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A novel approach for the production and isolation of tulipalin-A from bulbous tulip biomass.

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A NOVEL APPROACH FOR THE PRODUCTION AND ISOLATION OF TULIPALIN-A FROM BULBOUS TULIP BIOMASS

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Abstract

Biobased technologies have the potential to play a crucial role in many challenges posed to mankind. This research aims to develop a novel route for the production and isolation of α-Methylene-γ-butyrolactone (tulipalin-A) from bulbous tulip biomass. To analyse the biorefinery potential of tulip bulbs, a composition analysis was made by using a variety of analytical tools. This could account for approximately 96 wt% of fresh tulip bulbs. To induce tulipalin-A production, a novel pathogen stressing step is introduced without the need for any chemicals or additional treatment steps. Stressing for two to three days by cross contamination of pathogens, followed by overnight extraction with toluene and rotary evaporation were most successful and yielded a product of 3.9 g/L. Size reduction of the biomass prior to stressing increased the tulipalin-A production up to 30fold. The novel method proved to be successful but the final product concentration is low. Tulipalin-A contents decrease post-harvest over time, following research should be done on freshly harvested bulbs to increase the product yield. Finally, the product stability should be investigated throughout the whole process.

Contents

1 Introduction

A growing world population and increasing welfare enlarge the human pressure put on resources provided by planet earth. Not only fossil resources are being depleted, also food, water and chemicals are subject to exhaustion. It is therefore required to make more efficient use of all resources that are available to mankind. Part of this can be achieved by utilizing streams that are currently considered to be waste. A main principle that can play a role is the biobased economy and its related technologies, in which biomass is used as a source for a wide spectrum of products ranging from fuels to high-end chemicals [1]. In the light of the biobased principle, this thesis will focus on the potential of tulip bulbs as a source for a variety of products. Prior to designing a biorefinery system, it is required to get a proper overview of all the available components. Subsequently, a trade-off between volume and value can be made to choose the most optimal product(s). Therefore, this introduction will provide an insight into the available knowledge on tulip cultivation, the content of the biomass and available waste streams.

Tulips are bulbous flowers that originate from a region stretching east from Turkey towards central Asia [2]. Since the seventeenth-century people cultivated the flowers as their appearance was much admired and valued. This trend of cultivation continued and has reached a peak production in our modern society. With the introduction of greenhouses and improved cultivation techniques it is now possible to grow the tulip in every climate region, independently of seasons.

In the Netherlands alone approximately 2 billion tulips are grown each year [3]. This large scale tulip production is mostly done in two ways. First, there is the cultivation that aims at multiplying the bulbs. The wide colourful fields that most people associate to tulip cultivation are not for growing flowers but solely for the production of bulbs. As can be seen in Figure 1 the tulip undergoes several stages in its development. In the last two stages (E&F) there are smaller "daughter" bulbs formed, attached to their "mother" bulb.

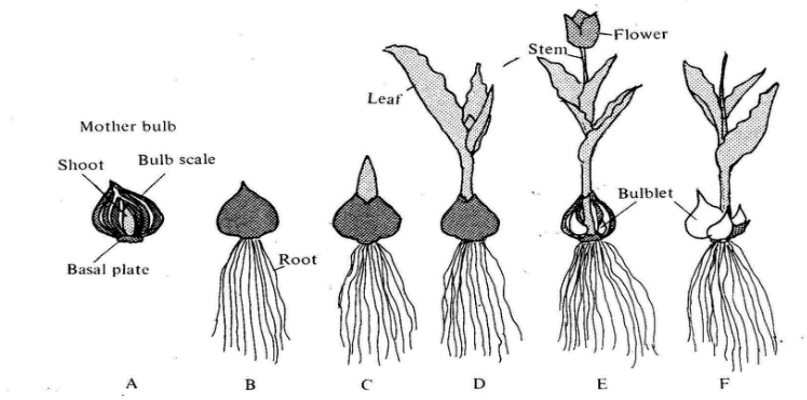


Figure 1; Developmental stages of the tulip [43]

Once this stage has arrived, around early summer, the farmer cuts off the flowers. They are left on the fields and the bulbs are harvested. These bulbs are then washed, sorted and either used on the field for next year's reproduction, sold in retail or used in greenhouses to produce flowers. This cycle is performed once a year as the bulbs need a time of dormancy before they can flower again [4].

Secondly, there is the production that aims at growing flowers. This is a large scale industry and since production is done in greenhouses, it is not bound to seasonal varieties. Also, the time till flowering is much lower than on the fields, meaning that this cycle is performed multiple times per year.

From this large-scale agricultural industry it is estimated that, in the Netherland alone, 10,000 tonnes of bulb dry matter is discarded annually (Figure 2). Each farmer has different reasons to discard bulbous matter, however, it is mostly related to an undesired quality or loss throughout the production chain [5]. It is unknown how this large quantity of waste is currently being handled and what value the farmers get for the rest stream.

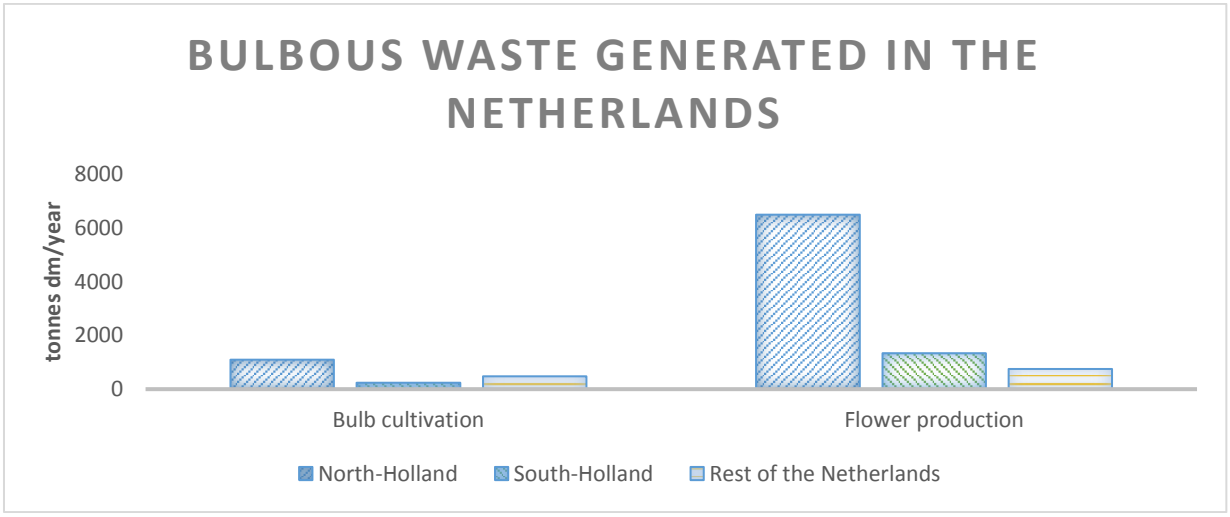


Figure 2; Geographical origin of bulbous tulip waste in the Netherlands [5]

When looking at the geographical origin of the waste (Figure 2), it can be seen that most waste is originating from the two provinces North-, and South-Holland. This also corresponds to the location where most cultivation takes place. From a biorefinery perspective, this can be very advantageous as long distance transportation of the waste is not required. Ultimately, the number of bulbs that is being discarded on a yearly basis is essential to determine the feasibility of a biorefinery plant as a minimum throughput is required.

In existing literature, little information can be found on the composition of tulip bulbs. However, there has been some research on specific aspects of the bulbs such as enzyme content and the effects of different storage techniques on carbohydrate content [6][7]. The main goal of these studies was to gain a better understanding of the processes that influence the flower growth, thereby enabling tulip farmers to be more efficient in growing their flowers and obtaining higher gains. In combination with research on other types of bulbous flowers or crops, such as onions, it is possible to estimate what components can be expected to be present in the bulbs.

As the function of a bulb is to store nutrients during time of dormancy it can be expected that different nutrients are present in relatively high concentrations [4]. A very abundant component in bulbous crops is carbohydrates, especially starch is often present as an energy source for the plant. When looking at onion bulbs, it is estimated that carbohydrates make up to 12 % of the fresh weight [8]. Apart from carbohydrates, protein, oil/fat and flavonoids can be expected in the tulip bulb in the range of 1-5 wt%. The moisture content will most probably be highest, with fractions going up 90 wt% for some onion cultivars [9].

Two components are of particular interest for the biorefinery of tulip bulbs; namely the tulipalins and their precursors tuliposides. These components have several uses in industry and are currently being synthesised via extensive routes from different starting materials [10]. However, both were also found in tulip biomass in concentrations of up to 2 wt% [11]. Under natural circumstances, the components mainly occur as a mix of tuliposides. Tuliposides are glycosides, consisting of one glucose unit with one or more α-methylene-γ-butyrolactone side chains. Most common are tuliposide-A (R=O) and tuliposide-B (R=OH), which are depicted in Figure 3.

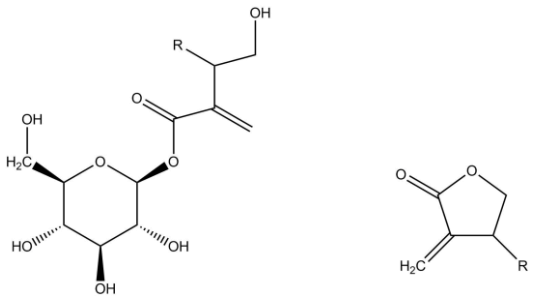


Figure 3; Left: tuliposide-A (R=H), tuliposide-B (R=OH). Right: tulipalin-A (R=H), tulipalin-B (R=OH).

Under threatening conditions, such as pathogenic attacks, the tuliposides are converted as follows: the side chains are separated from the glucose and form ring structures known as tulipalins. These are considered to be the active component against the pathogens and mainly occur as tulipalin-A and tulipalin-B (Figure 3).

Tulipalins and tuliposides have shown to possess antimicrobial properties against a broad range of strains of bacteria and fungi, even outside of the bulb [12][13]. Therefore, both components are considered to be a defence mechanism of the tulip. When extracted and purified, these components might have the potential to be used in agriculture as bio-pesticides or bio-fungicides [14][15].

The production of tulipalins from tuliposides is done via an enzyme-mediated conversion but the exact mechanism will not be a main part of this research. However, it is important to mention that tulipalins and tuliposides ratios and concentrations differ between different tulip cultivars [14][15].

Moreover, several researchers attempted to promote the production of these components. Research by *Lubbe et al.* has shown that bringing the bulb in contact with ethylene gas can induce gum formation. In natural systems, ethylene gas is often produced by pathogen-infected or damaged bulbs. To prevent themselves from infection, healthy surrounding bulbs detect the ethylene and start producing gum, which contains tuliposide and tulipalin concentrations up to 25 wt% [16]. Besides, an extracellular enzymatic conversion has been subject of research; the tuliposides and tuliposide-converting enzymes were extracted from different parts of the tulip and consecutively brought together in a reactor vessel where conversion to tulipalins took place [14].

These approaches might be interesting when designing a biorefinery system for tulip bulbs. After inducing the tulipalin production, this compound could be extracted and purified, thereby valorising the bulbous waste stream.

As there is little information on the exact composition of tulip bulbs the first step is a thorough analysis. With the use of both quantitative and qualitative analytical experiments, which are mentioned in Table 1, a profile of the bulb will be made. This information is useful for future research on the biorefinery possibilities of tulip bulbs.

Table 1; Analytical experiments used to quantitatively and qualitatively determine the composition of tulip bulbs

	Quantitative techniques	Qualitative techniques
Moisture/ash content	Drying	
Protein content	Kjeldahl/Bradford	
Oils/fat content	Soxhlet extraction	
Carbohydrate content	DuBois/Enzyme treatment	HPLC
Organic acid content	HPLC	HPLC
Amino acid content	HPLC	HPLC
Tuliposide/Tulipalin content		LCMS

With this information in mind, a biorefinery system for tulip bulbs can be designed. When making a trade-off between volume and value, the decision was made to focus on the tuliposides and tulipalins. Concentrations of up to 2 wt% are not very high but their value as bio-pesticide or –fungicide could be profitable. Due to time constraints, it is not possible to design and test the system for each tuliposide and tulipalin. Moreover, to properly quantify the efficiency of each biorefinery step, a commercially available pure standard is required. Tulipalin-A is the only component that has such a standard and will thus be the subject of this research.

As mentioned before, tulipalin-A is mainly present as its precursor tuliposide-A and the conversion takes place under stressful conditions. To have an optimal production and isolation, these conditions have to be mimicked.

Therefore, different stressing methods will be investigated to determine their efficiency. This research proposes a novel approach that makes use of cross contamination of pathogens from diseased bulbs to healthy bulbs. Consecutively, a downstream process has to be designed and tested to isolate and purify the product. Pre-treatment of the tulip bulbs could have a positive effect on the stressing and thus different approaches will be tested. The overall approach can be seen in Figure 4. Experiments will be performed to compare different techniques but not to evaluate the performance and yield of the process as a whole

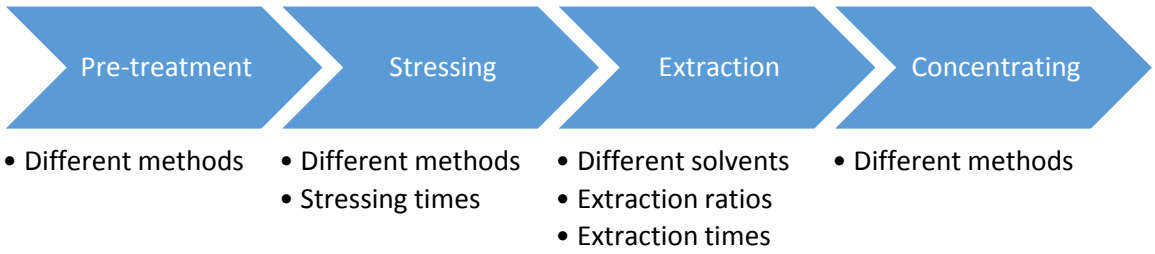


Figure 4; Approach for the production and downstream processing of tulipalin-A from tulip bulbs.

All these individual research subjects are then combined in the following research question:

What is the metabolite profile of tulip bulbs and what is the best approach for inducing tulipalin-A production with consecutive downstream processing for an isolated and concentrated product?

2 Materials and methods

This chapter will give an overview of the materials and methods used to quantitatively and qualitatively determine the composition of tulip bulbs. Both class I (high quality) and class II (poor quality) bulbs were investigated. Both classes were obtained from JUB Holland and can be seen in Figure 5. Class I consists of healthy *Royal Anthos* bulbs that are ready for retail. Class II is a mix of sick bulbs from different cultivars that were about to be discarded by the farmer. If not mentioned separately, all experiments were performed in duplicate.

Figure 5; Left: class I (healthy) bulbs. Right: class II (diseased) bulbs

Thereafter, experiments were performed to evaluate different stressing and downstream processing techniques. The experiments aimed for an optimal production and isolation of tulipalin-A from bulbous tulip biomass. Experiments were performed to compare different techniques but not to evaluate the performance and yield of the process as a whole. If not mentioned separately, these experiments were performed in triplicate.

2.1 Moisture and ash content

Fresh tulip material (both class I and class II) was cut into smaller pieces of approximately 5 mm. About 2.5 g of sample was weighed into a pre-weighted empty crucible and then placed in a drying oven at 105 °C, this was done in triplicate. After 2, 4 and 6 h the crucibles were taken out of the oven, put in a desiccator to cool and weighted. The loss in weight over time was noted until a constant weight was reached. To determine the ash content, the same approach was used with an oven of 550 °C.

2.2 Freeze drying

To limit the hindrance of water, most analytical experiments were done on freeze-dried biomass. In order to prepare this starting material, fresh tulip bulb material (both class I and class II) was cut into smaller pieces of approximately 5 mm and put in a freezer at -20 °C overnight. Consecutively, the frozen material was placed in a freeze drier (Christ, Germany) for 48 h. After this procedure, the samples were stored in a sealed container in a dark and dry place.

2.3 Oil/fat content

The combined oil and fat content was measured with the use of a Soxhlet apparatus (Thermo Fischer, USA). Freeze dried samples (both class I and class II) were grounded, 15 g was weighed and brought to extraction tubes. Boiling stones were added to round bottom flasks and the combined weight was noted. 200 mL petroleum ether was added to the flask to act as solvent, the extraction took place for 4 h. Consecutively, the petroleum ether was vaporized thereby leaving the oil and fat behind. The round bottom flasks were weighed again to determine the extracted oil and fat. The defatted material was stored for later analysis.

$$oil\ \&\ fat\ content\ (\%\ dw) = \frac{Remaining\ weight\ after\ soxhlet\ (g)}{Starting\ weight\ (g)} * 100$$

2.4 Protein content

Two methods were used to determine the protein content of tulip biomass. The first method for quantitative analysis was Bradford. The freeze-dried bulb material (both class I and class II) was well grounded and added to Tris-SO_4 (1M) buffer in order to create a 10 g/L solution. The mixtures were put on a magnetic stirring plate for 1 hour and consecutively centrifuged for 10 min at 14,000 rpm (Eppendorf, Germany). A 1 g/L BSA stock solution was made and used to generate a dilution range. 1 mL of Bradford reagent was transferred to cuvettes and subsequently 33 µL of water, BSA standard or sample was added. The solutions were well mixed and incubated for 5 min at room temperature. The cuvettes were mixed once again and the absorbance was measured against the blank with the use of a photo spectrometer at 595 nm. The same approach was used for a method in which Tris-SO_4 buffer was substituted by water as solvent.

Secondly, the Kjeldahl method was used to determine the amount of protein. As opposed to the Bradford method, Kjeldahl does not only measure the soluble protein content but the entire nitrogen content of the sample. The experiments were performed according to AOAC 2005 method 955.04 [17]. As there is currently no information available on the protein composition of tulip bulbs the general factor 6.25 was used to convert the amount of nitrogen to protein.

$$Protein\ content\ (\%\ dw) = \frac{Protein\ concentration\ measured\ against\ blank\ \left(\frac{g}{L}\right)}{Biomass\ concentration\ start\ \left(\frac{g}{L}\right)} * 100$$

2.5 Free amino acids

The free amino acid content was determined with the use of HPLC. Freeze dried material (both class I and class II) was grounded and brought to a 4.5 g/L solution in demi water. The mixture was stirred for 2 h at room temperature and consecutively a triplicate of samples was centrifuged at 14,000 rpm (Eppendorf, Germany) for 10 min. 500 µL of supernatant was brought to an Eppendorf tube and 400 µL of methanol was added for protein precipitation. Then 100 µL demi-water was added. The mixture was centrifuged at 14,000 rpm for 10 min and the supernatant was put through a 0.2 µm filter into a HPLC vial for analysis.

HPLC was performed with a Thermo Fischer, Dionex Ultrimate 3000 equipped with an acquity UPLC BEH C18 1.7 µm column and variable wavelength detector employed at 263 and 338 nm. Separations were performed at 10 °C, by gradient elution with solvent A (10 mM disodium phosphate, 10 mM sodium tetraborate, 2 mM sodium azide in MilliQ) and B (Methanol-Acetonitrile-MilliQ water 20:60:20). Elution profile : 0-6.9 min gradually to 80% A, 6.9-10 min gradually decrease to 44.7% A, 10-10.2 min decrease to 0% A, 10.2-12.49 min 0% A, 12.49-12.72 min gradually increase to 45% A. Flow 0.4 mL/min.

2.6 Carbohydrate content

Carbohydrates are present in many different forms. This research focused on soluble carbohydrates, total starch and fibre content of the tulip bulbs.

2.6.1 Soluble carbohydrates

The amount of soluble sugars was determined with the use of the DuBois method, also known as the phenol-sulphuric acid approach. 1 g of freeze-dried and defatted bulb material (both class I and class II) was grounded and made into a solution of 1 g/L in demi-water. This solution was brought to boil on a stirring plate with magnetic stirrer. Upon boiling, the heat was turned off and the mixture was left to cool down for one hour. A glucose stock solution of 0.5 g/L in demi water was made and used to generate a dilution range. From each dilution, 250 µL was brought to an Eppendorf tube. The cooled down samples were centrifuged for 10 minutes at 14,000 rpm and 250 µL of supernatant was transferred to an Eppendorf tube. To each Eppendorf, 125 µl of 0.5 g/L phenol solution was added, followed by 625 µL of concentrated sulphuric acid. The mixtures were incubated under dark conditions for 40 min and the absorbance measured in a photo spectrometer at 429 nm.

To qualitatively analyse the soluble sugar content the same 1 g/l solution was centrifuged at 14,000 rpm for 10 min and put through a 0.4 µm filter into a HPLC vial for analysis.

2.6.2 Total starch

The total starch content was determined with the use of a total starch kit from ©Megazyme. The following materials and methods were taken from the instruction manual. Freeze dried material (both class I and II) was grounded with mortar and pestle. 100 mg sample was weighted and put in a glass tube. 5.0 mL of aqueous ethanol (80%) was added and the tube was incubated at 80 °C for 5 min. The contents were mixed on a vortex stirrer and another 5 mL of ethanol (80%) was added. The tube was centrifuged at 3,000 rpm for 10 min and the supernatant was discarded. The pellet was then re-suspended in 10 mL ethanol (80%) and stirred on a vortex mixer. The mixture was centrifuged again as described before and the supernatant discarded. 3 mL of thermostable α-amylase in sodium acetate buffer (100 mM) was added and the mixture was incubated in a boiling water bath for 6 min. After 2, 4 and 6 min the tube was stirred vigorously. The tube was then placed in a bath at 50 °C and 0.1 mL of amyloglucosidase (330 U) was added, the tube was mixed an incubated for 30 min. The entire content was transferred to a 100 mL volumetric flask and the volume was adjusted with distilled water. An aliquot of this solution was centrifuged at 3,000 rpm for 10 min. Duplicate aliquots (0.1 mL) of the diluted solution were brought to glass test tubes and 3.0 mL of GOPOD reagent was added. The tubes were incubated at 50 °C for 20 min. The absorbance of each sample was read against a blank at 510 nm with the use of a UV-VIS spectrophotometer. D-glucose and pure starch samples were used as a reference.

$$Starch\ (\%) = \Delta A * \left(\frac{F}{W}\right) * FV * 0.9$$

Using:

$$\Delta A = Absorbace\ of\ sample\ \ read\ against\ reagent\ blank$$

$$F = \frac{100\ (\mu g\ of\ D\_glucose)}{absorbance\ for\ 100 \mu g\ of\ D\_glucose}$$

$$W = The\ starting\ weight\ of\ the\ sample\ (mg)$$

$$FV = final\ volume\ of\ the\ sample\ (100\ mL)$$

$$0.9 = \frac{162}{180} = adjustment\ from\ free\ D\_glucose\ to\ anhydro\ D\_glucose$$

2.6.3 Fibre

1 g of defatted and grounded biomass was weighed into an Erlenmeyer with stirrer (both class I and II). 100 mL demi water was added and the mixture was brought to boil. Upon boiling the heat was turned off and 0.5 mL of heat resistant α-amylase was added while mixing continued. After 45 min, 0.5 mL of amyloglucosidase was added and the mixture was incubated for a period of 4 h at 45 °C and 600 rpm in a thermomixer.

Thereupon, the Erlenmeyers were taken out of the thermomixer, put on a stirring plate and 100 mL ethanol (100%) was added. Stirring was done for 30 min and then the mixture was put through a pre-weighted vacuum filter. The Erlenmeyer was rinsed with 50 mL ethanol (100%) and contents were put through the filter. Consecutively, 50 mL ethanol (85%) and acetone (100%) were used to rinse the filter. Finally, the filter was placed in a fume hood to air-dry. Weight was noted after 2 and 24 h. The remaining weight is noted as the fibre content.

$$Fibre\ content\ (\%\ dw) = \frac{Remaining\ biomass\ on\ filter\ (g)}{Starting\ material\ (g)} * 100$$

2.7 Tulipalin-A and –B content

LCMS was used to assess the presence of both tulipalin-A and tulipalin-B. Freeze dried material of both class I and II was grounded and made into a solution of 50 g/L in both demi-water and ethanol (50%). The mixtures were vortexed for 1 min and stored overnight at 4°C. The samples were taken out of the fridge, centrifuged at 10,000 rpm for 10 min and brought to a LCMS vial for analysis.

Commercially available tulipalin-A was made into a 1 g\L solution in both water and ethanol (50%). Aliquots of the mixtures were brought to LCMS vials

Racemic tulipalin-B was chemically synthesised as follows: SeO2 (0.5 mmol) was added to a stirred solution of tulipalin-A (0.5 mmol) in acetonitrile (1 ml), the mixture was heated to 81 °C under reflux for 1 h to carry out the allylic hydroxylation reaction [14]. The reaction mixture was diluted 50 times in water and ethanol (50%) and aliquots were brought to LCMS vials.

A LCQ-Fleet (Thermo Scientific) equipped with an Acquity UPLC BEH C18 2,1x150mm 1,7µm (Waters 186002353) column was used. Separations were performed by gradient elution at 40 °C, with solvent A (53 mM Formic acid) and solvent B (ACN-H2O 70:30). Elution profile: 0-0.2 min 93% A, 0.2-14 min gradually to 20% A, 15-18 min gradually to 0% A. 18-20 min to 93% A. Flow 0.35 mL/min. Diode array detector was employed at 210, 280 and 350 nm. A spray volt of 5kV was used in a scan range of 50-600 m/z.

2.8 Organic acids content

Freeze dried material of both class I and II was grounded and made into a solution of 50 g/L in demi-water. The mixture was vortexed for 1 min and stored overnight at 4°C. The samples were taken out of the fridge, centrifuged at 10,000 rpm for 10 minutes and 1 mL supernatant was brought to a HPLC vial. Dilution ranges were made from commercially available ferulic-, protocatechuic-, caffeic-, gallic- and 4-hydroxybenzoic acid to act as reference and 1 mL of each reference was brought to a HPLC vial. 0.1 mL of phthalic internal standard (2 g/l) was added to the reference and sample vials and analysed by HPLC. Analysis was performed with a Thermo Fisher Ultrimate 300 equipped with an Aminex HPX-87H, 300x7.8 mm (Bio-Rad 125-0140) column and a variable wavelength detector employed at 210 nm. Separation was performed at 35 °C, by elution with solvent A (5 mM sulfuric acid). The flow was 0.5 mL/min.

2.9 Gum production

Healthy tulip bulbs were punctured 8 times at even distances and brought to an air-tight container, which was then flushed with N_2 for 1 min. Pure ethylene was brought to the container to obtain a concentration of approximately 100 ppm.

The system was placed in the dark at room temperature for 4 days. The gum was separated, the bulbs blended and both were brought into a 100 g/L solution in demi water. The solutions were placed in the refrigerator at 4 °C and stored overnight. Consecutively, the samples were taken out of the fridge, centrifuged at 10,000 rpm for 10 min and 1 mL supernatant was transferred to a LCMS vial via a 0.2 µm filter. The same LCMS equipment was used as in 2.7.

2.10 Bulb stressing

Two different bulb stressing methods were assessed for bulbs of the cultivars *Royal Anthos* and *Apeldoorn*. First of all the inductive effect of ethylene on tulipalin-A production was tested: 150 g healthy tulip biomass was cut in 4 and brought to an air tight container, as depicted in Figure 6, which was then flushed with N_2 for 1 min. Pure ethylene was brought to the container to obtain a concentration of approximately 100 ppm.

Figure 6; Air-tight container made for ethylene stressing

The system was placed in the dark at room temperature for 4 days. The stressed bulbs were then blended for 45 sec and brought into a 100 g/L solution in demi water. The solutions were placed in the refrigerator at 4 °C and stored overnight. Consecutively, the samples were taken out of the fridge, centrifuged at 10,000 rpm for 10 min and 1 mL supernatant was transferred to a HPLC vial via a 0.2 µm filter.

Secondly, the effect of pathogen infection on tulipalin-A production was tested:
150 g healthy tulip biomass was cut in 4, placed in a plastic container and brought in contact with diseased bulbs that were cut in 4 at a 1:1 weight ratio. Both types of bulbs were separated by a small paper barrier to prevent mixing, this barrier didn't prevent the two kinds of biomass to get in contact. The container was sealed, placed in the dark at room temperature for 4 days. The previously healthy biomass was then processed similarly to the bulbs from the ethylene stressing experiment. A dilution range of pure tulipalin-A in water was made and all samples were analysed by HPLC. An Acquity UPLC BEH C18 2,1x150mm 1,7µm (Waters 186002353) column was used. Separations were performed by gradient elution at 40 °C, with solvent A (53 mM Formic acid) and solvent B (ACN-H2O 70:30). Elution profile: 0-0.2 min 93% A, 0.2-14 min gradually to 20% A, 15-18 min gradually to 0% A. 18-20 min to 93% A. Flow 0.35 mL/min. Diode array detector was employed at 210, 280 and 350 nm.

Additionally, the same procedure was repeated and samples of the stressed biomass were taken after 0, 1,2,3,4 and 7 days to determine the tulipalin-A production over time. Moreover, the same procedure was used to test reference, non-stressed bulbs.

2.11 Pre-treatment

Class I tulip biomass was pre-treated in four different ways. Bulbs were either left intact, cut in 4 pieces, cut into small pieces of approximately 1 cm^3 or blended for 10 seconds. Immediately after pre-treatment, the biomass was placed in a plastic container and brought in contact with bulbs of class II that were cut in 4. This was done at a weight ratio between class I and II of 1:1. To prevent the two types of biomass from mixing a paper barrier was added. The plastic container was sealed with a plastic lid and placed in the dark at room temperature for 4 days. The bulbs were taken from the containers, blended for 30 seconds and added to demi water at 150 g/L. The mixture was placed in a refrigerator at 4 °C for a period of 24 h. Aliquots of the mixtures were taken, centrifuged for 10 min at 10,000 rpm, and 1 mL of supernatant was put through a 0.2 µm filter into a HPLC vial. Commercially available tulipalin-A (Sigma Aldrich) was used to make a dilution range. HPLC analysis, as described in 2.10, of both the references and samples was performed to assess the concentration of tulipalin-A in the extract.

2.12 Extraction

Water and toluene were tested for their capability to extract tulipalin-A from tulip biomass. Pathogen infected bulbs, as described under 2.10, were used as starting material. The infected bulbs were blended for 45 seconds and brought into a 250 g/L solution in both demi-water and toluene, placed in the refrigerator at 4 °C and stored overnight. To determine the effect of the biomass concentration in solution as well as the extraction time, infected bulbs were brought into 100, 200 and 300 g/L solutions and samples were taken after 0,5,30,120 minutes and 24 hours. HPLC samples were prepared and measured as described in 2.10.

2.13 Concentrating

To isolate and concentrate tulipalin-A from the extract, solid-liquid separation and concentrating experiments were performed. Pathogen infected bulbs, as described under 2.10 were blended for 45 sec, extracted with toluene at a concentration of 200 g/L and stored overnight at 4 °C. Samples were taken to determine the tulipalin-A concentration after extraction and the extract was then put through a vacuum filter to separate the solids and liquid. Samples were taken from the filtrate for HPLC analysis to determine the effect of filtration on the tulipalin-A concentration. The excess toluene was evaporated by means of distillation and rotary evaporation. Distillation was performed at 120 °C and rotary evaporation at 70 mbar and 40 °C until all toluene was evaporated. The concentrated tulipalin was then diluted 100 times in toluene and brought to a HPLC vial for analysis. All HPLC analysis was performed as described in 2.10.

The concentrated tulipalin from the rotary evaporator and the commercially available standard were diluted in $CDCl_3$ and analysed by ^{1H} NMR to confirm that tulipalin-A was actually the component in the extract.

3 Results and discussion

3.1 Composition analysis

Table 2 shows the results from the experiments as described in the materials and methods. Both class I and II bulbs were subject to investigation. As can be seen, the experiments that were performed can account for 96 % of the fresh weight for both classes and 91 % and 95% of the dry weight for class I and II respectively. The main differences between the two classes can be found in the water, starch and soluble carbohydrates content.

Table 2; Composition of tulip bulbs as a result of the analytical experiments performed (n.d. = not detected).*

	Class I			Class II	
	% fresh weight	% dry weight		% fresh weight	% dry weight
Water	53.97 ± 1.40			41.44 ± 4.24	
Ash	0.93 ± 0.07	1.72 ± 0.14		1.54 ± 0.06	3.72± 0.14
Oil/fat	0.15 ± 0.00	0.33 ± 0.00		0.42 ± 0.30	0.71 ± 0.51
Carbohydrates (soluble)	17.92 ± 0.91	38.92 ± 0.92		34.38 ± 0.96	58.71 ± 0.97
Carbohydrates (starch)	16.30 ± 0.39	35.41 ± 0.39		6.62 ± 0.05	11.31 ± 0.05
Carbohydrates (fibre)	3.16	6.87		5.73 ± 0.00	9.79
Protein (Kjeldahl)	2.89 ± 0.05	6.28 ± 0.06		5.35 ± 0.43	9.14 ± 0.46
Organic acids	1.03	2.23		1.30 ± 0.00	2.23
Tulipalins	n.d*.	n.d.		n.d.	n.d.
Total	96.35 ± 2.83	91.77 ± 1.51		96.79 ± 6.03	95.60 ± 2.13

The difference in moisture content could already be observed when the bulb was cut in half, as shown in Figure 7. The poor quality class II bulbs were dried out, probably caused by the diseases that they contained. This is in accordance with research by *Mor et al.*, that showed similar effects on pathogen infected narcissus bulbs [18].

Figure 7; Observed difference in moisture content between class I bulbs (left) and class II bulbs (right)

For the carbohydrate content it can be seen that the class II bulbs contain more soluble carbohydrates while their starch content is lower than that of the class I bulbs. However, when looking at the overall carbohydrate content (including fibres) the concentrations are almost similar, namely 81.2 wt% and 79.8 wt% for class I and II respectively. Research on the change in carbohydrate content in tulips over time was performed and concluded that indeed storage could lead to a decrease of starch accompanied by an increase in (soluble) oligosaccharide content [7]. As the class II bulbs have been stored for a longer time than the class I bulbs, this could give an explanation for the shift in carbohydrate composition. Although this change is in accordance with literature, the shift is bigger than described. It is expected that the presence of pathogens speeds up the starch degradation, as the smaller carbohydrates act as a substrate for the pathogens [19][20].
Moreover, *Ohyama et al*. described that starch is degraded in preparation of flower growth [21]. However, if no flower is produced by the diseased bulbs, the starch content cannot be completely restored.

The soluble carbohydrate fraction was investigated by means of HPLC to qualitatively determine what sugars were solubilized. These results however, only showed a fraction of the soluble carbohydrate content found by the DuBois method [22]. Most likely this is caused by the fact that only small mono- or di-saccharides are detected by the HPLC method. Thus, most soluble carbohydrates are probably present as oligosaccharides.

The fibre content was determined by attempting to solubilize all other components from the freeze dried biomass by consecutive heating, washing and enzymatic hydrolysis. The remaining weight was noted as the fibre content. However, it might be that the treatment didn't succeed to remove all other components, thereby over-estimating the fibre content.

To determine the protein content different methods were used and consequently different results were obtained. The results, as depicted in Table 2, were obtained from the Kjeldahl method and gave higher results than the Bradford approach. As Kjeldahl measures all nitrogen containing species the result is most likely an overestimation of the actual amount of protein present [23]. However, the other main constituents of the bulb biomass are not expected to contain considerable amounts of nitrogen and therefore chances off double-counting some of the components are low. When using the Kjeldahl method a conversion factor has to be used to convert the obtained results into protein concentrations [24]. An often used conversion factor is 6.25, which takes the average N content of proteins [24]. In this case 6.25 is used as it is comparable to the factor used for some onion cultivars [25]. In reality, this conversion is most likely not completely accurate and for a more precise result the protein composition of tulip bulbs has to be assessed.

In order to gain better understanding of the proteins that are present, the individual free amino acids were identified. From Table 3, it can be seen that the free amino acid content is approximately 1 wt% for both classes, with arginine being the most abundant.

Table 3; Free amino acids in bulbous tulip biomass, detected by HPLC.

	Class I	**Class II**
	% dry weight	% dry weight
Aspartic acid	0.09 ± 0.00	0.14 ± 0.00
Glutamic acid	0.13 ± 0.00	0.14 ± 0.00
Glutamine	0.35 ± 0.01	0.19 ± 0.00
Arginine	0.85 ± 0.02	0.42 ± 0.01
Total	1.42	0.88

From a biorefinery point of view, it is relevant to see what organic acids are present in the biomass. If there is a relatively high amount of one of the components it could be interesting to extract them to be used as food supplements [26]. Table 4 gives an overview of the detected organic acids, with citric acid being the most abundant in class I bulbs and formic acid for class II.

Table 4; Organic acid composition of bulbous tulip biomass, detected by HPLC.

	Class I	**Class II**
	% dry weight	% dry weight
Formic acid	0.17	1.96
Propionic acid	0.35	0.29
Lactic acid	0.01	0.02
Citric acid	0.92	n.d.
Glycoxalic acid	0.16	n.d.
Total	1.62	2.28

Although organic acids are often subject of research in biotechnological systems (e.g. fermentation processes), the quantities detected in tulip bulbs are most probably not sufficient for an economically feasible process [27].

To qualitatively determine the presence of both tulipalins, LCMS was used with a pure standard of tulipalin-A in both water and ethanol (50%). Tulipalin-A, which has a molecular mass of 98, was detected at a retention time of approximately 1.75 minutes, as can be seen in Figure 8 (A&B). Synthesised tulipalin-B, which has a molecular weight of 114, gave a strong peak at a retention time of 1.15 minutes, as can be seen in Figure 8 (C).

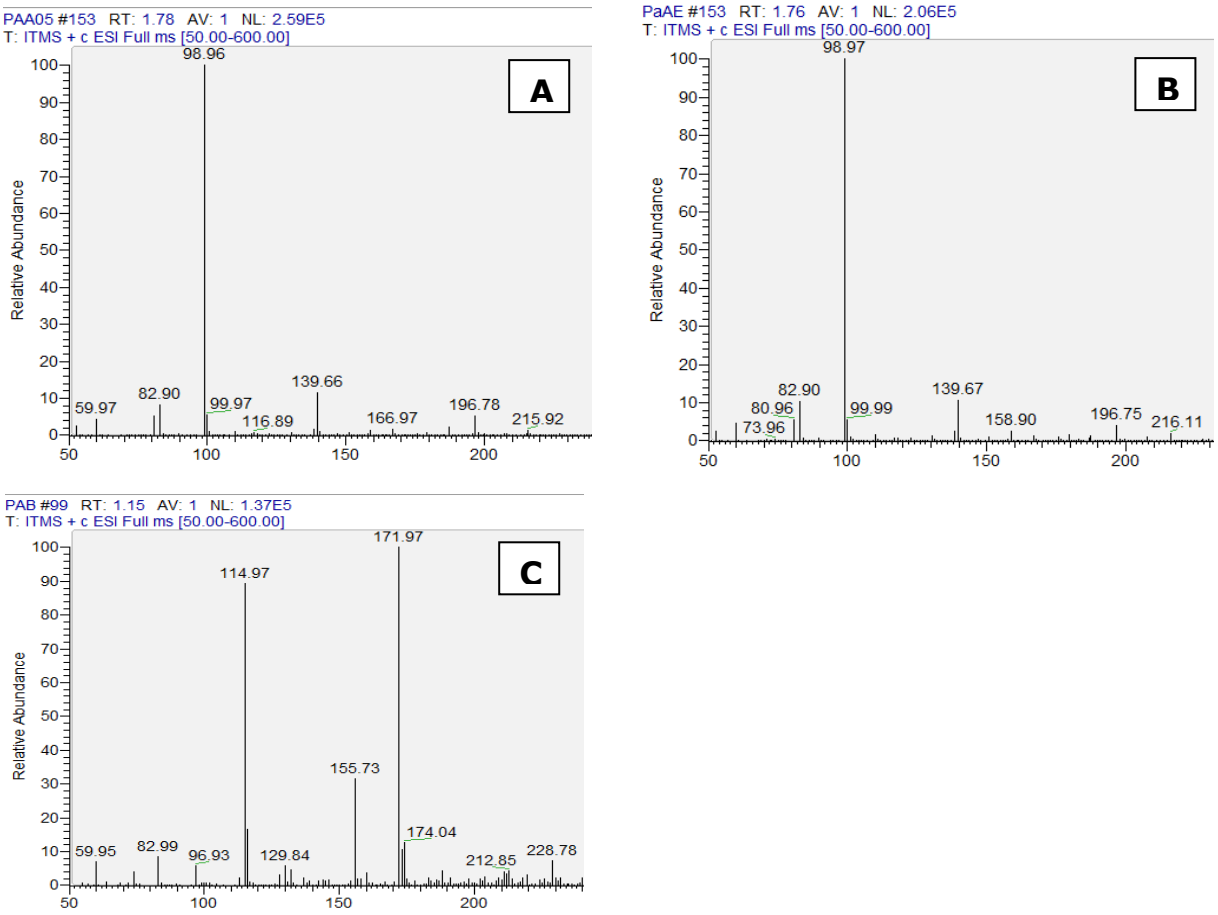


Figure 8; A: full ms spectrum of 0.5 g/L tulipalin-A in water at a retention time of 1.78 minutes, B: full ms spectrum of 1 g/l tulipalin-A in ethanol (50%) at a retention times of 1.76 minutes C: full ms spectrum of chemically synthesised tulipalin-B at a retention time of 1.15 minutes

LCMS measurements on the extracts of class I and II did not give a result at the given retention times, as can be seen in Appendix 1. Consequently, their amounts are annotated as not detected (n.d.), which can be seen in Table 2. Literature shows that different tulip cultivars contain different concentrations of tuliposides and tulipalins. Therefore, it could be that the cultivar of choice is not suited for the extraction of tulipalins [28][14]. Alternatively, the tulipalins might only be present as their precursor tuliposides. Only under stressful or enzyme-mediated circumstances, these precursors would be converted to their active counterparts [29]. In order to qualitatively determine the presence of tuliposide-A, a dilution range should be made and analysed by HPLC. This will be done for the stressing experiments.

3.2 Gum production

Stressing the bulbous biomass by placing it in an ethylene containing environment did lead to the production of gum, as can be seen in Figure 9, which is in accordance with research by *Lubbe et al.* [16].

Figure 9; Punctured and ethylene stressed tulip bulbs. Gum production indicated by red circles.

The gum was tested for its tuliposide and tulipalin content by LCMS, but the components appeared to be absent. This is not in accordance with previous research, a potential cause could be the difference in the stressing method. *Lubbe et al.* flushed the container with ethylene gas every 24 hours with approx. 30 ppm ethylene for a period of three days, whereas this research only added ethylene once. Moreover, the container might not have been completely air tight, resulting in a leakage of ethylene over time. Gummosis in tulip bulbs is a poorly understood mechanism, thus the gum formation might only be caused by puncturing the bulbs while tuliposide and tulipalin were not detected because of the absence of ethylene [30]. Finally, the choice in tulip cultivar could be a reason for the absence of the components. Previous research was done on the *Apeldoorn* cultivar, whereas this researched used *Royal Anthos*. Different cultivars might have other defence mechanisms when it comes to ethylene stress.

An additional observation was made; after the gum was dissolved in water and ethanol (50%), it was observed that the gum in ethanol (50%) gave a gel-like substance as can be seen in Figure 10. This is a remarkable property as there are only few components known that are able to produce gels in organic solvents [31]. Potential applications are not in the scope of this thesis. However, it would be interesting to perform additional research on this topic.

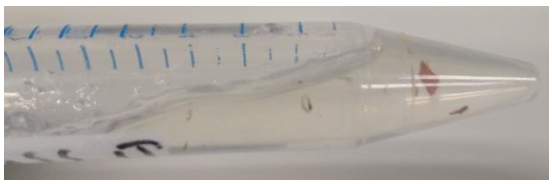


Figure 10; Tulip gum dissolved in ethanol (50%)

3.3 Stressing strategy

As described before tulipalin-A is a natural defence mechanism against microbial attacks. Tuliposide-A, the precursor of tulipalin is stored in the plant tissue and converted via an enzymatic mediated conversion when threatening conditions occur. Artificial stressing of the bulbs triggers the mechanism that would lead to this conversion under natural conditions. Literature gives a method in which the bulbs are exposed to ethylene in an air-tight container, as depicted in Appendix 2. Ethylene is a natural signal hormone that is used by many plants, including tulips, to indicate a microbial attack or damage to its biomass [16]. Healthy, surrounding, biomass detects the ethylene and starts producing tulipalin-A as a defence mechanism.

This report proposes a novel method to trigger the defence mechanism; by bringing healthy biomass in contact with diseased bulbs, cross-contamination of the pathogens infects the healthy bulbs thereby promoting the production of tulipalin-A.

Both methods were examined and the results, as depicted in Figure 11, were obtained by HPLC analysis.

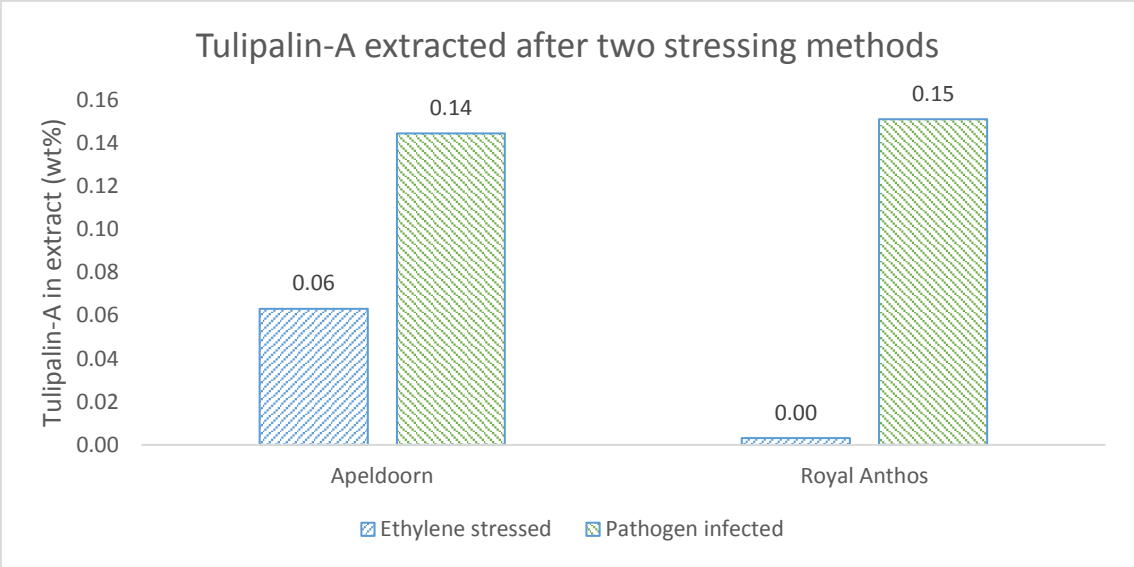


Figure 11; Tulipalin-A content of Apeldoorn and Royal Anthos tulip bulbs after stressing by ethylene treatment and pathogen infection. Bulbs were cut in 4 and extracted with water.

It was observed that infection by pathogens yields higher extracts of tulipalin-A for both the *Apeldoorn* and *Royal Anthos* cultivar. Apart from HPCL analysis, visual observation provided some clear differences between the 2 methods. Ethylene treatment didn't leave any visual trails on the biomass whereas the pathogen infected bulbs clearly show changes, as can be seen in Figure 12. Although these observations do not provide additional information on the tulipalin-A content they do prove that pathogen infection takes place when the biomass is brought in contact with diseased bulbs.

Figure 12; Left: pathogen infected bulbs. Right: ethylene stressed bulbs

Potentially, the lower amount of tulipalin-A in ethylene stressed bulbs could be caused by a different method as compared to literature for the same reasons as those listed at chapter 3.2, gum production [16].

The tulipalin production in pathogen infected bulbs was compared to that of reference non-pathogen infected bulbs. The results are depicted in Figure 13.

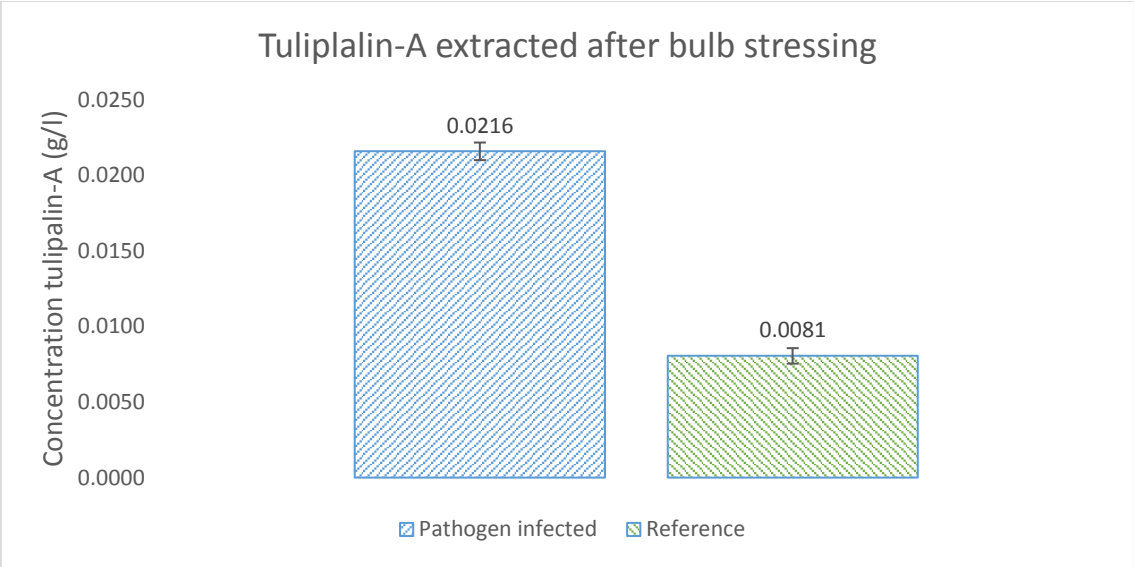


Figure 13; Tulipalin-A in pathogen infected and reference bulbs. Both cut in 4 and toluene extracted

The pathogen infected bulbs show a higher amount of tulipalin-A, which indicates that the stressing method was successful. Remarkable is the presence of tulipalin-A in the reference bulbs while the component was not detected during the composition analysis earlier. Visual inspection also shows biofilm formation on the reference bulb. This leads to the hypothesis that pathogens were present on the outer skins of the reference bulbs, which is accordance with literature [32]. Probably, the pathogens did not get the opportunity to form a biofilm and further infect the bulb due to the protective nature of the outer layers [33][34]. When the bulb was damaged, these pathogens got the chance to spread, thereby inducing tulipalin-A production.

To determine the product formation in stressed bulbs over time, samples were taken at several intervals. The samples were extracted and analysed with HPLC, these results can be found in Figure 14.

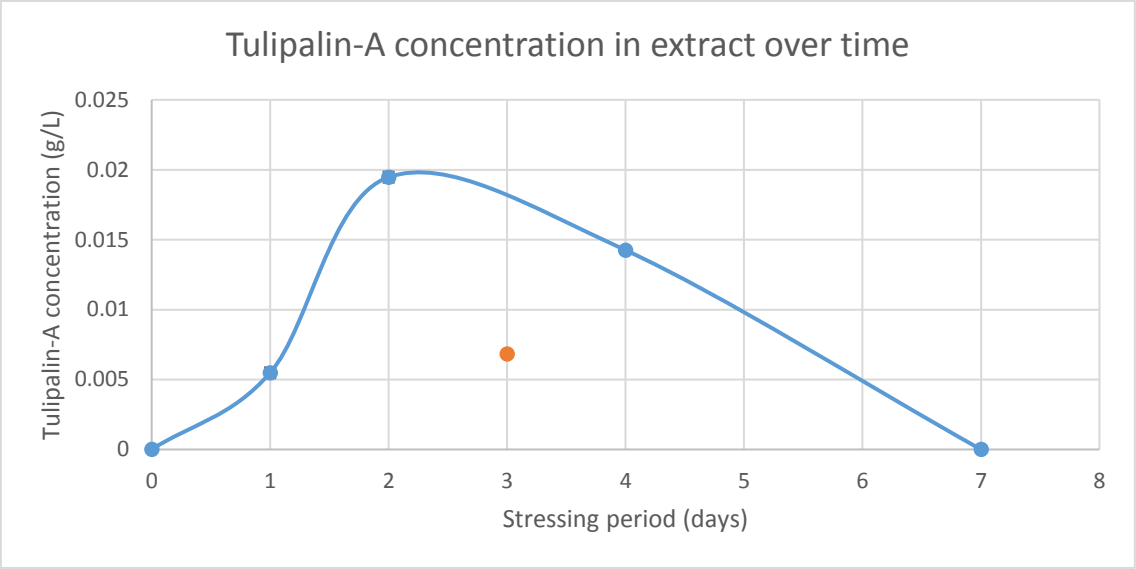


Figure 14; Tulipalin-A content in stressed bulbs over time. Bulbs were cut in 4 and toluene extracted

Initially (t=0) there is no product detected in the samples. As time proceeds, the extracted concentration gradually increases and reaches a peak value after approximately two days. After three days a sudden dip was detected, which is most likely caused by a measuring error. The blending of the biomass prior to extraction might not have been sufficient, resulting in a less efficient extraction step. However, the product concentration continues to decrease and reaches 0 g/L after seven days.

The absence of tulipalin-A at the beginning of the experiment was expected as the product is still present as its precursor tuliposide-A. Only when there is need for the active compound, due to the presence of pathogens, conversion will take place. As the degree of infection increases over the first days, the amount of tulipalin-A increases as well. Indicating that the defence mechanism of the bulb is activated.

The decrease in product during the stressing period might be caused by the interaction between tulipalin and pathogen. This exact mechanism has not yet been studied. However, the structure of tulipalin-A might be altered when the interaction takes place, resulting in inactivation of the product [29][35]. As there is only a limited amount of tuliposide and tulipalin available, this would ultimately lead to a decrease in concentration.
Alternatively the enzyme, responsible for the conversion, could inactivate over time. Different aspects such as temperature, pH and moisture content can have an effect on the stability of enzymes, resulting in denaturation and a loss in activity [36].

3.4 Pre-treatment strategy

Different pre-treatment methods were assessed to determine their effect on the overall tulipalin-A production. It was expected that an increased surface area results in a higher yield, consequently, the tulip bulbs were either left untouched, cut in 4, cut in small pieces or blended before they were exposed to diseased bulbs. Pathogen infection could be observed for all pre-treated bulbs (Appendix 3) and HPLC analysis gave the following results:

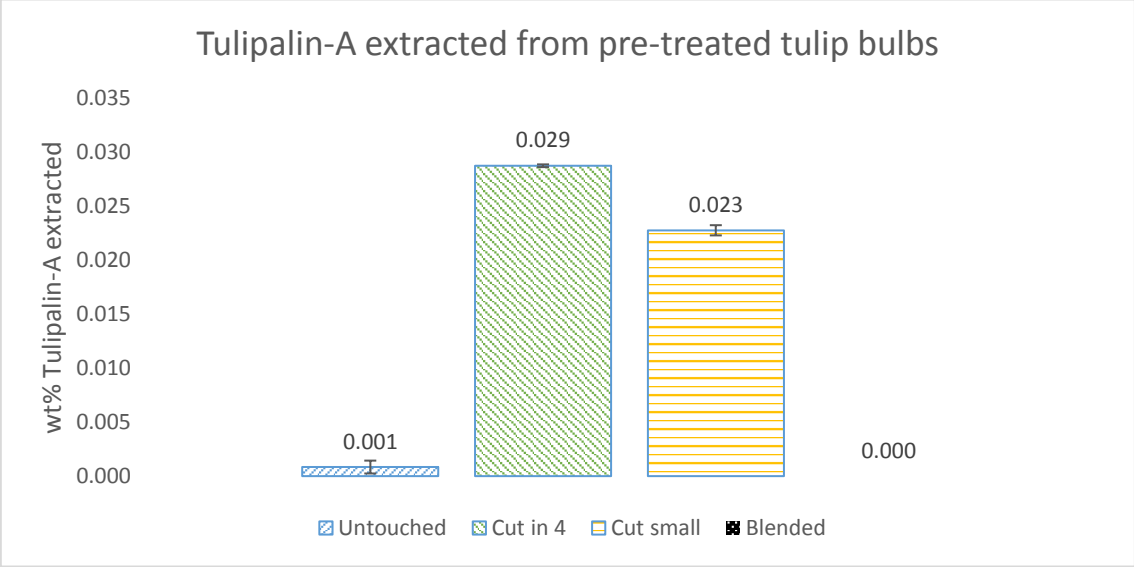


Figure 15; Tulipalin A content of tulip extracts subject to four different pre-treatment methods

As can be seen in Figure 15, the two pre-treatment methods resulting in the highest yield of tulipalin-A are cutting the bulbs in 4 or cutting them into smaller pieces (approx. 1 cm^3). When the bulbs remain untouched or are blended, the product is only present in trace amounts.

The outer white skin of the bulb provides a natural defence against pathogens [34]. Once the bulb is harmed, it is expected that pathogens can access the inner, unprotected, layers of the bulb. Consequently, the production of tulipalin-A is triggered to prevent further contamination. As the untouched bulbs still have this protective layer, it provides an explanation for the limited tulipalin content in the untouched bulbs.

When the bulb is harmed, this protective layer is circumvented and infection of the bulb can occur. Pathogens originating from the diseased bulbs are transferred via air and have the potential to form a biofilm on any nutrient containing surface they encounter [37][38]. When the available surface area increases, the biofilm can potentially grow larger. For the tulip bulbs this would result in an increased tulipalin-A production, thereby providing a relation between an increased surface area and increased tulipalin concentrations.

However, this contradicts with the results found for blended biomass. Although the surface area has increased, the tulipalin-A content has diminished. The enzymatic conversion from tuliposide-A to tulipalin-A occurs intracellularly [39]. Possibly, the cellular structure of the biomass is destroyed to such extend due to blending that the conversion from tuliposide-A to tulipalin-A can no longer take place.

Often, biotechnological processes require a cell disruption step such as blending, as this facilitates further downstream processing and allows for higher yields of intracellular products [40]. However, for tulipalin-A production it is important that the cell structure is still intact before stressing takes place. For further downstream processing, blending could be required.

The difference between "cutting in 4" and "cutting small" is insufficient to draw conclusions and thus the experiments were repeated to assess the influence of both pre-treatment methods, as can be seen in Figure 16.

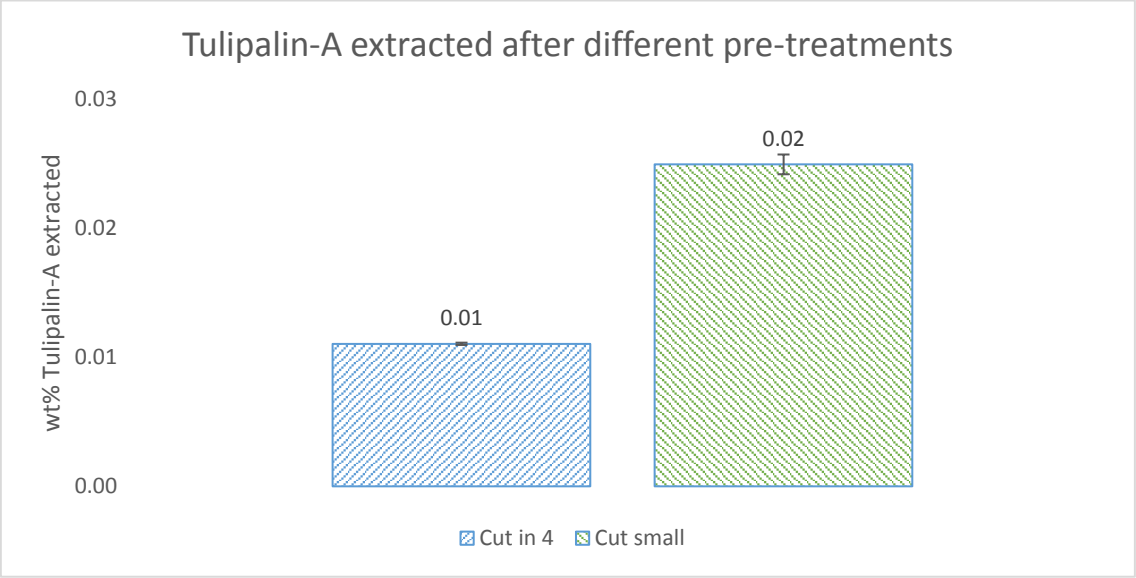


Figure 16; Tulipalin-A content of tulip extracts subject to two different pre-treatment methods

The small cut bulbs give the highest weight percentage of tulipalin-A extracted. Although the weight percentages are relatively low, cutting the bulbs into small pieces yields double the amount of product. Based on literature, it was expected that the small cut bulbs should give a higher wt% of tulipalin-A. As long as the cell structure remains intact, an increased surface area should lead to a higher yield. Therefore, one should aim to increase the surface area as much as possible without applying to much cell-destructive force.

3.5 Extraction strategy

Whereas pre-treatment and stressing aim at increasing the available amount of tulipalin-A, extracting and concentrating aim at a maximum concentration and purity of the component. After stressing, the biomass will contain a mixture of tuliposide-A and B and tulipalin-A and B [11]. To obtain a pure end product it is required to use a selective solvent for the extraction step as depicted in Figure 17. *Kato et al.* have shown that tulipalin-A can best be extracted with the use of aromatic hydrocarbons, whereas polar solvents are more suited for the extraction of tulipalin-B [11]. Consequently, toluene was selected as solvent, which was also recommended by *Kato et al.* [11].

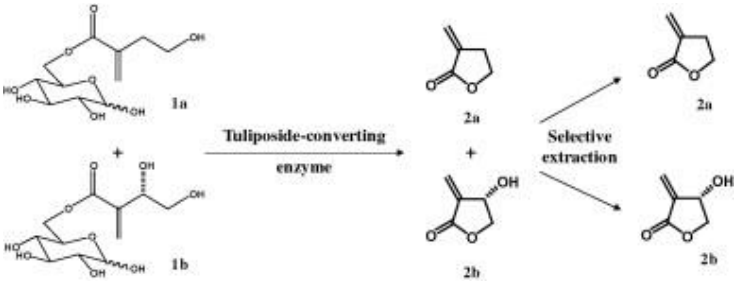


Figure 17; The need for a selective solvent [11]

A comparison with water was made to assess the different extraction capacities, as can be seen in Figure 18.

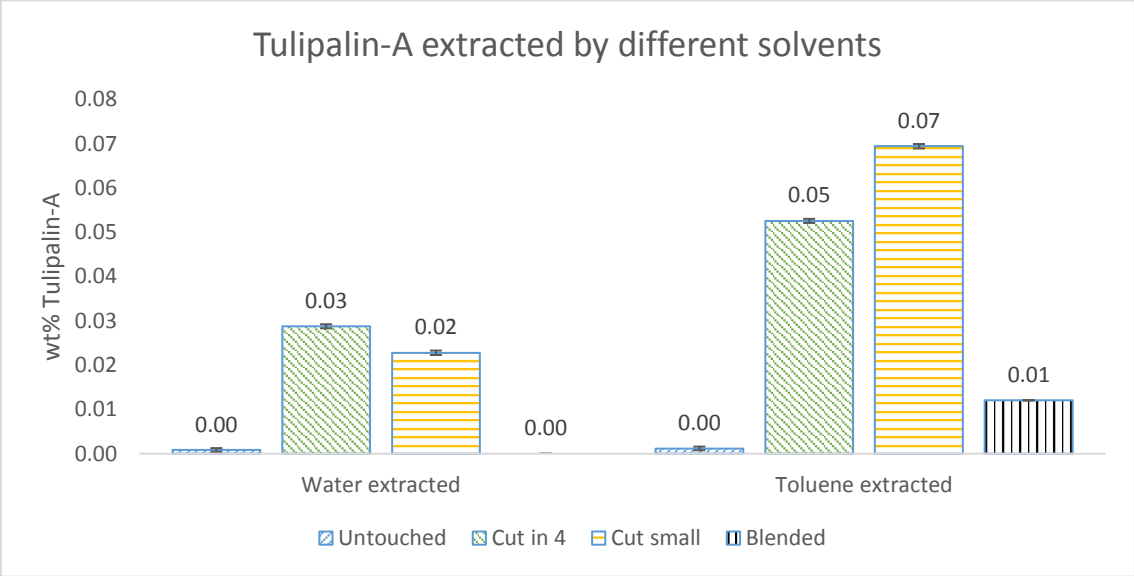


Figure 18; Tulipalin-A content of extract using water and toluene as solvents.

As expected, toluene extracts higher weight percentages of tulipalin-A, yielding approximately 3 times more product than water.

When looking at the solvent-solute interactions, the absence of an additional hydrogen atom in tulipalin-A as opposed to tulipalin-B might lead to a decrease in polarity. The decrease in polarity results in a higher affinity for non-polar solvents. Consequently, tulipalin-A would be better soluble in non-polar solvents such as toluene.

Appendix 4 and Appendix 5 show the retention time for the reference tulipalin-A and tulipalin-B during HPLC analysis. When water was used as solvent, many other molecules were extracted along with tulipalin-A, as can be seen in Appendix 7. However, toluene only showed one other component in the extract at a retention time of 15 minutes (Appendix 6). This would indicate that toluene is a more selective solvent. The nature of this additional component is unknown but the large peak indicates a large affinity for toluene.

The effects of the extraction time and biomass:solvent ratio were subject of research. Stressed bulbs were mixed with toluene at concentrations of 100, 200 and 300 g/L, and aliquots were taken at different time intervals. The increase in tulipalin-A extracted can be seen in Figure 19.

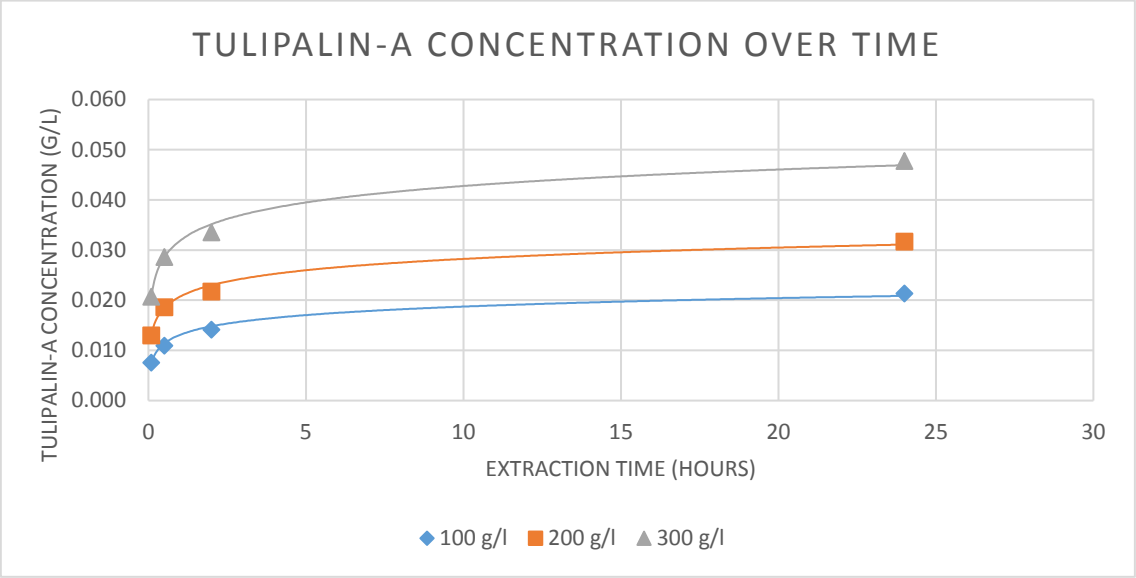


Figure 19; Tulipalin-A concentration at different time intervals for different biomass concentrations. All cut in 4 and toluene extracted.

A concentration of 300 g tulip bulbs per litre of toluene gave the highest concentration in the extract. This was expected as an increase in biomass also increases the amount of tulipalin available. When corrected for the initial amount of biomass the graph looks as follows, see Figure 20.

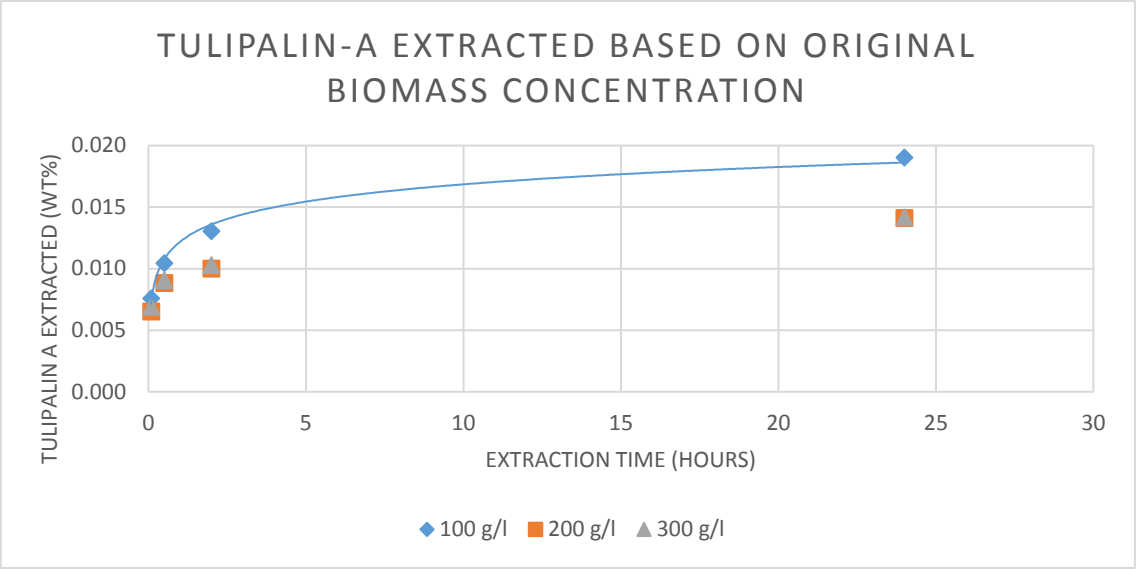


Figure 20; wt% tulipalin extracted based on original amount of bulbous biomass. All cut in 4 and toluene extracted.

A concentration of 100 g/L now gives the highest extraction efficiency. Due to the relative low weight percentages extracted, this is unexpected as it is unlikely that the toluene is saturated with tulipalin-A. Rather, overlapping curves such as those for 200 and 300 g/L were expected. Potentially, the unidentified component detected by HPLC is present in such high concentrations at 200 and 300 g/L that the toluene becomes saturated, resulting in a lower extraction capacity for tulipalin-A.

3.6 Concentrating strategy

Filtration and concentrating steps were performed to obtain a product that is as pure as possible. Vacuum filtration did not lead to any complications and thus biomass and liquid were separated successfully, resulting in a clear, slightly yellowish liquid as depicted in Figure 21.

However, the distillation step did pose some operational challenges. Toluene has a lower boiling point (bp=110 °C) then tulipalin-A (bp=180 °C) but not all toluene could be evaporated off. Consequently, too much liquid remained and concentrating was not done sufficiently. With the use of rotary evaporation most toluene could be evaporated, resulting in a yellowish, viscous product as seen in Figure 21.

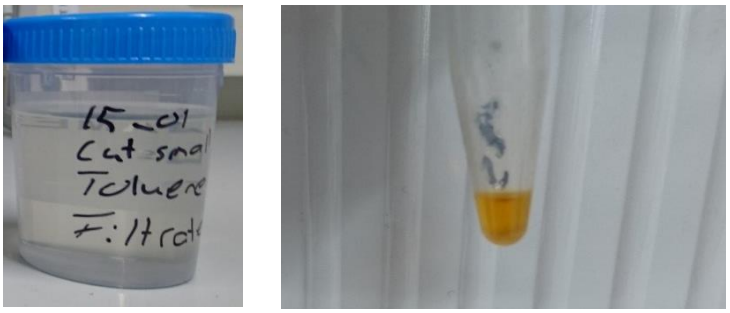


Figure 21; Left: filtrate of extract from pathogen infected tulip biomass. Right: concentrated tulipalin-A after rotary evaporation

HPLC analysis was performed right after extraction, filtration and rotary evaporation. The results are shown in Table 5.

Table 5; Tulipalin-A concentration after downstream processing steps

Treatment	Concentration (g/L)	STDEV
Extraction	0.0216	0.0006
Filtration	0.0225	0.0006
Rotary Evaporation	3.9128	0.2018

Analysis shows that there is no significant difference between the tulipalin-A content in the extract and the filtrate, meaning that the downstream process step does not lead to a decrease in yield. By rotary evaporation the concentration was brought to 3.9 g/l, which is approximately 175 times more concentrated than the filtrate.

Analysis of the concentrate by ^{1}H NMR gave similar results as previous research and thus confirmed the presence of tulipalin-A [14][15]. However, compared to tulipalin, a major fraction toluene was detected as well (Appendix 8). This means that the rotary evaporation step did not get rid of all solvent. Besides, several other components were detected. This indicates that toluene is not a completely selective solvent. The nature of these other peaks could not be determined.

3.7 Tulipalin-A degradation

If the development of tulipalin-A content is analysed over time, as shown in Figure 22, a steady decrease can be observed.

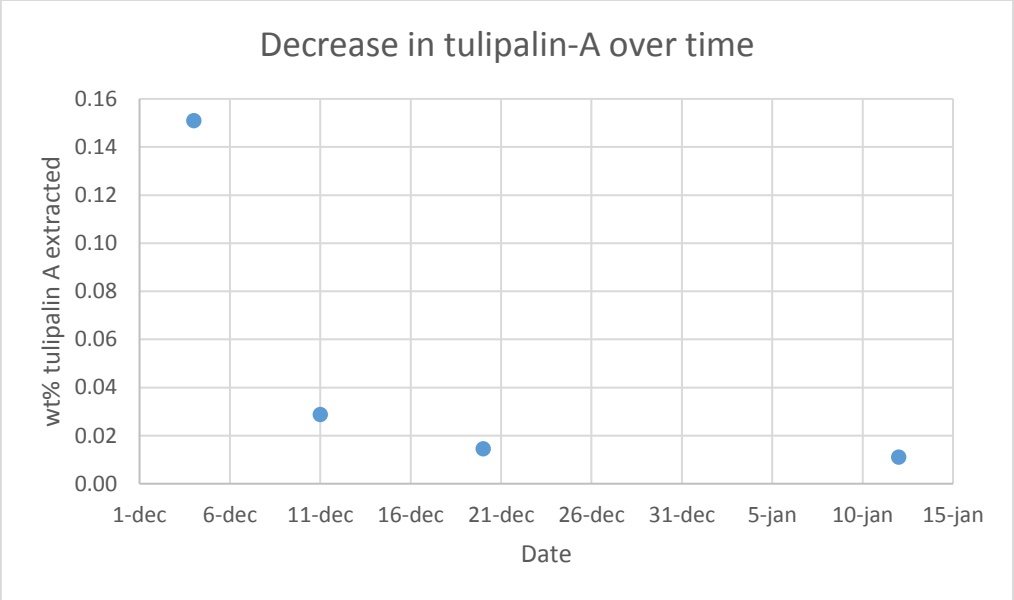


Figure 22; Tulipalin-A decrease over time in tulip biomass. Royal Anthos, pathogen-infected, cut in 4 and water extracted.

Early December the wt% of tulipalin-A extracted from stressed bulbs was approximately 0.15 as compared to 0.01 in early January, which is a decrease of more than 10 times. The bulbs were harvested in late July, meaning that there could already be a 10fold decrease in the period July-December, resulting in an overall 100fold decrease. After rotary evaporation, a concentration of 3.9 g/l was obtained. If the stressing and downstream process were performed right after harvesting, this might result in concentrations of up to 400 g/l.

Potentially, the decrease in tulipalin-A is caused by an inactivation of the enzyme responsible for the conversion from tuliposide to tulipalin. Research on the enzyme activity during storage of onions concluded that some enzymes inactivate over longer storage times [41]. Especially at room temperature inactivation is more common, storage at 5 °C increased enzyme stability for a longer time [41].

Moreover, the stability of tulipalin-A under different treatment conditions has not been a topic of research. Long-term storage might lead to structural changes of the component. Besides, each downstream processing step has the potential to alter the structure or activity of tulipalin-A. Changes in pH, pressure or temperature could, for example, lead to the opening of its ring structure [42].

4 Conclusion and recommendations

A variety of analytical technologies could account for 96 wt% of both class I and II tulip bulbs. Class I tulip bulbs have a higher moisture content than those of class II with weight percentages of 53 and 41 respectively. Although present in different compositions, both classes contain approximately the same amount of carbohydrates (80 dw%). The presence of pathogens leads to a decrease of starch and increase in oligosaccharides content. Proteins, amino acid and organic acids were detected ranging from 1 to 5 dw%. The combined oil/fat fraction makes up to 0.5 dw% and tulipalins were not detected.

A new stressing strategy to induce tulipalin-A production in tulip bulbs was designed and tested, the method proved to be successful. Bringing the healthy biomass in contact with diseased bulbs for a period of two to three days lead to pathogenic cross-contamination and promoted tulipalin-A production. Increasing the surface area of the bulb prior to stressing increased the tulipalin-A content up to 30 times as compared to untouched bulbs, until the point that too much cell disruptive force was applied. Overnight extraction with toluene, solid-liquid separation by vacuum filtration and rotary evaporation lead to a tulipalin-A concentrate of 3.9 g/l. Analysis by ^{1}H NMR showed that indeed tulipalin-A was extracted, but still a considerable fraction toluene along with some other unidentified biomolecules remained. The tulipalin-A content in the bulbous biomass decreased over time, resulting in a lower concentration in the final product.

Based on the research performed, several recommendations can be made to improve the proposed biorefinery system and increase the purity and yield of tulipalin-A:

1. As the stability of tulipalin-A and the enzyme responsible for its production are not well understood, research should be performed to analyse the underlying mechanisms. Knowledge on these topics helps to optimize storage conditions and downstream processing methods.
2. As the tulipalin-A content appeared to decreases over time, the proposed biorefinery system should be performed right after harvest to increase the amount of tulipalin-A extracted.
3. ^{1}H NMR showed a considerable fraction of toluene in the extract, additional drying steps should be tested to analyse if this fraction can be decreased.
4. HPLC and ^{1}H NMR showed the presence of other components in the toluene extract. Consequently, toluene is not a completely selective and different solvents or additional isolation steps should be tested.
5. Bulbs of the *Royal Anthos* cultivar were used for the biorefinery process. However, this cultivar might not have the highest tuliposide and tulipalin concentrations. Thus the same method should be tested for different cultivars.

5 References

[1] J. H. Clark, “Green chemistry for the second generation biorefinery—sustainable chemical manufacturing based on biomass,” *J. Chem. Technol. Biotechnol.*, vol. 82, no. 7, pp. 603–609, 2007.

[2] A. A. De Hertogh, L. H. Aung, and M. Benschop, “The Tulip: Botany, Usage, Growth, and Development,” in *Horticultural Reviews*, John Wiley & Sons, Inc., 1983, pp. 45–125.

[3] Royal Flora Holland, “Jaarverslag 2016,” 2016. [Online]. Available: http://jaarverslag.royalfloraholland.com/?&_ga=2.36131687.659601956.1504615618-1730818215.1504524123#/feiten-en-cijfers/snijbloemen?_k=ldlmsl.

[4] A. R. Rees, “Concepts of dormancy as illustrated by the tulip and other bulbs,” *Ann. Appl. Biol.*, vol. 98, no. 3, pp. 544–548, 1981.

[5] P. F. M. M. Roelofs and H. Gude, “Omvang en aard van reststromen uit de bloembollensector,” 2013.

[6] R. Kamenetsky *et al.*, “Water status and carbohydrate pools in tulip bulbs during dormancy release,” *New Phytol.*, vol. 158, no. 1, pp. 109–118, 2003.

[7] R. Moe and A. L. F. WickstrØm, “The Effect of Storage Temperature on Shoot Growth, Flowering, and Carbohydrate Metabolism in Tulip Bulbs,” *Physiol. Plant.*, vol. 28, no. 1, pp. 81–87, 1973.

[8] I. Majid, A. S. Dhatt, S. Sharma, G. A. Nayik, and V. Nanda, “Effect of sprouting on physicochemical, antioxidant and flavonoid profile of onion varieties,” *Int. J. Food Sci. Technol.*, vol. 51, no. 2, pp. 317–324, 2016.

[9] L. Abhayawick, J. C. Laguerre, V. Tauzin, and A. Duquenoy, “Physical properties of three onion varieties as affected by the moisture content,” *J. Food Eng.*, vol. 55, no. 3, pp. 253–262, 2002.

[10] K. Shigetomi, T. Kishimoto, K. Shoji, and M. Ubukata, “First total synthesis of 6-tuliposide B,” *Tetrahedron: Asymmetry*, vol. 19, no. 12, pp. 1444–1449, 2008.

[11] Y. Kato, H. Yoshida, K. Shoji, Y. Sato, N. Nakajima, and S. Ogita, “A facile method for the preparation of α-methylene-γ-butyrolactones from tulip tissues by enzyme-mediated conversion,” *Tetrahedron Lett.*, vol. 50, no. 33, pp. 4751–4753, 2009.

[12] K. Shigetomi, S. Omoto, Y. Kato, and M. Ubukata, “Asymmetric Total Synthesis of 6-Tuliposide B and Its Biological Activities against Tulip Pathogenic Fungi,” *Biosci. Biotechnol. Biochem.*, vol. 75, no. 4, pp. 718–722, Apr. 2011.

[13] P. K. Datta, C.-S. Kim, T. Hara, E. Itoh, and M. Horiike, “Insecticidal Activity of α-Methylene-γ-butyrolactone against Several Insect Pests,” *Biosci. Biotechnol. Biochem.*, vol. 63, no. 4, pp. 760–761, 1999.

[14] T. Nomura, E. Hayashi, S. Kawakami, S. Ogita, and Y. Kato, “Environmentally benign process for the preparation of antimicrobial α-methylene-β-hydroxy-γ-butyrolactone (tulipalin B) from tulip biomass,” *Biosci. Biotechnol. Biochem.*, vol. 79, no. 1, pp. 25–35, Jan. 2015.

[15] L. P. Christensen and K. Kristiansen, “Isolation and quantification of tuliposides and tulipalins in tulips (Tulipa) by high-performance liquid chromatography,” *Contact Dermatitis*, vol. 40, no. 6, pp. 300–309, 1999.

[16] A. Lubbe, R. Verpoorte, H. Gude, and M. Dijkema, “Tuliposides and tulipalins in tulip gum,” in *Acta Horticulturae*, 2013, no. 1002, pp. 333–338.

[17] AOAC international, *Official Methods of Analysis of AOAC international*, 18th ed. AOAC international, 2005.

[18] M. Mor and Y. Spiegel, “Infection of Narcissus Roots by Aphelenchoides subtenuis,” *J. Nematol.*, vol. 25, no. 3, pp. 476–479, Sep. 1993.
[19] U. Kües, “Fungal enzymes for environmental management,” *Curr. Opin. Biotechnol.*, vol. 33, pp. 268–278, 2015.
[20] T. Manavalan, A. Manavalan, and K. Heese, “Characterization of Lignocellulolytic Enzymes from White-Rot Fungi,” *Curr. Microbiol.*, vol. 70, no. 4, pp. 485–498, 2015.
[21] T. Ohyama, T. Ikarashi, T. Matsubara, and A. Baba, “Behavior of carbohydrates in mother and daughter bulbs of tulips (Tulipa gesneriana),” *Soil Sci. Plant Nutr.*, vol. 34, no. 3, pp. 405–415, Sep. 1988.
[22] M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. t Rebers, and F. Smith, “Colorimetric method for determination of sugars and related substances,” *Anal. Chem.*, vol. 28, no. 3, pp. 350–356, 1956.
[23] S. K. C. Chang and Y. Zhang, “Protein Analysis BT - Food Analysis,” S. S. Nielsen, Ed. Cham: Springer International Publishing, 2017, pp. 315–331.
[24] A. M. Magomya, D. Kubmarawa, J. A. Ndahi, and G. G. Yebpella, “Determination of plant proteins via the kjeldahl method and amino acid analysis: a comparative study,” *Int J Sci Technol Res*, vol. 3, pp. 68–72, 2014.
[25] S. Fujihara, A. Kasuga, and Y. Aoyagi, “Nitrogen-to-Protein Conversion Factors for Common Vegetables in Japan,” *J. Food Sci.*, vol. 66, no. 3, pp. 412–415, 2001.
[26] E. C. Y. Li-Chan, “Bioactive peptides and protein hydrolysates: research trends and challenges for application as nutraceuticals and functional food ingredients,” *Curr. Opin. Food Sci.*, vol. 1, pp. 28–37, 2015.
[27] J. Becker, A. Lange, J. Fabarius, and C. Wittmann, “Top value platform chemicals: bio-based production of organic acids,” *Curr. Opin. Biotechnol.*, vol. 36, pp. 168–175, 2015.
[28] L. P. Christensen, “Tuliposides from Tulipa sylvestris and T. turkestanica,” *Phytochemistry*, vol. 51, no. 8, pp. 969–974, 1999.
[29] G. Suazo Jimenez, “Pathological Studies Of Fusarium Oxysporum (Schlecht.) F. Sp. Tulipae Apt. In Tulip (Tulipa Gesneriana L.) Bulbs After Harvest,” 2012.
[30] G. A. Kamerbeek, A. L. Verlind, and J. A. Schipper, “Gummosis of tulip bulbs caused by ethylene,” in *Acta Horticulturae*, 1971, no. 23, pp. 167–172.
[31] A. G. Marangoni, “Organogels: an alternative edible oil-structuring method,” *J. Am. Oil Chem. Soc.*, vol. 89, no. 5, pp. 749–780, 2012.
[32] R. J. McGovern and W. H. Elmer, “Diseases of Tulip,” *Handb. Florists’ Crop. Dis.*, pp. 1–26, 2017.
[33] N. Tang *et al.*, “Genetic mapping of resistance to Fusarium oxysporum f. sp. tulipae in tulip,” *Mol. Breed.*, vol. 35, no. 5, p. 122, 2015.
[34] B. H. H. Bergman, “Presence of a substance in the white skin of young tulip bulbs which inhibits growth of Fusarium oxysporum,” *Netherlands J. Plant Pathol.*, vol. 72, no. 3, pp. 222–230, 1966.
[35] J. C. M. Beijersbergen and C. B. G. Lemmers, “In vitro inactivation of tulipalin A by Fusarium oxysporumf. sp. tulipae,” *Acta Bot. Need*, vol. 24, p. 250, 1975.
[36] P. V Iyer and L. Ananthanarayan, “Enzyme stability and stabilization—aqueous and non-aqueous environment,” *Process Biochem.*, vol. 43, no. 10, pp. 1019–1032, 2008.
[37] O. O. Alegbeleye, I. Singleton, and A. S. Sant’Ana, “Sources and contamination routes of microbial pathogens to fresh produce during field cultivation: A review,” *Food Microbiol.*, vol. 73, pp. 177–208, 2018.
[38] A. Bridier, P. Sanchez-Vizuete, M. Guilbaud, J.-C. Piard, M. Naïtali, and R. Briandet,

"Biofilm-associated persistence of food-borne pathogens," *Food Microbiol.*, vol. 45, pp. 167–178, 2015.

[39] T. Nomura, "Function and application of a non-ester-hydrolyzing carboxylesterase discovered in tulip," *Biosci. Biotechnol. Biochem.*, vol. 81, no. 1, pp. 81–94, Jan. 2017.

[40] J. F. Buyel, R. M. Twyman, and R. Fischer, "Extraction and downstream processing of plant-derived recombinant proteins," *Biotechnol. Adv.*, vol. 33, no. 6, Part 1, pp. 902–913, 2015.

[41] N. Benkeblia, "Phenylalanine ammonia-lyase, peroxidase, pyruvic acid and total phenolics variations in onion bulbs during long-term storage," *LWT-Food Sci. Technol.*, vol. 33, no. 2, pp. 112–116, 2000.

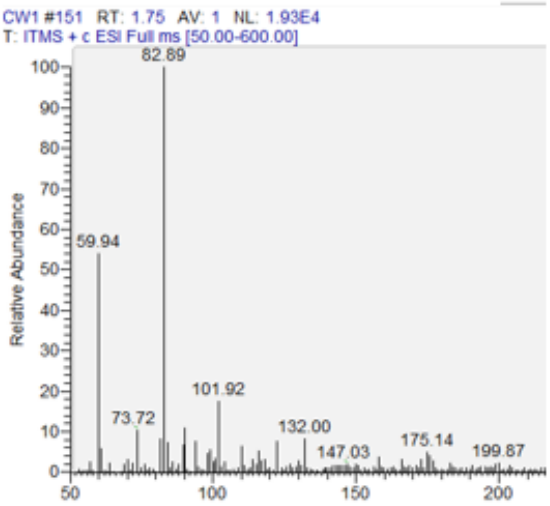
[42] M. Hong and E. Y.-X. Chen, "Coordination ring-opening copolymerization of naturally renewable α-methylene-γ-butyrolactone into unsaturated polyesters," *Macromolecules*, vol. 47, no. 11, pp. 3614–3624, 2014.

[43] T. Ohyama *et al.*, *Physiology and Genetics of Carbon and Nitrogen Metabolism in Tulip*. 2006.

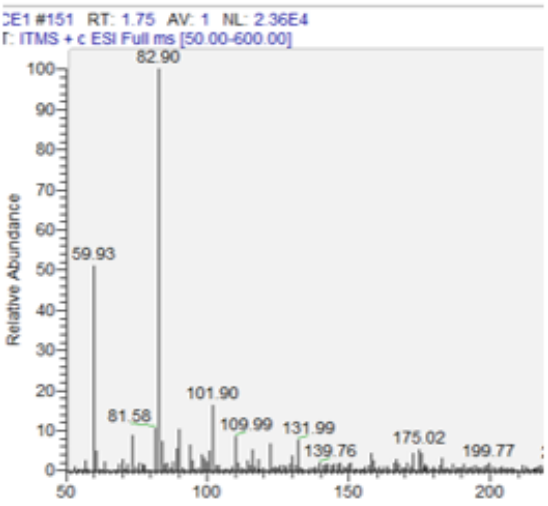
6 Appendices

Appendix 1; Full ms spectra at a retention time of 1.75 minutes for different stress factors and extraction methods of class I tulip biomass.

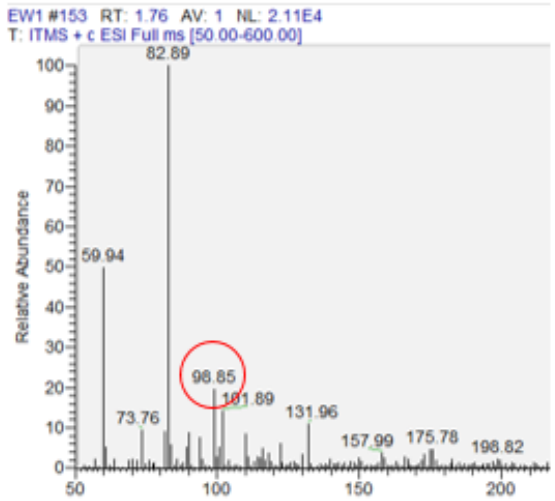
A) Control bulbs, water extracted



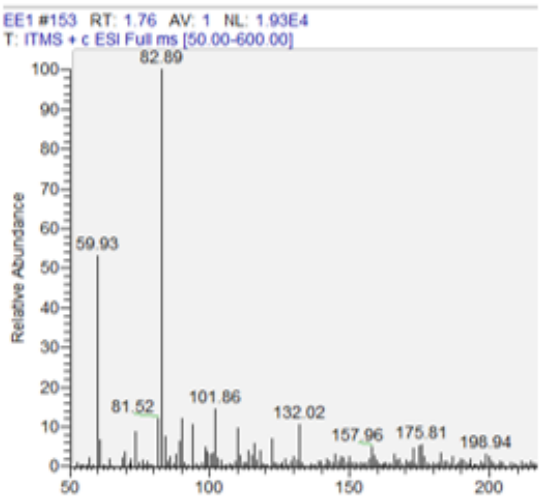
B) Control bulbs, ethanol extracted



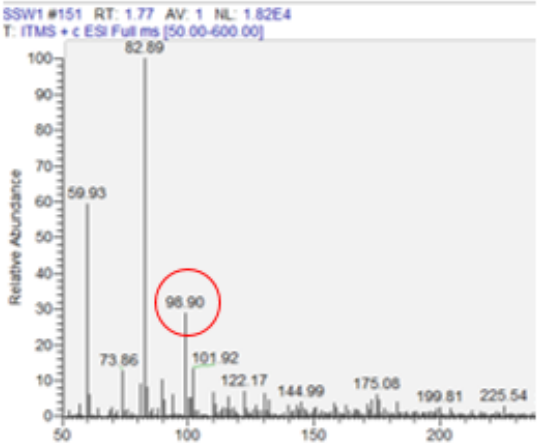
C) Ethylene stressed, water extracted



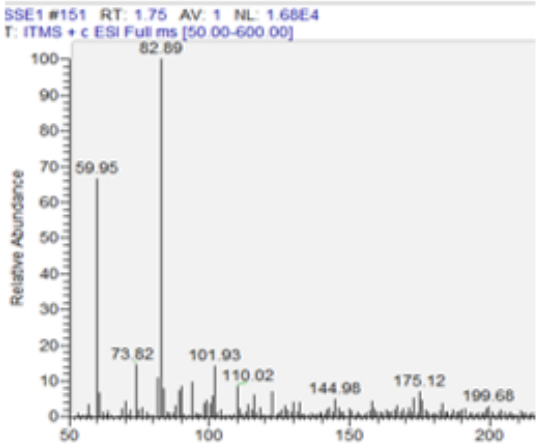
D) Ethylene stressed, ethanol extracted



E) Fungus infected, water extracted

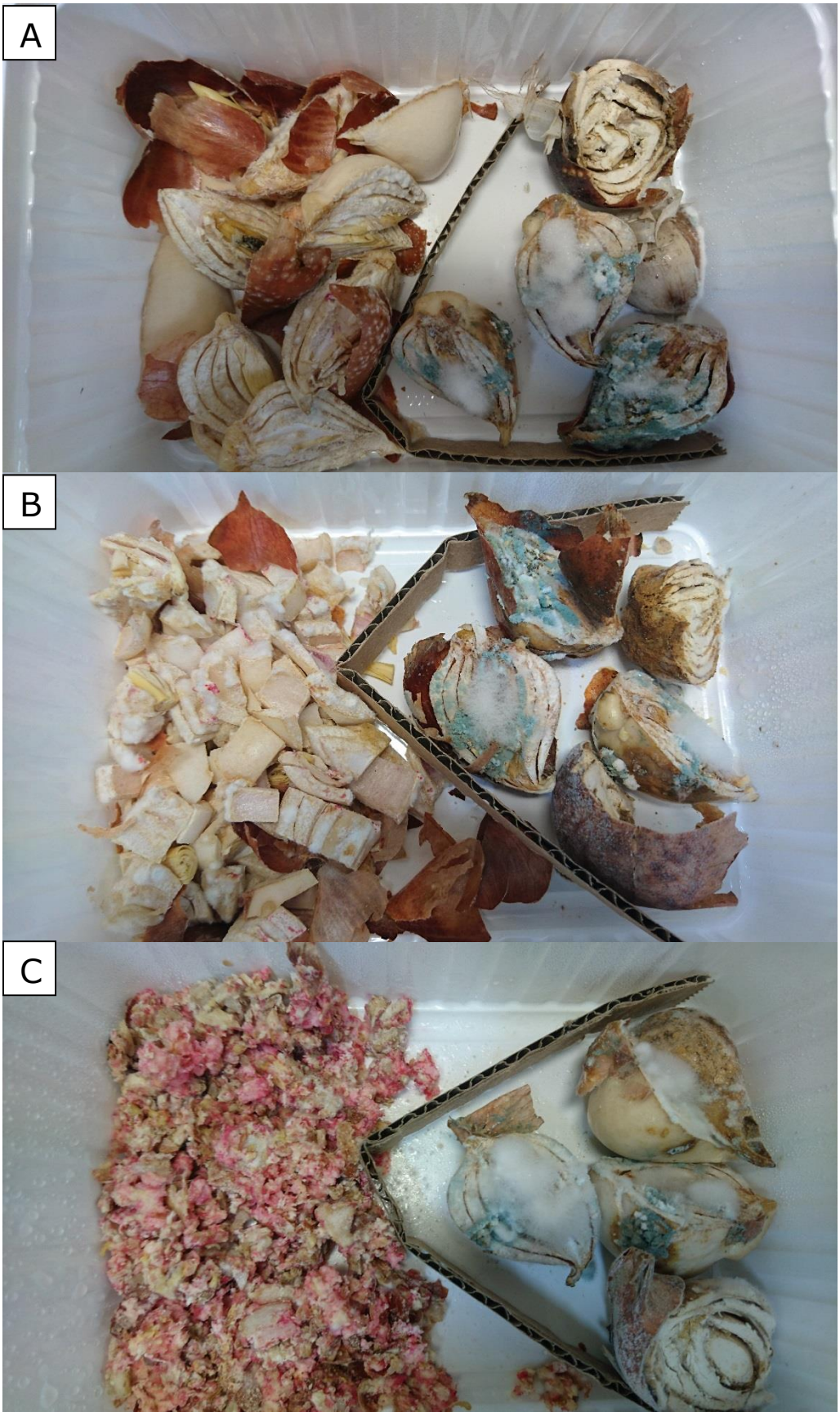


F) Fungus infected, ethanol extracted

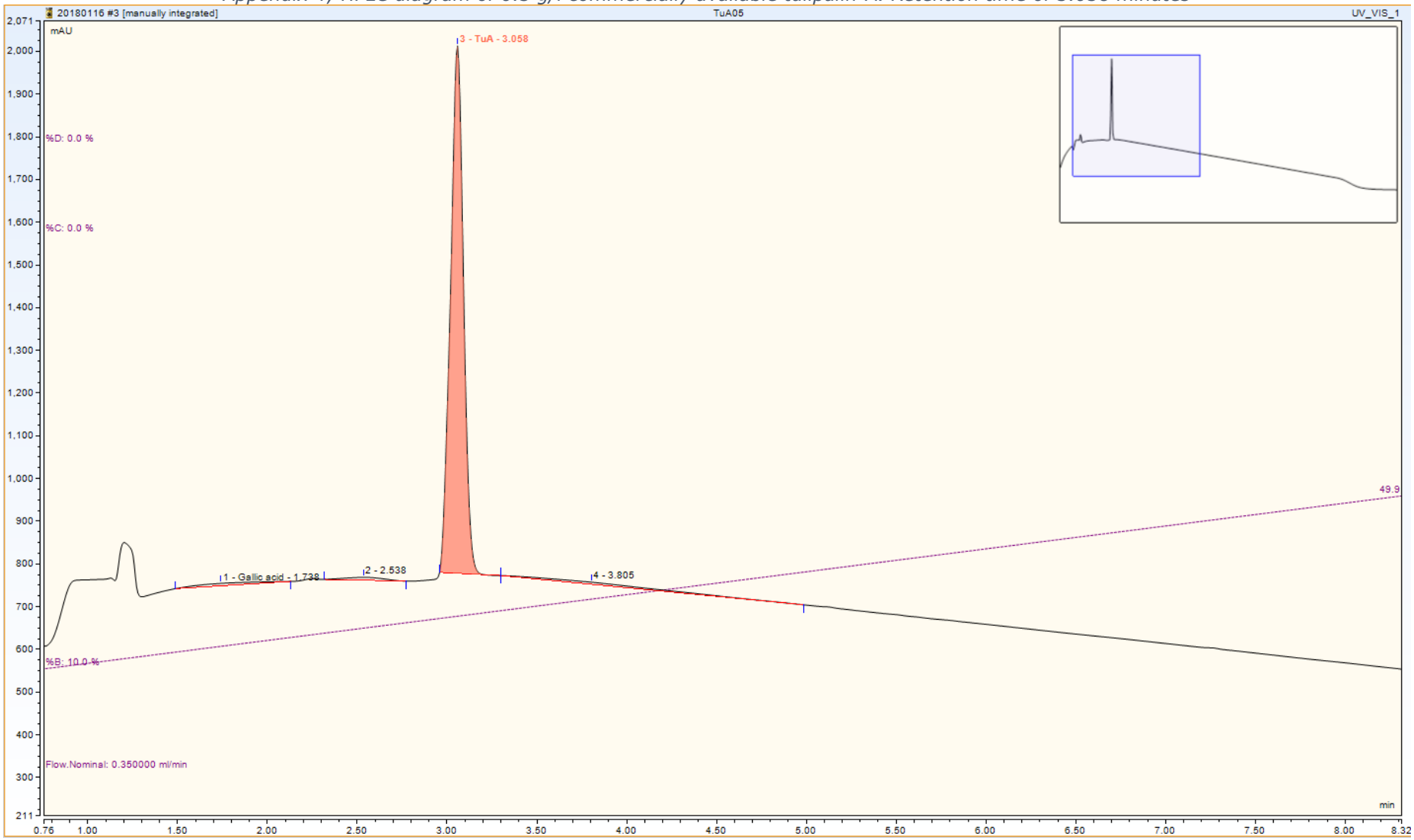


Appendix 2; Air-tight containers for ethylene stressing of both Royal Anthos and Apeldoorn tulip biomass

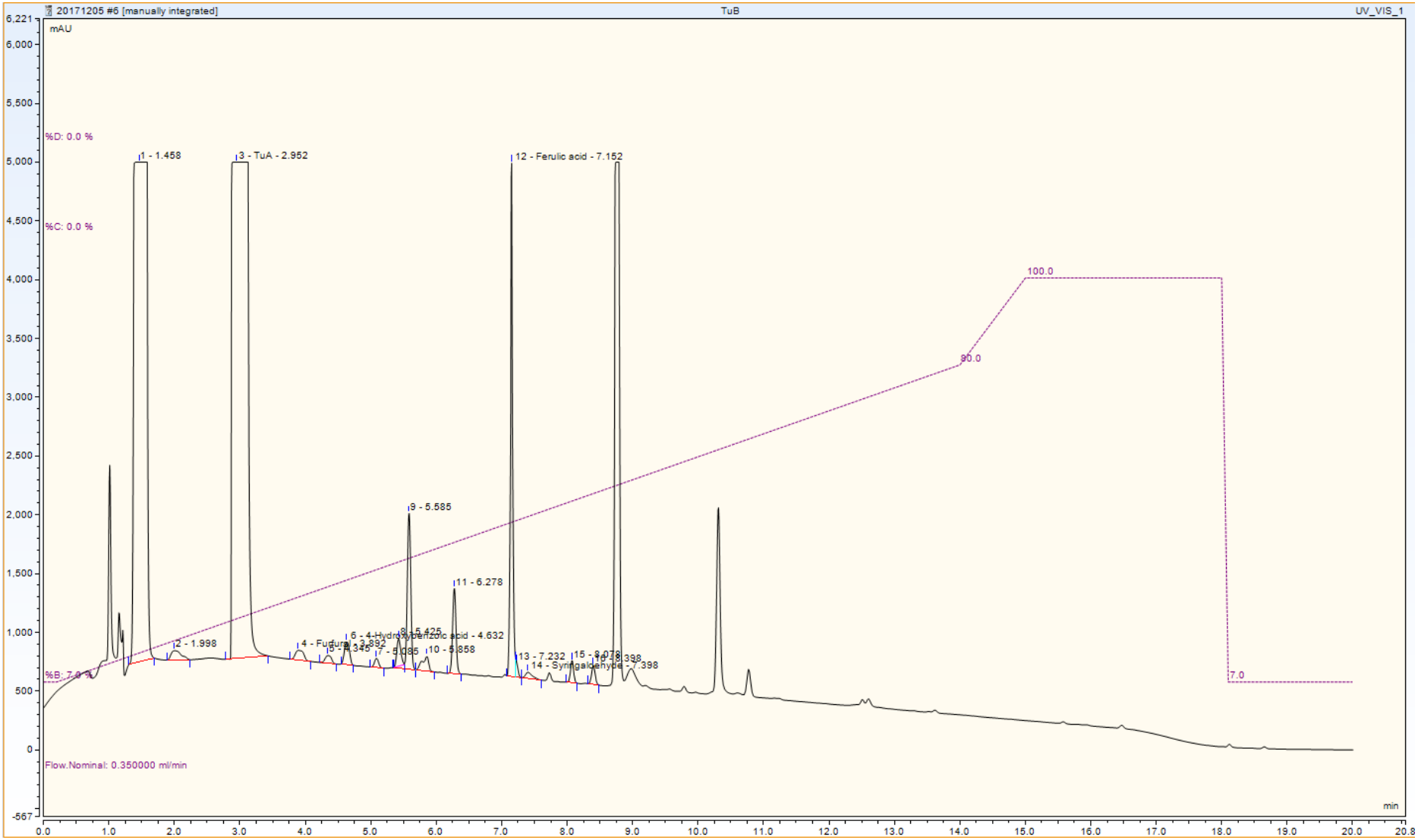
Appendix 3; Pathogen infected tulip biomass after different pre-treatment methods: cut in 4 (A), cut small (B), blended (C). Left: class I. Right: class II.



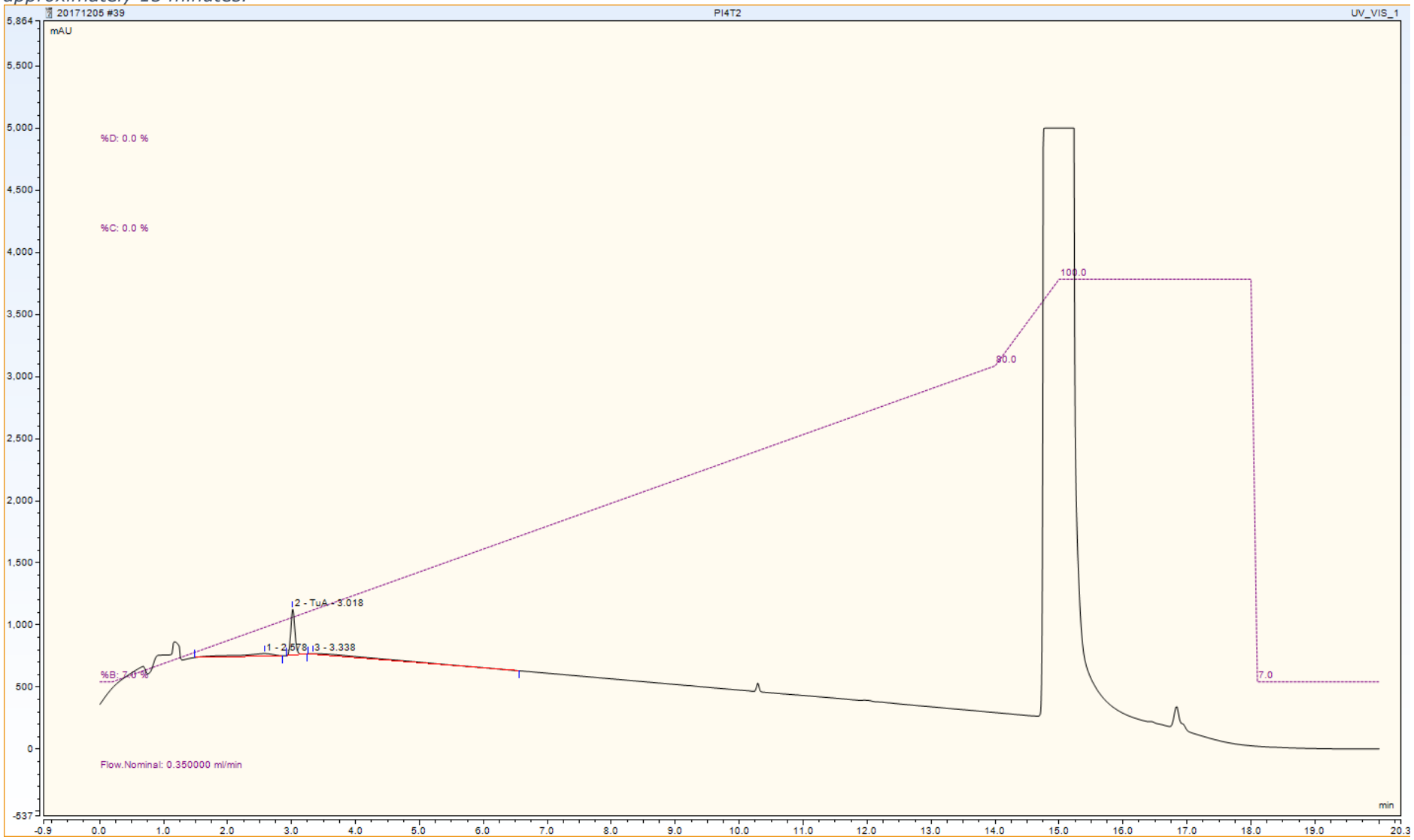
Appendix 4; HPLC diagram of 0.5 g/l commercially available tulipalin-A. Retention time of 3.058 minutes



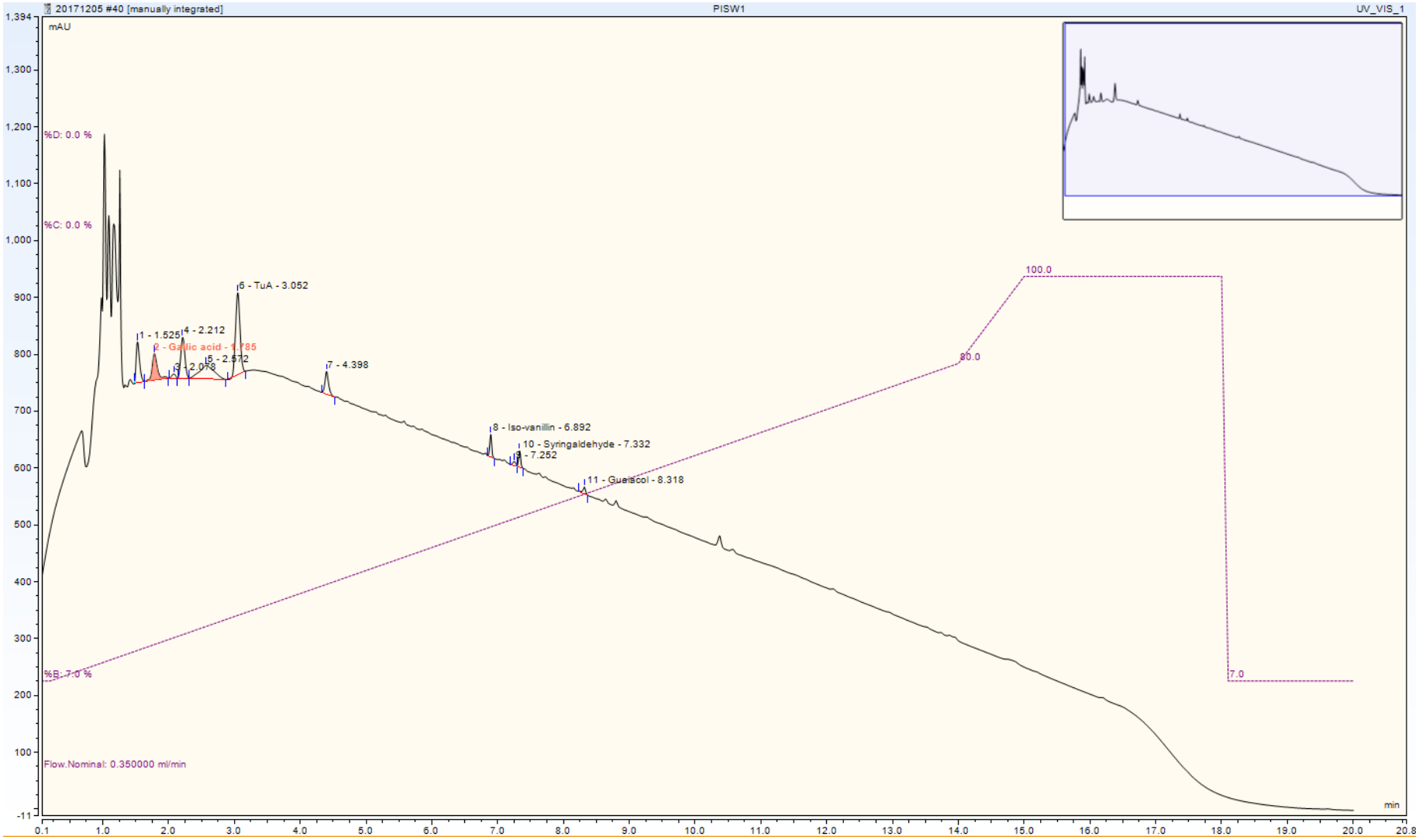
Appendix 5; HPLC diagram of chemically synthesised tulipalin-B from commercially available tulipalin-A. As described in literature, tulipalin-B has a retention time of 1.456



Appendix 6; HPLC profile of bulbous tulip biomass extracted in Toluene. Tulipalin-A has a retention time at 3.018 minutes. Unidentified peak at approximately 15 minutes.



Appendix 7; HPLC profile of bulbous tulip biomass extracted in water. Tulipalin-A has a retention time at 3.052 minutes. Several unidentified peaks at different retention times.



Appendix 8; Top: 1H NMR (CDCl3) spectrum of pure tulipalin-A. Bottom: 1H NMR (CDCl3) spectrum of extract, tulipalin-A indicated by squared areas. Peaks of toluene and other components detected as well.