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W.W. Hilhorst - Composition-caused inaccuracies of the QUENCHER method: a model system

The influence of pectin and sugar on the detectability of vitamin C and β-carotene in the QUENCHER method.

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Abstract

The QUENCHER method, a method for measuring antioxidant capacity, still needs optimizing. For that, it is important to know the factors that could cause inaccuracy in measurements. In this research, a model system is created based on the composition of mango. The influence of both pectin and sugar was looked at, as well as the difference in measuring accuracy of vitamin C and β-carotene, as those are the most important antioxidants present in mango. The goal of this research is to get a better understanding of the possibilities and the limitations of the QUENCHER method in terms of accurately measuring antioxidant activity. For this, a model system was used, using the composition of mango. From this research it can be concluded that pectin in mango decreases the TEAC measured by the QUENCHER method by at most 30.41%. Sugar in mango decreases the TEAC measured by the QUENCHER method by at most 5.99%. It can be concluded that the combination of HM-pectin and sugar has the largest negative influence, decreasing the TEAC that was measured by at most 85.77%. Furthermore, it can be concluded that β-carotene cannot be accurately measured with this method as it is now. Additionally, both 20% acetone and 20% THF are not suitable DPPH solvents for this method to measure β-carotene.

Keywords:

QUENCHER, antioxidant activity, pectin, sugar, vitamin C, β-carotene, trolox

List of abbreviations:

ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), a radical that can be used in antioxidant assays.
DPPH: 1,1-diphenyl-2-picryl-hydrazyl, the radical that is used in the QUENCHER method
ET: electron transfer
HAT: hydrogen atom transfer
QUENCHER: QUick, Easy, New, CHEap and Reproducible
TAC: total antioxidant capacity
TEAC: trolox equivalent antioxidant capacity
THF: tetrahydrofuran

Contents

Problem definition

Antioxidants are food components that are able to react with free radicals, in order to remove those. They are called antioxidants because they delay or prevent oxidation (Becker et al., 2004). While free radicals are important for humans, especially in clearing infections, they also increase the chance of several diseases. For example, several types of cancer and atherosclerosis are shown to have a connection with free radicals. Also, the process of ageing in general is accelerated by free radicals. As those free radicals are removed by antioxidants, the latter serve an important function in preventing those diseases (Lobo et al., 2010). Many food products contain antioxidants, and as they serve this function, it is important to be able to detect the antioxidant capacity of food products.

The QUENCHER method is a good method for determining the antioxidant activity in a product, as it is less time-consuming than alternative methods. Regular TAC-measuring (TAC: Total Antioxidant Capacity) methods combine values of the soluble and insoluble antioxidant fractions. This is done by first measuring the soluble fraction in one sample, and taking a second sample of the same product, which is solubilized and measured. As the QUENCHER method can measure both of those at the same time, it is a more accurate method to determine TAC levels. Therefore, the method seems suitable to be used to build a database containing antioxidant activities of many different products (Serpen et al. 2012). However, the procedure still has some flaws. That is why different ways to overcome said flaws should be tested. To be able to look for ways to increase the accuracy of the method, the first thing that should be known is under which conditions the accuracy decreases. This research focuses on researching the measuring inaccuracies that occur in the method caused by the ingredients of mango, as mango is a product that is used in many researches by the Food Quality and Design department. In mango, two possible influencers are pectin and sugars. Therefore, different concentrations of those should be tested.

Additionally, different processing steps have influence on the measured antioxidant capacity, not only in the QUENCHER method, but also in other methods (de Souza et al., 2015, Gültekin-Özgüven, Berktaş and Özçelik, 2016 and Ma et al., 2013). To get a better understanding of the reasons for this, a model system of the influence of different components is needed first.

Literature review

Different Antioxidant assays

There are two main types of antioxidant assays that are used now: those based on hydrogen atom transfer (HAT) and those based on electron transfer (ET). HAT methods are seen as more relevant to radical chain-breaking antioxidant capacity, as the hydrogen transfer reaction forms a key step in the chain reaction of radicals. The ET methods, however, do give useful information about antioxidant capacity. The reducing capacity that is measured with ET methods is not directly related to the radical scavenging capacity, but it still is an important parameter of antioxidants.
Most HAT methods make use of a synthetic free radical, an oxidizable molecule and an antioxidant. The HAT methods can be split into three main groups (Huang et al., 2005):

1. Methods based on inhibiting oxygen uptake: the amount of diene peroxide is used to determine the capacity of an antioxidant. The more diene peroxide that is formed, the lower is the antioxidant capacity
2. Methods based on inhibiting induced lipid autoxidation: the antioxidant capacity is measured in the same way as with methods based on inhibiting oxygen uptake. However, with this method, lipid autoxidation is artificially induced with UV light. Because of this, less initiator and substrate is needed and the method is more sensitive.
3. Assays using molecular probes: A thermal radical generator is used to create a constant flow of peroxyl radicals. The antioxidant that is added competes with the substrate, and will inhibit or retard the oxidation of the substrates. After that, colour measurement is generally used to determine the amount of oxydated substrate. Examples of assays that use this are the total radical trapping antioxidant parameter (TRAP) assay and the oxygen radical absorbance capacity (ORAC) assay. It is not certain, however, that this kind of method can give values that are translatable to food applications. This is the case because in foods the antioxidant concentration is usually much lower than the concentration of substrate. While measuring with this methods, this is the other way around.

According to Huang et al., the ET methods are split into five main groups:

1. Total phenols assay: This method measures the reducing capacity of a sample by letting it react with a Folin-Ciocalteu reagent. It is not actually a specific measure of antioxidant capacity, but it shows excellent correlation with different ET methods for determining antioxidant capacity. It is a convenient, simple and reproducible method, and is therefore the most used measure for antioxidant capacity as of now.
2. Trolox equivalent antioxidant capacity (TEAC) assay: A radical, e.g. ABTS, is allowed to react with potassium persulfate. After that, a small amount of this coloured radical is added to an antioxidant sample. Next, the absorbance is measured over time. The concentration of antioxidant that gives the same regression as trolox possesses the trolox equivalent antioxidant capacity. This can also be performed with DPPH, and the QUENCHER method is also measured via this principle.
3. Ferric ion reducing antioxidant power (FRAP) assay: Works very similarly to the TEAC assay, except it is performed under acidic conditions, and a ferric salt is used as an oxidant.
4. Total antioxidant potential assay using Cu(II) as an oxidant: As the name suggests, this assay uses Cu(II) as an oxidant, which is reduced to Cu(I). It is not commonly used.
5. DPPH radical scavenging capacity assay: DPPH is used as an oxidant. It is reduced, and a colour measurement is done until the absorbance is stable. The remaining percentage is calculated with this, and the concentration that would reduce half of the DPPH is determined.

There are also some methods that are based on measuring the capacity of a sample to react with one specific reactive oxygen species, like hydrogen peroxide, the superoxide anion and hydroxyl radicals.

All of the methods mentioned above do just give results based on in vitro chemical reactions. This is a good predictor of the mechanisms in vivo, but it is not valid to make claims about bioactivity based on results of these tests. Furthermore, none of this methods measure the insoluble antioxidants bound to macromolecules. (Huang et al., 2005)

A lot of difference can be observed between TAC levels measured with DPPH assays compared to ABTS. The cause of this is that lipids cannot act as a physical barrier for DPPH, while they can for ABTS. That is why lipid-rich products will get a higher TAC level when measured with DPPH assays than when measured with ABTS ones. (Serpen et al., 2012)

The QUENCHER method

The principle of the QUENCHER method is to directly place the radical reagent solution and the solid food in contact. In comparison, other popular method use an extraction step. The difference with other popular methods to measure total antioxidant capacity (TAC) is that the QUENCHER method also lets its reagent react to antioxidants on the surface of a polymer (Gökmen et al., 2009). As the total surface area influences the reactivity of the antioxidants on the surface of the macromolecules, the solvent that is chosen should make sure the surface area is as big as possible.
One of the flaws of the QUENCHER method is that it does not work with pure 100% ethanol as a solvent, that is used in the standard ABTS procedure. A solution for this problem was found by Serpen et al.. A solution of 50% ethanol with 50% water gave the most correct results for fruits and vegetables, with the highest TAC values (Serpen et al. 2012). This is because the solvent plays an important role in solubilizing the food matrix. A possible explanation is that ethanol causes big macromolecules like starch, cellulose and proteins to shrink, creating a physical barrier for free radicals to react with the insoluble antioxidants that are inside those macromolecules. Water favours swelling and opening of those macromolecules. For some food products, a water content of more than 50% gave even better results. However, this decreased the stability of the DPPH solution.
When the QUENCHER method was previously tested, it was shown that it did not measure the TAC of carotenoïds like β-carotene correctly. In an ethanol:water (50:50) mixture, the TAC found was 6 times lower than that in literature. This worked better with 100% ethanol, but still not truly accurate. This means other solvents should possibly be tried before carotenoid-rich food products can be measured correctly (Serpen et al., 2008).
Furthermore, for many moist products like some fruits and vegetables, a time consuming freeze drying step is needed for the direct QUENCHER procedure. Also, for products with high TAC values, an extra diluting step is performed, a step that sometimes causes variability between samples. Particle size could also influence the results, as the surface area is an important influencer of reactivity. (Serpen et al., 2012).
More importantly for this research, some food matrices might physically prevent the reaction between antioxidant and radical (Gökmen et al., 2009). However, there is still a gap of knowledge regarding the question which ingredients make the method measure less accurately. That last flaw is where this research will focus on.

Different components

To be able to make a good model system for measuring accuracy of the QUENCHER method, the influence on said accuracy should be tested for many different components of food. Starting with mango, there are some important components that should be tested. This contains two types of antioxidants, vitamin C and β-carotene. Also, pectin and sugar are used. Trolox is used as a reference.

Trolox

Trolox is used in this research to make a calibration curve for this method. Trolox is a water soluble analogue of vitamin E (Lucio et al., 2009). As explained before, it is often used to determine the antioxidant capacity

of different products. When trolox is used as a standard, all other products can be measured in TEAC (trolox equivalent antioxidant capacity).

Antioxidants

Compounds that are able to prevent or delay oxidation by reacting with free radicals are called antioxidants (Becker et al., 2004). In mango, two important antioxidant are vitamin C and β-carotene.
Vitamin C, or L-ascorbic acid, is a water soluble vitamin. It is one of the most well-known antioxidants in human food. Humans cannot produce vitamin C endogenously, which makes it an essential component of human diet (NIH, 2017). In mango, the amount of vitamin C that is present varies between 91 and 186 milligrams per kilogram of product (Franke et al., 2004). The TEAC value of vitamin C is 0.665 mmol/mL, as calculated using the ratio between all TEAC and VCEAC values in a research conducted by Almeida et al. (Almeida et al., 2011).
β-carotene, or provitamin A, is a natural colorant found in many vegetables, that gives an orange colour. It is a known anti-oxidant. Furthermore, in the body, it converts into vitamin A (University of Maryland Medical Centre, 2017). Mango contains 5-30 milligrams of β-carotene per kilogram of product (Manthey et al., 2009). The TEAC value of β-carotene is 1.9 mmol/mg (Re et al., 1999).
The addition of β-carotene is expected to give higher results in an antioxidant measuring assay, as it serves an antioxidative function (Paiva et al., 1999). However, in fruits they normally form complexes with proteins. When added to a solution in free form, it is possible that they do not increase the TAC (Serpen et al., 2012)

Sugar and pectin

Sugars are defined as follows: "All monosaccharides and disaccharides (includes naturally occurring sugars as well as those added to a food or drink, such as sucrose, fructose, maltose, lactose, honey, syrup, corn syrup, high-fructose corn syrup, molasses, and fruit juice concentrate); any oligosaccharides present in these compounds are not counted" (Hess et al., 2012).
In mango, 11.5-25 grams sugar per 100 grams product are present (Tharanathan et al. 2006). Of this, more than half is sucrose, one third is fructose and the last sixth of the sugars consists of glucose (USDA, 2017).

Pectin is a branched heteropolysaccharide that is naturally present in cell walls of plant materials, such as fruits. Pectin exists with different degrees of methyl-esterification (Sharma et al., 2006). The pectin form that is present in mango is considered high methylesterified (HM) pectin, as the degree of methylation is between 50 and 80% (Lima et al., 2010). In mango, 0.38-1.86 grams of pectin are present per 100 grams of product (Yashoda et al. 2005).
In mango, pectin does form a matrix (Vásquez-Caicedo et al., 2007). This means it can possibly be a physical barrier for the QUENCHER method.
Pectin can form hydrogen bonds with ascorbic acid, creating a complex. This makes free radicals less accessible for the ascorbic acid, and the TAC measured can possibly decrease with high pectin concentrations (Christina et al., 2015).

Both sugars (at least a 80% decrease for 50% w/w glucose sugar added) and pectin (at least a 90% decrease for 0.5% w/w pectin added) are shown to decrease the accuracy of the QUENCHER method, in the thesis of Abhiroop Anja Annayavari, 'Detection and Quantification of flavonoids in Cape Gooseberry.', in 2014.

Sugars in the solution make a HM-pectin matrix more stable and more solid, because synergy between the two components occurs. (Al-Ruqaie et al., 1997). Furthermore, when sugars are added to a pectin solution, the viscosity of said solutions rises. This shows that the pectin will be more rigid with sugars present. (Sato et al., 2004)

Research objectives

The goal of this research is to get a better understanding of the possibilities and the limitations of the QUENCHER method in terms of accurately measuring antioxidant activity.

The questions that need to be answered to reach this goal are the following:

1. What is the influence of pectin or sugar solutions with different concentrations on the accuracy of the QUENCHER method in terms of measuring antioxidants naturally present in mango using a model system?
2. Do interactions between pectin and sugars limit the measurement accuracy of the QUENCHER method?
3. Do β-carotene and vitamin C differ in the measurement accuracy of the QUENCHER method?

This questions need to be answered to get to know more about the food components that make the QUENCHER method measure less accurately. This is needed to make further research possible that is aimed at processing products in such a way that the QUENCHER measurement will become more accurate. Four hypotheses are stated for these questions:

1. The more pectin, the less accurate the QUENCHER method will be, as it can possibly hinder the measurement physically.
2. Sugar should not form any physical hinder for the method, so higher concentrations of sugar are not expected to lower the accuracy of the method.
3. Due to the interaction between sugar and pectin, the combination of these two will make the QUENCHER method even less accurate than with only pectin.
4. β-carotene will be less accurately measured with the QUENCHER method than vitamin C, like Serpen et al. found.

Materials and methods

Materials

All chemicals and solvents that were used can be seen in table 1.

Table 1: All chemicals and solvents that were used in the experiments

Material	Purchased from	Ordering code
HM-pectin from apple	Sigma-Aldrich	76282-500G
Sucrose	Sigma-Aldrich	S7903
Cellulose	Sigma-Aldrich	22182-1KG
Vitamin C	Sigma-Aldrich	95210-250G
6-hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic acid (trolox)	Sigma-Aldrich	238813
1,1-diphenyl-2-picryl-hydrazyl (DPPH)	Sigma-Aldrich	D9123-1G
β-carotene	Sigma-Aldrich	C9750
Fructose	Merck	105321
Glucose	Merck	108337
Ethanol	Merck	100983
Tetrahydrofuran (THF)	Biosolve	202206
Methanol	Biosolve	136835
Acetone	VWR	1.00012.1000
MilliQ water	Purelab	

Methods

The method that was used was the QUENCHER method, established by the FQD department as protocol 41: 'Total antioxidant capacity of foods using Quencher procedure'. Every step was performed according to this protocol, except when stated otherwise.

In this method, different concentrations of the sample that needs to be tested are brought into contact with DPPH radicals, and the absorbance values that are measured are compared to a trolox calibration to come to a TEAC value. For measuring the absorbance, a Cary 50 spectrophotometer was used, at a wavelength of 525 nanometres.

The research was performed in two parts: the first part was aimed at optimization of the QUENCHER method. The second, and main, part, was aimed at researching the effect of concentrations of sugar and pectin on antioxidant measuring capacity using the QUENCHER method.

Optimization of the QUENCHER method

Four experiments were performed aimed at improving the QUENCHER method. 1. A dilution series was created. 2. The effect of sonication and stirring on the absorbance of the DPPH solutions was researched. 3. The effect of time on the absorbance of DPPH solutions was researched. 4. The effect of time on the absorbance of the samples in the incubation step in the Heidolph Shaker was researched.
The results of the first, second and fourth experiment were used in the execution of the experiments researching the influence of sugar and pectin on antioxidant measuring accuracy using the QUENCHER method.

Dilution series of DPPH solution

A dilution series of DPPH solution was made to check which dilution factor was best to consistently reach the absorbance value of 0.6 after an 23 hour incubation step at room temperature, to make sure the QUENCHER method could be performed. The 10 mg/mL DPPH solution in 50% ethanol was diluted 2, 4, 8, 16, 32, 40, 64, 80 and 164 times in 50% ethanol, in triplo, and the absorbance values were set out in a graph.

Effect of sonication and stirring on the absorbance of DPPH solutions

An experiment was performed to research the influence of sonication in a HBM ultra-sonicator on the absorbance levels reached by the DPPH solution. A DPPH stock solution was prepared according to the protocol, and sonicated for 15 minutes prior the making of working solution. The working solution itself was also sonicated for 15 minutes. Additionally, in this experiment, the influence of stirring was researched, as well as the difference between making a fresh working solution directly from stock and making a working solution 23 hours in advance, as the protocol suggests. It was performed by measuring the absorbance of a DPPH solution after performing all different treatments. The DPPH solution was diluted 41.7 times instead of the 125 times in the current protocol. This was the case, because this resulted in a high enough absorbance value to be able to see differences. The experiment was performed in triplo.

Incubation time

Effect of type of solvent and incubation time of DPPH solution on measured absorbance value

The influence of the 23 hour incubation step of the DPPH solution was tested by making a working solution in 50% ethanol and measuring the absorbance of that solution after 0, 1, 2, 4, 6, 8, 24, 26, and 48 hours. This was done from 2 different stocks, 1 in ethanol and 1 in methanol. This was the case, because methanol is used to dissolve DPPH in an older antioxidant measuring protocol, protocol number 5: 'Trolox equivalents using DPPH assay'. Both of the working solutions were still in 50% ethanol, as that solvent has the best balance between dissolving DPPH and solubizing the food matrix (Serpen et al., 2012). The absorbance should be consistent and the maximum should be obtained in the shortest time possible. The experiment was performed in duplo.

Duration of incubation of samples prior to measurement

In the protocol, an incubation step of 100 minutes in a Heidolph shaker is present. For both trolox and vitamin C, however, the reaction seemed to occur faster than that. That is why an experiment was performed to research the influence of incubation time on the absorbance values of the trolox and vitamin C curves. For this, the amounts that were used can be seen in table 2. The protocol was performed with this amounts, and the absorbance values were measured after 0, 25, 50, 75 and 100 minutes of incubating in the Heidolph shaker.

Table 2: Amounts of trolox and vitamin C used

Number	Trolox (mmol)	Vitamin C (mg)
1	0.0005	0.0065
2	0.0010	0.013
3	0.0015	0.026

Effect of sugar and pectin on the detectability of vitamin C and β-carotene with the QUENCHER method

The main aim of this research was to investigate the way in which different components affect the detectability of the antioxidants in mango. The influence of pectin and sugar on the detectability of antioxidant activity of vitamin C was investigated. Additionally, the ability of the QUENCHER method to measure β-carotene in different solvents was investigated.

Effect of sugar and pectin on the detectability of vitamin C with the QUENCHER method

Only vitamin C

The protocol was followed using a sample with only vitamin C. A 2.6 mg/mL solution of vitamin C in milliQ water was used, and this was diluted in 100% ethanol. The amounts of vitamin C that were in the different samples can be seen in table 3. The curve was compared to a trolox calibration curve created following the protocol. With this, the trolox equivalent antioxidant capacity (TEAC) could be determined. The experiment was performed in duplo.

Table 3: Amount of vitamin C in assay

Sample name	Amount vitamin C in assay (mg)
V1	0.001625
V2	0.00325
V3	0.0065
V4	0.013
V5	0.026

Pectin and vitamin C

Solutions with the same concentrations of vitamin C as in the experiment before and the pectin/vitamin C ratios that can be seen in table 4 were produced. Pectin was wetted with ethanol, and dissolved in water of 60 °C (a sufficient amount of water to dissolve all of the pectin should be used). After that, it was stirred while in a water bath at 60 °C. Cellulose was added to all samples to prevent stickiness and to make sure the amounts that needed to be weighed for the assays were not too small. The way to calculate the amount of cellulose added can be seen below. All of this was produced in an aluminium tray (8x11x5 cm). After that, the samples were freeze dried in a Christ freeze dryer for at least 72 hours. After that, a 0.425 mm sieve was used to make a powder with equal particle size. Then the QUENCHER method was performed with 10, 20, 40, 80 and 160 milligrams of mixture, to make the right amounts of vitamin C, as can be seen in table 4. With the measured values, the TEAC values of the different mixtures were determined and set out in a graph. The experiment was performed in duplo.

Table 4: Amounts of vitamin C and pectin/vitamin C ratios that were used

Cellulose/vitamin C ratio	Amount vitC (mg) → Ratio pectin/vitC ↓	0.001625	0.00325	0.0065	0.013	0.026
6132.4	20.4	P1.1	P1.2	P1.3	P1.4	P1.5
6105.9	47	P2.1	P2.2	P2.3	P2.4	P2.5
6079.4	73.5	P3.1	P3.2	P3.3	P3.4	P3.5
6052.9	100	P4.1	P4.2	P4.3	P4.4	P4.5

Amount of cellulose (x) that is needed:

$$10\left(\frac{m}{(0.01625 + 0.01625p * 5)}\right) - (m * v(1+p)) = x$$

$$with\ x \geq 4 * pw$$

In this formula, *p* is the pectin/vitamin C ratio that is chosen. *m* is the total mass of vitamin C, pectin and cellulose that is needed. The chosen pectin/vitamin C ratio is used, as well as a cellulose/pectin ratio of 4, to prevent stickiness. *m* consists of a vitamin C fraction, a pectin fraction and a cellulose fraction. *v* is the vitamin C fraction. If this formula is used to calculate the amount of cellulose that needs to be weighed, the lowest sample weight that is needed in the assay will always be exactly 10 milligrams. *x* always needs to be higher than 4 times *pw* (the total amount of pectin), to prevent the pectin to become sticky while dissolving, based on the thesis of Christine Hartman, 'The effect of heating on the total antioxidant capacity in Gape gooseberries detected with the Quencher method.', from 2014.

Sugar and vitamin C

For the sugar/vitamin C samples, the ratios can be seen in table 5. The sugar that was used consisted of 50% sucrose, 33.3% fructose and 16.7% of glucose, based on the sugar composition of mango (USDA, 2017). Cellulose was added for the same reason as with the pectin samples, and the entire sample was transferred to an aluminium tray (8x11x5 cm). The formula for calculating the amounts of cellulose can be seen below. All of those samples were also freeze dried in a Christ freeze dryer for at least 72 hours and then sieved. 10, 20, 40, 80 and 160 milligrams of sample were used to perform the QUENCHER method with. The TEAC values of the different mixtures were determined from the measurement, and set out in a graph. The experiment was performed in duplo.

Table 5: Amounts of vitamin C and sugar/vitamin C ratios that were used

Cellulose/vitamin C ratio	**Amount vitC (mg) → Ratio sugar/vitC ↓**	**0.001625**	**0.00325**	**0.0065**	**0.013**	**0.026**
5534.6	618.3	S1.1	S1.2	S1.3	S1.4	S1.5
5292.6	860.2	S2.1	S2.2	S2.3	S2.4	S2.5
5050.7	1102.1	S3.1	S3.2	S3.3	S3.4	S3.5
4808.8	1344.1	S4.1	S4.2	S4.3	S4.4	S4.5

Amount of cellulose (x) that is needed:

$$10\left(\frac{m}{(0.01625 + 0.01625s)}\right) - (m * v(1+s)) = x$$

This formula is the same as the one with pectin and vitamin C, except that the amount of cellulose does not have to be 4 times as high as the amount of sugar, because sugar does not get sticky like pectin does. Furthermore, *s* is the sugar/vitamin C ratio.

Pectin, sugar and vitamin C

The samples with sugar, pectin and vitamin C all had the same pectin/vitamin c ratio, which is 73.5. The pectin was dissolved in the same way as mentioned before, and the sugar was added and dissolved after that. The sugar/pectin ratios of these samples can be seen in table 6. All samples were transferred to aluminium trays (8x11x5 cm). After adding cellulose, all of these samples were also freeze dried in a Christ freeze dryer for at least 72 hours and sieved. The formula to calculate the right amount of cellulose can be seen below. To create the right amounts of vitamin C, the freeze dried samples were used in the QUENCHER method in the following amounts: 10, 20, 40, 80 and 160 milligrams. All TEAC values were determined, and they were compared with values that were expected based on the previous experiments with pectin and vitamin C, and those with sugar and vitamin C. The experiment was performed in duplo.

Table 6: Amounts of vitamin C and sugar/pectin ratios that were used

Cellulose/vitamin C ratio	**Amount vitC (mg) → Ratio sugar/pectin ↓**	**0.001625**	**0.00325**	**0.0065**	**0.013**	**0.026**
5461.1	8.4	SP1.1	SP1.2	SP1.3	SP1.4	SP1.5
5219.2	11.7	SP2.1	SP2.2	SP2.3	SP2.4	SP2.5
4977.2	15	SP3.1	SP3.2	SP3.3	SP3.4	SP3.5
4735.3	18.3	SP4.1	SP4.2	SP4.3	SP4.4	SP4.5

Amount of cellulose (x) that is needed:

$$10\left(\frac{m}{(0.01625 + 0.01625p * 5 + 0.01625s)}\right) - (m * v(1 + p + s)) = x$$

$$with\ x \geq 4 * pw$$

This formula is a combination of the previous two formulas.

Effect of sugar and pectin on the detectability of β-carotene with the QUENCHER method

For β-carotene, the experiment aimed to check whether solvents other than 50% ethanol would work better to test β-carotene. 20% acetone and 20% THF were used, based on the thesis of Mauro Wijnands, 'Antioxidant activity of carotenoids (lycopene and beta carotene) in heated and unheated tomatoes', from 2014. For this, the solvents that were used for the DPPH working solutions, as well as the amounts of β-carotene, can be seen in table 7. The experiment was performed in duplo.

Table 7: Amounts of β-carotene and solvents that were used

Solvent	**0.001625**	**0.00325**	**0.0065**	**0.013**	**0.026**
50% ethanol	C1.1	C1.2	C1.3	C1.4	C1.5
20% acetone	C2.1	C2.2	C2.3	C2.4	C2.5
20% THF	C3.1	C3.2	C3.3	C3.4	C3.5

Statistical analysis

For the statistical analysis of the experiments, two tests were used. For all correlations that were tested, a Pearson's correlation test was used. For all mean comparisons, independent sample t-tests were used. For both analyses, SPSS statistics was used.

Results and discussion

Dilution series of DPPH solution

A dilution series was produced from the stock solution of 10 mg/mL DPPH in ethanol. The aim of this experiment was to find a suitable dilution factor of the stock solution for the rest of the experiments. This was used as a short term solution for the problem that the DPPH solution did not become stable at an absorbance of 0.6. In figure 1, the dilution series that was measured is depicted. As can be seen, the value of 0.6 is reached around a dilution factor of 64. The standard deviations range from 2.78% to 37.51% of the mean.

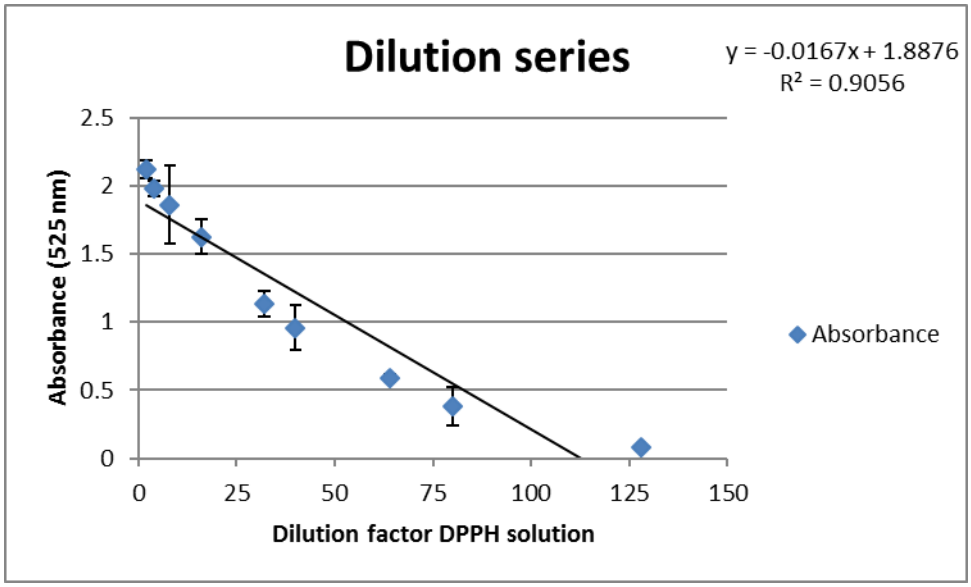


Figure 1: Results of the dilution series produced with DPPH solution. Values are means of duplicates ± SD.

Based on the result in figure 1, a dilution factor of 62.5 was used for the experiments with vitamin C and β-carotene and the trolox calibrations. This solution was convenient to make, and would always reach an absorbance between 0.55 and 0.7, which made it most suitable. According to the current protocol, a solution with a dilution factor of 125 should reach 0.6. Because of that, and some of the standard deviations being more than 10% of the mean, using the dilution factor of 62.5 is not a long term solution.

Effect of sonication and stirring on the absorbance of DPPH solutions

In this experiment, the influence of a few different processing steps on the absorbance of the DPPH working solution was tested. In figure 2, the results of the sonication experiment for the DPPH solution are shown. The sample from the working solution that was sonicated shows an absorbance value that is significantly higher than the one without sonicating or stirring. Additionally, it showed a significantly higher absorbance than the ones that are stirred ($p \leq 0.05$). The aim of this experiment was to reach an absorbance as high as possible. This was because, except for not being stable, the absorbance values for DPPH solution all turned out to not reach the desired absorbance of 0.6 after 23 hours of incubation. The samples from a working solution that was freshly prepared from stock showed higher standard deviations (2.76%-14.71% of the mean) than the ones from the working solution that was incubated for a day (0.23%-2.23% of the mean). Based on these results, the decision was made to use a working solution prepared one day in advance. Furthermore, the stock solution is sonicated before preparing the working solution. The working solution

itself will also be sonicated before use. Furthermore, while pipetting from the working solution, the working solution will be stirred, as stirring also increased the absorbance.

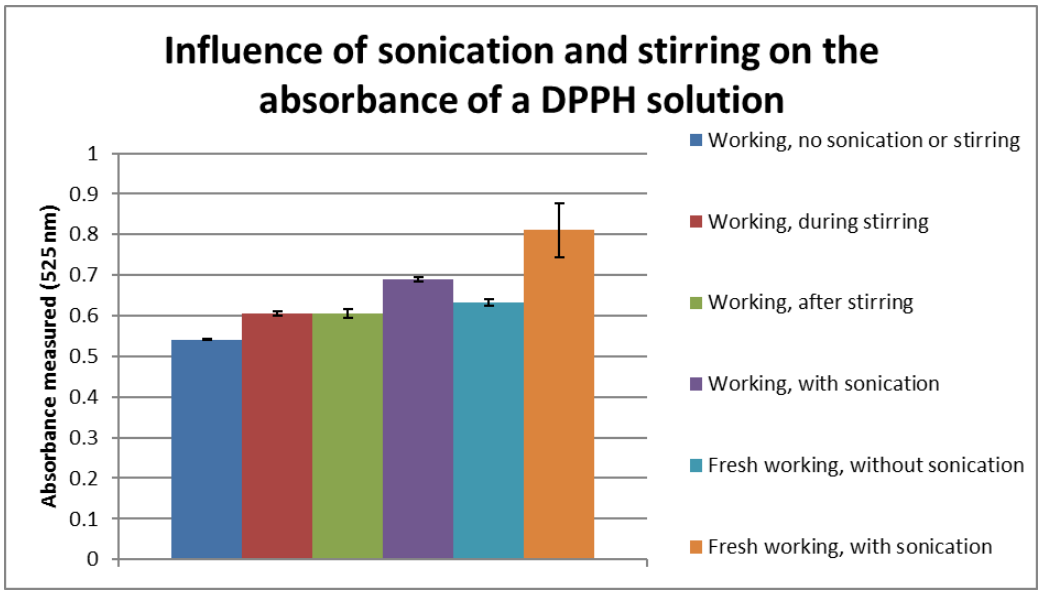


Figure 2: Results of different processing steps on the absorbance of DPPH solution. Values are means of triplicates ± SD.

Ultra-sonication is used to dissolve substances in a quick and efficient way (Hielscher, 2017). This means the result in figure 2 was to be expected, as dissolving the DPPH powder more efficiently makes the sample more homogenous. Additionally, when more DPPH is dissolved, more radical molecules will be available in the sample, increasing the absorbance.

Incubation time

Effect of type of solvent and incubation time of DPPH solution on measured absorbance value

In this experiment, the 23-hour incubation step of the DPPH solution was researched. Figure 2 shows the influence of time on the absorbance value of two DPPH solutions, 1 made from a stock of 10 mg/mL DPPH in methanol, and one with the same concentration in ethanol. Comparing both samples, a difference is seen at 0 h. The sample in ethanol shows a lower absorbance value than the sample in methanol does. The absorbance of the sample in ethanol increases over time. At 48 h, the absorbance values of the sample prepared in ethanol and the sample prepared in methanol do not differ significantly ($p \leq 0.05$). The standard deviations of the duplicates range from 0.01% to 1.02% of the mean.

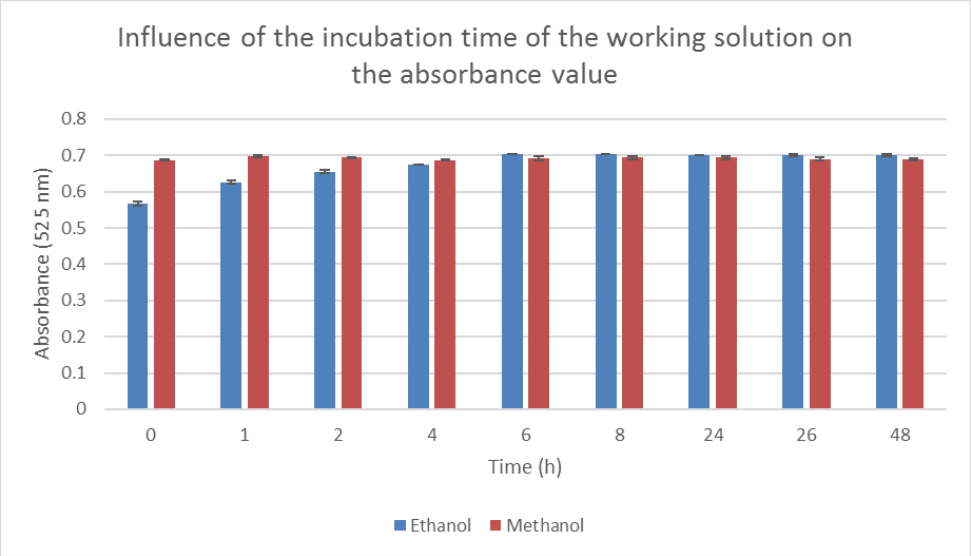


Figure 3: The influence of incubation time on the absorbance of DPPH solutions. Working solutions are made from 10 mg/mL stocks in ethanol (blue) and methanol (red). Values are means of duplicates ± SD.

In the FQD laboratories, methanol was already used as DPPH solvent for protocol 5: 'Trolox equivalents using DPPH assay'. The reason for this was that the DPPH solution in methanol could be used instantly to measure antioxidant activity with. This means this result was to be expected.

Duration of incubation of samples prior to measurement

The aim of this experiment was to test if the 120 minute incubation step in the Heidolph shaker is necessary for all antioxidants. The influence of incubation time, in the Heidolph shaker, on the absorbance values of trolox and vitamin C is shown in figure 4 and 5 respectively.

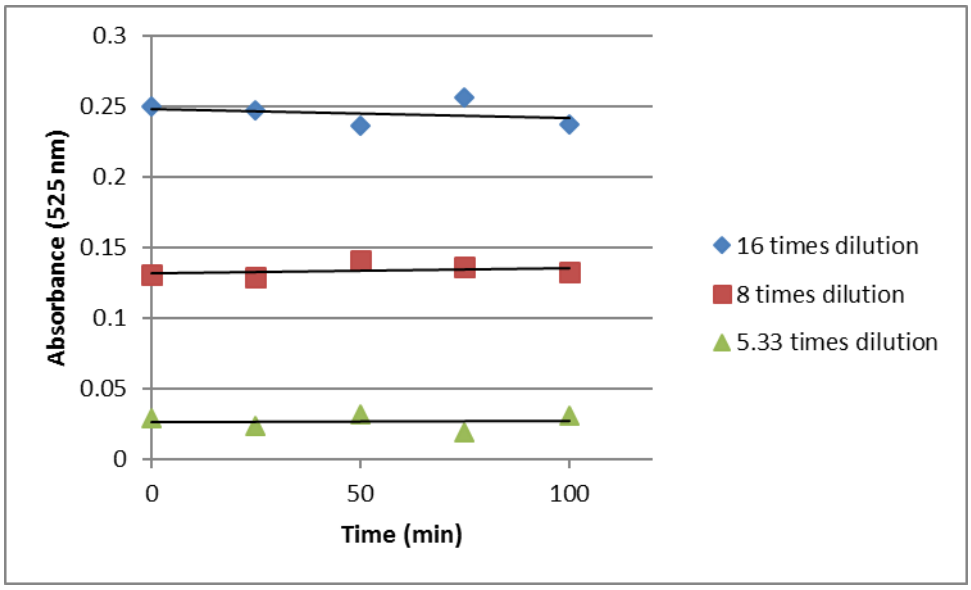


Figure 4: Influence of incubation time in a Heidolph shaker on the absorbance of trolox samples in DPPH.

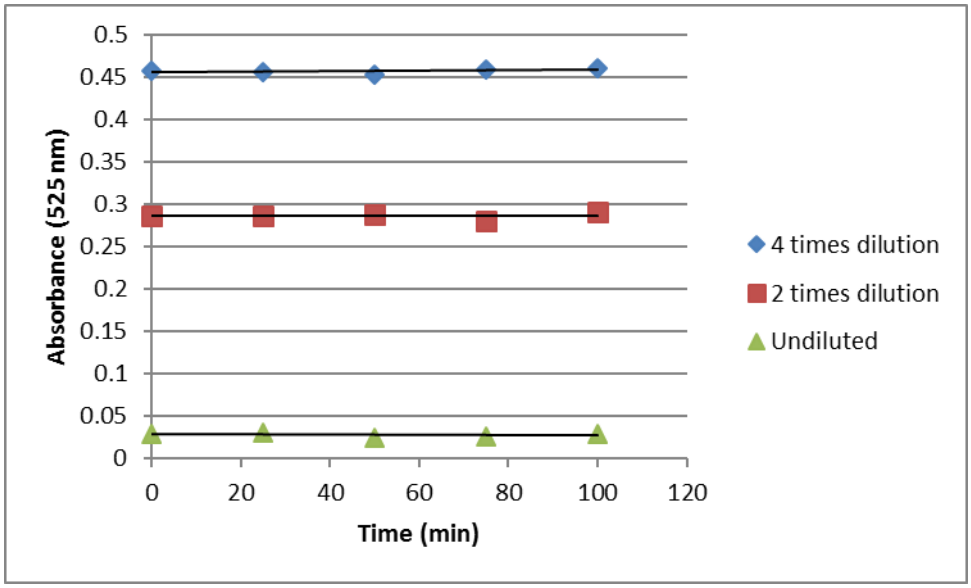


Figure 5: Influence of incubation time in a Heidolph shaker on the absorbance of vitamin C samples in DPPH.

In both figures, at all three concentrations, the incubation time does not change the absorbance significantly ($p \leq 0.05$). This means that for vitamin C and trolox, this incubation step is not necessary. This was in line with the visual observation on which this experiment was based, as both antioxidants seemed to show a colour change in the first seconds of incubation.

Effect of sugar and pectin on the detectability of vitamin C with the QUENCHER method

The following experiments were meant to test the influence of sugar and pectin on the ability of the QUENCHER method to measure vitamin C. The experiment with only vitamin C is used for comparison with the experiment with pectin and vitamin C and the experiment with sugar and vitamin C. Both of those experiments combined were used for comparison with the experiment with pectin, sugar and vitamin C.

Only vitamin C

The results of the experiment with exclusively vitamin C in the sample are shown in figure 6. The TEAC value that was determined from this figure was 0.687 mmol/mg. The standard deviations range from 0.39% to 21.47% of the mean. However, the value of 21.47% is the standard deviation of the absorbance at the highest concentration of vitamin C. Therefore, the absolute standard deviation at this point is small.

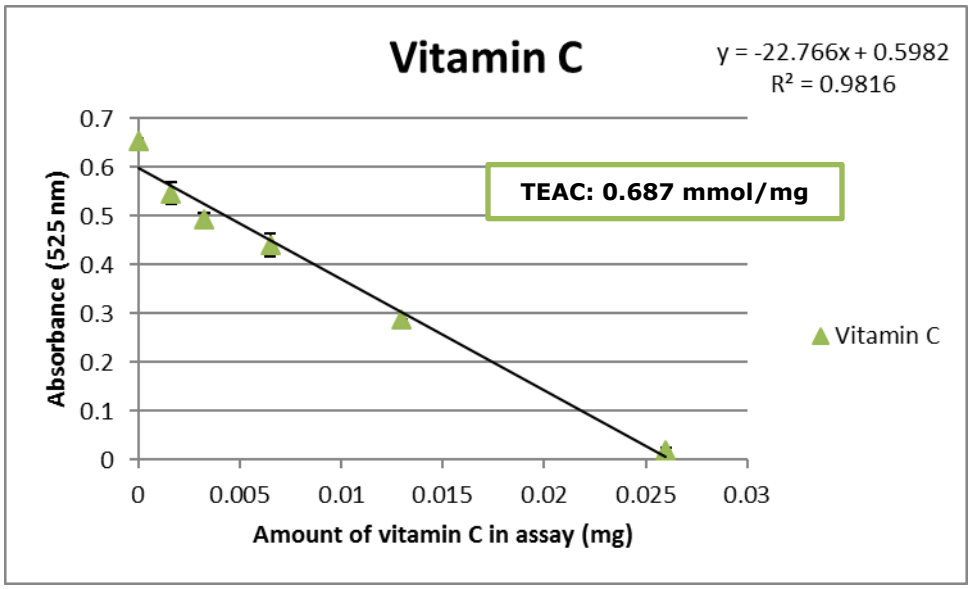
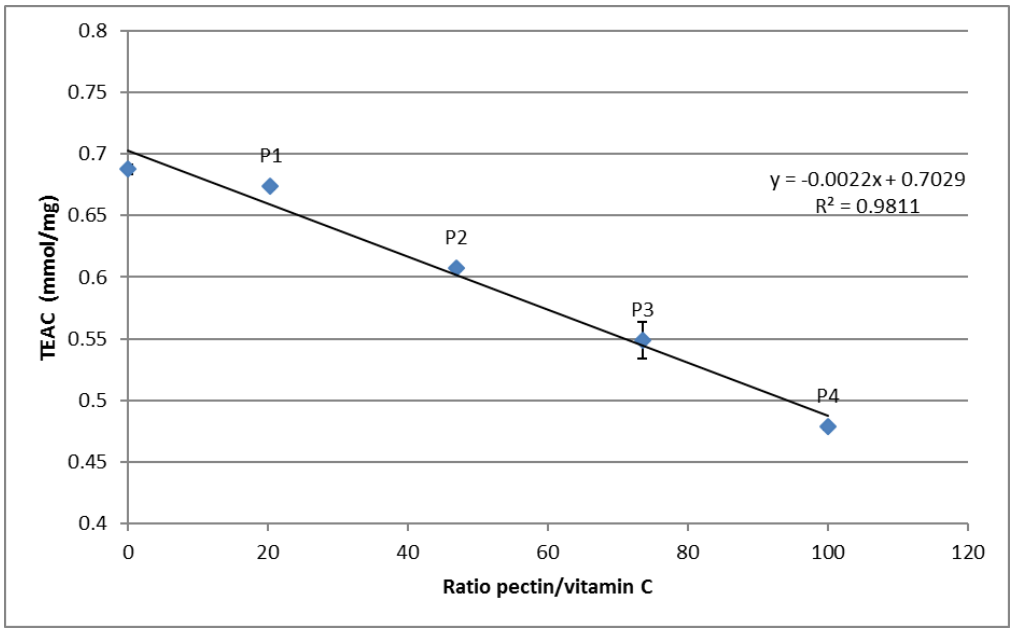


Figure 6: Antioxidant activity of different concentrations of vitamin C as measured by the QUENCHER method. Values are means of duplicates ± SD.

As the literature value of the TEAC of vitamin C is 0.665 mmol/mg (Almeida et al., 2011), and the R^2 of this result is 0.9816, this result can probably be trusted and used for the analysis of the other samples.

Pectin and vitamin C

Figure 7 shows the influence of the pectin/vitamin C ratio on the measurement of the TEAC value. The percentual decrease in TEAC value of every sample is shown next to it. The standard deviations range from 0.38% to 2.70% of the mean.



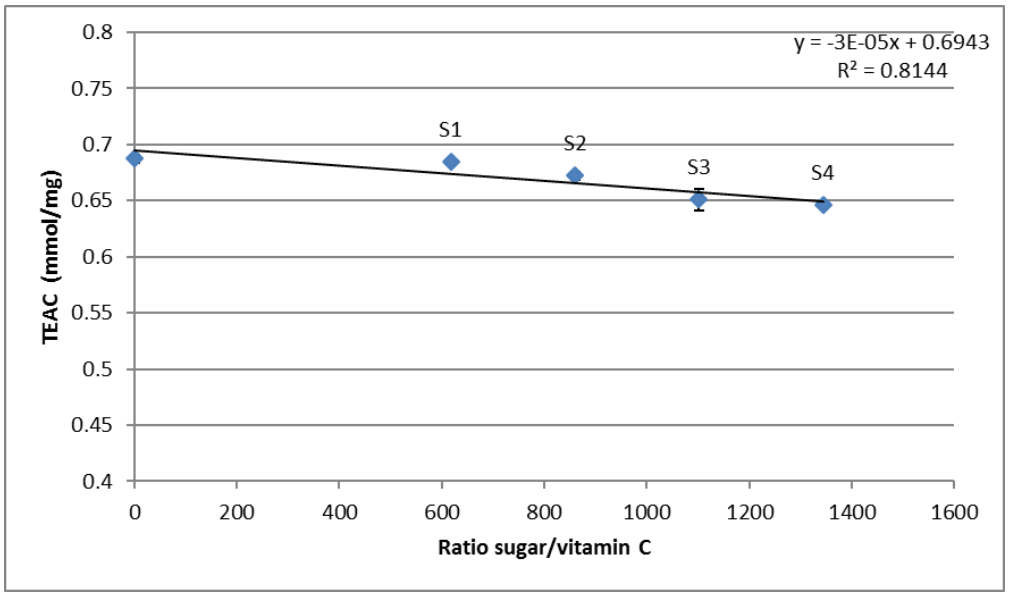
Percentual decrease in TEAC compared to 'only vitamin C' sample:
P1: 2.03%
P2: 11.71%
P3: 20.17%
P4: 30.41%

Figure 7: TEAC values of samples with different pectin/vitamin C ratios. Values are means of duplicates ± SD.

The pectin/vitamin C ratio and the TEAC value do negatively correlate ($p \leq 0.05$). Furthermore, from pectin/vitamin C ratio 47 and higher, the TEAC values significantly differ from the sample with only vitamin C ($p \leq 0.05$). This means the pectin influences the accuracy of the method. An explanation is that pectin hinders the antioxidant and the radical physically from colliding together (Gökmen et al., 2009) (Vásquez-Caicedo et al., 2007). It is possible that hydrogen bonds that are formed between pectin and vitamin C are the cause of this (Christina et al., 2015). However, the exact interaction that causes this measuring inaccuracy still needs to be investigated more. Additionally, this results means an extra step will have to be implemented in the method to prevent the physical hinder from happening for pectin-rich fruits and other food products, while keeping the antioxidants intact.

Sugar and vitamin C

The influence of sugar on the TEAC value is depicted in figure 8. The standard deviations range from 0.05% to 0.57% of the mean.



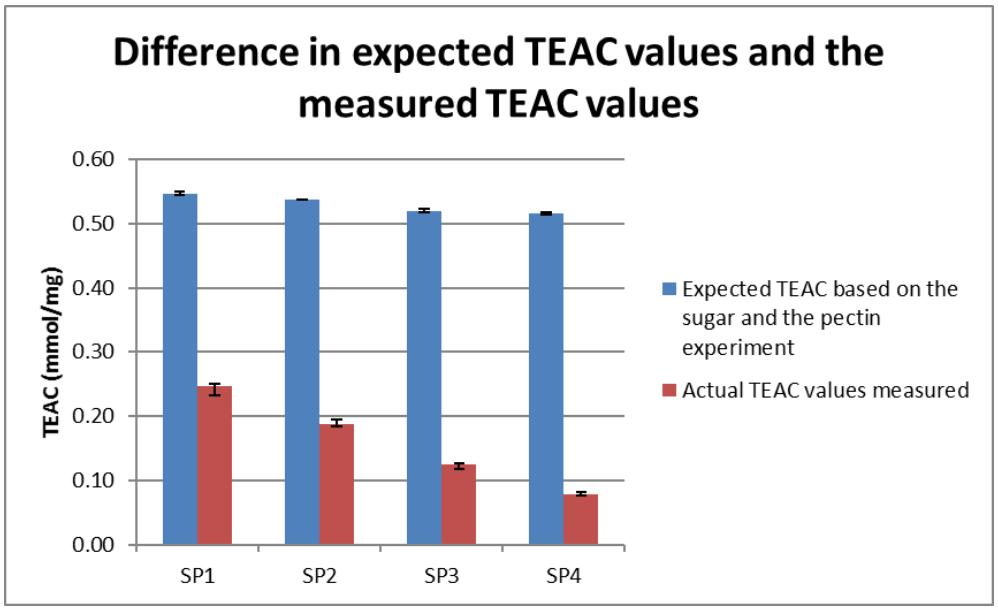
Percentual decrease in TEAC compared to 'only vitamin C' sample:
S1: 0.39%
S2: 2.22%
S3: 5.39%
S4: 5.99%

Figure 8: TEAC values of samples with different sugar/vitamin C ratios. Values are means of duplicates ± SD.

The sugar/vitamin C ratio and the TEAC value correlate negatively ($p \leq 0.05$). Additionally, the TEAC values of the TEAC values of the samples with sugar/vitamin C ratios of 1102.1 and higher do differ significantly with the sample without sugar ($p \leq 0.05$). This means the sugar influences the accuracy of the method, even though the influence is smaller than that of pectin. It is possible that the sugar molecules physically hinder the reaction for the same reason as the pectin molecules did. However, because the sugar molecules are smaller than the pectin molecules, the effect will be of smaller importance. As to which interactions lead to this influence on the accuracy, further research will be needed.

Sugar, pectin and vitamin C

Based on the experiment with pectin and vitamin C, and the experiment with sugar and vitamin C, a certain decrease in TEAC value could be expected in the experiment with sugar, pectin and vitamin C. In figure 9 that expected value can be seen in blue, while the actual measured values are shown in red. The expected value is determined using the fourth data point in figure 7, 'P3', and subtracting the percentual decreases that were determined for every sugar/vitamin C ratio in figure 8. The pectin/vitamin C ratio of all four samples is 73.5, and the sugar/pectin ratios are shown to the right of the figure. The standard deviations of the measured samples range from 1.33% to 4.51% of the mean.



Sugar/pectin ratio
SP1: 8.4
SP2: 11.7
SP3: 15
SP4: 18.3

Percentual decrease in TEAC compared to 'P3' sample:
SP1: 54.93%
SP2: 65.77%
SP3: 77.41%
SP4: 85.77%

Figure 9: Difference between expected TEAC of sugar, pectin and vitamin C samples (blue) and actual measured values of those samples (red). Values are means of duplicates ± SD.

All of the actual values differ significantly from the predicted value at the same sugar/pectin ratio ($p \leq 0.05$). This was to be expected, as the sugar increases the strength of the HM-pectin matrix (Sato et al., 2004). This can also be the explanation for measuring inaccuracies with mango samples, as the same sugar/pectin ratios are used.
Looking critically at these results, it should be taken into account that the duplicates used in the pectin and vitamin C, sugar and vitamin C and pectin, sugar and vitamin C experiments were taken after the freeze drying step. This means for further research it is important to investigate the variability of this freeze drying step itself.

Effect of sugar and pectin on the detectability of β-carotene with the QUENCHER method

In this experiments, the ability of the QUENCHER method to measure β-carotene was tested with three different solvents: 50% ethanol, 20% THF and 20% acetone.

In figure 10, the results of the tests with β-carotene in 50% ethanol is depicted. The TEAC value is given next to it.

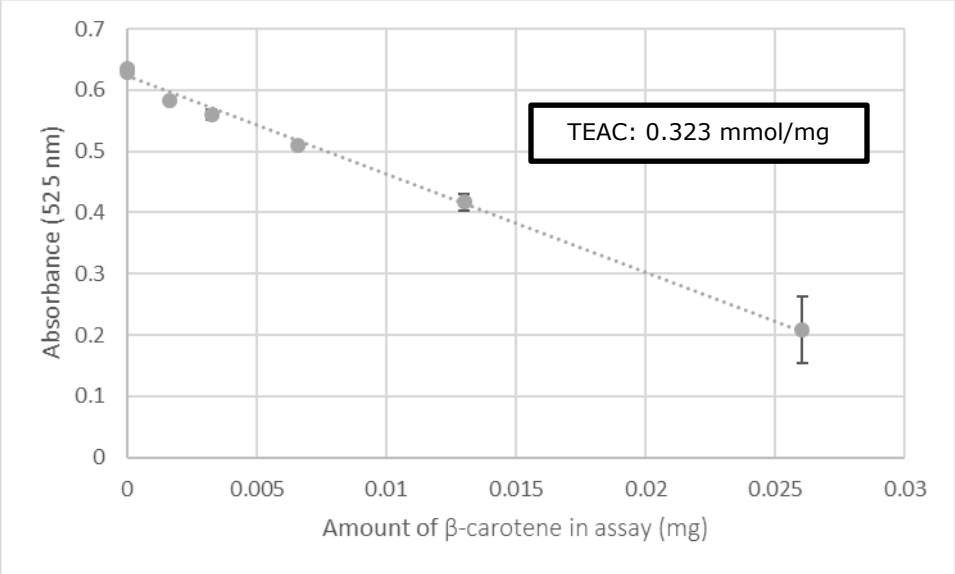


Figure 10: Antioxidant activity of different concentrations of β-carotene as measured by the QUENCHER method, using 50% ethanol as a solvent. Values are means of duplicates ± SD.

The sample that was used in 50% ethanol showed a TEAC value of 0.323. This is in line with the result of Serpen et al., as it is nearly 6 times as low as literature values for the TEAC of β-carotene (Serpen et al., 2008) (Re et al., 1999).

The tests with 20% acetone and 20% THF did not give any results, because the DPPH did not dissolve in both of those solvents. It was visible with the naked eye that a powder was at the bottom of the beaker, with a colourless liquid on top. In figure 11, the trolox calibrations of the experiments with those solvents are shown.

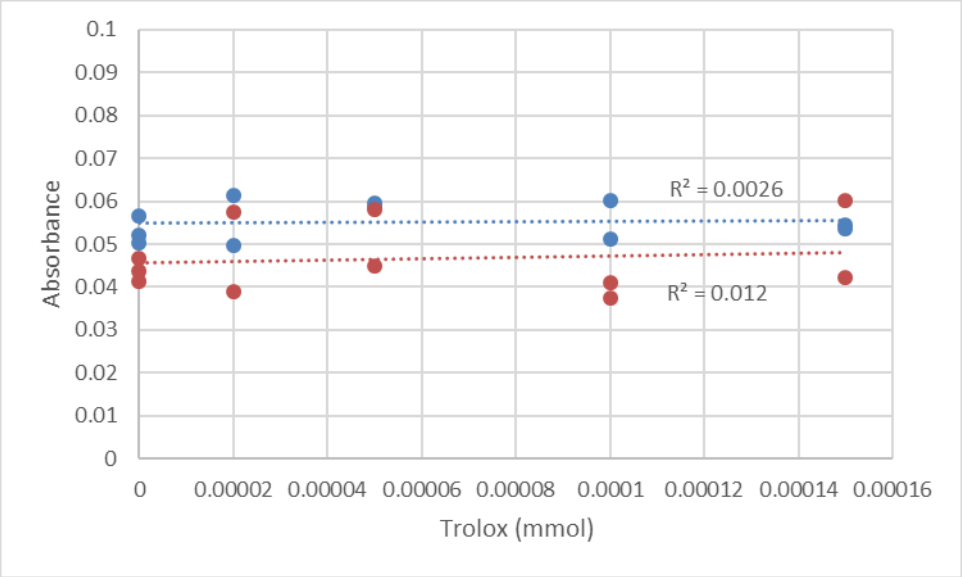


Figure 11: Trolox calibrations of QUENCHER method using 20% acetone (blue) and 20% THF (red) as solvents.

The values measured do not correlate with the amount of trolox, and the absorbance measured did not come close to the desired 0.6. This means both 20% acetone as 20% THF are not suitable solvents to replace 50% ethanol while measuring carotenoïds.

Conclusion and recommendations

Protocol improvements

From the experiment with the dilution series, it can be concluded that a dilution factor of 62.5 has good enough results to perform the rest of the experiments in this thesis with, but is not a long-term solution, as it cannot be completely trusted to have a stable absorbance.

From the experiment about sonication and stirring, it can be concluded that a working solution with an incubation is justly used in the protocol, as making the working on the spot makes the variability rise. Furthermore, the experiment shows that sonication both the stock solution and the working solution makes the absorbance value higher, which makes the result better. Additionally, the working solution will be stirred while adding it to the samples.

The conclusion that can be derived from the experiment regarding the incubation of the DPPH solution is the following: to make sure that the DPPH working solution can be used directly after it is created, it is better to use methanol as a solvent for the stock solution than ethanol.

From the experiment regarding the incubation time in the Heidolph shaker, it can be concluded that both trolox and vitamin C would show the same result with a drastically lower incubation time, or even no incubation time at all. From this experiment, nothing can be concluded about samples other than vitamin C or trolox.

Main research

In the main research, the aim was to find an answer to three research questions.
It was found that TEAC values of vitamin C that are measured by the QUENCHER method are decreased with the addition of pectin (30.41% decrease at most) or sugar (5.99% decrease at most). Therefore, it can be concluded that the presence of either pectin or sugar limits the measuring accuracy of the QUENCHER method.
It was found that the combination of pectin and sugar has a larger influence the measurement accuracy of the QUENCHER method more than pectin or sugar separately (85.77% decrease at most in comparison to the expected 5.99%). Therefore, it can be concluded that interactions between sugar and pectin limit the measuring accuracy of the QUENCHER method.
The TEAC value that was measured for vitamin C does not significantly differ from literature values. Meanwhile, the TEAC value measured for β-carotene is nearly six times lower than literature values. Therefore, it can be concluded that vitamin C is more accurately measured by the QUENCHER method than β-carotene.
Looking at all those conclusions, the main conclusion is that the QUENCHER method is not yet suited to measure antioxidant activity in mango samples, due to the combination of pectin and sugar, and the presence of β-carotene. However, looking at the possibilities of this method, some recommendations can be made to try to explore the full potential of the method.

Recommendations

Carotene solvents

Looking for a suitable solvent for the method when measuring β-carotene, or carotenoid-rich products, should be a priority. As long as the method cannot be trusted when measuring these kinds of products, the method cannot be used for those products. That means the big advantages that the QUENCHER method has cannot be fully exploited until that happens.

Incubation time tests for every sample

As the time in the Heidolph shaker did not seem to make a difference for both vitamin C and trolox samples, testing the time needed for each kind of food product before actually measuring the samples can save time. Additionally, the time needed for trolox calibrations can be reduced in the protocol.

Keep looking for stability DPPH

Looking for a way to get a stable absorbance value from the DPPH working solution is also a priority. It is important to be able to trust the stability of this solution, if this method becomes more widely used.

Research a way to deny the effect of sugar and pectin, and the combination of the two

As the main result of this result was that sugar and pectin, and more importantly the combination of the two, decrease the measuring accuracy of this method, looking for a way to eliminate that influence is important. The first option is that processing steps can be tested that keep the antioxidants intact, while breaking the structure of the other components. The second option is researching the influence of every different food components on every different antioxidant more precisely. This way all these effects can be taken into account while calculating the TEAC, neutralizing the effect. However, as this second option seems to be extremely time-consuming, the first option will be more ideal.

Research the exact interactions that influence the method

To be able to make a better prediction of the detectability of different antioxidants in different food products, it is important to know the specific interactions that influence the accuracy. Polymers with different chemical properties can be tested to reach this goal.

Try other polymers and other antioxidants to test their effects for food products other than mango

If the ultimate goal is to make a precise database of antioxidant activities of many food products, testing the influence of sugar and pectin on vitamin C is just the first of many important researches. Different kinds of pectin, as well as other polymers that could influence the method, should be tested, to make sure the method becomes truly accurate for testing many different food products.

Add sonication to protocol

Sonicating the stock solution before making the working solution, and sonicating the working solution before adding it to the samples, increased the absorbance of the DPPH solution. Because of that, the sonication steps should be added to the protocol.

Dissolve DPPH in methanol instead of ethanol

When using methanol as a solvent for the DPPH stock solution, the working solutions reached the desired absorbance value faster than when using ethanol. As different protocols are already using methanol, it is an idea to make this change in the QUENCHER protocol as well.
If anyone still prefers to use ethanol, at least 8 hours of incubation is necessary before the working solution can be used.

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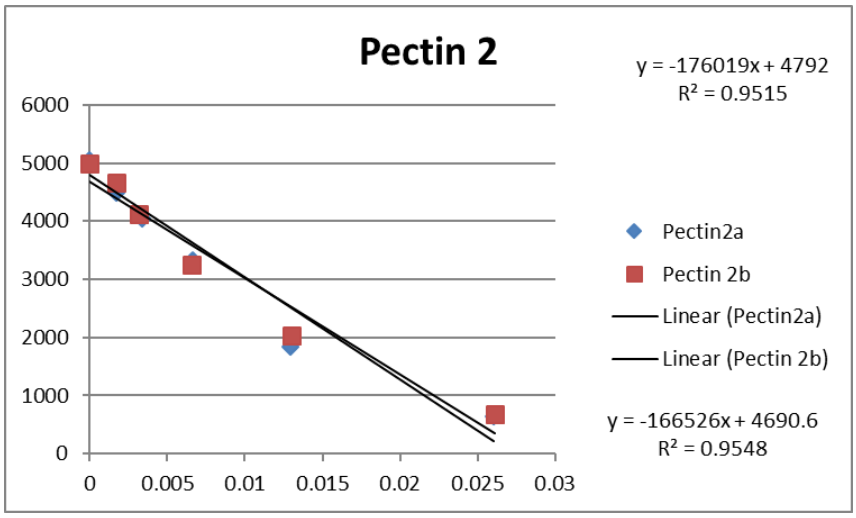
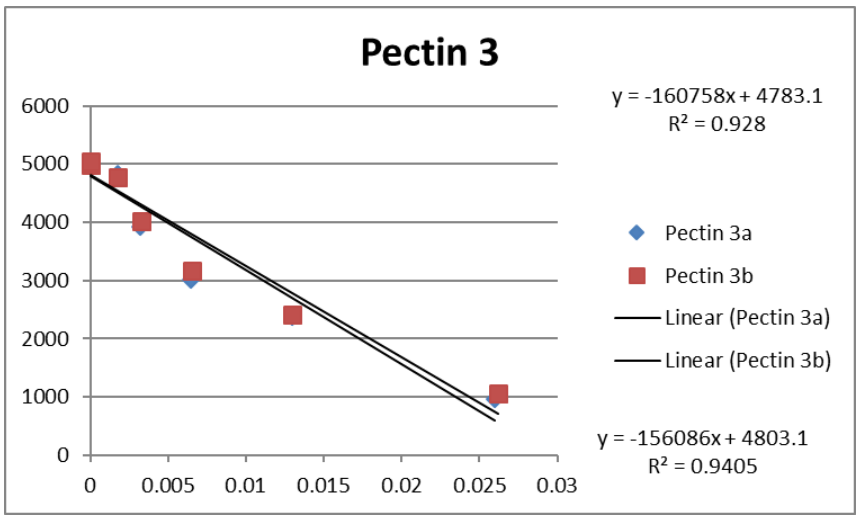
Annex

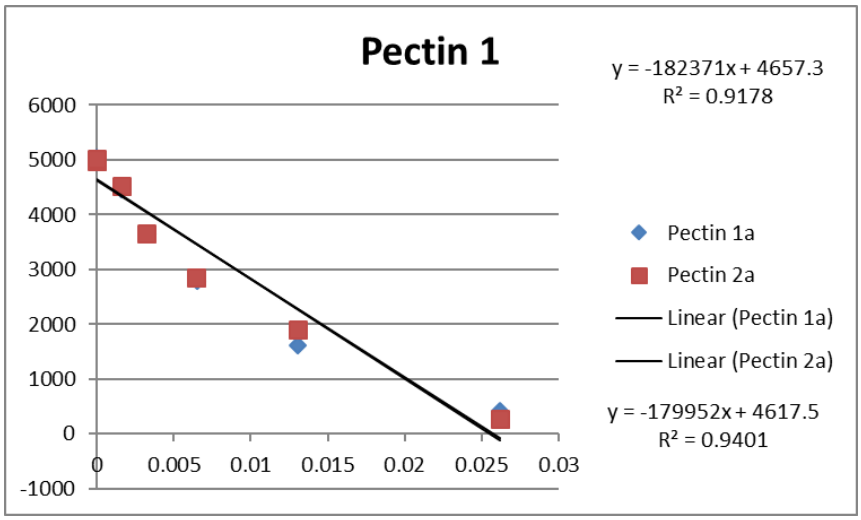
Graphs individual samples



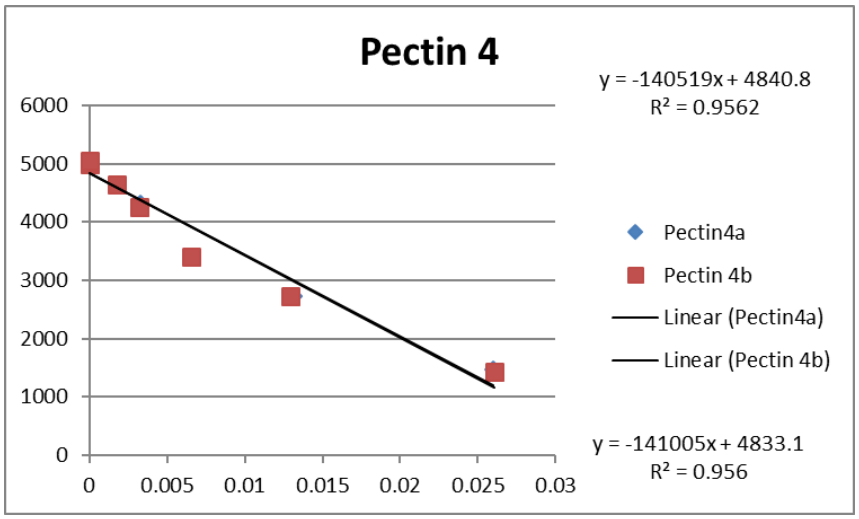
Annex 1: Absorbance measured in Pectin 2 sample compared with vitamin C concentration



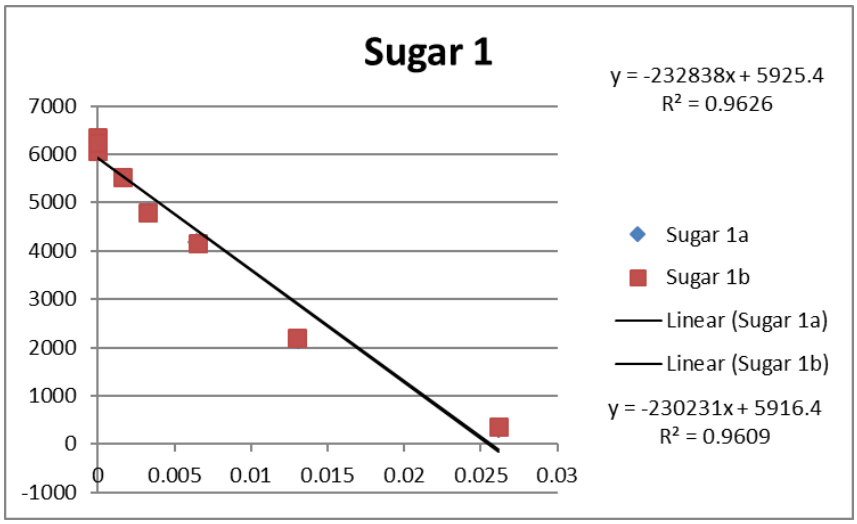
Annex 2: Absorbance measured in Pectin 3 sample compared with vitamin C concentration



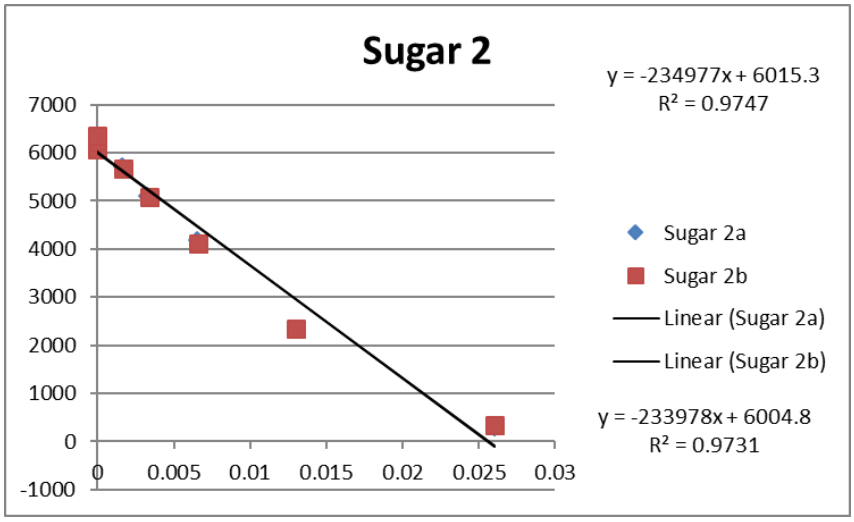
Annex 3: Absorbance measured in Pectin 1 sample compared with vitamin C concentration



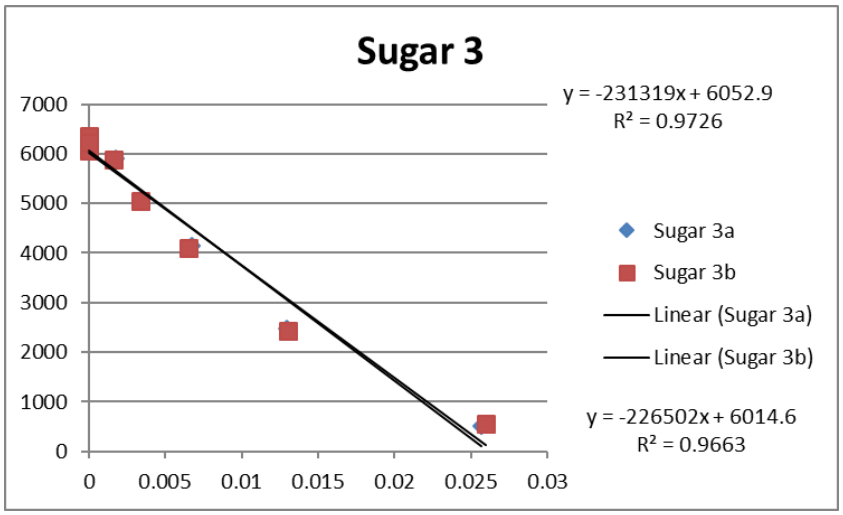
Annex 4: Absorbance measured in Pectin 4 sample compared with vitamin C concentration



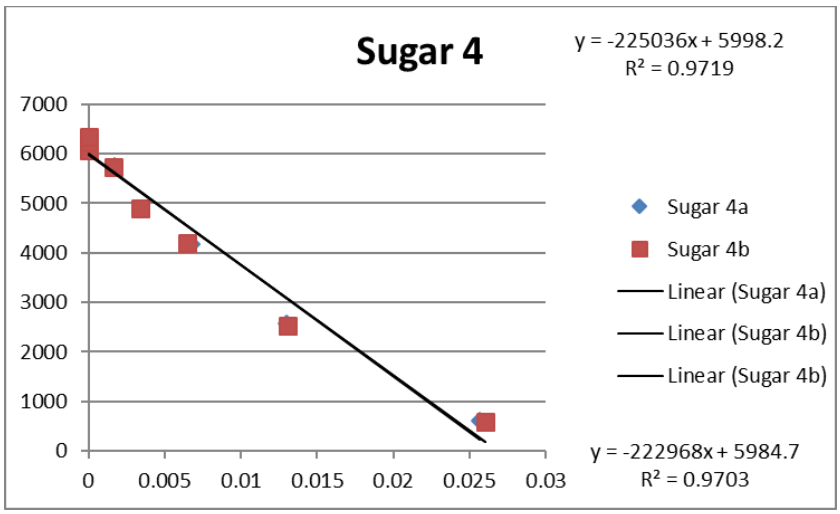
Annex 5: Absorbance measured in Sugar 1 sample compared with vitamin C concentration



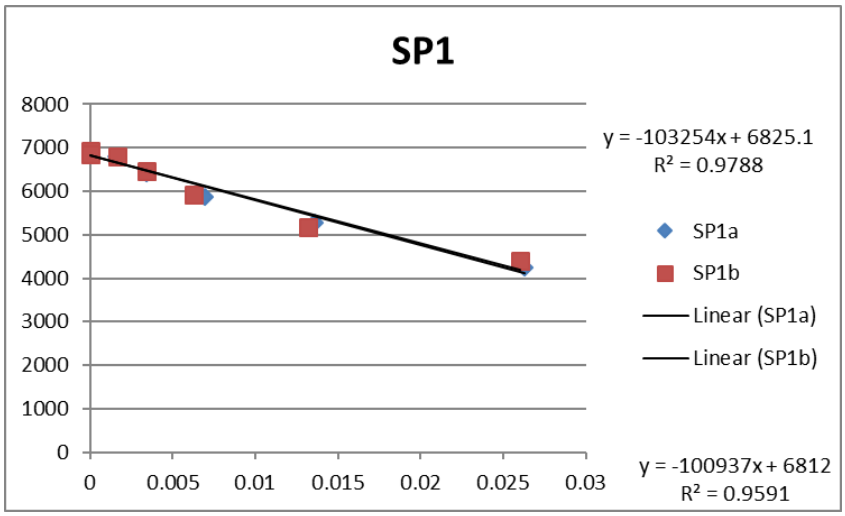
Annex 6: Absorbance measured in Sugar 2 sample compared with vitamin C concentration



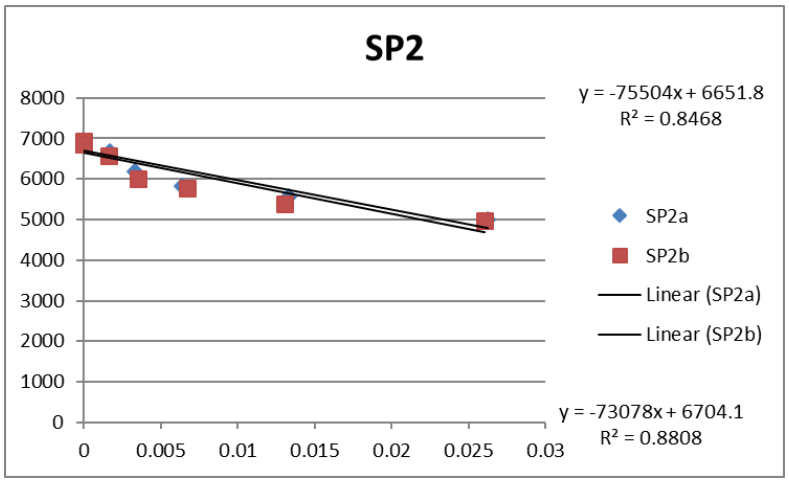
Annex 7: Absorbance measured in Sugar 3 sample compared with vitamin C concentration



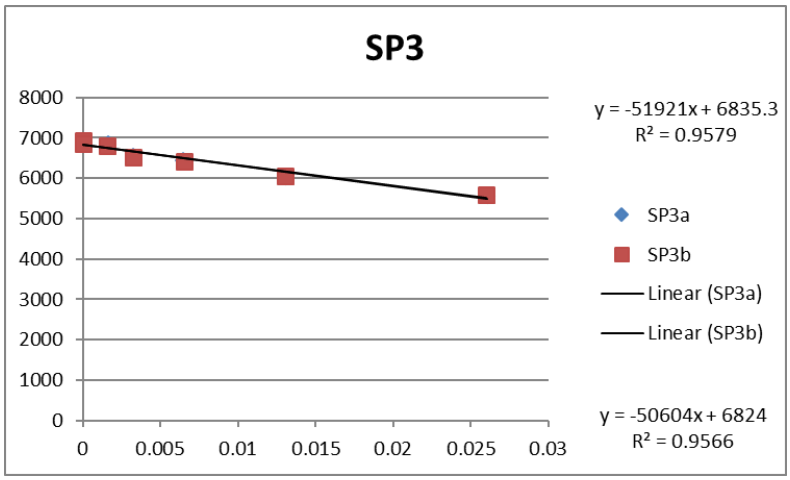
Annex 8: Absorbance measured in Sugar 4 sample compared with vitamin C concentration



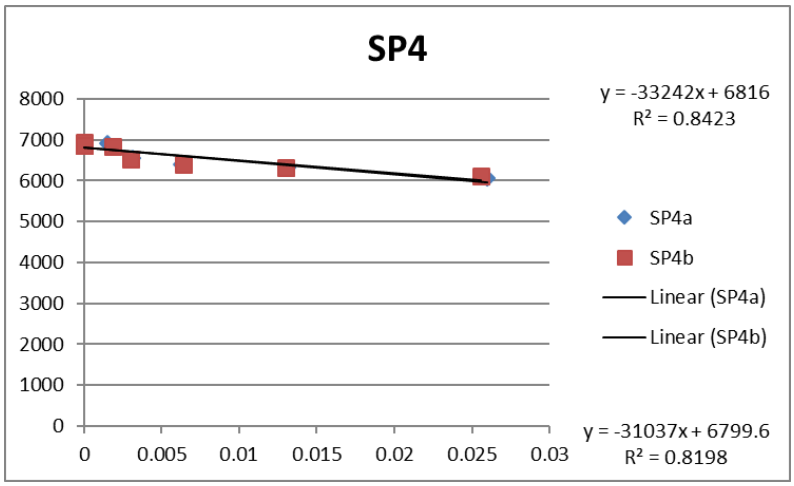
Annex 9: Absorbance measured in SP1 sample compared with vitamin C concentration



Annex 10: Absorbance measured in SP2 sample compared with vitamin C concentration

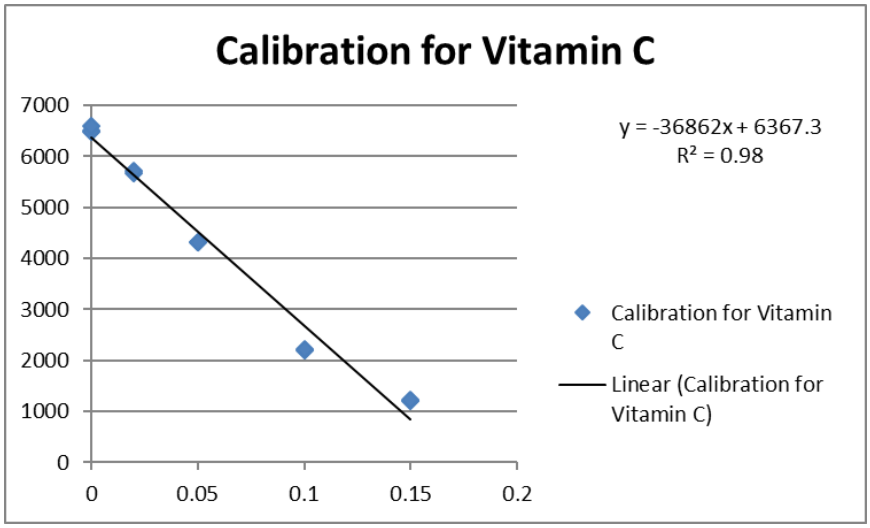


Annex 11: Absorbance measured in SP3 sample compared with vitamin C concentration

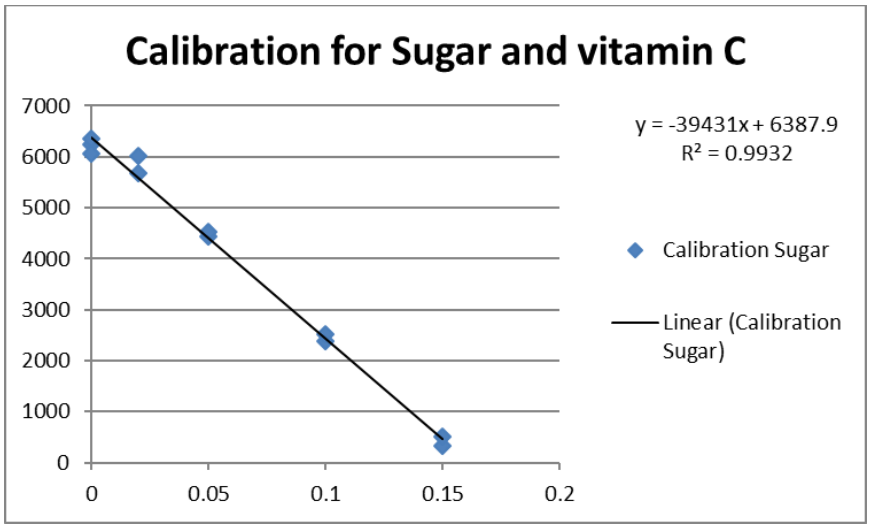


Annex 12: Absorbance measured in SP4 sample compared with vitamin C concentration

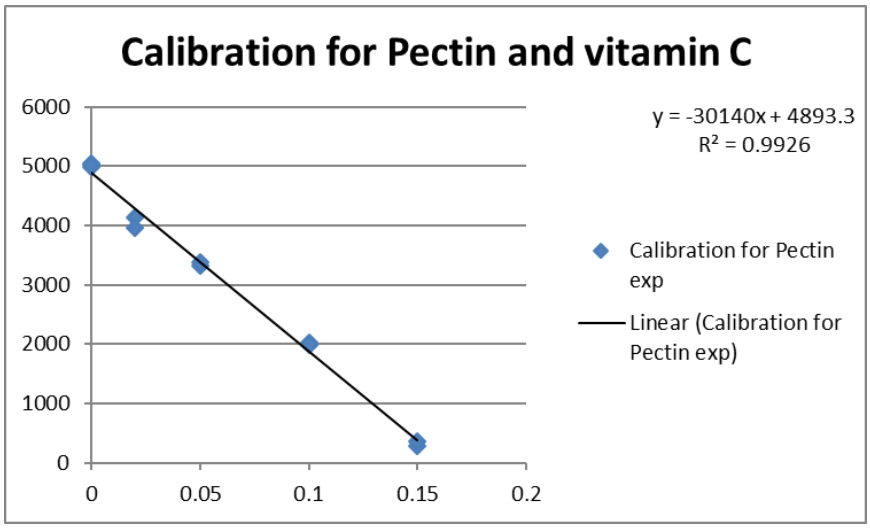
Calibration curves



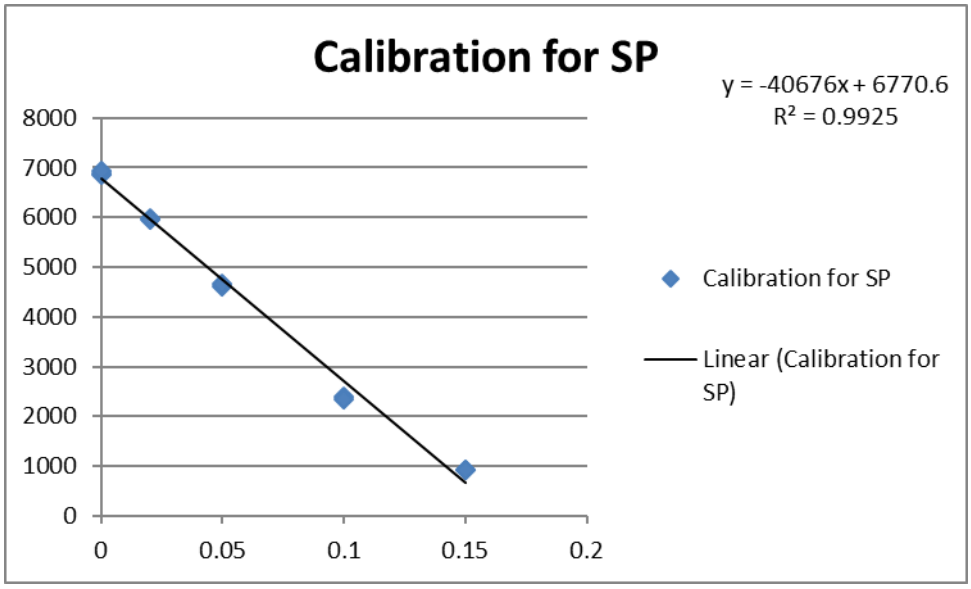
Annex 13: Trolox calibration for the experiments with exclusively vitamin C



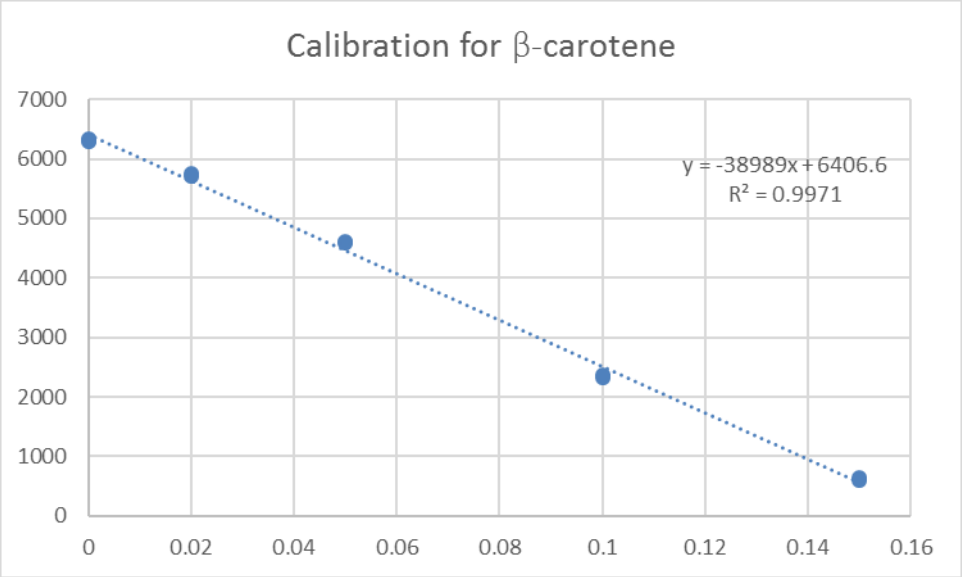
Annex 14: Trolox calibration for the experiments with sugar and vitamin C



Annex 15: Trolox calibration for the experiments with pectin and vitamin C



Annex 16: Trolox calibration for the experiments with sugar, pectin and vitamin C



Annex 17: Trolox calibration for the measurement with b-carotene in 50% ethanol

QUENCHER method

Protocol Nr: 41 Antioxidant Measurement Quencher Procedure Nov 2016 22-11-2016

Total antioxidant capacity of foods using Quencher procedure.

1. *Introduction*

Antioxidant activity according to the free-radical scavenging capacity can be measured with radical cations like DPPH* (1,1-diphenyl-2-picrylhydrazyl). In its radical form DPPH* has an absorption band at 525 nm, which disappears upon reduction by an antioxidant (AH) or radical (R*)
The following reaction occurs:

DPPH* + AH ⟶ DPPH-H +A
DPPH* +R* ⟶ DPPH-R

This method describes the measurement of total antioxidant activity of foods by Quencher procedure. Instead of dissolving the anti-oxidant compound in a extraction solvent, the surface reaction is monitored between the solid (bound antioxidant compound) and the liquid (soluble free radical).

2. *Reagents*

DPPH (2,2-diphenyl-1-picrylhydrazyl)	Sigma D 9132	AFSG.BZ.118.FQD.L2-11.REF
Trolox (6 hydroxy 2,5,7,8 tetramethylchroman 2-carboxylic acid)	Aldrich 23881-3	AFSG.BZ.118.FQD.L2-11.VCC
THF	BioSolve	AFSG.BZ.118.FQD.L2-09.FVC
Ethanol (pure)	Merck	AFSG.BZ.118.FQD.L2-09.FVC
Cellulose from spruce (high purity) (particle size: 0.02-0.15 mm)	Fluka 22182	AFSG.BZ.118.FQD.L2-11.VCC
Corn sample	Italy	AFSG.BZ.118.FQD.L2-11 cupboard 11.6a
MilliQ water		AFSG.BZ.118.FQD.L2-06

3. *Equipment and disposable materials*

Spectrophotometer Cary 50	Organic solution probe	Lab 2.08
Eppendorf centrifuge		Lab 2.07/2.10
Heidolph shaker		Lab 2.10/2.09
Freezer (-20°C)		Lab 2.07/2.10
Analytical balance		Lab 2.11
Balance		Lab 2.07
5 ml Eppendorf tube	Order number 0030 119 401	Lab 2.10 cupboard 10.8u
Round aluminum weighing dish (20 mL/⌀ 43mm)		Lab 2.11 cupboard 11.9a
10 mL Greiner tube		

For preparation of powder sample:

Small aluminum container	Lab 2.07 cupboard 12a
Christ freeze dryer	Bordes 2nd fl
IKA A11 Basic Batch Mill or	Contact Xandra de Haan
Planetary Ball Retch Miller	Lab 2.07
Lab. Test sieve 80 mesh/02.mm (or 120 mesh/0.125 mm)	Lab 2.07 cupboard 7.28u
Mortar and pestle	Lab 2.07 cupboard 7.33u/ Lab 2.11 cupboard 11.8a

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4. *Method*

Solutions:

DPPH (MW= 394,3): 0.1 mM in 50% Ethanol. Prepare 10 mg/ml stock solution in 100% Ethanol (*e.g.* 100 mg in 10 mL Greiner tube). This stock can be stored in -20 °C. Dilute solution to 5 mg/ml in milliQ water and subsequent dilution of 125x by adding 200 ul stock solution to 25 ml 50% ethanol*. Prepare solution 1 day in advance. Store overnight at room temperature in the dark to activate it. Check the absorbance next day. (Absorbance should reach a value of at least 0.6 at 525 nm after 23 hours of incubation). *Note: it is suggested to have similar absorbance values , if it is too high (e.g. 0.9), it can be diluted with 50% ethanol. Calculate the volume of DPPH solution needed, we need 5 mL/sample.*

**50% ethanol was made by dilution of pure ethanol with milliQ water.*

Calibration curve Trolox range 4-30 uM:

- Prepare 20 mg/mL Trolox (MW=250.29) stock solution in 100% Ethanol (EtOH). This stock solution can be stored in -20 °C. Subsequently dilute 5.33x/ 8x/16x/40x in 100% EtOH to get four points calibration curve of 3.75/2.5/1.25/0.5 mg/mL. In addition, prepare also a blank of 100% EtOH in triplicates.

Table 1. Trolox calibration curves dilution

Trolox (mL)	Ethanol/EtOH (mL)	Dilution factor	Concentration (mg/mL)	End Concentration (uM)	Concentration in test (mmol)	Calibration curve
-	1.0	-	0.00	0	0	Blank**
0.05	1.95	40x	0.50	4	0.00002	St1 (40x)
0.1	1.5	16x	1.25	10	0.00005	St2 (16x)
0.2	1.4	8x	2.50	20	0.00010	St3 (8x)
0.3	1.3	5.33x	3.75	30	0.00015	St4 (5.33x)

**Absorbance of the blank , which is the average value of its triplicates, was taken as the initial concentration in test (0.00 mmol)

- Weigh 7 * 10 mg cellulose in 5 ml Eppendorf tube, add 10 ul of Trolox dilutions (or 10 uL of ethanol for 3 blank samples).

Samples and controls:

Dry corn control sample: prepare 0.5 mg corn/mg total powder by mixing well 100 mg cellulose with 100 mg sieved corn on weighing paper using spatula. Note the exact weight. Weigh 10 mg in 5 ml Eppendorf.

Samples: prepare 0.5 mg material/mg total powder by mixing well 100 mg cellulose with 100 mg sample in a small aluminum dish using spatula. Perform in duplicates for each sample. Weigh 10 mg/20 mg/40 mg in 5 ml Eppendorf tube***. Note the exact weights.

**** If the samples turn white (discolor) during a few seconds incubation (of 100 minutes duration), it may because the antioxidant activity/absorbance value is out of the linear response range of the radical discoloration solution. It may need a less concentrated sample; mixing 200 mg cellulose with 100 mg sample (2:1 dilution factor) can be an alternative ratio. Inhibition range should fit between 20-80%.*

Protocol Nr: 41 Antioxidant Measurement Quencher Procedure Nov 2016 22-11-2016

Measurements

- Add 5 ml of DPPH solution to each sample
- Cover tubes with aluminum foil
- Incubate 100 minutes at room temperature in Heidolph shaker
- Centrifuge 5 min 9000 g at room temperature
- Measure at 525 nm in the spectrophotometer.

Calculation:

1. Construct the calibration curve of Trolox (X = concentration in test (mmol); Y = absorbance. Calculate the linear regression equation, (R^2 at least 0.98).
2. Calculate the average absorption value of the three replicates of blank samples. Determine the 50% absorption value in mmol (named it as Cr50 Trolox).
3. For each sample, construct the calibration curve (X = concentration in test (mmol); Y = absorbance. Calculate the linear regression equation, (R^2 at least 0.98).
4. For each sample, calculate concentration needed to reduce 50% inhibition of DPPH in mg, by applying Cr50 Trolox value in the respected linear regression equation (named it as Cr50 Sample).
5. Calculate the Trolox equivalent:
   Trolox equivalent (mmol/mg) = Cr50 Trolox (mmol)/ Cr50 sample (mg)

5. Remarks

- How to use centrifuge see FQD Protocol Nr. 17B.
- If the sample contain high water content, perform freeze-drying (it may takes 3-7 days depend on the sample), see FQD Protocol Nr. 50 (contact Frans Lettink). The sample used in this method should be in a homogenous powder form: mill freeze dried sample (with IKA A11 Basic batch mill) and sieve them (with lab. test sieve 80 mesh/0.2 mm). Keep them from moisture and light in a sample bottle in a desiccator or in a freezer (-20 °C) prior to analysis.
- It may be necessary to freeze dry sample that has high sugar content in the presence of cellulose. This is because some foods, especially fruits which have high sugar content on dry basis, became sticky after freeze drying process, which caused some difficulties in subsequent grinding and in solid cellulose dilution steps.
- Always cover tubes or cuvettes to prevent evaporation of ethanol.
- DPPH is a light sensitive reagent, work in the red room.

6. Literature.

Serpen, A., Gokman, C., Fogliano, V., *Journal of Food Composition and Analysis,* 26, 52-57, 2012

SPSS analyses

Incubation time samples in Heidolph shaker

Correlations

		Time	Dilute5.33x	Dilute8x	Dilute16x
Time	Pearson Correlation	1	,023	,319	-,295
	Sig. (2-tailed)		,970	,601	,630
	N	5	5	5	5

Annex 18: Correlation between incubation time in Heidolph shaker and absorbance of different samples

Incubation time DPPH solution

Group Statistics

	VAR00002	N	Mean	Std. Deviation	Std. Error Mean
VAR00001	1,00	10	7020,8000	24,74672	7,82560
	2,00	10	6916,3000	41,70545	13,18842

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality			
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference
VAR00001	Equal variances assumed	3,374	,083	6,814	18	,000	104,50000
	Equal variances not assumed			6,814	14,639	,000	104,50000

Annex 19: DPPH incubation difference after 48 hours between methanol (2) and ethanol (1)

Sonication and stirring experiment

Group Statistics

	Sonication	N	Mean	Std. Deviation	Std. Error Mean
working_no_S	,00	3	,541233	,0015567	,0008988
	1,00	3	,689633	,0098805	,0057045

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality			
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference
working_no_S	Equal variances assumed	10,410	,032	-25,698	4	,000	-,1484000
	Equal variances not assumed			-25,698	2,099	,001	-,1484000

Annex 20: Mean difference between sonicated sample (1) and non-sonicated sample (0)

Pectin and vitamin C

Correlations

		Pectin	Ratio_PV
Pectin	Pearson Correlation	1	-,990**
	Sig. (2-tailed)		,000
	N	10	10
Ratio_PV	Pearson Correlation	-,990**	1
	Sig. (2-tailed)	,000	
	N	10	10

Annex 21: Correlation between pectin/vitamin C ratio and TEAC

Group Statistics

	Ratio_PV	N	Mean	Std. Deviation	Std. Error Mean
Pectin	47,0	2	,6069000	,00428507	,00303000
	,0	2	,6896200	,00649124	,00459000

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality			
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference
Pectin	Equal variances assumed	.	.	-15,040	2	,004	-,08272000
	Equal variances not assumed			-15,040	1,732	,008	-,08272000

Annex 22: Mean difference between 'only vitamin C'-sample (0) and 'P2'-sample (47)

Sugar and vitamin C

Correlations

		Ratio_SV	Sugar
Ratio_SV	Pearson Correlation	1	-,909**
	Sig. (2-tailed)		,000
	N	10	10
Sugar	Pearson Correlation	-,909**	1
	Sig. (2-tailed)	,000	
	N	10	10

Annex 23: Correlation between sugar/vitamin C ratio and TEAC

Group Statistics

	Ratio_SV	N	Mean	Std. Deviation	Std. Error Mean
Sugar	1102,1	2	,6508050	,00365574	,00258500
	,0	2	,6896200	,00649124	,00459000

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality			
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference
Sugar	Equal variances assumed	.	.	-7,368	2	,018	-,03881500
	Equal variances not assumed			-7,368	1,576	,033	-,03881500

Annex 24:Mean difference between 'only vitamin C'-sample (0) and 'S3'-sample (1102,1)

Sugar, pectin and vitamin C

Group Statistics

	Ratio_SP	N	Mean	Std. Deviation	Std. Error Mean
SugarPectin	8,4	2	,2474300	,00329512	,00233000
	108,4	2	,5468600	,00311127	,00220000

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality			
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference
SugarPectin	Equal variances assumed	.	.	-93,440	2	,000	-,29943000
	Equal variances not assumed			-93,440	1,993	,000	-,29943000

Annex 25: Mean difference between SP1 (8,4) and the expected value (108,4)

Group Statistics

	Ratio_SP	N	Mean	Std. Deviation	Std. Error Mean
SugarPectin	11,7	2	,1879050	,00648417	,00458500
	111,7	2	,5367950	,00024749	,00017500

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality			
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference
SugarPectin	Equal variances assumed	.	.	-76,038	2	,000	-,34889000
	Equal variances not assumed			-76,038	1,003	,008	-,34889000

Annex 26: Mean difference between SP2 (11,7) and the expected value (111,7)

Group Statistics

	Ratio_SP	N	Mean	Std. Deviation	Std. Error Mean
SugarPectin	15,0	2	,1239950	,00215668	,00152500
	115,0	2	,5197950	,00292035	,00206500

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality			
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference
SugarPectin	Equal variances assumed	.	.	-154,184	2	,000	-,39580000
	Equal variances not assumed			-154,184	1,841	,000	-,39580000

Annex 27: Mean difference between SP3 (15) and the expected value (115)

Group Statistics

	Ratio_SP	N	Mean	Std. Deviation	Std. Error Mean
SugarPectin	18,3	2	,0781300	,00352139	,00249000
	118,3	2	,5161200	,00165463	,00117000

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality			
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference
SugarPectin	Equal variances assumed	.	.	-159,201	2	,000	-,43799000
	Equal variances not assumed			-159,201	1,421	,001	-,43799000

Annex 28: Mean difference between SP4 (18,3) and the expected value (118,3)

Ethische appendix

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Dagorde

Vorige week zat ik op de fiets te denken aan mijn onderzoek. Door mijn onderzoek kunnen we preciezer meten hoe veel antioxidanten er in eten zitten, en daarmee voorkomen dat mensen kanker krijgen (Voedingscentrum, 2017). Maar op dit moment kunnen we nog niet zeker weten of antioxidanten wel goed voor je zijn (Hendriks, 2013), dus is het dan wel zo goed om producenten claims te laten maken op producten? Kunnen we de consument niet beter autonoom laten beslissen over antioxidanten en het risico ontlopen dat het niet zo goed is als we denken?

Dilemma

Door mijn onderzoek kunnen we antioxidanten preciezer meten, zonder dat we genoeg weten van de werking daarvan. Zo kunnen producenten meer claims plaatsen op hun producten, die niet per se volledig gegrond zijn. Aan de andere kant, als het onderzoek niet wordt gedaan, is er helemaal geen noodzaak om de effecten van antioxidanten verder te onderzoeken, en zijn de claims die al wel gemaakt worden helemaal ongegrond.

Matrix

Belanghebbenden
Producenten, consumenten, wetenschappers

Op basis van de hierboven genoemde belanghebbenden is de ethische matrix ingevuld (Mepham, jaar onbekend). Daaronder is voor elk van de 9 vakken beschreven waarom tot deze beoordeling is gekomen. Naar mijn mening is de matrix een vrij goede beoordeling van een dilemma, al moet er nog wel een klein deontologisch puntje extra worden meegenomen. Deontologie gaat er namelijk vanuit dat er bepaalde normen zijn die onder geen enkele voorwaarde mogen worden overschreden (Weijers, 2016). Nadat de uitslag van de matrix wordt bekeken, zal dus ook nog even gekeken worden of er geen belanghebbenden zijn die meer negatieve gevolgen ondervinden dan nodig.

Respect voor	Welzijn	Autonomie	Eerlijkheid
Producenten	++	+/-	+
Consumenten	+/-	-	++
Wetenschappers	+	-	+/-

Producenten

Welzijn
Het welzijn van de producenten stijgt, doordat zij preciezer weten wat er in hun producten zit aan antioxidanten. Op deze manier kunnen zij gerichter adverteren. Daarnaast worden antioxidanten ook gebruikt voor de conservering van producten. Als producenten meer weten over de werking, kunnen ze hun producten makkelijker goed houden voor de gewenste tijd.

Autonomie
De autonomie van de producenten blijft gelijk, aangezien zij nog steeds dezelfde vrijheid genieten om al dan niet claims te maken over de werking van hun product.

Eerlijkheid
De eerlijkheid ten opzichte van producenten neemt marginaal toe. Op het moment dat zij beter weten wat hun product doet, zullen ze er waarschijnlijk iets meer geld aan kunnen verdienen, wat ervoor zorgt dat meer producenten genoeg verdienen voor hun werk.

Consumenten

Welzijn
In eerste instantie zal voor het welzijn van de consumenten niks veranderen. Zij zullen nog steeds dezelfde producten kopen met dezelfde ingrediënten. Op de lange termijn is het natuurlijk juist het welzijn van de consument dat hierdoor kan verbeteren. Als we de informatie uit deze thesis kunnen combineren met beter en gerichter onderzoek naar de invloed van antioxidanten in eten, kunnen we ziekten bij consumenten voorkomen. Dit vergroot hun welzijn, omdat gezondheid volgens de definitie een van de pijlers van welzijn is (Ensie.nl, 2017).

Autonomie
De autonomie van diezelfde consumenten neemt op termijn af. Als blijkt dat antioxidanten goed voor je zijn, en we kunnen ze na dit onderzoek preciezer meten, zullen producenten er meer gebruik van gaan maken in producten waarin origineel geen, of minder, antioxidanten zitten. Hierdoor krijgen consumenten, zonder dat ze daar zelf een beslissing in hebben gemaakt, meer antioxidanten binnen.

Eerlijkheid
De eerlijkheid ten opzichte van consumenten neemt enorm toe. Doordat de hoeveelheid antioxidantactiviteit preciezer gemeten kan worden, kan het ook preciezer op verpakkingen staan, waardoor consumenten beter weten waar ze aan toe zijn. Voorwaarde hiervoor is wel dat de regels voor producenten over hoe precies antioxidantactiviteit vermeld moet zijn op de verpakking streng zijn en blijven.

Wetenschappers

Welzijn
Het welzijn van wetenschappers zal toenemen, omdat dit onderzoek een volgende stap is naar het compleet begrijpen van antioxidanten. Hierdoor komt de wetenschap dichter bij een objectief oordeel hierover.

Autonomie
De autonomie van wetenschappers, echter, neemt wat af. Als we antioxidanten preciezer kunnen meten, stijgt de noodzaak voor onderzoek naar de werking van antioxidanten. Dat betekent dat de vrijheid van wetenschappers om te kiezen welk onderzoek ze gaan uitvoeren wat afneemt.

Eerlijkheid
De eerlijkheid ten opzichte van wetenschappers blijft gelijk. Dit onderzoek zal op dit aspect geen significante veranderingen teweegbrengen.

Conclusie en bijltjesmoment

In principe blijkt natuurlijk uit de matrix dat er meer argumenten voor het onderzoek zijn dan ertegen, op het moment dat alle plussen en minnen worden opgeteld. Buiten de matrix om is het interessant om te kijken of er niemand meer leed aangedaan wordt dan nodig, een van de belangrijke punten van de deontologie (Jacobs, 2017). De twee negatieve punten die uit de matrix voorkomen, de autonomie van zowel consument als wetenschapper, zullen op dit punt gevoelsmatig worden getoetst. Bij beide punten wordt de keuzevrijheid van desbetreffende belanghebbenden ingeperkt. Echter, in allebei de gevallen blijft de mogelijkheid bestaan om voor iets anders te kiezen. De autonomie wordt dus niet zo ver ingeperkt dat een situatie van dwang ontstaat. Hieruit volgt de conclusie dat geen van de drie belanghebbenden in dit ethische

dilemma te grote negatieve gevolgen zal ondervinden van het uitvoeren van het onderzoek. De keuze die dus op het bijltjesmoment gemaakt wordt, is om het onderzoek wel uit te voeren.

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