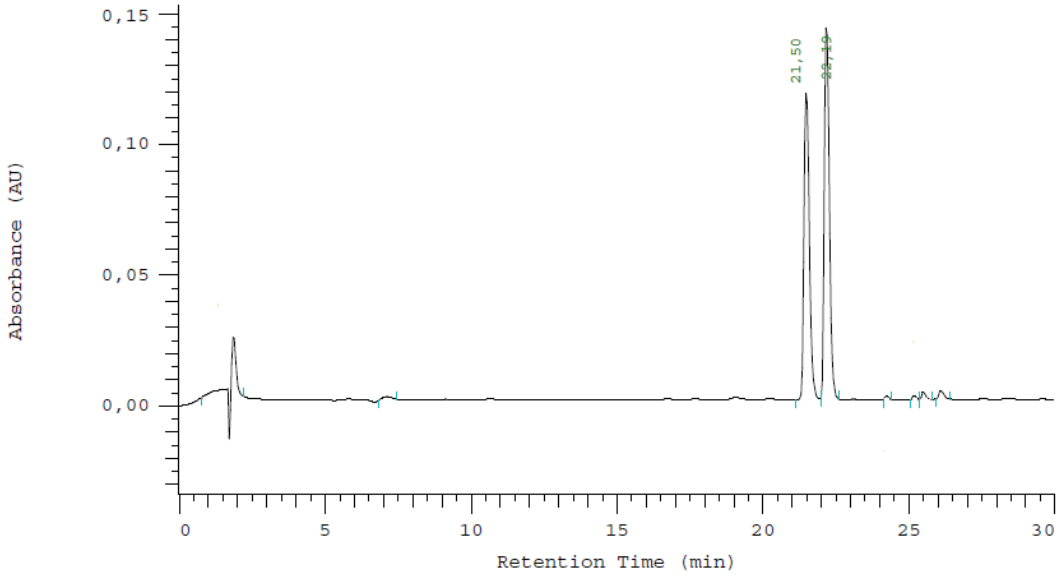



**Figure 52: Chromatogram of sinapic acid (100 mg/l) at 280 nm (CPC)**

In analyzing sinapic acid with the TEC, two peaks were observed, as well (figure 53). It is conspicuous that both peaks offer the shape of a shoulder. In contrast to the peaks seen in figure 52, the peaks detected with TEC are approximately of the same height and also width. Once again, the correlation coefficients were very good.

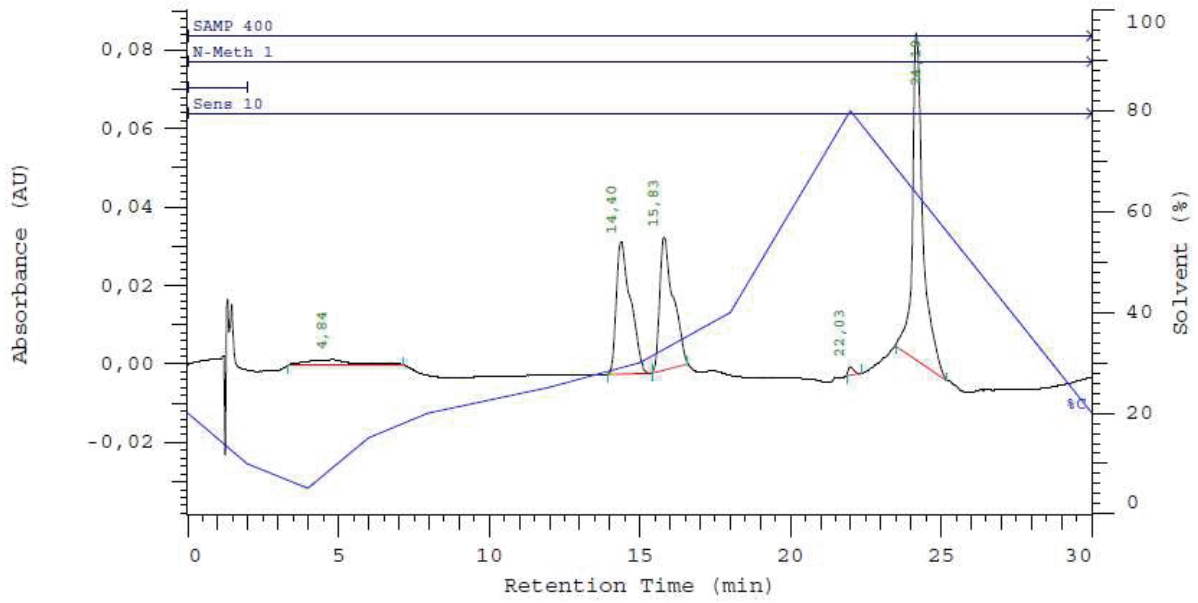



**Figure 53: Chromatogram of sinapic acid (100 mg/l) at 280 nm (TEC)**

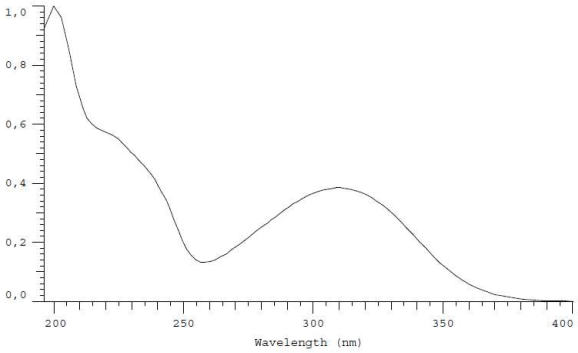



**Figure 54: Specrum of sinapic acid at RT 21,50 min (CPC)**

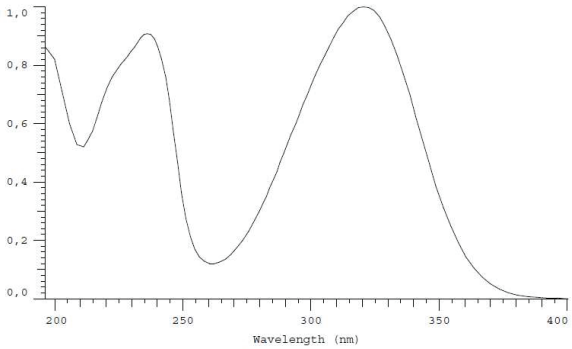



**Figure 55: Spectrum of sinapic acid at RT 22,19 min (CPC)**

The spectra curves seen in figure….are different from each other.

The retention times of rosmarinic acid and sinapic acid are very close together, but through analysis of the spectra it was possible to distinguish both acids in the juice samples. But because of the possibility that peaks of ferulic acid could also be representative of sinapic acid it must be concluded that the concentrations of sinapic acid cannot be identified clearly. Therefore, this acid is counted among the phenolic compounds, as well.

The spectra of the sinapic peaks, measured with TEC, were completely different from those shown in figures 54 & 55. Both curves display nearly similar shape. Therefore, both peaks were used for evaluating the concentrations of sinapic acid in the juice mixtures.

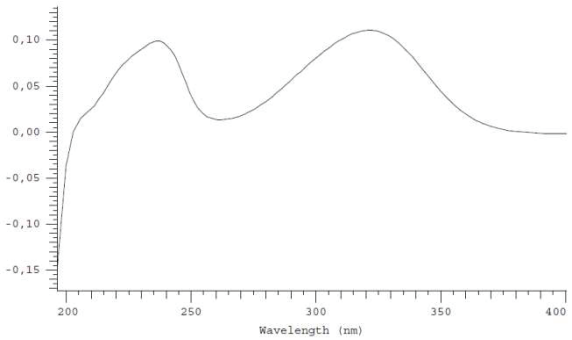



**Figure 56: Specrum of sinapic acid at RT 14,40 min (TEC)**

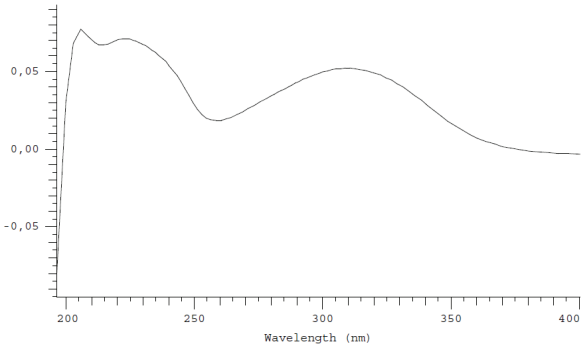



**Figure 57: Specrum of sinapic acid at RT 15,83 min (TEC)**

*Chlorogenic Acid*

For chlorogenic acid, the same standard concentrations were used as for cinnamic acid. Also this substance offered two peaks after HPLC analysis at 280 nm. In comparison to all other acids, which showed two peaks, the chlorogenic acid peaks are not split at the baseline and the second peak is smaller than the first one. Because of their unequal spectra curves, both peaks were treated as two different ones (figure 60). As with all other standard substances, chlorogenic acid also showed perfect correlation coefficients.

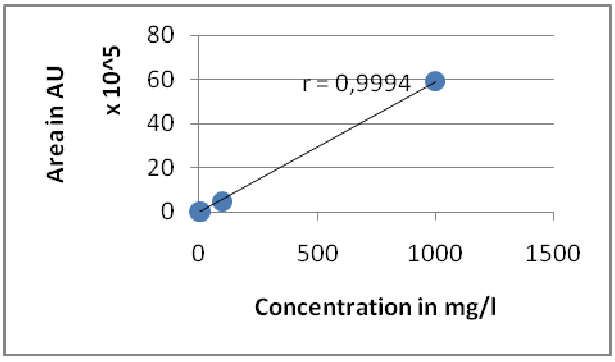



**Figure 58: Straight calibration line for chlorogenic acid peak 1 (CPC)**

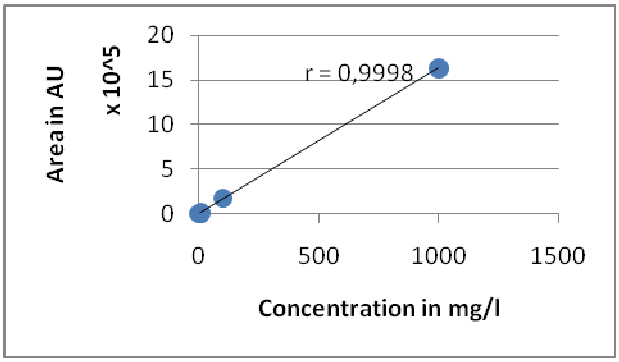



**Figure 59: Straight calibration line for chlorogenic acid peak 2 (CPC)**

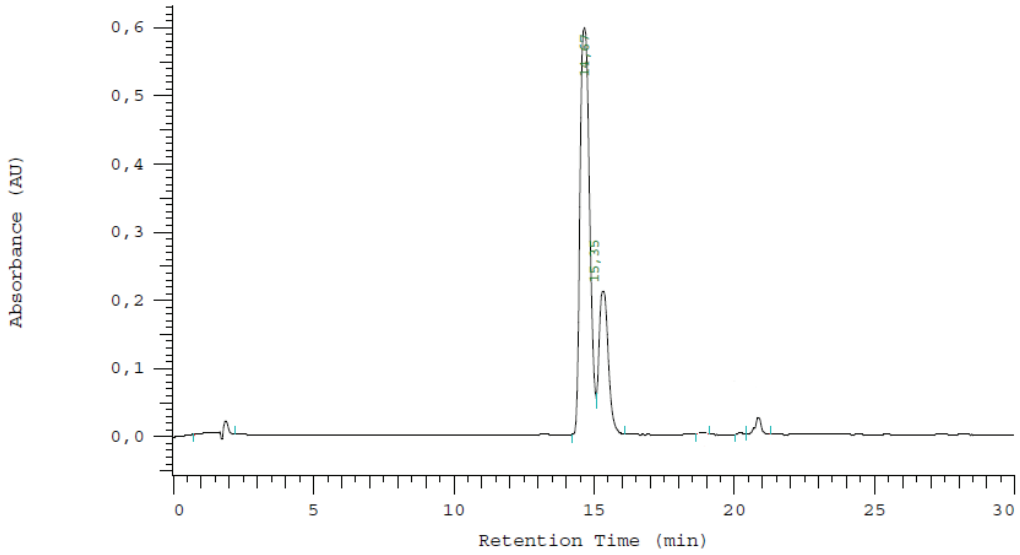



**Figure 60: Chromatogram of chlorogenic acid (1000mg/l) at 280 nm (CPC)**

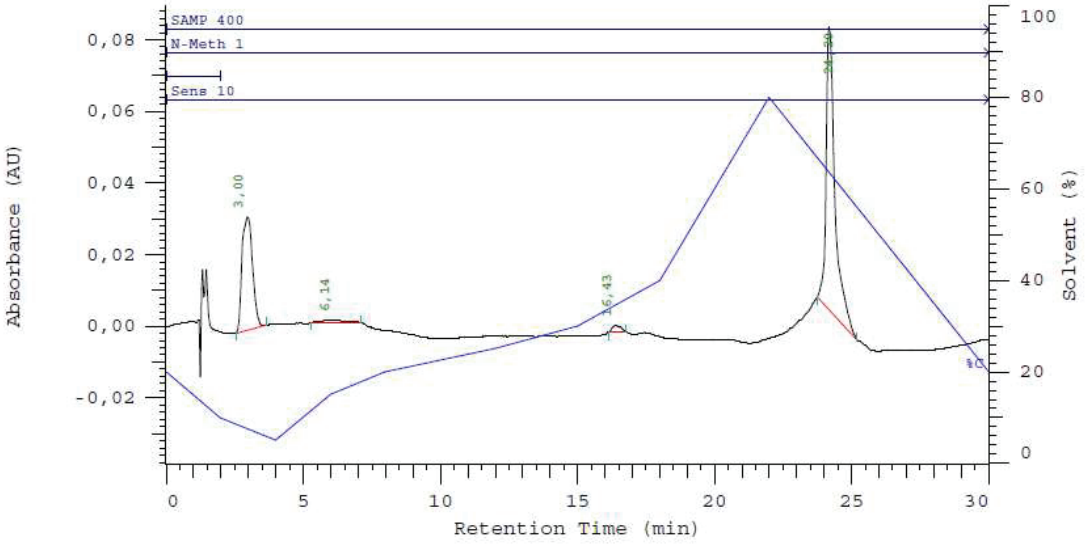



**Figure 61: Chromatogram of chlorogenic acid (1.000 mg/l) at 280 nm (TEC)**

With the TEC, there was only one peak detected for chlorogenic acid (figure below). The RT was around 3 min. Also the spectrum curve was different from those,analyzed with the CPC.

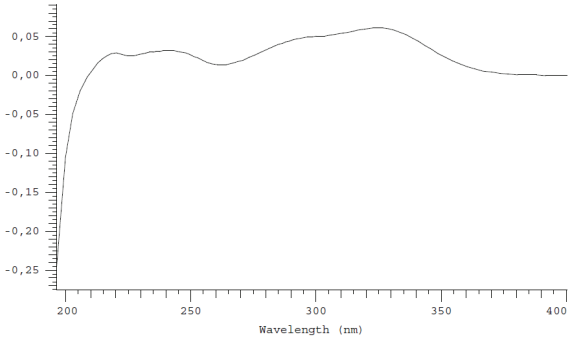



**Figure 62: Spectrum of chlorogenic acid at RT 3 min (TEC)**

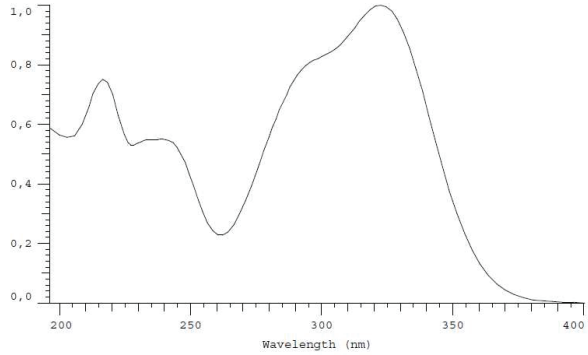



**Figure 63: Spectrum of chlorogenic acid at RT 14,67 min (CPC)**

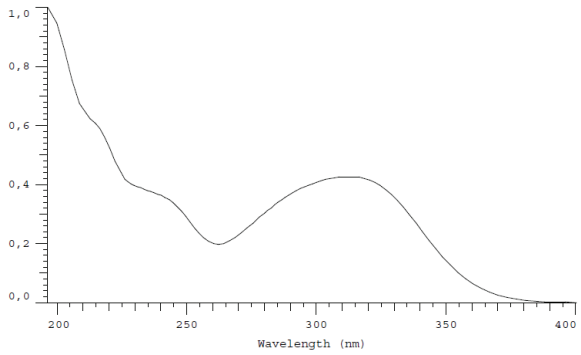



**Figure 64: Spectrum of chlorogenic acid at RT 15,35 min (CPC)**

The spectrum at RT 14,67 displays the same shape than caffeic acid. Chlorogenic acid is made of a unit of both caffeic acid and china acid. Due to that it seems that chlorogenic acid was split in caffeic acid and china acid. For evaluating the concentrations of chlorogenic acid in natural mediums, both peak areas were summated together.

The spectrum of chlorogenic acid at 15, 35 min also presents similarly as the spectrum of sinapic acid at RT 21,50 min. In figure 65, the spectra curves of chlorogenic acid can be recognized very well.

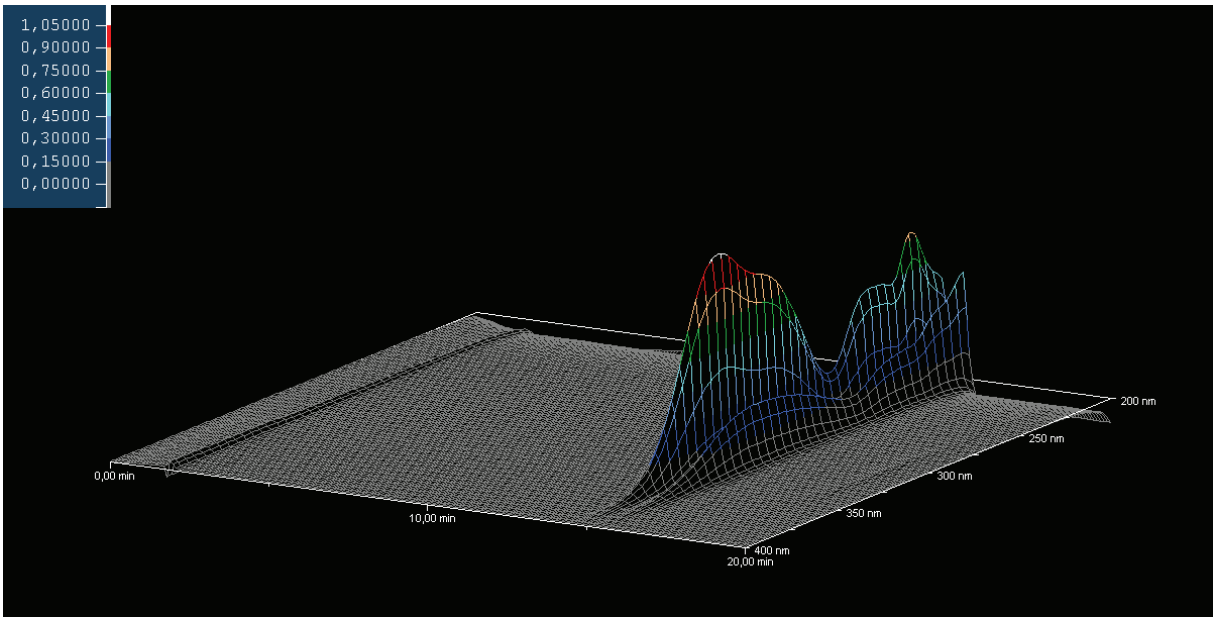



**Figure 65: 3-D spectrum of chlorogenic acid (CPC)**

*Minimal medium and biomass*

A minimal yeast fermented medium was analyzed to see if yeasts produce phenolic acids during fermentation.

The following figure offers that no cinnamic acid derivatives were detected with the TEC. The same results were reached with the CPC (data not shown).

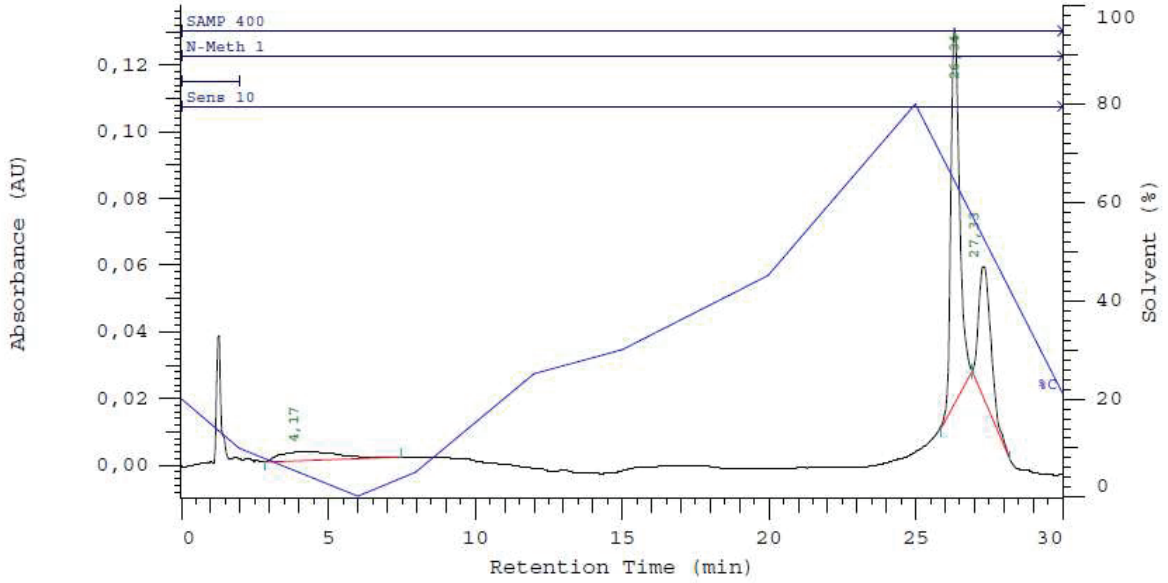



**Figure 66: Chromatogram of minimal medium detected at 280 nm, unconcentrated (TEC)**

The figure…shows the chromatogram of the concentrated minimal medium. Like in the unconcentrated medium, no peaks of hydroxycinnamic acids were detected.

Also a repeat analysis of the unconcentrated and concentrated medium did not show any other results (figures 94 & 95, appendix).

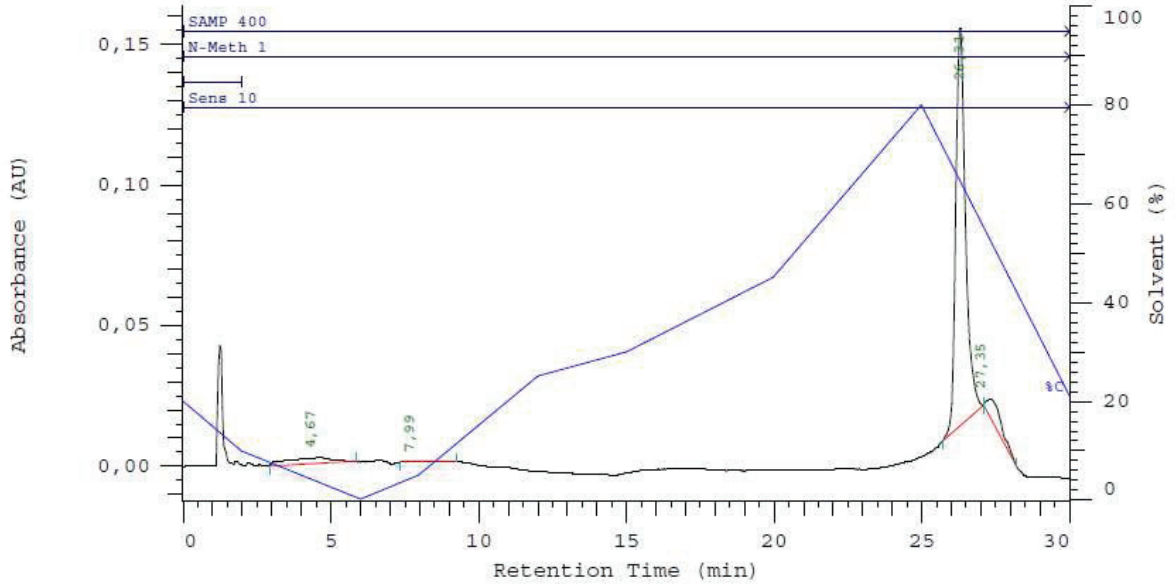



**Figure 67: Chromatogram of minimal medium detected at 280 nm, concentrated 3x (TEC)**

The following table shows the results of the remained biomass from the juice fermentation. The biomass was concentrated until mass was constant.

**Table 11: Balanced biomass in g (value includes the Eppendorf-tube contains the biomass)**

| Number of tubes | Blueberry | Cranberry | Apple | Sea buckthorn berry |
|---|---|---|---|---|
| 1 | 1,0288 | 1,0378 | 1,0389 | 1,0442 |
| 2 | 1,0193 | 1,0383 | 1,0259 | 1,0321 |
| 3 | 1,0248 | 1,0277 | 1,0373 | 1,0367 |
| 4 | 1,0283 | 1,0386 | 1,0387 | 1,0298 |
| 5 | 1,0217 | 1,0337 | 1,0295 | 1,0376 |
| 6 | 1,0236 | 1,0296 | 1,0283 | 1,0391 |
| 7 | 1,0199 | 1,0419 | 1,0344 | 1,0399 |
| 8 | 1,0321 | 1,0429 | 1,0239 | 1,0289 |
| 9 | 1,0196 | 1,0234 | 1,0412 | 1,0341 |
| 10 | 1,0232 | 1,0374 | 1,0328 | 1,0438 |
| 11 | 1,0387 | 1,0249 | 1,0324 | 1,0421 |
| 12 | 1,0165 | 1,0184 | 1,0282 | 1,0357 |
| 13 | 1,0314 | 1,0379 | 1,0249 | 1,0369 |
| 14 | 1,0237 | 1,0412 | 1,0388 | 1,0371 |
| 15 | 1,0269 | 1,0363 | 1,0436 | 1,0384 |
| **Average** | **1,0252** | **1,0340** | **1,0333** | **1,0371** |

It is seen, that the biomass of the fermented blueberry juice achieved the lowest value of all four juices. The yeast cells of apple juice, cranberry juice and sea buckthorn berry juice offered 1,0333 g, 1,0340 g and 1,0371 g, respectively. The discrepancy from the blueberry fermented biomass to the yeast cells of the other fermented juices is approximately 0,0080 g.

### 4.3 Juice Samples

In the following chapter, chromatograms of differently treated juices, analyzed with the CPC, show the presence of cinnamic acid and its derivatives.

All samples were detected at 280 nm.

As described in the chapter of standard substances, the acids ferulic, sinapic, coumaric and caffeic acid cannot distinguished as such. In the following section, the concentrations of these substances are described as “phenolic compounds”.

Because of faulty results, caused on the damaged lamp in the DAD, the data, measured with the TEC are not published in the following part.

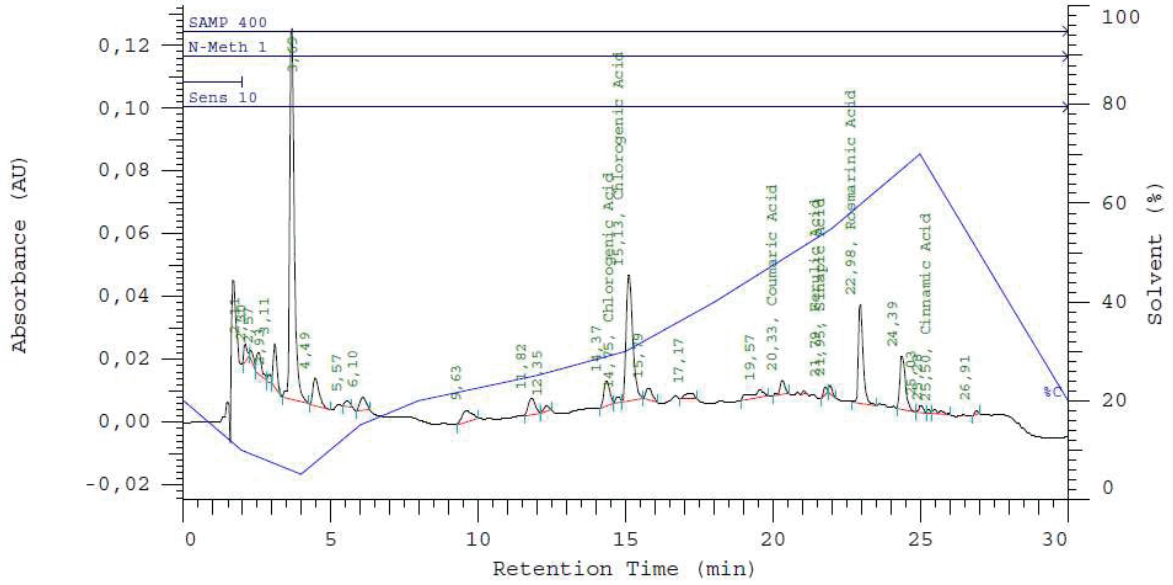



**Figure 68: Chromatogram of blueberry juice – unfermented, unconcentrated**

In the first chromatogram blueberry juice was directly analyzed from the juice box, without any treatment. Predominantly, chlorogenic acid and rosmarinic acid were found, with concentration of 164,62 mg/l and 29,26 mg/l, respectively. The other phenolic acids, except cinnamic acid, accounted for 2,89 mg/ml. Cinnamic acids offered 0,39 mg/l.

A replication of this analysis showed 62,88 mg/l chlorogenic acid, 29,96 mg/l rosmarinic acid, 0,75 mg/l cinnamic acid and 3,72 mg/l for the rest of the hydroxycinnamic acids. All concentrations but rosmarinic acid were either much higher or quite lesser.

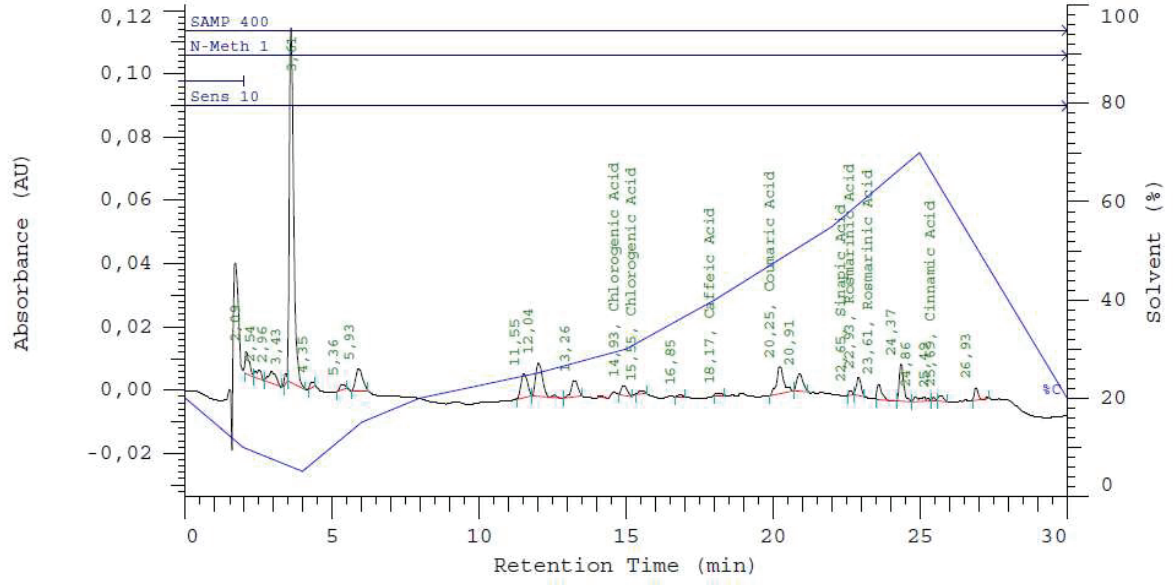



**Figure 69: Chromatogram of cranberry juice – unfermented, unconcentrated**

The analysis of untreated cranberry juice showed fewer concentrations then the blueberry juice. The content of chlorogenic acid amounted to 6,57 mg/l. The combined phenolic compounds could reached 4,59 mg/l.

The second attempt showed approximately double the concentration for chlorogenic acid (11,47 mg/l). The phenolic compounds achieved 3,20 mg/l.

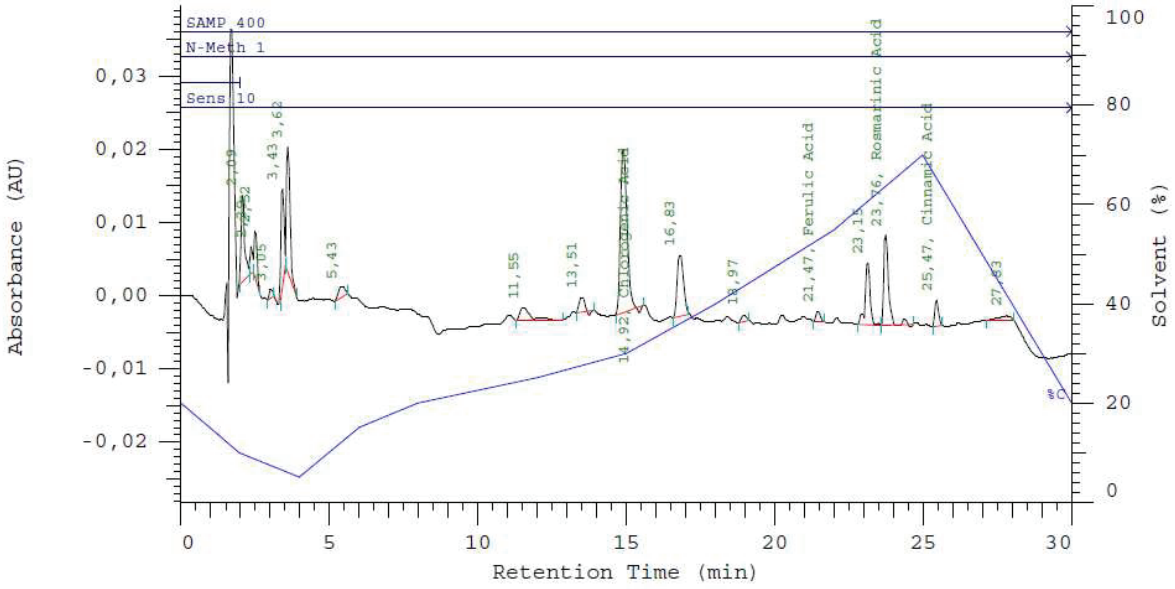



**Figure 70: Chromatogram of apple juice – unfermented, unconcentrated**

The pure apple juice offered a concentration of 24,54 mg/l for chlorogenic acid an 4,68 mg/l for rosmarinic acid. Cinnamic acid showed a concentration of 0,61 mg/l.

The duplication received around 10 mg/l more for chlorogenic acid (34,63 mg/l). Only one fifth was analyzed for cinnamic acid (0,58 mg/l) after the second pass.

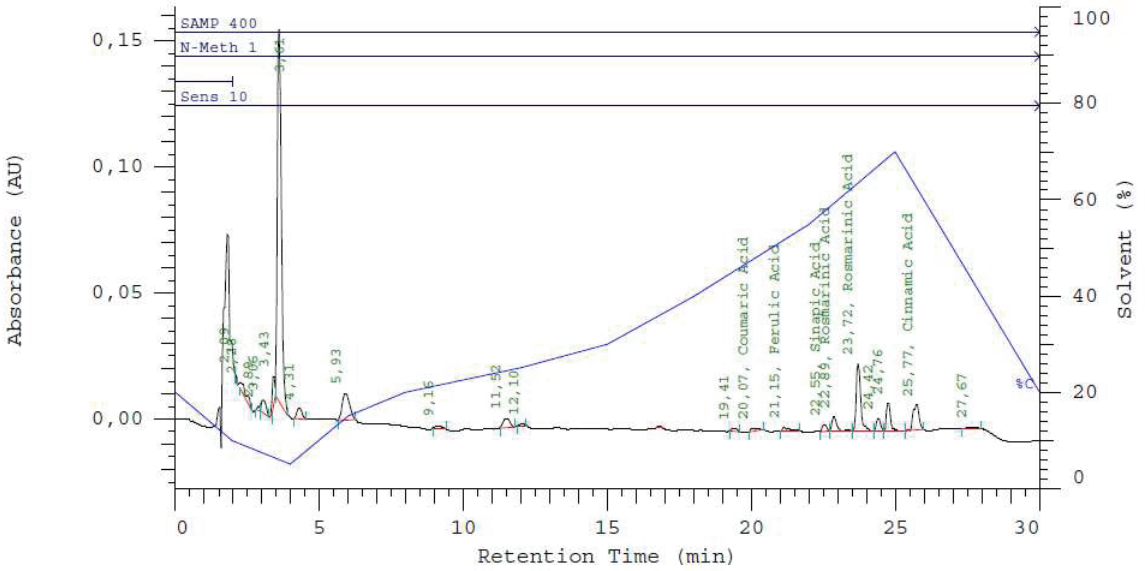



**Figure 71: Chromatogram of sea buckthorn berry juice – unfermented, unconcentrated**

In figure 71, it is seen, that no chlorogenic acid was detected. The major component in sea buckthorn berry juice was rosmarinic acid with a concentration of 16,60 mg/l. Cinnamic acid had reached 3,05 mg/l, similar to the phenolic compounds with 3,07 mg/l.

The second analysis reached 2,72 mg/l for the phenolic compounds and 2,90 mg/l for cinnamic acid. The concentration of rosmarinic acid was 16,18 mg/l. Comparing the particular data with each other, the duplication resulted in a good reproducibility.

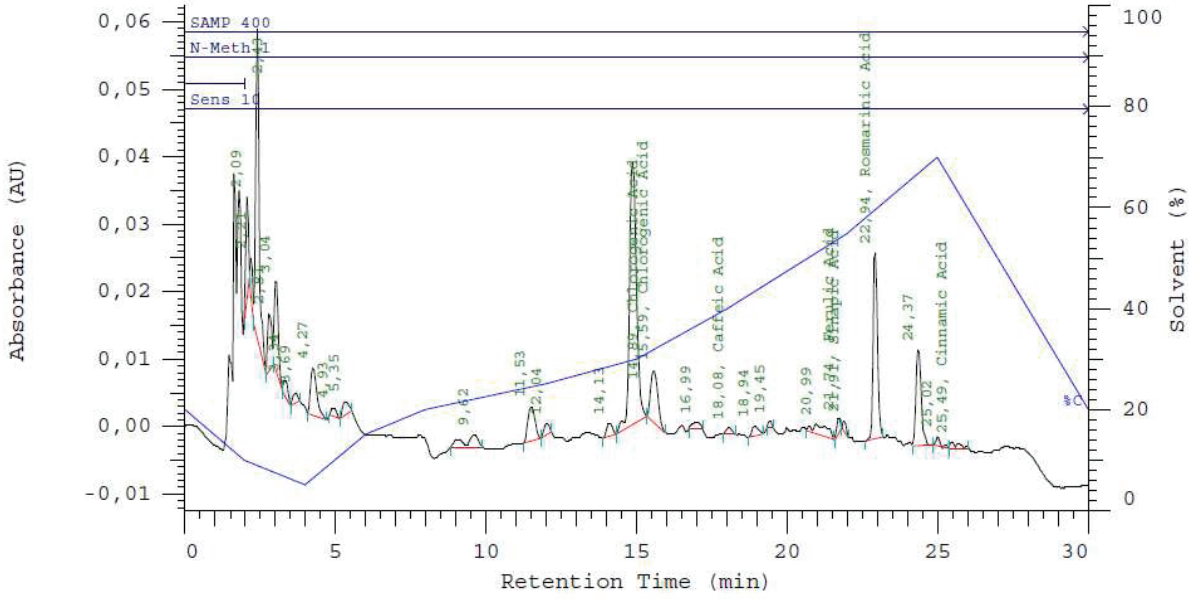



**Figure 72: Chromatogram of blueberry juice – fermented, unconcentrated**

As in the unfermented and unconcentrated blueberry juice, chlorogenic acid and rosmarinic acid were the major components with 79,96 mg/l and 23,20 mg/l, respectively. Compared to the unfermented blueberry juice, the concentrations were reduced. The second analysis showed concentrations of 103,15 mg/l for chlorogenic acid and 28,03 mg/l for rosmarinic acid. Conversely rosmarinic acid had the same level found in the unfermented juice. The values for chlorogenic acid displayed a wide range, as seen in figure 72. The concentrations for the other phenolic compounds increased with the duplication, from 1,67 mg/l to 2,25 mg/l.

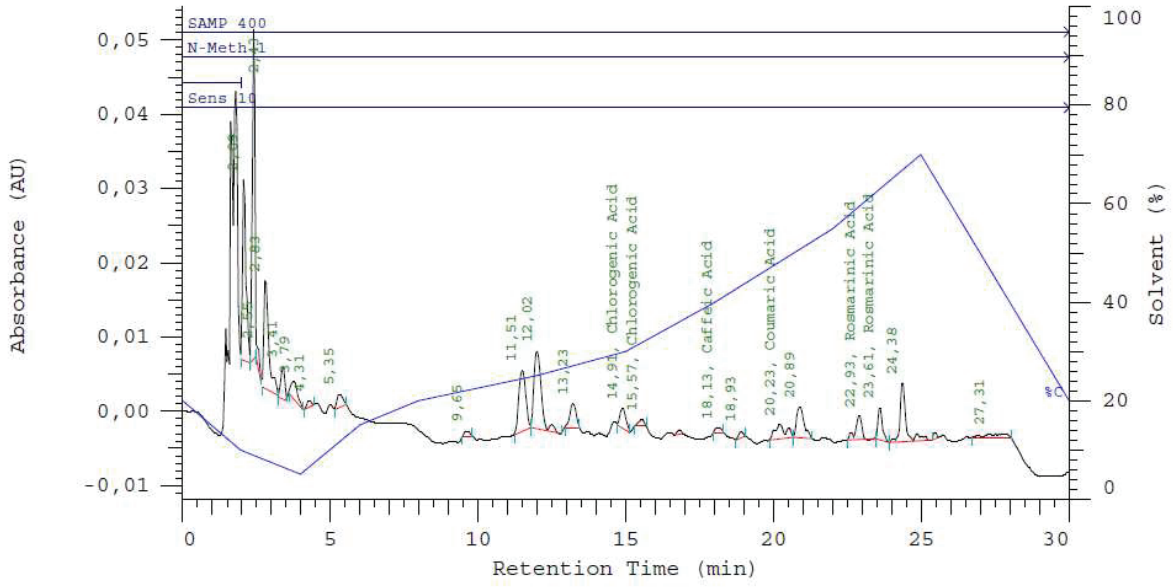



**Figure 73: Chromatogram of cranberry juice – fermented, unconcentrated**

The fermented unconcentrated cranberry juice achieved concentrations of 5,85 mg/l for chlorogenic acid, 5,82 mg/l for rosmarinic acid and 1,52 mg/l for the other phenolic compounds. Cinnamic acid could be not found in both analyses. A concentration of 8,96 mg/l for chlorogenic acid and 4,10 mg/l for rosmarinic acid were seen in the second run.

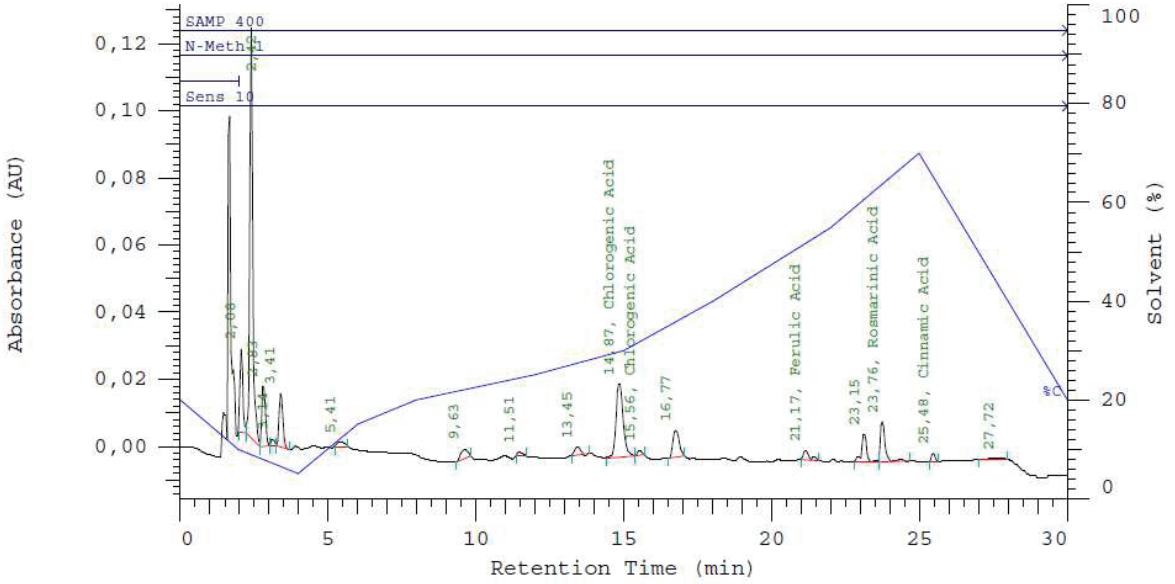



**Figure 74: Chromatogram of apple juice – fermented, unconcentrated**

With a concentration of 30,39 mg/l for chlorogenic acid, it was one fifth more than that found in the unfermented apple juice. Rosmarinic acid offered a value of 4,42 mg/l and cinnamic acid of 0,47 mg/l. The replication showed a concentration of 4,23 mg/l for rosmaric acid and 0,45 mg/l for cinnamic acid. For that reason, a good reproducibility could be reached, for both of these acids.

The value for chlorogenic acid offered around 2 mg/l more (32,13 mg/l) in the second run.

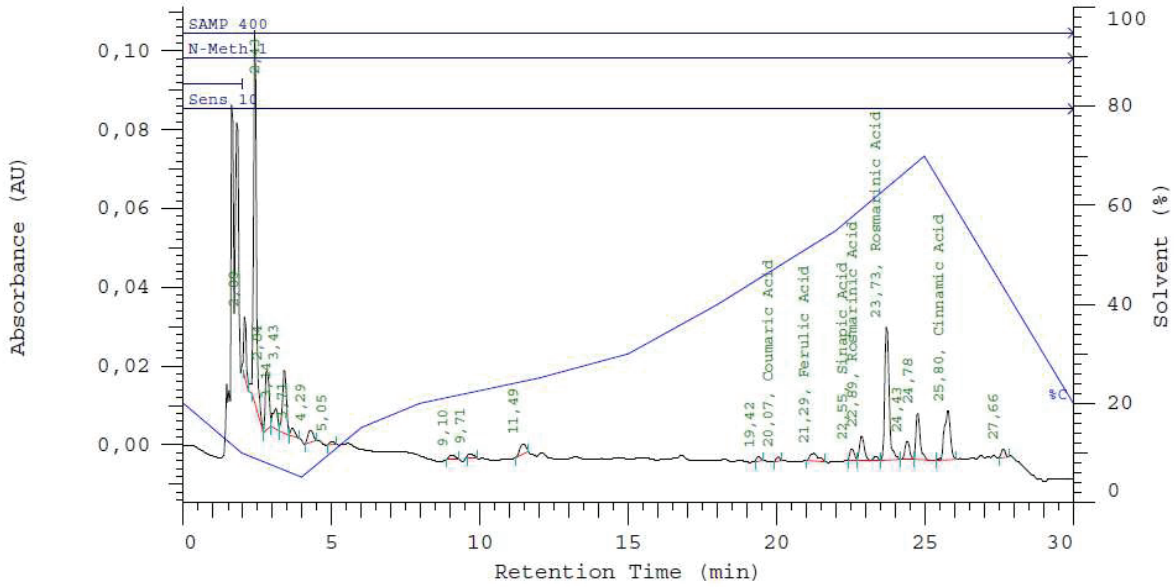



**Figure 75: Chromatogram of sea buckthorn berry juice – fermented, unconcentrated**

As already seen in the chromatogram of the unfermented sea buckthorn berry juice, no chlorogenic acid was found. The major component, rosmarinic acid, could achieve a concentration of approximately 20 mg/l. In contrast, cinnamic acid was presented at 3,56 mg/l, as in the unfermented and unconcentrated juice. The duplication showed values of 18,97 mg/l and 3,59 mg/l for rosmarinic acid and cinnamic acid, respectively. Apart from the concentrations of the phenolic compounds (1.: 3,42 mg/l; 2. try: 2,31 mg/l), the second run proved to be highly replicative.

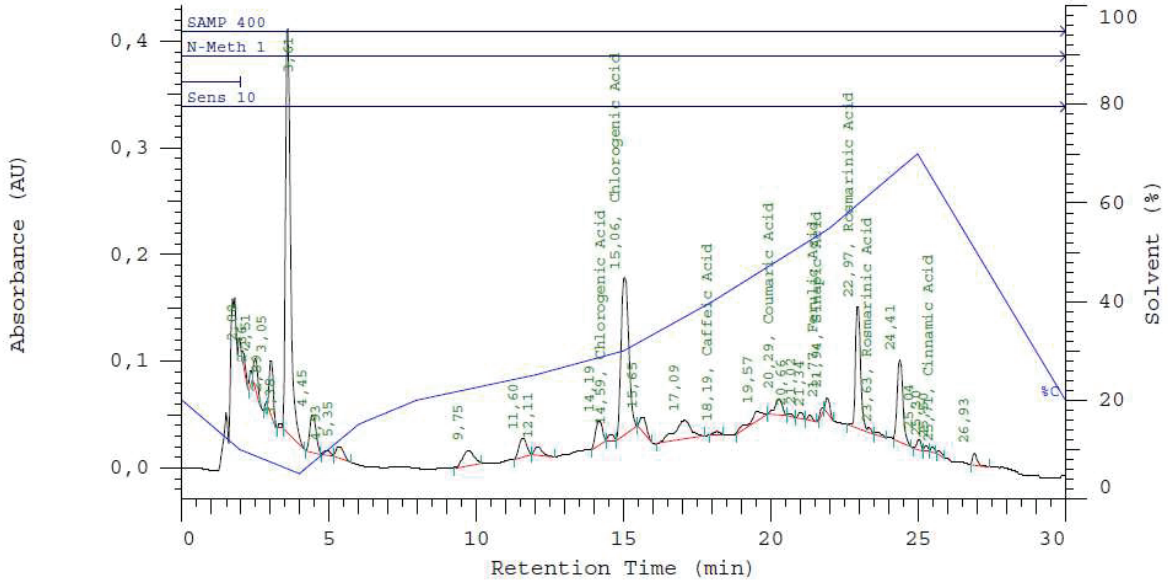



**Figure 76: Chromatogram of blueberry juice – unfermented, concentrated (2x)**

The double concentrated and unfermented blueberry juice contained high concentrations of its major components chlorogenic acid and rosmarinic acid. With values of 728,61 mg/l and 125,41 mg/l, the concentrations were more then four times as high, compared with the unfermented and unconcentrated blueberry juice. With 16,90 mg/l for the phenolic compounds, the increase was quintuplicate.

The duplication offered a lower concentration for rosmarinic acid (113,93 mg/l). The concentration for chlorogenic acid reduced to 74,09 mg/l. The phenolic compounds were observed to rise up to 17,44 mg/l. The concentration of 0,88 mg/l for cinnamic acid increased to 1,19 mg/l in the repeated analysis.

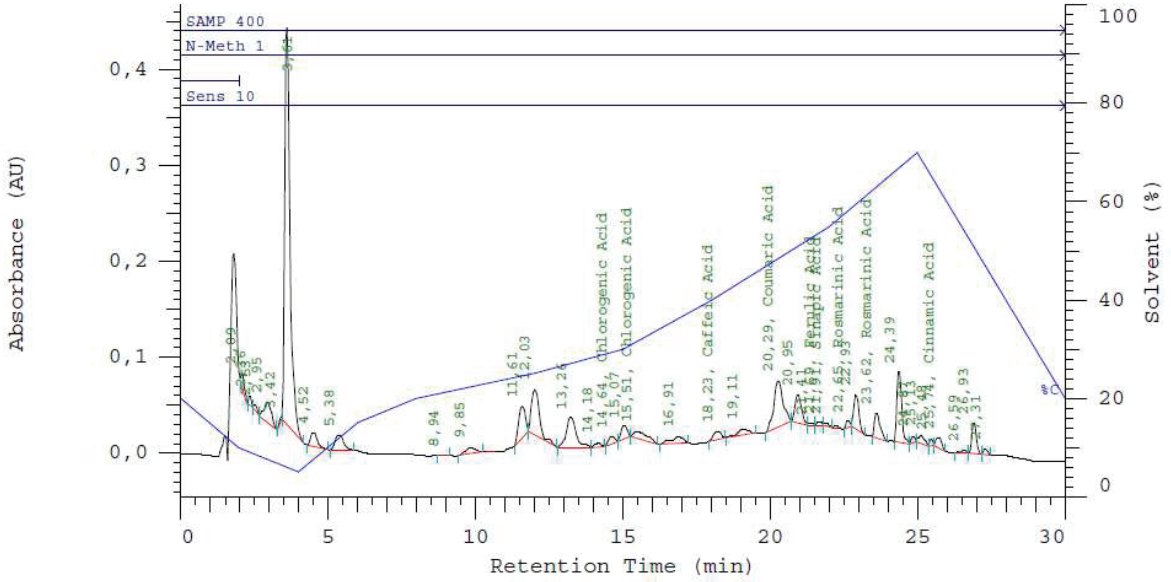



**Figure 77: Chromatogram of cranberry juice – unfermented, concentrated (2x)**

Compared to the unfermented and unconcentrated cranberry juice, the concentrated one displayed higher concentrations for chlorogenic acid (16,78 mg/l), rosmarinic acid (18,69 mg/l), cinnamic acid (2,94 mg/l) and the combined phenolic compounds (32,42 mg/l).

With the second HPLC analysis, a much higher concentration for chlorogenic acid was seen. The levels for cinnamic acid, rosmarinic acid and the phenolic compounds nearly remained constant.

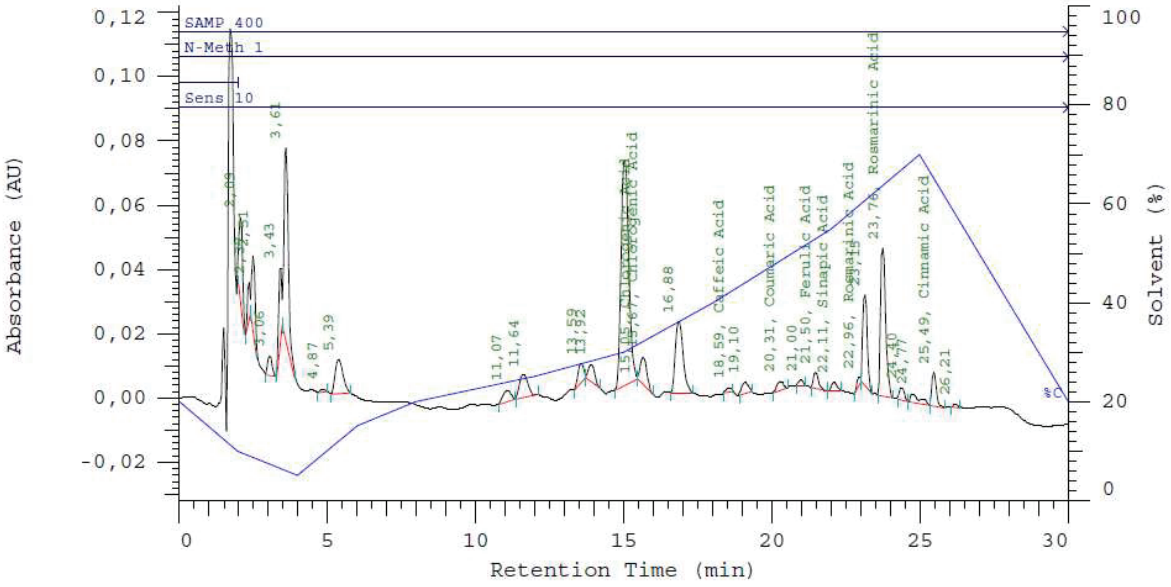



**Figure 78: Chromatogram of apple juice – unfermented, concentrated (2x)**

The concentrations for the concentrated apple juice were also higher, as in all other concentrated juices. From chlororgenic acid to cinnamic acid over to rosmarinic acid and finally the phenolic compounds, much higher values were observed (figure 78).

The duplication showed different results then the first analysis, but also achieved higher concentration compared to the unconcentrated apple juice.

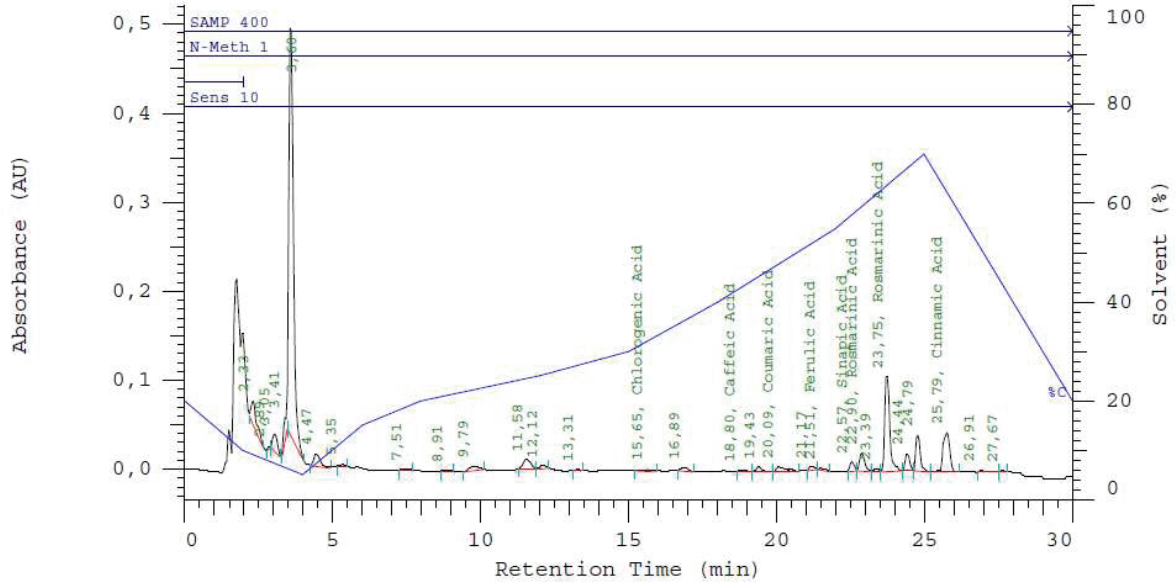



**Figure 79: Chromatogram of sea buckthorn berry juice – unfermented, concentrated (2x)**

For the first time, the concentrated version of sea buckthorn berry juice offered a low level of chlorogenic acid (9,28 mg/l). But still, the major component in sea buckthorn berry juice was present as rosmarinic acid (67,98 mg/l). But also all other hydroxycinnamic acids increased, compared to the unconcentrated juice. Similarly the replication showed the same results.

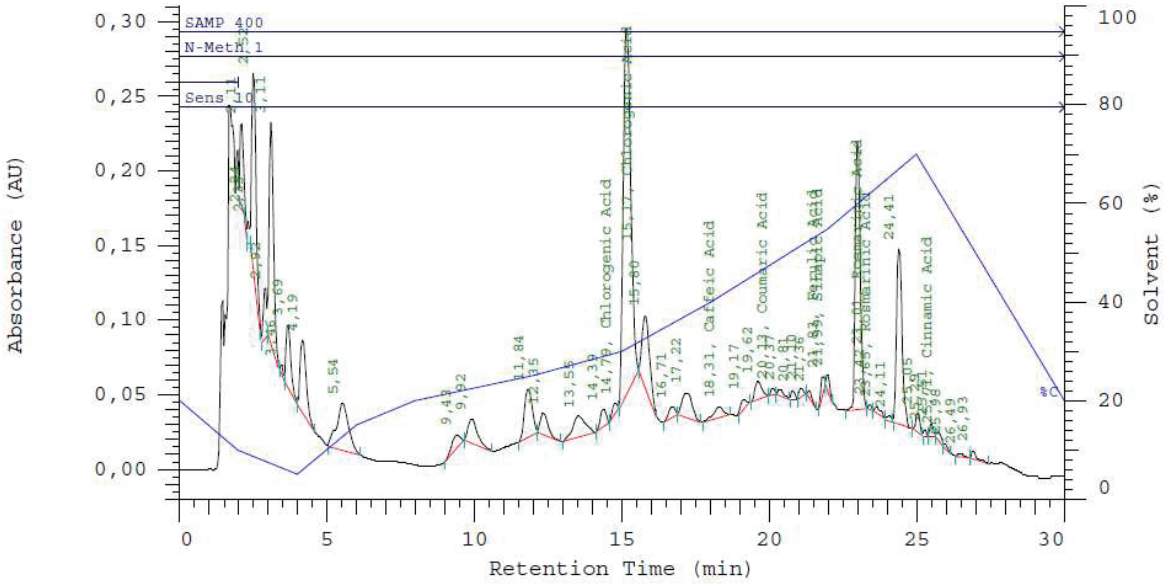



**Figure 80: Chromatogram of blueberry juice – fermented, concentrated (3x)**

As seen in figure 80, the major components of blueberry juice are still chlorogenic acid and rosamarinic acid. Due to a three-fold higher concentration the value for chlorogenic acid and rosmarinic acid increased to almost 2200 mg/l and 187,76 mg/l, in each case. Also the concentration of cinnamic acid rises from 0,88 mg/l to 1,31 mg/l.

In contrast, the second analysis offered only a concentration of 30,13 mg/l for chlorogenic acid. For the other cinnamic derivatives, the concentrations were either similar or rather higher.

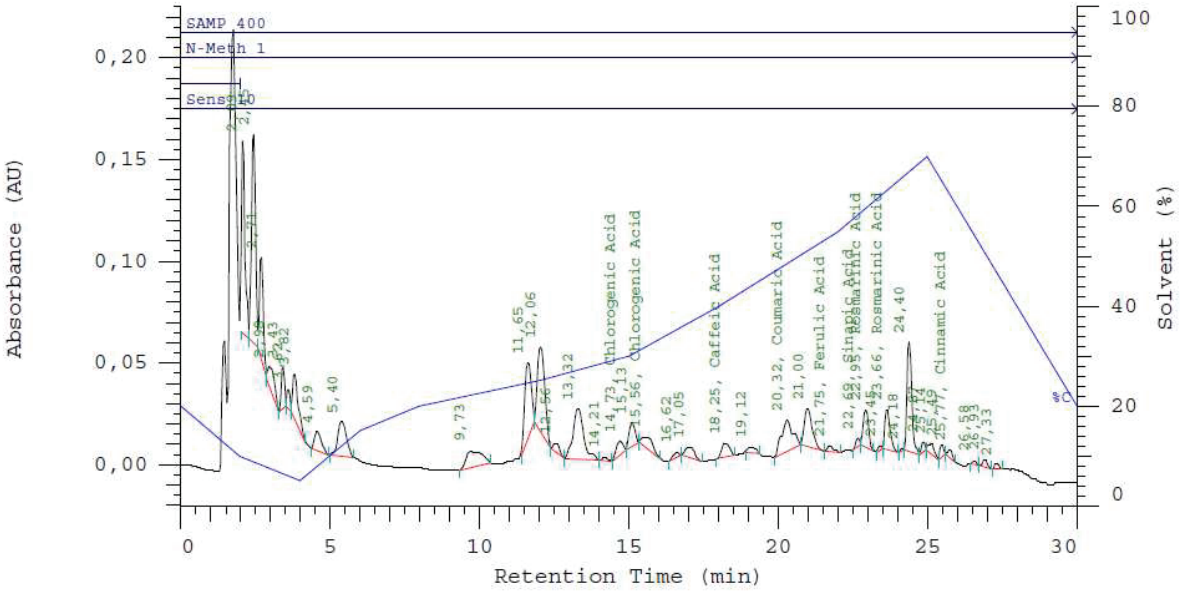



**Figure 81: Chromatogram of cranberry juice – fermented, concentrated (3x)**

The chromatogram in figure 81 shows increased concentrations for chlorogenic and rosmarinic acid, compared to the double concentrated unfermented cranberry juice. On the other hand, the analysed concentrations for cinnamic acid and the phenolic compounds were reduced. The duplication achieved the same results as the first analysis.

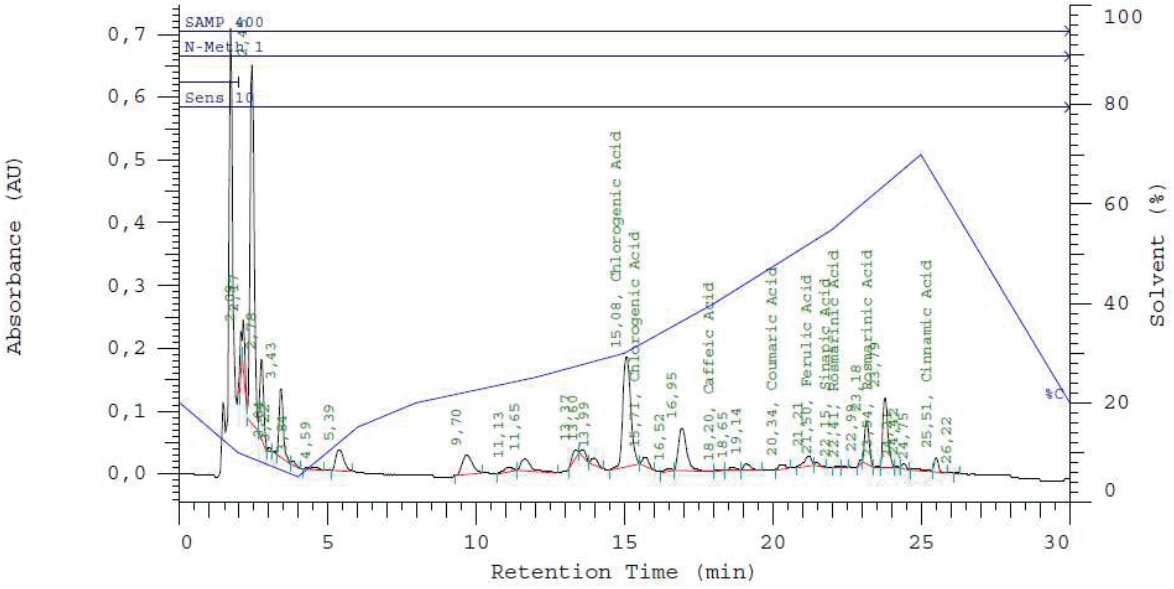



**Figure 82: Chromatogram of apple juice – fermented, concentrated (3x)**

The apple juice ranked second highest as compared to all other fermented and concentrated juices, with reference to the concentration for chlorogenic acid (306,31 mg/l). However, the values for rosmarinic acid are the lowest of these four juices (1,46 mg/l). Cinnamic acid achieved 4,49 mg/l, the remaining phenolic compounds had 6,59 mg/l.

The replication showed well reproduced concentrations for the fermented and triple concentrated apple juice.

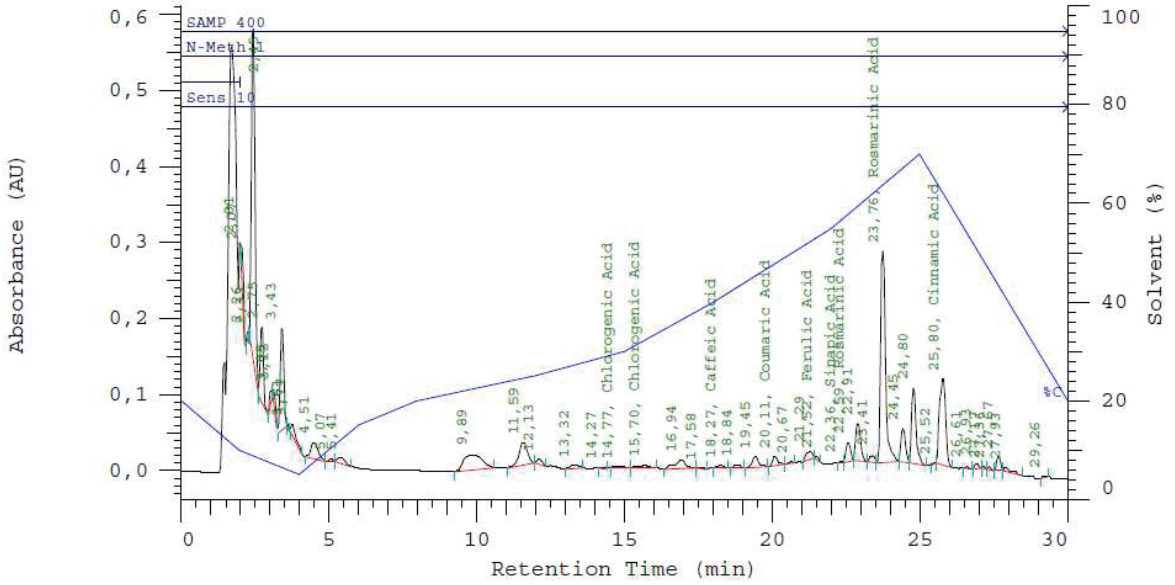



**Figure 83: Chromatogram of sea buckthorn berry juice – fermented, concentrated (3x)**

With the triplicate concentration, sea buckthorn berry juice showed a concentration of 23,20 mg/l for chlorogenic acid. This was the highest concentration of this cinnamic acid derivative as measured for sallow thorn juice. As predicted, the data for rosmarinic acid rose up to 141.81 mg/l. With 33,80 mg/l, cinnamic acid displayed its highest concentration of all juices and analyses that were done for this work.

With discrepancies of few mg/l, the duplication reached the same results as described above.

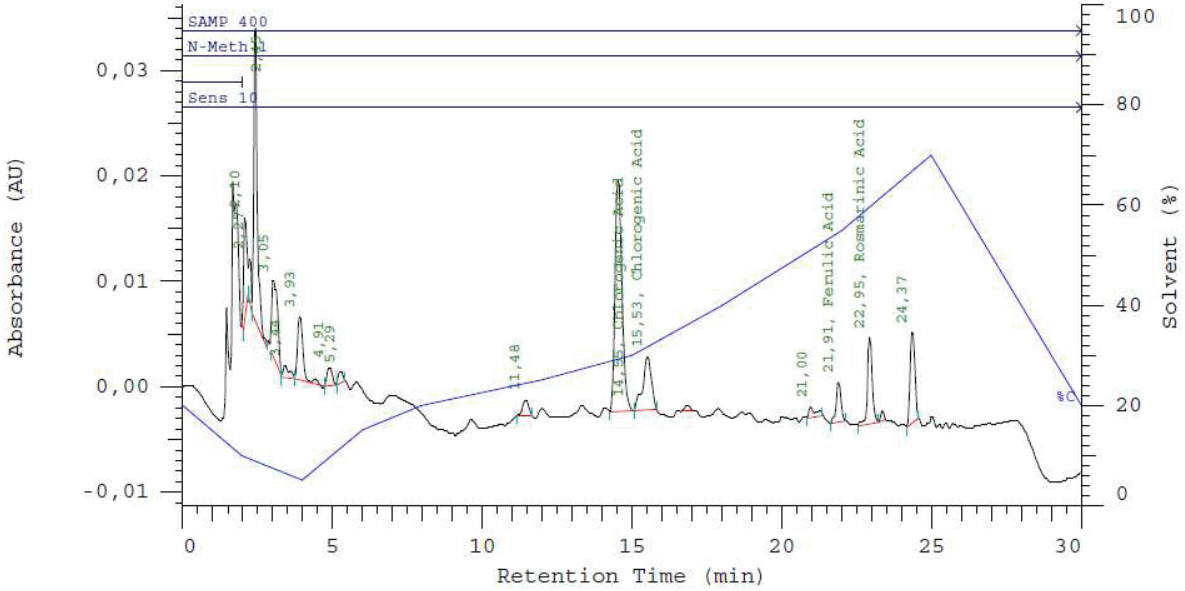



**Figure 84: Chromatogram of blueberry juice – extracted, unconcentrated**

The extracted blueberry juice had only presented 54,92 mg/l of chlorogenic acid. For the other substances, there was practically no existence verified. However, rosmarinic acid was present at 7,62 mg/l.

But within the second run, rosmarinic acid was no longer detected. Also the concentration of chlorogenic acid declined to 12,36 mg/l. In this case, the phenolic compounds displayed 5,29 mg/l.

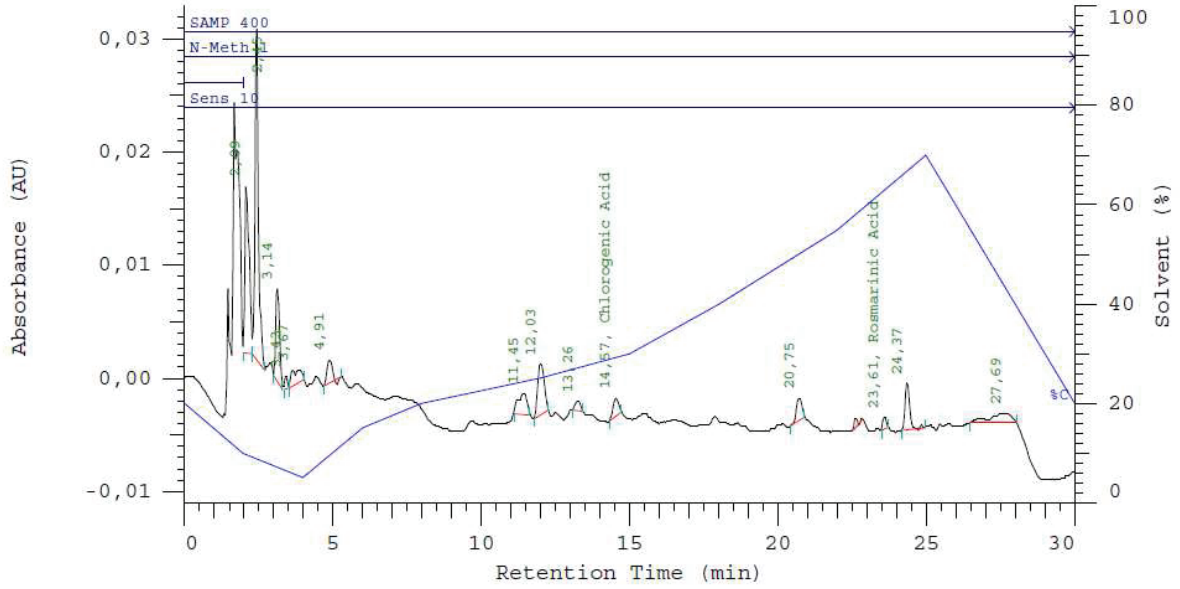



**Figure 85: Chromatogram of cranberry juice – extracted, unconcentrated**

The phenomenon of scarcely no concentrations of phenolic acids was witnessed again in the extracted cranberry juice. Only a few peaks of chlorogenic acid and rosmarinic acid were seen.

Reproducing this analysis, the concentration of chlorogenic acid increased to 0,94 mg/l (before 1,49 mg/l). Simultaneously, rosmarinic acid could not be found in the second run anymore.

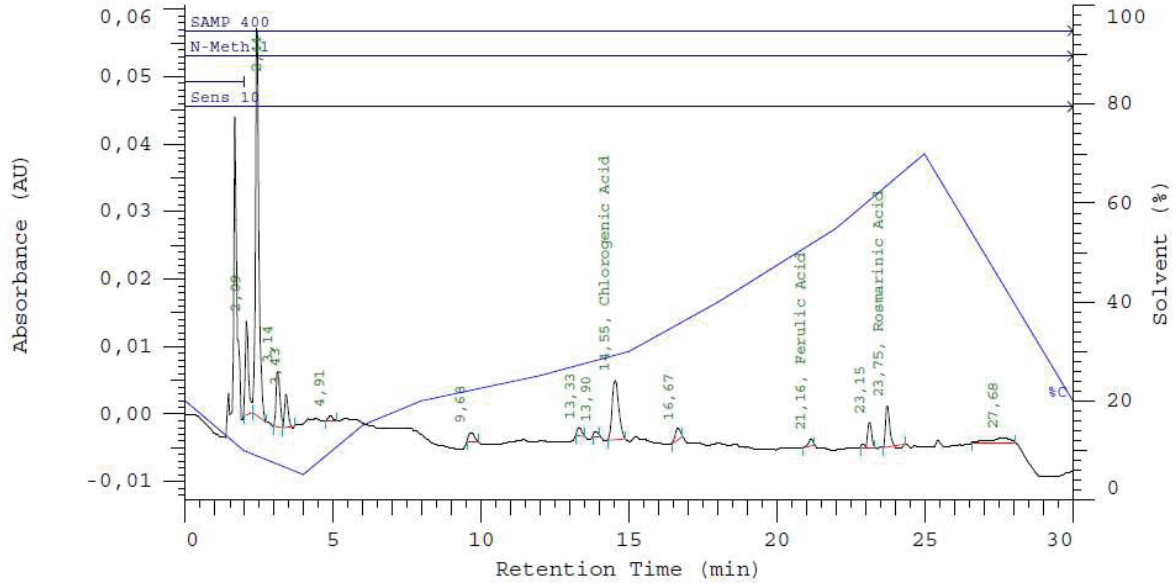



**Figure 86: Chromatogram of apple juice – extracted, unconcentrated**

In apple juice, the concentrations of chlorogenic acid (10,11 mg/l) and rosmarinic (1,77 mg/l) acid were little higher than in the extracted cranberry juice. At the same time another phenolic compound was observed on a small scale (0,351 mg/ml).

But after repeated analysis, the compound was not present any longer. However the levels of chlorogenic and rosmarinic acid remained constant.

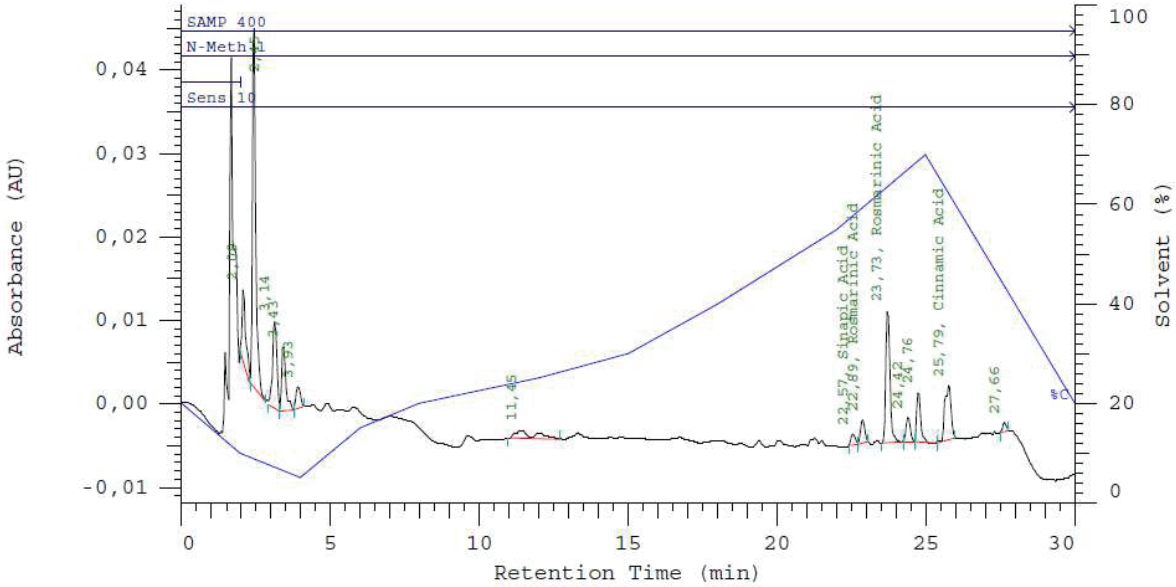



**Figure 87: Chromatogram of sea buckthorn berry juice – extracted, unconcentrated**

Like in the fermented, unconcentrated and unfermented unconcentrated sea buckthorn berry juice, no concentrations of chlorogenic acid were found in the extracted juice mixture. Yet still, cinnamic acid (1,86 mg/l) and rosmarinic acid (7,76 mg/l) showed the highest levels of all extracted, unconcentrated juices.

A good reproducibility was optained for this juice. The differences between first and second analysis were 0,34 mg/l for rosmarinic acid and 0,08 mg/l for cinnamic acid.

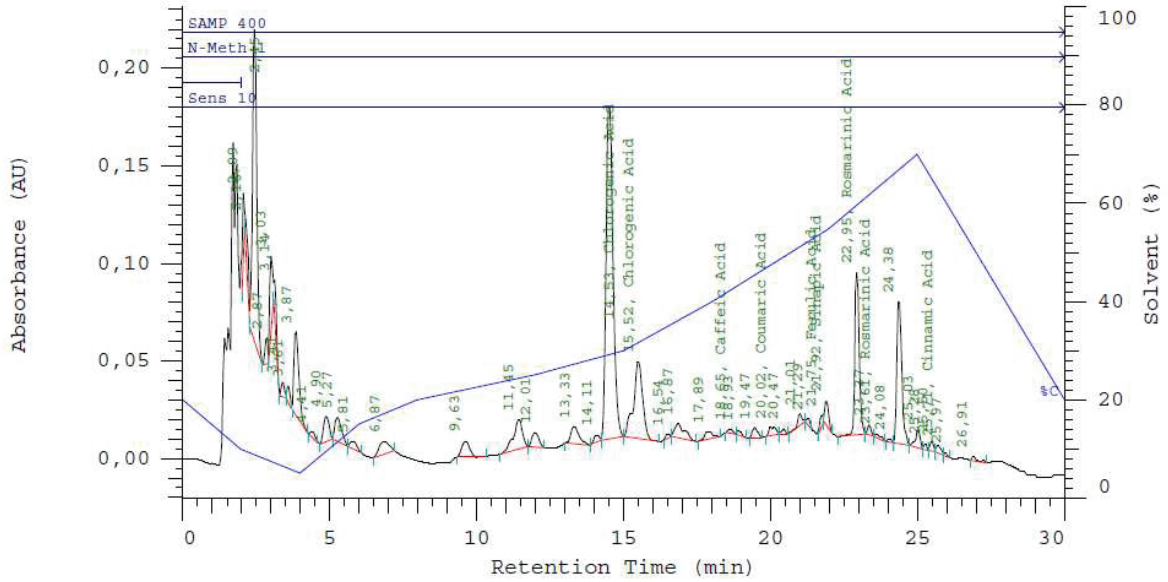



**Figure 88: Chromatogram of blueberry juice – extracted, concentrated (3x)**

The treatment of concentrating the extracted juices showed higher concentrations of hydroxycinnmic acid and derivatives, as predited. Compared to the rest of the four juices, blueberry juice had the highest level of chlorogenic acid (446,11 mg/l), like usual. Rosmarinic acid came a close second with 75,59 mg/l. It must be pointed out also that the phenolic compounds reached a level of 9,44 mg/l.

The duplication achieved approximately the same concentrations.

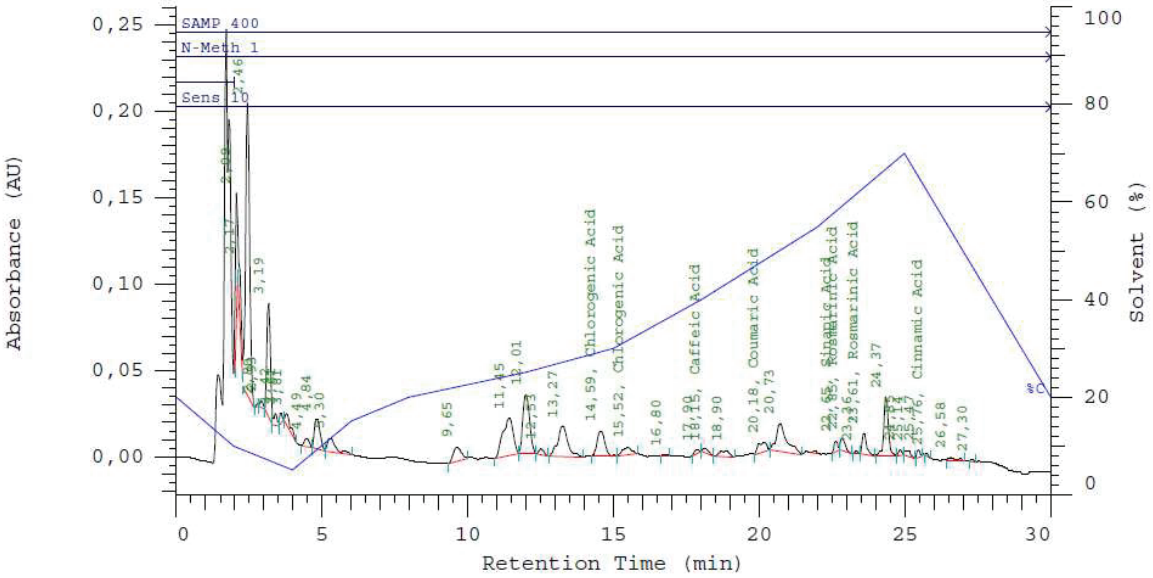



**Figure 89: Chromatogram of cranberry juice – extracted, concentrated (3x)**

The extracted, concentrated cranberry juice showed concentrations of all hydroxycinnamic acids. Like usual, the major component is presented through chlorogenic acid with a level of 47,16 mg/l. Rosmarinic acid showed 12,04 mg/l. The phenolic compounds are signified as 6,55mg/l.

The second analysis offered only 38,43 mg/l for chlorogenic acid and 4,70 mg/l for the group of the phenolic compounds. For rosmarinic acid a consistent level of 11,23 was detected. The biggest difference was seen for cinnamic acid. With a concentration of 2,04 mg/l, the value was 1,80 mg/l higher than in the first run.

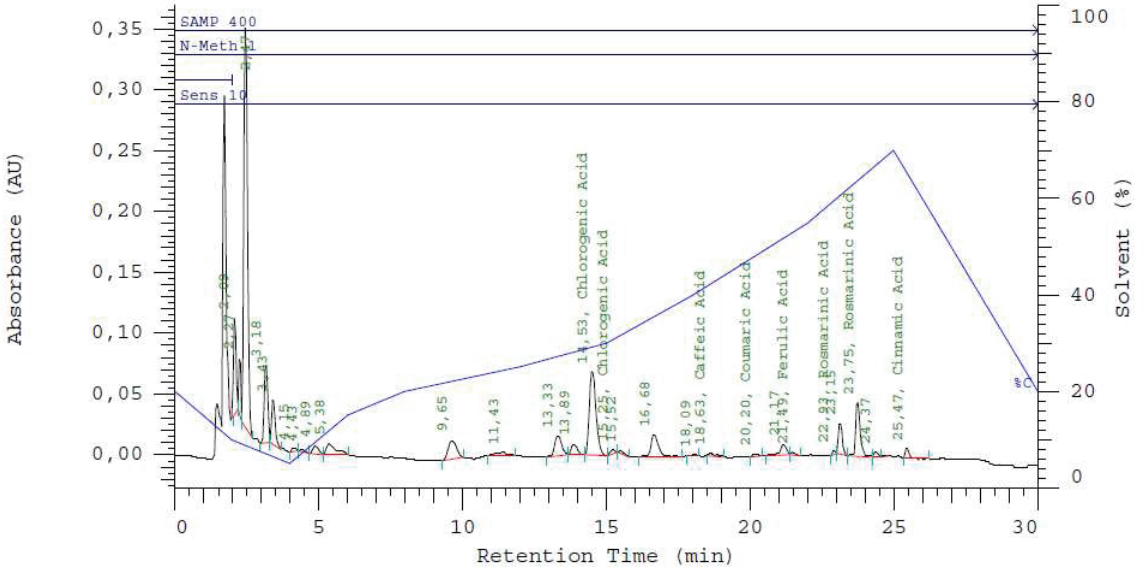



**Figure 90: Chromatogram of apple juice – extracted, concentrated (3x)**

In figure 90, it is still seen that chlorogenic acid was the major component of apple juice. The level of 92,74 mg/l was followed by 19,08 mg/l of rosmarinic acid. The phenolic compounds achieved 2,18 mg/l, cinnamic acid 1,17 mg/l.

In replicaton, the concentration of chlorogenic acid increases around 27 mg/l. The phenolic compounds increased to 8,48 mg/l. Also cinnamic acid recorded 0,22 mg/l more then before.

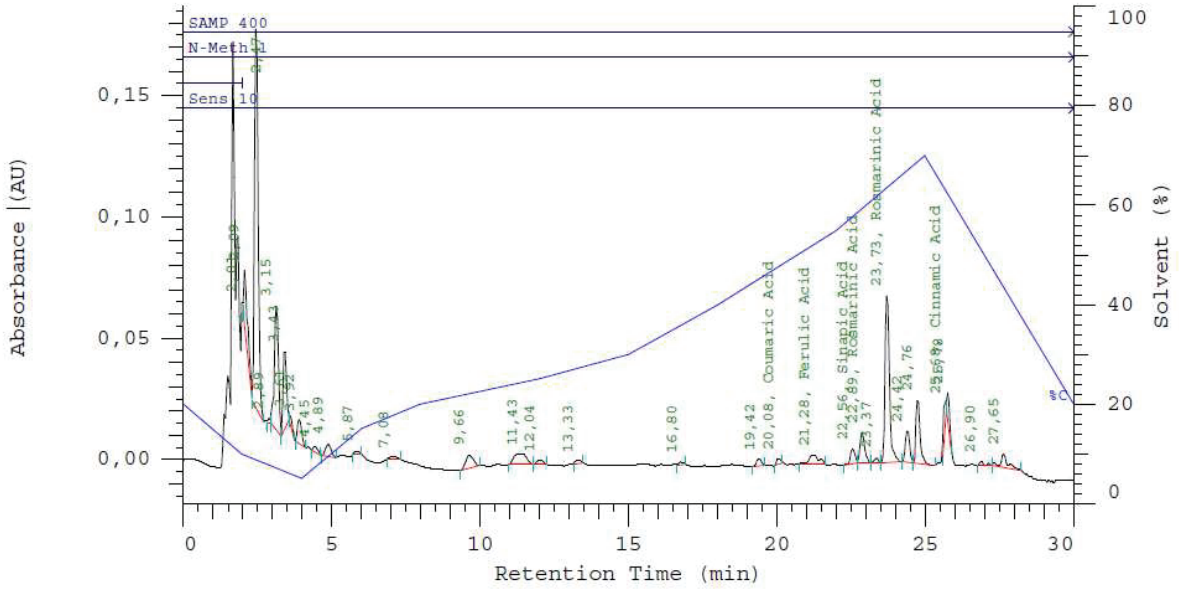



**Figure 91: Chromatogram of sea buckthorn berry juice – extracted, concentrated (3x)**

In contrast to the results achieved with the extracted, unconcentrated sea buckthorn berry juice, the concentrations of rosmarinic acid and the phenolic compounds increased. The level of cinnamic acid acid dropped from 1,86 mg/l to 0,80 mg/l.

The duplication of this analysis certified the findings described above.

*Summary of the results*

The main detected substances in the samples were chlorogenic acid and rosmarinic acid. Sea buckthorn berry juice was the only natural medium, which contains such low amounts of chlorogenic acid that it could be only detected in concentrated samples. However, sea buckthorn berry juice offered the highest concentrations of cinnamic acid, in most cases. All in all, blueberry juice was the substance offered the highest amount of chlorogenic acid and total hydroxycinnamic acids, as well.

In giving an overview, all concentrations that were analyzed for the juice mixtures are shown in following table. Table 13 offers the content of the total phenolic acids in each juice.

**Table 12: Concentration of phenolic compounds**

| | Chlorogenic Acid in mg/l | | Rosmarinic Acid in mg/l | | Cinnamic Acid in mg/l | | Phenolic Compounds in mg/l | |
|---|---|---|---|---|---|---|---|---|
| | 1. Analysis | 2. Analysis | 1. Analysis | 2. Analysis | 1. Analysis | 2. Analysis | 1. Analysis | 2. Analysis |
| B-Juice unferm. unconc. | 164,62 | 62,88 | 29,26 | 29,96 | 0,40 | 0,75 | 2,89 | 3,722 |
| C-Juice unferm. unconc. | 6,57 | 11,47 | 1,99 | 7,39 | 0,42 | 0,44 | 4,59 | 3,203 |
| A-Juice unferm. unconc. | 24,54 | 34,63 | 4,68 | 5,09 | 0,61 | 0,58 | 0,51 | 0,678 |
| S-Juice unferm. unconc. | 0,00 | 0,00 | 16,60 | 16,18 | 3,05 | 2,91 | 3,07 | 2,719 |
| B-Juice ferm. unconc. | 79,96 | 103,15 | 23,20 | 28,03 | 0,35 | 0,00 | 1,67 | 2,249 |
| C-Juice ferm. unconc. | 5,85 | 8,96 | 5,82 | 4,10 | 0,00 | 0,00 | 1,52 | 0,447 |
| A-Juice ferm. unconc. | 30,39 | 32,13 | 4,42 | 4,23 | 0,47 | 0,45 | 1,33 | 0,792 |
| S-Juice ferm. unconc. | 0,00 | 0,00 | 19,58 | 18,97 | 3,56 | 3,59 | 3,42 | 2,314 |
| B-Juice unferm. conc. | 728,26 | 74,09 | 125,41 | 113,92 | 0,88 | 1,20 | 16,90 | 17,437 |
| C-Juice unferm. conc. | 16,78 | 40,17 | 18,69 | 19,18 | 2,94 | 3,03 | 32,42 | 32,512 |
| A-Juice unferm. conc. | 129,06 | 131,37 | 22,29 | 23,07 | 2,10 | 2,11 | 5,32 | 5,325 |
| S-Juice unferm. conc. | 9,28 | 6,68 | 67,98 | 22,81 | 14,40 | 15,21 | 11,39 | 10,989 |
| B-Juice ferm. conc. | 1185,43 | 30,13 | 187,76 | 185,13 | 1,31 | 1,71 | 13,97 | 17,49 |
| C-Juice ferm. conc. | 47,16 | 45,84 | 29,31 | 29,13 | 1,01 | 0,98 | 17,95 | 18,264 |
| A-Juice ferm. conc. | 306,39 | 302,86 | 1,46 | 1,45 | 4,49 | 4,52 | 6,59 | 8,132 |
| S-Juice ferm. conc. | 23,20 | 24,09 | 141,81 | 142,74 | 33,80 | 34,31 | 6,31 | 7,24 |
| B-Juice extrac. uncon. | 54,92 | 12,36 | 7,62 | 0,00 | 0,00 | 0,00 | 1,44 | 5,293 |
| C-Juice extrac. uncon. | 1,49 | 0,94 | 0,28 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| A-Juice extrac. uncon. | 10,11 | 9,21 | 1,77 | 2,13 | 0,00 | 0,00 | 0,35 | 0,00 |
| S-Juice extrac. uncon. | 0,00 | 0,00 | 7,76 | 7,42 | 1,86 | 1,78 | 0,80 | 0,00 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| B-Juice extrac. conc. | 446,11 | 437,54 | 75,59 | 78,03 | 0,82 | 0,90 | 9,44 | 14,756 |
| C-Juice extrac. conc. | 47,16 | 38,43 | 12,04 | 11,23 | 0,25 | 2,04 | 6,55 | 4,698 |
| A-Juice extrac. conc. | 92,74 | 119,98 | 19,08 | 16,78 | 1,17 | 1,39 | 2,18 | 8,476 |
| S-Juice extrac. conc. | 0,00 | 0,00 | 37,54 | 36,48 | 0,80 | 8,87 | 7,69 | 5,178 |

**Table 13: Total hydroxycinnamic acids of all juices calculated from the first analysis (CPC)**

| | Total HCA in mg/l |
|---|---|
| B-Juice unferm. unconc. | 197,16 |
| C-Juice unferm. unconc. | 13,56 |
| A-Juice unferm. unconc. | 30,34 |
| S-Juice unferm. unconc. | 22,73 |
| B-Juice ferm. unconc. | 105,18 |
| C-Juice ferm. unconc. | 13,19 |
| A-Juice ferm. unconc. | 36,60 |
| S-Juice ferm. unconc. | 26,56 |
| B-Juice unferm. conc. | 871,45 |
| C-Juice unferm. conc. | 70,83 |
| A-Juice unferm. conc. | 158,77 |
| S-Juice unferm. conc. | 103,05 |
| B-Juice ferm. conc. | 1388,47 |
| C-Juice ferm. conc. | 95,42 |
| A-Juice ferm. conc. | 318,92 |
| S-Juice ferm. conc. | 205,10 |
| B-Juice extrac. uncon. | 63,98 |
| C-Juice extrac. uncon. | 1,77 |
| A-Juice extrac. uncon. | 12,23 |
| S-Juice extrac. uncon. | 10,42 |
| B-Juice extrac. conc. | 531,96 |
| C-Juice extrac. conc. | 66,00 |
| A-Juice extrac. conc. | 115,18 |
| S-Juice extrac. conc. | 46,03 |

### 4.4 Reproducibility

For the juice samples, the reproducibility of the HPLC analysis was carried out with the retention times and the peak areas. Due to lack of time the juice samples were analyzed two times. Because of that, it was not necessary nor expedient to define any statistical analysis, such as coefficient of variation (CV). The standard substances were only measured one time.
On the basis of the results shown in figures 12 & 13, it is seen that the analysis method displayed good reproducibility, but this predication should be accepted with reservation.

## 5 Discussion

According to RECHNER 2000, apples contain a high amount of chlorogenic acid. Based on the procedure of juice processing and the sort of apples, chlorogenic acid in apple juices amounted from 2,3 to 557,5 mg/l. In the analysed mixtures, contents of circa 30 mg/l in both, unfermented and fermented apple juice was observed. Also caffeic acid and coumaric acid are present in apple juices. But because of the described problems about the RT´s and spectra, unfortunately it was not possible to analyze the particular contents of these acids.

Looking of the concentrations of chlorogenic acid in the juices, it is conspicuous that blueberry juice was the sample with the highest amount of this acid. Already, SZAJDEK et al. 2008, reported the high value of chlorogenic acid in their article regarding the health-promoting properties of berry fruits. CLIFFORD 2000, declared that blueberries are an important supplier of chlorogenic acid. No other acid could reach comparable concentrations to this hydroxycinnamic acid. The contents amongst the different treated, but unconcentrated blueberries juices vary, especially when considering the values of both runs. Taking the average of both analyses, it is seen that the fermented juice contained a lower concentration of around 20 mg/l. Therefore it could be, that *S.cerevisiae* absorbs phenolic compounds during fermentation.

In advance, fermentations with different yeasts showed, that no differences can be found in the behavior of phenolics during fermentation. Because of that the classical baker´s yeast *Saccharomyces cerevisiae* was used for fermentations.

With regard to the concentrated blueberry mixtures, this fact of lower acids concentrations in fermented juices was not observed within duplicate concentrated unfermented juice and the triplicate concentrated fermented juice. For a comparison, a uniform concentration should be taken. As mentioned above, the values for the first and second analysis were practically very different. Differences between the first and the second run for the concentration of chlorogenic acid in the unfermented and fermented mixtures ranged from 400 % to 1000 %. Because of that, no statistically reliable claims can be made. Therefore, the predication that the fermented blueberry juice contains less chlorogenic acid than the unfermented one is not supported. Anyhow, there were no reasons cited in the literature for less concentration of polyphenols in fermented foodstuffs. Rather, information about inhibiting fermentation by yeasts due to polyphenols were published by CHAMBEL et al 1999. Also KLENAR et al. 2004, emphasized that phenolic compounds affect the production of yeast cells. This effect is seen on the dried biomass of the fermented juices. The juice with the highest level of chlorogenic

acid and other hydroxycinnamic acids, even blueberry juice, showed the lowest presence of yeast cells compared to all other juices. In contrast to blueberry juice, all other juices offered a total hydroxycinnamic acid amount between 14 and 30 mg/l for unfermented and unconcentrated juices, as well. In opposition to that, blueberry juice observed a total acid content of circa 200 mg/l. With the treatments of fermentation and concentration, respectively, blueberry mixtures always offered significant higher levels of total cinnamic acid derivatives. So, the biomass of cranberry juice, apple juice and sea buckthorn berry juice reached concentration higher than for blueberry juice, but were all around the same level.

Whether unfermented or not, cranberry juice was the substance with the lowest amount of hydroxycinnamic acids. Already CLIFFORD 2000, identified that the content of total phenols is fundamental lower in cranberry juice than in blueberry juices.

Next to chlorogenic acid, which was found in all blueberry mixtures, it attracted attention that rosmarinic acid was the substance with high concentrations, followed by chlorogenic acid. The highest contents of rosmarinic acid were found in blueberry juices, as well. In that case, it was also noticed that the content of rosmarinic acid was little higher in unfermented juices. Possibly, the speculation of absorbing hydroxycinnamic acids by yeast in procedure of fermentation is not absurd as thought. Also cinnamic acid and the group of the "phenolic compounds" showed fewer concentrations in fermented juices. The duplication certified this, as well.

In classical meaning, the fermentation method used in this thesis, caused to a "bad" wine. An adaptation of parameters used for "real" wine fermentation should be taken for having authentic results.

The extracted samples showed only very low concentrations of hydroxycinnamic acids. Anyway, contents of each acids were found, which were good detected after triplicate concentration. Cinammic acid itself was only found in sea buckthorn berry juice, what supports the predication of ARIMBOOR et al. 2007, that cinnamic acid is one of the typical hydroxycinnmic acids found in sea buckthorn berry juice. A confirmation about the fact, that sea buckthorn berry juice showed rosmarinic acid as its main component, could not be found.

The unfermented and unconcentrated apple mixtures observed a phenolic acid level of 30,34 mg/l. This amount is just half of it, presented by KAHL et al. 2005, with a concentration between 56,8 and 67,7 mg/l. Sea buckthorn berry juice was penultimate with its level of cinnamic acid derivatives. Characteristically for sea buckthorn berry juice was, that chlorogenic acid was presented in very low concentrations, that it was only detectable in the concentrated mixtures. ARIMBOOR et al. 2007 referred that the main acids in sea buckthorn

berry juice are presented through cinnamic, caffeic, ferulic and coumaric acid. As shown in table 12, the concentrations of cinnamic acid in all fermented and unfermented juices extended to thirty times higher amounts than the other juices. Because of the compression of ferulic, caffeic, coumaric and sinapic acid to “phenolic compounds”, there can be formed no closer evidence for the several acids. By analyzing the values for “phenolic compounds” of sea buckthorn berry juice, no signs for higher concentrations of these acids could be determined.

According to CLIFFORD 2000, also caffeic acid is typical for apples and blueberries. The highest concentrations for the “phenolic compounds” were contained in cranberry, blueberry and sea buckthorn berry mixtures, in equal measure. For apple juices, values were only found in lower concentrations. Following LEE 2003, caffeic acid is not a typical acid for apple juices. Potentially, the content of caffeic acid was higher than the other acids, including in “phenolic compounds”. But therefore it would be necessary to separate the peaks more precise.

The chosen parameters, like flow rate and injection volume, but also the taken solvents were capable for the detection of the standard substances. However, the gradient required an optimization. Furthermore, more solutions should be used for the calibration of the standard substances. In this work a linear dilution series was established, whereupon three of four measuring points were at close quarters. Therefore TOLONEN & UUSITAB 2005 presented the usage of eight different standard solutions. FANG et al. 2007 used five concentration levels for detecting flavonoids.

With the help of the data formed by calibration, it is seen that both colums, TEC and CPC offered advantages and disadvantages. Compared to the TEC, the CPC observed better defined peaks , without shoulders, as it was seen for ferulic acid. Furthermore, the peaks, analyzed with CPC, were detected at higher absorbance, by using the same HPLC conditions for each column. This effect dues to the damaged lamp in the DAD. The TEC observed a better peak separation alongside the gradient. With the CPC the first standard substance was detected after 14 min at earliest. Another advantage for the TEC was the detection of chlorogenic acid in one peak. The CPC determined chlororgenic acid in two separated peaks. However, the problematical detection of coumaric, ferulic and sinapic acid was the same with both columns. For better results, the gradient need to be optimized, especially in reference to the last named acids.

Another offer for successive analysis could be, to take juices which consist of 100 % pure juice. But also with involvement of the other juice parts in the fruit juices, here grape juice and orange juice, would be helpful to achieve significant results.

With the help of the developed method and the CPC, it was able to demonstrate the differences for the different concentrations of hydroxycinnamic acid and its derivatives or rather an acid group, in commercial fruit juices and yeast fermented mixtures.

## 6 Summary

During the last ten years, the awareness of health-promoting substances has increased. Therefore the polyphenols were of particular interest. These substances are important for humans, but also for plants themselves.

Methodologies for analyzing polyphenols have bee tested and improved by several scientists. Specifically the high-performance liquid chromatography has grown to be one of the best analytical operations available.

For the basis of the presented Bachelor thesis, the fate and behavior of phenolic compounds, specifically the hydroxycinnamic acid and its derivatives, has been analyzed. As objects for analysis, four different commercial fruit juices were examined. Moreover, not only the pure juices, but also the influence of yeast fermentation was investigated or the presence of phenolic acids. These acids were chlorogenic acid, caffeic acid, rosmarinic acid, ferulic acid, coumaric acid, sinapic acid and cinnamic acid.

Polyphenols affiliate to the secondary metabolites and play a major role in human healthcare. Because of anti-oxidative, anti-carcinogenic and also anti-inflammatory properties, phenolic compounds are exceptionally important substances in foodstuffs. Easily accessible sources for polyphenols are fruits, vegetables, crops and juices.

Another important role affecting daily nutrition is food processing, which can result in longer storage life, but also better food quality of edibles. Aside from the fermentation of lactobacilli (kefir, sauerkraut), the alcoholic fermentation by yeasts are fundamental for society; Otherwise there would be no beer or wine. For finding out which influence yeasts are having on the polyphenols, the analysis of products fermented by yeast is included in this work. For this blueberry juice, cranberry juice, apple juice and sea buckthorn berry juice are considered. Each of these juices have been analyzed unfermented as well as fermented. For better detection of the phenolic compounds, the juices have been concentrated. The remaining biomass of the fermentation by *Saccharomyces cerevisiae* has been extracted during the process. Furthermore, the yeast cells have beeb dried until mass constancy and adducted for the evaluation of the phenolic content of the extracted sample mixtures. Accordingly, 24 juice samples have been analyzed with the HPLC.

With the help of different machines (centrifuge, concentrator) and substances, the samples were prepared for the HPLC analysis. The sample mixtures were detected with a gradient system, using the solvents methanol and purified water (pH3).

On the basis of the results, it is seen that chlorogenic acid was the most highly detected phenolic compound. Due to the amount of chlorogenic acid in blueberry juice (unfermented, fermented, extracted), this juice contained the highest concentration of total phenolic acids. Next to chlorogenic acid, rosmarinic acid was the second highest observed substance, especially found in sea buckthorn berry juice, which barely contain measurable chlorogenic acid. Cinnamic acid was obsrved in all juice mixtures at lower concentrations. A precise evaluation of ferulic, sinapic, caffeic and coumaric acid was infeasible, due to specific detection problems. All of these acids were categorized in the group of “phenolic compounds”.

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## 8 Figure Register

## 9 Table Register

## 10 Abbreviation Register

| | |
|---|---|
| HPLC | high performance liquid chromatography |
| NP | normal phase |
| RP | reversed phase |
| DAD | diode array detector |
| ATP | adenosintriphosphate |
| AU | absorbance units |
| LDL | low-density lipoproteins |
| RT | retention time |
| 3-D | 3-dimension |
| CV | coefficient of variation |
| UPLC | ultra performance liquid chromatography |
| MS | mass spectrometry |
| THF | tetrahydrofran |
| TEC | thermo electron column |
| CPC | chromolith performance column |
| rpm | revolutions per minute |
| min | minute |
| mg | milligramm |
| ml | milliliter |
| µl | mikroliter |
| nm | nanometer |
| r | correlation coefficient |

# 11 Appendix

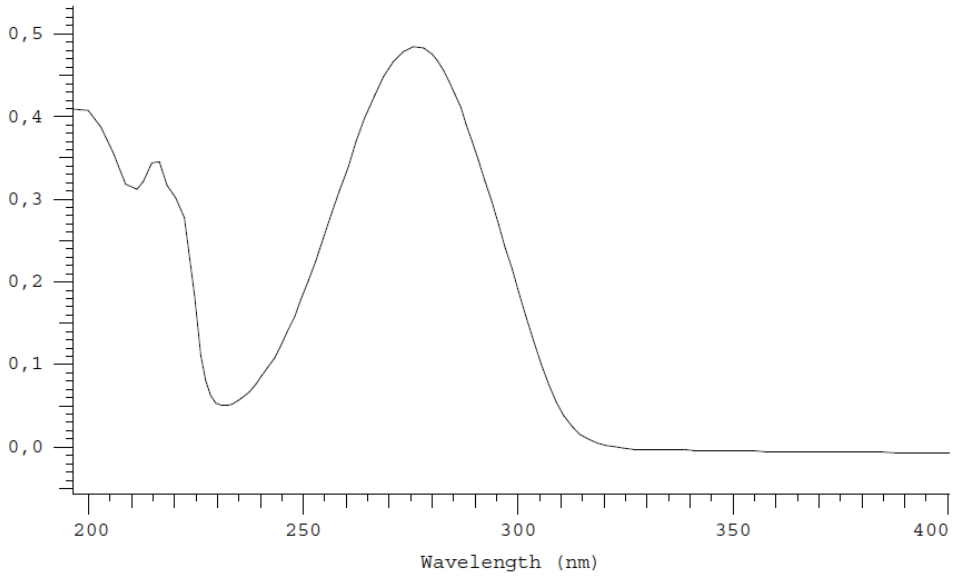


**Figure 92: Spectrum of cinnamic acid at RT 22,01 min (TEC)**

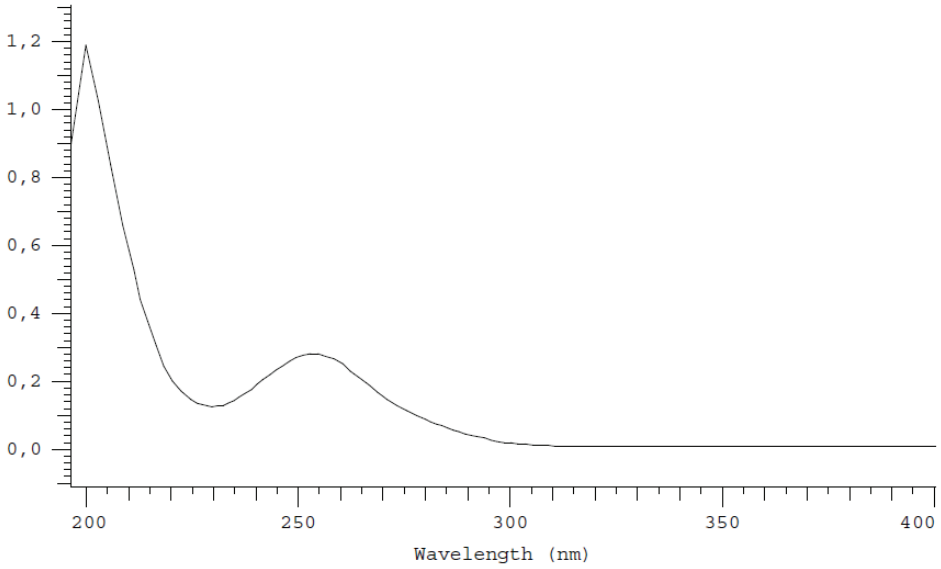


**Figure 93: Spectrum of caffeic acid at RT 3,72 min (TEC)**

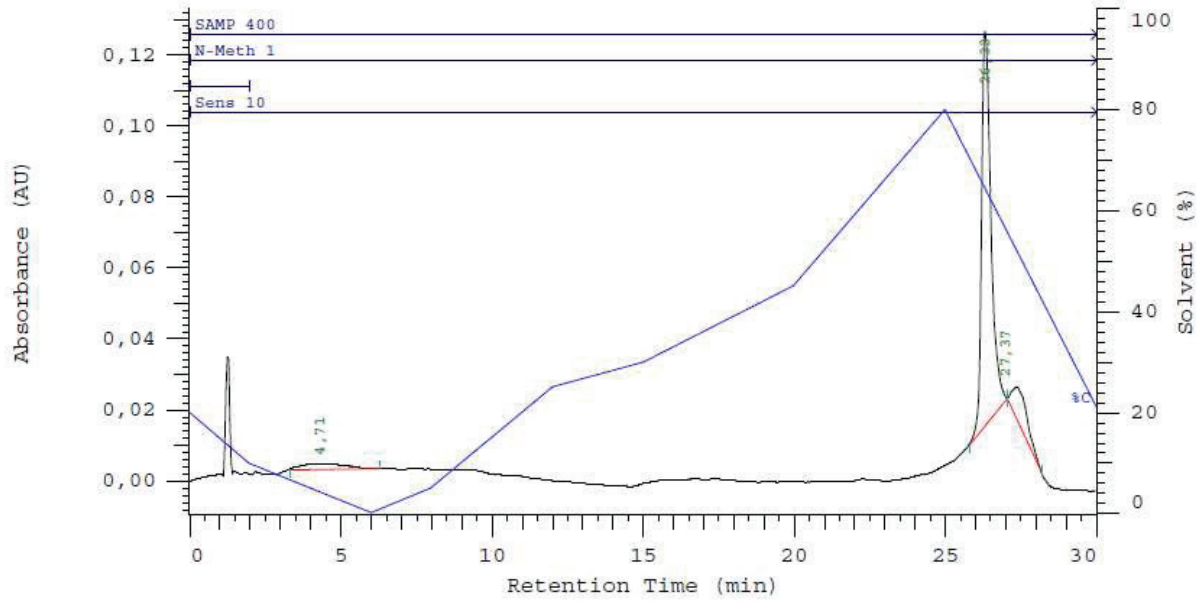


**Figure 94: Chromatogram of minimal medium detected at 280 nm, unconcentrated (TEC)**

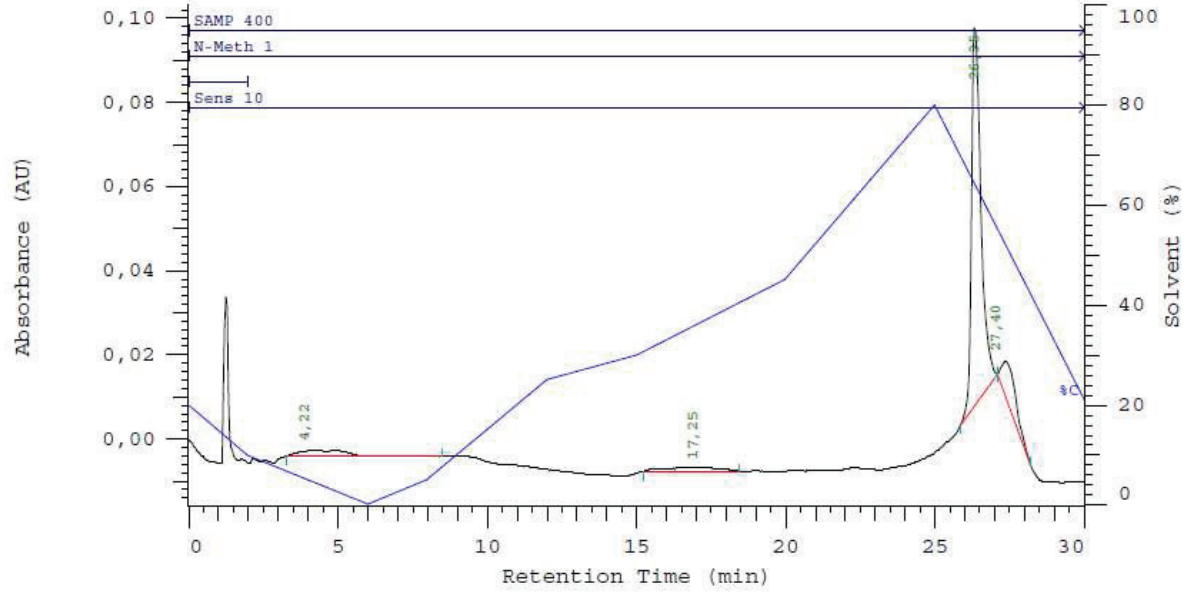


**Figure 95: Chromatogram of minimal medium detected at 280 nm, concentrated 3x (TEC)**

## 12 Declaration of autonomous making of this thesis

Herewith I promise, that I wrote this Bachelor Thesis autonomous, without using other sources and additives than declared.

Neubrandenburg, 27.08.2010 _____

Place, Date Carolin Ehrhardt