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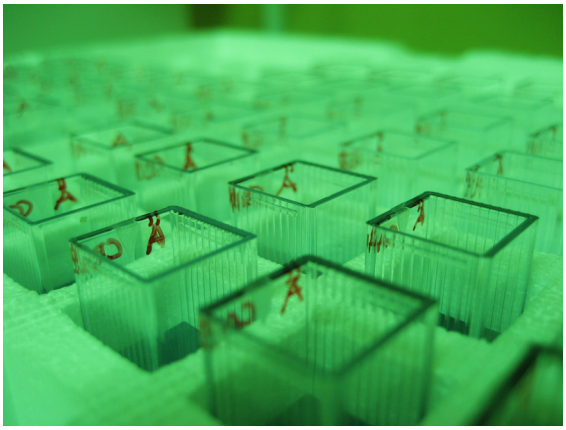
Interferences during analysis of polyphenols in fruit juices



Jennifer Sandström

Fakulteten för landskapsplanering, trädgårds- och jordbruksvetenskap

SLU-Alnarp

Sveriges Lantbruksuniversitet, SLU
Fakulteten för landskapsplanering, trädgårds- och jordbruksvetenskap (LTJ)
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Författare (author): Jennifer Sandström

Handledare (Supervisor): Kimmo Rumpunen (forskare)

Biträdande handledare (Co-supervisor): Anders Ekholm (forskningsingenjör)

Examinator (Examiner): Hilde Nybom (professor)

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Område Växtförädling och bioteknik (Department of Plant Breeding and Biotechnology)

Contact address: Balsgård, Dept. of Plant Breeding and Biotechnology
Swedish University of Agricultural Sciences (SLU)
Fjälkestadsvägen 459, 291 94 Kristianstad

Cover illustration: Cuvettes analyzed with the Folin-Ciocalteu method. Photo: Jennifer Sandström

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Abstract

One of the most commonly used methods for analysis of polyphenols is the Folin-Ciocalteu (FC) method, where the FC reagent has been reported to interfere with some substances in fruit juices. The interfering substance that is present in the highest amount in most fruit juices is ascorbic acid. In this study, the total phenolic content (TPC) of three fruit juices was analysed by the FC method as well as with an enzymatic method with hydrogen peroxide oxidoreductase type II (HRP) for comparison. To investigate how the interfering ascorbic acid affects the TPC, juice samples were analysed after removal and addition, respectively, of ascorbic acid. The samples were analysed with HPLC both before and after the extraction phase of the FC method. The results show a decrease in ascorbic acid of approximately 21 % after extraction compared to before. This study showed that the FC method and the HRP method differ in sensitivity regarding various groups of polyphenols. The analysis concerning cyanidin-*3*-*O*-*β*-glucoside and tannins yielded higher values with the FC method than the HRP method whereas procyanidin B2 instead had a lower value. Furthermore, it was shown that heat treatment of fruit juice could result in degradation of polyphenols. Since fruit juices have very complex matrices, TPC values should be compared for each type of fruit juice separately.

Sammanfattning

En av de vanligaste analysmetoderna för att detektera polyfenoler, Folin-Ciocalteu (FC)-metoden, innehåller reagens som visat sig interferera med ämnen som finns i fruktjuicer. Det interfererande ämnet är framförallt askorbinsyra vilket förekommer i höga mängder i juice. I denna studie har tre fruktjuicer analyserats med FC-metoden och jämförts med en enzymatisk metod baserad på enzymet väteperoxid-oxidoreduktas (HRP). För att se hur interferensen av askorbinsyra påverkar totalfenolhalten (TPC) i juicerna, gjordes analyser där askorbinsyra avlägsnades respektive tillsattes. Proverna analyserades med HPLC både före och efter det steg i FC-metoden där extraktionen sker. Resultaten visar att koncentrationen askorbinsyra minskar med 21 % efter extraktion jämfört med före extraktion. Denna studie visade att FC-metoden och HRP-metoden skiljer sig åt i känslighet för olika polyfenoler. Analysen avseende cyanidin-*3-O-β*-glukosid och tanningruppen gav högre värden vid FC-metoden än vid HRP-metoden medan värdet för procyanidin B2 var lägre vid FC-metoden. Dessutom påvisades att värmebehandling av fruktjuice kan resultera i nedbrytning av polyfenoler. Fruktjuicer är komplext sammansatta och därför bör totalfenolresultaten jämföras sortvis (samma typ av fruktjuice) och inte med varandra (olika typer av fruktjuicer).

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1. Aim

Polyphenols are considered to be important natural antioxidants in different foods. The aim of this study was to compare an enzymatic method (Stevanato *et al.,* 2004; 2008) and the Folin-Ciocalteu (FC) method (Singleton *et al.,* 1999) for analysis of the total phenolic content (TPC) in fruit- and berry juices. Both methods are straightforward and based on spectrophotometric analysis. The FC method is the most common method presently used for such analyses. However, interferences with different substances have been reported (Singleton *et al.,* 1999; Stevanato *et al.,* 2004) and therefore may bias the results. When considering fruit juices the most likely interference is with ascorbic acid that is commonly present in various juices. The enzymatic method was chosen for comparison because the many different substances usually present in fruit- and berry juices that could produce an overestimated result should not affect this method.

This study will thus consider advantages and disadvantages of the different TCP analyses, which interfering substances that have to be considered when performing these analyses and how large impact these compounds will have on the results of TPC analysis.

2. Background

2.1 Polyphenols – natural antioxidants

Polyphenols are a group of natural antioxidants that are abundant in all plant organs (Bravo, 1998) and are often found in the cell walls (Dragsted, 2003). Polyphenols protect the cells from reactive free radicals and have also other beneficial effects to the plant. Free radicals are produced during normal metabolism in humans. These radicals can react with the human DNA and could induce mutations, which can lead to several cardiovascular diseases and together with other factors, also cancer. Antioxidants, especially polyphenols, have a strong capacity to trap free radicals and can therefore protect the human cells (Dragsted, 2003). A high intake of polyphenols in our daily food could thereby prevent such diseases (Manach *et al.,* 2004; Dragsted, 2003; Bravo, 1998). At the moment the content of polyphenols in different foods is not frequently declared but this may be more common in the future. To be able to identify and find out which of the polyphenols that have antioxidant actions on several biological processes in the body will be very important in upcoming studies. If such knowledge is achieved, there will be possibilities to affect the daily intake and understand how cardiovascular diseases can be prevented by a proper diet.

2.1.1. Subclasses of polyphenols

Polyphenols can be subdivided into four groups; phenolic acids, flavonoids, stilbenes and lignans. Polyphenols are often present in conjugated forms; i.e. with one or more sugar residues linked to hydroxyl groups or carboxylic acids, organic acids, amines or lipids (Bravo, 1998). The content and composition of different polyphenols vary between plant species and organs. The simple phenolic acids (e.g. ferulic acid, vanillic acid and pyrogallol) are abundant in most fruits and berries. An important subgroup of flavonoids is the anthocyanins. Anthocyanins are water-soluble plant pigments found in red wine, some root vegetables and fruits (Manach *et al.,* 2004). Anthocyanins are known for their red and blue colour of flower and fruit parts and are mostly found in the skin of berries and fruits where concentration increases with maturity; the stronger the colour, the higher the concentration of anthocyanins in the berry or fruit. The most common anthocyanidin (aglycone) in food is cyanidin and this will, like other anthocyanidins, change colour with pH (Manach *et al.,* 2004).

Plant polyphenols can be analysed by many different methods. For screening purposes a simple spectrophotometric method such as the FC method is usually preferred but also enzymatic methods are available.

2.2. The Folin-Ciocalteu method

The FC method is based on a reagent that consists of sodium tungstate, sodium molybdate, phosphoric acid and lithium sulphate. The similar molybdenum compounds are yellow whereas the isopolyphosphotungstates are colourless in the reagent. These compounds are present in an acidic solution. When the molybdenum compounds are reduced by one or more electrons the structure changes and the compound will turn blue. The FC method is used for measuring TPC in e.g. juices, proteins, plant tissues, wines and phenol-contaminated water. During analysis the sample is put in a cuvette, and then FC reagent, Na_2CO_3 and water are added. Standards that are commonly used are catechin and gallic acid (Singleton *et al.,* 1999).

2.2.1. Interferences between different substances

The FC reagent is not specific but will mainly bind to the polyphenols in the sample. There are reports that prove that also other substances such as ascorbate, benzoate, and citrate could bind to the reagent (Stevanato *et al.,* 2004). This means that when TPC is measured with the FC method it can be overestimated due to interference with such substances.

Ascorbate and L-ascorbic acid (vitamin C) is an antioxidant that is present in most fruits and vegetables. The daily intake of ascorbic acid is obtained from different sources; approximately 53 % of the ingestion comes from berries and juice and 24 % from vegetables (Livsmedelsverket, 2009). The concentration of ascorbic acid in juices depends on the production procedure of the juice. In apple juice made from concentrate there is approximately 1 mg/100 ml vitamin C, in pasteurised orange juice from concentrate there is approximately 35 mg/100 ml and in freshly squeezed orange juice about 50 mg/100 ml (Miljömat, 2009).

2.3. The enzymatic method with hydrogen peroxide oxidoreductase (HRP)

An enzymatic method that has been used for analysis of plant polyphenols is based on the Trinder reaction where hydrogen peroxide (H_2O_2) is determined. H_2O_2 reacts with phenols and 4-aminoantipyrine (4-AP) is catalysed by peroxide (Fig.1). The reaction proceeds in two steps. First the enzyme HRP oxidizes and reacts with phenols that form phenoxyl radicals, then these radicals can react with aromatic amine groups and will bind to 4-AP which is present in excess amount in the solution. This reaction results in a quinone-imine coloured product with H_2O as a by-product. Most phenols can be aromatic donors and this enzyme has a large potential for catalyzing a variety of reactions (Stevanato *et al.*, 2004). The enzymatic method is further referred to as the HRP method.

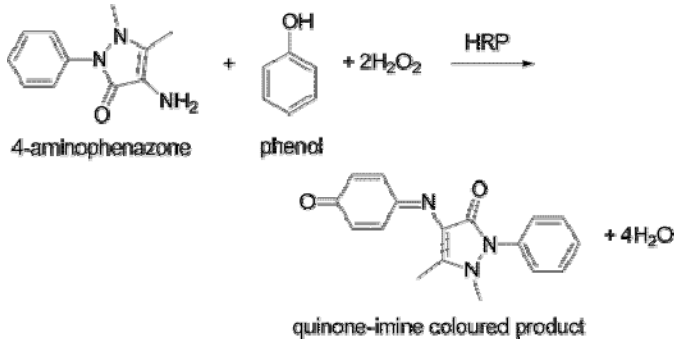

Fig.1. Reaction between 4-aminophenazone, phenol and hydrogen peroxide catalysed by the enzyme HRP.

3. Material and methods

First, the enzymatic method was optimized. The enzymatic method was then compared with the Folin-Ciocalteu method on different samples. High performance liquid chromatography (HPLC) as well as high performance liquid chromatography-mass spectrometry (HPLC-MS) was also used in different parts of the study. The experiments were planned and performed based on the successively obtained results (Fig. 2). For both methods, catechin was used as a standard and the results were presented as concentration of catechin equivalents (CE).

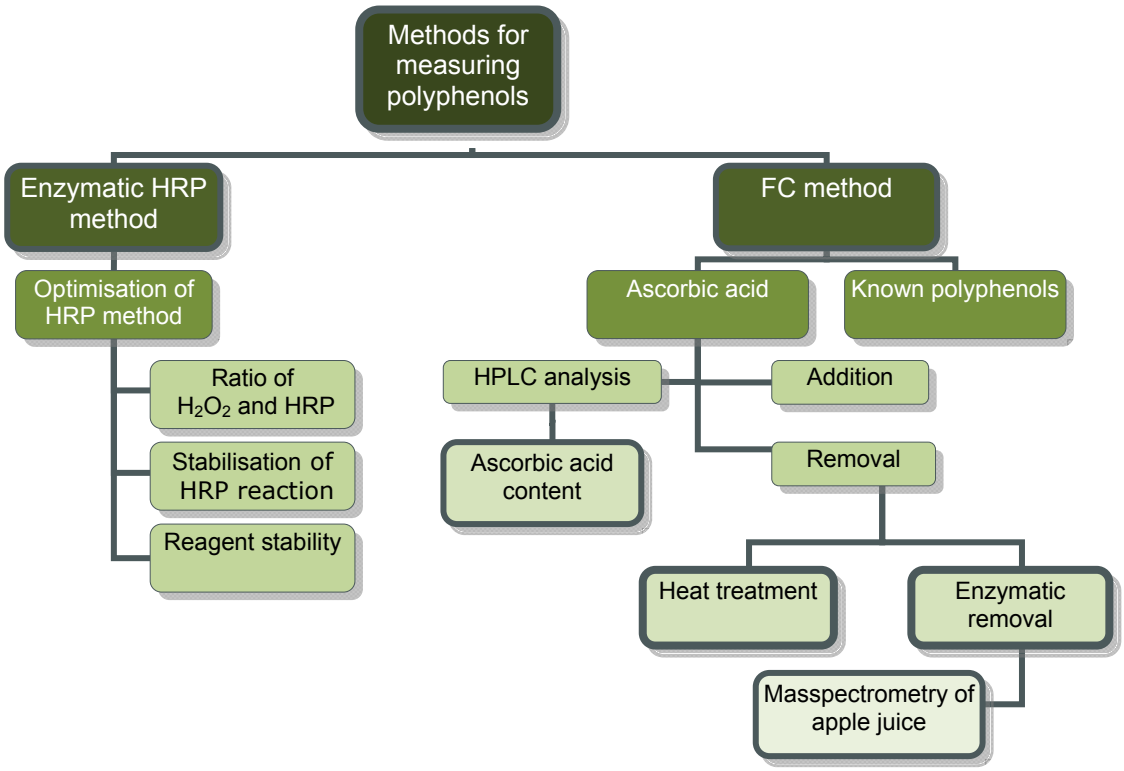

Fig. 2. Experimental overview.

3.1 Material

3.1.1. Chemicals

FC reagent, Na_2CO_3, 4-aminoantipyrine, H_2O_2, α(+) catechin, ascorbic acid, enzymes hydrogen peroxide oxidoreductase type II (HRP) from horseradish peroxide and ascorbate oxidoreductase were purchased from Sigma Aldrich. Cyanidin-3-O-glucoside, chlorogenic acid, tannins and procyanidin B2 where purchased from Extrasynthese.

3.1.2. Samples

Three different juices were tested; apple, orange and grape/apple (as concentrates). These juices were chosen because I wanted to have as different juices as possible but still some of the most common on the market. I also wanted to have several different antioxidant compounds included. The juice samples were prepared by diluting the juice concentrate (1+4 according to the instructions on the packages) to juice ready to drink. The juices were of the brand 'JO' produced by JO bolaget, purchased in packages of 2 dl at a supermarket in the centre of Malmoe, Sweden.

Three sub samples (packages) from the same production date were used for each juice type and three replicates were used for each package (Fig. 3).

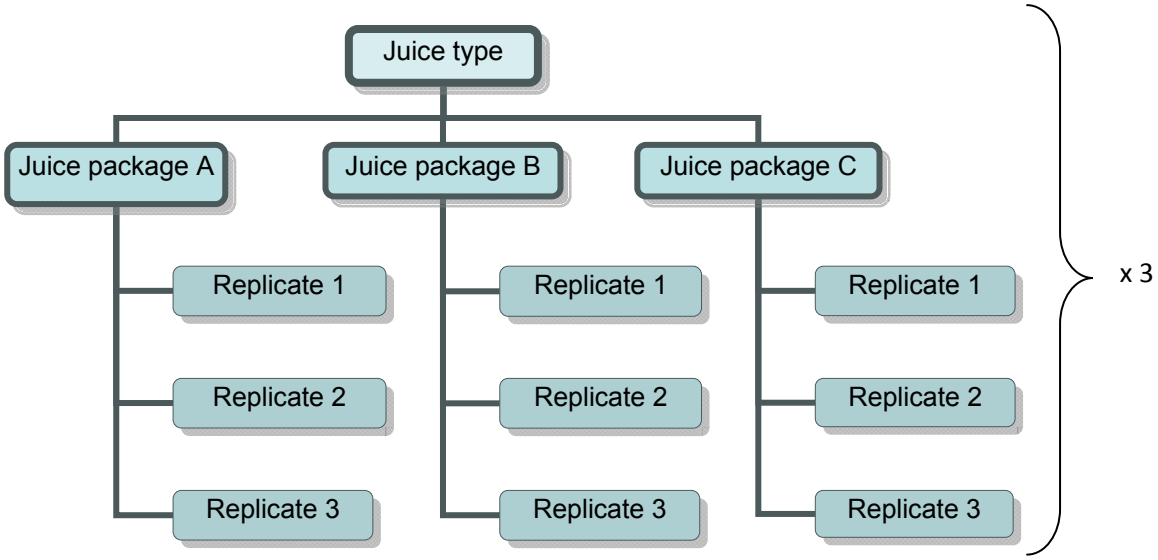

Fig. 3. Sampling overview.

3.2. Methods

3.2.1. Optimisation of the HRP method

The HRP method chosen has previously been used by Stevanato *et al.* (2004) on white and red wine along with various teas. Recently, the method was also tested on fruit, vegetables and fresh-pressed juice (Stevanato *et al.,* 2008). The method has also been used previously, but then in other areas such as contaminated water analysis.

The analyses were carried out according to the procedure reported by Stevanato *et al.* (2004) with some modifications following an initial optimisation of the method. First the enzymatic reagent (3 mM 4-aminoantipyrine (4-AP), 2 mM H_2O_2 and 0.33 µM hydrogen peroxide oxidoreductase (HRP)) was mixed in a potassium phosphate buffer.

Then 3 ml reagent was added to a 3 ml cuvette followed by 25 µl of the sample. The sample was stirred with a pipette and then analysed by a spectrophotometer at a wavelength of 500 nm after 5 minutes.

3.2.1.1. HRP reagent stability

The stability of the reagent was investigated by testing the reagent. Reagent prepared on the previous day was compared with reagent prepared the same day. Also pH was measured. Prior to adding the juice samples, some side-reactions took place when the reagent solution was mixed, which affected the spectrophotometer measurements. For that reason, it was decided to add both H_2O_2 and HRP directly into the cuvette just before running the sample in the spectrophotometer. The reagent solution further on only consisted of potassium phosphate buffer with 4-AP. In the cuvette, the compounds were added in the following order: reagent solution, juice sample, H_2O_2 and HRP. The enzyme HRP was dissolved in the same potassium phosphate buffer as used in the analysis. The cuvette content was stirred when the HRP was added, and then the sample was analysed.

3.2.1.2. Ratio of H_2O_2 and HRP

Different concentrations of H_2O_2 and HRP were tested to investigate the stability of the reagent. 10, 20 and 30 µl of H_2O_2 were added to the samples with 10 µl of HRP.

3.2.1.3. Stability of the HRP method

The stability of the reaction between HRP and H_2O_2 was tested at different time points. Measurements were made directly after stirring the content, and then after 10, 20, 50, 110, 170 and 290 minutes.

3.2.2. The FC method

The FC analysis was carried out according to the procedure in Singleton *et al.* (1999). 1 ml of the juice was transferred to a 15 ml test tube and 9 ml of a mixture of 50 % ethanol and 50 mM H_3PO_4 were added to extract the polyphenols in the sample. The test tube was shaken overnight, approximately for 14 hours, fastened to a vibration plate in cold storage, at about 5˚C. The test tube was then removed from the cold storage and put in a centrifuge at 10 000 rpm for 10 minutes. The samples with orange juice were centrifuged for 20 minutes because of a more viscous texture. 40 µl of juice sample was then pipetted in a cuvette and 60 µl of 5 % ethanol was added. 200 µl of FC reagent was then added, followed by 2 ml $NaCO_3$ and 1

ml deionised H_2O. The content of the cuvette was agitated and then incubated for 2 hours. The samples were run on a spectrophotometer at 765 nm. Each sample was measured twice.

3.2.3. Ascorbic acid removal

Since ascorbic acid interferes with the TPC in the juice when using the FC method, the ascorbic acid in the juices has to be removed to study possible interferences. This could be done either by heat or by enzymatic treatment.

3.2.3.1. Heating

Ascorbic acid was removed by gently heating the samples to 85˚C in a water bath during 2 hours according to the procedure in Georgé *et al.* (2005). The temperature was checked every five minutes during the first hour to ensure that it was stable. After heating, the samples were prepared according to the FC method.

3.2.3.2. Enzymatic removal

The enzyme ascorbate oxidase was used for enzymatic removal of ascorbic acid. Ascorbate oxidase reduces ascorbic acid to dehydroascorbic acid according to the following reaction:

$$2\ \text{L-Ascorbic acid} + O_2 \xrightarrow{\text{Ascorbate oxidase}} 2\ \text{L-Dehydroascorbic acid} + 2\ H_2O$$

Ascorbate oxidase was dissolved in a phosphate buffer consisting of 100 mM KH_2PO_4, 4 mM Na_2PO_4 and 5 mM EDTA and pH was set to 5.6. Different sets of samples were prepared; reference samples, samples with added ascorbic acid (conc. 30 mg/100 ml), samples treated with ascorbate oxidase and pH-adjusted reference samples. The pH was adjusted to 5.6 in the samples treated with ascorbate oxidase, and the reference samples, since this is the optimum pH for the enzyme. 10 µl of enzyme was added to the test tube containing only the sample and the sample was then left for 20 minutes.

3.2.4. Addition of ascorbic acid

Ascorbic acid, with the concentrations of 0, 10, 30, 50 and 100 mg/100 ml, was added to the samples and the TPC was evaluated using both the HRP method and the FC method.

3.2.5. HPLC analyses

HPLC was used to analyse the amount of ascorbic acid in the fruit juices. Samples were mixed with 2 % metaphosphoric acid and pipetted into vials. Analysis was also performed on heat- and enzymatically treated juice samples. Of the samples with added ascorbic acid, only orange juice samples were analyzed by HPLC.

3.2.6. Comparison between the HRP and the FC method

The results obtained with the HRP and FC methods were compared for different treatments: heat, enzymatic removal, ascorbic acid addition and reference samples. After comparing the results, further experiments were set up.

3.2.7. The FC and HRP method tested with known polyphenols

Five different types of polyphenols were used to study if there were differences between the methods in sensitivity to different compounds that are commonly present in different fruit juices. The polyphenols used were cyanidin-3-O-β-glucoside, chlorogenic acid, tannins and procyanidin B2 diluted in a buffer solution. These were chosen to cover most of the different groups of polyphenols present in the juices. Each of the four substances was tested in five replicates by both the HRP method and the FC method. The procedures were the same as previously described for pure fruit juices.

3.3. Statistical analysis

The results were analyzed with the statistical program Minitab 15 and one-way ANOVA analysis was carried out with a confidence interval of 95 %.

3.4. Technical equipment

The spectrophotometer used for TCP analysis with both the HRP and FC method was a Shimadzu UV-2101PC. HPLC analyses were carried out on a Shimadzu SIL-10A SPD-10AV.

4. Results

4.1 Optimisation of the HRP method

4.1.1. HRP reagent stability

HRP reagent was tested on the same day, immediately after being prepared (measurement 1) and 25 hours later (measurement 2). Considerably lower levels (32 %) of polyphenols were obtained at measurement 2 compared to measurement 1. The pH value of the reagent was however quite stable (a slight increase from 8.40 to 8.46).

4.1.2. Ratio of H_2O_2 and HRP

Different concentrations of H_2O_2 and HRP enzyme were tested in various combinations and the most relevant results are presented. Three concentrations of H_2O_2; 10, 20 and 30 µl were tested together with 10 µl of the enzyme HRP (Fig 4).

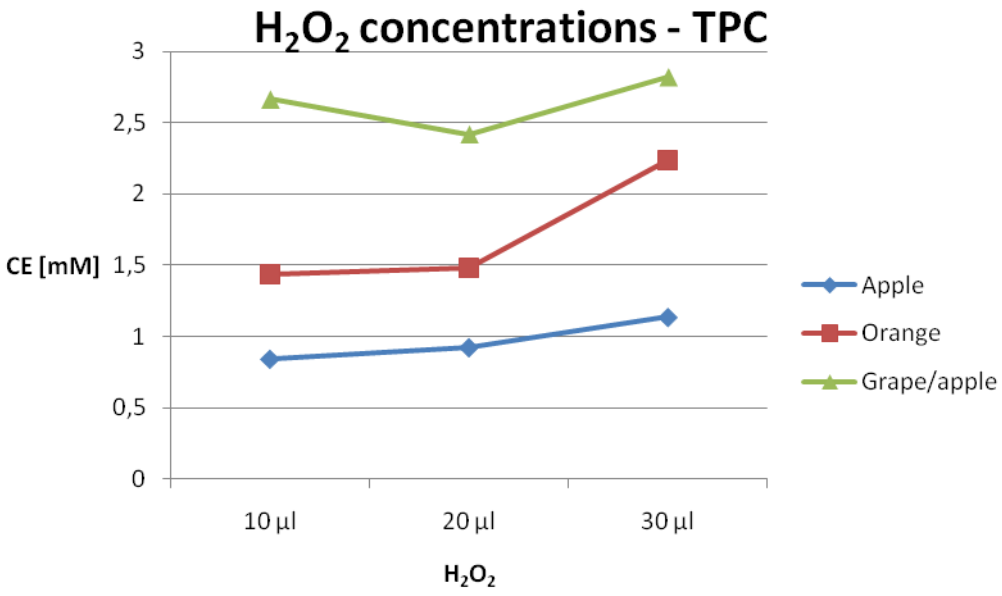

Fig. 4. TPC analysis with HRP method. The TPC results are reported in CE (catechin equivalents) and were studied for three concentrations of H_2O_2: 10 µl, 20 µl and 30 µl; on the three juices: apple, orange and grape/apple. The results presented in the graph are mean values of two replicates.

As shown in Fig. 4, increased concentration of H_2O_2 was associated with an increase also in TPC. During analysis of TPC, two lag phases were observed at the concentrations of 30 µl with H_2O_2. Consequently, this concentration was considered too high and unstable due to possible unknown reactions.

4.1.3. Stability of the HRP method

The stability of the HRP reaction was investigated at the time intervals 0, 10, 20, 50, 110, 170 and 290 minutes using a concentration of H_2O_2 and HRP of 20 µl and 60 µl respectively (Fig. 5). Two subsamples, i.e. two packages (I) and (II) of each juice were tested and reported separately. The concentration of TPC increased with time for all three types of juices studied. However, the last two measurements of orange juice in package II decreased in TPC, whereas juice in package I instead continued to increase. Apple and grape/apple juice showed more similar TPC values between the packages compared to the orange juice. The standard deviation is also highest for orange juice, ±0.170 in package I and ±0.267 in package II.

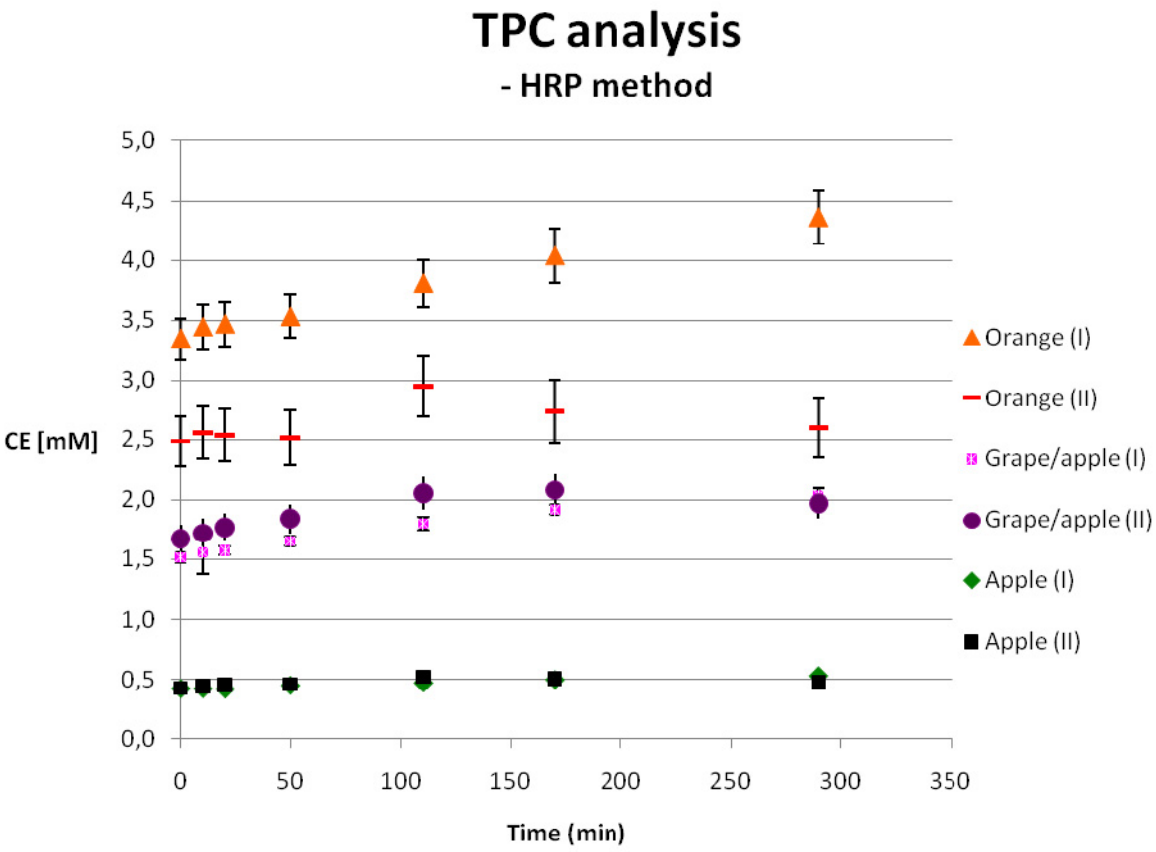

Fig. 5. TPC analysis of apple, orange and grape/apple juice using the enzymatic HRP method. The results are presented in CE (catechin equivalents). Two packages from each juice type from the same processing batch were tested (I) and (II). The CE values are plotted with standard deviations.

4.2. The FC method

The FC method was used to analyse the reference samples, the heat-treated (*procedure described in 3.2.3.1.*) samples, the references samples with adjusted pH, the ascorbate oxidase treated samples and the samples with ascorbic acid added. The samples, which were subject to enzymatic ascorbic acid removal, were pH adjusted to 5.6 because of improved action of the enzyme at this pH. Reference samples were also pH adjusted and included in the trial to study if pH affected the results.

4.2.1. Comparison between HRP and FC method

There was a significant difference ($P < 0.001$) between the two methods in obtained results of total phenolic content for all three juices when reference samples (juice samples without any treatment) were analyzed both by the HRP method and the FC method (Fig. 6).

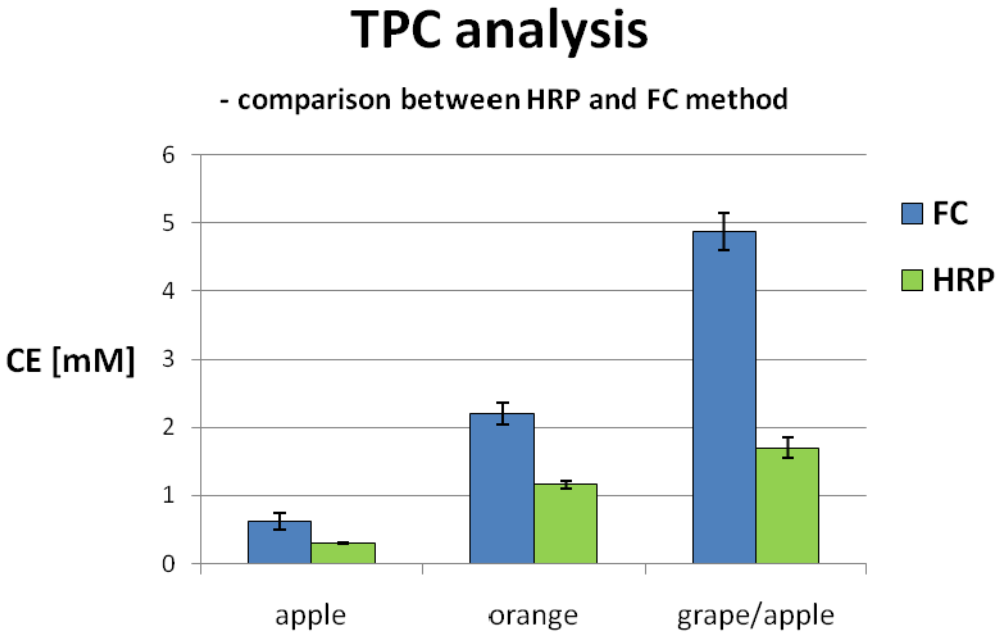

Fig. 6. The concentration of total polyphenols (as CE, catechin equivalents), in mM in the three juices tested, using the FC and HRP method respectively. Mean values of three subsamples and three replicates.

4.2.2. Ascorbic acid removal

4.2.2.1. Ascorbic acid removal by enzymatic treatment

The TPC measurements of reference samples and ascorbic acid treated samples are reported and compared in Fig. 7. There was no significant difference between the treatments for apple juice, and there was also no ascorbic acid present in the samples based on the results from

HPLC analysis. The obtained TPC of ascorbate oxidase-treated samples was significantly lower ($P < 0.05$) compared to the reference sample for orange juice. The TPC of grape/apple juice was instead significantly higher ($P < 0.001$) in the ascorbate oxidase-treated samples compared to the reference samples.

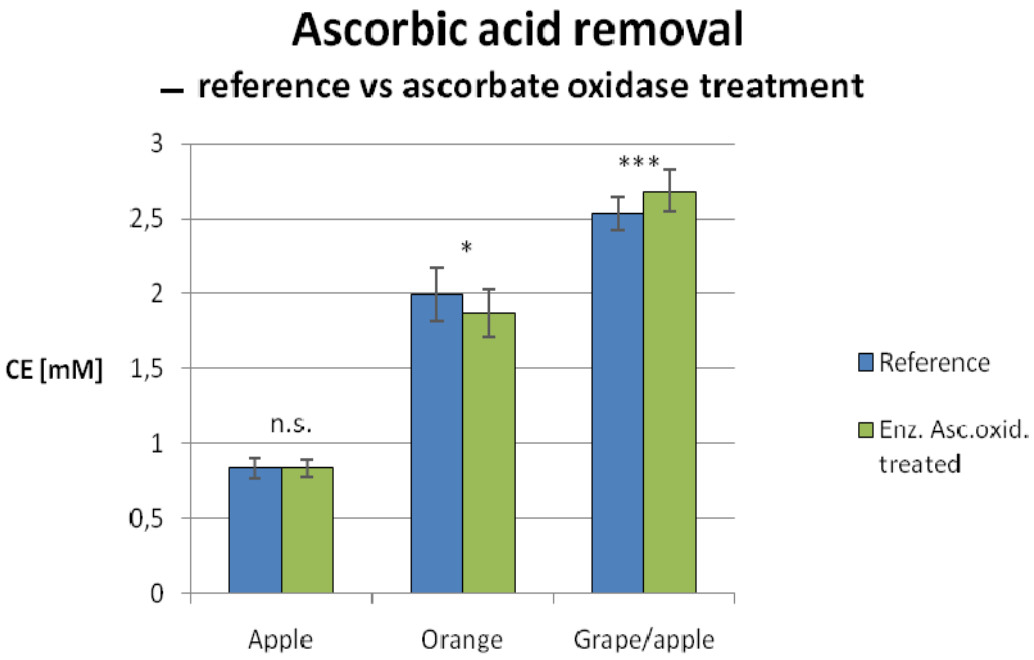

Fig. 7. TPC analysis with the FC method. Reference samples were compared with ascorbate oxidase-treated samples of apple, orange and grape/apple juice. All samples were pH adjusted.

4.2.2.2. Ascorbic acid removal by heat treatment

TPC analysis of reference samples and heat-treated samples are reported and compared in Fig. 8. There was no significant difference (n.s.) between the treatments in neither orange juice ($P = 0.112$) nor grape/apple juice ($P = 0.292$). On the contrary, there was a significant increase ($P < 0.001$) in TPC in gently heat-treated apple juice compared to reference samples of apple juice.

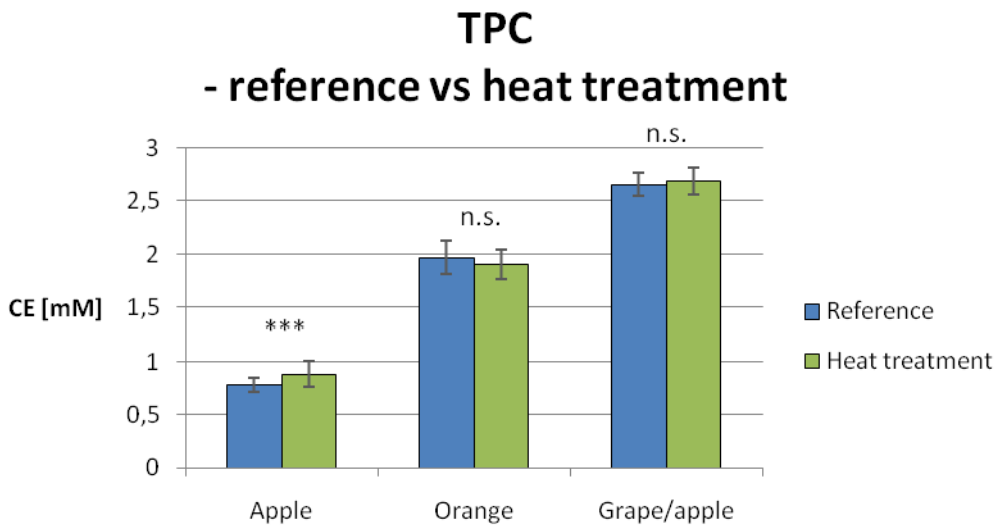

Fig. 8. TPC analysis with the FC method. Reference samples are compared with heat-treated samples in the three juices apple, orange and grape/apple.

4.2.2.2.1. Heat-treated samples of apple juice

Since heat treatment of the apple juice resulted in an increased estimate of TPC (from 0.78 mM to 0.88 mM) it was suspected that some of the polyphenols in the apple juice may have been broken down to other, more simple phenols. This was also confirmed by a HPLC-MS analysis in which a peak of chlorogenic acid derivatives had increased compared to the reference sample (Table 1).

Table 1. Peak with chlorogenic acid derivatives from heat treated (ht) and reference samples (ref) of apple juice. Results in cps (counts per second) of mass spectrometry analysis with three subsamples.

Sample ID	Peak area
Apple 1a (ht)	111000
Apple 1b (ht)	113000
Apple 1c (ht)	103000
Apple 1d (ref)	42600
Apple 1e (ref)	63400
Apple 1f (ref)	56200

4.2.3. Ascorbic acid addition

The results of TPC analysis made on orange juice samples with added ascorbic acid in the concentrations 0, 10, 30, 50 and 100 mg/100 ml are shown in Fig. 9, with and without previous overnight extraction. Overnight extraction reduced the TPC estimate independently of the amount of ascorbic acid added. The TPC value was thus clearly influenced by the amount of ascorbic acid added.

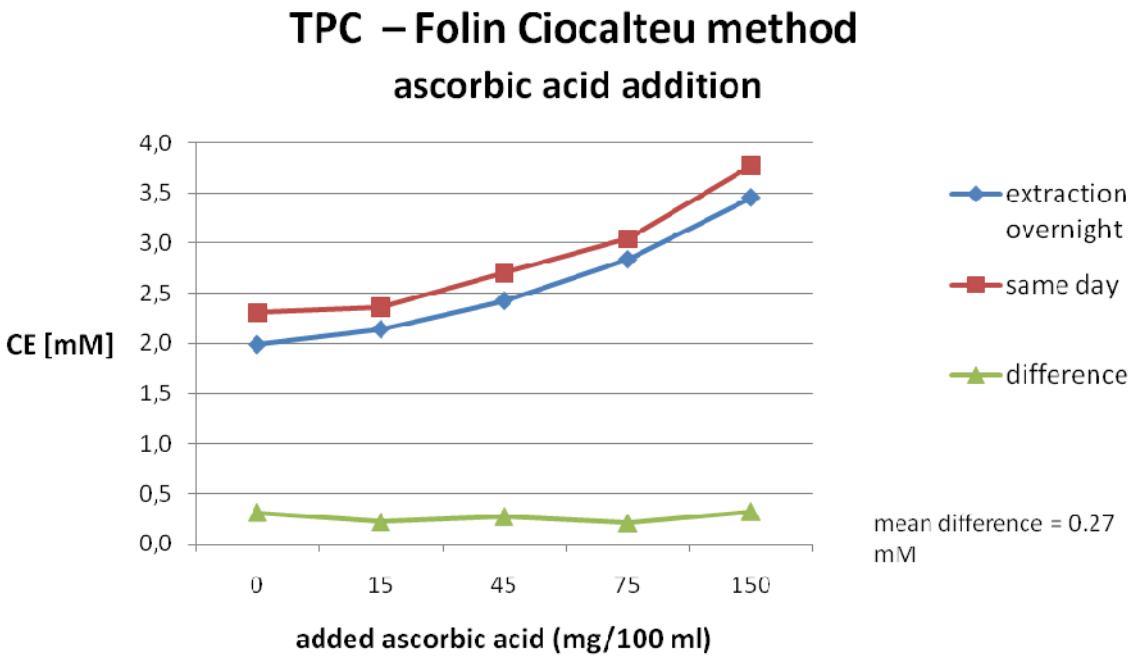

Fig. 9. TPC analysis with the FC method of orange juice with ascorbic acid addition; with and without overnight extraction. The difference between the two treatments is also plotted in the graph.

4.3. HPLC analyses

4.3.1. Ascorbic acid measurements of the juices

The ascorbic acid concentration was measured by HPLC analysis. Apple juice and grape/apple had no detected amount of ascorbic acid whereas the mean content of ascorbic acid was 27.3 mg/100 ml for orange juice.

4.3.2. Analysis of orange juice with ascorbic acid addition

In Fig. 10 it is shown that approximately 79 % of the ascorbic acid was left in the solution following an overnight extraction of polyphenols (using a solution of 50 % ethanol and 50 mM Na_2CO_3) of orange juice supplemented with different concentrations of ascorbic acid.

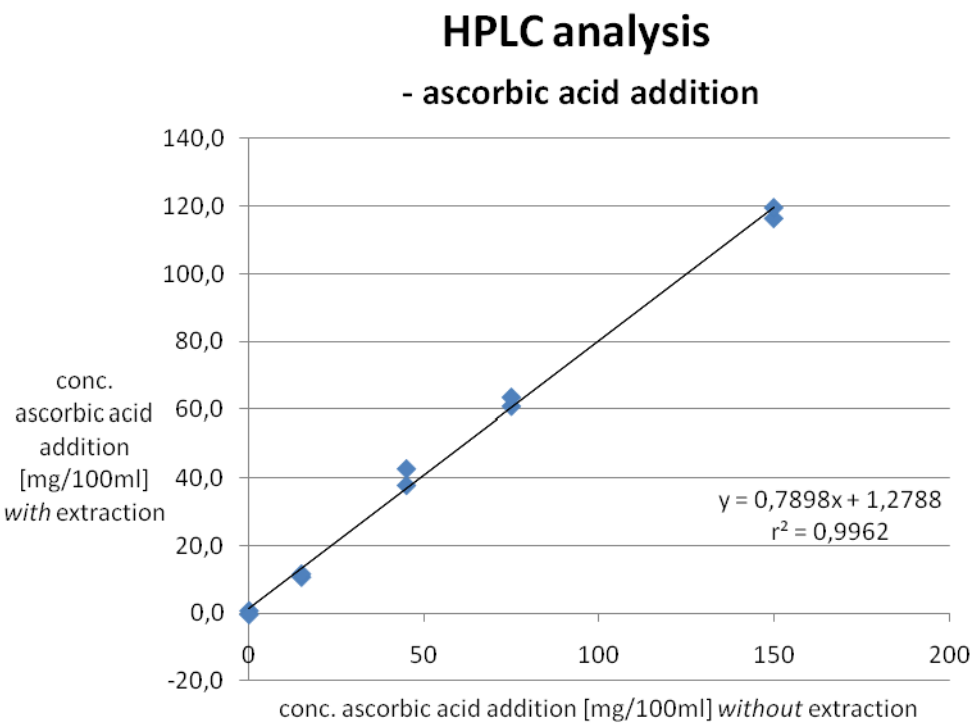

Figure 10. HPLC analysis of orange juice with addition of ascorbic acid. The y axis shows the concentration of added ascorbic acid *with* extraction and the x-axis shows the concentration of ascorbic acid *without* extraction overnight. Only the added concentrations of ascorbic acid are presented in the graph. The graph illustrates that only approximately 79 % (k-value 0.7898) of the ascorbic acid was present in the solution after extraction overnight.

4.4. TPC analysis of known polyphenols

CE concentrations differed significantly between the two methods for all tested polyphenols. As shown in Fig. 11, cyanidin-*3-O-β*-glucoside was to a very limited extent detected by the enzymatic HRP method; average CE concentration was only 0.30 mM for the HRP method as compared to 2.27 mM for the FC method. The HRP method resulted in a large variation among the replicates for cyanidin-*3-O-β*-glucoside (±0.152 mM) and procyanidin B2 (±0.106 mM). Even chlorogenic acid differed significantly between the two methods, in spite of rather similar averages, 1.48 mM for the HRP method and 1.52 mM for the FC method.

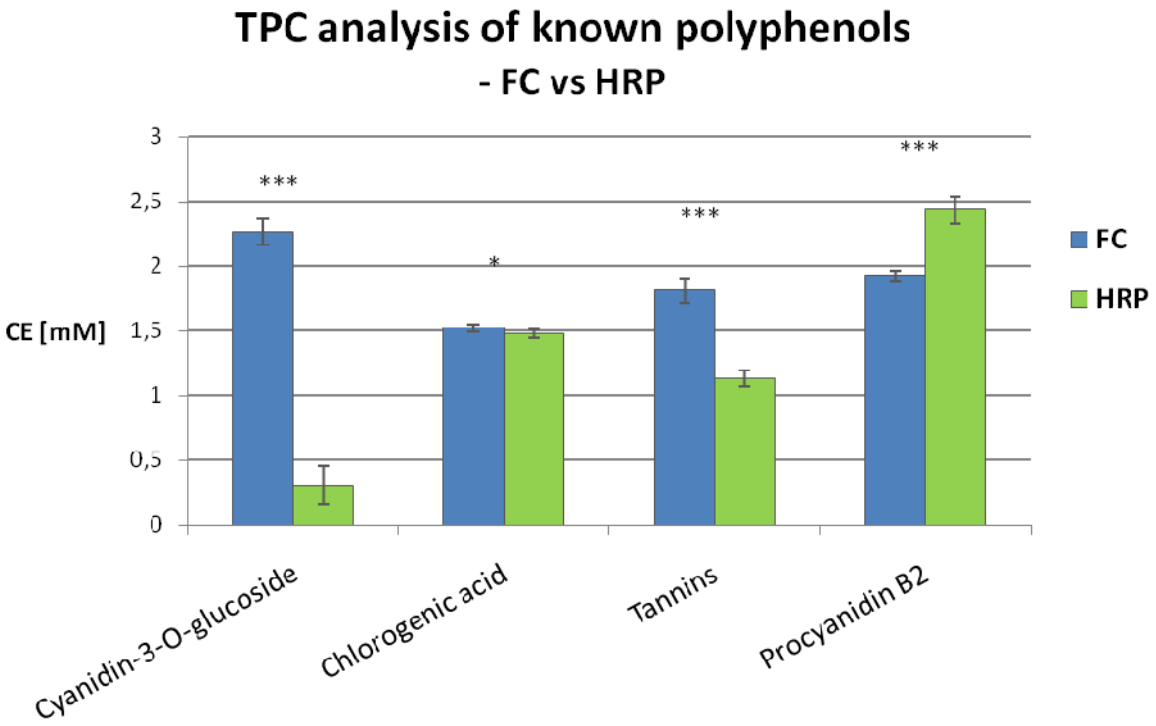

Fig. 11. TPC analysis of known concentrations of four polyphenols (cyanidin-3-O-β-glucoside, chlorogenic acid, tannins and procyanidin B2) estimated by HRP and the FC method respectively. The results and significances presented derive from five replicates.

5. Discussion

5.1. Optimisation of HRP method

After some initial experiments, it was soon realized that the HRP method needed to be optimized. The method had been used previously to study polyphenols, but then for different areas, e.g. contaminated water (Khan and Nicell, 2007). Phenols are a known issue in wastewater and analysis are made to be able to detect the level of abundant particles. Concentration of phenols in these kinds of solutions is much lower and not of the same complexity when it comes to antioxidants and different polyphenols present in the matrix. However, by studying the setup of the experiment (Khan and Nicell, 2007) some conclusions could be drawn about the ratio between the reacting substances, which were helpful for planning my experiments. Furthermore, the decision not to mix H_2O_2 and the enzyme HRP with the rest of the reagent was taken to avoid the substances being degraded or depleted in the solution before analysis. This procedure is probably not suited for routine laboratory work since it is inefficient and time consuming, but it was used in these experiments to minimize the source of errors.

To study the stability of the reagent, a trial with different time intervals was made. This was considered necessary since it is important to know how long before analysis the samples could be prepared without affecting the final result. These results suggest that TPC analysis should be performed within approximately one hour. This will facilitate for preparation of several cuvettes at a time before running the samples on the spectrophotometer. However sufficient 4-aminoantipyrine must be present so that the reaction between H_2O_2 and phenols can occur (Stevanato *et al.,* 2004; 2008).

5.2. Analyses with the FC method

Samples were prepared and analysed with the two different methods, optimized HRP and FC, which produced significantly different results. Knowing that the HRP method should not interfere with ascorbic acid, removal of ascorbic acid from the samples before using the FC method was a strategy to study if there was a presumed interference.

5.3. Ascorbic acid removal

The two methods chosen for removing ascorbic acid have both advantages and disadvantages. The heat treatment is more time-consuming but at the same time less expensive than using an enzyme. Since the results show that ascorbate oxidase needed a higher pH than naturally present in the fruit juices (enzyme pH 5.6 and juice pH 3.6-3.9) more adjustments in the experimental procedure were needed. Heat treatment showed an insignificant tendency to decrease the TPC of orange juice. The kind of heat treatment that should be performed could also be discussed. Two hours of heating is a rather long time when comparing to processing of juice. When processing juice, the heating normally lasts for about 15 seconds at about 91˚ (high pasteurization) or 70˚C at 20 seconds (light pasteurization). This is a process that is aimed to remove the bacteria in the juice but high pasteurization also has been proven to remove some amounts of ascorbic acid (Bates *et al.,* 2001). To enable such a short heating time, more advanced equipment is needed.

What also has to be taken in consideration is the fact that the chemical structures in the matrix of the juice could change during heat treatment; a trend that was seen in the apple juice samples. The increment of TPC in apple juice shows that something has changed since there is no ascorbic acid present in the apple juice. Heat treatment should not be used to remove ascorbic acid since this will give a misleading result of TPC of the juice. The HPLC analysis showed that only orange juice contained detectable amounts of ascorbic acid; a mean value of 27.3 mg/100 ml. By analysing the actual values of ascorbic acid, more precise conclusions of further results could be drawn.

5.3. Ascorbic acid addition

As mentioned previously, ascorbic acid interferes with the FC analysis and to find out to what extent it affects the results this was investigated further. Only orange juice was chosen in this part of the experiment because it was the only juice with ascorbic acid naturally present and thereby believed to give more reliable results, considering the matrix variations between the juices. Since approximately 21 % of ascorbic acid was degraded during the extraction phase of normal TPC analysis with the FC method, the interference in the analysis may be somewhat smaller than expected. When looking at these results of the TPC analysis of orange juice with and without extraction, there seems to be a mean difference of approximately 0.27 mM, independently of the concentration of added ascorbic acid. If this difference is reasonably constant, it could be subtracted from TPC results on at least orange juice. Future

studies should test other ascorbic acid-rich juices to see how much ascorbic acid that will be degraded with the same method and to see if there are large variations within the matrices of different juices. What also would be interesting is to compare some juices with both high content of ascorbic acid and polyphenols. If there is a high polyphenol content, the interference might be of less importance since the TPC value is as high anyhow.

5.4. TPC analysis of known polyphenols

When this experiment started, the hypothesis was that the TPC results from the two methods should be comparable when ascorbic acid had been removed. Only orange juice contained ascorbic acid in significant amounts and the results from the other juices tested should thus be directly comparable. However the TPC results were not identical and therefore it was thought that different sensitivity of polyphenols may influence the results.

It was noticed that the HRP method was not able to react with all groups of polyphenols; especially not cyanidin-3-*O*-*β*-glucoside. Since this anthocyanidin is a pH-indicator and in juices present as a flavylium cation that is red coloured, it could be a problem to detect it with the HRP analysis at the wavelength of 500 nm. This experiment also confirms that the HRP method may not be suitable for TPC analysis of fruit juices due to high matrix complexity.

5.5. Other studies

Previous studies with the HRP method also report of different CE values compared to values derived using the FC method (Stevanato *et al.,* 2008). Possibly there would be a similar explanation as in the experiments performed in this report; not all spectra of polyphenols are detected, but this could not be verified since there is no relation to the potential interfering substances.

Although the FC method is the most frequent analysis method used for fruit juices, there are also some studies using a terbium sensitised fluorescence method (Shaghaghi *et al.,* 2008), which is reported to be highly sensitive and selective and at the same time easy to use. The method is comparable to the FC method and could be an alternative to the HRP method. Another method that also has been used for TPC analysis of different fruit juices is the 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging method (Stevanato *et al.,* 2008). This method has unfortunately been found to be inert to many antioxidants and thus produces unreliable results.

6. Conclusions

Some more efforts should be done to optimize the HRP method to find the most efficient ratio between H_2O_2 and HRP. It could be that this method is more suitable for other analysis such as phenol contaminated water and matrices of low complexity.

When considering the different treatments for enzymatic removal, heat treatment is not to be preferred due to the degradation of some polyphenols. As it affects different types of juices in various ways, the results will be too varying. One could also ask how efficient the ascorbate oxidase is and if there are alternative methods to this enzyme since it did not succeed at removing ascorbic acid efficiently in the polyphenol-rich juice.

Improvements that could be done on the FC method can also be discussed. The extraction usually decreases the ascorbic acid content, which is positive for the results of the TPC analysis since it reduces the interference. Would longer extraction time decrease the ascorbic acid content further? However, it could also be a possibility that the polyphenol content will be influenced negatively. Juices have very complex matrices and therefore the results of different TPC analyses are most reliable when only juices of the same type are compared by the same method.

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