

Effects of different mycorrhizas on the quality of northern highbush blueberries

Effekter på kvalitet för trädgårdsblåbär vid odling med olika typer av mykorrhiza

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Anna Lundström 25 may 2017

Summary

Blueberries contain components like for instance triterpenes and stilbenes which have potentially protective roles against many human diseases. These substances are also important at threats from herbivores and fungi attacks to plants. Quality traits assessed in this study was the amount of healthy substances like triterpenes, stilbenes, but also sugar/acid ratio which are important for flavor.

In this work it was investigated if mycorrhiza inoculations on blueberry plants can increase the quality of the berries. Quality trait that was studied was the triterpenes ursolic acid and oleanolic acid, the stilbene pterostilbene and sugar/acid ratio of the blueberries. The result showed that ursolic acid, oleanolic acid and sugar/acid ratio was dependent on the variety of blueberry and not the mycorrhiza inoculation. Pterostilbene was not possible to detect with the methods that was used.

Sammanfattning

Blåbär innehåller ämnen som till exempel triterpener och stilbener som hjälper till att förebygga många humansjukdomar. Dessa ämnen är även viktiga vid hot från växtätare och svampangrepp mot växter. Kvalitetaspekter som bedömdes i denna studie var mängd hälsosamma ämnen som triterpener och stilbener, men även socker/syra-innehåll som är viktigt för smaken.

I detta arbete undersöktes om mykorrhiza-ympning på blåbärsplantor kan öka kvaliteten på bären. Kvalitetegenskaper som studerades var triterpenerna ursolic acid och oleanolic acid, stilbenen pterostilbene och socker/syra innehåll i blåbären. Resultatet visade på att mängd ursolic acid, oleanolic acid och socker/syra-innehåll beror på blåbärssort och inte mykorrhiza-ympningen. Pterostilbene var inte möjligt att detektera med de använda metoderna.

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1. Introduction

According to Szakály et al. (2012) consumers care about what they are eating; they want to eat healthy foods. Blueberries are healthy with health-promoting components like anthocyanins, triterpenes and stilbenes which have potentially protective roles against many human diseases (Neto, 2007).

Minimum requirements for quality of blueberries according to trade are that they should be (Quote from UNECE, 2010):

- *Intact*
- *Sound; produce affected by rotting or deterioration such as to make it unfit for consumption is excluded*
- *Clean, practically free of any visible foreign matter*
- *Practically free from pests*
- *Practically free from damage caused by pests*
- *Fresh in appearance*
- *Free of abnormal external moisture*
- *Free of any foreign smell and/or taste, including bitter taste in case of bilberries.*

When buying berries, the customer look at appearance, freshness and firmness (Kader, 1999). To get consumer buy the berries again the flavor and nutritional quality are important (ibid). The flavor is due to factors like soluble solids (sugar), organic acids, sugar/acid ratio and firmness. Nutritional quality means that the berries contain for instance vitamins and minerals, but also other compounds that are good for human health like anthocyanins, triterpenes and stilbenes (ibid).

To achieve good quality of blueberries, nitrogen, potassium and calcium are important nutrients because it determines for instance firmness, fruit size, yield, sugar and acid which are important for the quality (Retamales & Hancock, 2012).

Blueberries thrive in acidic soils with a pH of 4.0 to 5.5, but pH needs to be higher for many nutrients to be more easily available to the plant (ibid). This, in combination with that the blueberry plants has a lack of root hairs and have a shallow root system, limits the uptake of nutrients to the plant (ibid). In nature, blueberries have solved this by forming a symbiosis with fungus called ericoid mycorrhiza (formed within *Ericaceae* family), which helps the plant to make the nutrients in the soil available (Smith & Read, 2008; Retamales & Hancock, 2012). The fungus grows into the plant

roots epidermis cells and forms a hyphae coil (Smith & Read, 2008). The hyphae grow into the soil from the root and help to provide the plant with nutrition (ibid). In exchange the fungus gets carbon for energy to be able to live/survive and to its growth (ibid). Cultivated blueberries can also form mycorrhiza with fungi, but not to the same extent as blueberries living natural in the nature (Czesnik & Eynard, 1989; Stevens et al., 1997; Kasurinen & Holopainen, 2001; Scagel & Yang, 2005).

1.1 Background

A research project carried out mainly at the Department at Biosystems and Technology at SLU, but also including Department of Plant Breeding, was the background for this work. The overall aim of the project was to develop methods for optimizing organic production of northern highbush blueberries in the Nordic climate. In the project the establishment and growth of blueberry plants inoculated with mycorrhiza was studied. Further, the project also investigated how well the plants take up nutrition from organic fertilizers. Anthocyanins were also measured of the blueberries. The plant materials that have been used in the present study originated from the above mentioned blueberry project.

1.2 Triterpenes: Ursolic acid and oleanolic acid

Ursolic acid ($C_{30}H_{48}O_3$) and its isomer oleanolic acid ($C_{30}H_{48}O_3$) are triterpenes (see figure 1) that belongs to the secondary metabolite group terpenes (Gurevitch, Scheiner & Fox, 2006). Terpenes are for instance important for the plants as defense against herbivores and fungal pathogens (ibid). Ursolic acid and oleanolic acid have been suggested to have many good health effects (Neto, 2007). They have for instance been implicated to have anti-tumor/anti-cancer (Liu, 1995; Harmand et al., 2005; He & Liu, 2006; Ikeda, Murakami & Ohigashi, 2008; Liberty et al., 2009), anti-inflammatory (Liu, 1995; Ikeda, Murakami & Ohigashi, 2008), anti-ulcer (against stomach wound) (Liu, 1995), anti-hyperlipidemia (counteract raised levels of lipids in the blood) (ibid), anti-atherosclerotic (counteracts clogging of arteries / blood vessels) (ibid), anti-diabetic (Jang et al., 2009; Castellano et al., 2013), anti-bacterial (Fontanay et al., 2008), anti-hypertensive (counteracts high blood pressure) (Somova et al., 2003) and hepatoprotective (protect damage on the liver) effects (Liu, 1995;

Saraswat, Visen & Agarwal, 2000; Saravanan, Viswanathan & Viswanathan Pugalendi, 2006; Ikeda, Murakami & Ohigashi, 2008). Ursolic acid has been shown to reduce growth and cause cell death in colon tumor cells (Liberty et al., 2009). Growth of liver cancer cells and breast cancer cells has also been shown to be inhibited by ursolic acid (He & Liu, 2006). Ursolic acid also caused cell death of melanoma cells; a form of skin cancer (Harmand et al., 2005). Ursolic acid has been suggested to reduce liver damage when drinking ethanol (Saraswat, Visen & Agarwal, 2000; Saravanan, Viswanathan & Viswanathan Pugalendi, 2006). These compounds can be found in other fruits, berries and vegetables and in different concentrations (Belding et al., 1998; Griffiths et al., 2000; Bauer, Schulte & Thier, 2004). Oleanolic acid and ursolic acid are important components in the cuticular wax on the blueberry peel, which works as a protect layer against for instance biotic and abiotic stresses, attacks from fungi and reduces water loss, which is important for maintaining the blueberry quality (Laraa, Belgea & Goulaob, 2014; Silva-Moreno et al., 2016; Chu et al., 2017).

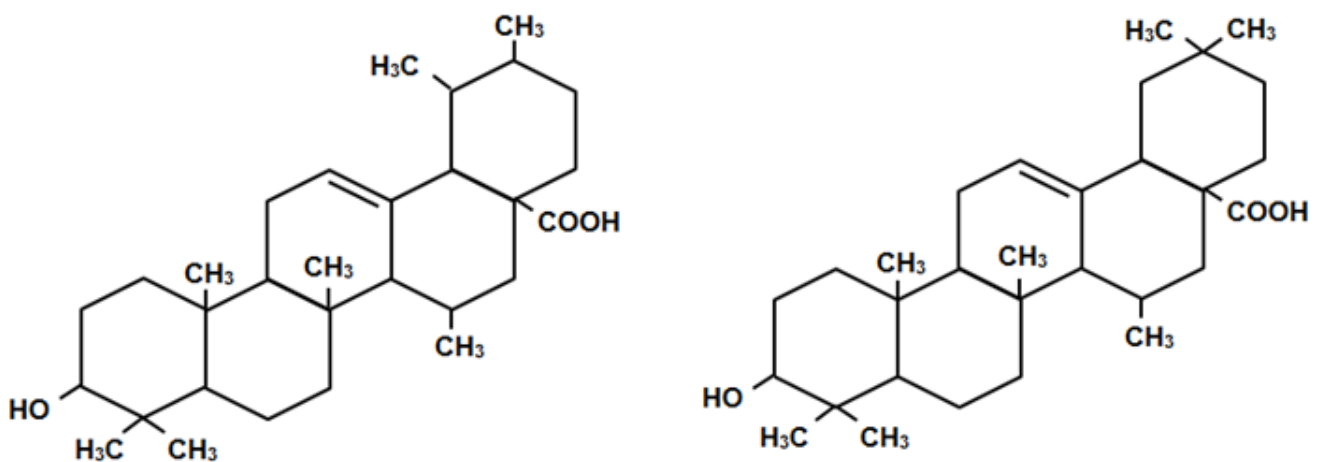


Figure 1. Structure formula of ursolic acid (left) and oleanolic acid (right)

1.3 Pterostilbene

Pterostilbene (C₁₆H₁₆O₃) is a stilbene (see figure 2) and can be found in the peel of blueberries (Adrian et al., 2000). Stilbene belongs to the secondary metabolite group phenolics (Gurevitch, Scheiner & Fox, 2006). Phenolics are for instance important for the plants as defense against herbivores and fungal pathogens (ibid). Many trials have been made to investigate the health benefits of pterostilbene. *In vitro* and *in*

vivo-trials have for instance shown anti-cancer (breast, colon, bladder, liver, lung, leukemia, melanoma, prostate, stomach, pancreas), anti-inflammation, anti-atherosclerotic (counteracts clogging of arteries / blood vessels), anti-hyperlipidemia (counteract raised levels of lipids in the blood), hepatoprotective (protect damage on the liver) effects of pterostilbene (Remsberg et al., 2008; McCormack & McFadden, 2012; McCormack & McFadden 2013). Pterostilbene have also shown that they could affect eyes and other ocular surface diseases (Li et al., 2016).

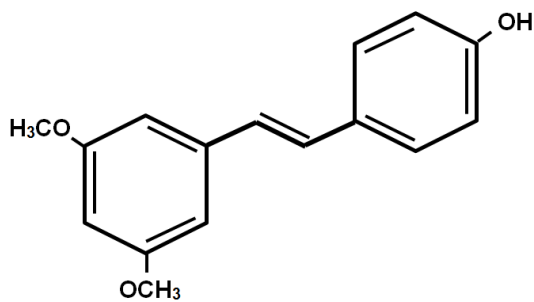


Figure 2. Structure formula of pterostilbene

1.4 Sugar/acid ratio

For the sensory quality of the berries, the balance between sugar and acid is important, as this have strong influence of your experience of the taste of the berry and tells if you want to eat it again (Kader, 1999). High sugar/acid ratio means the berries are sweeter and a low sugar/acid ratio means they are sourer.

To measure the sugar content a common method used is a refractometer to get a °Brix-value and thereby percent sugar that are in the fruit juice (Mitcham, Cantwell & Kader, 2003). Actually you measure the soluble solids content (SSC) when the Brix-value is measured, where organic acid, amino acids, phenolic compounds and soluble pectin are included (ibid). However, sugar is the main soluble solids in the fruit juice and can therefore be expressed as a measure of the sugar concentration.

Citric acid is the main acid in highbush blueberries, with an average 75 % of the total acids (Retamales & Hancock, 2012). Other acids that are present in the berries are malic (3 %), quinic (5 %) and succinic acid (17 %) (ibid). The sour taste in blueberries comes from citric and malic acid while succinic have a bitter taste (ibid).

1.5 Aim

The aim of the study was to measure some quality traits of blueberries inoculated with mycorrhiza. Quality traits that were measured were some substances considered as healthy for humans (ursolic acid, oleanolic acid and pterostilbene) and one important indicator for taste (sugar/acid ratio).

1.6 Research question

Does mycorrhiza affect the quality of blueberries?

1.7 Limitations of the study

Substances that were measured in the blueberries were the substances ursolic acid, oleanolic acid and pterostilbene. Sugar/acid ratio was also measured because of its importance for the taste. In the main project anthocyanin content was measured, but there was an interest of measuring more quality traits in the blueberries to see if there were some more effects of the mycorrhiza.

2. Material and Methods

2.1 Field trials

Blueberries from northern highbush blueberry (*Vaccinium corymbosum*) of the varieties 'Duke' and 'Reka' was tested. The blueberry plants were subjected to three different treatments: 1: Not inoculated with mycorrhiza (control), 2 and 3: Inoculated with two different kinds of commercial mycorrhiza preparations. The treatment number was: 2 (blueberry varieties) × 3 (mycorrhiza treatment/control) = 6 treatments. The experimental design was as follows: 6 treatments × 3 plants per treatment × 3 blocks/replicates = 54 plants. See figure 3 for an image of the experimental design. Harvest of the blueberries was performed at two times; week 30 and 32, in 2016. After harvest the blueberries were frozen at -80°C and was thawed about seven months after harvest for analyzes.

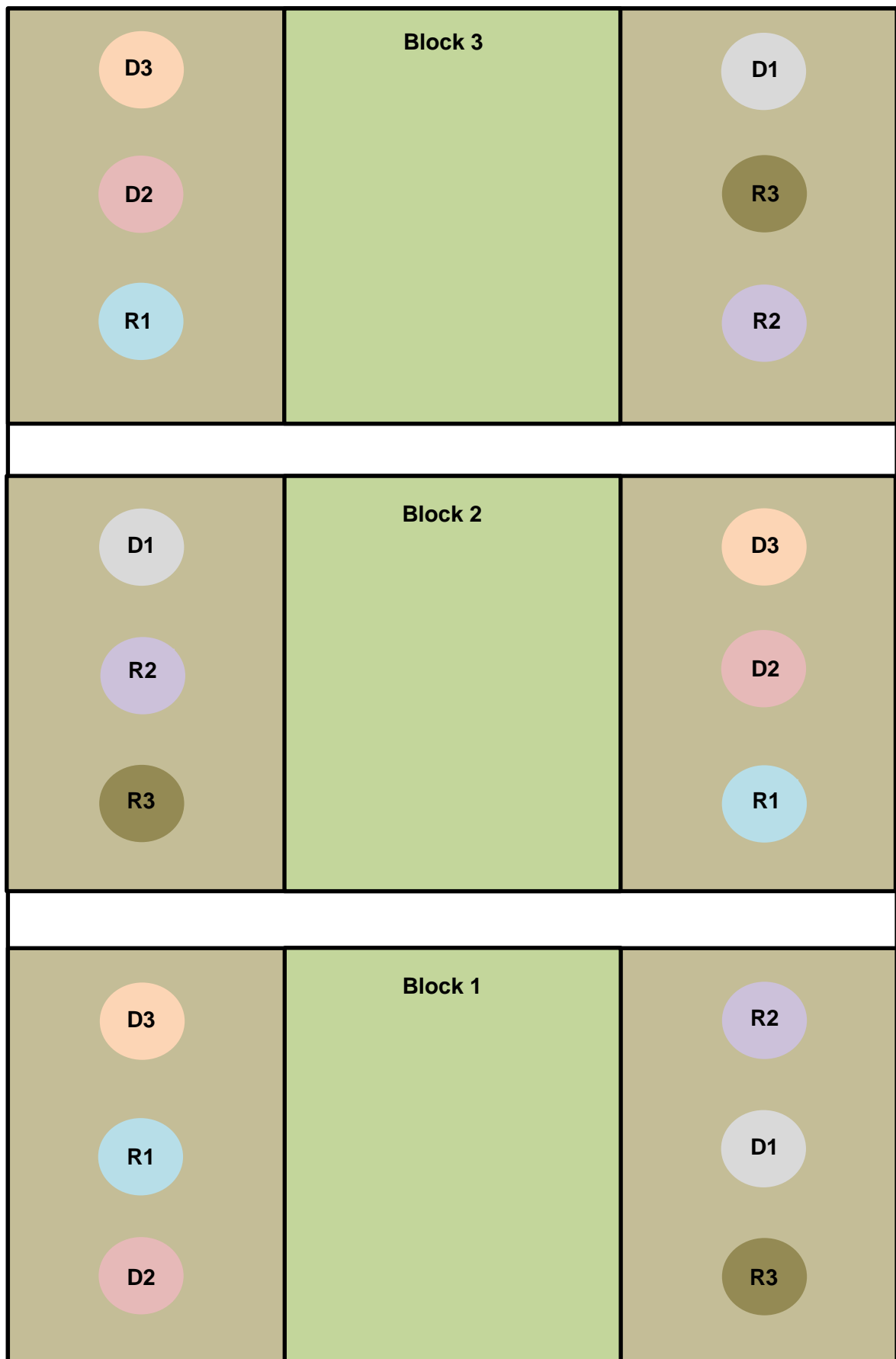


Figure 3. The experimental design. Of each treatment there are three plants on a vertical row. (D='Duke', R='Reka', 1= No mycorrhiza, 2=Mycorrhiza inoculation A, 3=Mycorrhiza inoculation B). The green in the figure represent grass. Proportions in the figure are approximate.

2.2 Analysis of triterpenes

2.2.1 Calculating the blueberry surface area

The substances investigated are found in the cuticular wax, so the surface area of the blueberries was calculated. Two diameters of the blueberries; equatorial (d_1) and polar (d_2) were measured with a caliper. The blueberries were assumed being spherical (Ketata, Desjardins & Ratti, 2013; Chu et al., 2017) and the surface area was calculated as $S = 4\pi r^2$ and $r = (d_1 + d_2)/4$.

2.2.2 Ursolic acid and oleanolic acid extraction

Seven (approximately 11-20g fresh weight) frozen blueberries from each plant was extracted with 15 mL 99.5 % ethanol (Solveco, Rosersberg, Sweden) for 30 minutes, occasionally shaken. 2 mL of each sample was transferred to new tubes and was kept in the freezer to the next day, when 1 mL was transferred to HPLC-vials.

2.2.3 Standard

Ursolic acid and oleanolic acid standards were purchased from Extrasynthese (Genay, France). The standards were used to calculate a standard curve. Because ursolic acid and oleanolic acid was hard to separate from each other on the chromatogram only ursolic acid standard curve was used to calculate the amount of both components. Ursolic acid and oleanolic acid almost gave the same dose response.

2.2.4 HPLC analysis

The samples were analyzed using Agilent 1100 Series HPLC value system (Waldbronn, Germany) running with chemstation ver8.3. A reversed phase column Vydac 201 TP54 250*4.6 (Apple Valley, USA) was used. The mobile phase consisted of acetonitrile (Merck, Kenilworth, USA), methanol (Merck, Kenilworth, USA) and high purity water 18.2 M Ω (Millipore) (2:2:1, v/v/v (isocratic elution)). The flow rate was 1.6 mL/min and the injection volume was 10 μ L. A Diode Array Detector was used to identify ursolic acid and oleanolic acid at λ 205 nm.

2.3 Analysis of Pterostilbene

2.3.1 Standard

Standards of pterostilbene and resveratrol (another stilbene that is more common than pterostilbene, which was interesting to compare the results of pterostilbene to) were purchased from Phytolab (Vestenbergsgreuth, Germany).

2.3.2 Pterostilbene extraction

Two different methods have been tested:

1. Seven (approximately 11-20 g fresh weight) frozen blueberries were extracted with 10 mL methanol (Merck, Kenilworth, USA) and formic acid (Merck, Kenilworth, USA) (100:1, v/v). They berries were mixed with an Ultra-Turrax (IKA, Germany) and then another 10 mL of the extraction solvent were added. Samples were extracted in freezer to the next day. Next day they were shaken for 60 minutes and after that centrifuged at 4000 g for 5 minutes (Eppendorf, Hamburg, Germany). The supernatant was collected to another tube and the pellet was extracted with another 10 mL extraction solvent and shaken for 15 minutes. It was centrifuged again 4000 g for 5 minutes before the supernatants were pooled together. Total volume was 30 mL extraction solvent + water content from berries.

5 mL of the solution was diluted with 40 mL high purity water 18.2 MΩ (Millipore) before it was loaded on a Sep-Pak C18 cartridge, (500 mg) (Waters, Milford, USA). 2 mL of methanol were used to elute pterostilbene from the cartridge. The eluate was centrifuged and 100 µL was transferred to HPLC-vials with 200 µL inserts.

2. Seven freeze dried blueberries were extracted with 25 mL methanol (Merck, Kenilworth, USA) and shaken for 21 hours in a +4°C dark room. The berries were mixed with an Ultra-Turrax (IKA, Germany) and 2 × 2 mL of the mixed berries and methanol was transferred to tubes and centrifuged (Eppendorf, Germany) in 4000 g for 5 minutes. The supernatant was transferred to one 2 mL tube. The solution was evaporated with a TurboVap LV (Biotage, Uppsala, Sweden) for around 60 minutes in 50°C. 100 µL methanol (Merck, Kenilworth, USA) was added to the tubes to solve the solids; 50 µL was transferred to HPLC-vials with 100 µL inserts.

2.3.3 HPLC analysis

There were two different HPLC analyses performed, one for extraction 1 and one for extraction 2:

1: This method was based on the method of Vrhovsek et al. (2012), with modifications.

The samples were analyzed using an Agilent 1260 HPLC-DAD-MS-ESI⁺ (Waldbronn, Germany). An YMC Triart C18 ExRS plus 150*3 mm column (Kyoto, Japan) was used. The substances were eluted with a binary gradient. Solvent A: 0.1 % HCOOH and solvent B: Acetonitrile with 0.1 % HCOOH. The binary gradient was 5-70 % solvent B (0-25 min), 70-95 % solvent B (25-27 min) and back to 5 % of solvent B in 10 minutes. The flow rate was 0.6 mL/min and the injection volume was 10 μ L. A Diode Array Detector at λ 320 nm where used for quantification of pterostilbene. Spectra where collected between 250 – 450 nm for identification of different stilbenes. An Agilent mass-detector 6120b (MS-ESI in positive mode) where used for determination of stilbenes molecular ions; resveratrol at 229 g/mol and pterostilbene at 257 g/mol.

2: This method was based on Phytolabs recommendations with modifications.

The samples were analyzed using Agilent 1260 HPLC-DAD-MS-ESI⁺ (Waldbronn, Germany). A Phenomenex (Luna C18 (2) 100*3mm) column (Torrance, USA) was used. The substances were eluted with a binary gradient. Solvent A: high purity water 18.2 M Ω (Millipore) and solvent B: methanol (Merck, Kenilworth, USA). The binary gradient was 15-95 % solvent B (0-30 min), 95-15 % solvent B (30-31 min) another 5 minutes for equilibration; total runtime 35 minutes. The flow rate was 0.5 mL/min and the injection volume was 5 μ L. A Diode Array Detector at λ 320 nm where used for quantification of pterostilbene. Spectra where collected between 250 – 450 nm for identification of different stilbenes. An Agilent mass-detector 6120b (MS-ESI in positive mode) where used for determination of stilbenes molecular ions; resveratrol at 229 g/mol and pterostilbene at 257 g/mol.

2.4 Analysis of sugar and acid

Blueberries with approximate 50 mL volume were thawed before they were pressed through a sieve. The juice was transferred to two 2 mL tubes and then centrifuged (Eppendorf, Hamburg, Germany) at 10 000 g for 5 minutes. The supernatant was transferred to a 2 mL tube from the two tubes. 1 mL of the juice was mixed with 9 mL of high purity water 18.2 MΩ (Millipore) and then the pH and titratable acid were measured in the blueberries. To measure the acidity in the blueberries the instrument TitroLine easy (Camlab, Cambridge, United Kingdom) was set on the end point 8.2 (pH). The titrator added X number of mL 0.05 M sodium hydroxide (NaOH) (Merck, Kenilworth, USA) until the pH value of 8.2 was reached.

Around 400 µL of the blueberry juice was transferred to the table refractometer (RMF 80, Bellingham and Standley Ltd., United Kingdom) and the Brix values of the samples were measured.

2.4.1 Calculation of titratable acidity and sugar/acid ratio

The X number mL of 0.05 M NaOH (Merck, Kenilworth, USA) that was needed to reach pH 8.2 was used to calculate total acid/titratable acidity (citric acid) in the berries. The equation was (Mitcham, Cantwell & Kader, 2003):

$$\text{Titratable acidity (\%)} = \frac{\text{mL NaOH} \times \text{M NaOH} \times \text{acid milliequivalent factor} \times 100}{\text{mL titrated juice}}$$

According to Mitcham, Cantwell & Kader (2003) the acid milliequivalent factor for citric acid is 0.064. To calculate the sugar/acid ratio the Brix value was divided with titratable acidity/total acid:

$$\text{Sugar/acid ratio} = \frac{\text{Brix}}{\text{Titratable acidity/Total acid}}$$

2.5 Data analysis

Significant differences were analyzed with Minitab 17 (Minitab, Inc., State College PA, USA) and ANOVA → General linear model. P-value < 0.05 was considered as a significantly different. To know if the weeks were significant from each other, the difference of the week's values was calculated. An ANOVA → General linear model test was done to see if the p-value was below 0.05. The average values and standard deviations were calculated using Excel 2010 (Microsoft, Redmond, USA).

3. Results

3.1 Triterpenes: Ursolic acid and oleanolic acid

The experiment showed that there was a significant difference between blueberry varieties, but not between the mycorrhiza inoculations. The highest amount of oleanolic acid and ursolic acid was found in the variety 'Reka' (figure 4 and 7). Considering the substances individually, there is also a significant difference between which substances is most dominant in the variety (figure 5-6 and 8-9). Oleanolic acid was found at the highest concentration in the variety 'Reka' with the level at around 14-17 $\mu\text{g}/\text{cm}^2$ week 30 and around 12-14 $\mu\text{g}/\text{cm}^2$ week 32 (figure 5 and 8). 'Duke' only had around 2-4 $\mu\text{g}/\text{cm}^2$ both weeks (figure 5 and 8). Further, 'Duke' contained significantly more ursolic acid with around 9-11 $\mu\text{g}/\text{cm}^2$ both weeks compared to 'Reka' 7-8 $\mu\text{g}/\text{cm}^2$ both weeks (figure 6 and 9). There was no interaction between blueberry variety and mycorrhiza in any of the different treatments. There was a significant difference between the weeks of the total and oleanolic acid, but not ursolic acid (figure 4-9). Week 30 had higher amount in total and oleanolic acid compared to week 32.

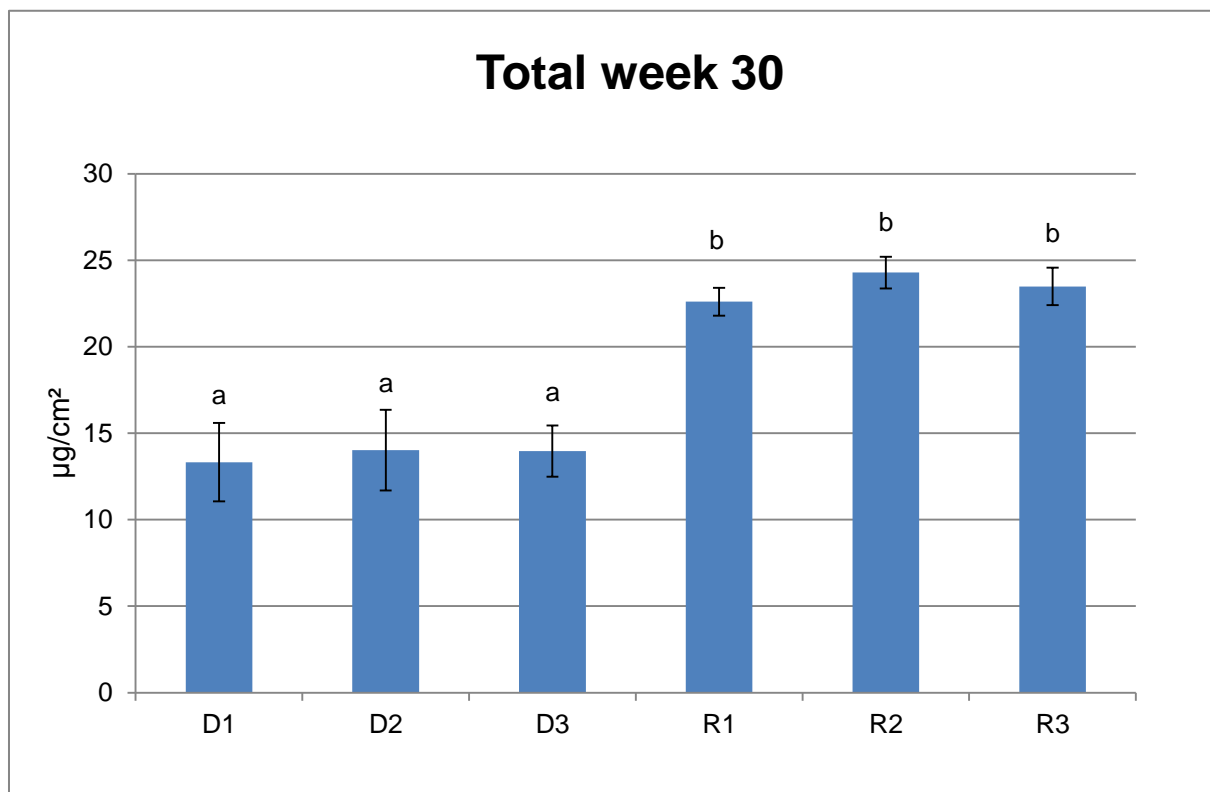


Figure 4. Total amount of ursolic acid and oleanolic acid ($\mu\text{g}/\text{cm}^2$) week 30 with standard deviations. (D='Duke', R='Reka', 1= No mycorrhiza, 2=Mycorrhiza inoculation A, 3=Mycorrhiza inoculation B). Values with same letter denotations are not significantly different.

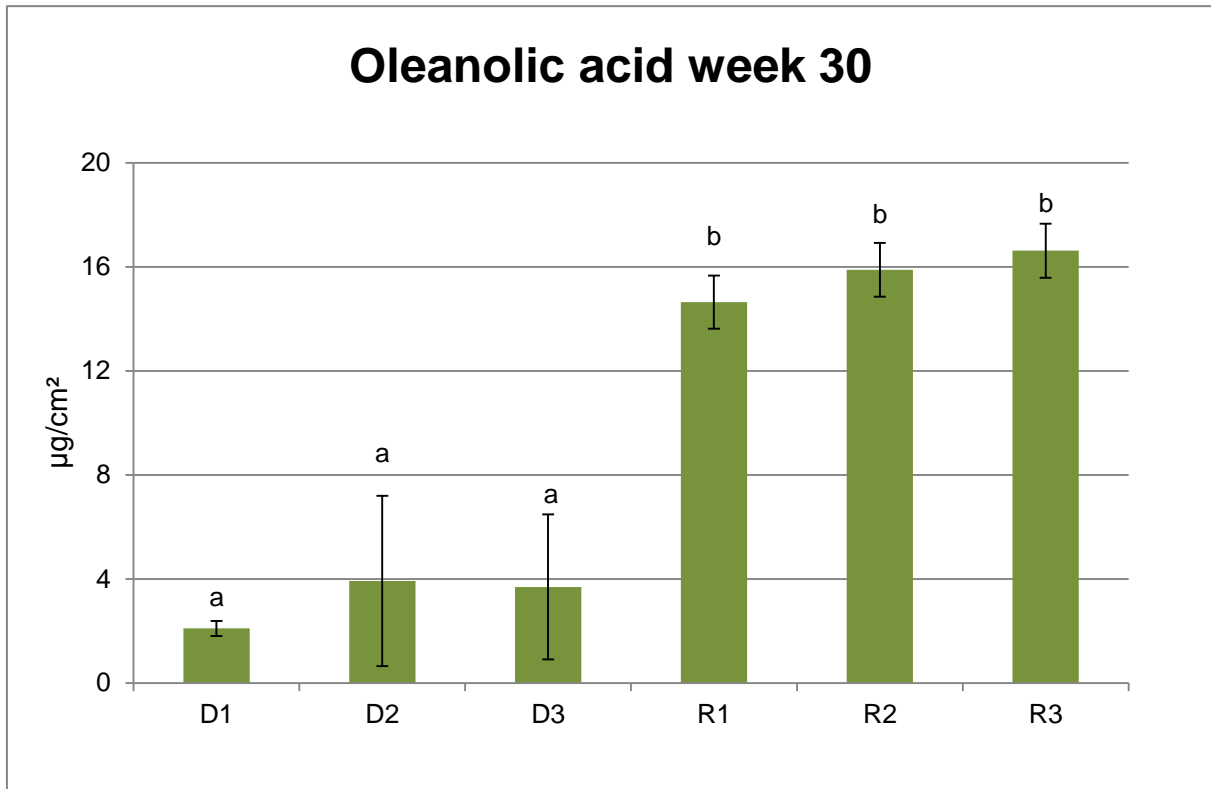


Figure 5. The amount of oleanolic acid ($\mu\text{g}/\text{cm}^2$) week 30 with standard deviations. (D='Duke', R='Reka', 1= No mycorrhiza, 2=Mycorrhiza inoculation A, 3=Mycorrhiza inoculation B). Values with same letter denotations are not significantly different.

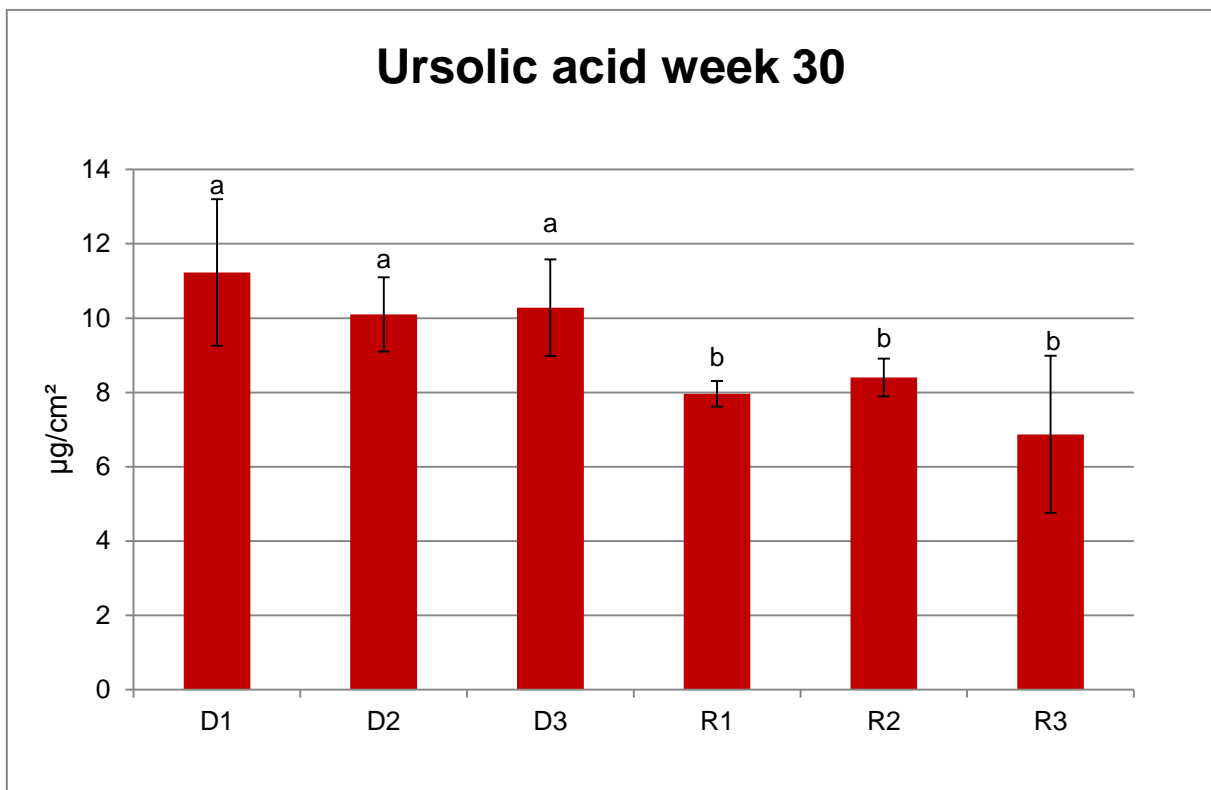


Figure 6. The amount of ursolic acid ($\mu\text{g}/\text{cm}^2$) week 30 with standard deviations. (D='Duke', R='Reka', 1= No mycorrhiza, 2=Mycorrhiza inoculation A, 3=Mycorrhiza inoculation B). Values with same letter denotations are not significantly different.

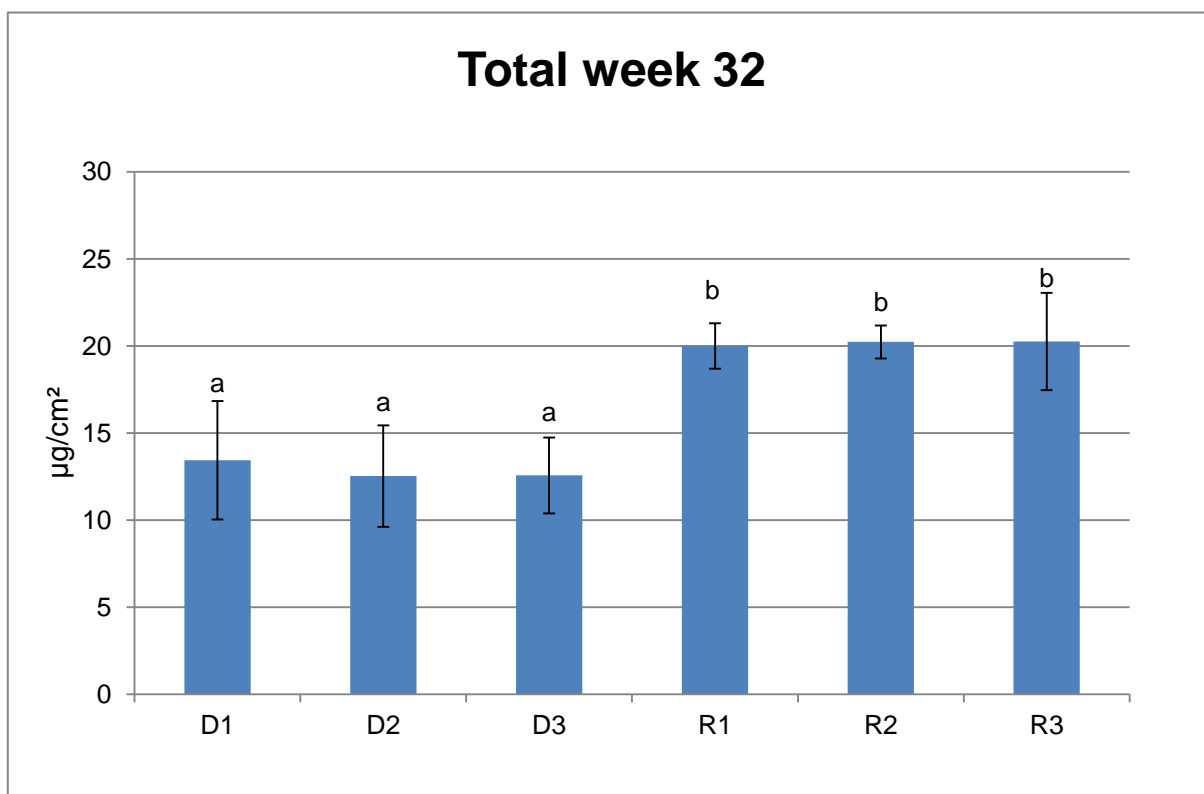


Figure 7. Total amount of ursolic acid and oleanolic acid ($\mu\text{g}/\text{cm}^2$) week 32 with standard deviations. (D='Duke', R='Reka', 1= No mycorrhiza, 2=Mycorrhiza inoculation A, 3=Mycorrhiza inoculation B). Values with same letter denotations are not significantly different.

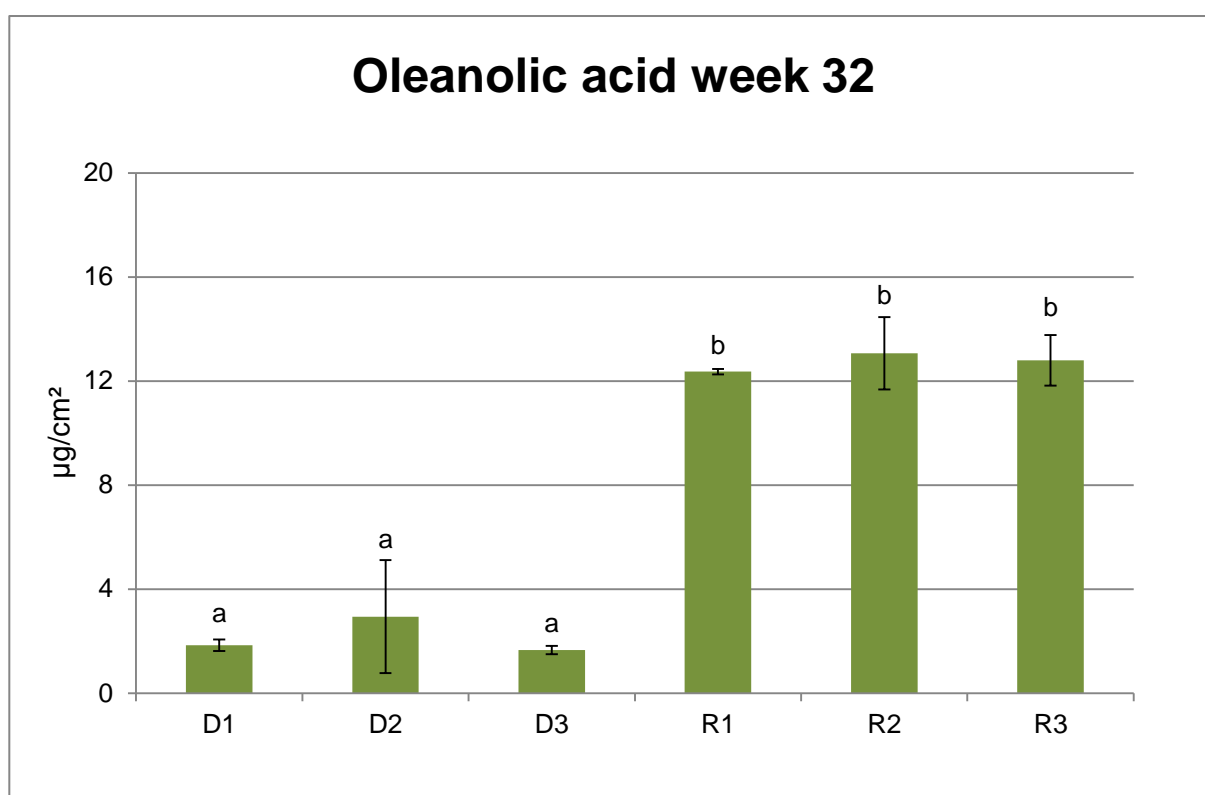


Figure 8. The amount of oleanolic acid ($\mu\text{g}/\text{cm}^2$) week 32 with standard deviations. (D='Duke', R='Reka', 1= No mycorrhiza, 2=Mycorrhiza inoculation A, 3=Mycorrhiza inoculation B). Values with same letter denotations are not significantly different.

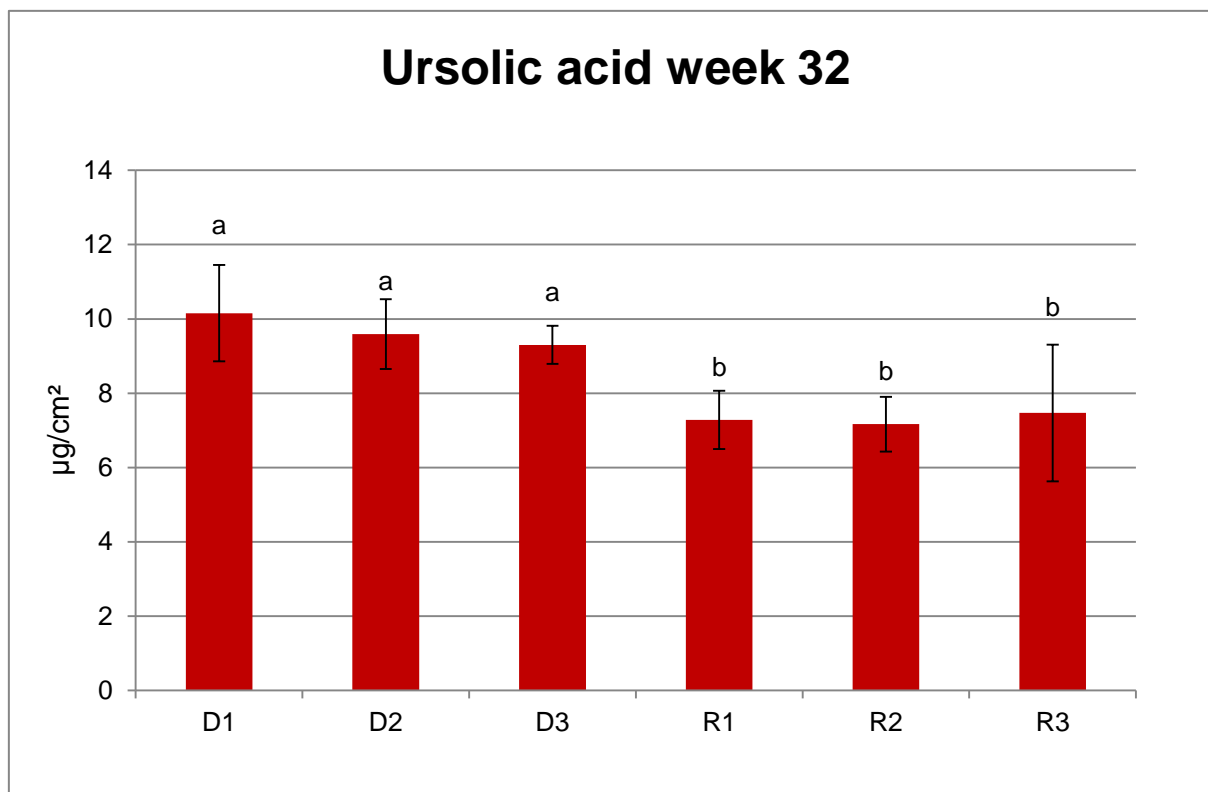


Figure 9. The amount of ursolic acid ($\mu\text{g}/\text{cm}^2$) week 32 with standard deviations. (D='Duke', R='Reka', 1= No mycorrhiza, 2=Mycorrhiza inoculation A, 3=Mycorrhiza inoculation B). Values with same letter denotations are not significantly different.

3.2 Pterostilbene

No pterostilbene was found in the blueberries with the methods that were tested. Figure 10 shows a representative sample of 'Duke' and standards of resveratrol and pterostilbene detected by a DAD-detector (Diode Array Detector) and figure 11 shows the same samples with a mass-detector. Figure 12 shows a representative sample of 'Reka' and standards of resveratrol and pterostilbene detected by a DAD-detector and figure 13 the same samples with a mass-detector. In figure 10 there is an indication that 'Duke' might contain resveratrol, but this could not be seen in the mass-detector chromatogram (figure 11).

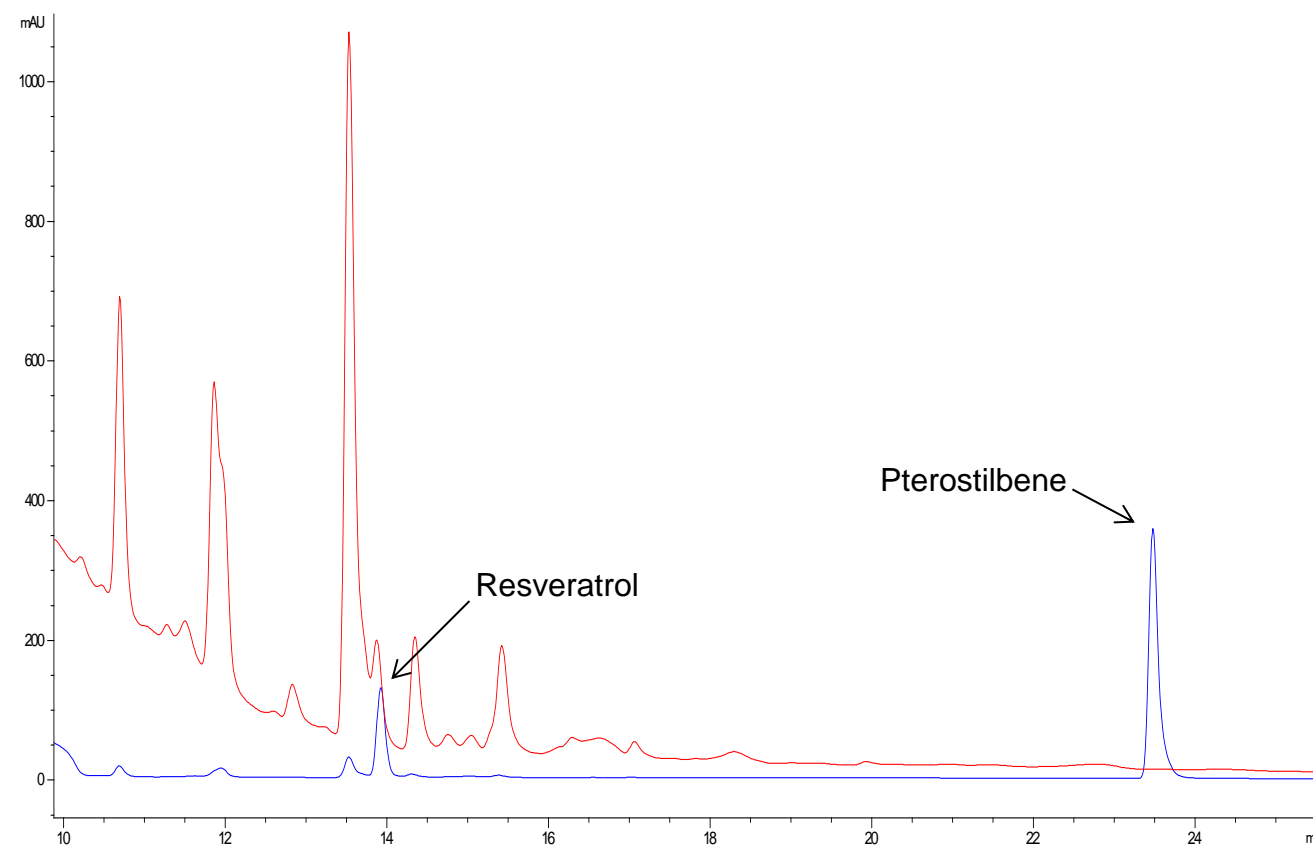


Figure 10. Chromatogram of standards (blue) of resveratrol and pterostilbene detected by a DAD-detector. Resveratrol peak was shown at 14 min and pterostilbene at 24 min. Sample of 'Duke' is red. y-axis: milli absorbance unit (mAU), x-axis: retention time (min).

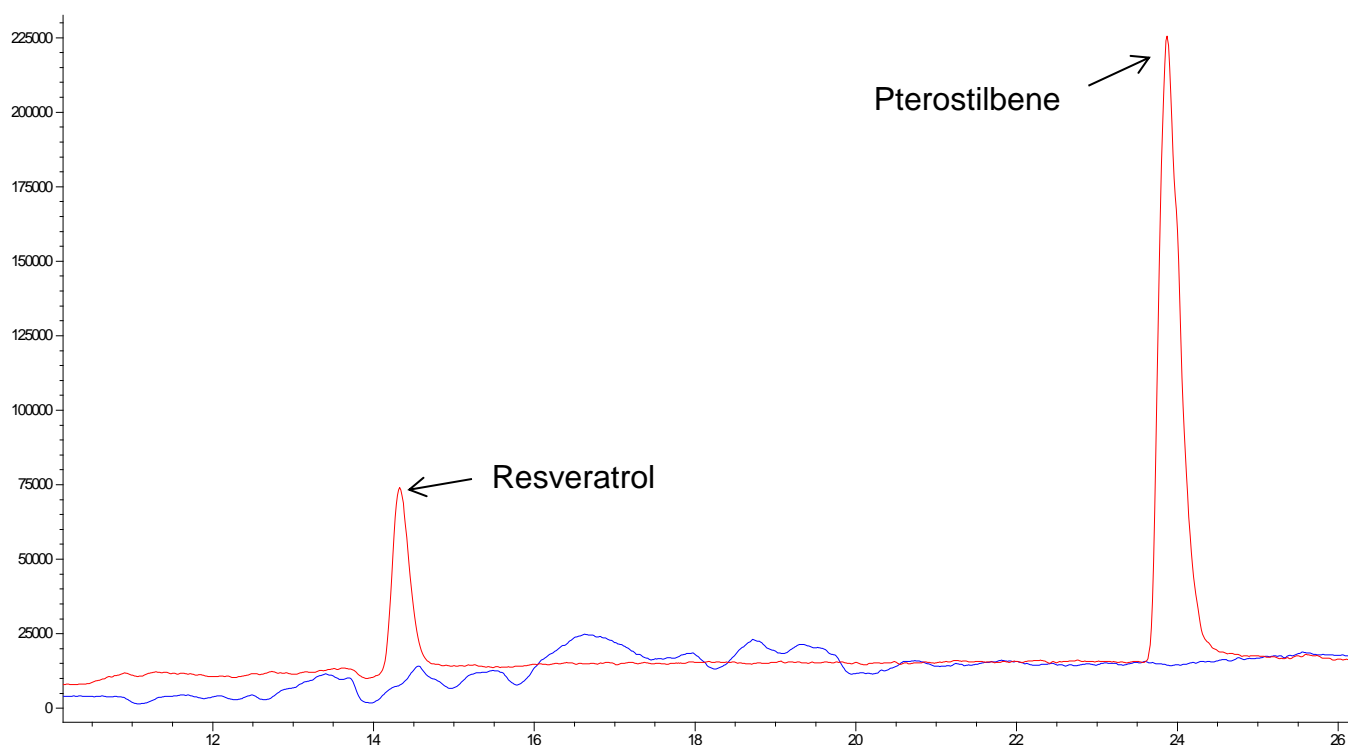


Figure 11. Chromatogram of standards (red) of resveratrol and pterostilbene detected by a mass-detector. Resveratrol peak was shown at 14 min and pterostilbene at 24 min. Sample of 'Duke' is blue. y-axis: milli absorbance unit (mAU), x-axis: retention time (min).

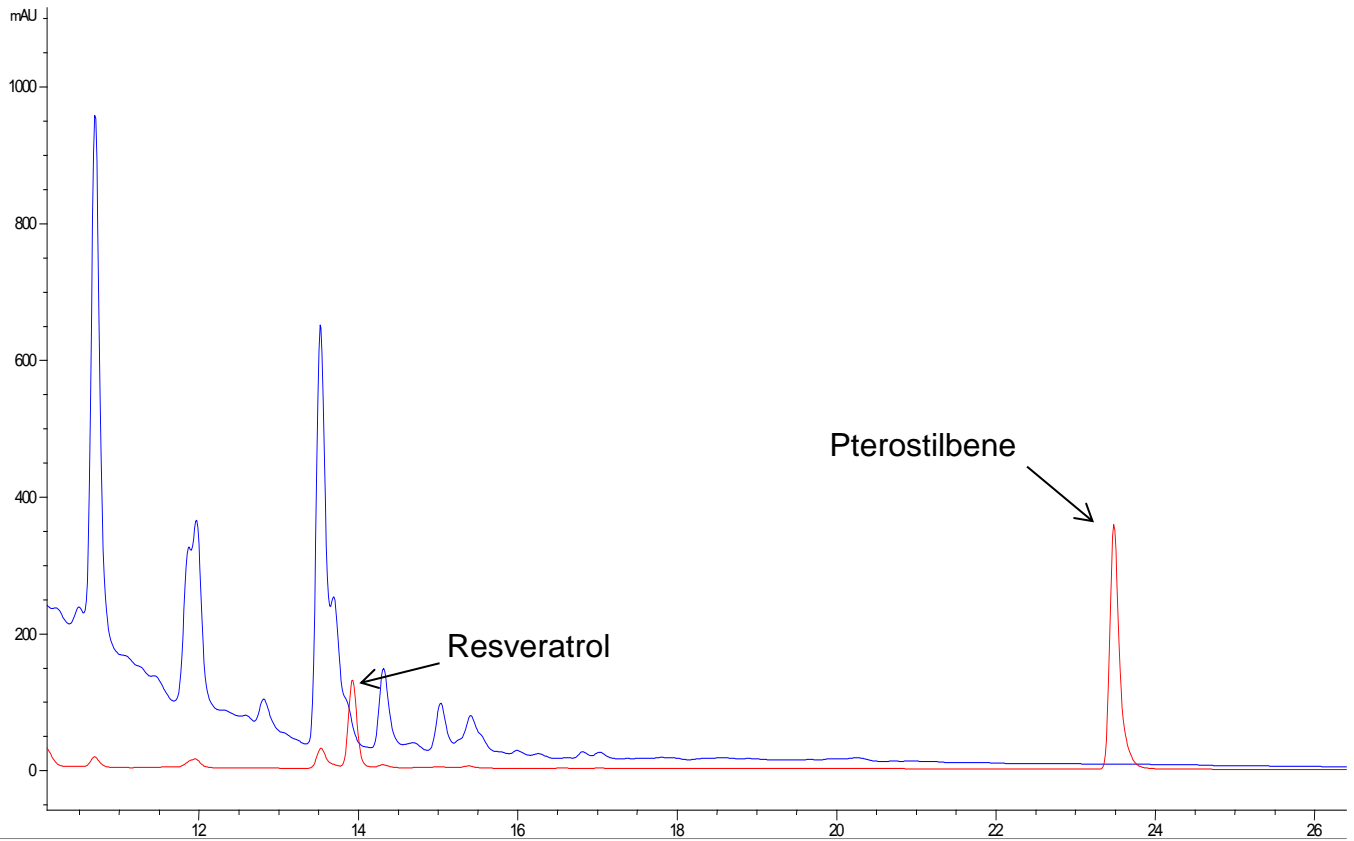


Figure 12. Chromatogram of standards (red) of resveratrol and pterostilbene detected by a DAD-detector. Resveratrol peak was shown at 14 min and pterostilbene at 24 min. Sample of 'Reka' is blue. y-axis: milli absorbance unit (mAU), x-axis: retention time (min).

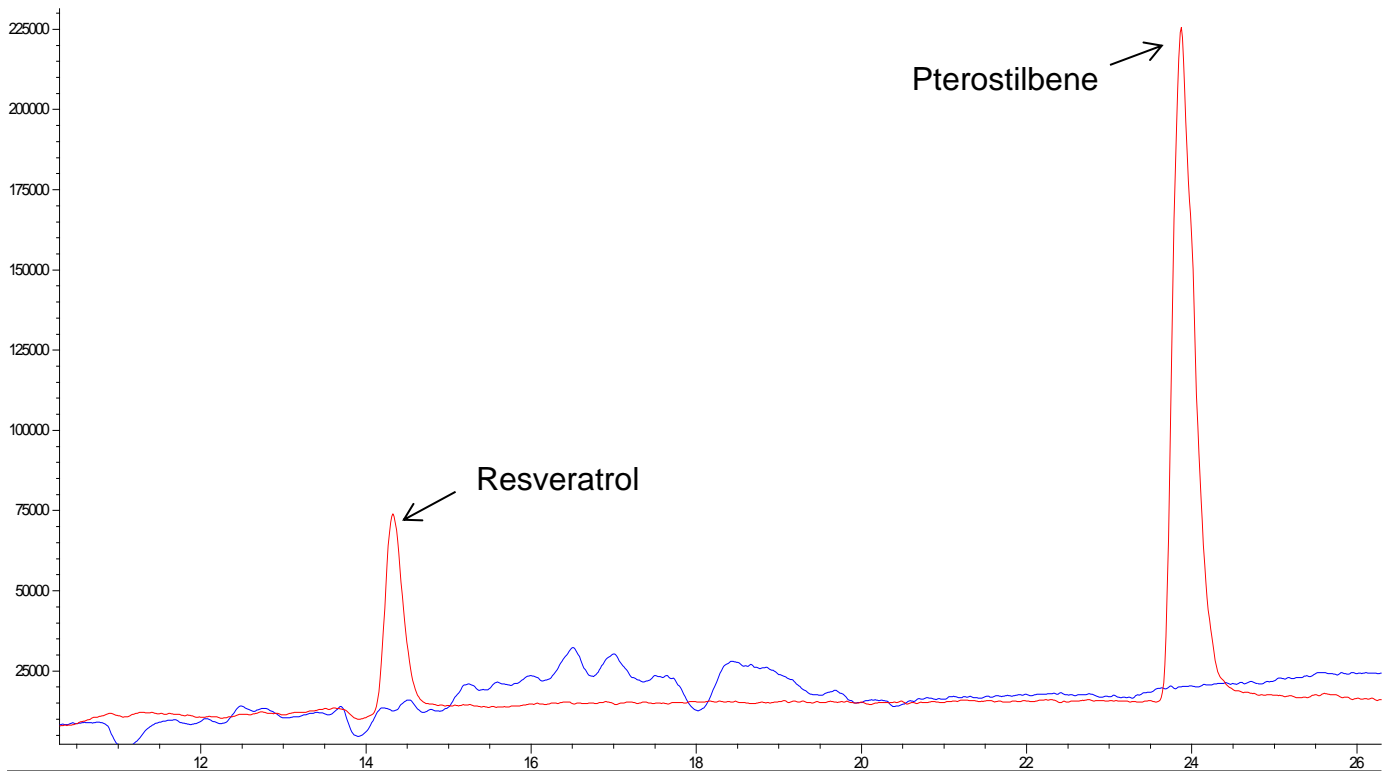


Figure 13. Chromatogram of standards (red) of resveratrol and pterostilbene detected by a mass-detector. Resveratrol peak was shown at 14 min and pterostilbene at 24 min. Sample of 'Reka' is blue. y-axis: milli absorbance unit (mAU), x-axis: retention time (min).

3.3 Sugar/acid

No significant difference between block, blueberry varieties and mycorrhiza was found measuring Brix and pH (figure 14 and 15), but a significant difference was found between the blueberry varieties measuring total acid and sugar/acid ratio (figure 16 and 17). 'Reka' contained most total acid with values around 1.4 % compared to 'Duke' 1.0-1.2 % (figure 16). Highest sugar/acid ratio was found in 'Duke' with 10-11 compared to 'Reka' 8 because 'Duke' contained less total acid (figure 17). There was no interaction between blueberry variety and mycorrhiza in any of the different treatments. Brix, pH, total acid and sugar/acid ratio was only measured week 30, not week 32.

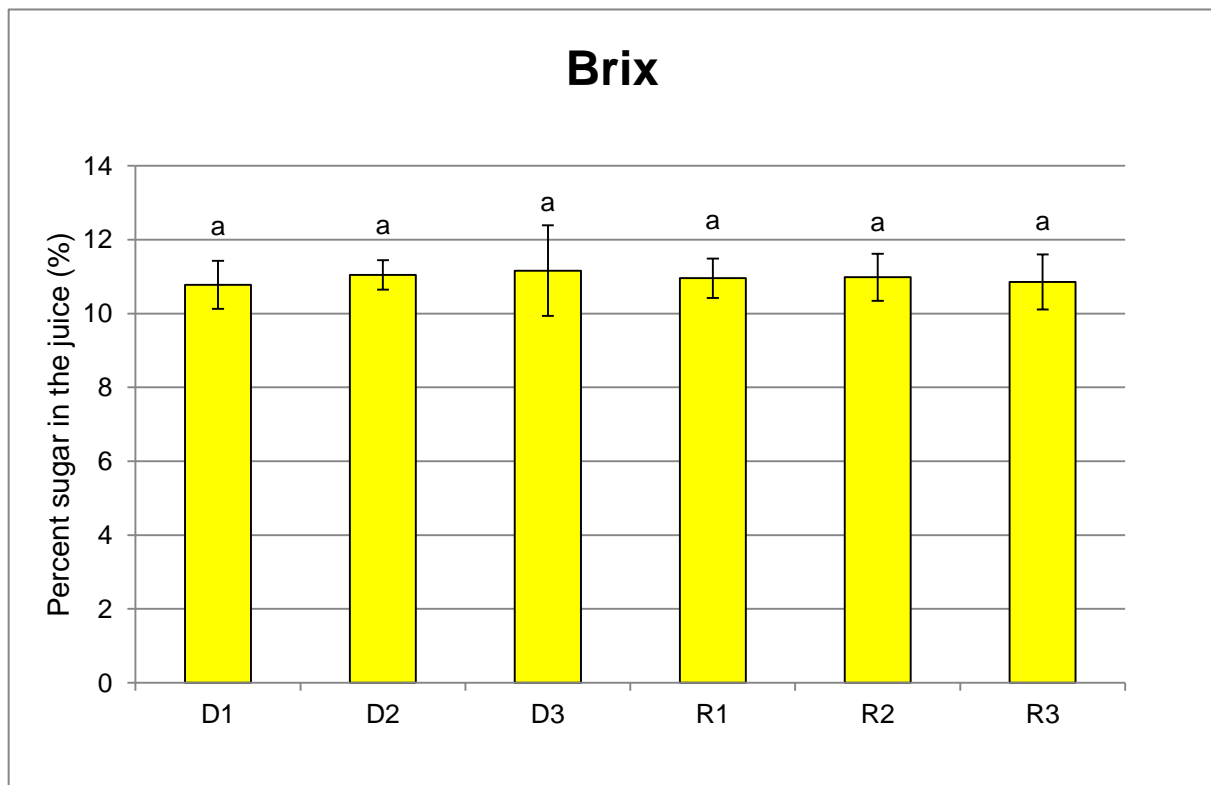


Figure 14. Brix (percent sugar in the juice) with standard deviations. Blueberries from week 30. (D='Duke', R='Reka', 1= No mycorrhiza, 2=Mycorrhiza inoculation A, 3=Mycorrhiza inoculation B). Values with same letter denotations are not significantly different.

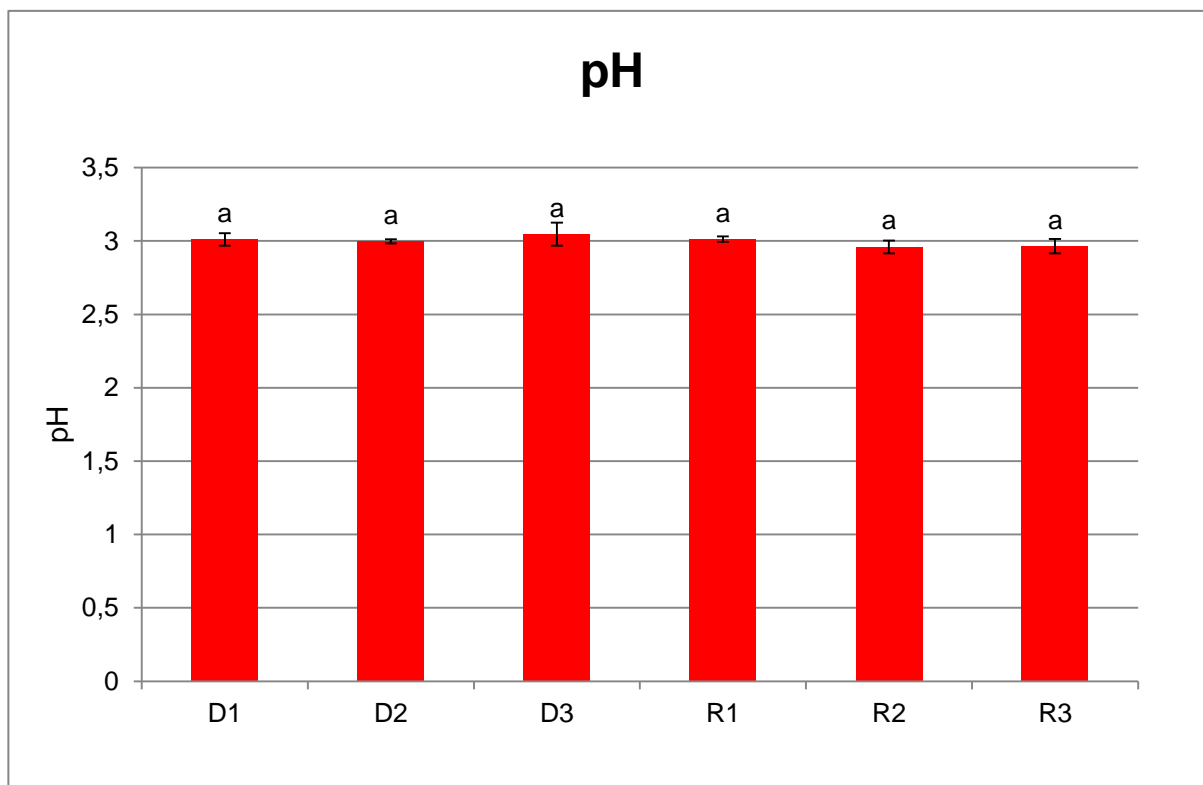


Figure 15. pH with standard deviations. Blueberries from week 30. (D='Duke', R='Reka', 1= No mycorrhiza, 2=Mycorrhiza inoculation A, 3=Mycorrhiza inoculation B). Values with same letter denotations are not significantly different.

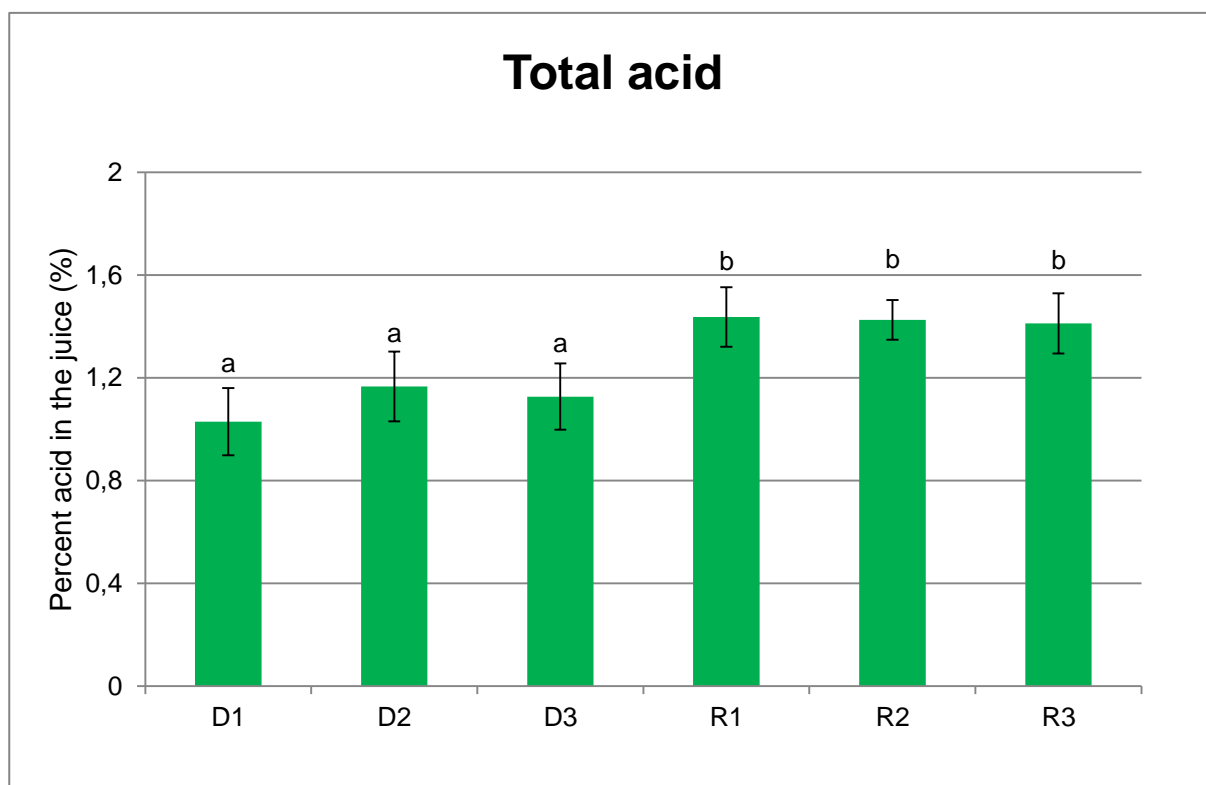


Figure 16. Total acid (percent acid in the juice) with standard deviations. Blueberries from week 30. (D='Duke', R='Reka', 1= No mycorrhiza, 2=Mycorrhiza inoculation A, 3=Mycorrhiza inoculation B). Values with same letter denotations are not significantly different.

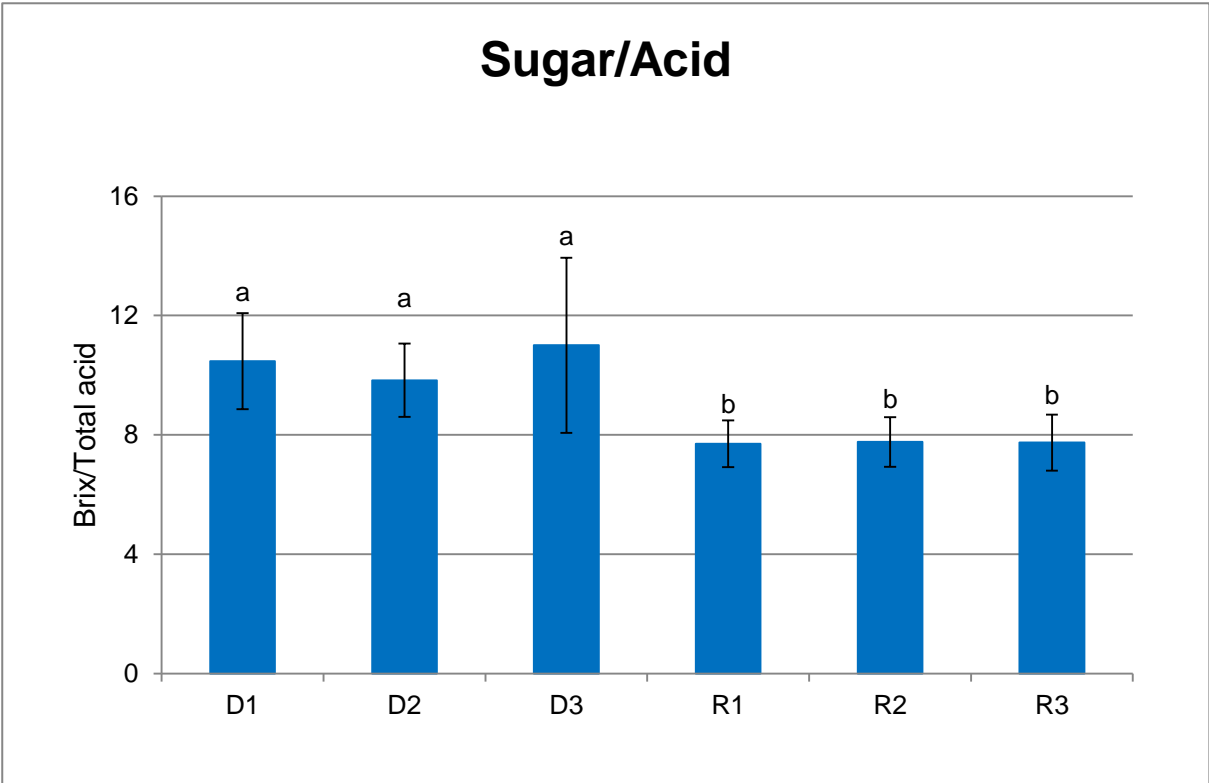


Figure 17. Sugar/acid ratio with standard deviations. Blueberries from week 30. (D='Duke', R='Reka', 1= No mycorrhiza, 2=Mycorrhiza inoculation A, 3=Mycorrhiza inoculation B). Values with same letter denotations are not significantly different.

4. Discussion

4.1 Ursolic acid and oleanolic acid

The results showed that 'Reka' contained significantly more oleanolic acid than 'Duke' while 'Duke' contained significantly more ursolic acid than 'Reka' (figure 5-6 and 8-9). 'Reka' contained significantly most triterpenes (ursolic acid and oleanolic acid) than 'Duke' (figure 4 and 7). Mycorrhiza did not affect the amount of ursolic acid and oleanolic acid. There seems to be no evidence in the literature that mycorrhiza effect ursolic acid and oleanolic acid content.

Studies on the amount of ursolic acid and oleanolic acid in the cuticular wax of blueberries showed that differences in quantity differ between years, species and varieties. Chu et al. (2007) measured oleanolic acid and ursolic acid from nine different varieties; three northern highbush (*Vaccinium corymbosum*), three southern highbush (*Vaccinium corymbosum*) and three Rabbiteye blueberry (*Vaccinium ashei*). The three varieties of northern highbush 'Misty', 'O'Neal', 'Sharpblue' contained more ursolic acid than oleanolic acid, which also was seen in the varieties of Rabbiteye: 'Britewell', 'Powderblue' and 'Premier'. The varieties of Southern highbush 'Brigitta', 'Darrow', 'Legacy' contained more oleanolic acid than ursolic acid. Chu et al. (2007) results differ from present study where two northern blueberry varieties 'Reka' and 'Duke' were studied. 'Reka' contained most oleanolic acid and 'Duke' contained most ursolic acid. 'Duke' showed the same result as Chu et al. (2007) where northern blueberries contained most ursolic acid, but 'Reka' showed the opposite. This showed that amounts of ursolic acid and oleanolic acid are depending of the varieties within the species.

Moggia et al. (2016) measured ursolic acid and oleanolic acid content in the blueberry variety 'Duke' and found that it contained higher amounts of ursolic acid than oleanolic acid, which the present study also concluded (figure 6 and 9). Moggia et al. (2016) also observed that the amount of ursolic acid and oleanolic acid in 'Duke' decrease with maturity. However, this was not shown in the other variety 'Brigitta' that was investigated, where the amount was the same throughout the maturity.

In present study a high standard deviation was found in treatment D3 ('Duke', Mycorrhiza B) week 30 for oleanolic acid, but was low week 32. Sugar/acid ratio

week 30 for D3 also had a high standard deviation which indicates maturity differences within the same treatment; when high sugar/acid ratio means more ripe berries. Moggia et al. (2016) observed that oleanolic acid and ursolic acid could decrease with maturity which maybe could explain the high standard deviation week 30 for D3 and oleanolic acid; the berries were different mature week 30 and not 32. At the same time ursolic acid did not showed any high standard deviation. Sugar/acid ratio was never measured week 32 so it is difficult to conclude if this theory can be true.

4.2 Sugar/acid

Percent sugar in the juice (Brix) and pH did not depend on the blueberry variety or mycorrhiza inoculation (figure 14-15). The amount of total acid and sugar/acid ratio, on other hand, showed a significant difference between the varieties (figure 16-17). 'Reka' contained more total acid than 'Duke' and 'Duke' had higher sugar/acid ratio (because 'Reka' had higher total acid) which could indicate differences in the quality related to taste between the cultivars (figure 16-17). Beaudry (1992) suggested that the values of sugar, pH, total acid and sugar/acid ratio could indicate the quality of blueberries. The values suggested were pH: 2.25-4.25; Total acid 0.3-1.3 %; Sugar: Higher than 10 %; Sugar/acid ratio: 10-33. When comparing these values with the results in present study it can be seen that values were almost in the same range. The results in the present study were higher than 10 % of sugar and pH was around 3. Total acid was within the range of 1.0 - 1.2 % in 'Duke' and slightly higher by 1.4 % in 'Reka'. Beaudry (1992) suggested values between 10 and 33 on sugar/acid ratio. 'Duke' was around 10, but 'Reka' was around 7. The values found in this investigation were lower than what Beaudry (1992) suggested. A value of sugar/acid ratio <18 are good for have a long shelf life of the berries and >32 not so good shelf life (Retamales & Hancock, 2012).

The values of sugar, pH, total acid and sugar/acid ratio are depending on species, varieties, culture climate and year (Bremer et al., 2008; Gündüz, Serçe & Hancock, 2015). Sugar and total acid were also depending of the maturity; higher maturity gives more sugar and less total acid (Moggia et al., 2016). Gündüz, Serçe & Hancock (2015) measured sugar, pH, total acid and sugar/acid ratio in 'Duke' and 'Reka' growing in Michigan and Oregon 2010 and Oregon 2012. The results varied between year and place. The values were between: Sugar: 11.7-13.3 %, pH: 3.4-3.7, Total

acid: 0.6-0.8, Sugar/acid ratio: 17-19 for 'Duke' and Sugar: 11.4-12.3 %, pH: 3.3-3.9, Total acid: 0.3-0.8 and Sugar/acid ratio: 14-37 for 'Reka'. Compared to results in the present investigation, it can be seen that sugar amount are almost the same or little higher. The pH-value was less than present study. Further, berries in this study had higher total acid than Gündüz, Serçe & Hancock (2015) and also lower sugar/acid ratio. 'Reka' contained significantly more total acid than 'Duke' in present study, and 'Duke' significantly more sugar/acid ratio than 'Reka' (because 'Reka' contained higher percent total acid); Gündüz, Serçe & Hancock (2015) showed the opposite. What possible could explain the differences is different weather/climate in USA (Gündüz, Serçe & Hancock, 2015) compared to Sweden (present study) when different climate affect the sugar and total acid amount. The maturity of the berries maybe was not the same at harvest. Another possible explanation is that the blueberry plant grown in USA (Gündüz, Serçe & Hancock, 2015) have received other amount of nutrition's than present study which can affect amount of sugar and total acid in the berries (Retamales & Hancock, 2012). The consequence is that same variety berries can taste different which might confuse the consumers.

Moggia et al. (2016) have also measured sugar, total acid and sugar/acid ratio for 'Duke' and it is depending on maturity. The more ripe the berries are the more sugar and less total acid is found, and also a higher sugar/acid ratio. Sugar ranged between 13.6 - 16.3 %, total acid: 1.11 - 0.51 % and sugar/acid ratio: 12.8 - 34.2. These berries contained more sugar, and little lower or the same level of total acids than berries in the present study, which indicate that present studies berries are less mature or grown in a colder climate when warmer climate means higher amount of sugar.

Bremer et al. (2008) performed a taste test on southern highbush blueberries, and it was concluded that consumers do not accept berries with low acidity like around 0.3 %, even if the sugar was around 10 % and the sugar/acid ratio was 33. Bremer et al. (2008) does not think that sugar/acid ratio is a good measure of quality when the total acid value is so low because consumers do not accept low acidity. According to Beaudry (1992) sugar/acid ratio of 33 is the highest value that is recommended and total acid of 0.3 % the lowest.

4.3 Mycorrhiza

Mycorrhiza did not affect the content of ursolic acid, oleanolic acid, sugar, pH, total acid and sugar/acid ratio. Mycorrhiza does not seem to be independent of the variety of the blueberry.

In the research project this study originated from it was found that blueberry plants of 'Reka' contained higher yield than 'Duke' both 2014 and 2016 (Caspersen, n.d)). Mycorrhiza B gave a lower yield (ibid). Shrub height of the blueberry plants was not depending on a special variety or mycorrhiza (ibid). 'Duke' contained higher amount of anthocyanin than 'Reka' (ibid). The nutrient content in the leaves was not affected by mycorrhiza or varieties (ibid). Further it could be seen that there was nutrition deficit in young leaves after the growing season 2016. Nutrients like nitrogen, phosphorus, sulfur, iron, zinc, copper and boron were at a bit too low concentrations in the plants. Mycorrhiza inoculations should help the plant with nutrition and in that way increase the quality of the berries (Smith & Read, 2008; Retamales & Hancock, 2012). Maybe this could explain why it was not seen any difference between the mycorrhiza treatments and the resulting quality because it was malnutrition in the plants.

Combination of varieties, nutrition and type of mycorrhiza inoculations are important for establishment of mycorrhiza (Scagel, 2005). Even in cultivated blueberries without inoculation, you can see different results for varieties and what soil they are grown in also affects the result (Czesnik & Eynard, 1989; Stevens et al., 1997).

4.4 Pterostilbene

When the amount of pterostilbene was measured it could never been detected. There seems to be no evidence in the literature that pterostilbene are present in highbush blueberries. Rimando et al., (2004) have found pterostilbene in Rabbiteye blueberry (*Vaccinium ashei*) and Deerberry (*Vaccinium stamineum*) but not in highbush blueberries (*Vaccinium corymbosum*). Further it was seen that resveratrol and piceannol (two other stilbenes) was found in highbush blueberries (ibid). Both standards from resveratrol and pterostilbene were analyzed in this investigation, but no substances could be identified with the HPLC-analyses and extractions that have been used. Figure 10-13 shows chromatogram from HPLC-analyses of representative sample of 'Duke' and 'Reka' with the standards resveratrol and

pterostilbene detected by a DAD-detector and mass-detector. It could be concluded that possibly resveratrol was found in 'Duke' (figure 10), but it was not seen in the mass-detector chromatogram. For pterostilbene it was clear that there was nothing detected in the samples (figure 10-13).

A problem that was encountered was that there was a lot of sugar in the berries that became like syrup in small amount of sample. Another problem was pigments (like anthocyanins) in the berries that gave several peaks on the chromatogram when trying to measure the stilbene which can disrupt the measure; mainly resveratrol, because they give peaks roughly at the same time on the chromatogram. What would be tested was to only have peel in the extraction to remove the sugar and found a way to remove the pigments. Maybe also have a higher amount of peel due because of the very small amounts of this substance in the blueberries. For *Vaccinium ashei* and *Vaccinium stamineum* the pterostilbene amount found was between 99-520 ng/g dry samples (Rimando et al., 2004). It had been helpful to have access to blueberries in which pterostilbene have been detected to be able to evaluate if the extraction methods were sufficient in the present study, and if there really is no pterostilbene in highbush blueberry or if the extraction method could be improved. 'Bluecrop' was the variety that was tested (Rimando et al., 2004) of pterostilbene in the previous investigation and maybe this is a variety that does not contain so much of this substance or none; but other highbush blueberries might.

In grapevine inoculated with arbuscular mycorrhizal fungi it could be seen that amount of stilbenoids in leaves increase when they was infected by *Plasmopara viticola*, but not so much in the non-mycorrhizal leaves (Bruissson et al., 2016). Maybe the blueberries have to be attacked by fungi to increase pterostilbene concentrations to measurable values?

5. Conclusions

In this study the amount of ursolic acid, oleanolic acid, total acid and sugar/acid ratio was depending on the blueberry variety but not the mycorrhiza inoculation. There were no differences between different treatments regarding Brix (sugar) and pH-values. Pterostilbene was not detected.

The research question was “Does mycorrhiza affect the quality of blueberries?” The answer was NO in this study, but only some quality traits have been studied. Further studies should investigate other quality aspects apart from those that have been measured here. Because taste is important for quality a taste test should be performed.

Mycorrhiza inoculations should help the plant with nutrition and in that way increase the quality of berries. The blueberry plant in present study had malnutrition which maybe explains why mycorrhiza did not affect the result. In further studied is should be concluded that mycorrhiza have been established so it is possible to measure the mycorrhiza inoculations impact of the quality for blueberries.

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