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Triterpenes in apple cuticle of organically and IP cultivated apples

by

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1 Summary

Apples are produced all over the world both for domestic consumption and for export. In Sweden apples are the most common grown fruit both in commercial production and in home gardens. The outermost cover of the fruit is the cuticle. The cuticle is interesting as it serves many different functions such as protecting the fruit from water loss and invasions of pathogens. The cuticle of apples is also interesting in a human health point of view, especially the triterpenes that have been indicated to have medical effects. To understand these different functions it is interesting to investigate the content of the cuticle. The objectives with this study were to investigate and identify the triterpenes in the apple cuticle. The content of triterpenes was also compared between apples produced according to two different cultivation methods, integrated production (IP) and organic production. In the first experiment that was conducted, a method was developed and in the second experiment effects of two different cultivation practices were compared. The results from the second experiment indicated that apples produced according to IP had a larger content of triterpenes compared with organically produced apples. The significance of the difference between the two cultivation methods was hard to evaluate due to the small selection of samples.

2 Sammanfattning

Äpplen produceras världen över för både inhemsk konsumtion och export. I Sverige är äpplen det vanligaste fruktslaget som odlas både för försäljning och i våra hemmaträdgårdar. Det yttersta skiktet på frukten kallas kutikula. Denna kutikula är intressant att studera då den uppfyller många funktioner som till exempel minska vattenavdunstning och skydd mot invasioner av patogener. Äpplens kutikula är även intressant ur en hälsosynvinkel, speciellt ämnesgruppen triterpener som har visat indikationer på att ha medicinska effekter. För att förstå dessa olika funktioner är det intressant att undersöka innehållet av denna kutikula närmare. Syftet med detta arbete var att studera och identifiera de olika triterpenerna i äpplets kutikula. Innehållet av triterpener har även jämförts mellan äpplen som har producerats enligt två olika produktionssystem, integrerad produktion (IP) och ekologisk produktion. I det första experimentet utvecklades en metod för analys av triterpeninnehållet och i det andra experimentet studerades skillnaderna mellan äpplen som producerats enligt de två olika produktionssystemen. Resultatet från det andra experimentet indikerade att äpplen som producerats enligt IP hade ett högre innehåll av triterpener jämfört med ekologiskt producerade äpplen. Den signifikanta skillnaden mellan de olika produktionssystemen kunde inte beräknas då urvalet var för litet.

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4 Introduction

4.1 Apple production

Apples are widely produced all over the world and are one of the fruit that has a significant role in the world trade with fresh produce. Year 2000, 63 millions tones of apples were produced worldwide. The largest producing country was China followed by USA, France, Germany and Iran. The largest apple exporting countries in 1999 were France, USA, Italy and Chile. (Karlsson et al., 2002). The consumption of apples in the European Union year 1999 was approximately 8.25 millions tones of apples (Karlsson et al., 2002) and in Sweden 100000 tones of apples were consumed in 2003 (Jordbruksverket, 2005). This indicates that the apple is an important fruit all over the world. The apple production in Sweden is small compared to the largest producing countries. In Sweden, 2003, 21 500 tones of apples were produced. The apple production in Sweden is not covering the amount of apples that are consumed in Sweden and the same year 92 800 tones of apples were imported (Jordbruksverket, 2005).

Apples are generally produced in Sweden according to two different growing systems, integrated production (IP) and organic production.

4.1.1 Integrated production, IP

Integrated production is a Swedish quality- and environmental management system, which is benchmarked to the European system EurepGAP. The system's goal is to minimize the production processes impact on the environment and the nature. Integrated production system implies that the production is adjusted according to the productions requirements. The environmental goals include objectives to minimize the use of pesticides, minimize the risk for humans and the environment when using the pesticides and minimize leakage of fertilizers. The IP policy is to work for a good working environment and a high standard of food quality (Grön Produktion, online; 2006).

4.1.2 Organic production

The main goal of organic production is to develop a production system that is effective in the use of resources and based on a recycling system that has a low impact on the environment and that produces food with high quality. Another goal of the system is to maintain and develop a great variety of species and biodiversity in the countryside.
The organic system is based on using local- and renewable resources and recycling by returning nutrients from waste products to the soil. The use of chemical pesticides and commercial fertilizers are not allowed at all in the organic production system (Jordbruksverket, online; 2006).

4.2 The outer coating of plants – the cuticle

All above-ground plant organs, such as apple fruits, that are exposed to the atmosphere are covered with an outer coating called cuticle. The cuticle consists of three layers; a top coating of surface wax, a thick middle layer consisting of cutin embedded in wax and a lower layer consisting of cutin and wax mixed with the cell wall substances pectin, cellulose and other carbohydrates (Taiz and Zeiger, 2002; Belding et al., 1998).

4.2.1 Cutin

Cutin is an insoluble macromolecule, a polymer. The polymer consists of many long-chain fatty acids that are liked to each other by ester linkages creating a matrix (Taiz and Zeiger, 2002, Kolattukudy, 1984). The cutin is synthesized by the epidermal cells that are the outermost layer of cells (Kolattukudy, 1984; Post-Beittenmiller, 1996). Cutin can not function alone as a barrier against the environment since the polymer is not an excellent waterproofing substance, therefore the cuticle is also consisted of epicuticular wax layers that is included on and in the cutin matrix (Kolattukudy, 1984; Post-Beittenmiller, 1996).

4.2.2 Wax

Waxes are composed of a complex mixture of long-chain acyl lipids that are extremely hydrophobic (Taiz and Zeiger, 2002). The wax consists of many different components and the most common components are straight-chain alkanes and alcohols of 25 to 35 carbon atoms, other components found in the wax are aldehydes, ketones, esters, free fatty acids and triterpenes, such as ursolic acid and oleanolic acid (Belding et al., 1998; Taiz and Zeiger, 2002; Verardo et al., 2003; Bringe et al., 2006).

Belding et al., 1998 report that the largest amount of components found in apple epicuticle wax are represented by triterpenes such as ursolic acid and the second largest amount of components are represented by alkanes. The composition of cuticle wax may vary with plant species, organ, age and environmental conditions (Post-Beittenmiller, 1996; Veraverbeke et al., 2001; Bringe et al., 2006). The cutical wax is also synthesized by the epidermal cells (Taiz and Zeiger, 2002; Post-Beittenmiller, 1996).

The outermost coating, the surface wax, is often crystallized into different patterns like rods, tubes or plates (Taiz and Zeiger, 2002). The characteristic crystalline shape differs between plant species and the surface wax on apples takes form as plate-like crystals (Kolattukudy, 1984). These crystals appear like powdery bloom on the fruit to the naked eye.

4.3 Triterpenes a secondary metabolite

Triterpenes belongs to terpenes which are one of the three major groups of secondary metabolites in plants. Triterpenes are a large terpene and consists of 30 carbon atoms. The carbon skeleton is based on six isoprene units. Most triterpenes are either alcohols, aldehydes or carboxylic acids. These compounds are often found in waxy coatings of leaves and on fruits such as apples. The triterpenes may serve a protective function in repelling attacks from insect and microbes (Taiz and Zeiger, 2002; Harborne, 1998).

4.4 The cuticle functions

The cuticle acts as a barrier between the plant and its environment. It protects the plant from water loss and invasions of pathogens. The waxes of the cuticle also provide the fruit with an attractive shine and overall good appearance (Veraverbeke et al. 2005; Jenks and Ashworth, 1999).

4.4.1 The cuticle and water loss

The waxes of the cuticle play an important role in water bearing. During storage of apples, water and weight loss is not desired. The cuticle functions as a natural barrier against water movement. When the apples are stored, the water transport from the apples into the atmosphere occurs through epidermal transpiration (transpiration through stomata, lenticels and across the cuticle). This transpiration occurs during the growth and continues after that the apples are harvested and is one of the main causes of water and weight loss during storage. When the conditions of temperature, light and gas are normal the epidermal

transpiration occur through the stomata. Under stress conditions the stomata close and the epidermal transpiration is limited to water transport through open lenticels and across the cuticle. This transpiration is called cuticular transpiration (Kerstiens, 1996; Araus et al., 1991; Veraverbeke et al., 2003).

The chemical constituents of epicuticular waxes create a continuous hydrophobic water barrier against water loss from plant organs, like apples. In response to water deficiency the plant can either produce more wax or alter the chemical compositions of their epicuticular waxes. By increasing the amount of hydrophobic components, like long-chain alkanes in the wax, the water loss can be reduced (Jenks and Ashworth, 1999). A study conducted by Veraverbeke et al., 2001, showed that controlled atmosphere (CA) storage and the following shelf life affected the wax properties and caused changes in the chemical composition, especially during the shelf life. The components responsible for the changes were mainly the alkane and ester fractions.

4.4.2 The cuticle and plant pathogens

The cuticle acts also as the first line of defence against invasions of plant pathogens. The cuticle serves as a physical barrier that the pathogens must penetrate to cause infection (Jenks and Ashworth, 1999).

Most fungal spores require free water or relative humidity above 95% in order to germinate. The requirement of water is also vital for bacteria to be able to multiply. The hydrophobic waxes on the fruit surface form a water repellent surface that prevents the formation of a water film which reduces the opportunities for germination or multiplication of pathogens (Agrios, 1997; Jenks and Ashworth, 1999).

The cuticle waxes may also play a role in plant-insect interactions. Plant surfaces with thick wax coat inhibit many insects feeding behaviour (Jenks and Ashworth, 1999).

4.5 The apple cuticle and human health

Compounds found in apple cuticle are also interesting in a human health point of view. Phenolic compounds are well known for its antioxidant activity which means that the phenols act as a free-radical scavenger. The free radicals are a natural part of the metabolism but can also be potentially harmful to humans. An overproduction of free radicals can cause

irreversible damages to cells and membranes of biological systems, which in turn have an important role in serious diseases such as cancer, and inflammation. Other compounds found in apples that have an antioxidant activity are flavonoid compounds and triterpenes such as ursolic acid, oleanic aldehyde and the corresponding acid, pomolic acid and betulic acid (Cefarelli et al., 2006).

Triterpenes like oleanolic acid, pomolic acid and betulic acid found in plant cuticle have shown effective growth inhibition of human leukaemia cell line K562 (Fernandes et al., 2003). Another triterpene, ursolic acid, extracted in large amounts in apple peal has shown growth inhibiting activities against tumour cell line (Ma et al., 2005).

A study, by Liu et al., 2005, has shown that an extract of a whole apple has inhibited mammary cancer growth in a rat. Two different studies have looked closer to the relation between intake of flavonoids and lung cancer and have reported that apple intake has an inhibiting effect on lung cancer (Knekt, et al., 1997; Le Marchand et al., 2000).

In an unpublished study, at SLU, Alnarp, the triterpene ursolic acid ability to inhibit cancer cell growth was investigated. The study indicated that further research on the apple cuticle components was of interest (Personal communication, Marie Olsson, 2006).

4.6 The objectives of the study

The objectives of this study were to investigate and hopefully identify triterpenes in apple cuticle. Apples cultivated according to IP (integrated production) and organic production were investigated and compared.

5 Materials and Methods

Two experiments have been conducted, one where a method was developed and one where two different cultivation methods for growing apples have been investigated and compared.

5.1 Experiment 1, Determination of n-hexane extraction time

5.1.1 Apple materials

For experiment one, a mixture of samples of 9 apple cultivars was used, Antonovka, Alexander, Boramleg, Bramely, Cox's orange, Kivik Coop 25, Kivik Rubinola, Kivik Vanda

and Wellington. All cultivars were organic produced except from Cox's. The apples were pealed and the peals were freeze-dried and stored in a freezer at -80ºC.

5.1.2 Extraction

Freeze-dried apple peal was grinded into a fine powder using a mixer and 0.500 ± 0.005 g of the apple powder were weighted into a centrifuge tube. The wax was extracted by adding 5 ml of n-hexane to the tube. In order to determine how long time it takes to extract the wax, the powder was extracted in n-hexane during 4 different time ranges, 1, 10, 30 and 120 min. The samples were then centrifuged for 4 min. The supernatants were transferred into new tubes and the remaining pellets were saved for ethanol extraction.

The supernatants were concentrated, by evaporating the samples with help of nitrogen gas, N_2 (g), into dried samples. The dried remaining samples were dissolved in 500 µl chloroform ($CHCl_3$). The final extracts were then transferred into glass HPLC (high pressure liquid chromatography) vials and saved for analysis.

The saved pellets were extracted in 5 ml ethanol (100%) during one hour and then centrifuged. The obtained supernatants were then transferred into new test tubes and diluted with 1.5 ml H_2O MillyQ water for the final concentration of 77 % ethanol for the extraction. To increase the concentration of the ethanol extract the samples were run through an IST Isolute SPE Column ENV+ 6 ml. An equilibration step, with first 5 ml of 100% ethanol followed by 10 ml of 77 % ethanol, was made between samples. 2000 µl of $CHCl_3$ were added to the columns to elute the extract. The samples were then evaporated with help of nitrogen gas until dry and then dissolved in 500 µl chloroform ($CHCl_3$). The final extract was then transferred into a vial and saved for HPLC analysis.

The experiment was conducted with three replicates per n-hexane extraction time resulting in 12 n-hexane extracts and 12 ethanol extracts for analysis by two different methods HPLC and a spectrophotometric method called Liebermann-Burchard method.

5.1.3 Liebermann-Burchard analysis

The extracts were analysed by a spectrophotometric method called Liebermann-Burchard to see if this method could be used as a quick method to analyse presence of triterpenes in a sample.

Liebermann-Burchard reagent was prepared by adding 15 ml of concentrated sulphuric acid drop by drop during 5 minutes to 60 ml of cooled (0ºC) acetic anhydride placed in an ice bath and then 25 ml of acetic acid was added (Gluzman, M. Kh., 1973).

The extracted samples were diluted 50 (0.02 g dw/ml) respectively 500 (0.002 g dw/ml) times to be able to use the standard curve which gives a relationship between the absorbance and known concentrations of ß–sitosterol. Pure ß-sitosterol standard was bought from Extrasynthese. A standard solution of the triterpene ß-sitosterol was prepared by dissolving approximately 5 mg (6.56 mg) ß-sitosterol in 50 ml of chloroform. The ß-sitosterol solution was then diluted into 5 different concentrations, 0%, 10%, 25%, 50% and 100%, to be able to conduct a standard curve.

2 ml of the sample to analyse and 1 ml of Liebermann-Burchard reagent were added to a cuvette. The absorbance was then measured after 60 min with help of a spectrophotometer (Cary 50 Bio UV-visible spectrophotometer) at the wavelength of 430 nm. The extracted samples were analysed as well as the standard solutions of ß-sitosterol.

To determine how long time the sample needs to react with the reagent and at what wavelengths to measure the absorbance at, several reaction times were tested at different wavelengths (results can be seen in appendix 1).

5.1.4 HPLC analysis

Several tests were made to develop a good method that could separate the four compounds from each other (method development can be found in appendix 2).

The extracted samples and the standard solutions were analysed by HPLC with two different eluents. Eluent B was a mix of 85.5% acetonitril, 4.5% methanol, 10% water and 0.1% acetic acid and eluent A, a washing eluent, consisted of a mix of 95% acetonitril, 5% n-hexane and 0.1% acetic acid. The tests were carried out at 210 nm and ran for 40 min at a flow of 1ml/min with an injection volume of 20 µl. The eluent program was: 0-29.9 min 100% of eluent B, 29.9-30 min the eluent was changed from 100 % of B to 100% of A, 30-35 min 100% of eluent A, 35-36 min the eluent was changed from 100% of A to 100% of B and 36-

40 min ended with 100 % of eluent B. The extracted samples were tested once together with a new set of standard samples.

Standard solutions of four known triterpenes, ursolic acid, oleanolic acid, uvaol and ß-sitosterol, were prepared by dissolving approximately 5 mg of pure triterpene (5.0 mg ursolic acid, 5.93 mg oleanolic acid, 5.0 mg uvaol and 6.84 mg ß-sitosterol) in 50 ml of ethanol. The standard solutions were prepared in 3 different concentrations, 100% (1µg/µl), 50% (0.5 µg/µl) and 10% (0.1 µg/µl), to be able to conduct a standard curve for each one of the compounds. Pure ursolic acid was bought from Sigma- Aldrich Sweden AB and pure oleanolic acid, uvaol and ß-sitosterol were bought from Extrasynthese.

The HPLC test system used was consisted of Merck Hitachi D-6000 Interface, Merck Hitachi L-4250 UV-VIS Detector, Merch Hitachi L-6200 Intelligent Pump, Merck Hitachi AS-2000 Autosampler and the software D-7000 HPLC System Manager. A Phenomenex Gemini 5 µ, C18, 110A, 250x4.60 mm 5 micron column was used.

5.2 Experiment 2

Quantification and identification of triterpenes by HPLC

5.2.1 Apple materials

For experiment 2, apple peals from the apple cultivar Cox's Holsteiner were used. The cultivar was grown according to the integrated production system, IP, and organically, to be able to compare the two different cultivation methods. The apples were cultivated at different farms around Kivik in the south part of Sweden. The apples were pealed and the peals were freeze-dried and stored in a freezer at -80ºC.

5.2.2 Extraction

The extractions were made in the same way as the previous experiment with some exceptions. Only 0.300 g ± 0.005 g from each apple were extracted in 3 ml of n-hexane followed by ethanol. The apple peals were extracted in n-hexane during 10 minutes and the n-hexane supernatants were discarded. The remaining pellets were extracted in ethanol as in previous experiment except that the samples were concentrated by running through a Sep Pak Plus C18 Column instead of a IST Isolute SPE Column ENV+ 6 ml and that the concentrated samples were finally dissolved in 300 µl of ethanol instead of $CHCl_3$ and saved for analysis by HPLC.

The reason why the ethanol was used instead of $CHCl_3$ was to avoid the samples to separate into to two phases. An experiment was made that resulted in a separation of the samples and the HPLC was not able to give any reliable results. It is known that the triterpenes are dissolved in ethanol and that's why it was used in experiment 2. Three repeats per apple sample were extracted as well as a control sample with only 3 ml of ethanol.

5.2.3 HPLC analysis

The samples were only analysed by HPLC. The HPLC analysis was conducted in the same way as in experiment one with the same eluents except that a different HPLC test system was used. The HPLC system used in experiment one measured only the absorbance at 210 nm and the reason why a different HPLC test system was used in experiment two was to gain light spectrum to clearly identify the separated compounds. The HPLC test system used was consisted of HP Agilent series 1100 interface, detector, pump and auto sampler. Software used was HP Chemstation v. 9.03. The column used was a Phenomenex Gemini 5 µ, C18, 110A, 250x4.60 mm 5 micron. The detection was carried out at 210 nm and light spectrum was measured in the interval of 200-600 nm. The tests were run for 40 min at a flow of 1ml/min with an injection volume of 5 µl instead of 20 µl.

The standard solutions used in the previous experiment were used in this experiment as well, with the exception of the ß-sitosterol standard solution that was remade.

6 Results

6.1 Experiment 1

6.1.1 Liebermann-Burchard analysis

With help of the absorbance values of the extracted samples and a standard curve with known concentration of ß-sitosterol the amount of triterpenes could be determined in the extracted samples. In figure 1 the amount of triterpenes in mg per g dry weight apple peal after extraction in n-hexane can be seen. Figure 2 illustrates the amount of triterpenes after the subsequent extraction in ethanol.

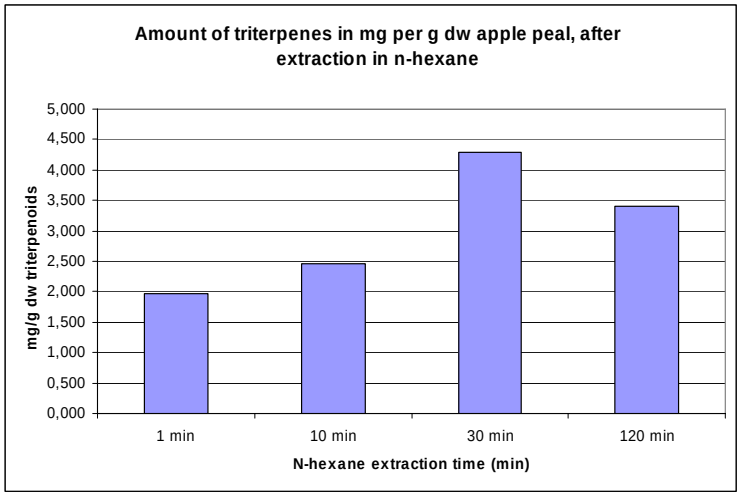

Figure 1: The amount of triterpenes in mg per g dw apple peal, after extraction in n-hexane.

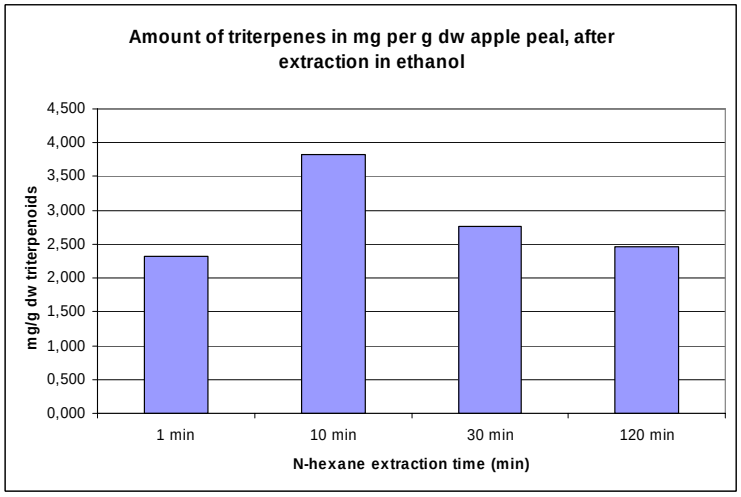

Figure 2: The amount of triterpenes in mg per g dw apple peal, after the subsequent extraction in ethanol.

Figure 1 and 2 show that when extraction in n-hexane for 10 min the largest amount of triterpenes is gained in the ethanol extraction. When the samples were extracted in n-hexane during more than 10 min the amount of triterpenes were increased in the n-hexane extraction and decreased in the ethanol extraction.

6.1.2 HPLC analysis

The extracted samples together with standard solutions of ursolic acid, oleanolic acid, uvaol and ß-sitosterol were analysed by HPLC and the amounts of the different triterpenes were calculated. The chromatograms from the n-hexane extraction did not show any distinct peaks that could be identified as known triterpenes, and therefore no data from these chromatograms are presented. The HPLC chromatograms of the ethanol extracted samples showed distinct

peaks that were identified as the known triterpenes. The retention time were noted and an average retention time with standard deviation was calculated for each of the known triterpenes, oleanolic acid 8.91 ± 0.06 min ursolic acid, 9.18 ± 0.06 min and uvaol 11.86 ± 0.085 min. With help of the data from the chromatograms of the extracted samples and the equations from the standard curves the amounts of the different triterpenes in the ethanol extraction could be calculated. The amounts of the different triterpenes are illustrated in figure 3. ß-sitosterol was not included in the calculations due to methodological problems.

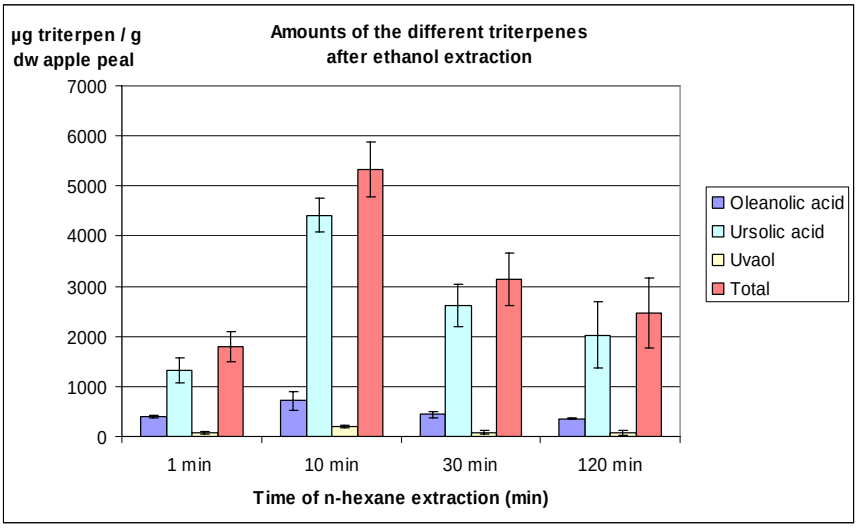

Figure 3: Amounts of different triterpenes after ethanol extraction, depending on how long time the samples were extracted in n-hexane.

* The figures were calculated from an average value out of three replicates except for extraction time 10 min that were calculated on an average of 2 replicates. The brackets are illustrating the standard deviation.

As can be seen in figure 3 the largest amount of triterpenes was gained in the samples that had been extracted in n-hexane during 10 min. The samples that had been extracted in n-hexane during 10 min had the largest contents of the three triterpenes.

6.2 Experiment 2

The extract of the apple cultivar Cox's Holsteiner and a set of standard solutions were analysed by HPLC and the amount of the known triterpenes oleanolic acid, ursolic acid and uvaol were calculated. In figure 4 the amounts of the three triterpenes in organic respectively IP produced Cox's Holsteiner apple are illustrated.

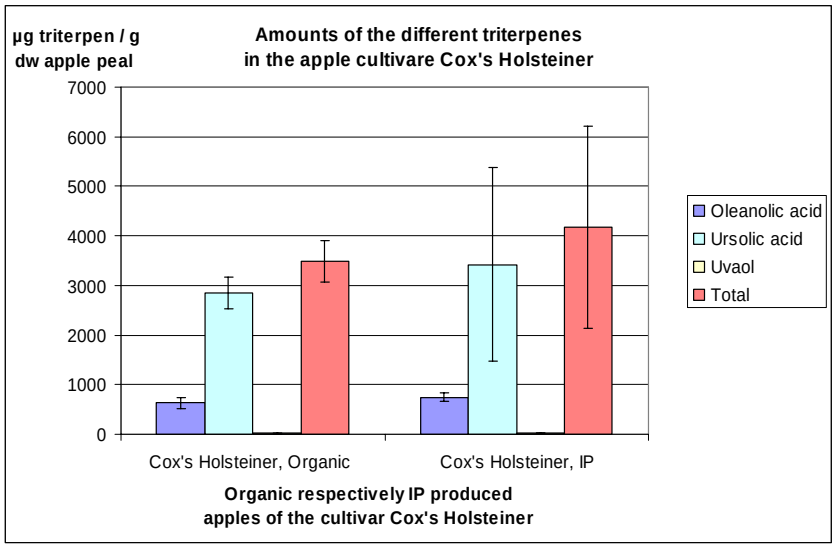

Figure 4: Amounts of different triterpenes in the apple cultivar Cox's Holsteiner grown according to organic respectively IP.

* The figures were calculated from an average value out of three replicates except for the sample grown according to IP that was calculated on an average of 2 replicates. The brackets are illustrating the standard deviation.

The amount of ursolic acid, that was the largest triterpene present in the samples, was 2842 µg/g dw respectively 3420 µg/g dw, the amount of oleanolic acid was 627 µg/g dw respectively 740 µg/g dw and the amount of uvaol was 12 µg/g dw respectively 17 µg/g dw, in organic respectively IP produced apples. This may indicate that the IP produced apples contained more triterpenes compared to organic produced apples, but due to the small sample selection no significance test have been conducted.

In figure 5, a HPLC chromatogram is illustrated, each peak in the chromatogram is representing a compound.

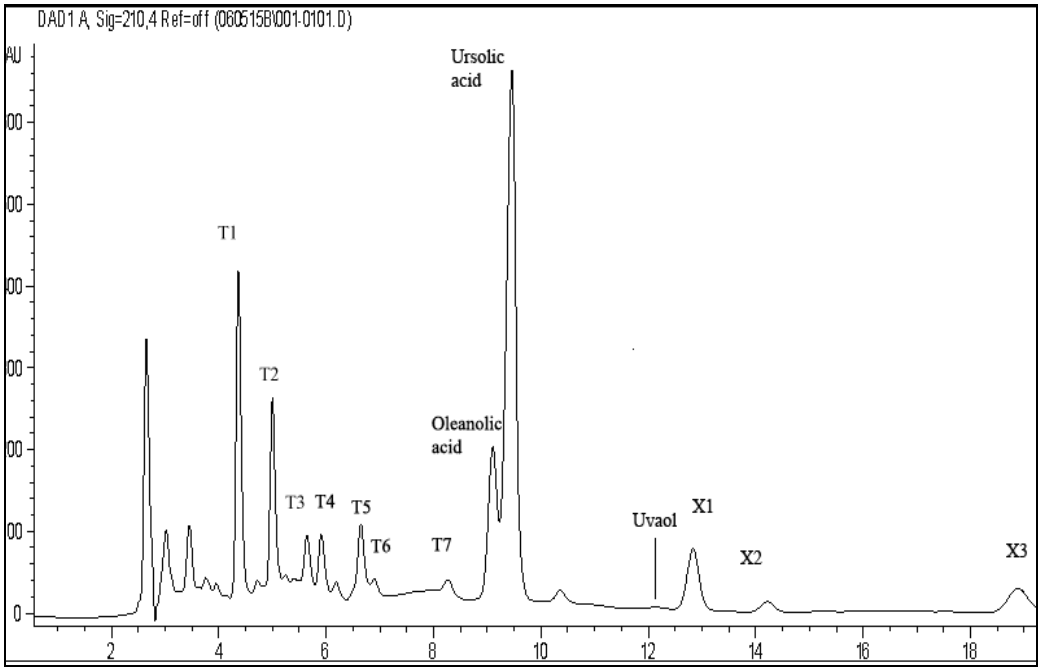

Figure 5: A chromatogram from the HPLC analysis of one of the organically grown samples. The different peaks represent different compounds. The peaks marked T were assumed to be unknown triterpenes due to that their spectra were similar to known triterpenes spectra and peaks marked X were assumed to be a phenolic fatty acid ester due to spectra similarities.

In the apple samples 10 different compounds have been found to have similar light spectra as triterpenes. Three of the compounds were known. The known triterpenes, oleanolic acid, ursolic acid and uvaol, are marked with their name in the chromatogram. Peaks marked with T are assumed to be triterpenes due to spectra similarities with known triterpenes, but not identified. Peaks marked with X are assumed to be esterified phenolic compounds of a coumaryl alcohol, coumaryl fatty acid esters, due to spectra similarities (Whitaker et al. 2001).

The retention times for the different compounds in the chromatogram in figure 5 are presented in table 1.

Table 1: The average retention time with standard deviation for respectively peak in the chromatogram in figure 5.

Peak	Retention time (min)
T1	4.36 ± 0.01
T2	5.00 ± 0.01
T3	5.64 ± 0.01
T4	5.91 ± 0.01
T5	6.64 ± 0.01
T6	6.90 ± 0.01
T7	8.03 ± 0.55
Oleanolic acid	9.10 ± 0.02
Ursolic acid	9.46 ± 0.06
Uvaol	12.11 ± 0.01
X1	12.81 ± 0.02
X2	14.20 ± 0.02
X3	18.86 ± 0.03

The amounts of different triterpenes as well as the total amount of triterpenes in the apple samples are presented in figure 7. The unknown triterpene marked T6 was not included in the calculation due to insufficient separation.

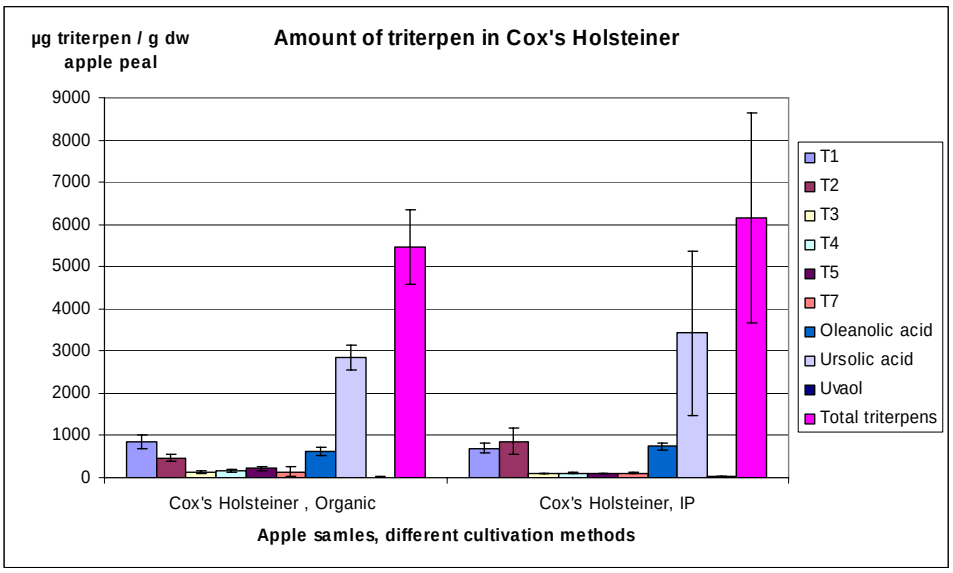

Figure 7: The amount of known and unknown triterpenes in the apple cultivar Cox's Holsteiner.

As can been seen in figure 7 ursolic acid was the main triterpene in the apple samples, followed by the unknown triterpene T1, T2 and oleanolic acid. The amounts of the different

triterpenes were slightly larger in the apples produced according to the integrated production system compared to the organic produced apples, though the significance could not be tested.

7 Discussion / Conclusion

7.1 Experiment 1

In the first experiment a good method was developed by investigating how long time the apple peal needed to be extracted in n-hexane to give the largest amount of triterpenes in the following ethanol extraction. The apple peals were extracted two times due to previous studies which have indicated that the best extraction of triterpenes is gained after the surface wax has been removed by a pre-extraction (Jetter R. et al. 2000, Chandramu, C. et al., 2003). Hexane has successfully been used to extract the surface wax and was therefore used in this experiment (Veraverabeke et al. 2001; Whitaker et al., 2001; Verardo et al., 2003)

When the extracted samples were analysed two different methods were used to see if they gave the same result. A spectrophotometric method, Lieberman Burchard analysis and analysis by HPLC were conducted.

The Lieberman-Burchard analysis gives a picture of the amount of triterpenes. This method can not be used to get an exact figure of the amounts of triterpenes in a sample. It can neither separate the different triterpenes from each other. The method is used foremost as a quick method to investigate if triterpenes are present in a sample.
If a more exact figure of the amount of different triterpenes is desired the HPLC method is better to use. Depending on what column and eluent that is used in the HPLC analysis the different triterpenes can be separated and identified. Wax components, including triterpenes, have been separated and analysed by HPLC (Andisano et al., 1995; Whitaker et al., 2001; Ma, et al., 2005).

Both the Lieberman-Burchard and the HPLC analysis showed the same result. The amounts of triterpenes in the ethanol extract were largest in the sample that had been extracted in n-hexane during 10 min. The total amounts of triterpenes gained after 10 min of n-hexane extraction followed by ethanol extraction were with the Liebermann-Burchard method approximately 3.8 mg/g dw and with the HPLC analysis the total amounts of the three known triterpenes were 5.3 mg/g dw. The difference in the figures shows that the Liebermann-

Burchard method can be used as an indication method but should not be used for quantification of triterpenes.

One of the replicates of the samples that hade been extracted during 10 min in n-hexane was not included when the average of the amount of triterpenes of the 10 min extraction was calculated after the analysis by HPLC. The reason why this sample was excluded was that the values were divergent from the other two probably due to an error in the extraction procedure. During the ethanol extraction of the 1 and 10 min n-hexane extracted samples a leakage from one of the repeats was observed but the leakage source were not identified. No data deviations were observed from the Liebermann-Burchard analysis.

The conclusion from the experiment was that the extraction time of 10 min in n-hexane gave the largest amount of triterpenes after the ethanol extraction, but to be able to draw certain conclusion the experiment should be repeated with more replicates. In the following experiment the apple peals were extracted in 10 min to gain the largest amount of triterpenes in the ethanol extract.

7.2 Experiment 2

In experiment 2, two different cultivation methods were compared. Samples of the apple cultivar Cox's Holsteiner produced organically and according to integrated production were extracted and analysed by HPLC. The chromatograms from the HPLC were studied and known triterpenes were identified and unknown peaks were investigated by observing and comparing the light spectrum of the different peaks. The amounts of the known respectively the unknown triterpenes were calculated.

7.2.1 The chromatogram

In the apple samples 10 different compounds have been found to have similar spectra as known triterpenes. Three other peaks were observed to have a different spectrum which was investigated by comparing the spectrum with literature references.

Cefarelli et al. 2006, report that several different triterpenes have been found in the apple cultivar Annurca. The triterpenes found in the apple were two lupine triterpenes (betulinic aldehyde and betulinic acid), two oleanic triterpenes (oleanic aldehyde and oleanic acid) and nine usane triterpenes (ursanaldehyde, ursolic acid, pomolic acid, uvaol, uvaol acetate,

annurcoic acid, annurconic acid, 2-oxopomolic acid and 3-epi-2-oxopomolic acid). Two different sterols were also reported found in the apple, ß-sitosterol and 7 ß-hydroxystigmast-4-en-3-one as well as 12 different fatty acid esters.

In this experiment three of these triterpenes have been identified, oleanolic acid, ursolic acid and uvaol. Seven other compounds have been observed to have the same spectra as the known triterpenes marked T1-7 in figure 5. The unknown triterpenes have not been identified due to that no reference with retention time or chromatogram from a HPLC analysis has been found to compare with.

The sterol/triterpene, ß-sitosterol, was expected to be identified but this was not possible. The standard solution of ß-sitosterol did not behave as expected. When the ß-sitosterol were analysed by HPLC the first time a signal were observed close to the signal of uvaol but when the standard solution was analysed again no signal could be observed not even after the standard solution was remade. This could be due to degradation or crystallisation of the ß-sitosterol standard. The ß-sitosterol was therefore not included in the calculations.

Three other substances were observed in the chromatogram that hade identical spectrum. In an article by Whitaker et al. 2001, the light spectrum of an esterified phenolic compound of a coumaryl alcohol (coumaryl fatty acid esters) is illustrated. The spectra of the three unknown compounds named X1-3 (see figure 5 and 6) gained from the HPLC chromatogram of the Cox's Holsteiner apple sample correspond to the spectra illustrated in the article and therefore the three samples could be assumed to be coumaryl fatty acid esters.

7.2.2 Organic versus Intergraded Production

When comparing the amounts of triterpenes in the apple produced according to the two different cultivation methods a small difference in the amount of triterpenes can be seen between the organic and the IP produced apples. The result indicates that IP produced apples have a larger content of triterpenes compared to organic produced apples of the cultivar Cox's Holsteiner. The content of ursolic acid was 17%, oleanolic acid 15% and uvaol 29% larger in the IP-produced apples compared to the organic produced apples. Due to the small selection of samples it was not possible to investigate if the difference in content between the two cultivation methods was significant. The amounts of triterpenes in the IP-samples were calculated only from two replicates compared to three due to that one of the replicates results

were twice as large as the other two and were assumed to be unreliable. The divergent result can be explained by a lab problem that resulted in that the sample in question were extracted in n-hexane during a longer time compared to the other samples.

When comparing the amount of triterpenes in the samples of experiment 1 and 2 the amount of uvaol was remarkably higher in experiment 1 (190 µg /g dw) compared to the amount in experiment 2 (12 µg /g dw and 17 µg /g dw in organic respectively IP). The apple samples in experiment 1 consisted of a mixture of several different apple cultivars and the apple samples in experiment 2 were only apple peal from the cultivar Cox's Holsteiner. This can indicate that Cox' Holsteiner has a small content of uvaol compared to other cultivars, but to draw a more certain conclusion more studies and experiment are needed.

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10 Appendix 1

10.1 Liebermann-Burchard analysis

To determined how long time the sample needs to react with the Liebermann-Burchard reagent and at what wavelengths to measure at, a test was made to develop a good method.

Standard solutions of ß-sitosterol with the concentrations 10%, 25%, 50% and 100% were used in this test. The absorbance was then measured after 15, 30, 60 and 90 min with help of a spectrophotometer (Cary 50 Bio UV-visible spectrophotometer) at the wavelengths 430, 460, 560, and 630 nm.

10.1.1 Results

The absorbance values of ß-sitosterol standard solutions measured at the different wavelengths are presented in figures 1-4. At the wavelengths of 430 nm and 460 nm (figure 1 and 2) the absorbance was increasing with time compared to at the wavelengths of 560 nm and 630 nm (figure 3 and 4) the absorbance was decreasing with time. The absorbance values at 430 nm and 460 nm (figure 1 and 2) are most stabile between 60 and 90 min.

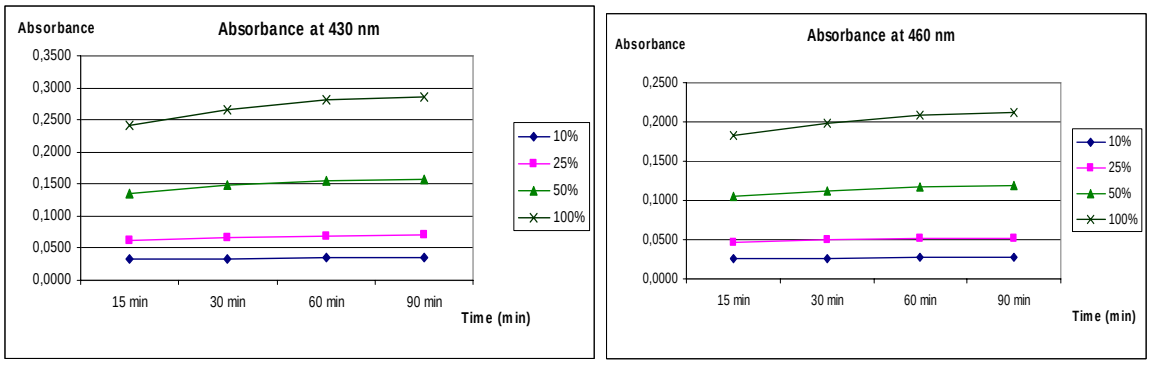

Figure 1 and 2: The absorbance measured at 430 nm respectively at 460 nm after different time ranges.

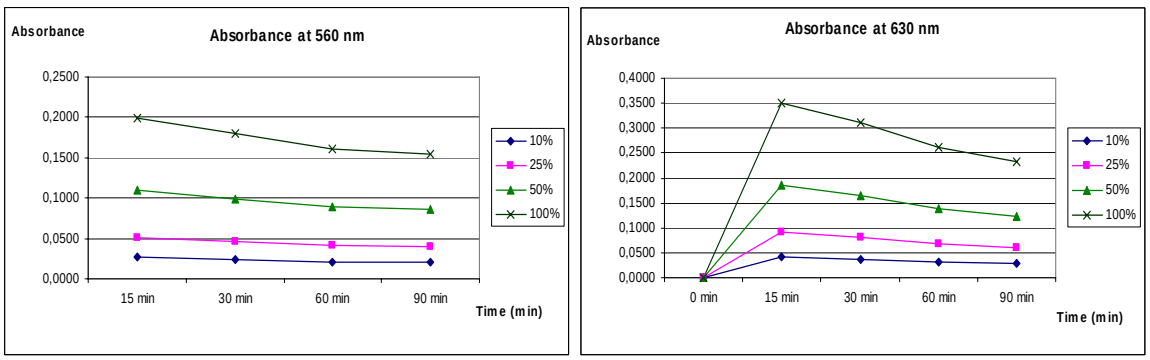

Figure 3 and 4: The absorbance measured at 560 nm respectively at 630 nm after different time ranges.

The absorbances measured after 60 minutes at different wavelengths are presented in figure 5. The highest absorbance value is measured at wavelengths of 430nm.

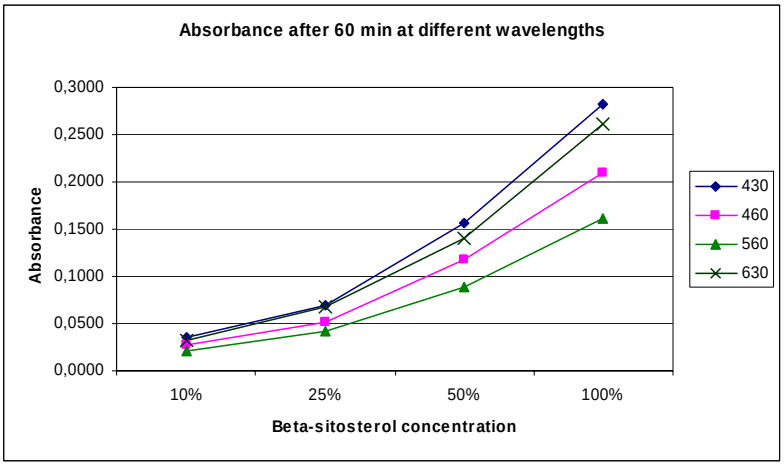

Figure 5: The absorbance measured after 60 min at different wavelengths.

10.1.2 Conclusion

The most stabile and highest absorbance value of the ß-sitosterol standard solutions was gained after 60 minutes of reaction and at the wavelengths of 430 nm. These parameters were used in analysis of the extracted samples.

11 Appendix 2

11.1 HPLC- method development

Several columns, eluents, flow rates and temperatures were tested to gain good separation of the triterpenes by the HPLC analysis.

1. Column used: Astec cyclobond I2000 betacyclodextrin 250*4.6 mm, 5 μm particle.
   Eluent: n-hexane: isopropanol / 79%: 21% (v/v).
   Flow rate: 1 ml/min
   Tested sample: standard ursolic acid 1μg/μl.
   Result: The ursolic acid had too short retention time (Retention time: 4.21 min).

2. The eluent was changed to: n-hexane: isopropanol / 70%: 30% (v/v).
   Result: No difference, ursolic acid had too short retention time (Retention time: 4.67 min).

3. The eluent was changed to: n-hexane: isopropanol: HAC / 50%: 50%: 1% (v/v).
   Result: No difference, ursolic acid had too short retention time (Retention time: 4.20 min).

4. Technical problems with the HPLC machine and the Astec column.

5. Another column and eluent was tested:
   Column:Phenomenx Luna 5 μm C18(2) 100A.
   Eluent: ACN: MeOH / 95%: 5% (v/v).
   Flow rate: 1 ml/min
   Tested sample: A mixture of all the standard triterpenes were used, (standard ursolic acid, oleanolic acid, uvaol and β-sitosterol (1 μg/μl)).
   Result: One peak with a split top was gained from the mixed sample.

6. 10% H_2O was added to the eluent.
   Eluent: ACN: MeOH: H_2O / 85.5%: 4.5%: 10% (v/v)
   Tested sample: A mixture of all four standard triterpenes.
   Result: The peak was separated into two distinct peaks.

7. The column was changed.
   Column: Phenomenex Gemini 5 µ C18 110A 250x4.60 mm 5micron.
   Eluent: ACN: MeOH: H_2O / 85.5%: 4.5%: 10% (v/v)
   Tested sample: A mixture of all four standard triterpenes.
   Result: The first of the two peaks gained a split top. Three different compounds were observed.

8. 0,1% HAC was added to the eluent.
   Column: Phenomenex Gemini 5 µ C18 110A 250x4.60 mm 5micron.
   Eluent: ACN: MeOH: H_2O: HAC / 85.5%: 4.5%: 10%: 0.1% (v/v)
   Tested sample: A mixture of all four standard triterpenes.
   Result: The split of the first peak was more distinct.

9. The flow rate was changed to 0,5 ml/min
   Result: No difference was obtained.

10. The concentration of HAC was increased to 0,2%
    Eluent: ACN: MeOH: H_2O: HAC / 85.5%: 4.5%: 10%: 0.1% (v/v)
    Result: No difference was obtained.

11. Change of column.
    Column: Phenomenex Synersi 4 µ Hydro-RP 80A 250x4.60mm 4µ micron
    Eluent: ACN: MeOH: H_2O: HAC / 85.5%: 4.5%: 10%: 0.1% (v/v)
    Tested sample: A mixture of all four standard triterpenes.
    Result: One peak with a split top was gained, same result as method no 5. No improvements.

12. The column was changed to one that was previously tested, and the H_2O concentration was increased to 15%.
    Column: Phenomenex Gemini 5 µ C18 110A 250x4.60 mm 5micron.
    Eluent: ACN: MeOH: H_2O: HAC / 80.75%: 4.25%: 15%: 0.1% (v/v)
    Result: The two peaks were dislocated in time but no improvements of the separation were obtained.

13. Increase in temperature to 40ºC temperature. The column was placed in 40ºC temperature.
    Column: Phenomenex Gemini 5 µ C18 110A 250x4.60 mm 5micron.
    Eluent: ACN: MeOH: H_2O: HAC / 85.5%: 4.5%: 10%: 0.1% (v/v)
    Tested sample: A mixture of all four standard triterpenes.
    Result: No improvements in the separation were made.

14. Increase in temperature to 60ºC temperature.
    Result: No differences were obtained.

15. A gradient of two eluents was tested.
    Eluent A: ACN: MeOH: H_2O: HAC / 85,5%: 4,5%: 15%: 0,1% (v/v)
    Eluent B: ACN: MeOH: H_2O: HAC / 47,5 %: 2,5 %: 50%: 0,1% (v/v)
    The gradient was: 4 min of 100 % of eluent A followed by 20 min of 100% of eluent B, 2 min of 100% eluent B and then 4 min of 100% eluent A.
    Result: No improvements in the separation.

11.1.1 Comment:

The best separation was obtained with method no. 8, with a Phenomenex Gemini 5 µ C18 110A 250x4.60 mm 5micron column and an eluent consisting of, 85.5 ACN, 4.5% MeOH, 10% H_2O and 0.1% HAC and a flow rate of 1 ml/min.

11.2 Identification of triterpenes in extracted sample

Standard solutions of triterpenes were added to the unknown extracted sample to identify the different compounds. The extracted sample was diluted five times (150 µl of extracted sample were diluted with 600 µl $CHCl_3$) to lower the concentration of substances. 50 µl of a standard triterpene solution was added to 100 µl of the diluted extracted sample. Each triterpene was tested separately. The different identification sample, a pure extracted sample and a set of standard series of the known triterpenes were analysed by HPLC.

11.2.1 Comment

The different triterpenes were identified, and retention times was determined; oleanolic acid 9.03 min ursolic acid 9.30 min and uvaol 12.11 min. The triterpene β-sitosterol was suspected to have a retention time at 12.63 min but the results were unclear and therefore assumed to be unreliable.