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Biomarkers as indicator for Erwinia infection on potato tuber

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Summary

The Dutch Ministry of Agriculture, Nature and Food Quality has financially supported the research programme named "Total use of unrefined and unprocessed food(crops)". The programme consisted of a number of projects and one of the projects was entitled "Control of Restrictive Supply Chains; biomarkers as indicator for shelf life" (KB 33 002 011). The aim of the project is to build up knowledge needed to optimize and re-design restrictive supply chains of fruit, vegetables and flowers to avoid postharvest losses, maintain high resource use efficiency and connect consumer demands to production and supply chain restrictions. The objective of this project is to measure the production of volatiles in healthy and rotten (infected) potatoes and as such identify volatiles that may be used as a biomarker for rot development. A key aspect of the envisaged experimental design is to measure the development of these volatiles over time. This will give insight in the potential of this idea as an early detection method to avoid the further expansion of a rot infection during storage of potatoes.

An experimental set up was built to allow the production of volatiles and respective sampling. The potatoes were placed in a glass jar and an air flow was applied. The volatiles were sampled and measured in the air flow. Three treatments were applied: potatoes wounded and infected with the bacteria *Pectobacterium polaris*, wounded potatoes (without infection) and healthy potatoes. Each treatment was applied in duplicate. The six glass jars with the potatoes were kept at room temperature for maximal 17 days and the production of volatiles was daily monitored. The volatiles in the out coming air were measured with a thermo-desorption GC, a PTR-ToF-MS and a GC-MS (via a SPME). In addition, the amount of oxygen consumed and carbon dioxide produced was also measured in the air flow with a CompactGC. The amount of infection was visually quantified (through the glass jar).

The following has been concluded:

- Clear differences in volatile production between infected and non-infected potatoes were found.
- Those differences are measurable very early upon the infection. Even when the infection is not yet visible. This indicates that the production of volatiles is a suitable biomarker for bacterial infection in potatoes.
- Next to the volatile production, the effect of bacterial infection on the production of CO_2 and the consumption of O_2 (respiration rate) was studied. The respiration rate of infected potatoes is much higher than that of healthy or wounded potatoes. The tubers react prompt to the infection by increasing the production of CO_2 and consumption of O_2. Also this change in metabolism may be used as biomarker.
- Several methods have been explored for the measurement of the volatile production. The measurements were done with a Thermo-Desorption GC, a PTR-ToF-MS and a GC-MS. Despite the differences in the type of gas analysis the results of the different methods show a good agreement with each other and seem to be suitable for measurement of produced volatiles.
- Another important result of the project is the development of a suitable inoculation protocol for the bacteria *Pectobacterium polaris* (an *Erwinia* infection has been chosen for this project because this is an aggressive type of microorganism and commonly found in potatoes). The developed protocol makes it possible to successfully infect the tubers in a controlled manner and create a suitable model system. The results are directly relevant for the industry as this kind of micro-organism is responsible for a large amount of infections during storage hence contributing for product waste.

The volatiles measured with the Thermo-Desorption GC and the PTR-ToF-MS have not yet been identified. The GC-MS results do not allow to identify all the volatile compounds as the column and the sampling methods differ too much from the ones used with the GC with desorption unit. Only 10 of the 45 volatiles were identified with their chemical names via GC-MS analysis. At this time, not all compounds have been identified with certainty.

1 Introduction

1.1 Total use of resources programme

The Dutch Ministry of Agriculture, Nature and Food Quality has financially supported the research programme named "Total use of unrefined and unprocessed food (crops)". The programme consisted of a number of projects and one of the projects was named "Control of Restrictive Supply Chains; biomarkers as indicator for shelf life" (KB 33 002 011). The aim of the project is to build up fundamental knowledge needed to optimize and re-design restrictive supply chains of fruit, vegetables and flowers to avoid postharvest losses, maintain high resource use efficiency and connect consumer demands to production and supply chain restrictions. This document reports the research work carried out independently within this project.

1.2 Background

Fruits and vegetables undergo many metabolic changes after harvest. These processes affect the storability and shelf life of all fruit and vegetable products. Knowledge of these processes can both be used to improve the quality of fruit and vegetables (for instance by managing the ripening process) but also to detect and control quality issues as chilling injury, the development of rot and other defects.

In several of the referred metabolic changes volatiles are produced. These volatiles might be used as biomarkers for ripening but also for the development of undesirable quality features as rot. These are complex processes. Moreover they are also crop and chain conditions dependent, therefore the project needs to focus on specific crops and quality issues.

Potatoes are harvested after the summer and kept in large storage facilities for many months. One of the relevant issues in the storage of potatoes is the development of rot. Storage conditions leading to temperature abuse and high relative humidity play an important role in the development of rot. One or two infected tubers are enough to trigger a spreading infection. Often such infections start on the bottom of the potatoes bulk and are thus not visible leading to extensive product losses. Hence the identification of volatile compound(s) that could indicate if an infection has started would be valuable.

1.3 Objective and research questions

Potatoes produce a large amount of volatiles. Which volatiles depend on several factors as the cultivar, maturity stage, growing conditions, etc. Pathological attacks from for example fungi, bacteria, insects, nematodes, viruses and viroid's lead to large changes in the volatile production. These volatiles reflect the defence mechanism that the tubers put in place to deal with the infection. Volatiles can also be directly produced by the pathogen itself.

The objective of this project is to measure the production of volatiles in healthy and infected potatoes and as such identify volatiles that may be used as a biomarker for rot development. A key aspect of the envisaged experimental design is to measure the development of these volatiles over time. This will give insight in the potential of this idea as an early detection method to avoid the further expansion of a rot infection during storage of potatoes.

Rot in potatoes can be induced by different micro-organisms[1] among other *Erwinia (Pectobacterium), Pseudomonas or Clostridium.*

[1] Meeting with Jan van der Wolf, Ernst Woltering and Suzan Gabriëls (19-06-2018)

According to the industry, *Erwinia (Pectobacterium)* is often the cause of soft rot infections during long storage of potatoes. The research is therefore focussed on this microorganism. An infection with *Erwinia* does not necessary lead to the production of off-odours, however several volatiles are induced upon an infection.

The following hypothesis were formulated and tested in the experimental work carried out within the project:
Hypothesis 1: Infected potatoes produce a higher concentration of (specific) volatiles
Hypothesis 2: The ratio between produced volatiles is different between healthy and infected potatoes
Hypothesis 3: The production rate of CO_2 of infected potatoes is higher than by healthy potatoes.

2 Material and methods

2.1 Pre-test

Pre-experiments were carried out prior starting the inoculation of the potatoes. In these pre-experiments, focus on the respiration rate methodology and infection protocol was applied.
The following actions were taken:

- Determinate the respiration rate of healthy and wounded potatoes in order to select the optimal synthetic medical air flow. It was decided that using a flow of 10mL/min of synthetic medical air, our Air-GC (equipment) was able to determine the oxygen consumption and the carbon dioxide production rates of healthy and wounded potatoes.
- A cleaning protocol for the glassware was set-up in order to be sure that no volatile compounds pollute the experimental setup.
- The different channels connected to the thermo-desorption unit and to the Air-GC instrument were tested (leakage, clean, ...)
- Inoculation of the potato with *Erwinia* bacteria was practiced two weeks in advance. The amount of wounding per potatoes, as well as the amount of potatoes per glass jar was tested and optimised. During this pre-test, criteria for the visual quality evaluation were identified and a score scale was proposed.

The methodology and the results of these pre-tests are not presented in the present document.

2.2 Material and inoculation method

Potatoes (cv. Innovator) were harvested in September 2018, and stored as consumption potatoes (1 month at 15°C followed of 1 month at 5.5°C) before the start of the experiment. Potatoes were first wash and kept at room temperature for 4 days in order to acclimatize to the new storage temperature. Before treating the potatoes, special attention was taken to select only potatoes without bruising or infections. Groups of 6 potatoes with approximately the same weight (around 700g) were made. All potatoes were wetted prior to putting then in the glass jar to create a moisty environment.

The following treatments were applied:

- Healthy potatoes were first immersed in clean demi water (Figure 1-a) and put in the glass jars (Figure 1-d).
- Wounded potatoes were first immersed in demi water and then punched on two locations per potato with the tip (2cm) of a 2 mL pipet (Figure 1-b). In order to detect any volatile compounds that may be released by agar medium itself, each potato was inoculated with sterile agar medium (medium used for the growth of *Erwinia* bacteria).
- Infected potatoes: first the potatoes were immersed in demi water, punched at two locations per potato (similar method used for wounded treatment) and infected with *Erwinia* bacteria Figure 1-c). *Erwinia* bacteria were directly collected on agar medium with sterile puncture equipment.

For each treatment, two glass jars with 6 potatoes per glass jar were prepared and connected to the air flow system. (see paragraph 2.3.2).

Erwinia bacteria (*Pectrobacterium polaris* IPO1948) was provided by Wageningen Plant Research (Jan van der Wolf). Bacteria were recovered from a stock library and grown on agar plate (nutrient agar, Oxoid) for two days at 27°C. Extra agar plate, non-inoculated, were also provided in order to investigate any volatile compounds that be released from the agar during the experiment.

Figure 1: Preparation and inoculation of potatoes: a) potatoes are wetted in demi-water; b) potatoes are punched on two locations; c) potatoes are inoculated with sterile agar medium or Erwinia bacteria (Pectobacterium polaris IPO1948); d) potatoes are carefully transferred in glass jar.

2.3 Experimental set up

In order to avoid any air cross-contaminations between the samples, a special experimental set-up was built up. In an effort to avoid temperature fluctuation during the measurement, the complete set up was placed in the same room where the Thermo-desorption GC and Air-GC were located (Figure 2). Where possible, glass jars and GC's were continuously connected to each other in order to automatize the measurement.

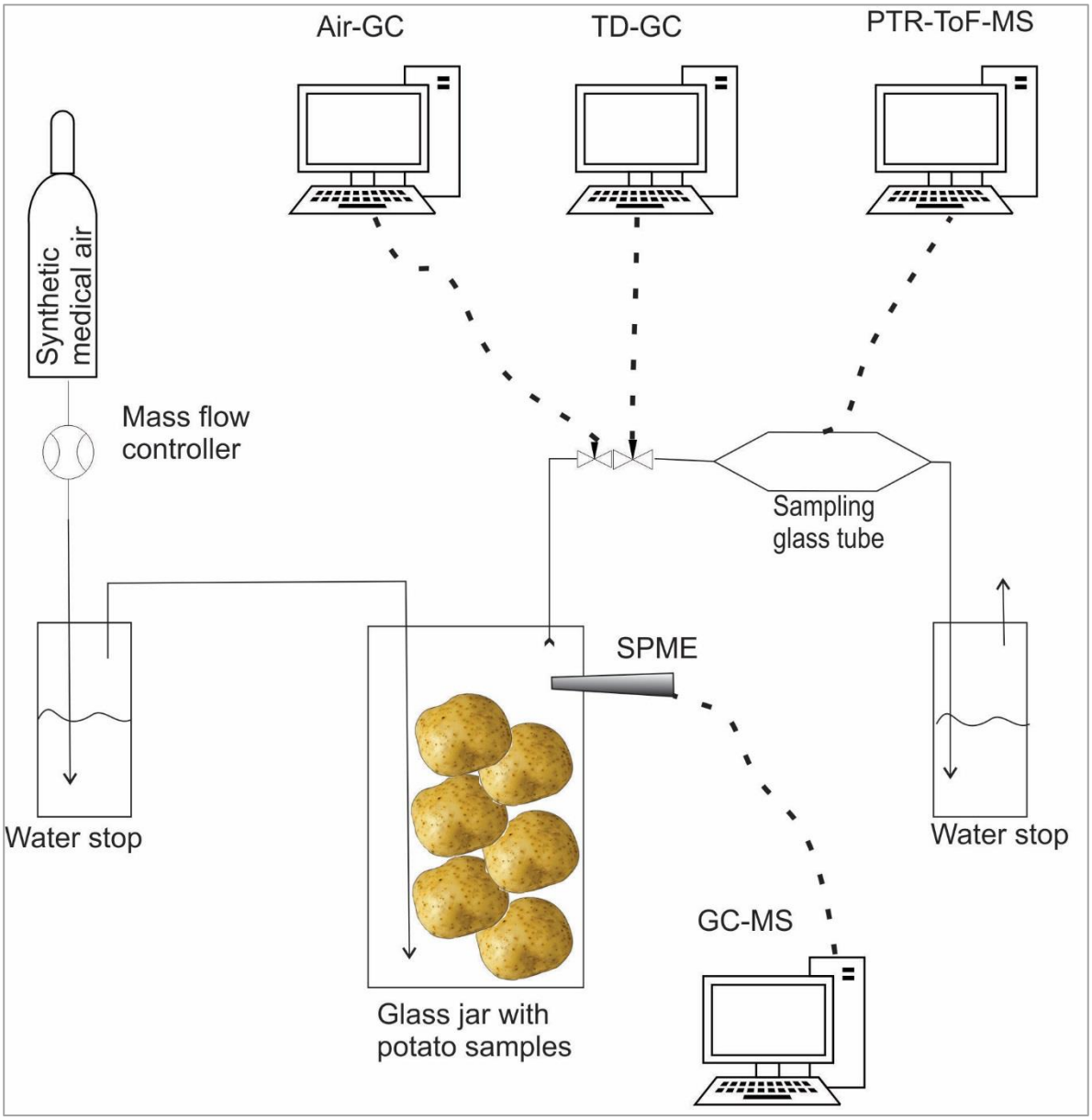

Figure 2: Schema of experimental set-up.

2.3.1 Cleaning of set up

The glass jars were cleaned to assure that no volatile compounds were present when starting the experiment.
The glass jars, rubber rings and lids were cleaned as following:

- Remove green pipe, rubber and septum.
- Flush out 3 times with hot water (Temperature > 50°C).
- Add 20g Alconox® (10 mg/mL = 20 gram per glass jar) with hot water and scrub intensively with brush.
- Rinse at least 3 times with hot water.
- Rinse three times with demi-water.
- After drying the glass jar, the septum and inlet/outlet valves were flushed with nitrogen to remove any remaining water.
- (New) septum was placed; glass jars were closed and flushed with medicinal air until use.

2.3.2 Setup flow-system

In order to assure a continuous air-flow through the potato samples, an extra pipe was installed inside the glass jar to bring the inlet flow at the bottom position. The outlet is located on the top of the glass jar. Septum for sampling headspace gas were located next the outlet (Sampling with SPME) or in the outlet pipe (Thermo-desorption GC, and air GC).
Prior setting the potatoes into the glass jars, these ones were cleaned as described in paragraph 2.3.1, and connected to a high flow (>100mL/min) of synthetic medical air via inlet in order to further clean these glass jars. Potatoes were then with care set into the glass jar and the glass jar was closed with the top lid. After 1 minute, flow of humidified synthetic medical air was reduced to 10 mL/min. One glass jar was used as blank (no potato).
To avoid any flush back of the gas sample with air from the laboratory and to assure a high relative humidity inside the glass jars, water stops were installed at the inlet and outlet of each glass jar. The water stop consisted of bubbling the synthetic medical air through a bottle of demi water. Air flow was continuously supplied via Mass Flow controller (5850 TR series, BrooksInstrument, Hatfield, PA) and checked every few days with the ADM-flow meter (Agilent, Santa Clara, CA). Figure 3 gives an overall impression of the set-up during the experiment.

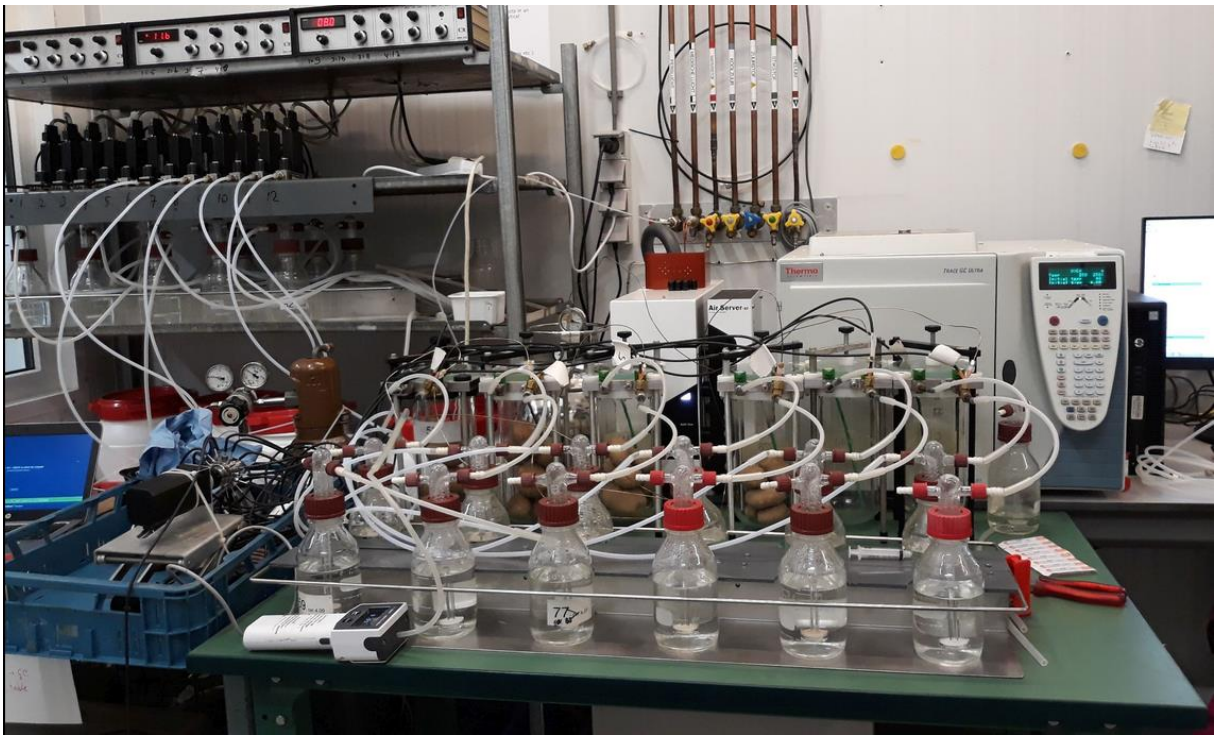

Figure 3: Overview picture of the setup of the flow-system, air-GC (left) and thermo-desorption GC (right); mass flow controller (on the left back).

2.3.3 SPME and PTR-ToF-MS sampling

The sampling for GC-MS and PTR-ToF-MS were done on day 1, 4, 8 and 16. The SPME's were carefully inserted via the septum into the glass jar (Figure 4). The sampling period consisted of 17 hours at room temperature to allow the absorption of the volatile compounds by the SPME's material.
For the PTR-ToF-MS sampling, 800 mL glass tubes were placed at the outlet of the glass jar. Air flow was interrupted for a short time (< 1 min.) when connecting and removing the glass tubes from the tubing between the glass jar outlet and the bottle of water (water stop). The PTR-ToF- MX sampling tubes were connected to the jar outlet overnight (17 hours).

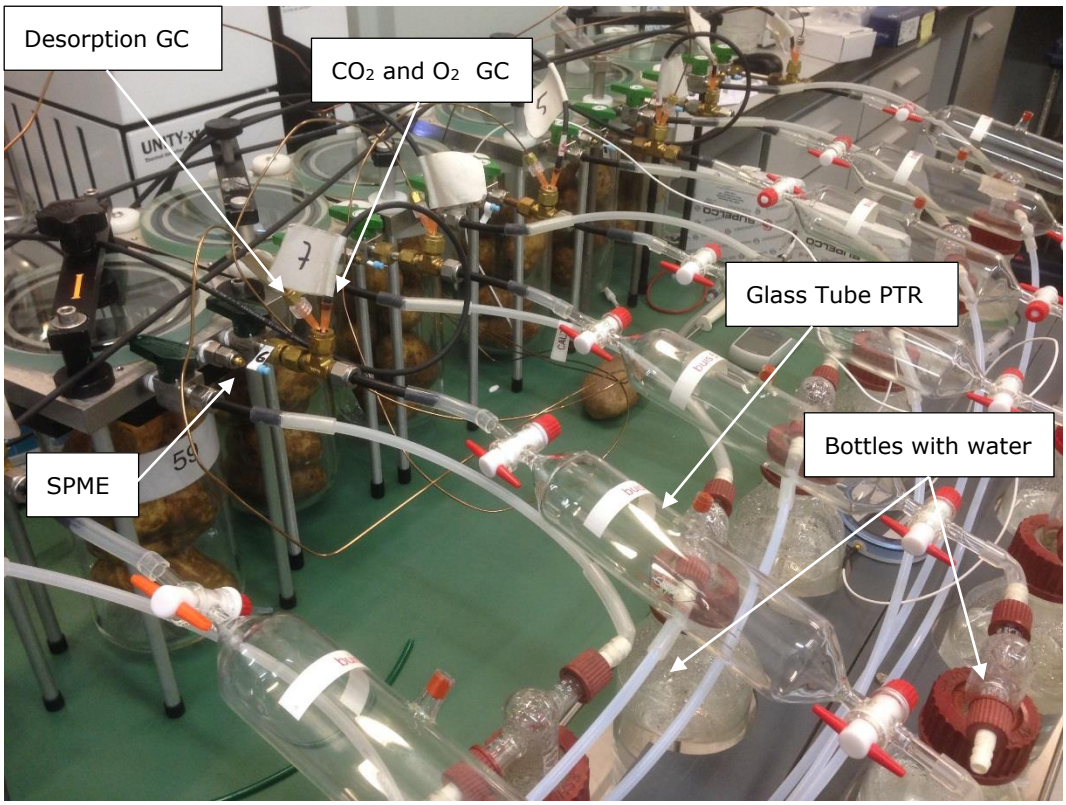

Figure 4: Experimental setup with location of the different samplings.

2.4 Thermo-desorption GC with FID detector

Glass jar headspace were several times per day sampled in order to investigate the volatile compound production rate in function of the treatment applied to the potatoes.
The gas headspace was analysed with a Trace GC Ultra (Interscience, Breda, NL) coupled with a FID detector (temperature: 250°C; cycle time: 20 min.). The Trace GC Ultra was equipped with a column type Restek Rtx-5Ms (30m x 0.32mm, 1µm Restek coating) and a thermal desorber Unity Xr (Markes international, Llantrisant, UK) with an Air Toxics/TO-14 cold trap tube. The oven temperature profile was programmed as follow: initial temperature 40°C, holding time: 4 min.; final temperature: 250°C; ramp: 15°C/min.; Hold time: 2 min. The injection was done at a temperature of 230°C. The settings of the thermal desorber are summarized in Table 1.
GC and thermal desorber unit were controlled by Chromeleon Software (version 7.2.8) from ThermoScientific.

Table 1: Thermal desorption settings

Parameter	Settings
On Line sampling	
Line Flush time	2 min.
Line Flush flow	20 ml/min.
Sample time	10 min.
Sample flow	10 ml/min.
Trap desorb	
Trap low	0°C
Trap heating rate	Max
Trap high	300°C
Trap hold	5 min.
Split on	5 ml/min.
Flow path temp	180°C

The most promising volatile compounds were selected on the chromatograph and the area under the peak were calculated.
As these components were not chemically identified and no calibration per volatile compounds were applied, the area under the chromatograph peak was used as final results. The volatile compound production rate was calculated according to the Equation 1 and expressed in (mV.min) kg^{-1} h^{-1}.

Equation 1: Volatile compound production rate

$$R_{Vol\ comp_x} = \frac{\left(F_{cvt} \times y^{cvt}_{Vol\ comp_x} - F_{med\ air} \times y^{med\ air}_{Vol\ comp_x}\right)}{M \times V_{sampling}}$$

Where F_{cvt} and $F_{med\ air}$ are the flow in L/hour of synthetic medical air flushed continuously through the glass jar with potato samples and the empty glass jar respectively. M is the potato weight in kg. $V_{sampling}$ is the volume in litre of glass jar air headspace sampled by the unity instrument. $y^{in}_{Vol\ comp_x}$ is the area under the chromatograph peak (in mV.min) for specific volatile compound. Superscript $^{med\ air}$ indicated that sampling was made on empty glass jar flushed with synthetic medical air, superscript ^{cvt} indicates that sampling was made on glass jar with potatoes.

2.5 (Air) GC with TCD

Oxygen and carbon dioxide content of glass jar headspace was analysed by an Interscience CompactGC system (Interscience, Breda, NL) equipped with a RT-QBond column (0.32mm diameter, 10m) for detecting CO2 and a MolSieve 5A (0.32 mm diameter, 5m) coupled with a back pressure column type RT-QBond (0.32mm diameter, 3m) for the detection of oxygen. Both columns lead into Thermal Conductivity Detectors (TCD) with 64x input gain. CGCeditor software (v1.5.5, 2008) was used to control the setting of the CompactGC. GC was continuously connected to the samples via tubing connected to a VICI valve (model EMTMA-CE). Valve and CompactGC were coordinated by EZChrom Elite software (v3.32 SP2).
Before starting experiment, oxygen and carbon dioxide were calibrated using two calibration gas mixes supplied by LindeGas. These calibration gases were regularly measured in order to check the stability of the system.
Sampling of the gas was done at the outlet of each glass jar. The gas content measured in the empty glass jar was used as inlet value for the respiration rate calculation.
The samples were continuously flushed with humidified synthetic medical air gas. The flow was regulated using mass flow controller (5850 TR series, BrooksInstrument, Hatfield, PA) and calibrated using the ADM-flow meter (Agilent, Santa Clara, CA).

The oxygen consumption rate and the carbon dioxide production rate were calculated according to Equation 2 and Equation 3 (Fonseca et al., 2002).

Equation 2: Oxygen consumption rate

$$R_{O_2} = \frac{\left(y^{in}_{O_2} - y^{out}_{O_2}\right) \times F}{100 * M}$$

Equation 3: Carbon dioxide production rate

$$R_{CO_2} = \frac{\left(y^{out}_{CO_2} - y^{in}_{CO_2}\right) \times F}{100 * M}$$

Where:

Symbol	Description
R	Respiration rate (mL $kg^{-1}h^{-1}$)
Y	Volumetric concentration (%v/v)
F	Flow rate (mL h^{-1})
M	Mass (kg)
In	Inlet
Out	Outlet

2.6 GC-MS

Gas chromatograph coupled with a mass spectrometer (GC-MS) was used to identify the most important volatile components produced by the healthy, wounded and infected potatoes. During the pre-test experiment, blue SPME (Polydimethylsiloxane/Divinylbenzene (PDMS/DVB) 65µm) and grey SPME (StableFlex Divinylbenzene/Carboxen/PDMS (DVB/CAR/PDMS) 50/30µm) were compared in order to determine which one was able to absorb volatile compounds in the same region of interest of the ones that were analysed with the thermo-desorption GC. The grey SPME was selected for the test. SPME were introduced inside the glass jar via a septum (close by the glass jar outlet) and the absorption was done over the night for a period of about 17 hours at room temperature.

The SPME was then analysed with a Trace GC (Interscience, Breda, NL) coupled with a Mass Sequencing detector type DSQ II (source temperature: 300°C; massa range: 29-250; method: Full scan). The Trace GC was equipped with a column type Restek RTX (30m x 0.25mm, df=0.25µm) and a PTC injector (injector temperature: 230°C). The oven temperature profile was programmed as follow: initial temperature 40°C, holding time: 2 min; final temperature: 315°C; ramp: 25°C/min.; Hold time: 1 min. the injection was manually done at a temperature of 230°C.
GC-MS was controlled by Thermo X-Calibur version 3.0.63 software.

2.7 PTR-ToF-MS

Volatile organic compound (VOC) production was determined by sampling glass jar headspace into glass tubes of 800 mL. These tubes were connected to the outward flow of the glass jars holding the potato samples. After 15 hours of flushing, the tubes were locked and sampled using a Proton-Transfer-Reaction Time-of-Flight Mass Spectrometer (PTR-ToF-MS). The PTR-ToF-MS 8000 instrument (Ionicon Analytik GmbH, Innsbruck, Austria) had a drift voltage of 1000V at 60°C and 3.8 mbar, resulting in an E/N of 134 Td. Sampling flow rate was 60 mL/min and the mass range was 20 – 512 m/z. Samples were taken from the tubes by direct injection into the PTR-ToF-MS drift tube through a heated (110 °C) peek inlet connected to a syringe needle. Sampling was done every second for 60 seconds total; the first 5-10 seconds consisted of ambient air, followed by 35 seconds of sample headspace and 20 seconds of carbon-filtered air. PTR-ToF-MS data were analysed per measurement day using the program PTRwid (Holzinger, 2015), after which the data per sample were averaged over the sampling period (from second 15-35).

2.8 Statistical analysis

The correlations between the volatile compounds production rate and the different treatments applied on the potatoes were analysed via a PCA (Principal Component Analysis) analysis. It was chosen to keep all the data in the data set, and so not to work with the average data per evaluation day. A SVD (Singular Value Decomposition) was applied on the data-set. Bi-plots as well as correlation loading (X) plots were created as output. All the statistical analysis were performed using the Unscrambler X software (CAMO software AS, Oslo, N).

3 Results and discussion

3.1 Thermo-desorption GC

In total more than 500 injections were assessed over the 18 days period. A quick overlay study of the most pronounced samples permitted to point out the most prominent volatile compounds on the chromatogram. In total more than 48 volatile components were separated and appointed for each chromatograph. As the thermo-desorption GC was connected to a FID detector and not to a MS detector, it was not possible to identify volatile compounds on basis of a MS-library. The volatile compounds were therefore numbered as component 1, 2 ..., 49. Table 2 summarizes the peak name and its retention time.

Table 2: List of components identified on the chromatograph and retention time (min)

Name	Retention time (min)	Name	Retention time (min)
Comp 1	2.897	**Comp 26**	9.845
Comp 2	3.306	**Comp 27**	9.963
Comp 3	3.567	**Comp 28**	10.086
Comp 4	3.866	**Comp 29**	10.393
Comp 5	4.279	**Comp 30**	10.539
Comp 6	4.457	**Comp 31**	10.845
Comp 7	4.636	**Comp 32**	11.134
Comp 8	5.258	**Comp 33**	11.404
Comp 9	5.853	**Comp 34**	11.533
Comp 10	5.942	**Comp 35**	11.643
Comp 11	6.030	**Comp 36**	11.723
Comp 12	6.100	**Comp 37**	11.977
Comp 13	6.400	**Comp 38**	12.320
Comp 14	6.613	**Comp 39**	12.619
Comp 15	6.684	**Comp 40**	12.749
Comp 16	7.290	**Comp 41**	12.997
Comp 17	7.453	**Comp 42**	13.092
Comp 18	7.969	**Comp 43**	13.203
Comp 19	8.460	**Comp 44**	13.337
Comp 20	8.610	**Comp 45**	13.491
Comp 21	8.681	**Comp 46**	13.597
Comp 22	8.899	**Comp 47**	13.782
Comp 23	8.991	**Comp 48**	14.039
Comp 24	9.114	**Comp 49**	14.694
Comp 25	9.482		

3.2 Respiration rate

According to Suslow and Ron (1998), respiration rate of potatoes stored at 20°C falls within the range of 9 and 23 mL CO_2/kg.hour. Similar respiration rate patterns were observed in the healthy potatoes samples during the first 15 days of storage (Figure 5 and Figure 6).
A slight increase of respiration rate in the first 2-3 days after starting the experiment was observed for the wounded potatoes and to a lesser extend for healthy samples. This extra activity results of the biological reaction called wound healing that occurs when potato tubers have been wounded (Borchert et al., 1974).
The increase of respiration rate from day 14 until day 17 for the healthy treatment relates to a bacterial infection (potentially *Erwinia*) of tubers in one of the two glass jars.

No correlation was found between the sprouting process observed in the potatoes from day 9 (Table 3) and any increase of respiration activities.
The inoculation with *Erwinia* bacteria (infected samples) leads to an increase in oxygen consumption and carbon dioxide production. In the first 24-30 hours after inoculation, no difference between the infected and the wounded potatoes activities can be detected. The slight increase in respiration rate can be explained by the wounding. The first 30 hours after inoculation relate to the lag phase in the bacterial growth. After this delay, bacteria grow at an exponential rate on the subtrate (potato). The higher oxygen consumption and carbon dioxide production rates measured for these samples combine both the higher activity of potato tuber and the respiration rate of the bacteria itself.

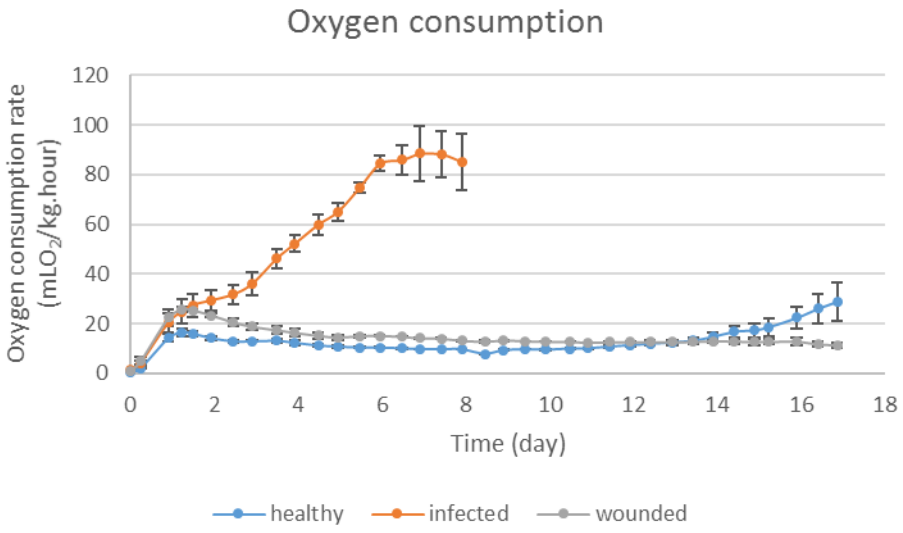

Figure 5: Oxygen consumption rate over time of healthy, wounded and Erwinia infected potato. Error bar shows standard deviation. (n=2)

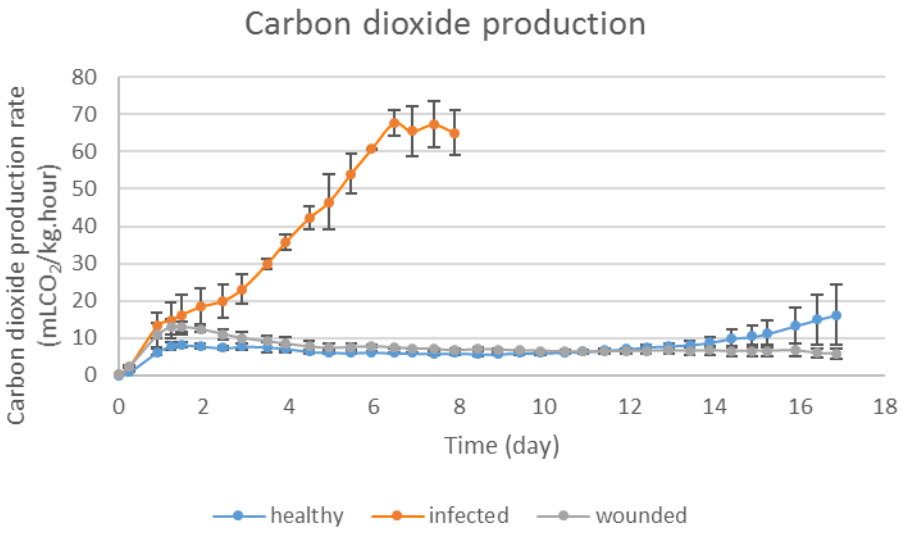

Figure 6: Carbon dioxide production rate over time of healthy, wounded and Erwinia infected potato. Error bar shows standard deviation. (n=2)

A visual quality evaluation was performed over time. The results are given in Table 3, pictures of each glass jar over time can be found in Annex 1. It is clear from the visual quality evaluation that *Erwinia* bacteria was able to grow on the potatoes. From day 3, foam coming from infected potatoes was observed in one of the two infected glass jars. The second infected sample showed similar infection symptoms the following day.

Table 3: Qualitative description of infection symptoms in the potatoes in glass jars over time

	Day 4		Day 7		Day 9		Day 14		Day 16	
	infection	sprouting	infection	sprouting	infection	sprouting	infection	sprouting	infection	sprouting
Healthy A	-	-	-	-	+/-	+/-	+	+	++	+
Healthy B	-	-	+/-	-	+/-	+	+	+	++	+
Wounded A	-	-	-	-	+/-	+/-	-	+	-	++
Wounded B	-	-	-	+/-	+/-	+	+/-	++	+/-	++
Infected A	++	-	+++	-	+++	-	n.d.	n.d.	n.d.	n.d.
Infected B	+	-	+++	-	+++	-	n.d.	n.d.	n.d.	n.d.

- = not present; +/-: maybe present but not clear; + present but still small; ++ clearly present; +++ infection: present with large white areas on potatoes dus to infection.

3.3 GC-MS

From the pre-test, the chromatographs obtained with the two SPMEs were compared and the most important peaks were identified (Figure 7). With both SPMEs, the volatile compound named Isoproturon was detected and identified. This chemical is a systemic herbicide used as weed control (Kebede et al., 2016). This herbicide may have been used during the cultivation of the potato to control the growth of the weed.

Regarding the amount of volatile compounds absorbed by both SPMEs, the grey SPME showed better absorption properties than the blue one for the specific volatile compounds produced by infected potatoes. For this reason, grey SPMEs were used later on to compare the volatile compounds produced by healthy, wounded and infected potatoes over the time.

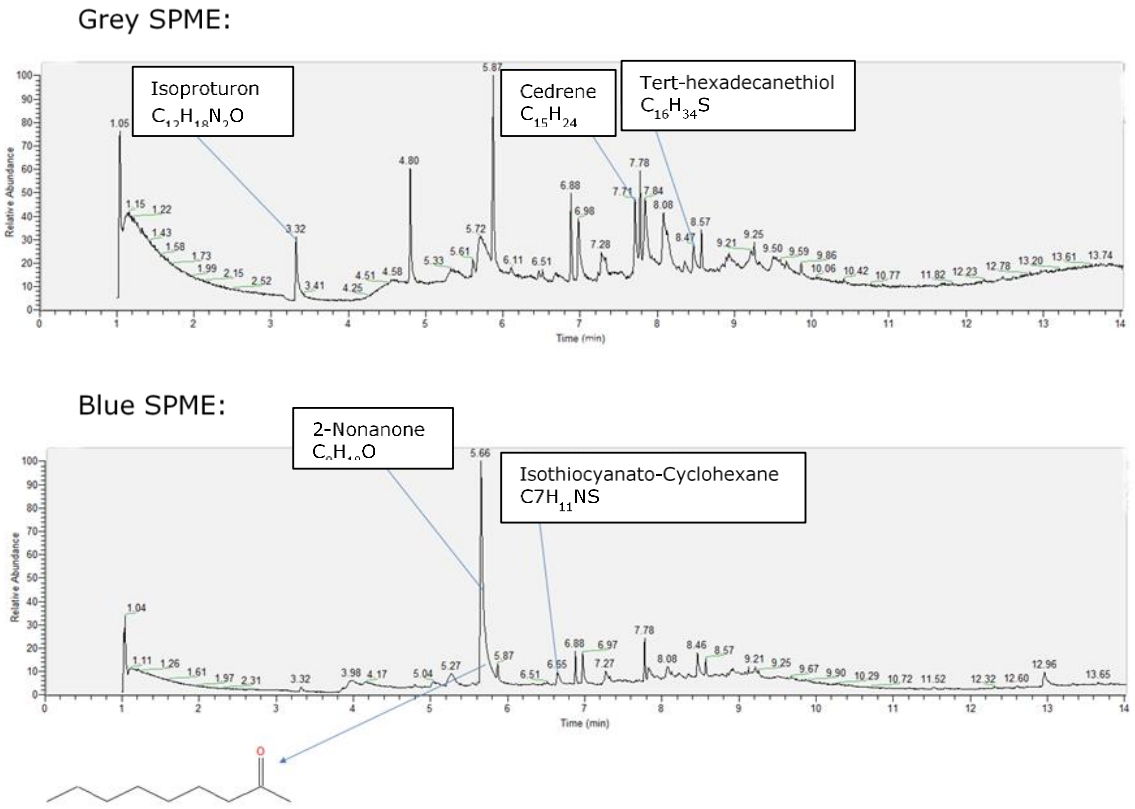

Figure 7: Chromatographs obtained after desorption of grey SPME (top figure) and blue SPME (down figure) during pre-test. Both SPMEs were introduced in same glass jar with infected potatoes.

During the test with the healthy, wounded and infected potatoes, the analysis with GC-MS method was performed at four distinct time points in order to screen the complete range of volatile compounds produced/released by potatoes and/or bacteria during the several phases of infection. The Table 4 summarizes the most important and relevant volatile compounds that were present. With exception of the last day for one sample of the healthy treatment (in which bacterial infection was noticed on the last day of the experiment), all volatiles were of very low abundancy in the glass jars with healthy and wounded treatments and for this reason GC-MS analysis was not able to measure them.
On basis of the detection of the volatile compounds per evaluation day, it was possible to observe a dynamic in the production/release of volatile compounds that may be correlated with the infection status of the potatoes by the *Erwinia* bacteria. The volatile compounds with the highest abundancy were identified on the last sampling day for the infected sample (day 8).

Table 4: List of volatile compounds detected and identified by the GC-MS test. Each component was detected at least in one of the four sampling moments (day 1, 4, 8 and 16). Infected samples were not analysed on day 16.

Treatment	Component #[1]	Evaluation day	Chemical group	Mass (g mol^{-1})	Comments[2]
Healthy only detected in one glass jar on last day	Non-identified	16	organosulfur	94	garlic like off-odour
Wounded	Non detected[3]				
Infected	Non-identified	1	ester	74	pleasant glue like smell
	Comp 13	1	ester	88	sweet smell
	Comp 18	4	amine	101	strong fishy odour
	Comp 11	4	ketone	72	sharp sweet odour
	Comp 5	4-8	ketone	58	fruity smell
	Comp 17	4-8	alcohol	74	NA[4]
	Comp 7	4-8	organosulfur	62	cabbage like odour
	Comp 47	4	alcohol	130	heavy, earthy and slightly floral odour
	Non-identified	8	arene	150	?

[1]: As the present results may be used for scientific publication, it was decided to not disclose the specific name of the component identified by the MS library.
[2]: Comments about specific odour are provided by Wikipedia.
[3]: All volatiles compounds were at too low abundancy to be measured.
[4]: Odour characteristics of this volatile compound not available.

3.4 PTR-ToF-MS

3.4.1 Analysis of relative abundance

Volatile production of potatoes was assessed on day 1, 4 and 8 using PTR-ToF-MS. Relative comparison of the volatile production on day 1 showed that healthy and wounded potatoes produced slightly more volatiles than are present in a sample container without potatoes (Blanco; Figure 1a). Infected potatoes showed a strongly increased production compared to the healthy and damaged potato samples, particularly in the lower mass/charge range. Clean air, sampled from the source air used to flush the entire setup, showed the lowest volatile presence over the entire mass/charge range.

Regarding the diversity of volatiles in the outgoing air-flows of the different potato samples, healthy potatoes show a number of large peaks in the mass/charge range below 125 Da (Figure 1b). Mass charge ratios that showed elevated levels of production were 43, 59, 61, 93, 107 and 111 Da. These elevations increased slightly between days 1 and 4. Wounded potatoes showed a volatile pattern very

similar to that of the healthy potatoes for all the days (Figure 1c). Infected potatoes showed a different pattern compared to healthy and wounded potatoes, mainly due to a very strong presence of a number of volatiles, namely mass/charge ratios at 33, 43, 61, 75 and 89. These volatiles were more than 75 times higher for the infected samples compared to the healthy and wounded samples (Figure 1d). On day 4, analysis of the infected samples was no longer possible due to saturation of the PTR-ToF-MS detector.

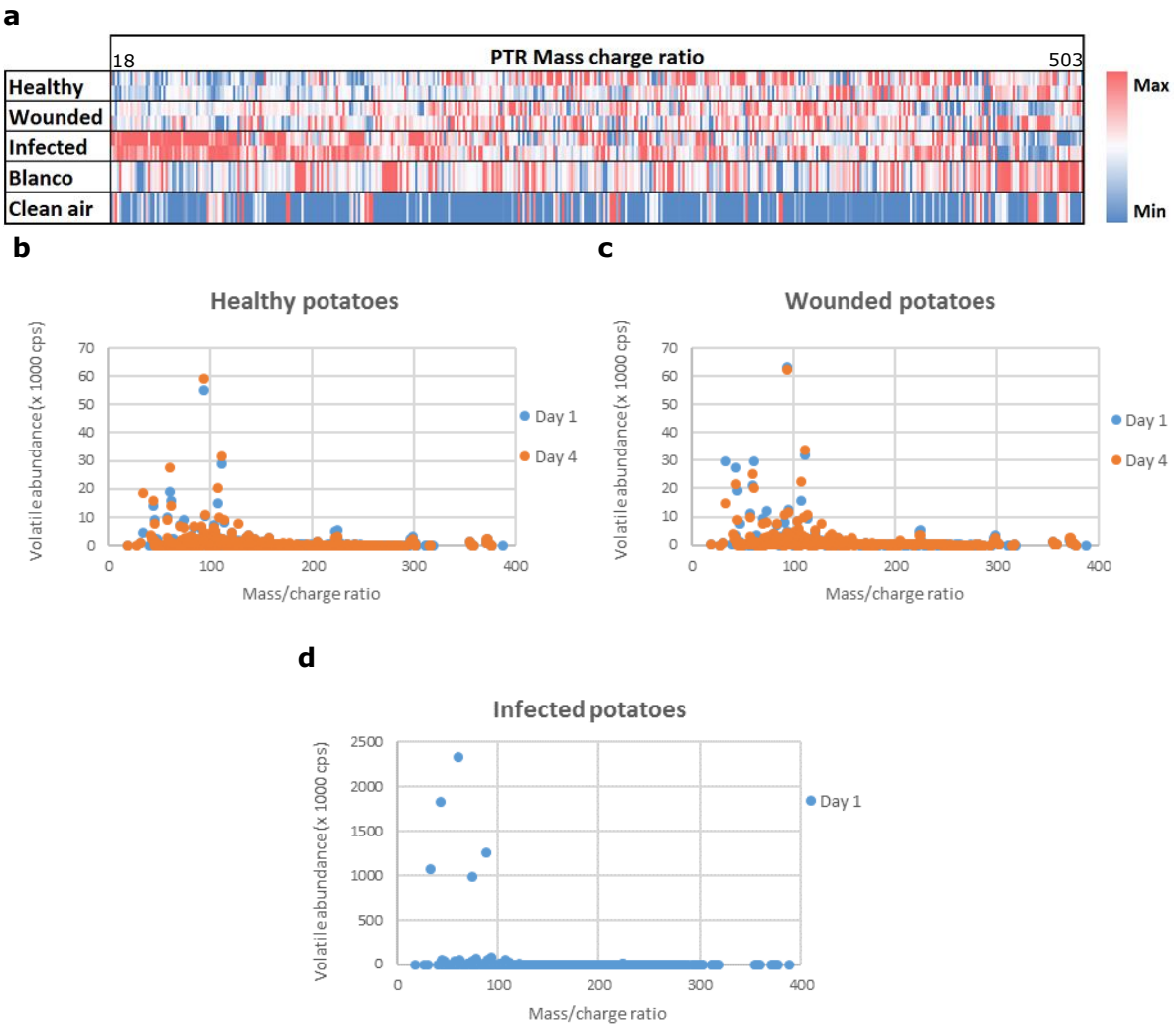

Figure 8: Volatiles produced by potatoes that were either healthy, wounded or infected. Measured were the outgoing air-flows from containers with potatoes that were healthy, wounded or infected with Erwinia or without potatoes (Blanco), as well as the incoming air-flow (Clean air). Relative abundance in the different sample types per volatile on day 1 is represented using a heatmap (a). Here, the highest production is shown in red, medium production in white and the lowest production in blue. Different volatiles are indicated by differences in their mass/charge ratio. Presence of the volatiles (in 1000 counts per second) over time is shown for healthy (b), wounded (c) and infected potatoes (d). Sampling occurred day 1 (blue) and 4 (orange). Data represent means, n=2.

Tentative identification of the more abundant volatiles from figure 7 shows that more than half of these mass/charge ratios belong to acids or esters, alcohols, acetone and acetaldehyde (Table 5). Among these volatiles, methanol and the different acids/esters are particularly abundant in infected potatoes. For the majority of these volatiles, similar abundances were measured for healthy and wounded potato treatments. Methanol and acetaldehyde abundance was significantly higher in wounded potatoes compared to the healthy potatoes. These two volatiles are presumably related to processes induced by wounding.

Table 5: Tentative identification of volatiles produced by the potatoes on Day 1. Per mass charge, blue and red colours schematize respectively the minimum and maximum count per seconds between healthy, damaged and infected potato samples (blue is lowest, white is medium and red is highest abundance). Values represent mean counts per second. Abbreviations: n, unknown.

Mass/charge ratio (Da)	Tentative identification	Healthy	Wounded	Infected
33.033	Methanol	4549	29821	1064882
43.018	Acid or Ester fragment	14019	27661	1827305
43.054	Propanol	2891	3243	16109
44.021	n	331	638	38178
45.033	Acetaldehyde	9167	19478	50521
47.049	Ethanol	2144	7564	39473
57.07	Butanol	9794	11187	33077
59.049	Acetone	18907	21224	46028
61.028	C2 acid or ester	15788	29676	2330835
62.027	C2 acid or ester istope	2005	2130	47723
69.07	n	7094	9172	13041
73.064	n	9106	12151	22700
75.026	n	1087	1319	33738
75.042	C3 acid or ester	2265	2700	978743
76.045	C3 acid or ester istope	204	215	34172
79.053	n	3105	3808	63422
87.044	n	2210	2844	10529
89.024	n	260	316	9647
89.06	C4 acid or ester	911	1189	1251675
90.062	C4 acid or ester isotope	57	62	56032
91.057	n	5864	7778	12155
93.037	n	55318	63231	81851
95.017	n	10245	12713	13793
107.048	n	2647	3150	18190
107.085	n	14830	15612	53530
111.047	n	28875	31768	30310
113.029	n	8208	9470	9536

Min Max

3.4.2 Targeted PTR analysis

A targeted analysis was applied on the ten compounds identified by GC-MS (Table 4). PTR data from day 1 and day 4 of healthy, wounded and infected samples were compared between each other. Ratio of count/s was calculated and reported in Table 6.

Wounded and healthy potatoes showed similar patterns regarding volatile presence on the first evaluation day (ratio around 1 for Wounded/Healthy day1). For healthy and damaged potatoes, component 11 content decreased during the inoculation period of 4 days (ratio of 0.67 between day 4 and day 1). Two volatiles had a significant higher abundance for infected potatoes compared to wounded and healthy treatments (ratio> 362 for component non-identified 2 and > 1000 for component 13). These two compounds were also detected on day 1 during the GC-MS analysis. The other top 10 components were not detected or below detection level on day 1. Both sampling methods were able to detect these two volatiles.

Table 6: Ratio of volatile compounds abundance of a selected list of volatiles identified by GC-MS. Abundance ratio between samples measured on similar day or over 4 days inoculation period. n.d.: not detected by the PTR-ToF-MS detector. b.a.: detected but below accuracy level (< 3000 counts/s)[1].

Component #	Mass (g.mol^{-1})	Infected/ Healthy Day 1	Infected/ Wounded Day 1	Wounded/ Healthy Day 1
Non-identified 1	94	n.d.	n.d.	n.d.
Comp 7	62	3	3	1.41
Non-identified 2	74	**432**	**362**	1.19
Comp 13	88	**1375**	**1053**	b.a.
Comp 18	101	n.d.	n.d.	n.d.
Comp 11	72	2	2	1.33
Comp 5	58	2	2	1.12
Comp 17	74	n.d.	n.d.	n.d.
Comp 47	130	n.d.	n.d.	n.d.
Non-identified 3	150	n.d.	n.d.	n.d.

3.5 Volatile compounds production rate

3.5.1 Thermo-desorption GC

The volatile compounds produced by the potatoes were analysed 3 to 4 times a day during the complete duration of the experiment. In total 49 peaks were separated and integrated. On basis of the area under each peak and the air supply flow, the production rate of each volatile compound was calculated and used for further statistical analysis. As the majority of the volatile compounds were not chemically identified, no calibration was made. The volatile compound production rate is expressed in (mV.min)/kg.hour. The production rate over the time of some of the measured volatile compounds is summarized in Annex 2.
Based on the produced volatiles profile, a clear distinction between the infected samples and the two other treatments can be done. Component 5 was the most dominant volatile compound produced by the infected potatoes (Figure 9). The production rate of this compound followed an exponential curve from day 3 until day 5. A lag phase can be identified corresponding to the microbial lag phase of the *Erwinia* bacteria. Other volatiles remained at lower peak level such as component 6.

On the last days of the experiment, infection was visually observed on potatoes of both samples that started the test as healthy potatoes. An increase in the production rate of the component 5 was observed over the same time period that the bacterial infection was visually noticed (Figure 9-B). Furthermore, a small increase in the production rate of the component 5 was also observed on day 4 and 5. This seems to be a cross-contamination from the infected samples. Sampling for Thermo-desorption measurement was done in series where sample "Healthy B" was tapped off just after the sample "Infected A". The component 5 content in this sample was so high that it is possible that component 5 remained on the absorption tube and piping of the Thermo-desorption instrument, resulting in cross contamination between the samples.

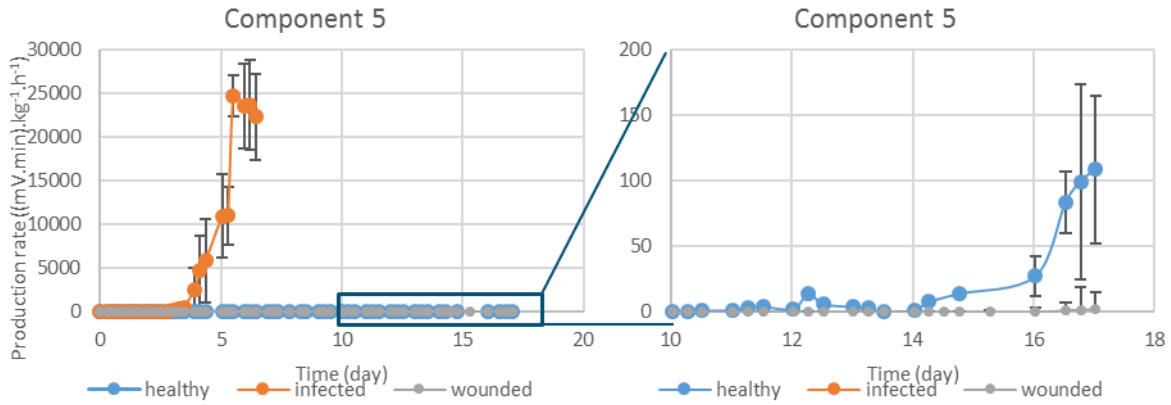

Figure 9: Production rate of component 5 over the time. Figure A displays the average production rate of the three treatments. Figure B focus on the production for healthy and wounded samples from day 10 till day 17 (y-axis down sized till 200 (mV.min)/kg.h). Error bar shows standard error. (n=2)

3.5.2 PCA analysis

In order to identify patterns in the volatile compounds production of potatoes, a PCA analysis was performed on the data set collected from the thermo-desorption analysis. As the infected potatoes were removed from the experimental set-up after 5 days of experiment, the comparison between the three treatments was performed on the data set collected from day 1 to 5. The PCA score plot (Figure 10) shows a clear distinguishing between the measurements made on the infected samples comparing to the ones done on the two other potato treatments (healthy and wounded). The Principal Component 1 explains 96% of the variation observed between the samples whereas the second PC explains only 2%. When regarding the effect of the evaluation day, no clear distinction between the evaluation day in general can be done (Figure 11). The principal component 1 explains partly the evaluation time when regarding only samples from the infected potatoes: samples move to the right of the figure (higher PC1 value) when reaching the end of the evaluation period. Due to the exponential increase of the volatiles production in the infected samples, the effect of the latest days is large and dominates the data variation. For this reason, the effect of the evaluation time for the samples taken on healthy and wounded potatoes is not visible on the score plot: dots representing these samples stay within a distinct cloud (middle-left) whatever the evaluation day.

The correlation loadings plot presented in Figure 12 should be analysed on basis of the conclusion drawn from Figure 10. This figure indicates that the infected potatoes samples can be mainly separated from the two other treatments via the first component. Volatile compounds on the right side of the plot, between the two outside circles have high correlation with the infected potatoes. From the 49 volatile compounds identified on the chromatographs, more than the half are located between the two circles. This indicates that the compounds located on the right side between the two circles are exclusively produced by infected potatoes and/or bacteria. Component 5 is produced in all infected samples and is far more related to the infection than other volatiles. For some infected samples there is a considerable production of other volatiles on days 3, 4 and 5. On basis of the correlation loading (X) figure (Figure 12), a ranking of the top 10 of volatile compounds has been established and summarized in Table 7. From this top 10, only two components were detected by the GC-MS analysis: one ketone (MW: 48g/mol) and one organosulfur (MW: 62g/mol).

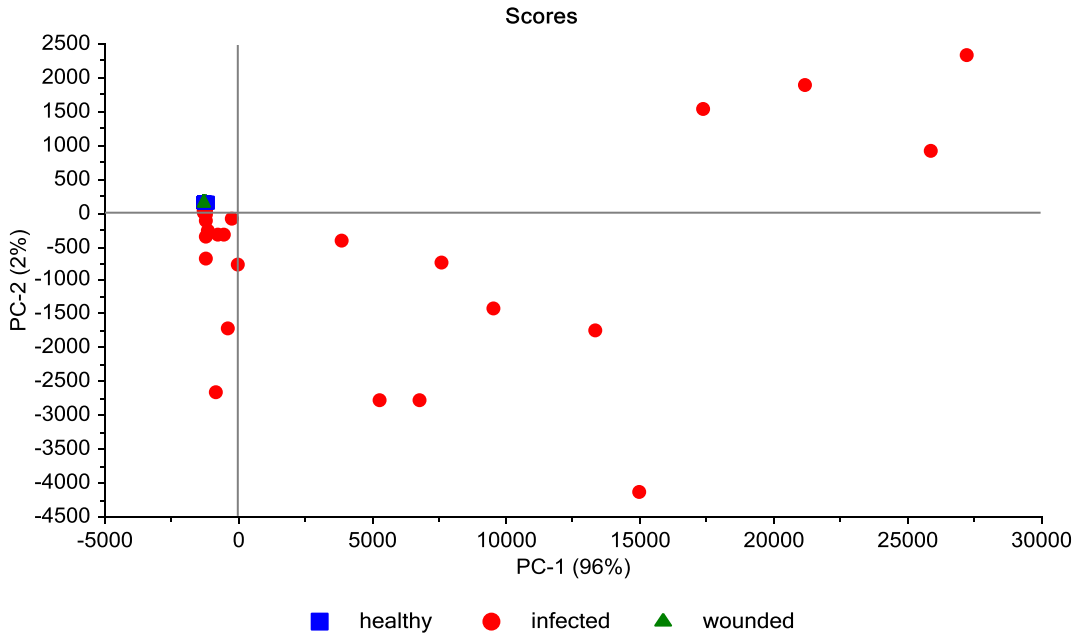

Figure 10: PCA score plot of the volatile compounds production rate of three treatments applied on potatoes over a period of 5 days. Blue dots summarize volatile compounds production rate of healthy potatoes, green dots of wounded potatoes and red one of potatoes infected with Erwinia bacteria. PC-1 explains 96% of the variation and PC-2 2%.

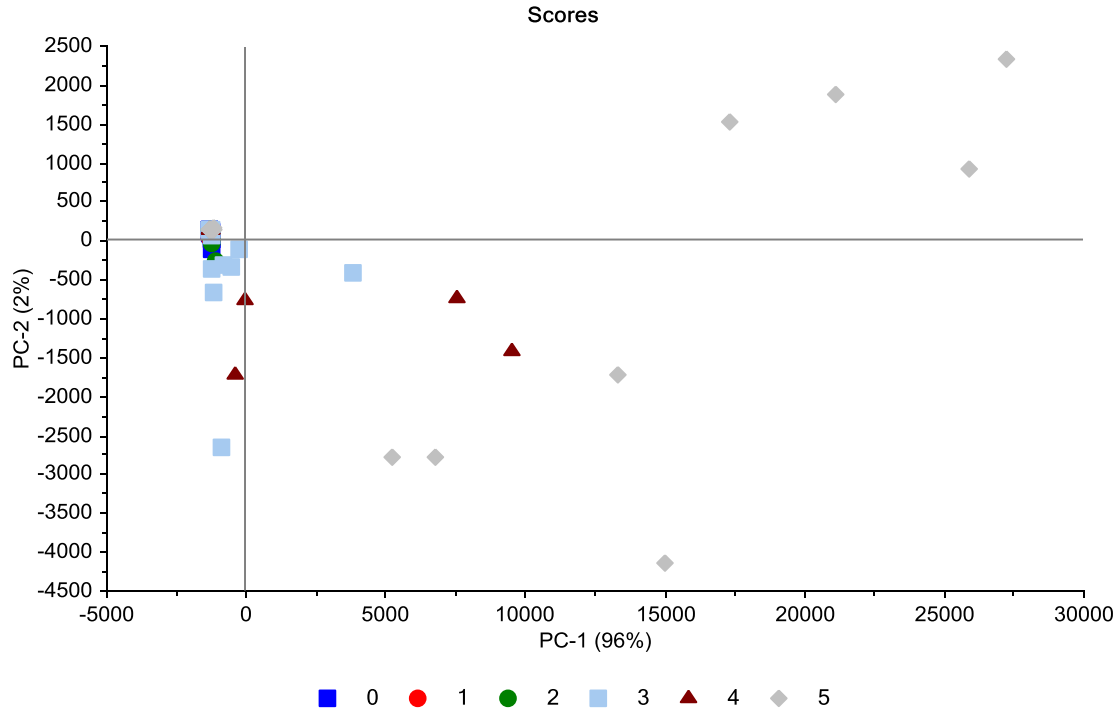

Figure 11: PCA score plot of the daily volatile compounds production rate of potatoes over a period of 5 days. Different dot colours represent different evaluation moment (see legend). PC-1 explains 96% of the variation and PC-2 2%.

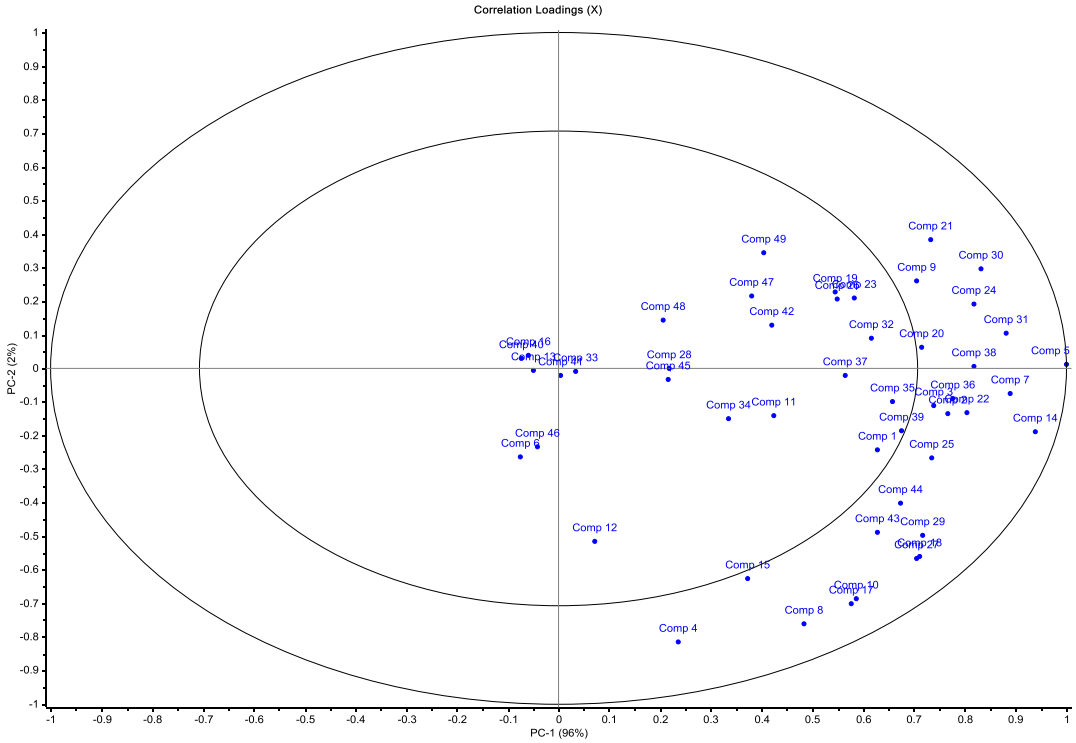

Figure 12: Correlation loadings (X) of volatile compounds production rate of potatoes stored up to 5 days. Component outer circle have significant contribution to the variation in the data.

Table 7: Top-10 volatile compounds ranking on basis of correlation loading (x) outputs corrected by the percentage of each principal component.

Ranking	Volatile compound	PC-1	PC-2	$0.96*PC1^2+0.02*PC2^2$
1	Comp 5	0.9999	0.0133	0.9598
2	Comp 14	0.9380	-0.1885	0.8454
3	Comp 7	0.8888	-0.0751	0.7585
4	Comp 31	0.8822	0.1062	0.7473
5	Comp 30	0.8313	0.2977	0.6651
6	Comp 38	0.8187	0.0051	0.6435
7	Comp 24	0.8172	0.1916	0.6419
8	Comp 22	0.8048	-0.1309	0.6222
9	Comp 36	0.7756	-0.0891	0.5777
10	Comp 2	0.7673	-0.1336	0.5656

On basis of the top-10 ranking of volatile compounds described in Table 7, it was decided to analyse one more time the dataset with only healthy and wounded samples over the complete duration of the experiment. The volatiles produced by the infected potatoes strongly dominate the volatile production, hence relevant volatiles but produced in lower amounts may be missed in the data analysis. The infected potatoes were excluded from the new analysis, and the data set was extended until day 17. Due to potential cross-contaminations on day 4 and 5 (volatile compounds produced by infected potatoes may have remained in the sampling system when measuring healthy samples), four data points (healthy samples on day 4 and 5) were removed from the data set. The rejected data are marked with a circle on the score plot figure (Annex 3). On the last three days of the experiment, bacterial infection was observed on potatoes stored in the glass jar coded as healthy (Table 3).

Figure 13 summarizes both PCA score plots and the correlation loadings (X) plot. The score plot figure (Figure 13-A) shows that it is possible to differentiate both treatments from each other: the PC-1

describes mainly the healthy samples where the bacterial infection at the end of the experiment was observed, whereas the PC-2 describes the wounded samples on the first day. Similar PCA analysis expressed over time instead of per treatment is summarized in Figure 13-B. The majority of the wounded samples taken on the first days are explained by the second principal component (25%), whereas the samples made on the last days of the experiment are explained for 70% by the first principal component (PC-1). The PC-1 explains a larger part of the data variation, indicating that the infection has a stronger effect on the volatile production than the healing process. The volatiles produced by the healthy samples after becoming infected are in agreement with volatiles measured by the infected samples during the first days of the test.

Taking into account this partitioning and the position of each volatile component on the correlation loadings (X) bi-plot (Figure 13-C), it is possible to correlate the components 5, 38, 14 and 25 with the bacterial infection observed on the healthy potatoes at the end of the storage period. On the other hand, components 2, 4 and to a lesser extend component 9 can be correlated to the wounding and healing reactions observed on wounded potatoes in the first day(s) of the experiment.

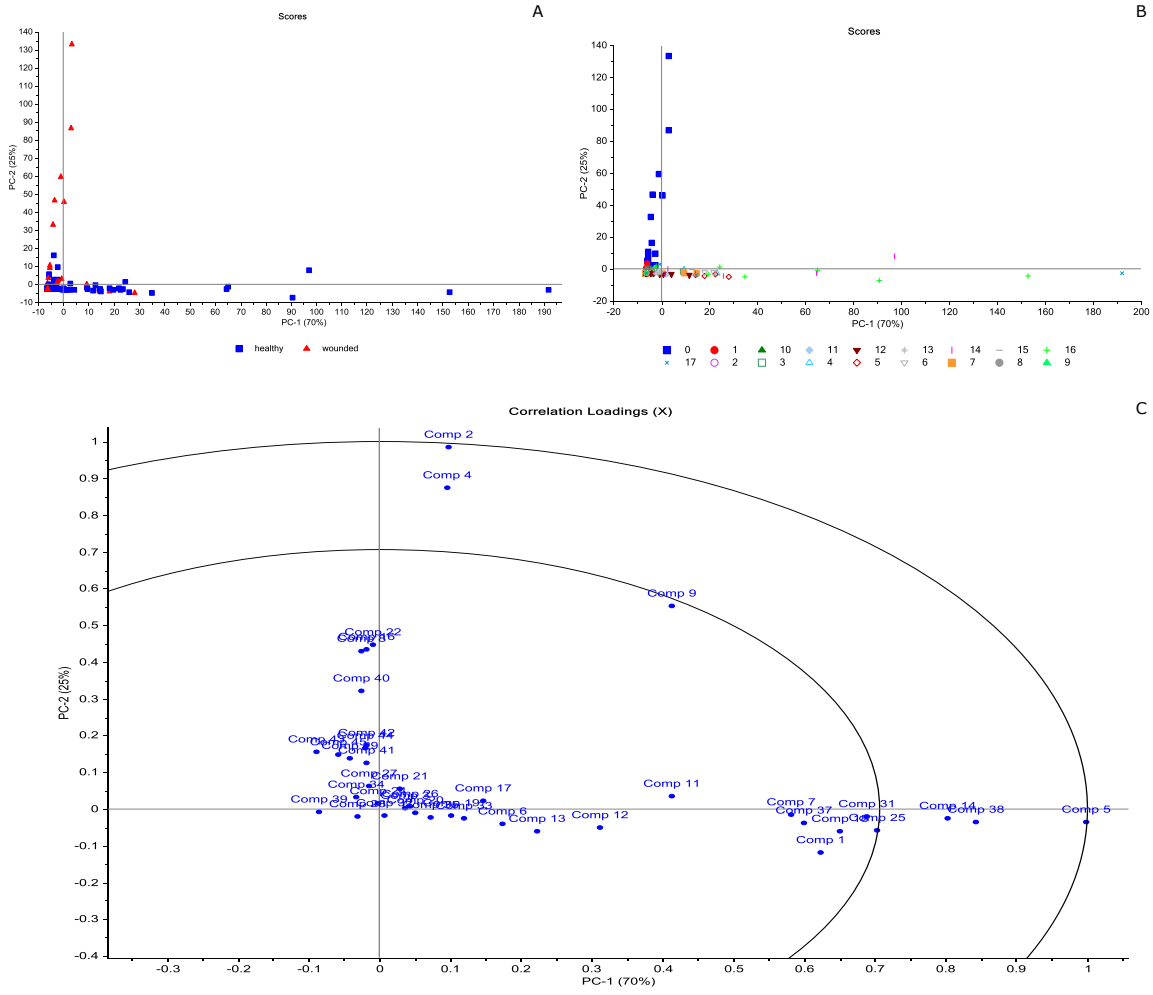

Figure 13: A) PCA Bi-plot of the volatile compounds production rate per treatment. In blue the volatile compounds produced by healthy potatoes and in red produced by wounded potatoes both over period of 17 days. B) PCA Bi-plot of the volatile compounds production rate over the time. C) Correlation loadings (X) of volatile compounds production rate. Components outer circle have a significant contribution to the variation in the data.

Besides the multivariate analysis performed on the total volatile compounds production rate data-base, an extra multivariable analysis was also performed to assess the relationship between the volatile compounds profiles and the respiration rate activities of the potatoes (Figure 14). The analysis showed that both respiration rate variables (oxygen consumption rate and carbon dioxide production rate) are strongly correlated to each other but also positively correlated to a group of volatile

compounds. Taking into account that both respiration rate variables are fitting between the two correlation circles, it is possible to conclude that respiration rate is also a good indicator to detect any infected potatoes. With other words, infected potatoes have significant higher respiration rate activities. Respiration rate activities can be used as bio-marker to identify any infection patterns. However the PCA with both respiration rate variables show a lower percentage of explained variance than the PCA based on the produced volatiles. This indicates that the volatiles production is more related to the infection than the respiration rate. Several factors may influence the respiration rate whereas the production of certain volatiles is more specific to the bacterial infection.

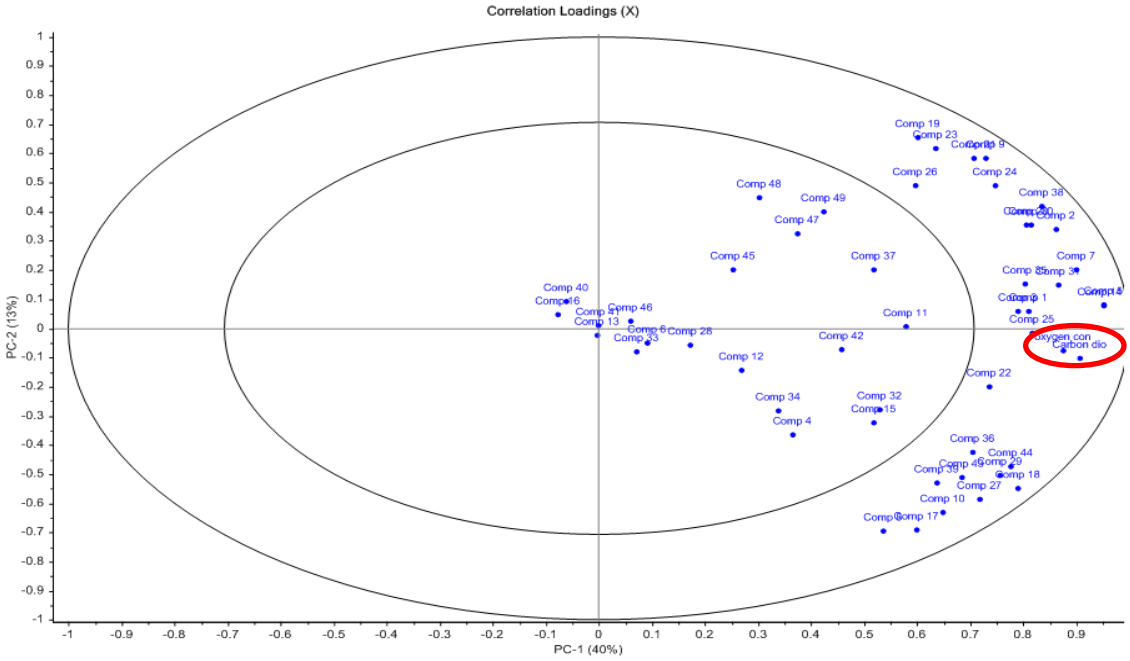

Figure 14: Correlation loadings (X) of potatoes volatile compounds production rate and potato respiration rate activities. Potatoes were healthy, wounded or infected with Erwinia bacteria. Blue spots represent different volatile compounds production rate measured with the Thermo-Desorption GC from day 0 until day 5 of the inoculation period. Two dots pinned down with a red circle represent the oxygen consumption rate and carbon dioxide production rate measured at similar interval than volatile compounds.

4 Discussion

This study shows clearly that specific volatiles are produced during a bacterial infection with *Erwinia* on potatoes. The production rate of several volatiles is significantly higher by infected potatoes than by healthy or wounded potatoes. This result has been confirmed by three assessment techniques, thermo-desorption GC, GC-MS and PTR-ToF-MS, applied in this research. The volatiles are most probably produced both by the potato as a response to the infection but also by the bacteria as the result of their metabolism. With the current set up it was not possible to distinguish between these two physiological processes.

The PCA analysis of the thermo-desorption GC data shows that component 5 is clearly correlated to the infected samples, and has the highest PCA loading. The results of the GC-MS analysis are in good agreement, as the same compound 5 was also measured both on day 4 and on day 8 after infection. Hence this compound, a ketone, seems to be a good biomarker for the studied *Erwinia* infection. Moreover compound 7, an organosulfur molecule, has also been found both on thermo-desorption GC top 10 most relevant compounds and measured on day 4 and day 8 after infection with the GC-MS. This compound may also be a relevant biomarker for the infection.

It is not possible to link directly the molecular weights of the volatile compound found on the GC assessments and the mass/charge ratio of the PTR-ToF-MS measurements and therefore a comparison between the results is not yet possible. The PTR-ToF-MS shows a high amount of counts per second for lower molecular weight compounds (below mass/charge ratio 45) which were not measured in the GC-MS. However the sampling method applied for GC-MS is not completely optimised for volatile compounds with short carbon chain which might explain the mismatch on the lower molecular weight range. The highest peak has a mass/charge ratio of 61.028 and a similar mass (around 60g/mol) was not found in the GC-MS results. On the other hand, the compound with mass/charge ratio 75.042 could be the same as compound 17 which has been detected in the GC-MS results on day 4 and 8 after infection. On basis of the targeted analysis, it was possible to identify similar volatile compounds for both GC-MS and PTR-ToF-MS on day 1. These compounds were strongly linked with the *Erwinia* infection of the potatoes. Each analysis method showed advantages and inconveniences: GC-MS was able to identify the volatiles thanks to the MS-library; however, it was not possible to quantify both compounds over the time. On the contrary, PTR-ToF-MS was able to detect and separate masses and give an indication about their abundance level, but was not able to identify precisely their chemical identity. Special attention should be taken when comparing the PTR-ToF-MS data between different evaluation days as for unexplained reason, the sensitivity of the PTR detector may fluctuate drastically over the day.

The application of a SPME to trap (and afterwards release) the produced volatiles seems to have limitations as the number of volatiles measured with the thermo-desorption GC and the GC-MS differ significantly. After 3 days, the oxygen consumption of the infected samples is already twice as high as the oxygen consumption of the healthy potatoes. This difference increases exponentially during the test period. This indicates that the respiration rate variables are also a good indicator for the infection. The PCA analysis show accordingly a good correlation between the relevant infection volatiles and the respiration rate variables. An increased respiration rate upon a bacterial infection may be expected since a bacterial infection increases the potato metabolism. The volatiles production seems however to be more related to the infection than the respiration rate. Several factors may influence the respiration rate whereas the production of certain volatiles is more specific to the bacterial infection.

The PCA analysis of the thermo-desorption GC data indicates that specific volatiles are produced in non-infected tubers during healing. Dastmalchi et al. (2016) identified nine constituents with high antioxidant properties during the wound healing process. These molecules have a high molecular weight and were extracted/measured with liquid chromatography. These molecules were not measured in our experiment as the sampling and the analysis methods differed from the ones used by Dastmalchi et al. (2016).

5 Conclusions

The mains conclusion of this research are:

- Specific volatile compounds are produced by an infection with the bacteria *Erwinia* on healthy potatoes.
- Each detection technique/method used in this study (SPME sampling method coupled to GC-MS, thermo-desorption sampling method coupled with GC and PTR-ToF-MS) was able to detect all or a part of the volatiles produced by infected potatoes.
- GC-MS was able to detect the most relevant/abundant volatile compounds. The sampling method is not completely optimised for volatile compounds with short carbon chain.
- PTR-ToF-MS was also able to detect volatile compounds related to the infection but the identification requires complementary work that was not carried out in the fame of this project.
- Thermo-desorption sampling method was able to capture and condense the most relevant volatiles produced by infected potatoes. The identification of the several peaks remains a challenge, which may be solved by coupling the thermo–desorption instrument to a mass spectrometer. The other advantage of the thermo-desorption sampling method is the possibility to sample air samples directly in-situ (by commercial potatoes storage company) through a sampling tube.
- In the present study, a list of the most relevant volatile compounds related in the infection of potato by *Erwinia* bacteria was identified. Despite the differences in technique and methodology, some of the compounds were assessed by two or even all techniques. Further research is required in order to quantify these volatile compounds and correlate them to the infection level of the potatoes. In parallel, investigations around sensible and cheap detector should be carried out to allow the use of this detection technology at industrial level.
- It is possible to distinguish two groups of volatile compounds: one related to the wounding-related physiological processes and one related to the *Erwinia* infection. The volatile compounds produced by the infected potatoes and/or by the bacteria follow an exponential production curve.
- Respiration rate activities were also correlated to the infection.

Furthermore, this experimental set-up can be used for the detection of specific volatile compounds for other bacterial/fungal infections of fresh product during their storage.

Recommendation and further work:

- Chemical identification of the volatiles selected in the top-10 ranking list drawn in this study.
- Optimise the methodology to detect volatiles from the top 10-ranking list at early infection stage (inoculate with different counts levels) or at really low volatile compounds threshold level.
- Optimise the measurement method and try out new detector technologies that may be able to detect at early stage these compounds
- Optimise a sampling method that allow air sampling from storage rooms: using air-toxic sampling tube that can be thermo-desorbed in our GC system or sampling air samples for PTR-ToF–MS analysis.

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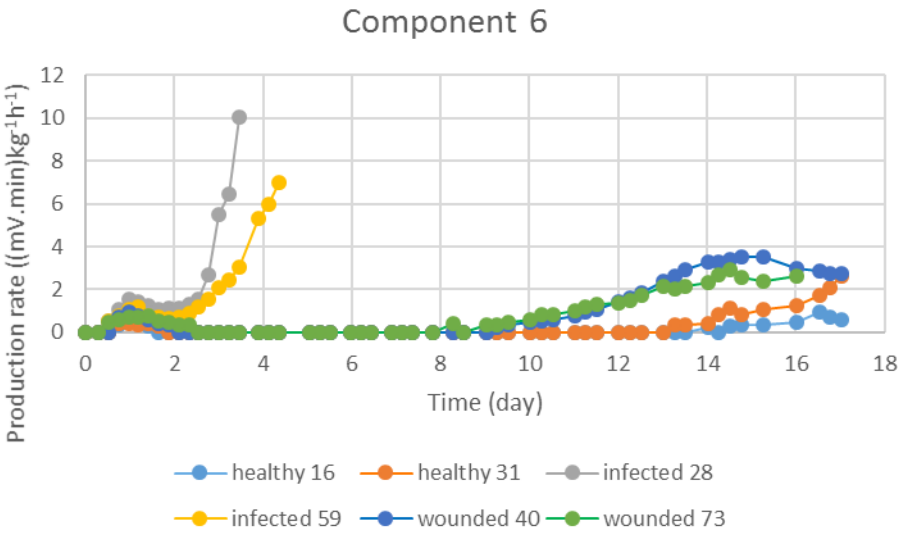
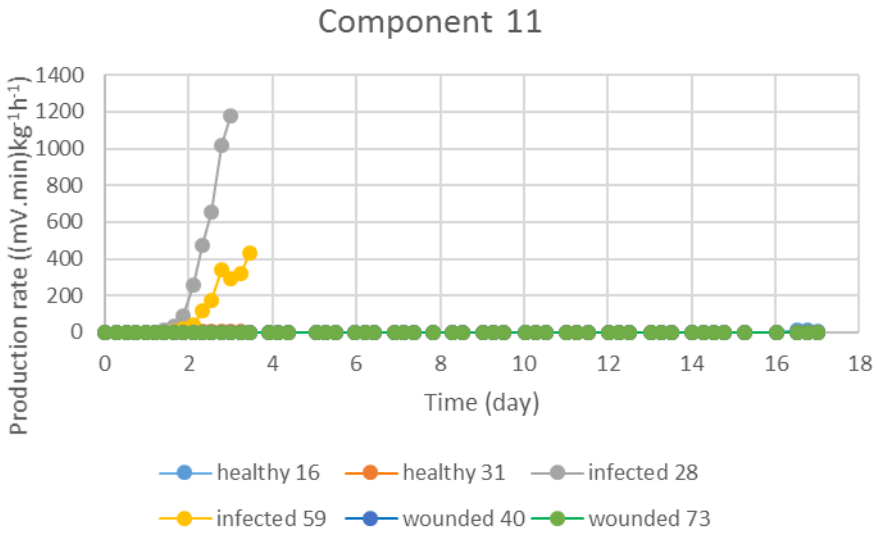
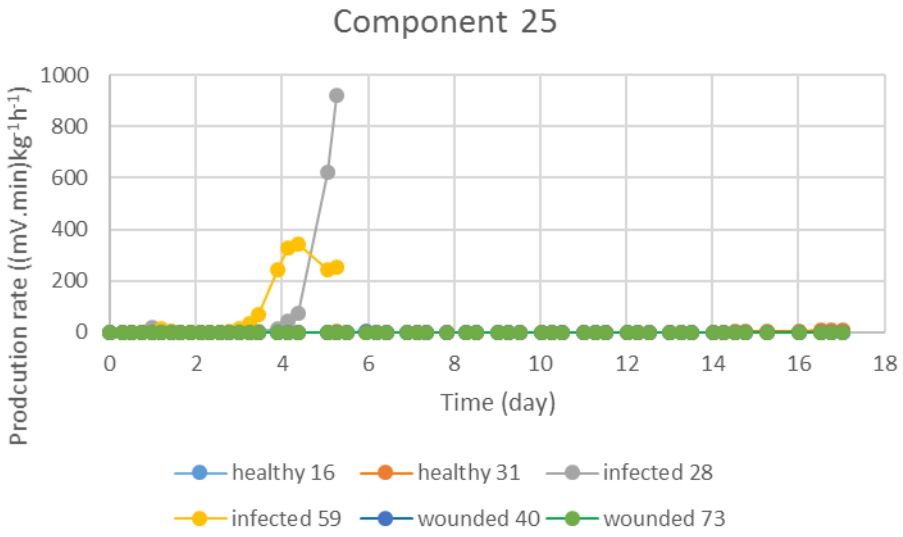
Annex 1 Pictures of each glass jar taken during the 16 days storage at 20°C

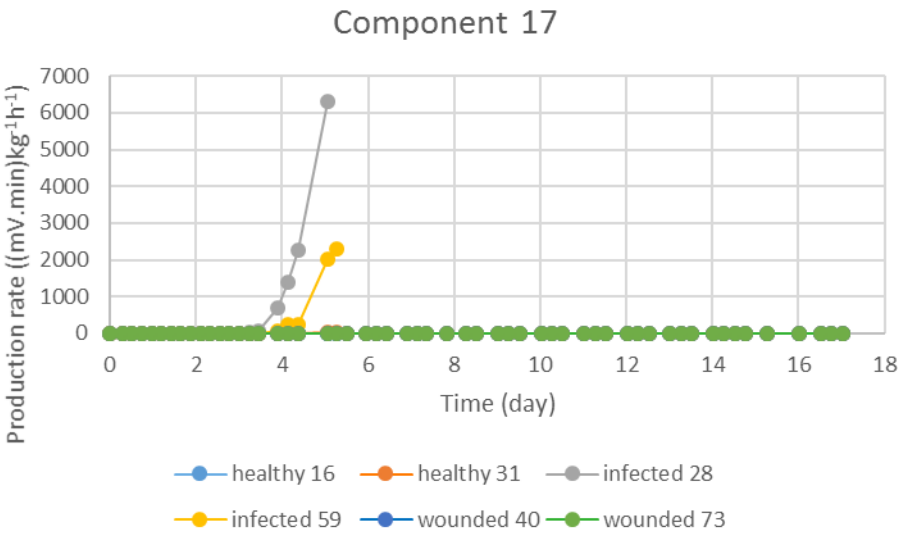

Wounded
Healthy
Wounded
Healthy
Day 9
Day 14
Day 16

Annex 2 Production rate of volatile compounds over the time

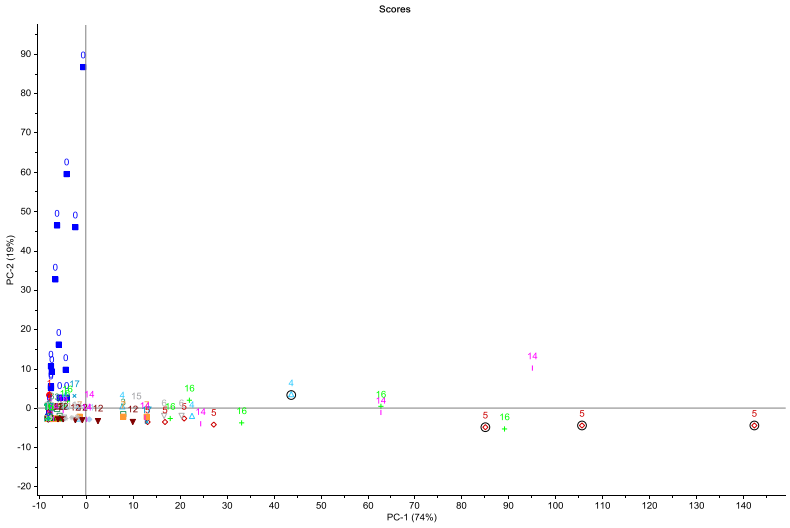





Component 25
Prodcution rate ((mV.min)kg-1h-1)
1000
800
600
400
200
0
0
2
4
6
8
10
12
14
16
18
Time (day)
healthy 16
healthy 31
infected 28
infected 59
wounded 40
wounded 73


Component 17
Production rate ((mV.min)kg-1h-1)
7000
6000
5000
4000
3000
2000
1000
0
0
2
4
6
8
10
12
14
16
18
Time (day)
healthy 16
healthy 31
infected 28
infected 59
wounded 40
wounded 73

Annex 3 PCA Score plot



PCA score plot of the volatile compounds production rate of healthy and wounded potatoes over a period of 17 days storage at 20°C. Dots marked with a circle are samples that may have been cross-contaminated during the analysis.

To explore the potential of nature to improve the quality of life

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