Finding the Markers of Fungal and Bacterial Infections in *Allium cepa* L.

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M.Sc. Amir Balash

aus Aleppo, Syrien

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Erstgutachter: Prof. Dr. Michael Keusgen

Zweitgutachter: Prof. Dr. Klaus Reuter

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I hope you enjoy your reading.

Amir Balash
Marburg 12.09.2018

Table of Contents

List of tables

List of figures

List of abbreviation

°C	Degree celsius
XTT	2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide
A.	*Allium*
cm	Centimeter
cfu	Colony-forming unit
COSY	Correlated spectroscopy
J	Coupling constant
CSO	Cysteine sulfoxide
DMDHF	Dimethyl dihydrofuranone
DMSO	Dimethyl sulfoxide
DPDS	Dipropyldisulfide
DPTS	Dipropyltrisulfide
EI	Electron ionization
EleNa	Electronische Nase
ESI	Electrospray ionization
EtOAc	Ethyl acetate
XIC	Extracted ion chromatogram
FID	Flame ionization detector
FAO	Food and agriculture organization
GC	Gas chromatography
g	Gram
HMPC	Heteronuclear multiple bond correlation
HPLC	High performance liquid chromatography
HR	High resolution
hr	Hour(s)
T24	Human bladder carcinoma cell line
UMUC3	Human bladder carcinoma cell line
IR	Infra red
IMS	Ion mobility spectrometry
JKI	Julius Kühn institut
kPa	Kilopascal

LF	Lachrymatory factor
LFS	Lachrymatory-factor synthase
L	Liter(s)
MS	Mass spectrometer / Mass spectrometry
m/z	Mass to charge ratio
MeOH	Methanol
µ	Micro
mg	Milligram
ml	Milliliter(s)
min	Minute(s)
nm	Nanometer
N_2	Nitrogen gas
NMR	Nuclear magnetic resonance
NOESY	Nuclear overhauser effect spectroscopy
PBS	Phosphate buffer salt
PDA	Potato dextrose agar
Rt	Retention time
RPMI	Roswell park memorial institute medium
sec	Second
SD	Standard deviation
ssp.	Subspecies
TDS	Thermal desorption spectroscopy
TLC	Thin-layer chromatography
UV	Ultra violet
UNO	United nations organization
H_2O	Water
λ_{max}	Wavelength of maximum absorbance

ABSTRACT

Common onion (*Allium cepa* L.) is considered as one of the most important vegetables due to the extensive usage in industrial food production. The production of dried onions is more than 88 million tons every year. However, onions are prone to several microbial infections reducing their yield and quality within a short period. The early stages of infection cannot be detected in onions by the conventional analytical methods. It can be assumed that the pattern of chemical compounds is altered by the action of pathogenic bacteria and fungi. In this project, we aim to identify the markers of infection in *Allium cepa* L. which can help to provide early detection of common onion diseases.

Five microbial strains including: *Fusarium oxysporum*, *Fusarium proliferatum, Penicillium sp., Botrytis aclada,* and *Erwinia carotovora,* were used to infect onion bulbs. Healthy and infected bulbs were extracted with ethyl acetate. The extracts have been analyzed using HPLC to define potential infection markers. Two new peaks were observed and suggested to be markers for the infections. Fractions were further investigated to charactarize their chemical structures by HPLC/MS, HRMS, UV, IR, and NMR. For the quantitative determination of both marker compounds, dimethyl dihydrofuranone (DMDHF) was served as an internal as well as external standard. Seven other *Allium* species were also

studied to investigate the presence of the same markers. In addition, some experimental work was done to synthesize the markers and detect them using GC and IMS. The bioactivity of the new substances in infected onion extracts was also studied.

The structures of the two markers obtained after infection were confirmed as 2-hexyl-5-methyl-3(2H)-furanone and 2-octyl-5-methyl-3(2H)-furanone. These compounds could only be detected after infection, and they could be considered as markers of fungal and bacterial infections in *Allium cepa* L. The concentration of the markers varied between 1 and 30 ppm depending on the fresh mass of onions. The exact concentration might depend on the grade of infection. These investigated markers also appeared in *A. altaicum* Pall., *A. pskemense* B. Fedt., *A. cornutum* Clementi ex Vis., *A. fistulosum* L., and *A. porrum* L., whereas they could not be detected in *Allium sativum* L. In bioactivity tests, no significant antifungal or antitumor activity could be recorded. The results showed also increases in free quercetin in infected onion bulbs in comparison to the healthy ones.

The 3(2H)-furanones have already been reported in previous studies as normal aroma substances located in *Allium cepa*. The production of 3(2H)-furanones in plants after infection could be an indirect defense strategy to confuse the quorum sensing in bacteria and other microorganisms.

ZUSAMMENFASSUNG

Die Zwiebel (*Allium cepa* L.) gilt als eines der wichtigsten Gemüse. Die Produktion von getrockneten Zwiebeln beträgt mehr als 88 Millionen Tonnen pro Jahr. Der umfangreiche Einsatz von Zwiebeln für die industrielle Lebensmittelproduktion erfordert gute Lagereigenschaften. Zwiebeln sind jedoch anfällig für mehrere mikrobielle Infektionen, die ihren Ertrag und ihre Qualität innerhalb kurzer Zeit verringern. Die frühen Stadien der Infektion lassen sich bei Zwiebeln mit herkömmlichen Analysemethoden nicht nachweisen. Es ist davon auszugehen, dass das Muster der chemischen Verbindungen durch die Einwirkung von pathogenen Bakterien und Pilzen verändert wird. In diesem Projekt wollen wir die Infektionsmarker in *Allium* cepa L. identifizieren, die zur Früherkennung häufiger Zwiebelkrankheiten beitragen können.

Fünf Arten von Mikroorganismen, nämlich: *Fusarium oxysporum, Fusarium proliferatum, Penicillium sp., Botrytis aclada* und *Erwinia cartovora,* wurden in Zwiebeln getestet. Gesunde und infizierte Zwiebeln wurden mit Essigsäureethylester extrahiert. Die Extrakte wurden mittels HPLC analysiert, um die Infektionsmarker zu bistimmen. Wir haben zwei verschiedene neue Peaks in den Chromatogrammen identifiziert, die sich auf

Markerverbindungen beziehen. Die Fraktionen wurden weiter untersucht, um ihre chemische Struktur mittels HPLC/MS, HRMS, UV, IR und NMR aufzuklären. Für die quantitative Bestimmung beider Verbindungen wurde Dimethyldihydrofuranon (DMDHF) als interner und externer Standard etabliert. Sieben weitere *Allium-Arten* wurden ebenfalls getestet, um das Vorhandensein der gleichen Marker zu untersuchen. Einige zusätzliche Experimente wurden durchgeführt, um die Marker zu synthetisieren, sie mit GC und IMS nachzuweisen und die Bioaktivität der neuen Substanzen in infizierten Zwiebeln zu testen.

Die Struktur zweier neuer Marker wurde nach der Infektion als 2-Hexyl-5-methyl-3(2H)-furanon und 2-Octyl-5-methyl-3(2H)-furanon bestätigt. Diese Verbindungen konnten erst nach einer Infektion nachgewiesen werden und sie konnten als Marker für eine Pilz- oder Bakterieninfektion bei *Allium cepa* L. angesehen werden. Die Konzentration der Marker variierte zwischen 1 und 30 ppm, bezogen auf die Frischmasse der Zwiebeln. Die genaue Konzentration kann vom Infektionsgrad abhängen. Diese untersuchten Marker traten auch in *A. Altaicum* Pall.*, A. . pskemense* B. Fedt*, A. cornutum* Clementi ex Vis.*, A. Fistulosum* L. und *A. porrum* L. auf, während sie in *Allium sativum* L. nicht nachgewiesen werden konnten. In Bioaktivitätstests konnte keine signifikante antimykotische oder antitumorale Aktivität festgestellt werden. Die Ergebnisse zeigten ebenfalls einen Anstieg des freien Quercetins in infizierten Zwiebeln im Vergleich zu den gesunden Zwiebeln.

Die 3(2H)-Furanone wurden bereits in früheren Studien als normale Aromastoffe in *Allium cepa* L. nachgewiesen. Die Produktion von 3(2H)-Furanonen in Pflanzen nach einer Infektion könnte eine indirekte Abwehrstrategie sein, um das Quorum sensing in Bakterien und anderen Mikroorganismen zu stören.

1. INTRODUCTION

1.1 The Genus *Allium*

The genus *Allium* is considered as a large genus which covers more than 750 species. *Alliums* consist of 15 monophyletic subgenera [1] (Figure 1-1). *Allium* species spread through the holarctic region between the boreal zone and the subtropics, especially in the area between Middle Asia, Pakistan, Afghanistan, and the Mediterranean Basin. In these areas, a very high diversity of *Allium* species can be found. Western North America can be considered as a second center of *Allium* species diversity [2], [3]. Generally, *Alliums* grow in sunny, open and dry areas in an arid and moderate-humid climate [4].

Allium cepa L. (onion) and *Allium sativum* L. (garlic) are considered as the most common and vital *Allium* species. The importance of some other cultivated species depends on the local cultivation and usage like *A. porrum* L., *A. schoenoprasum* L., *A. fistulosum* L., *A. tuberosum* L. and *A. chinens* L. Despite the extensive usage of *Allium* species as vegetables, seasonings, and medical plants, the information about their different properties are not complete [5], [6].

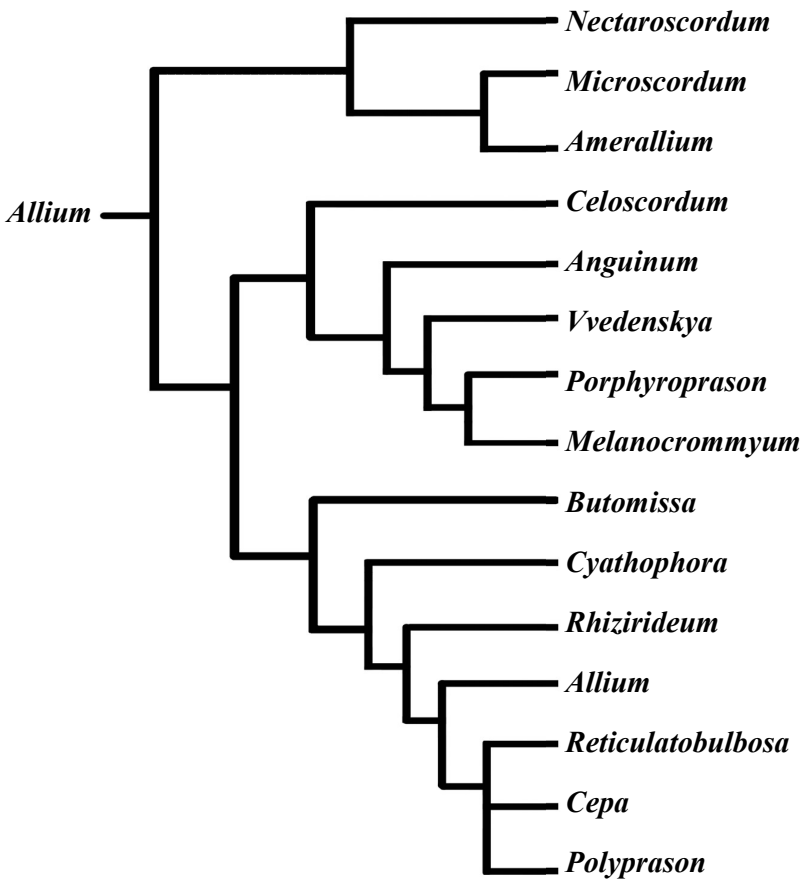


Figure 1-1 Subgenera classification of the genus *Allium* [1]

Allium species could be adapted to different environmental niche causing diversity of a lot of different morphotypes, which leads to some difficulties in the taxonomy and classification of the genus *Allium* [5].

The first description of the chemistry in genus *Allium* was summarized in 1844. Aqueous distillation of garlic bulbs gave a strongly smelling sulfur-containing oil. Later on, similar experiments were conducted with onions, which led to isolating 1-propenyl propyl disulfide. In the middle of the 20th century, some researchers could elucidate the flavoring compounds in onion and garlic. They could also understand the mechanism of their formation [7].

Allicin has been isolated from *A. sativum* L. (garlic) [8]. The lachrymatory factor in onion (LF, C_3H_6SO) was described first in 1963 [9]. The role of enzymes in forming the volatile compounds in *Allium* species got evident at the same time. Therefore, Alliinase, which is the primary enzyme in *Alliums*, got more emphasis on the analysis and characterization of

its precursor compounds. This theory was approved by discovering the precursor molecule Alliin, which is a derivative of the amino acid cysteine. Alliin could be isolated and synthesized [7], [9].

After the first findings, intensive research approaches started to elucidate the complicated chemical properties of *Allium* volatile compounds. Especially to find secondary aroma compounds and to investigate the health benefits of them. The sulfides of onion and garlic oils are considered as secondary aroma compounds. Many types of research on these molecules, and their chemistry and biological effects are still ongoing [7].

1.2 *Allium cepa*

Onion is one of the most popular food ingredients in the world [10]. According to FAO data, the production of dry onion bulbs in 2014 was more than 88 million tons. The leading producers are China 25%, India 22%, United States 4%, Egypt 3%, and Iran 2% [11] (Figure 1-2).

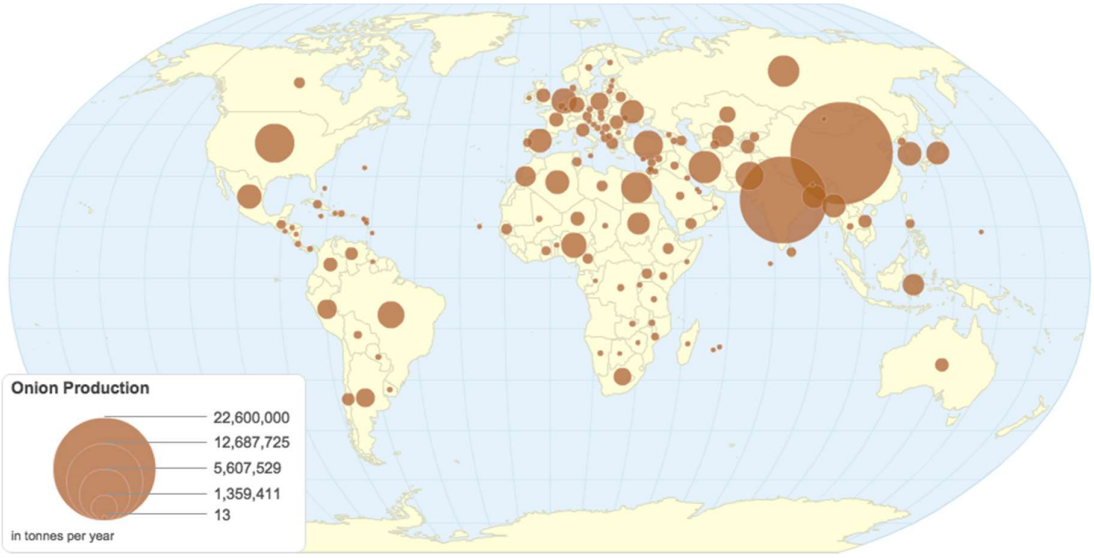


Figure 1-2 Onion production over the world [11]

Onion in history

Onion is cultivated since ancient times and was used in herbal medicine besides its importance in flavoring and nutrition [12]. Red, yellow and white onions can be eaten raw, cooked, fried, dried, baked or roasted. Onion is also used as a seasoning [12]–[14].

The name "onion" is derived from a Latin word *"unio"* which means: one large pearl [12]. The ancient Egyptians draw onions on pyramid walls and in tombs. They used them for funeral offering and embalming [15]. Onion is also mentioned in the Bible, Talmud, and Quran [12]. In some cultures, such as Jains and Buddhists, they avoid eating or using onion [16]. There are lots of stories, articles, and even jokes regarding onion's benefits. However, many reports do not provide evidence or scientific investigations to link onion's chemical substances with proven medical effects.

The plant onion

Allium cepa is a perennial plant with a fibrous root system, having 3-18 large and tubular leaves often covered with a waxen bloom. Leaves have a base of juicy, fleshy and proliferative scales [17], [18]. Onion leaves cover each other and form the bulb. The outer scales of the bulb are dry (Figure 1-3). Bulb's weight is 20-250 g, and it can reach 1000 g [12], [18]. The flower stalk is about 150 cm in height. It is inflated, hollow, and terminates in multi-flowered inflorescence. The optimum growth temperature for *Allium cepa* is 22-28 °C. The plant tolerates up to minus 6-7 °C, but it needs a long day to flower [12].

Figure 1-3 *Allium cepa* – the plant [19]

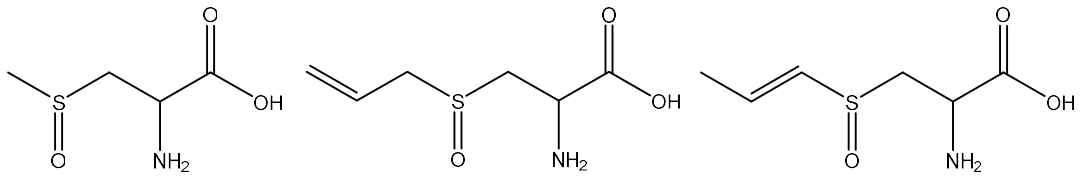
There are more than 150 onion varieties. They differ from each other by the color of their external scales (white, yellow, cream, brown, violet), and bulb's form (spherical, flat-spherical). Bulbs also differ by the number of rudiments germs, duration of the vegetative period, and taste [18], [20]–[22].

The bulbs contain 8-14 % sugars, 1.5-2 % protein, and 2-14 mg of vitamin C, carotin, vitamins B1, B2, PP, U, mineral salts, and amino acids. Onion also contains essential oils formed upon alliinase reaction (0.012-0.162 %), which give it a specific smell and pungent taste [12], [20].

Onion is considered as an important source of phenols, flavonoids, fructo-oligosaccharides and sulfur compounds [23], [24]. Many of these components have beneficial bioactivity [23]–[25]. Onion bulb extract is potential to be used in the treatment of keloids and hypertrophic scars [26]. Moreover, the bulbs are active as hypocholesterolemic, hypolipidemic, anti-hypertensive, anti-diabetic, antimicrobial, antioxidant and prebiotic remedies [23].

Volatile compounds in common onion (*Allium cepa*)

Most of the *Alliums* have the same precursors of aroma compounds; cysteine sulfoxides (S-alk(en)yl-l-cysteine-S-oxide) (Figure 1-4) which are located in the cytoplasm. Methiin is located in many *Allium* species in more or less high amounts. Garlic contains alliin, while onion has isoalliin. This difference cause a complete diverse pattern of their aroma compounds. Cysteine sulfoxides amounts are also different, while they can reach 1.2 % in garlic related to its fresh weight, onion contains about 0.25 %. Therefore, in order to yield "onion oil" using steam distillation high amounts of onions are required [7].



Methiin Alliin Isoalliin

Figure 1-4 The main cysteine sulfoxides in *Alliums.* Methiin, Alliin, and Isoalliin [7]

When *Allium* plant material is crunched, cysteine sulfoxides are subjected to alliinase enzyme, a C-S lyase located in the vacuoles, giving sulfenic acids. Despite the difference between alliinase isoenzymes in onion and garlic, they catalyze basically the same

reactions. Alliinase targets the cysteine sulfoxides in their substituents (methyl-, ethyl-, propyl-, 1-propenyl-, or 2-propenyl-group). As a result, the primary aroma substances will be thiosulfinates or mixed thiosulfinates [1], [7], [23].

Cutting fresh onion causes a particular odor, and the eyes will tear. This is related to reactive sulfur compounds which are released through rupturing onion cell structure (Figure 1-5) [7], [27], [28]. The enzymatic reaction between cysteine sulfoxides and alliinase produces pyruvate, ammonia and several sulfenic acids according to the S-attached substituents in the cysteine sulfoxide [29].

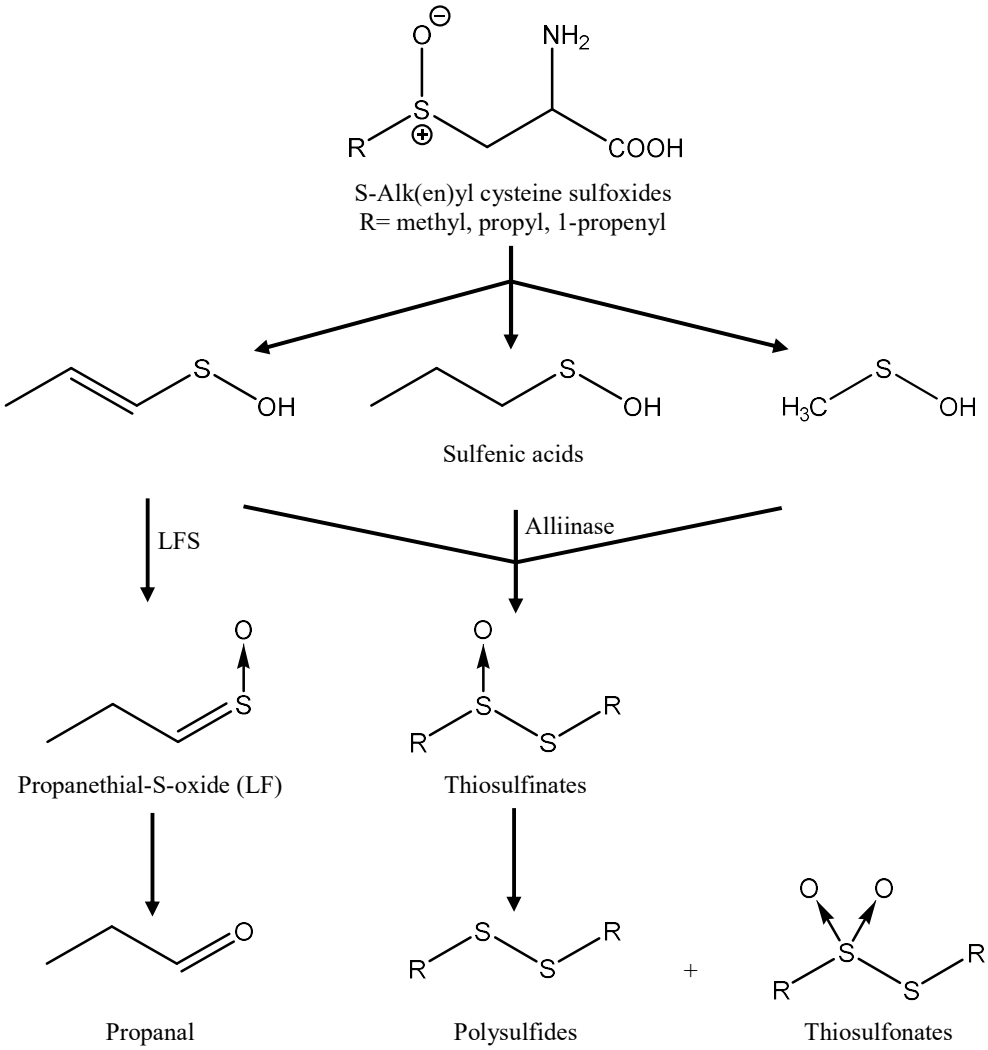


Figure 1-5 Alliinase and LF synthase reactions [7], [30]

1-Propenyl sulfenic acid is mostly transformed into propanethial S-oxide by LFS, propanethial S-oxide is known as the lachrymatory factor (LF) [31], LF starts to appear directly after cutting the onion with almost complete disappearance in half an hour [32]. Water presence causes LF degradation into propanal (Figure 1-5) [30], [32].

The odor of fresh cut onions can come from other sulfenic acids which condense into thiosulfinates [7], [27], [32]. The thiosulfinates usually are unstable and turn into the secondary aroma; thiosulfonates, polysulfides, and other compounds [27], [29].

Summary of the secondary aroma compounds in onion could be Figure 1-6. The main products are cepaenes, disulfides, polysulfides, and zwiebelanes[28]. There are also some organoselenium compounds that occur in onion and some other *Alliums*[7].

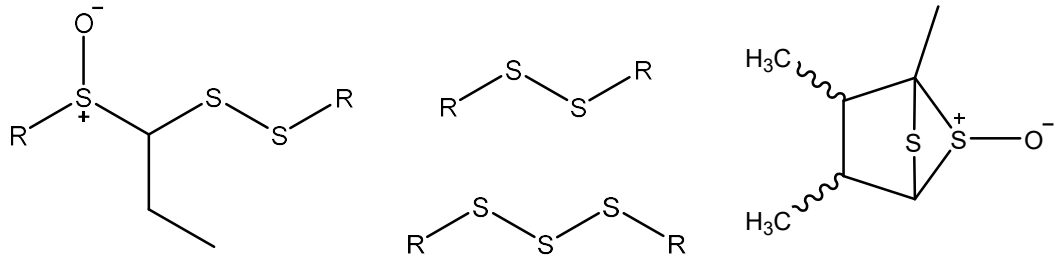


Figure 1-6 The main sulfur secondary aroma compounds following tissue disruption in onion [28]

1.3 *Allium cepa* diseases

As other vegetable crops, onions are also susceptible to numerous foliar. Pathogens can attack onion's bulb, root or leaves. Such diseases can reduce onion's yield and quality, which can be caused by fungi, bacteria, viruses, insects, mites and nematodes [33]. Some of the most common onion diseases and their causative agents are listed in the following:

Basal rot (Bottom rot)

Causative agent: *Fusarium oxysporum, Fusarium proliferatum.*

Basal rot is present in most parts of the world where onions are grown. It can attack onions at all stages of their growth, from seeding to stored bulbs and can cause up to 90% loss of the seeding [34]. The incidence of basal rot is more in the area where onion crops are grown

continuously. The temperature of 22 to 28°C is considered optimal for basal rot development [33].

Neck rot

Causative agent: *Botrytis aclada (B. allii), B. squamosal,* and *B. cinerea.*

Neck rot is one of the common bulbs destroying diseases. The fungus usually infects the mature plants through their neck tissues or wounds in the bulbs [33].

Blue mold

Causative agent: *Penicillium polonicum*, *P. glabrum,* and *P. expansum.*
The infection starts with yellowish plots and watery soft spots on the bulbs, followed by a blue-green mold of spore-producing conidiophores on the infected place. *Penicillium* species usually attack bulbs through tissues damaged by bruises, wounds, sunscald or freezing [35].

Damping-off

Causative agent: *Pythium sp, Phytophthora sp, Rhizoctonia solani,* and *Fusarium spp.*

Damping-off is an important disease of onion during nursery stage. It causes about 60 to 75% damage to the crop. It can delay seedling emergence in addition to root and basal rot. Moderate temperature and high soil moisture along with high humidity especially in the rainy season increase the development of this disease [33].

Purple blotch

Causative agent: *Alternaria porri.*

Purple blotch is an important disease of onion and garlic prevalent in all the onion growing areas over the world. Temperature range from 21 to 30°C and relative humidity 80 to 90% favors the development of this disease [33].

Stemphylium blight

Causative agent: *Stemphylium vesicarium*

Stemphylium produces significant damage either alone or with *Alternaria porri*. In some areas, foliage losses of 80 to 90% have been recorded [33].

Downy mildew

Causative agent: *Peronospora destructor*

Downy mildew is a widely distributed destructive disease of onion crops. Considerable losses of seed and bulb production of onion crops have been reported due to onion's infection with *Peronospora destructor* [36].

White rot

Causative agent: *Sclerotium cepivorum*

White rot is present in many areas of the world where *Alliums* are cultivated [37].

Onion smut

Causative agent: *Urocystis cepulae*

Onion smut is probably present in most *Allium* cultivating areas. The fungus spores have the ability to survive in the soil for many years [38].

Black mold

Causative agent: *Aspergillus niger*

Black mold is one of the most important postharvest diseases in hot climates. It is a very common disease wherever onion or garlic are stored [39].

Anthracnose/Twister /Seven curl disease

Causative agent: *Colletotrichum gloeosporiodes.*

This infection is reported to be widespread over the world, but it is more common in the tropics and subtropics [33].

Pink root rot

Causative agent: *Phoma terrestris.*

Pink root rot is present in areas with high soil temperatures. It mainly infects onion and garlic. Usually, it occurs in association with *Fusarium* basal rot. In these situations, it can be difficult to determine the relative importance of each disease [33].

Bacterial soft rot

Causative agent: *Erwinia sp, E. carotovora, E. chrysanthemi, E. herbicola,* and *E. rhapontica.*
Soft yellow to brown rot starts in bulb center by releasing fetid-smelling, viscous and watery fluid. It can infect most of the *Allium* species [35].

Sour skin

Causative agent: *Pseudomonas cepacia.*

Sour skin is reported from all over the world. Damages often appear in the stored onions, but infection usually begins in the fields. Sour skin can be serious causing 5–50% yield loss [40].

Bulb canker-skin blotch

Causative agent: *Embellisia allii*.

Bulb canker is one of the major diseases in onion and garlic bulbs during storage [41].

Bacterial brown rot

Causative agent: *Pseudomonas aeruginosa.*

Bacterial brown is a severe disease of onions during storage. The infection usually occurs through the wounds [42].

Root-knot nematode

Causative agent: *Meloidogyne spp.*

Root-knot nematodes are common pathogens of vegetable crops all over the world. They affect both the quantity and quality of the yields. They can also interact with other plant pathogens leading to increased damage caused by other diseases. In onions, this infection may cause bulb weight reduction by 50 to 70% [33].

Thrips

Causative agent: *Thrips tabaci.*

Thrips are spread around the world. These pests attack onions, garlic, and several other crops [43].

Onion maggot

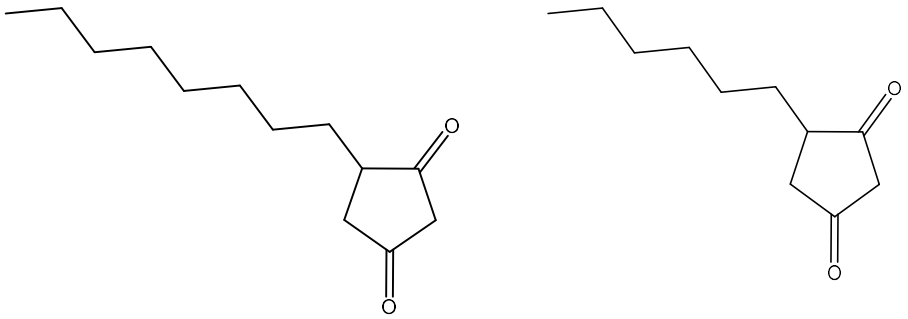
Causative agent: *Delia antique,* and *D. patura*

Maggot is one of the onion's pests. It can cause 20 - 90% losses in high temperate regions [44].

1.4 Detecting infection markers in onion bulbs

Finding new changes and substances in infected onions was the target of some projects in the past three decades. The results have been varied using different pathogens and different analytical methods.

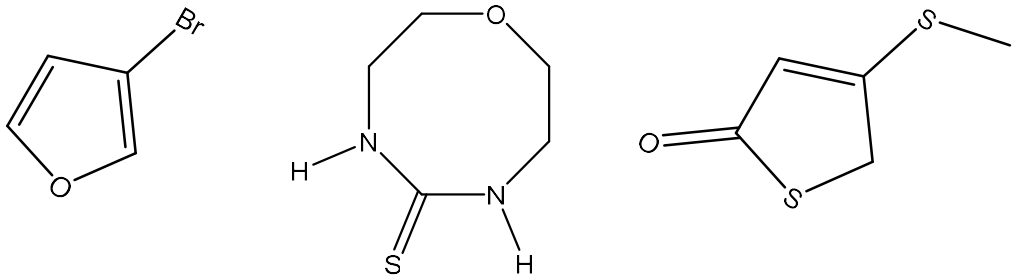
In 1990, infected onion bulbs by *Botrytis cinerea* were extracted by ethanol and tested using TLC and HPLC. 1,3-Dion-5-octyl-cyclopentane (tsibulin 1d) and 1,3-dion-5-hexyl-cyclopentane (tsibulin 2d) (Figure 1-7) were isolated from the extracts as phytoalexins accumulated in onion bulb scales in response to *B. cinerea* infection [45].



1,3-dion-5-octyl-cyclopentane 1,3-dion-5-hexyl-cyclopentane

Figure 1-7 The structure of Tsibulin 1d and Tsibulin 2d

Another study published in 2004 used GC-MS to analyze the volatile metabolites of onion bulbs headspace gas. In this study, the onions were inoculated with three different pathogens, *Erwinia carotovora* ssp*. carotovora, Fusarium oxysporum*, and *Botrytis allii*. The study showed that 3-bromofuran was specific to *E. carotovora* ssp. *carotovora*. 1-Oxa-4,6-diazacyclooctane-5-thione and 4-mercapto-3-(methylthio)-ς-(thiolactone)-crotonic acid (Figure 1-8) were located only in onions inoculated with *F. oxysporum* [46].



3-Bromofuran 1-Oxa-4,6-diazacyclooctane-5-thione 4-Mercapto-3-(methylthio)-ς-(thiolactone)-crotonic acid

Figure 1-8 The structure of substances found in *E.carotovora* and *F.oxysporum* infections

Acetone, propyl carbamate, acetic acid-hydrazide, thiirane, 1-Bromo-1-propene, 1-(methylthio)-1-propene and 1-ethenyl-4-ethyl-benzene (Figure 1-9) were considered specific to *B. allii* [46].

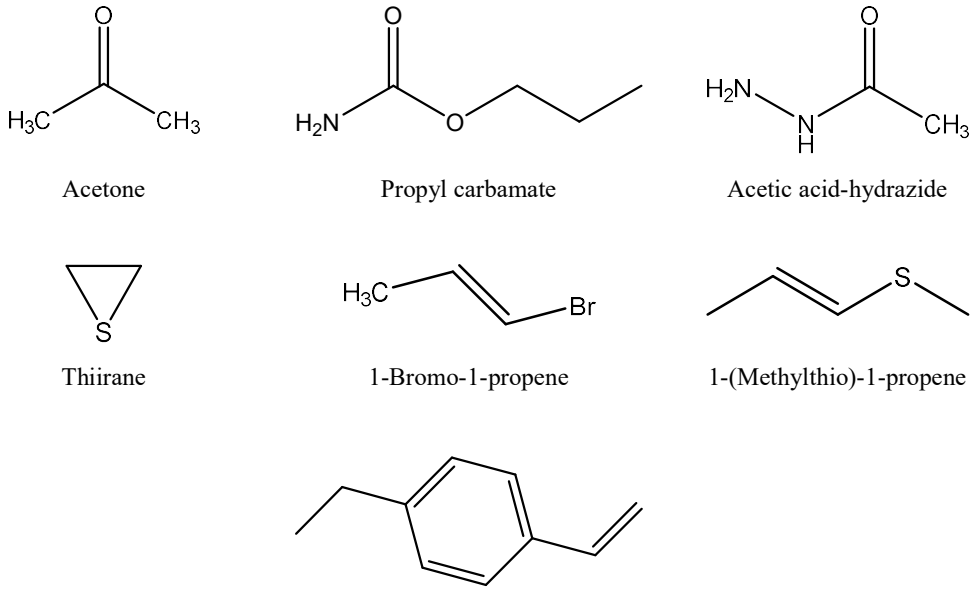


Figure 1-9 The structure of substances found in *B.allii* infection

Three substances: 1,1-dimethylethyl-urea, 4,4-dimethyl-1,3-diphenyl-1-(trimethyl silyloxy)-1-pentene and 3,7,7-trimethyl-, bicyclo[4.1.0]hept-4-en-3-ol (Figure 1-10) were found in all infected bulbs, without presence in control bulbs [46].

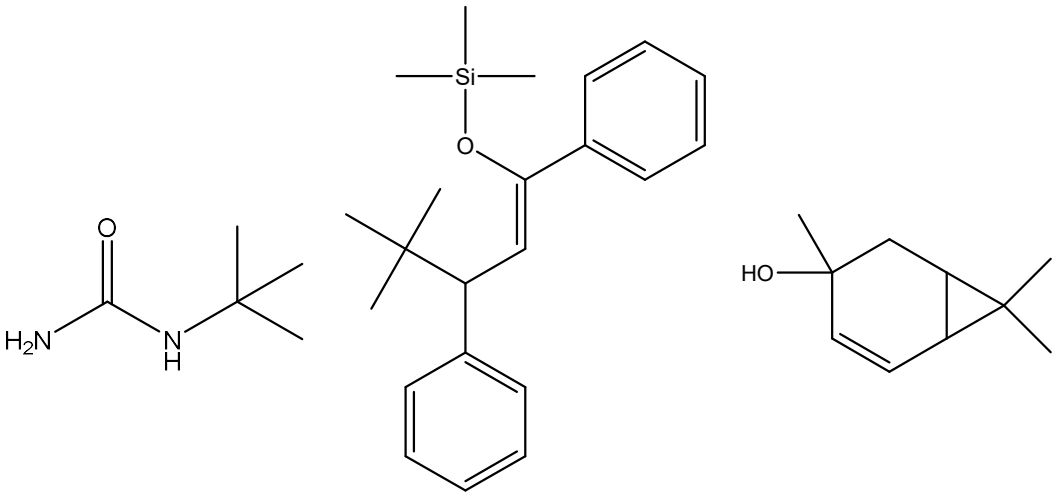
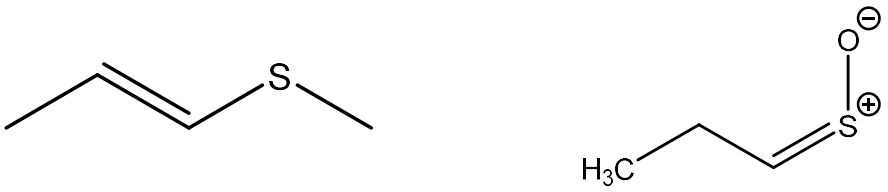


Figure 1-10 The structure of substances found in *B.allii, E.carotovora* and *F.oxysporum* infections

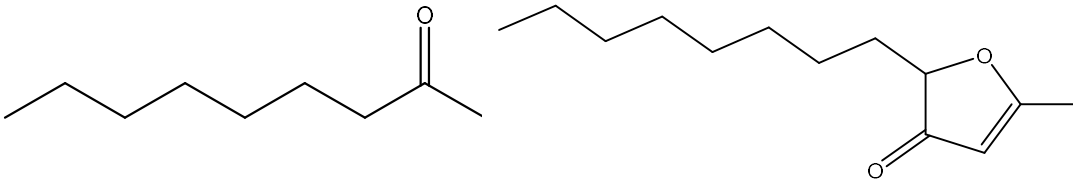
In 2011, Li and Schmidt tried to analyze the headspace volatiles using GC-MS and gas sensor array [47]. Bulbs were inoculated with *Botrytis allii* and *Burkholderia cepacia*. 1-(methylthiol)-1-propene and Z-propanethiol-S-oxide (Figure 1-11) were detected only in *B. allii* infection.



1-(Methylthio)-1-propene Z-Propanethiol-S-oxide

Figure 1-11 The structure of substances found in *Botrytis allii* infection

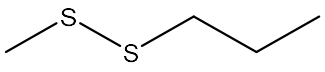
2-Nonanone and 2-octyl-5-methyl-3(2H)-furanone (Figure 1-12) were found only in *B. cepacia* infections [47].



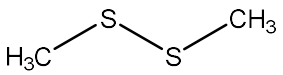
2-Nonanone 2-octyl-5-methyl-3(2H)-furanone

Figure 1-12 The structure of substances found in *B. cepacia* infection

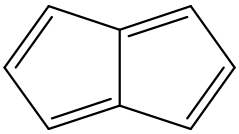
Eleven other substances (Figure 1-13) were found in both the *B. cepacia* and *B. allii* infected bulbs (methylpropenyldisulfide, dimethyldisulfide, methylpropyldisulfide, pentalene, 2,5-dimethylthiophene, 1-nonanol , 4-(1,2-dimethyl-cyclopent-2-enyl)-butan-2-one, n-dodecane, dipropyltrisulfide, 2-tridecanone, aromadendrene) [47].



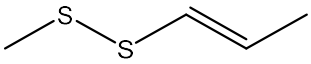
Methylpropyldisulfide



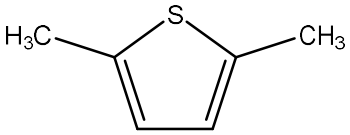
Dimethyldisulfide



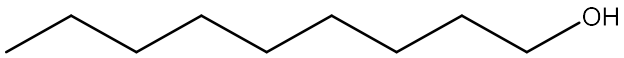
Pentalene



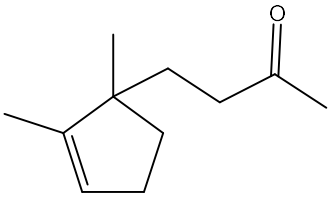
Methylpropenyldisulfide



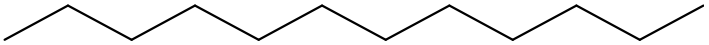
2,5-Dimethylthiophene



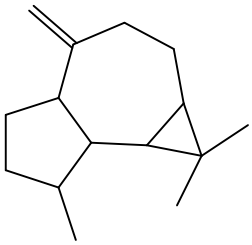
1-Nonanol



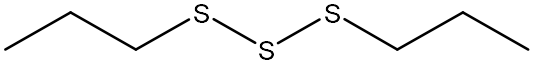
4-(1,2-Dimethyl-cyclopent-2-enyl)-butan-2-one



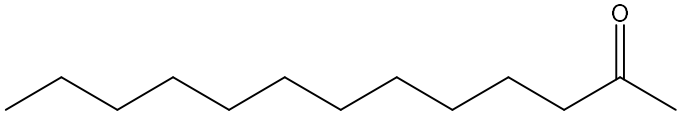
n-Dodecane



Aromadendrene



Dipropyltrisulfide



2-Tridecanone

Figure 1-13 The structure of substances found in *B. cepacia* and *B. allii* infection [47]

The same study also found a group of compounds including dipropyldisulfide (DPDS), 1-propanethiol, 2-hexyl-5-methyl-3(2H)-furanone, 2,4-octanedione, and 2-undecanone (Figure 1-14). These compounds were detected in healthy and infected bulbs, but the concentrations in infected bulbs were relatively higher [47].

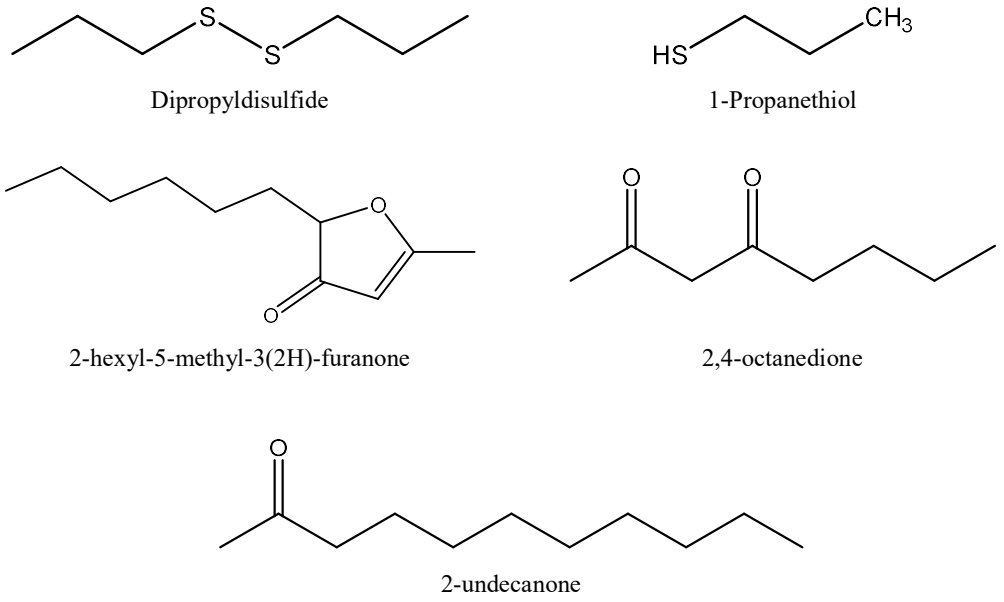


Figure 1-14 Compounds detected in healthy and infected bulbs with different concentrations [47]

In 2012, infected bulbs by *Pseudomonas aeruginosa* were tested using GC-MS to find the unique compound in infected bulbs. Pantolactone, 4,5-dihydro-4,5-dimethylfuran-2(3H)-one, myristic acid, and linoleic acid (Figure 1-15) were found only in infected bulbs [48].

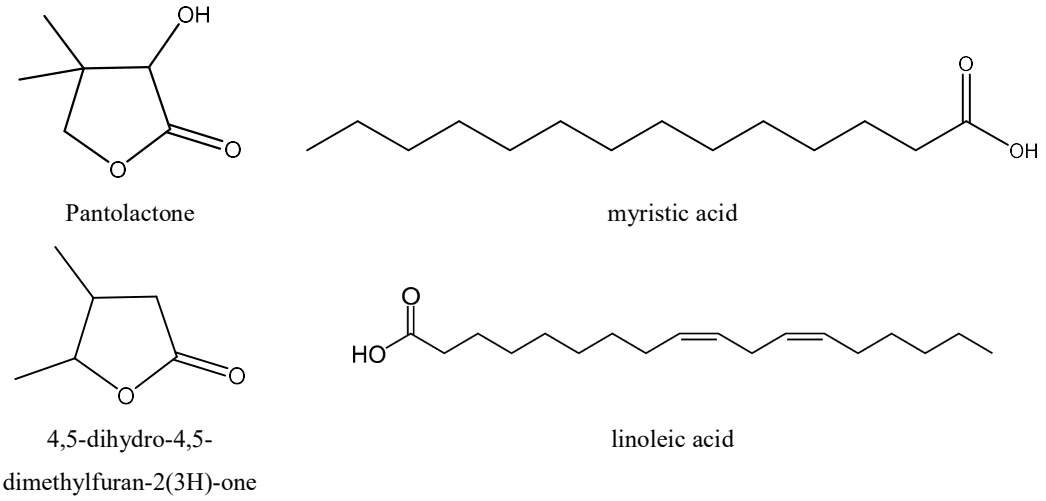


Figure 1-15 Compounds detected in infected bulbs by *Pseudomonas aeruginosa* [48]

1.5 Electrical Nose – Elektronische Nase (EleNa)

The human nose has been considered for a long time as the best tool to distinguish the smell or taste of the different substances. Recently, new electrical devices have been developed to imitate the ability of human nose [49]. This development was important especially to find a fast, inexpensive and mobile analyzer to detect volatile compounds in complex mixtures [50]. The technical imitation of the human nose and developing an electrical one were started in 1982 [51]. In their research, Persaud and Dodd explained the principles of the chemosensor array, which is connected to an electrical data processor and a pattern recognition system [51] (Figure 1-16).

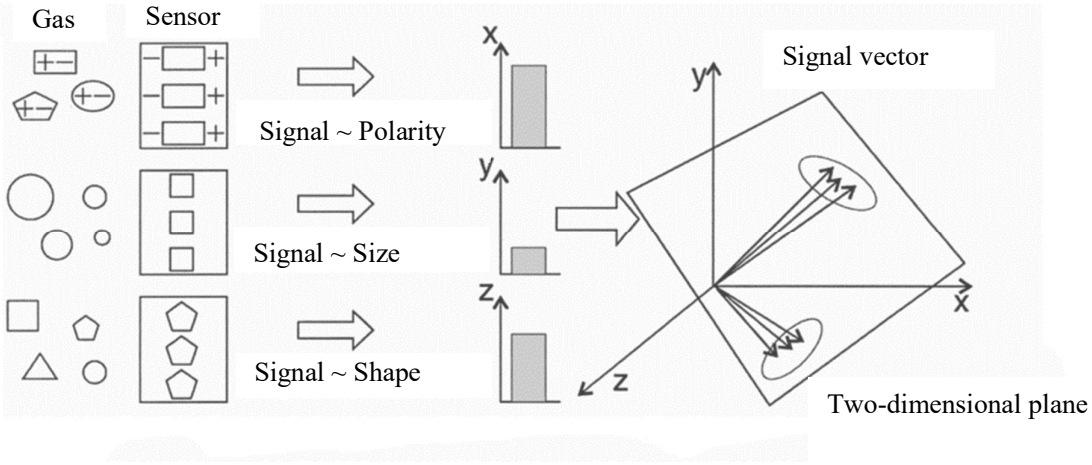


Figure 1-16 The function of the chemosensor array [52]

Three different sensors react to different chemical properties of volatile components like polarity, size or shape. The signal strength of each sensor is determined according to the molecular properties of the volatile compounds. Thus, each gas mixture has a characteristic signal pattern, which can be detected by gas sensor array [53]. The signal vector refers to the components of the mixture. Its direction defines the composition, and its length defines the total concentration [54]. In the case of several sensors, the signals are processed according to mathematical methods, and the information are transferred to a two-dimensional plane [55].

The similar chemical compounds can be detected by similar signal type and signal strength. The human smelling-cell has a filter function, which can only perceive odorous substances. In the produced gas sensors, there are no filters for odors. So, they can detect odor-active as well as odorless substances [55].

In summary, the concept of electronic noses is the detection of volatile organic compounds using special sensors. The sensors generate a characteristic fingerprint spectrum, which is compared with the previously stored patterns in the database [56].

Some established analytical methods like mass spectrometry and ion mobility spectrometry (IMS) can be used as an electronic nose. By these spectrometric methods, it is possible to determine substances when they have already been saved in the database [57].

Some of the potential applications of electronic noses are in the food industry, detection of explosive or harmful gases, ventilation systems or early detection and diagnosis of some diseases in the medical field. [53], [54], [57].

1.5.1 The electronic nose concept in developing the stores

The special ability of the electronic nose to detect volatile substances can be used in vegetable storage. For instance, detection of new compounds in onion stores can play an important role in saving the storage [58]. According to UNO's estimations, the global population will approximately be 9.7 billion in 2050. Even today with 7.6 billion, we are not able to provide enough food for every human being [59]. So, it is very important to store the existing foodstuffs correctly and to develop the efficiency of the stores.

In Germany, over 10,000 hectares are cultivated with onions. The onion harvest is over 460,000 tons per year (as in 2014) [11], and it should be stored efficiently from the late summer to the next harvest season without any change in the taste, the smell or the appearance. One important issue is that even small injuries on onion bulbs can significantly alter their properties. In this case, it would be possible to detect the volatile substances resulting from infection by the electronic nose. When it is integrated into the ventilation system, the system would be able to detect sources of contamination and warn the warehouse operator regarding the damage.

The application of such an electronic nose would be applicable by the use of thermal desorption spectroscopy (TDS) or ion mobility spectrometry, coupled with a gas chromatograph. This would allow a sufficient selection and sensitive detection, whereby even small traces in the air, which indicate an attack, would be detectable.

2. AIMS AND OBJECTIVES

One of the onion's characteristics is releasing volatile compounds. Detection of some compounds in damaged bulbs by analytical devices can protect the rest of the crop. The detection of defective onions due to substances in the air could also be attributed to further storage shortcomings, e.g., microbial contamination or fungal infection. This project aims to find, identify and test the main volatile infection markers.

2.1 Objectives

- Finding a specific pattern of volatile compounds for the infection in *Allium cepa* L. using HPLC.
- Finding the markers of onion infections caused by four fungal and one bacterial pathogen.
- Structural identification of the main infection markers.
- Testing the bioactivity of the new substances (antifungal and antitumor activities).
- Investigating the markers in other *Alliums* infections.
- Quantitive analysis of the markers.
- Testing the markers using GC and IMS for early detection of infections.
- Synthesizing the markers.

3. MATERIALS AND METHODS

3.1 Plant materials

Plant materials (control and infected *Allium cepa* L. bulbs) were mainly provided by JKI (Julius Kühn Institut, Quedlinburg, Germany). The bulbs were extracted and tested as control and infected samples by four types of fungal infections (*Fusarium oxysporum, Fusarium proliferatum, Penicillium sp,* and *Botrytis aclada*) and one type of bacterial infection (*Erwinia carotovora*).

Similar experiments using *Allium cepa* L.*, Allium porrum* L.*, Allium fistulosum* L. and *Allium sativum* L. provided from supermarkets (Tegut, Bioland trade mark) in Marburg city were also conducted. Control and infected bulbs of each species were extracted and tested.

Wild Allium species (*Allium altaicum* Pall., *Allium cornutum* Clementi ex Vis.*., Allium bastard* and *Allium pskemense* B. Fedt.) were cultivated and harvested in IPK Gatersleben (Germany). These species were also extracted and tested as control and infected samples. The list of tested species and infecting pathogens are shown in Table 3-1.

Plant name (Cultivar/Tax nr.)	Pathogen	Infection place/date	Disease	Extracted samples	
				Healthy	Infected
Allium cepa L. (Hytech, Sturon)	*Fusarium oxysporum* (fungi) DK-Nr. 287	JKI 2014-2018	basal rot	25	54
Allium cepa L. (Hytech, Sturon)	*Fusarium proliferatum* (fungi) DK-Nr. 582	JKI 2015-2018	basal rot	6	24
Allium cepa L. (Sturon, Stuttgarter Riesen)	*Penicllium sp.* (fungi) DK-Nr. 901	JKI 2015-2018	blue mold	9	19
Allium cepa L. (Sturon, Stuttgarter Riesen)	*Botrytis aclada* (fungi) DSM 6281	JKI 2016-2018	neck rot	8	21
Allium cepa L. (Wellington, Hytech)	*Erwinia carotovora* (Bacteria) GSPB-Nr. 426	JKI 2016-2018	bacterial soft rot	17	29
Allium cepa L. (Tegut market)	*Fusarium oxysporum* (fungi) DK-Nr. 287	Our laboratory 2016-2017	basal rot	6	38
Allium porrum L. (Tegut market)	*Fusarium oxysporum* (fungi) DK-Nr. 287	Our laboratory May 2017	basal rot	7	8
Allium fistulosum L. (Tegut market)	*Fusarium oxysporum* (fungi) DK-Nr. 287	Our laboratory May 2017	basal rot	3	3
Allium sativum L. (Tegut market)	*Fusarium oxysporum* (fungi) DK-Nr. 287	Our laboratory May 2017	basal rot	8	12
Allium altaicum Pall. (5456)	*Fusarium oxysporum* (fungi) DK-Nr. 287	Our laboratory May 2017	basal rot	3	3
Allium pskemense B. Fedt. (0514)	*Fusarium oxysporum* (fungi) DK-Nr. 287	Our laboratory May 2017	basal rot	3	3
Allium cornutum Clementi ex Vis. (1748)	*Fusarium oxysporum* (fungi) DK-Nr. 287	Our laboratory May 2017	basal rot	3	3
Allium bastard (1077)	*Fusarium oxysporum* (fungi) DK-Nr. 287	Our laboratory May 2017	basal rot	3	3

Table 3-1 List of *Allium* species which were extracted and analyzed.

3.1.1 Preparation of infected bulbs in our laboratory

Fusarium oxysporum was obtained from (JKI) Julius Kühn Institute. PDA plates were prepared in the presence of small pieces of the fungi.

3,9 g of PDA were dissolved in 100 ml of distilled water. The mixture was heated to boil and sterilized by autoclaving at 121°C for 15 minutes. 10 ml of PDA media was added to petri plate and 0.05 g of *Fusarium oxysporum* was spread on it. *Fusarium* plates were tightly closed incubated at 28°C for further use.

Three small wounds were made at the base of healthy bulbs (Figure 3-1) using a sterilized needle. Small amounts (5-10 µL) of the cultivated fungi on PDA were placed in contact with the wounds. The wounded bulbs were stored in closed containers to achieve high humidity and cultivated at 28°C for five days.

Figure 3-1 The three wounds place for *Fusarium oxysporum* inoculation in the lab.

In some bulbs, cultivated fungi were in direct contact with the root without making wounds. In this method, more time (about ten days) was required for the infection to start. It should also be mentioned that some bulbs did not develop the fungal infection.
The three wounds method was considered for all *Alliums* infection in our laboratory for a fast and effective process.

3.1.2 Preparation of ethyl acetate extracts of plant material

Ethyl acetate can help to extract many polar and non-polar compounds. After preliminary extractions using different solvents (methanol, ethyl acetate, diethyl ether, cyclohexane), extraction using ethyl acetate was considered in this project to get phenols, flavonoids as well as sulfur compounds.

Fresh healthy and infected onion bulbs were cleaned with removing dry parts, weighted (50-75 g) and cut in small pieces and grounded in a mortar. About 10-20 ml of phosphate buffered saline (PBS) were added 0.01 M (pH=7.4) to provide the optimal environment for alliinase reaction and the biosynthesis of volatile compounds. After one-hour of incubation at room temperature 20-25 °C, the mixture was extracted four times using ethyl acetate; 200 ml, 100 ml, 100ml, 100ml (total of 500 ml) and transferred carefully into a bottle. The organic phase was dried using magnesium sulfate ($MgSO_4$). The solvent was evaporated carefully by rotary evaporator at 28°C up to ca. 20 ml residue. This was then divided into 2-3 vials in order to be evaporated under nitrogen gas until to an oily residue (Figure 3-2). The extracts were stored at -20°C until further processing (generally HPLC).

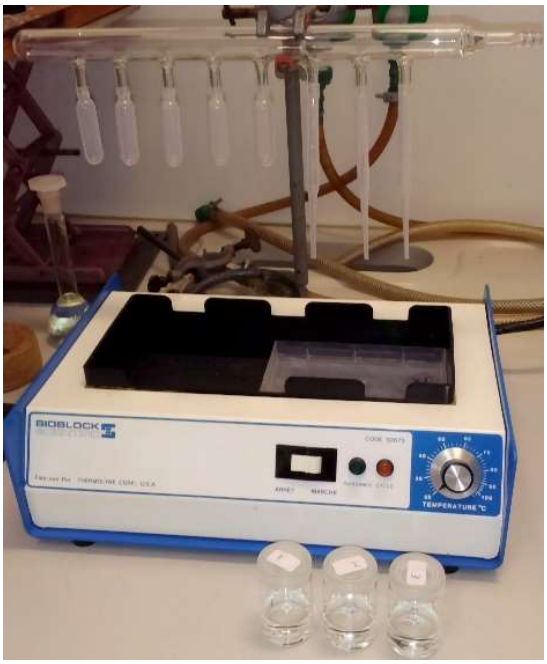


Figure 3-2 The vials are ready for evaporation using nitrogen gas.

3.1.3 Preparation of plant material extract with alliinase inhibition

Alliinase mediated reactions are very critical in onion chemistry. The investigated markers could be some products of these reactions. In order to understand if alliinase played any roles in the markers production, fresh infected onion bulb was cleaned with removing dry parts, weighted and grounded carefully in a mortar in the presence of liquid nitrogen to inhibit alliinase during this process. The next step was powder lyophilization overnight to obtain dry onion powder (Figure 3-3).

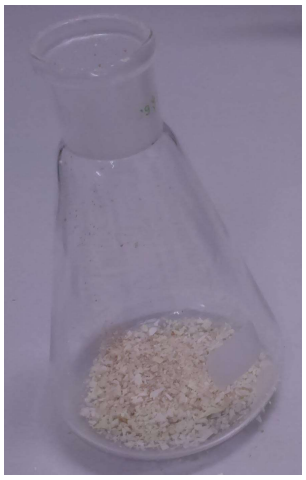


Figure 3-3 Dry onion powder after alliinase inhibition.

The powder was extracted four times using ethyl acetate; 200 ml, 100 ml, 100ml, 100ml (total of 500 ml) and transferred carefully into a bottle. The solvent was dried using magnesium sulfate ($MgSO_4$) and evaporated carefully by rotary evaporator at 28 °C up to ca. 20 ml residue. This residue was then divided into 2-3 vials in order to be evaporated under nitrogen gas until to oily drops. The extract was kept at -20 °C until further processing.

3.1.4 Preparation of *Fusarium pxysporum* extract

Extraction of infected bulbs can also include extraction of some fungal or bacterial products. To ensure that our investigated markers are related to natural onion products, we obtained *Fusarium oxysporum* from (JKI) Julius Kühn institute and prepared PDA plates in the presence of small pieces of the fungi. *Fusarium* plates were tightly closed incubated at 28 °C for 72 hr.

The PDA containing growing *Fusarium oxysporum* was extracted four times using ethyl acetate (total of 500 ml) and transferred carefully into a bottle. The organic solvent layer was dried using magnesium sulfate and evaporated carefully. The extract was saved at -20 °C until further processing.

3.1.5 Preparation of plant material extracts using distillation apparatus

To get the oily volatile extract from *Allium cepa* L. bulbs, Clevenger apparatus was employed. Infected onions were cleaned with removing dry parts, weighted (150-250 g) and cut into small pieces. Plant material and demineralized water (500 ml) were cooked until boiling and distilled over Clevenger apparatus.
Because oily drops could not be noticed during distillation, 2 ml ethyl acetate was added to the collecting part to catch the oily drops (Figure 3-4).

Figure 3-4 Extracting onion bulbs using a Clevenger apparatus.

After one hour of distillation, the organic phase (ethyl acetate) was collected, dried via magnesium sulfate, filtered, and evaporated under nitrogen gas up to an oily residue. The extracts were analyzed fresh or stored at -20 °C until further processing (preparative HPLC).

3.2 Chemicals, equipment, and devices

Chemicals, equipment and devices used in this research are listed in the following tables.

Chemical	**Source**
PBS	Sigma-Aldrich
Ethyl acetate	Merck, Darmstadt
Magnesium sulfate	Merck, Darmstadt
Methanol	Merck, Darmstadt
Dichloromethane	Merck, Darmstadt
DMDHF	Abcr GmbH, Karlsruhe, Germany
PDA	Sigma-Aldrich
PRMI-1640 medium	Sigma-Aldrich
Cyclopentandione	Sigma-Aldrich
Quercetin	Merck, Darmstadt
Spiraeoside	Abcr GmbH, Karlsruhe, Germany
XTT cytotoxicity proliferation assay kit	AMS Biotech, UK
Fetal bovine serum	Gibco BRL, Grand Island, NY, USA
H_2O_2 35%	Acros Organics, New Jersey, USA
Diethyl ether 99,5%	Grüssing GmbH, Filsum
Heptanal	Sigma-Aldrich
Butynol	Sigma-Aldrich
Butyl lithium	Sigma-Aldrich

Table 3-2 Chemicals used in this project

Equipment or device	**Source**
Agilent 1100 HPLC	Agilent, Santa Clara United States
Waters Preparative HPLC system (600E System controller, 991 Waters PDA)	Waters, Milford, USA
VP 250/2 Nucleodur 100-5 C18 EC HPLC Column	Macherey-Nagel, Düren
VP 250/4 Nucleodur 100-5 C18 EC HPLC Column	Macherey-Nagel, Düren
VP 250/10 Nucleodur 100-5 C18 EC HPLC Column	Macherey-Nagel, Düren
Shimadzu UV-2401 spectrometer	Shimadzu Suzhou Instruments, Suhzhou, Jiangsu, China
Shimadzu SCL10Avp HPLC system	Shimadzu Suzhou Instruments, Suhzhou, Jiangsu, China
Shimadzu GC 2014AFsc	Shimadzu Suzhou Instruments, Suhzhou, Jiangsu, China
ATR-FTIR Bruker spectrometer	Brucker, Billerica, Massachusetts
IMS	IUT Medical GmbH/Berlin, Germany
Emax Microplate reader	Molecular Devices, Sunnyvale, CA, USA
JEOL-ECA 500 NMR spectrometer	Jeol, Tokyo, Japan

Table 3-3 Equipment and devices used in this project

3.3 HPLC preparations and analyses

Healthy and infected *Allium* bulb samples were tested using HPLC methods developed to detect the markers fast and easy. Another method was developed to fractionate onion extracts for further tests as well as structure elucidation analyses.

3.3.1 Preparation of extract samples for HPLC analysis

Allium bulbs were extracted using method 3.1.2 or method 3.1.5 to get an oily residue. For HPLC analysis, the oily residue was weighted. Methanol was added to prepare a methanolic solution (4 mg/ml). Ultrasonic shaker was used to shake the extract solution for 1 min. The methanolic solution was filtered using a 0.25 µm syringe filter to get rid of any possible precipitation. The filtrate was filled into 2 ml HPLC vials. The samples were tested directly after preparation using HPLC and saved at -20 °C for further analyses.

3.3.2 HPLC analysis of healthy and infected *Allium* extracts (100 min)

To detect new compounds appeared in onion extracts after infection, a 100 min-method was developed and used to scan polar and nonpolar substances in *Allium* extracts. This method was applied to Agilent HPLC. Methanol/water gradient method was used with a constant flow rate of 0.8 ml/min through Nucleodur 100-5 C18 ec column (250 × 4 mm, 5 microns). Twenty µl of the sample were injected with auto-sampler. UV detector was set to 254 nm. The gradient used to analyze the samples is displayed in Table 3-4.

Time (min)	Flow rate (ml/min)	Methanol %	Water %
0	0.8	5	95
5	0.8	5	95
95	0.8	95	5
100	0.8	95	5

Table 3-4 The gradient program used in Agilent device for 100 min method.

3.3.3 HPLC analysis of healthy and infected *Allium* extracts (35 min)

To detect the investigated markers which seem to be nonpolar compounds, a 35 min-method was developed and used to test *Allium* extracts faster. This method was used in Agilent HPLC. Methanol/water gradient was used with a constant flow rate of 0.8 ml/min through Nucleodur 100-5 C18 ec column (250 × 4 mm, 5 microns). Twenty µl of the sample were injected. UV detector was set to 254 nm. The gradient used to analyze the samples is displayed in Table 3-5.

Time (min)	Flow rate (ml/min)	Methanol %	Water %
0	0.8	50	50
5	0.8	50	50
30	0.8	95	5
35	0.8	95	5

Table 3-5 The gradient program used in Agilent device for 35 min method.

This method was also used to test the purity of the markers fractions after preparative HPLC, and to test reference substances.

3.3.4 Preparative HPLC analysis of healthy and infected *Allium* extracts

For preparative separation, different isocratic methods were used to develop fast and effective separation of infection markers in infected *Allium* extracts. Waters HPLC system (600 E System controller and Waters 991 PDA) was used to collect the targeted peaks. Isocratic methanol/water flow was used with a constant flow rate of 7 ml/min through Nucleodur 100-5 C18 ec column (250 × 10 mm). 500-1000 µl of the sample were injected manually for each separation. The UV detector was set to 254 nm. The method used to analyze the samples is displayed in Table 3-6.

Time (min)	Flow rate (ml/min)	Methanol %	Water %
0 - 25	7	87	13

Table 3-6 Isocratic flow program used in preparative HPLC device (WATERS).

Extract fractions were collected using 100 ml flasks. The collected fractions were tested in Agilent HPLC system using method 3.3.3 to ensure their purity. Oily pure compounds were extracted from the fractions for the further investigations.

3.3.5 Extracting the pure compound for structure identification

After preparative separation (Method 3.3.4), the fractions were dissolved in methanol/water (87:13). In some cases, the further analysis required different solvents or dry materials. Therefore, the fractions were extracted using an organic solvent. For this extraction, three different solvents were tested; n-hexane, ethyl acetate, and dichloromethane. Dichloromethane was considered the best solvent for this step.

300 ml of water was added to each fraction in order to reduce the solubility of the investigated markers in water/methanol solution and to get good separation (without adding water, we get one mixture of methanol/water/dichloromethane). The markers were extracted three times using dichloromethane; 100 ml + 100 ml + 100 ml (total of 300 ml using a separation funnel).

After extraction, dichloromethane was dried using magnesium sulfate, and evaporated using rotary evaporator at 28°C up to ca. 2 ml volume. The residue was then transferred into 2 ml vials and evaporated under nitrogen gas carefully. This step was critical because the pure compound could evaporate easily during the process. The oily pure compounds were dissolved directly using deuterated dichloromethane for NMR analysis, or tightly closed for other further analyses.

3.3.6 HPLC-MS analysis of healthy and infected *Allium* extracts

To detect the investigated markers and to have a primary idea about the molecules, HPLC-MS System was used. The method was applied in Shimadzu HPLC and Sciex Q-Trap 2000 mass spectrometer. Methanol/water gradient (Table 3-7) was used within a constant flow rate (0.5 ml/min) through Nucleodur 100-5 C18 ec column (250 × 2 mm, 5 microns). 20 µl of the sample were injected by autosampler. The UV detector was set to 254 nm.

Time (min)	Flow rate (ml/min)	Methanol %	Water %
0	0.5	50	50
5	0.5	50	50
30	0.5	95	5
35	0.5	95	5

Table 3-7 The gradient program used in HPLC/MS device.

The triple quadrupole mass spectrometer was used with positive ionization to determine the mass to charge ratio for separated peaks using the settings in (Table 3-8). HPLC-MS analysis was also valuable to ensure the presence of the markers in the other *Allium* species after detecting the peaks in Agilent system.

Source type	ESI
Ion polarity	Positive
Curtain gas	10
Ion spray voltage	5500
Gas 1 (nebulizer gas)	20
Gas 2 (turbo gas)	0
Interface heater	200
Declustering potential	110
Entrance potential	11

Table 3-8 MS device's settings for HPLC-MS analysis

3.4 High resolution mass spectrometry (HRMS)

To have an idea about the elemental structure of the markers, high-resolution MS system was used. After preparative HPLC separation (Method 3.3.4 and Method 3.3.5), pure compound fractions were dissolved in 2 ml methanol. The samples were applied on AutoSpec double focusing magnetic mass spectrometer using positive ionization, heteroatom max: 20.

In AutoSpec system, positive electron ionization could also be done. EI^+ spectrums of the same samples were obtained for further structure interpretation using the settings in (Table 3-9).

Source type	ESI
Ion polarity	Positive
Scan begin	50 m/z
Scan end	500 m/z
Capillary	4500 V
End plate offset	-500 V
Collision cell RF	180.0 Vpp
Nebulizer	2.5 Bar
Dry heater	200 °C
Dry gas	6.0 l/min

Table 3-9 HRMS settings

3.5 Ultra violet spectroscopy (UV)

For a full structure identification, UV spectrums were obtained for the markers using Shimadzu UV-2401PC device. Samples were dissolved in Methanol and scanned in the range of 200-800 nm. A methanol reference sample was also used in this device.

3.6 Infra-red spectroscopy (IR)

Infrared spectroscopy is one of the most important methods to prove the functional groups in a substance structure. Infrared spectrums were achieved for the markers using Bruker-ALPHA ATR-FTIR device. Because it was impossible to apply the oily sample directly, 1 ml methanol was added to the oily substance (prepared in Method 3.3.5). Drops of the methanolic solution were applied on Bruker IR device. The sample was scanned after waiting for 10 sec to allow methanol to be evaporated.

3.7 Nuclear magnetic resonance spectroscopy (NMR)

Nuclear magnetic resonance spectroscopy is a very efficient method to get details about the electronic structure of the molecules and their individual functional groups. One and two dimensional NMR spectroscopy were performed for the investigated compounds using a JOEL 500 MH NMR system.

The performed NMR analyses for the markers were:

1. Proton ^{1}H NMR.
2. Carbon ^{13}C NMR.
3. COSY (COrrelated SpectroscopY) NMR.
4. HMBC (Heteronuclear Multiple Bond Correlation) NMR.
5. NOESY (Nuclear Overhauser Effect SpectroscopY) NMR.

To prepare the samples for NMR, pure oily substances (3-6 mg) were dissolved in deuterated dichloromethane CD_2Cl_2 (750 µl) and filled in NMR tubes. Masternova NMR software was used to read the resulted spectrums.

3.8 Bioactivity tests

To test the biological efficacy of the new compounds in infected onions, the following two experiments were applied. In this regard, healthy and infected onion bulb extracts were used to test the antifungal and antitumor activity.

3.8.1 Evaluating the antifungal activity

This experiment was a preliminary rapid trial to evaluate the sensitivity of fungi to the new compounds produced by the onion's bulb after infection. It was repeated three times to be sure if there is a potential efficacy.

The antifungal activity was tested against six different fungal microorganisms:

- *Aspergillus niger* Tiegh. (IRAN 1354)
- *Aspergillus flavus* Link (IRAN 1426)
- *Penicillium italicum* Wehmer (IRAN 1049)
- *Mucor hiemalis* Wehmer (IRAN 911)
- *Cryptococcus neoformans* (San Felice) Vuill. (DSM 15466)

- *Basidiobolus ranarum* Eidam (DSM 957)

Preparation of the fungal suspension was as follows: the surface of the seven-day-old fungi plate grown over a PDA media was rubbed smoothly with 10 ml sterile distilled water containing 0.01% Tween 20 in order to collect the spores. The suspension was then vortexed, centrifuged and washed three times using sterile distilled water. The fungal suspension was standardized to 10^6 cfu/mL.

1 mL of each fungal suspension was spread uniformly onto the surface of PDA agar plates (without filter). The plates were allowed to dry for 30 min. The oily extracts of healthy and infected bulbs were weighed and dissolved in DMSO to give the concentration of 10 mg/ml. 5 µl of each extract was dropped over the plates to form spots of infected onion extract (F) and others of healthy onion extract (H) (Figure 3-5). The Petri-dishes were sealed with parafilm and incubated at room temperature 24±2°C, with readings taken after 72h.

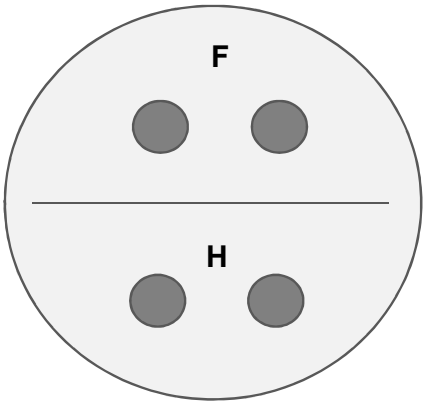


Figure 3-5 Spots of healthy and infected onion bulb extracts

3.8.2 Evaluating the antitumor activity

To assess the antitumor activity of the new substances in the infected *Allium cepa* L. extracts, a pre-clinical drug discovery process was applied. Healthy and infected *Allium cepa* L. ethyl acetate extracts were screened using XTT cell proliferation assay to see if there is superior activity in the infected onion extracts. Human bladder cancer cell lines T24 and UMUC3 were obtained from the department of urology and child urology at the university hospital of Giessen and Marburg for this assay.

3.8.2.1 Preparing human cell lines T24 and UMUC3

The human bladder cancer cell lines T24 and UMUC3 were kept in standard conditions in the department of urology and child urology at the university hospital of Giessen and Marburg. Cells were cultured in tissue culture flasks at 37°C. They were grown under humidified 5% CO_2 atmosphere in medium (RPMI 1640). The medium was supplemented with 10% fetal bovine serum and 2% penicillin-streptomycin (10,1000 U/ml penicillin and 10 mg/ml streptomycin)[60].

3.8.2.2 XTT cell proliferation assay

To check if there is any potential anticancer activity of the new substances produced in infected onions, T24 and UMUC3 cells were cultured in triplicates in 96-well plates with 2500 cells/well for T24 and 6000 cells/well for UMUC3. They were incubated for 24h before the next treatment.

Healthy onion extract, infected onion extract, Doxorubicin as a positive control, and the media as a negative control were added to wells in a dose-dependent manner (1.67, 1.33, 1.00, 0.67, 0.33, 0,27, 0.20, 0.13, 0.07, 0.03 mg/ml) (Figure 3-6**)**. After 48 hr of treatment, the cell supernatant was removed, XTT reagent was added. The plates were incubated with the reagent for 4 hr. By using a microtiter-plate reader, the absorbance was measured at 405 nm with 650 nm as a reference. The values were processed using Microsoft Excel.

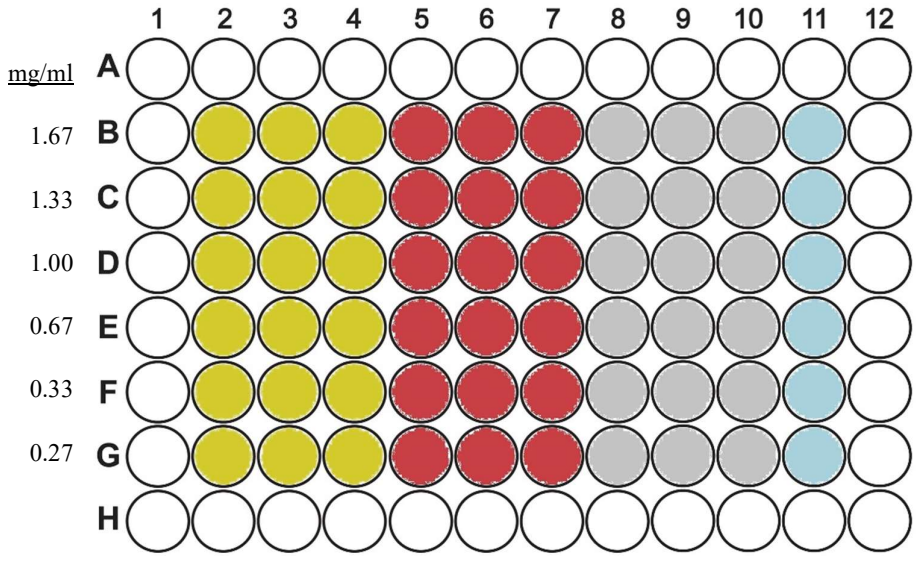


Figure 3-6 Well plate diagram for the antitumor activity test. Yellow color for healthy bulb extract, Red for infected one, gray for Doxorubicin as a positive control, and blue for media as a negative control.

3.9 Quantitative analysis of the markers

Because of the volatile nature of the investigated substances and not having the possibility to purchase them, dimethyl dihydrofuranone, a similar compound having a similar chromophore, could be used as an internal and external standard to generate the calibration curve. Because of the volatile nature of the markers and DMDHF, both internal and external standard methods were necessary to calculate the markers quantities in onion bulbs.

3.9.1 External standard

Dimethyl dihydrofuranone is a comparable substance of dihydrofuranones in the chemicals market, which has a similar chromophore in order to be used as an external standard. To make a calibration curve, 94% pure dimethyl dihydrofuranone was purchased from ABCR GmbH. Different concentration were prepared (1 , 0.75 , 0.5 , 0.25 mmol/l) using methanol as a solvent. Each concentration was measured three times by an Agilent HPLC using a 60 min method (Table 3-10).

The mean peak area for each concentration was calculated using Excel software to construct the calibration curve and to obtain the curve equation.

Time (min)	Flow rate (ml/min)	Methanol %	Water %
0	0.8	5	95
5	0.8	5	95
55	0.8	95	5
60	0.8	95	5

Table 3-10 The gradient program used in Agilent device for a quantitive analysis.

3.9.2 Internal standard

Infected onion bulbs by *Fusarium oxysporum, Fusarium proliferatum, Botrytis aclada, Penicillium sp*, and *Erwinia carotovora* were extracted using dimethyl dihydrofuranone as an internal standard to determine the amount of the investigated markers in the infected onion bulbs.

Three infected bulbs (150-300 g) were cleaned with removing dry parts and cut in small pieces and grounded in a mortar, with 5 ml of DMDHF solution (1 mmol/l). About 10-20 ml of phosphate buffered saline (PBS) were added (pH=7.4) to provide the optimal environment for alliinase reaction and the biosynthesis of volatile compounds. After one hour of incubation at room temperature 20-25 °C, the mixture was extracted four times using ethyl acetate (total of 500 ml) and transferred carefully into a bottle. The organic phase was dried using magnesium sulfate ($MgSO_4$). The solvent was evaporated carefully by rotary evaporator at 28°C up to ca. 20 ml residue. The residue was then evaporated under nitrogen gas until to an oily extract.

The oily extract was dissolved in 5 ml methanol and filtered using a 0.25 µm syringe filter to get rid of any possible precipitation. The samples were tested in Agilent HPLC three times. Peak areas for DMDHF and the investigated markers were recorded. The mean area value of each peak was replaced in the external standard equation to calculate its concentration (mmol/l). After extraction, the concentration of DMDHF (internal standard) was corrected to 1 mmol/l. The correction factor was used again for the concentrations of the markers. The concentration of each marker was calculated to the fresh onion weight W/W in ppm (mg/kg onion). This process was repeated three times regarding each infection.

3.10 Gas chromatography analysis (GC)

The investigated markers have been analyzed using Shimadzu GC 2014AFsc (AOC-5000 auto-sampler) with GC-solution Ver. 2.3 software. Heliflex column, 30 m long x 0.25 mm ID x 0.25 µm film thickness (5% phenyl- 95% methylpolysiloxane). GC device was equipped with a flame ionization detector (FID).

The available device was not coupled to a mass spectrometer. Therefore, each marker was scanned separately in order to determine its retention time. The pure oily marker samples were then dissolved in methanol. Other GC conditions were as in Table 3-11 and Table 3-12.

Carrier gas	N_2
Inlet temperature	200 °C
Detector temperature	300 °C
Equilibrating time	3 min
Split ratio	1:100
Column flow	1 ml/min
Average velocity	26.9 cm/sec
Pressure	84.2 kPa

Table 3-11 The GC analysis conditions

Rate	**Temperature**	**Hold Time**
-	70 °C	2 min
5 °C/min	200 °C	0 min

Table 3-12 GC operation mode. Running time was 28 min

The volatile compounds of infected onion bulb extracts by *Fusarium oxysporum* were analyzed via headspace GC scan using the parameters in Table 3-11, Table 3-12 in order to detect the markers of infection.

Needle	MSH 02-00B, Vol 2.5 ml, ID 28
Sample volume	2 ml
Incubtion tempraure	50 °C
Incubation time	5 min
Agi speed	500 rpm

Table 3-13 The headspace GC analysis settings

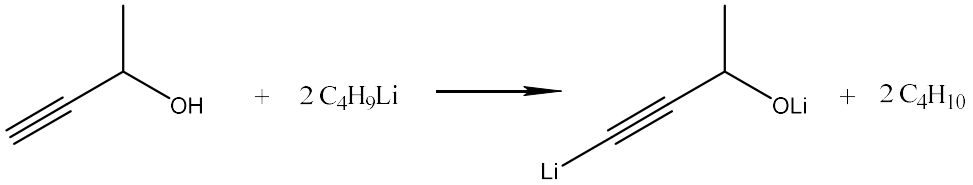
3.11 Ion Mobility Spectrometry (IMS)

Ion mobility spectrometry was used to test the investigated markers. The IMS device was obtained from IUT Medical GmbH / Berlin, Germany. IMS_1 software was used to review the data. The pure oily marker samples were tested by inserting the sampler tube inside the sample vial and closing it tightly. Each sample was scanned for 5 min three times. Infected onion bulb extracts were also scanned using the standard settings in the IMS device and software (Temperature was 60 °C).

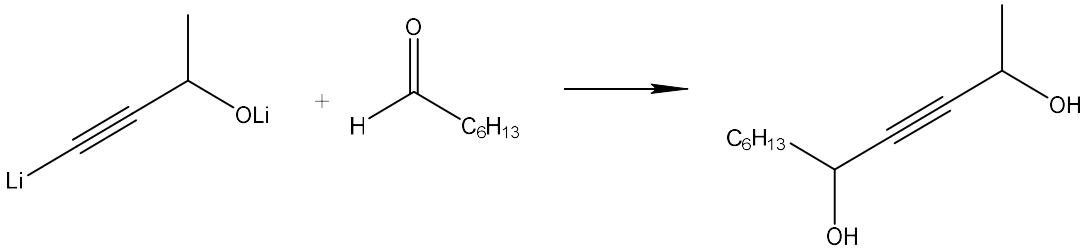
3.12 Synthesis of 2-hexyl-5-methyl-3(2H)-furanone

One of the possible methods to ensure the chemical structure of a substance is synthesizing the estimated structure and comparing it with the natural extract. Some trials have been done to synthesize 2-hexyl-5-methyl-3(2H)-furanone. The reaction was adapted from Thomas and Damm [61].

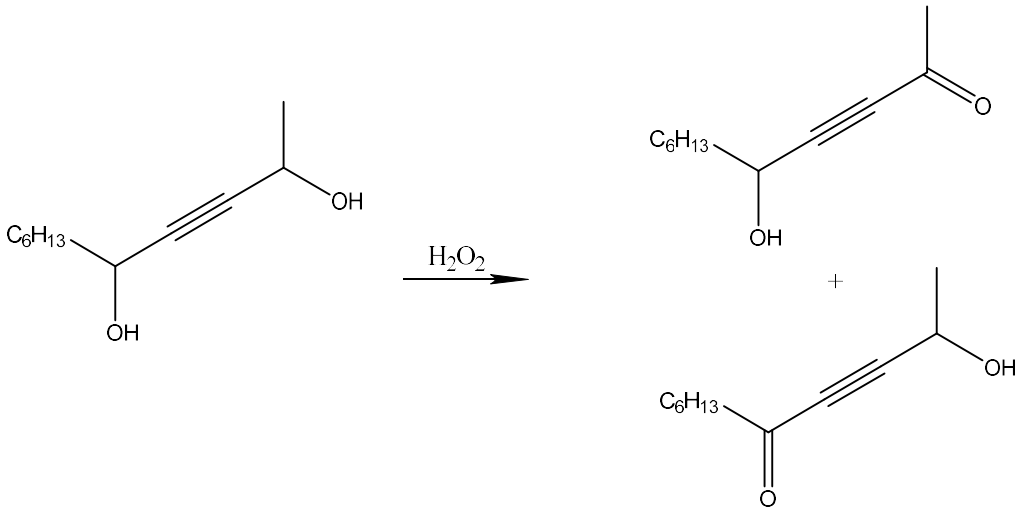
N-butyl lithium 3.3 g (0.052 mol) was added to 1.788 g (0.026 mol) of but-3-yn-2-ol (2 ml) in 20 ml anhydrous diethyl ether in a closed argon atmosphere. The mixture was cooled by a water bath, stirred and refluxed for two hours.



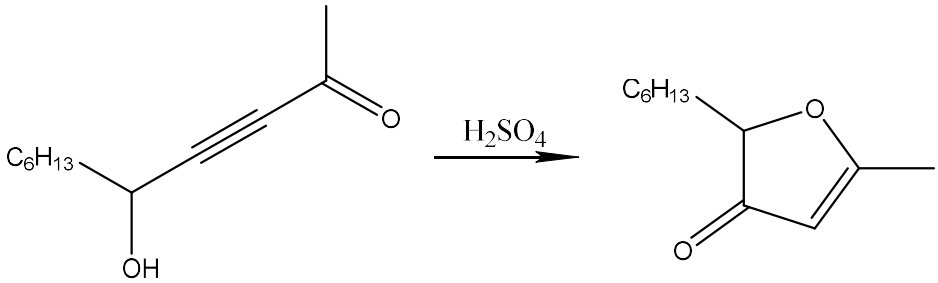
After that, 2.92 g (0.026 mol) heptanal was added to the mixture. Cooling, stirring and refluxing were kept for two hours. The product was washed with 1% acetic acid. The organic layer was separated, and most of the solvent was removed using a rotary evaporator under vacuum. The residue was purified by flash chromatography on silicagel using ethyl acetate/hexane (4:5) to afford undec-3-yne-2,5-diol.



After evaporating the solvents, 3 ml of 30% H_2O_2 has been added to undec-3-yne-2,5-diol in the presence of 0.2 g Na_2WO_4 with cooling by a water bath, stirring and refluxing for one hour to oxidize undec-3-yne-2,5-diol and get 2-hydroxyundec-3-yn-5-one and 5-hydroxyundec-3-yn-2-one.



After that, 10 ml of 1% aqueous sulphuric acid was added with cooling and stirring for 10 hours to cyclize 5-hydroxyundec-3-yn-2-one.



Flash chromatography on silica gel using ethyl acetate/hexane (3:5) was used to isolate 2-hexyl-5-methyl-3(2H)-furanone. The product was tested using MS analysis to detect the m/z of the furanone.

4. RESULTS

4.1 Detection of the markers in *Allium cepa* L. using HPLC

In the present project, it was assumed that the pattern of volatile compounds in *Allium cepa* L. was altered after infection. Infected onion bulbs have different odor and color. New chemical substances in the plant's extract could be detected by using different techniques of chromatography. HPLC is a powerful technique for quantitative and quantitative analysis. Comparing chromatograms obtained from healthy onion extracts to those from infected extracts could allow detecting some infection markers.

In order to analyze onion extracts in HPLC, onion bulbs were extracted using ethyl acetate. Ethyl acetate is an excellent and popular choice to extract many organic substances which have some polar or nonpolar functional groups like phenols, flavonoids, sulfur compounds or other substances. It can also be removed easily after extraction by using a rotary evaporator. In this project, healthy and infected onion bulbs by four fungal and one bacterial pathogens were extracted using ethyl acetate. Afterwards, extracts were compared using their HPLC chromatograms to detect new chemical substances.

4.1.1 Infection by *Fusarium oxysporum*

Healthy and infected onion bulbs obtained from (Julius Kuhn-Institut) were extracted using ethyl acetate and analyzed by analytical HPLC (Agilent) to compare the contents pattern.

Using the 100 min method (Method 3.3.2) did not show significant peaks during the first 50 minutes. The short method (Method 3.3.3) which starts with methanol/water (50:50) was considered in our results to compare healthy and infected bulbs.

The spectrum of a healthy onion bulb extract at 254 nm showed that we had a major peak at 10.2 ± 0.2 min, the peak A, and other small peaks at different retention times representing minor absorption (Figure 4-1). This pattern was similar in all tested healthy bulbs.

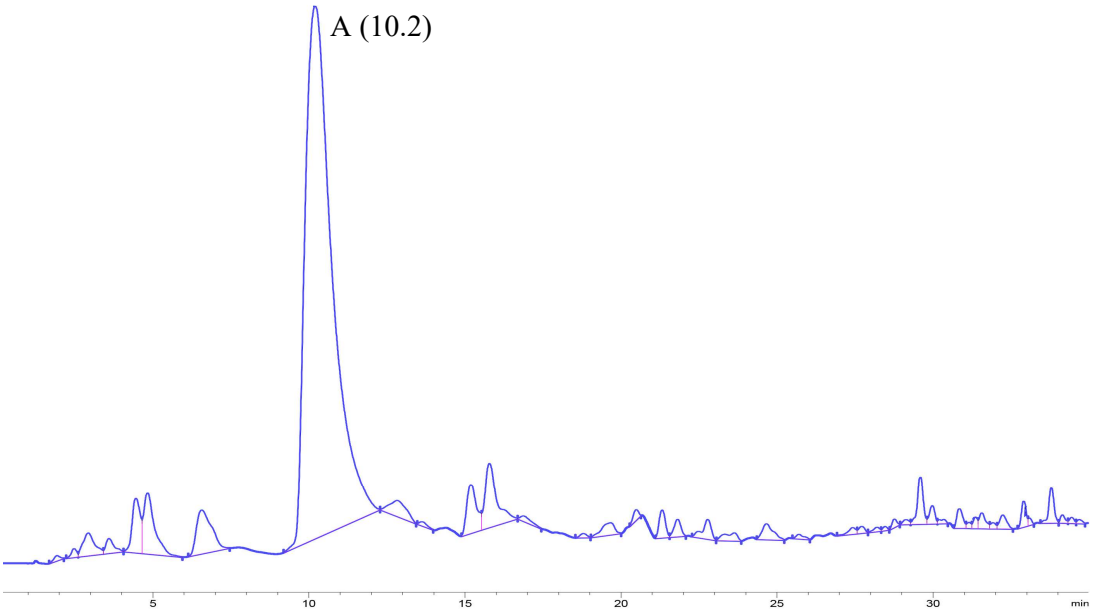


Figure 4-1 Healthy bulb's extract spectrum. The peak A appeared at 10.2 min

In the other hand, after infection by *Fusarium oxysporum*, spectrums of infected onion bulb extracts showed the peak A, which was found in the healthy bulbs and three new peaks, i.e. B, C, and D (Figure 4-2). This pattern was similar in all infected bulbs by *Fusarium oxysporum.*

The retention time of peak B was 15.5 ± 0.3 min. For peak C, the retention time was 25.0 ± 0.2 min. Peak D appeared at 30.0 ± 0.1 min.

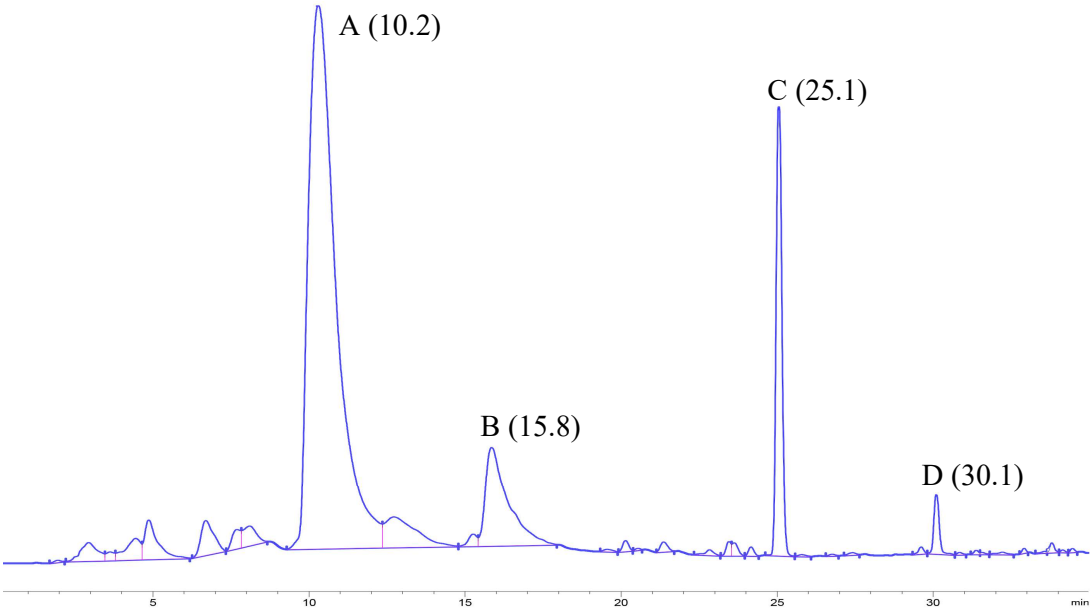


Figure 4-2 Infected bulb's extract spectrum (*Fusarium oxysporum)*. By comparing this figure with figure 4.1, three new peaks, i.e., B, C and D can be seen in this spectrum.

A sample of pure *Fusarium oxysporum* fungi obtained from Julius Kuhn-Institut was also extracted to ensure that the new peaks were coming only from onion and not related to fungal compounds (Method 3.1.4). The results showed no peaks at the retention time points for peaks A, B, C and D. The spectrum is presented in the Appendix chapter (Figure 8-3).

Another sample of infected onion extracts was analyzed after alliinase inhibition to understand if alliinase could mediate the markers biosynthesis in onions (Method 3.1.3). Peaks A, B, C and D could be noticed in the spectrum, which means that markers production was not related to alliinase reactions (Figure 8-4).

Two other samples were extracted from the same bulb having a partial infection in order to compare the infected and healthy parts belonging to the same bulb (Figure 4-3).

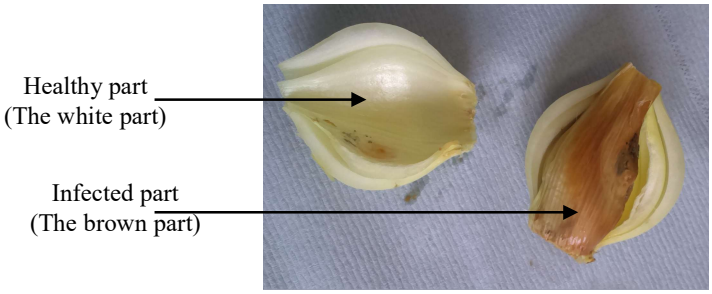


Figure 4-3 Infected part and healthy one in infected onion bulb by *Fusarium oxysporum*

Results obtained from this extraction method demonstrated the same pattern for both healthy bulb extract as well as healthy part of the infected bulb. Moreover, peaks B, C, and D, which were seen in the infected bulbs also appeared in the infected part of the infected bulb. The spectrum is presented in the Appendix chapter (Figure 8-5 and Figure 8-6).

More onion samples were investigated in different stages of infection. The peaks C and D appeared in all infected bulbs with *Fusarium oxysporum*. Peak B, on the other hand, only appeared in high amounts in some bulbs which were relatively old, while in the others only a small peak was seen.

4.1.2 Infection by *Fusarium proliferatum*

Infected bulbs with *Fusarium proliferatum* were obtained from (Julius Kuhn-Institut) and extracted using ethyl acetate (Method 3.1.2). The extracts were analyzed by analytical HPLC (Method 3.3.3) to find the nonpolar contents pattern in this infection.

In the results, the spectrum of an infected onion bulb extract at 254 nm showed peak A, which was already found in all healthy bulbs. Peaks C and D, which appeared in *Fusarium oxysporum* infection, could also be seen in the infected bulb spectrum with *Fusarium proliferatum* (Figure 4-4). It should also be mentioned that in this spectrum, peak B appeared small in most of the tested samples.

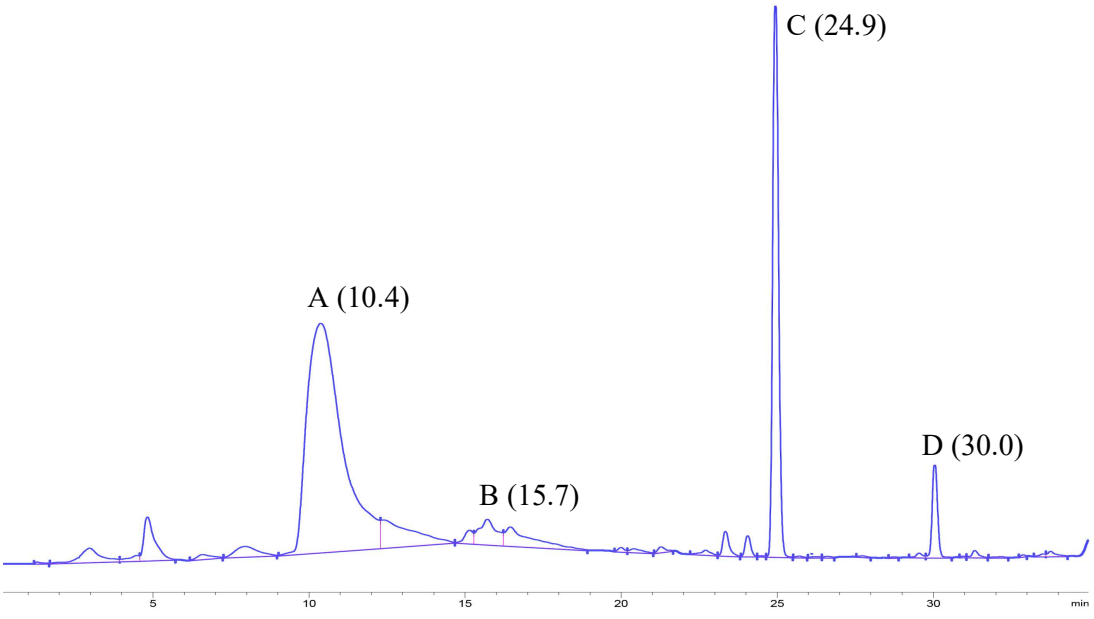


Figure 4-4 Infected bulb's extract spectrum (*Fusarium proliferatum)*. The peaks C and D appeared.

When a whole bulb having slight infection was extracted, peak A was higher while peaks C and D were relatively smaller. If the extracted bulb had a good infection or we tried to extract the infected part of the bulb, peaks C and D would be higher and peak A smaller (Figure 4-4). Peak B appeared more in older, bigger bulbs with or without infection.

4.1.3 Infection by *Penicillium sp.*

As in the previous infections, infected bulbs by *Penicillium sp* were obtained from (Julius Kuhn-Institut). The bulbs were also extracted using ethyl acetate and analyzed using HPLC (Method 3.3.3). The new infection did not show any new significant peaks in addition to the already described ones.

The spectrum of infected bulb extract at 254 nm showed the peaks A, C and D. Peak A was found in control healthy bulbs. Peaks C and D, that we found in the previous infections by *Fusarium oxysporum* and *Fusarium proliferatum,* also appeared in *Penicillium sp infection* (Figure 4-5).

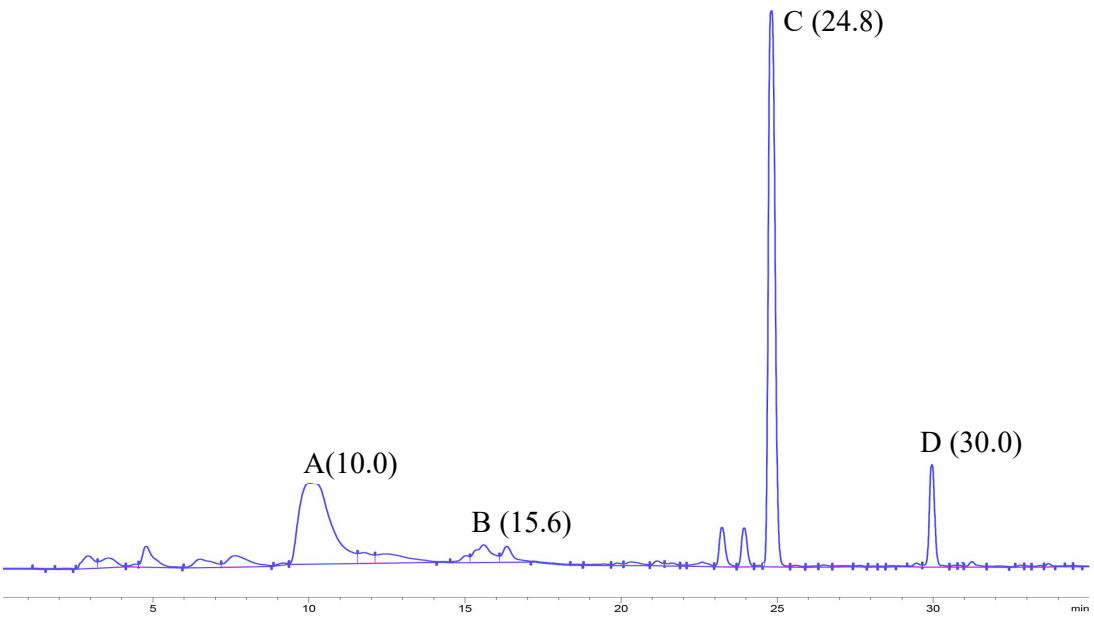


Figure 4-5 Infected bulb's extract spectrum (*Penicillium sp).* The peaks C and D appeared

By extracting several infected bulbs by *Penicillium sp* over two months. Peaks C and D could be observed in the early stage of infection. The peak B appeared later in the old

samples. Similar results were observed with *Fusarium oxysporum* and *Fusarium proliferatum.*

4.1.4 Infection by *Botrytis aclada*

HPLC analytical device was used to analyze onion bulb extracts after infection by *Botrytis aclada*. The tested bulbs were already extracted using ethyl acetate. The results were similar to the previous infections. No new differences were observed between healthy and infected bulbs. As a result, peak A appeared in healthy and infected bulbs. Peaks C and D were found in this infection showing similar markers of infection to the infection by *Fusarium oxysporum*, *Fusarium proliferatum,* or *Penicillium sp* (Figure 4-6). The peak B was found in healthy and infected bulbs in this batch.

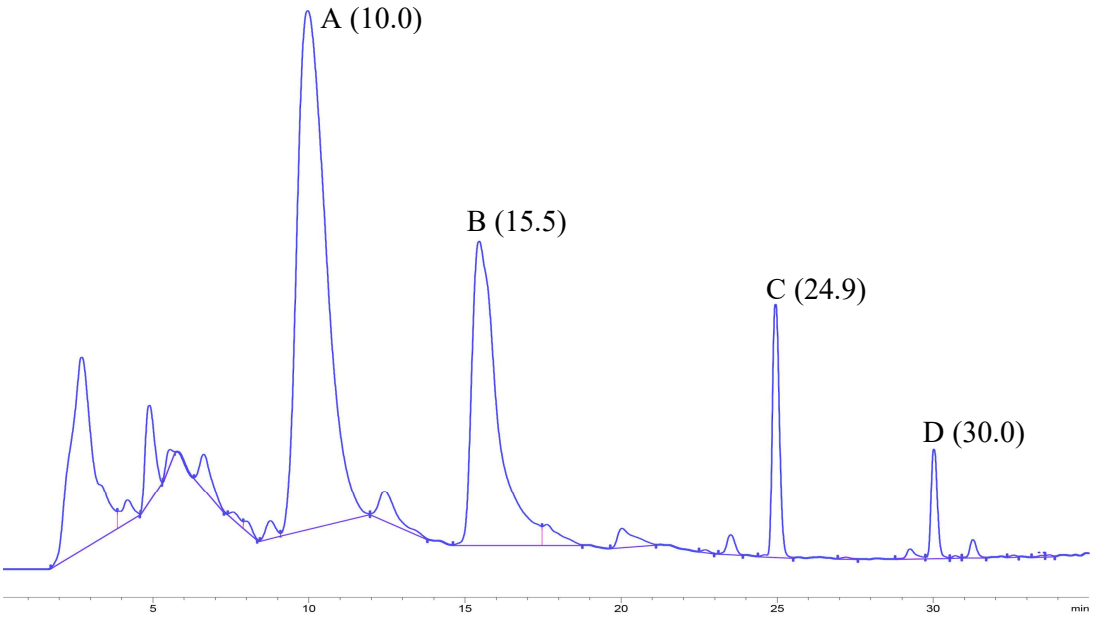


Figure 4-6 Infected bulb's extract spectrum (*Botrytis aclada)*. The peaks B, C and D appeared.

While the infection in *Fusarium oxysporum, Fusarium proliferatum*, and *Penicillium sp* started in the center of the bulbs, *Botrytis aclada* infection occured in the outer layers as shown in (Figure 4-7).

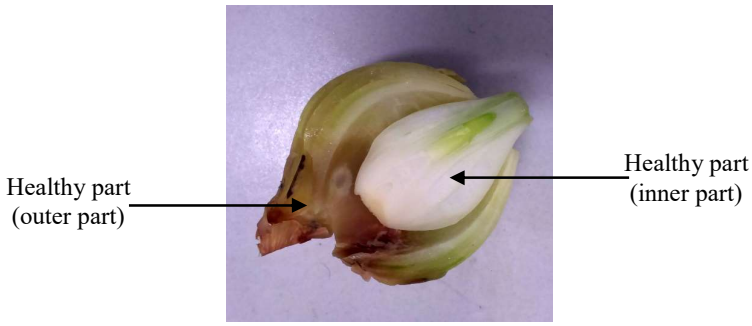


Figure 4-7 Infection by *Botrytis aclada*

4.1.5 Infection by *Erwinia carotovora*

As an example of bacterial infection, bulbs infected with *Erwinia carotovora* were obtained from (Julius Kuhn-Institut), extracted using ethyl acetate, and analyzed via analytical HPLC to find the volatile contents pattern.

The spectrum of an infected onion bulb extract demonstrated peaks A and B which were found in the healthy bulbs of this patch. Peaks C and D that we found in fungal infection with *Fusarium oxysporum*, *Fusarium proliferatum*, *Penicillium sp* and *Botrytis aclada,* were also observed in bacterial infection with *Erwinia carotovora* (Figure 4-8).

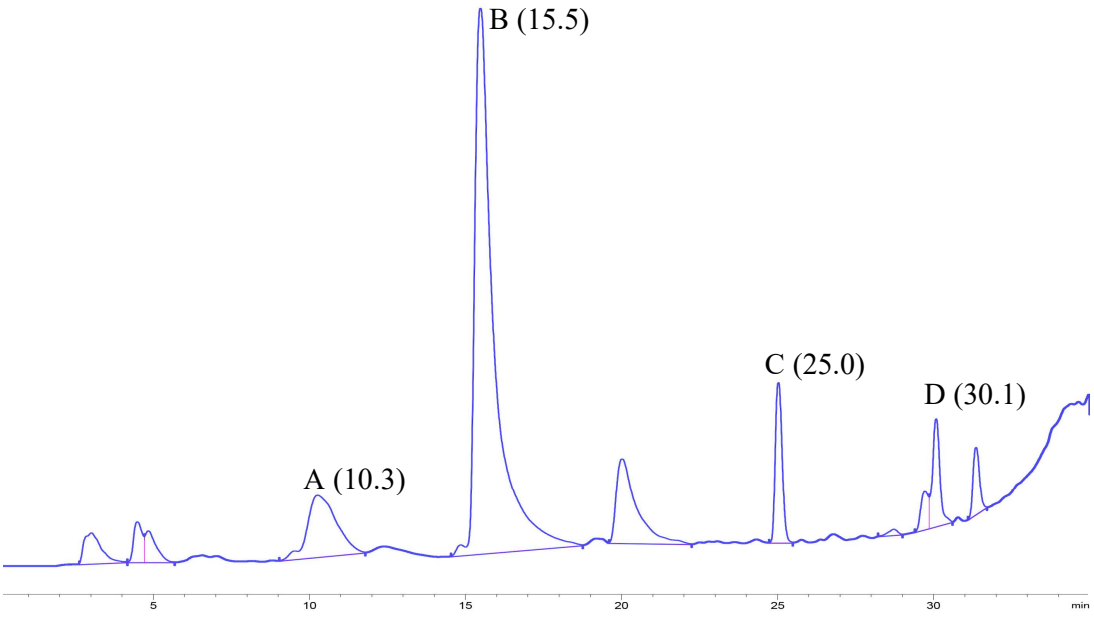


Figure 4-8 Peaks A, B, C and D in infected onions by *Erwinia carotovora*

Infection by *Erwinia carotovora* was more aggressive. The bulbs were totally damaged in one week (Figure 4-9).

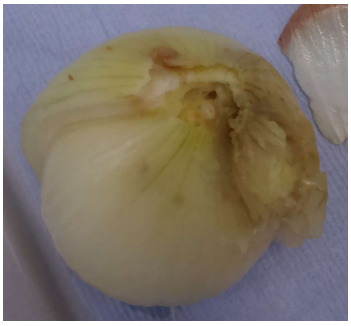


Figure 4-9 Infection by *Erwinia carotovora*

4.1.6 Onion from the market infected in our laboratory

Different onion batches (Bio) were bought from the local markets (Tegut) in Marburg, Germany. The bulbs were infected in the lab by *Fusarium oxysporum* and incubated at 28° C (Method 3.1.1). Tree days after incubation, the bulbs were extracted using ethyl acetate and analyzed by analytical HPLC. Healthy bulbs from the same market were kept under similar condition as a control. After 4 - 8 weeks, some of the healthy bulbs developed an infection by unknown pathogens. The symptoms were damping off, blue, black and white mold. These bulbs were also extracted using ethyl acetate and analyzed by analytical HPLC.

The infected bulbs from the market (by *Fusarium oxysporum* and the unknown pathogen) showed the same results as seen in Figure 4.2. Peaks A, B, C, and D were seen as major peaks. Peaks C and D were considered as markers for the infection.

4.1.7 Extracted bulbs by distillation

The previous results were obtained from extracted bulbs using ethyl acetate as mentioned before (Method 03.1.2). To get the oily volatile extract from *Allium cepa* L. bulbs, Clevenger apparatus was used as described before (Method 3.1.5). In this method, extracts were analyzed using HPLC to investigate the markers and to find the volatile contents pattern. The spectrum of an infected onion bulbs extract using distillation method could be seen in Figure 4-10. Peaks A and B could not be observed, while peaks C and D were clear

and almost pure. According to the purity of peaks C and D using this method, it was intended to be used for onion bulb extraction for further preparative separation by HPLC.

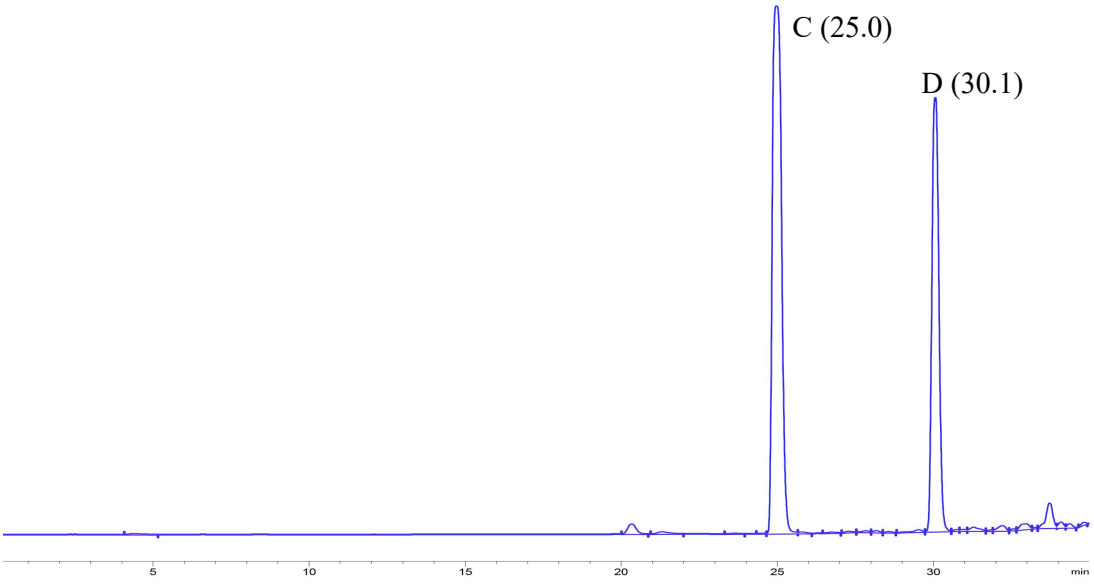


Figure 4-10 Spectrum of infected bulbs extract (distillation). Only the peaks C and D appeared

4.2 Structure Elucidation

4.2.1 ESI and HPLC-MS analysis of the extracts

To determine the masses of the investigated peaks, a liquid chromatography-mass spectrometry test was performed on healthy and infected extracts (Method 3.3.6) with positive ionization mode (Figure 4-11 and Figure 4-12).

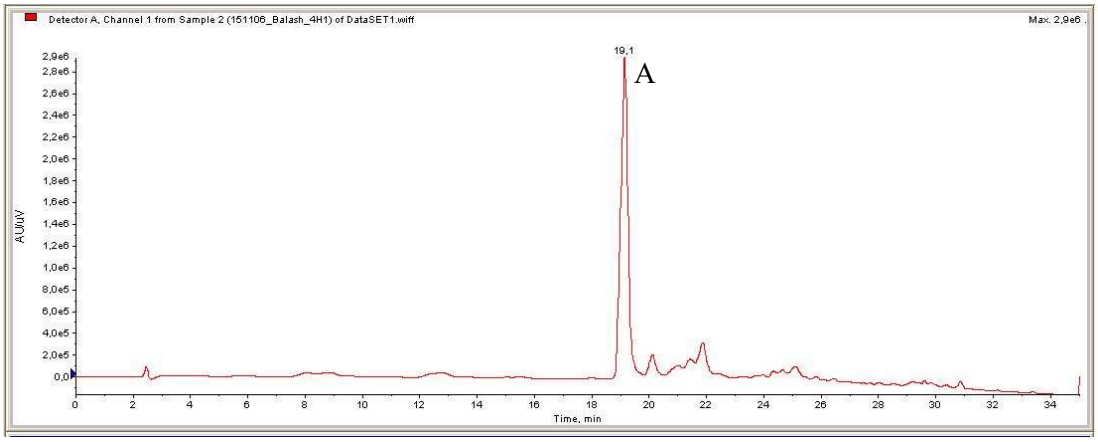


Figure 4-11 Healthy bulbs extract chromatogram using HPLC/MS at 254 nm

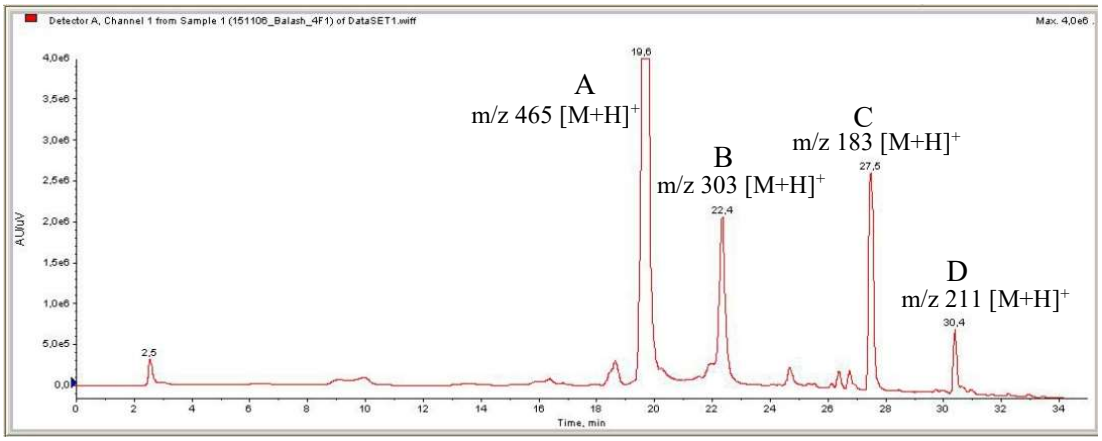


Figure 4-12 Infected bulbs extract chromatogram using HPLC/MS at 254 nm

The results showed the molecular mass 464 for peak A (RT = 19.3 ± 0.2 min). The molecular mass of peak B was 302 (RT = 22.2 ± 0.2 min). These peaks, as mentioned before, appeared in both healthy and infected bulbs. The new peaks which appeared only after infection were peak C with molecular mass of 182 (RT = 27.3 ± 0.2 min) and peak D with molecular mass of 210 (RT = 30.2 ± 0.2 min). XIC spectrums are presented in the appendix chapter.

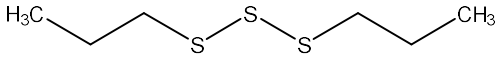
In infected bulbs, peak B was bigger and higher whereas peak A was smaller. m/z of peak A, which was 465 $[M+H]^+$, represented the quercetin monoglycosides. There were two quercetin flavonoids matching the molecular mass 464, i.e. quercetin-4′-*O*-glucoside and quercetin-3-*O*-glucoside [62]. Peak B had the m/z of 303 $[M+H]^+$, which represented free quercetin.

By comparing the retention times with reference materials using Agilent HPLC system, quercetin-4′-*O*-glucoside and quercetin aglycone were confirmed as the substances in peaks A and B respectively (Table 4-1**).** HPLC spectrums are presented in the appendix chapter (Figure 8-12 and Figure 8-13).

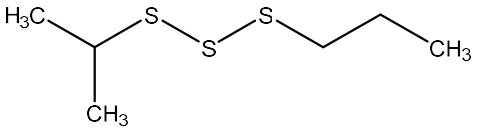
	Retention time RT = min
Peak A	10. 2 ± 0.2
Quercetin-4′-*O*-glucoside	10.2
Peak B	15.5 ± 0.3
Quercetin aglycone	15.2

Table 4-1 Comparing the retention times between the reference substances and the peaks A and B.

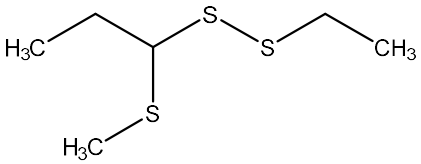
The m/z of peak C was 183 $[M+H]^+$. According to the literatures, This mass could represent five different substances: dipropyl trisulfide (DPTS)[2], [12], [47], [63]–[65], isopropyl propyl trisulfide[12], ethyl 1-(methylthio)propyl disulfide[66], 2-hexyl-5-methyl-3(2H)-furanone[12], [47], [63]–[69], or 4-hexyl-1,3-cyclopentanedione[45] (Figure 4-13).



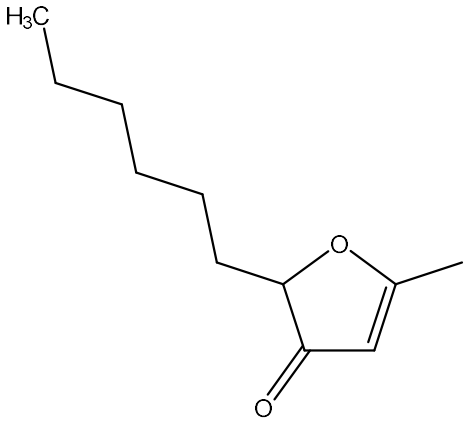
Dipropyl trisulfide



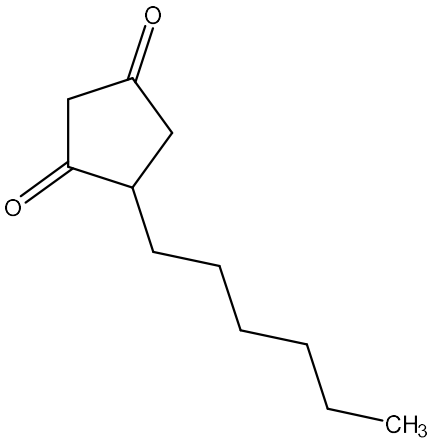
Isopropyl propyl trisulfide



1-(methylthio)propyl disulfide



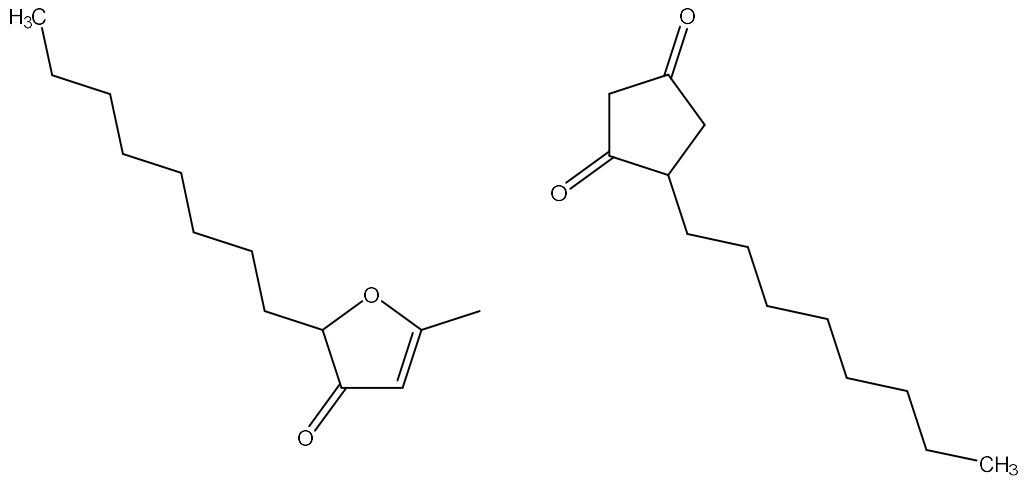
2-Hexyl-5-methyl-3(2H)-furanone



4-Hexyl-1,3-cyclopentanedione

Figure 4-13 Possible molecules for m/z 183 $[M+H]^+$

Peak D had m/z of 211 $[M+H]^+$. This mass represented either 5-methyl-2-octyl-3(2H)-furanone [12], [47], [63], [64], [68]–[70], or 4-octyl-1,3-cyclopentanedione [45] (Figure 4-14).



2-Octyl-5-methyl-3(2H)-furanone 4-Octyl-1,3-cyclopentanedione

Figure 4-14 Possible molecules for m/z 211 $[M+H]^+$

The mentioned substances were not commercially available in order to be used as reference substances. To focus more on peaks C and D, the peak fractions were collected using preparative HPLC using the extracts gained via distillation method because they were already isolated from the other peaks. Afterward, different analyses such as HRMS, EI^+, UV, IR, and NMR were done to clarify the structure of the two markers.

4.2.2 Preparative HPLC fractionation results

In order to provide a full structure elucidation of the infection markers in *Allium cepa* L., pure substances were required to perform the analyses. The extraction with distillation demonstrated clean C and D peaks. Therefore, the extracts, which we gained after distillation, were used for preparative separation.

Preparative fractionation was performed manually using Waters preparative HPLC system as described in method 3.3.4. RT for peak C was 9.4 min, whereas RT for the peak D was 15.5 min (Figure 4-15).

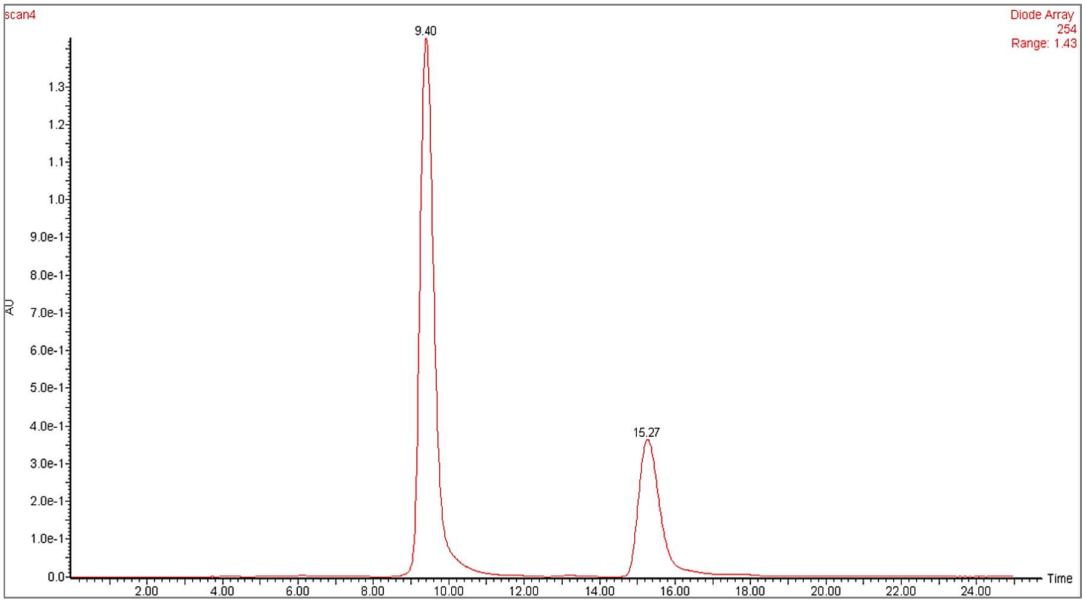


Figure 4-15 Distilled infected onion extracts in preparative HPLC

After fractionation, the purity of both two fractions was verified by injecting them again in Agilent HPLC. The relative purity of peaks C and D was 98-100%. The spectrum of the isolated fractions after preparative HPLC can be seen in appendix chapter (Figure 8-14 and Figure 8-15).

After preparative HPLC separation, the fractions were extracted using dichloromethane, dried carefully and saved for further analyses. Using ethyl acetate to extract the pure fraction was difficult because ethyl acetate, methanol and water were mixed together. Adding NaCl was not very effective solution here. Pure compounds were also very volatile, they could be lost during ethyl acetate evaporation.

We used n-hexane in some trials. It was better for extraction and evaporation, but its traces in the sample affected the quality of NMR spectrums especially in alkanes chemical shifts. Dichloromethane was the best choice in our experiments for this step.

4.2.3 High Resolution Mass Spectroscopy (HRMS) results

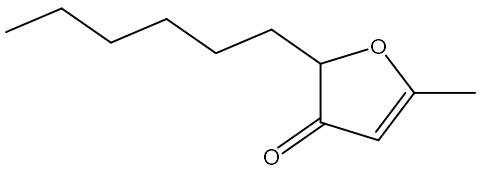
The elemental analysis using HRMS was done to get an idea about the elemental composition of the markers. After preparative HPLC separation, pure compound fractions were dissolved in methanol and applied on AutoSpec double focusing magnetic mass spectrometer using positive ionization.

The results showed the mass 182.1305 for peak C and the expected formula $C_{11}H_{18}O_2$. For peak D, the mass was 210.1613 and $C_{13}H_{22}O_2$ as an expected formula (Table 4-2)

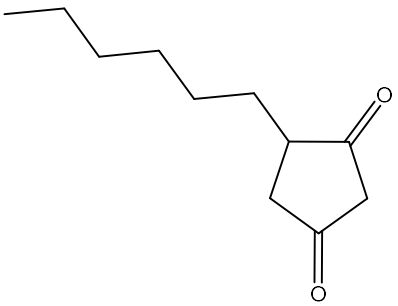
Peak	Mass	PPM	mDa	Calc. Mass	C	H	O	N	S
C	182.1305	0.5	0.1	182.1306	11	18	2		
		-6.8	-1.2	182.1293	9	16	1	3	
		62.2	11.3	182.1419	10	18	1	2	
		-68.5	-12.5	182.1181	10	16	2	1	
		-90.6	-16.5	182.1140	5	16	4	4	
		-97.0	-17.7	182.1129	11	18			1
D	210.1613	3.1	0.6	210.1619	13	22	2		
		-3.3	-0.7	210.1606	11	20	1	3	
		56.5	11.9	210.1732	12	22	1	2	
		-56.8	-11.9	210.1494	12	20	2	1	
		-75.9	-16.0	210.1453	7	20	4	3	
		-81.4	-17.1	210.1442	13	22			1

Table 4-2 HRMS results for peaks C and D

According to the results, the formula $C_{11}H_{18}O_2$ for peak C means that this compound might be 2-hexyl-5-methyl-3(2H)-furanone, 4-hexyl-1,3-cyclopentanedione or a new compound.



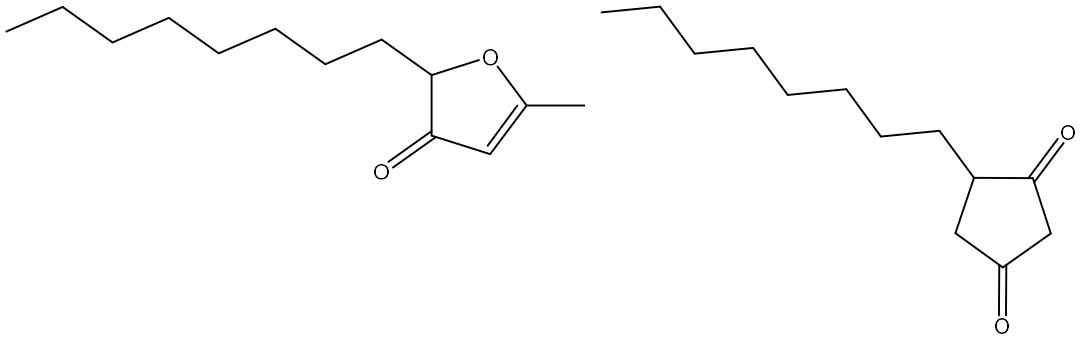
2-Hexyl-5-methyl-3(2H)-furanone



1,3-dion-5-hexyl-cyclopentane

Figure 4-16 The possible structures for the peak C

Results regarding peak D showed the formula $C_{13}H_{22}O_2$, showing that this peak can either be 2-octyl-5-methyl-3(2H)-furanone, 4-octyl-1,3-cyclopentanedione, or a new substance.



2-octyl-5-Methyl-3(2H)-furanone 1,3-dion-5- octyl-cyclopentane

Figure 4-17 The possible structures for the peak D

HRMS could give valuable information about the exact mass and the possible formula for our markers C and D, but it was not enough to elucidate their structures. More analyses were required for exact structure elucidation.

4.2.4 Electron ionization EI^+ results

In order to get more information regarding peaks C, D and their ion fractions, positive electron ionization, EI^+, was performed. The spectrums showed m/z of 98 as the basis mass peak in both of the molecules C and D (Table 4-3). EI^+ spectrums are presented in appendix chapter.

Peak	Molecular mass	Basis mass	Other ion fragments
C	182.1	98.0	111.0 - 149.0 - 85.2
D	210.2	98.0	111.0 - 134.1 - 85.2

Table 4-3 The positive electron ionization for peaks C and D

The positive electron ionization of peaks C and D could be explained by furanone as well as cyclopentanedione structures. The next table shows the possibility of ion fractions (Table 4-4).

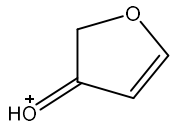
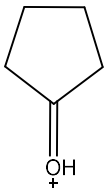
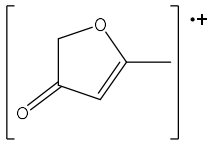
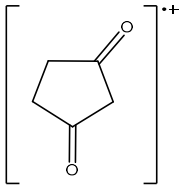
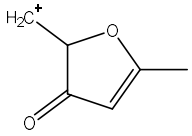
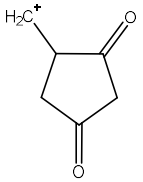
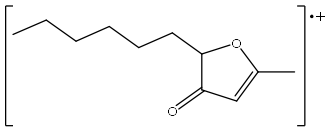
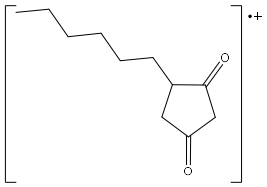
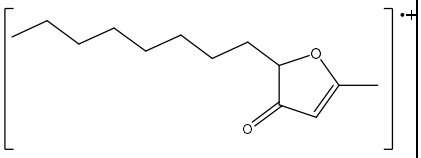
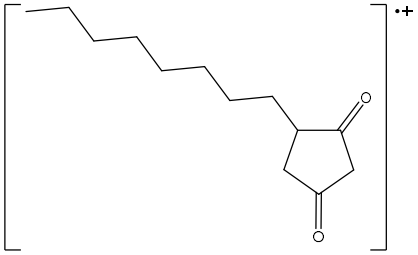
m/z value	**Furanone**	**Cyclopentanedione**
85		
98		
111		
182		
210		

Table 4-4 Possible ion fractions of peaks C and D

4.2.5 Ultra Violet spectroscopy (UV) results

To obtained UV spectrums for the markers, pure samples were dissolved in methanol and scanned in the range of 200-800 nm. Chromophores in 3(2H)-furanones differs from the ones in cyclopentanediones. Therefore, in order to compare the UV spectrums regarding peaks C and D, dimethyl dihydrofuranone (DMDHF) and 1,3-cyclopentanedione were used as references for 3(2H)-furanone and cyclopentanedione respectively.

UV spectroscopy results showed that the maximum absorption wavelength of DMDHF (λ_{max} = 257 nm) is more similar to the maximum absorption wavelengths of peaks C and D (λ_{max} = 262 and 261 nm), than 1,3-cyclopentanedione with a maximum absorption of λ_{max} = 241 nm (Figure 4-18).

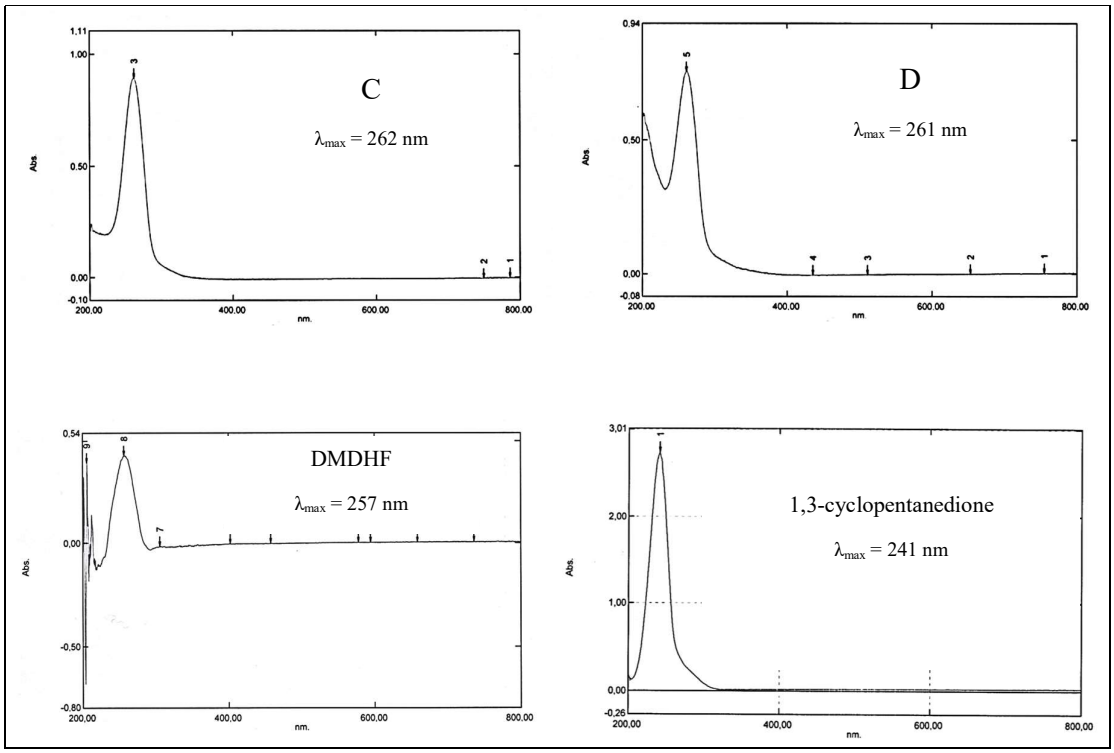


Figure 4-18 The UV spectrum for 1,3-cyclopentanedione, dimethyl dihydrofuranone, peaks C and D. λ_{max} for peak C was 262 nm. λ_{max} for peak D was 261 nm. λ_{max} for dimethyl dihydrofuranone was 257 nm. λ_{max} for 1,3-cyclopentanedione was 241 nm.

4.2.6 Infrared spectroscopy (IR) results

To prove the presence of 3(2H)-furanone functional groups in the markers structures, infrared spectrums of peaks C and D were gained using Bruker ATR-FTIR device.

The spectrums revealed several transmittance bands in the region of stretching vibrations for C-H, C=O, and C=C bonds. Dimethyl dihydrofuranone DMDHF and 1,3-cyclopentanedione were also scanned with the device in order to be compared with the investigated markers. IR spectrums for the markers and the reference substances were presented in appendix chapter.

Infrared spectrums of peaks C and D proved the structure of 3(2H)-furanone, especially when we compare the results to DMDHF spectrum (Table 4-5). These results approved that peaks C and D have similar structures, possibly 3(2H)-furanones, whereas the IR spectrum of 1,3-cyclopentanedione was completely different. C=O and C=C bonds could be considered characteristic bonds in this case.

	Peak C	**Peak D**	**DMDHF**
C-H stretching vibration	2955.43 cm^{-1} 2927.17 cm^{-1} 2857.78 cm^{-1}	2955.02 cm^{-1} 2924.24 cm^{-1} 2854.55 cm^{-1}	2984.79 cm^{-1} 2937.58 cm^{-1}
C=O stretching vibration	1698.84 cm^{-1}	1702.41 cm^{-1}	1694.52 cm^{-1}
C=C stretching vibration	1602.52 cm^{-1}	1603.82 cm^{-1}	1587.73 cm^{-1}

Table 4-5 The most prominent IR peaks of C, D, and DMDHF

4.2.7 Nuclear magnetic resonance spectroscopy (NMR) results

After using MS, UV and IR analyses, nuclear magnetic resonance spectroscopy was used to get details about the electronic structure of the molecules and their individual functional groups. Peaks C and D were scanned using JOEL 500 NMR system. NMR spectrums were presented in appendix chapter.

4.2.7.1 Proton ^{1}H and carbon ^{13}C NMR for peak C

The results of testing peak C in proton and carbon nuclear magnetic resonance after dissolving the oily pure fraction in deuterated dichloromethane CD_2Cl_2 were represented in Figure 4-19 and Table 4-6.

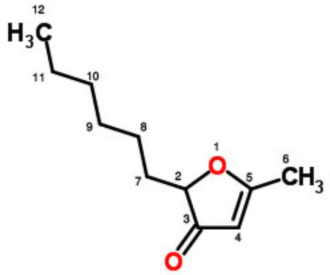


Figure 4-19 The structure of 2-hexyl-5-methyl-3(2H)-furanone

Atom number	^{1}H ppm	^{13}C ppm
2	4.39 (dd, J = 10.2, 2.9 Hz, 1H)	86.58
3	-	204.23
4	5.38 (s, 1H)	103.82
5	-	189.83
6	2.21 (s, 3H)	16.62
7	1.66 – 1.57 (m, 1H) 1.90 – 1.81 (m, 1H)	31.18
8	1.43 – 1.38 (m, 2H)	24.65
9, 10, 11	1.29 – 1.26 (m, 6H)	28.98, 31.57,22.60
12	0.88 (t, J = 7.0 Hz, 3H)	13.82

Table 4-6 ^{1}H and ^{13}C NMR spectrum data for peak C in CD_2Cl_2

Proton ^{1}H and carbon ^{13}C NMR spectrums for peak C confirmed the suggested structure of 2-hexyl-5-methyl-3(2H)-furanone.

4.2.7.2 Proton ^{1}H and carbon ^{13}C NMR for peak D

The results of testing peak D in proton and carbon nuclear magnetic resonance after dissolving the oily pure fraction in deuterated dichloromethane CD_2Cl_2 were given in Figure 4-20 and Table 4-7.

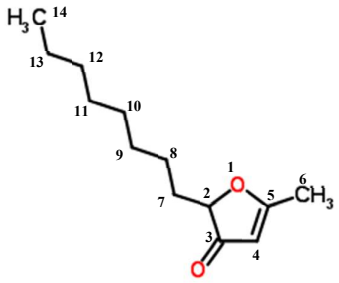


Figure 4-20 The structure of 2-octyl-5-methyl-3(2H)-furanone

Atom number	^{1}H ppm	^{13}C ppm
2	4.40 (dd, J = 8.1, 4.1 Hz, 1H)	86.67
3	-	204.76
4	5.38 (s, 1H)	103.95
5	-	189.99
6	2.21 (s, 3H)	16.64
7	1.90 – 1.81 (m, 1H) 1.65 – 1.61 (m, 1H)	31.85
8	1.43 – 1.40 (m, 2H)	29.34
9, 10, 11,12,13	1.28 – 1.26 (m, 10H)	29.37, 31.21 29.22, 24.70, 22.67
14	0.88 (t, J = 7.0 Hz, 3H)	13.93

Table 4-7 ^{1}H and ^{13}C NMR spectrum data for peak D in CD_2Cl_2

Proton ^{1}H and carbon ^{13}C NMR spectrums for peak C confirmed the suggested structure of 2-octyl-5-methyl-3(2H)-furanone.

4.2.7.3 COSY (COrrelated SpectroscopY) NMR

The results from Proton ^{1}H and carbon ^{13}C NMR spectrums confirmed that peaks C and D have very similar structures. The results of testing peak C in correlated NMR spectroscopy could also appear in peak D. The results of COSY NMR were summarized in Table 4-8.

	H2	**H4**	**H6**	**H7(1,2)**	**H8**	**H9, H10, H11**	**H12**
H2							
H4							
H6							
H7(1,2)							
H8							
H9, H10, H11							
H12							

Table 4-8 COSY NMR spectrum data for peak C in CD_2Cl_2

The spectrum indicated correlation peaks between proton H2 and H7(1,2) as well as between H7(1,2) and H8. This means that H7 was coupled to H2 and H8. H10 appeared in the same peak with H9 and H11.

There were correlation peaks between proton H8 and H9 and between H12 and H11. This means that H8 was coupled to H9, and H12 coupled to H11. There were no correlation peaks for H6 and H4, which means they were not coupled to other protons.

COSY NMR spectrum also confirmed the structures of 3(2H)-furanones for the investigated markers

4.2.7.4 HMBC (Heteronuclear Multiple Bond Correlation) NMR

The results of testing peak C in heteronuclear multiple bond correlation NMR confirmed the structure of 3(2H)-furanone. The spectrum indicated correlation peaks between Carbons and Protons as listed in Table 4-9

Carbon	Correlated protons
C2	H7(1,2), H8, H4
C3	H4, H2, H7(1,2)
C4	H6
C5	H6, H2, H4
C6	
C7	H2, H8, H9
C8	H2, H7, H9, H10
C9, C10, C11, C12	H7, H8, H9, H10, H11, H12

Table 4-9 The correlation between protons and carbons in peak C

4.2.7.5 NOESY (Nuclear Overhauser Effect SpectroscopY) NMR

This test was made to investigate the stereochemistry for carbon C2. The results showed correlations between H2 and the two protons in position H7(1 and 2). However, this test did not give sufficient information to clarify the stereochemistry of 3(2H)-furanones. Because of the possible keto-enol tautomery, the markers could have racemates after some time.

NMR spectrums confirmed the structure of 2-hexyl-5-methyl-3(2H)-furanone for peak C, and 2-octyl-5-methyl-3(2H)-furanone for peak D.

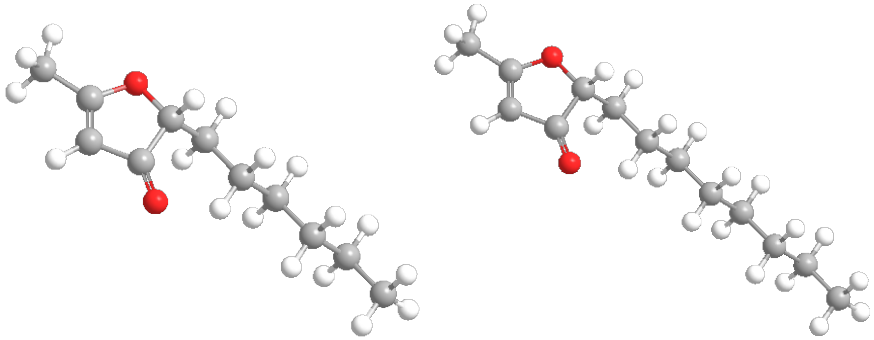


Figure 4-21 3D structure for the two markers using ChemDraw

4.3 Results regarding bioactivity tests

4.3.1 Antifungal activity results

In order to evaluate the sensitivity of fungi against the new compounds produced by onions bulbs after infection, preliminary drop test screening was performed on six fungal species as mentioned in (Method 3.8.1).

Results gained from this method demonstrated that infected onion extracts have no significant efficacy compared to the healthy onion extracts as shown in Figure 4-22. The extracts of infected bulbs (F) showed weak or no antifungal activity compared to the healthy onion extract (H) against all the tested fungi species. Because the primary results were not promising, no further analyses were done for this activity.

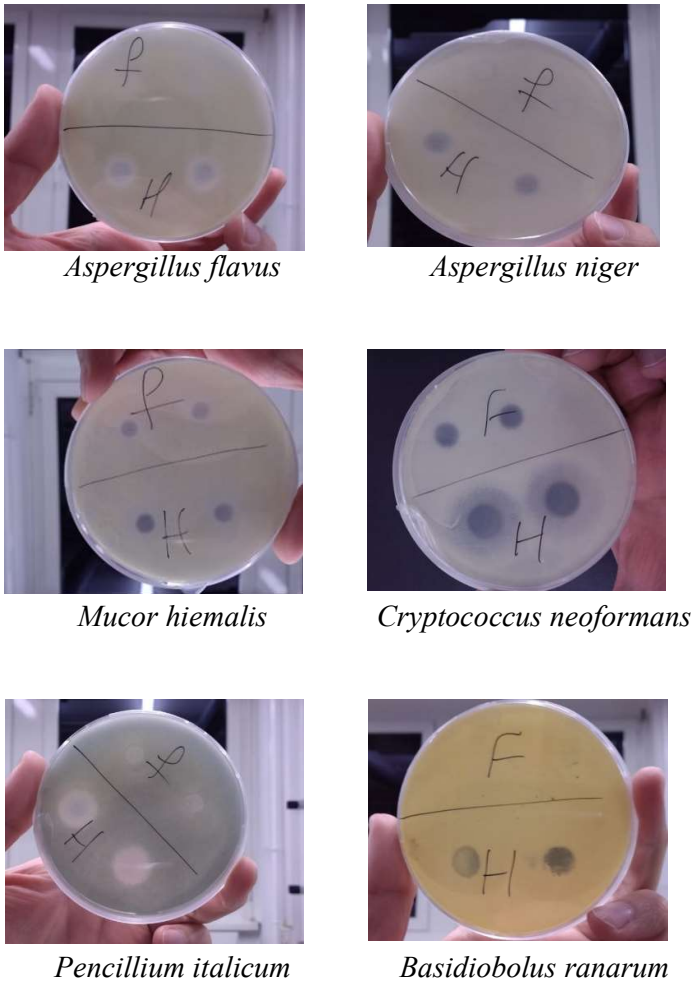


Figure 4-22 The antifungal activity test results. (F) Infected onion extract. (H) Healthy onion extract

4.3.2 Antitumor activity results

A preliminary experiment, using tumor cell lines T24 and UMUC3 cells, was done to characterize the effects of different concentrations of healthy and infected onion extracts as described in Method 3.8.2. After 48 hr of incubation, the cytotoxicity was assessed using XTT assay. The absorbance was measured at 405 nm with 650 nm as a reference. The results (Figure 4-23) didn't show superior activity for the infected onion extracts. This test was repeated three times. No more experiments for anticancer activity were performed.

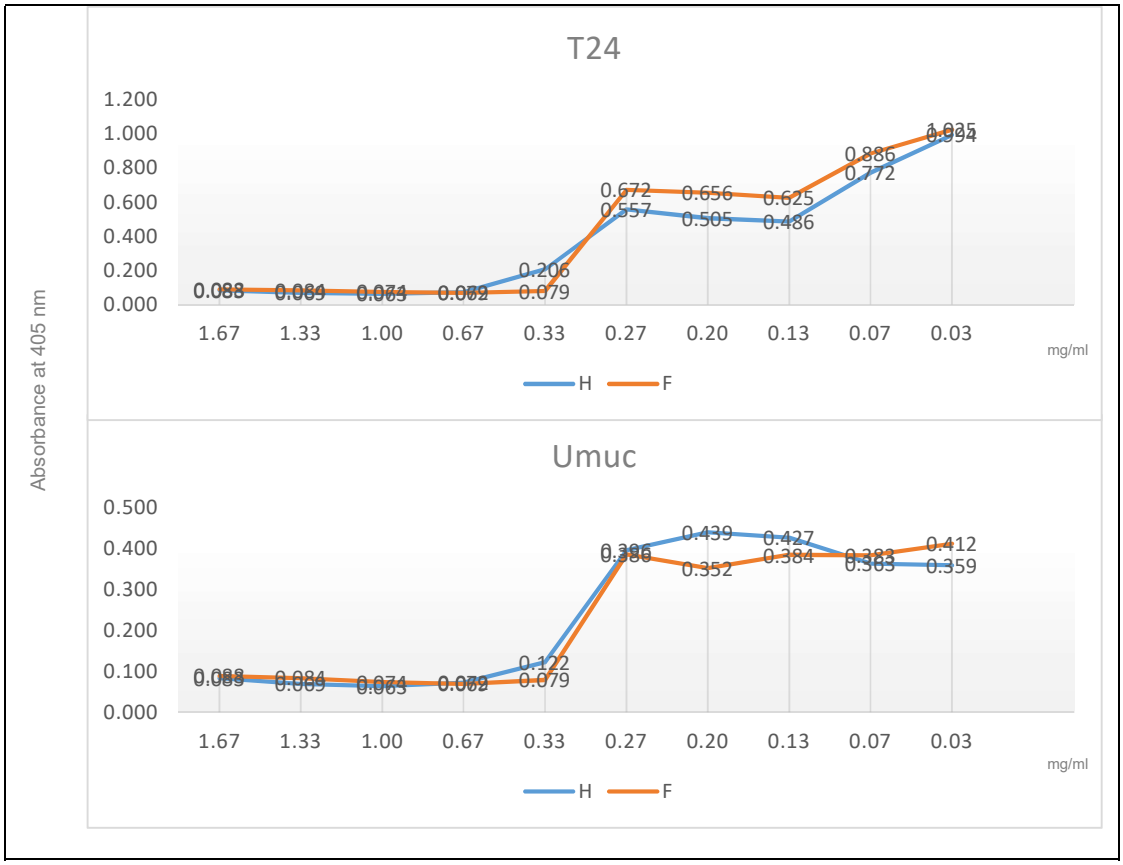


Figure 4-23 The results of the antitumor test in healthy and infected onion bulb extracts. The blue line represented a healthy bulb extract; Red line represented infected one. The two extracts showed week efficacy and no significant difference

4.4 Detection of the markers in other *Allium* species

In our previous results, 2-hexyl-5-methyl-3(2H)-furanone and 2-octyl-5-methyl-3(2H)-furanone were identified as infection markers in *Allium cepa*. Some other *Allium* bulbs could share the same markers, which were found in onion. Seven *Allium* species were infected by *Fusarium oxysporum* in our laboratory (Method 3.1.1) to investigate the presence of our furanone markers. Healthy and infected bulbs of each *Allium* were extracted using ethyl acetate and compared using HPLC (Method 3.3.3) and HPLC-MS systems (Method 3.3.6).

4.4.1 *Allium altaicum* Pall.

After extracting healthy and infected bulbs of *Allium altaicum* Pall. (Figure 4-24), the spectrum of infected bulbs extract was compared to the healthy ones at 254 nm (the spectrums are presented in appendix chapter), especially to find the furanone peaks. The results showed a relatively high peak at 29.6 min which might represent peak D. Another peak at 24.7 min seems to be peak C (Figure 8-17). The HPLC-MS data proved the presence of m/z 211 and 183 (M+1) for 2-octyl-5-methyl-3(2H)-furanone and 2-hexyl-5-methyl-3(2H)-furanone respectively.

Before infection After infection

Figure 4-24 *Allium altaicum* Pall. before and after infection

4.4.2 *Allium pskemense* B. Fedt.

Allium pskemense B. Fedt. bulbs (healthy and infected) were extracted using ethyl acetate. By comparing the spectrums at 254 nm (Figure 8-18 and Figure 8-19), two peaks could be seen at 24.7 min and 29.6 min, representing peaks C and D subsequently. Mass data could prove the presence of m/z 183 and 211 (M+1) for the investigated peaks, which means that 2-hexyl-5-methyl-3(2H)-furanone and 2-octyl-5-methyl-3(2H)-furanone could also be considered as markers of infections in *Allium pskemense* B. Fedt.

4.4.3 *Allium cornutum* Clementi ex Vis.

HPLC spectrums of extracted healthy and infected *Allium cornutum* bulbs did not show clear C or D peaks at 254 nm using Agilent analytical HPLC (Figure 8-20 and Figure 8-21). Whereas mass data, positive ionization, could prove the presence of m/z 183 and 211 (M+1) at the right retention times for 2-hexyl-5-methyl-3(2H)-furanone and 2-octyl-5-methyl-3(2H)-furanone. These results from different infected bulbs could list the two furanones as infection markers in *Allium cornutum* Clementi ex Vis..

4.4.4 *Allium bastard*

The spectrums of healthy and infected *Allium bastard* bulb extracts at 254 nm showed the appearance of peak C in the infected bulb extract (Figure 8-22 and Figure 8-23). Peak D was not clear in this extract using analytical HPLC. Anyway, mass data proved the presence of m/z 183 and 211 (M+1) for 2-hexyl-5-methyl-3(2H)-furanone and 2-octyl-5-methyl-3(2H)-furanone respectively.

4.4.5 *Allium fistulosum* L.

Allium fistulosum L. is one of the popular *Allium cepa* species. Fresh bulbs were infected in our laboratory and extracted using ethyl acetate. By comparing the spectrums of healthy and infected *Allium fistulosum* L. bulbs extracts (Figure 8-24 and Figure 8-25), two peaks appeared at 24.7 min and 29.8 min, which can be peaks C and D (2-hexyl-5-methyl-3(2H)-furanone and 2-octyl-5-methyl-3(2H)-furanone). Mass data could also prove the presence of m/z = 183 and 211 (M+1) for these peaks.

4.4.6 *Allium porrum* L.

Allium porrum L. is a popular *Allium* used widely as a food ingredient. As in the other species, healthy and infected *Allium porrum* L. bulbs extracts were compared using analytical HPLC at 254 nm. In infected bulbs, two peaks appeared at 24.6 min and 29.7 min, which could refer to our markers (Figure 8-26 and Figure 8-27). Mass data could also prove the presence of m/z 183 and 211 (M+1) for 2-hexyl-5-methyl-3(2H)-furanone and 2-octyl-5-methyl-3(2H)-furanone.

4.4.7 *Allium sativum* L.

After testing six *Allium* species and proving the presence of the investigated two markers after infection. It was interesting to see the results with *Allium sativum* L. (garlic). Infecting garlic was not easy as in the other bulbs. Many trials failed to start an infection in *Allium sativum* L.. By using more amounts of fungi and more time for incubation, infection symptoms started to appear. Healthy and infected garlic were extracted and tested in analytical HPLC (Figure 8-28 and Figure 8-29). The peaks C and D could not be noticed at their retention times in the chromatograms. The HPLC-MS data proved the absence of the m/z 183 and 211 (M+1) meaning that 2-hexyl-5-methyl-3(2H)-furanone and 2-octyl-5-methyl-3(2H)-furanone are not produced in *Allium sativum* L. after infection.

As a summary, both of 2-hexyl-5-methyl-3(2H)-furanone and 2-octyl-5-methyl-3(2H)-furanone were found in *A. altaicum* Pall.*, A. pskemense* B. Fedt.*, A. cornutum* Clementi ex Vis.*, A. bastard, A. fistulosum* L.*,* and *A. porrum* L. but *A. sativum* L. (garlic) didn't contain any of the tested furanones (Figure 4-24).

Plant name	3(2H)-furanones
Allium altaicum Pall.	+
Allium pskemense B. Fedt.	+
Allium cornutum Clementi ex Vis.	+
Allium bastard	+
Allium fistulosum L.	+
Allium porrum L.	+
Allium sativum L.	-

Figure 4-25 Detecting 3(2H)-furanones in some *Allium* species

In some samples, the markers were not clear in HPLC spectrums, but they could be detected using HPLC-MS device. The concentrations of the markers depended on the grade of infection. Most of the tested *Alliums* did not develop clear symptoms. Because these compounds appeared only after infection, they could be considered as infection markers in many *Allium* species as well as cultivars.

4.5 Quantitive analysis of 3(2H)-furanone

2-hexyl-5-methyl-3(2H)-furanone and 2-octyl-5-methyl-3(2H)-furanone have a volatile nature. The pure extracted substance was very hard to be collected and its weight could not give the exact content in the bulbs because we lose the substance through the extracting process. The investigated 3(2H)-furanones also are not available in markets to have standard references. Dimethyl dihydrofuranone, a similar substance having similar chromophore could be purchased and used as an internal and external standard.

4.5.1 External standard results

Different concentrations of Dimethyl dihydrofuranone DMDHF were prepared and measured three times using Agilent HPLC. An example chromatogram of DMDHF solutions in HPLC is presented in the Appendix (Figure 8-30). DMDHF peak appeared after 21.5 ± 0.2 min. Peak areas for three scans of each concentration were recorded (Table 4-10).

Mmol/l	Scan1	Scan2	Scan3	Mean	SD	SD%
0.25	4851	4875	4884.7	4870.2	17.3	0.4
0.5	10024.6	9872.6	10038.2	9978.5	91.9	0.9
0.75	15258.4	15009.9	15269.4	15179.2	146.8	1
1	20194.2	20550.7	20258.7	20334.5	189.9	0.9

Table 4-10 Peak Areas for dimethyl dihydrofuranone concentrations

Mean peak areas were represented to draw calibration curve and obtain the curve equation (Figure 4-26). The concentration of DMDHF could be calculated according to the curve equations.

Y= 20637X + 307.8

X= (Y-307.8)/ 20637

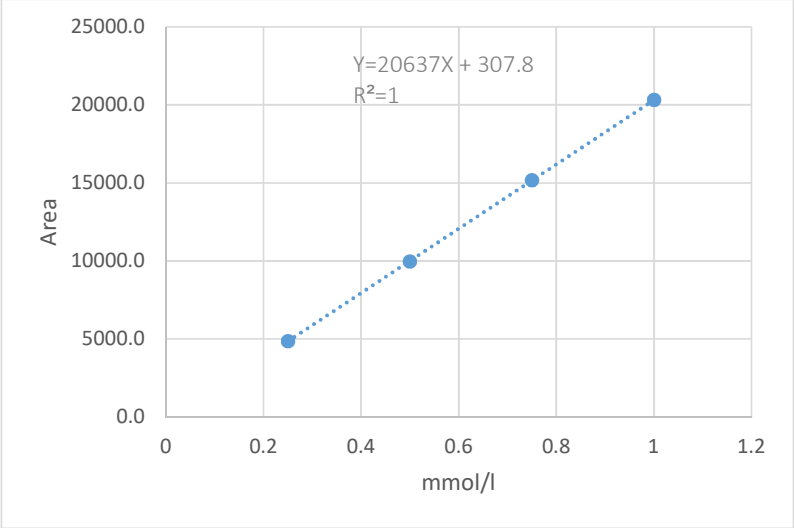


Figure 4-26 Dimethyl dihydrofuranone calibration curve

4.5.2 Internal Standard results

The infected *Allium cepa* L. bulbs by *Fusarium oxysporum, Fusarium proliferatum, Botrytis aclada, Penicillium sp*, and *Erwinia carotovora* were extracted using ethyl acetate. DMDHF was used as an internal standard to provide a quantitative analysis of 2-Hexyl-5-methyl-3(2H)-furanone (peak C) and 2-octyl-5-Methyl-3(2H)-furanone (peak D) in infected onion bulbs as mentioned in (Method 3.9.2). An example of the tested extract chromatograms was shown in Figure 4-27.

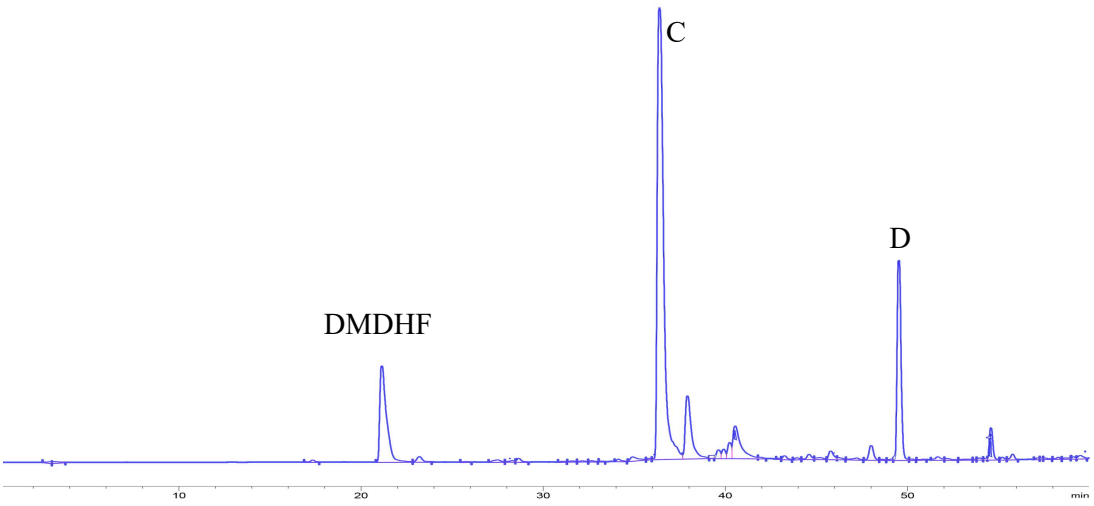


Figure 4-27 Spectrum of infected extract and DMDHF as an internal standard

A special HPLC method was used here to get isolated DMDHF peak (as mentioned in method 3.9.2). DMDHF appeared at 21.1 min. Peak C appeared at 49.5 min. RT for peak D was 54.6 min.

The mean area value of each peak was replaced in the external standard equation to calculate its concentration (mmol/l). The concentration of DMDHF after extraction was corrected to 1 mmol/l. The correction factor was used again for the concentrations of peaks C and D. The concentration of each marker was calculated to the fresh onion weight W/W in ppm. This experiment was repeated three times for each type of infection.

The next tables display the concentrations of 2-Hexyl-5-methyl-3(2H)-furanone (peak C), and 2-octyl-5-Methyl-3(2H)-furanone (peak D) in infected onion bulbs (Table 4-11, Table 4-12, Table 4-13, Table 4-14 and Table 4-15). The concentrations were calculated in ppm (mg/kg).

	Test1 ppm	Test2 ppm	Test3 ppm	Mean ppm	SD	SD%
2-Hexyl-5-methyl-3(2H)-furanone (peak C)	4.2	30	19.3	17.8	13.0	72.7
2-octyl-5-Methyl-3(2H)-furanone (peak D)	0.1	1.9	3.1	1.7	1.5	88.8

Table 4-11 Markers concentrations in the infected onions by *Fusarium oxysporum*

	Test1 ppm	Test2 ppm	Test3 ppm	Mean ppm	SD	SD%
2-Hexyl-5-methyl-3(2H)-furanone (peak C)	5.1	15.5	11	10.5	5.2	49.5
2-octyl-5-Methyl-3(2H)-furanone (peak D)	0.5	2.1	0.9	1.2	0.8	71.4

Table 4-12 Markers concentrations in the infected onions by *Fusarium proliferatum*

	Test1 ppm	Test2 ppm	Test3 ppm	Mean ppm	SD	SD%
2-Hexyl-5-methyl-3(2H)-furanone (peak C)	11	2.1	4.3	5.8	4.6	79.9
2-octyl-5-Methyl-3(2H)-furanone (peak D)	2.3	1	1.4	1.6	0.7	42.5

Table 4-13 Markers concentrations in the infected onions by *Botrytis aclada*

	Test1 ppm	Test2 ppm	Test3 ppm	Mean ppm	SD	SD%
2-Hexyl-5-methyl-3(2H)-furanone (peak C)	24.2	0.8	7.6	10.9	12.0	110.8
2-octyl-5-Methyl-3(2H)-furanone (peak D)	2.9	0.1	2.1	1.7	1.4	84.8

Table 4-14 Markers concentrations in the infected onions by *Penicillium sp*

	Test1 ppm	Test2 ppm	Test3 ppm	Mean ppm	SD	SD%
2-Hexyl-5-methyl-3(2H)-furanone (peak C)	21	13.3	5.4	21	13.3	5.4
2-octyl-5-Methyl-3(2H)-furanone (peak D)	7.4	2.7	5	7.4	2.7	5

Table 4-15 Markers concentrations in the infected onions by *Erwinia carotovora*

According to the previous results, 2-Hexyl-5-methyl-3(2H)-furanone concentrations were 0.8 – 30 ppm calculated to the fresh bulbs weight (mg/kg). 2-Octyl-5-methyl-3(2H)-furanone concentrations were 0.1 – 7.4 ppm. Some further experiments were done to compare furanones concentrations over time, but the concentrations were related to the rate of infection more than the time. Moreover, rates of infection varied extremely between bulbs from no infection to a totally destroyed bulb. It has to be mentioned that infection by *Erwinia carotovora* was much more aggressive than the other infections.

A summary of 3(2H)-furanones quantitative analysis could by displayed in Figure 4-28.

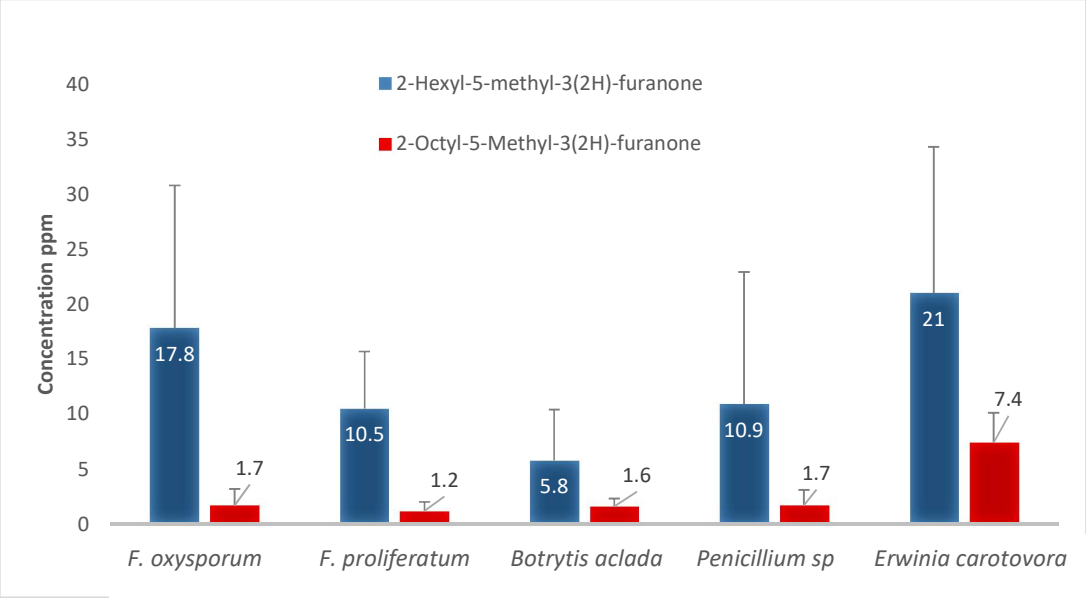


Figure 4-28 Markers concentrations in different types of infection

The calculations were made using a correction factor related to DMDHF. However, it should be concerned that the two markers are more volatile and nonpolar. The extraction process may lead to higher loses of the marker D than the marker C than DMDHF. So, it is recommended to reevaluate the quantities of the furanones when they are available commercially or after synthesis.

4.6 Gas Chromatography

GC analysis is a common method to test and detect volatile compounds. The available device in our institute (Shimadzu GC 2014AFsc) is not coupled to a mass spectrometer. That makes substances detection difficult without having reference substances. The two investigated markers (2-hexyl-5-methyl-3(2H)-furanone and 2-octyl-5-methyl-3(2H)-furanone) were analyzed using GC device to determine their retention time, which might allow to detect them later in a mixture or an extract.

Peak C appeared after 20.2 min (Figure 4-29). Peak D appeared after 25.4 min (Figure 4-30). Minor peaks appeared at 9.6 min and 15.2 min.

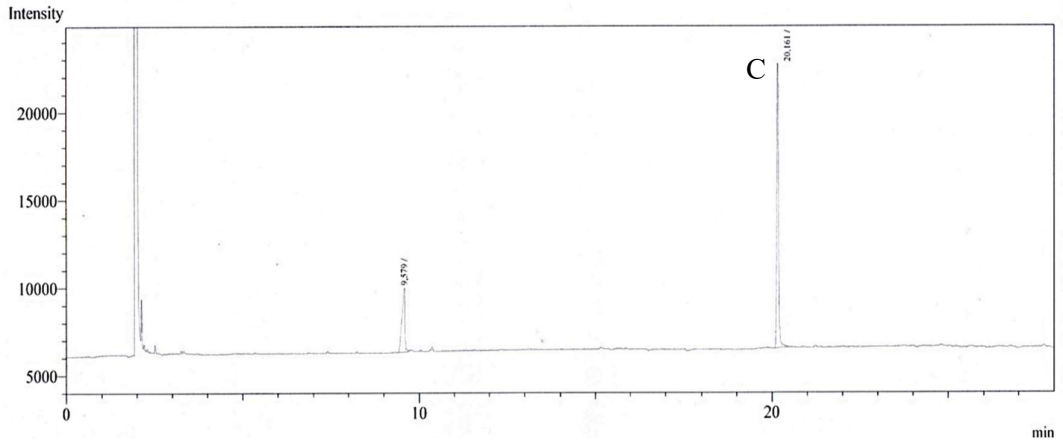


Figure 4-29 GC analysis for 2-hexyl-5-methyl-3(2H)-furanone. RT=20.161

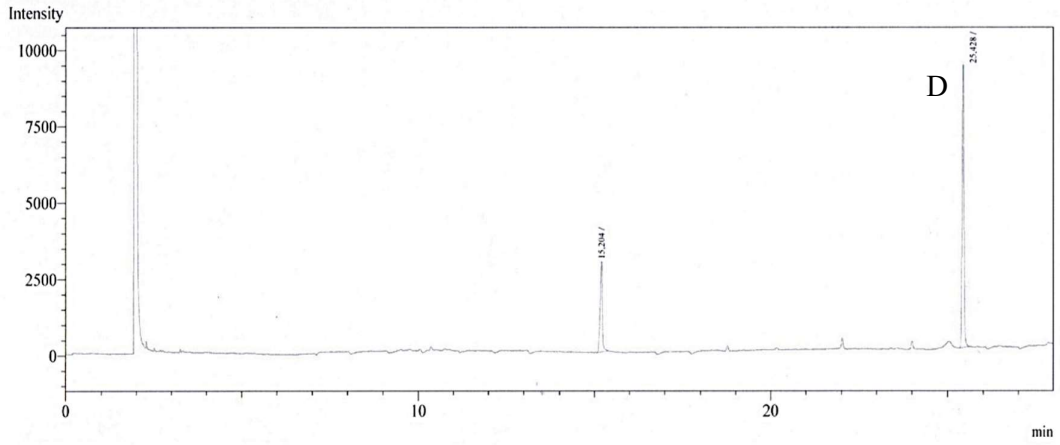


Figure 4-30 GC analysis for 2-octyl-5-methyl-3(2H)-furanone. RT=25.428

Because onion extract could contain nonvolatile compounds, Headspace analysis was used to test the volatile content in the extracts of infected onion bulbs by *Fusarium oxysporum.* The results showed high peaks which might represent volatile sulfur compounds. Peaks C and D showed tiny signals. Because of the differences in concentrations and volatility, other volatile substances in onion extract were relatively better remarkable (Figure 4-31). In our method (3.10) we used 50 °C for incubation, the markers might need higher incubating temperatures to increase their concentrations in the gas sample.

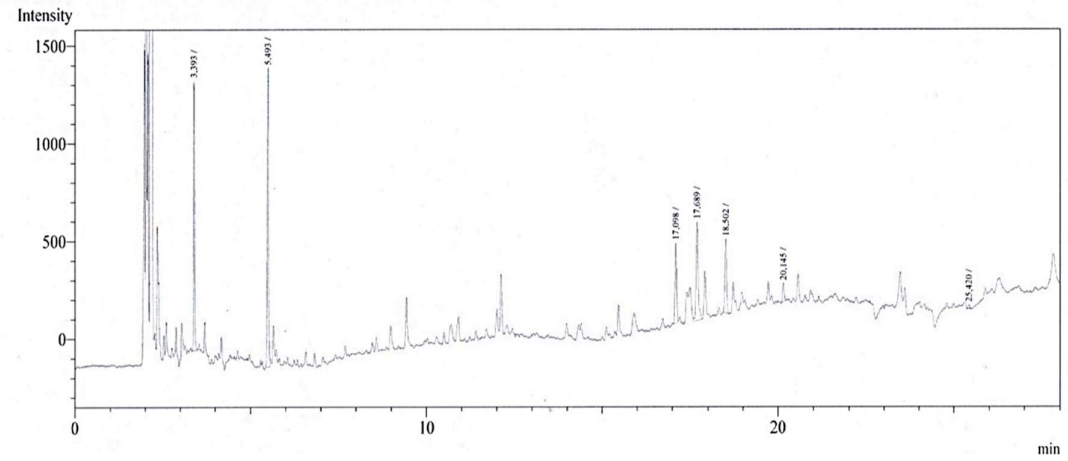


Figure 4-31 GC analysis for the headspace of an infected bulb extract

4.7 Ion mobility spectrometry

Ion mobility spectrometry might be the main application of detecting infection markers in *Allium cepa* L. Early stage detection of onion's infection in onion stores can save the healthy bulbs. IMS was used to test peaks C and D. Each isolated peak was scanned. IMS pattern for the peak C (2-hexyl-5-methyl-3(2H)-furanone) was shown in Figure 4-32.

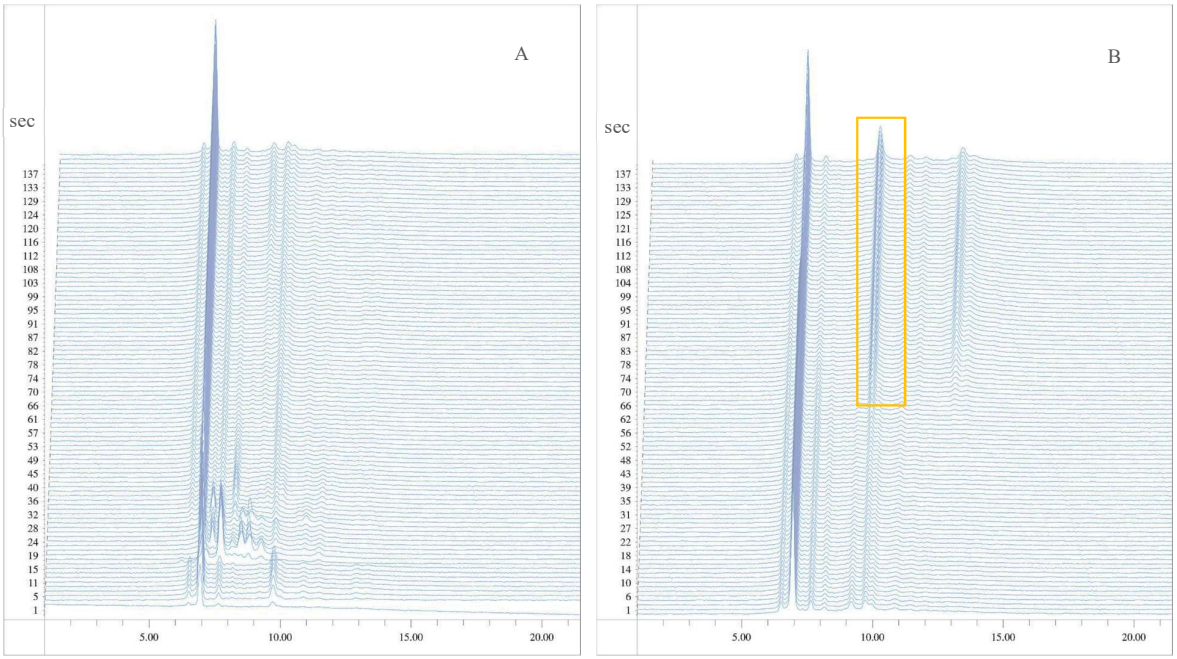


Figure 4-32 IMS analysis for 2-hexyl-5-methyl-3(2H)-furanone

IMS pattern for the peak D (2-octyl-5-methyl-3(2H)-furanone) is displayed in Figure 4-33.

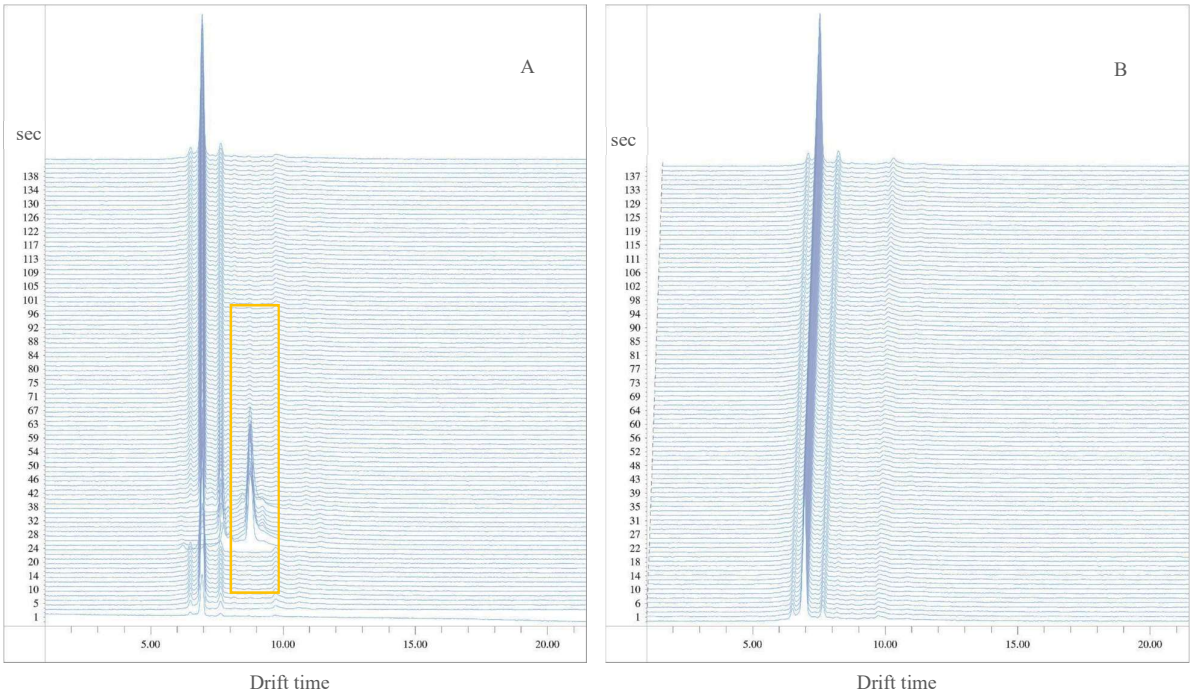


Figure 4-33 IMS analysis for 2-octyl-5-methyl-3(2H)-furanone

After testing the markers, infected onion bulb extract by *Fusarium oxysporum* was also tested using IMS. The resulted pattern is shown in Figure 4-34.

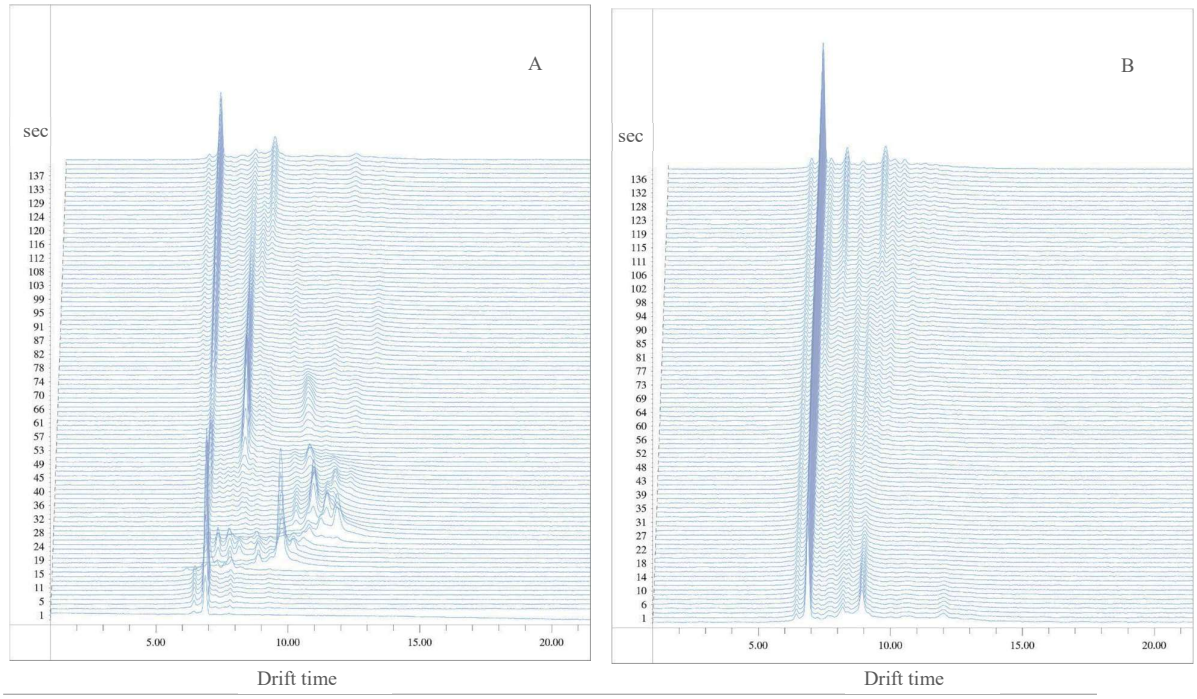


Figure 4-34 IMS analysis for an infected bulb extract

The signal of normal air content appears in all spectrums at drift time 6.8 msec. Characteristic signal for 2-hexyl-5-methyl-3(2H)-furanone appeared after 210 sec (Figure 4-32 B). For 2-octyl-5-methyl-3(2H)-furanone, the signal between 20-60 sec could be characteristic (Figure 4-33 A). Many signals appeared during the first 60 sec representing different very volatile substances in the extract (Figure 4-34 A). The signals of the two markers could not be noticed in the extract's spectrum. That might be related to their law concentrations in the total extract. Because analyzing IMS data was not easy, other colleagues in our group started to work on IMS as a separate project. The pattern of our two markers can also be used in that project as reference spectrums for pure compounds.

4.8 Synthesis of 2-hexyl-5-methyl-3(2H)-furanone

Isolating and collecting enough pure volatile markers was in somehow difficult. Another way to prove an assumed molecule structure is synthesizing it. Therefore, in this trial, we aimed to ensure the estimated 3(2H)-furanone structure by synthesizing and comparing it with the natural extract.

We tried a previously describes reaction to synthesize 2-hexyl-5-methyl-3(2H)-Furanone [67]. Actually, the reaction was not successful. This reaction had to be done in a closed argon atmosphere (Method 3.12). That did not allow to test the results step by step. The analysis of the final products by mass spectrometry did not confirm the synthesis success. The mass spectrometry results showed m/z 202.17 and 207.15 which could refer to $[M+NH_4]^+$ and $[M+Na]^+$ of undec-3-yne-2,5-diol (Figure 4-35). The mass spectrum is presented in appendix chapter (Figure 8-38).

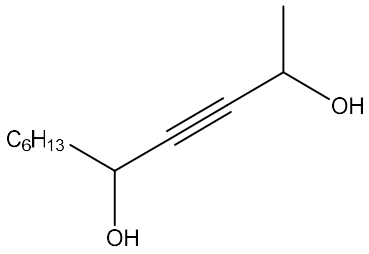


Figure 4-35 Undec-3-yne-2,5-diol

Synthesis experiments were not completed with other trials because it could be possible to confirm the markers structures by analytical methods. Besides that, one of our colleagues started a master project regarding 3(2H)-furanones synthesis[71]. Synthesis of 3(2H)-furanones might give further information about the markers properties and stereochemistry. It also allows testing their biological activity as isolated pure substances.

5. DISCUSSION

5.1 Detection of infection markers in *Allium cepa* L.

Despite the antimicrobial properties of common onion, as with other vegetable crops, they are susceptible to some fungi and bacteria. Pathogens can attack onion bulbs, roots or leaves. Onion diseases can reduce its yield and quality.

Infections in onion bulbs can persist without causing clear disease symptoms. This project aimed to find new phytochemicals produced in the infected onion bulbs to use them as infection markers for early detection of *Allium cepa* L. diseases.

By comparing ethyl acetate extracts from healthy and infected bulbs by HPLC using *Fusarium oxysporum* as a pathogen, four peaks at 254 nm were considered as significant peaks to focus on. While peak A appeared in all samples, the peaks C and D appeared only in infected samples. Peak B was noticed in some healthy and infected samples, especially in old bulbs.

It was supposed to find different markers for different pathogens. However, in our methods and results, we could see the same markers for the five microbial pathogens (*Fusarium oxysporum*, *Fusarium profileratum*, *Penicillium sp*, *Botrytis aclada* and *Erwinia carotovora*). These results mean that *Allium cepa* L. react similarly to all kinds of infection.

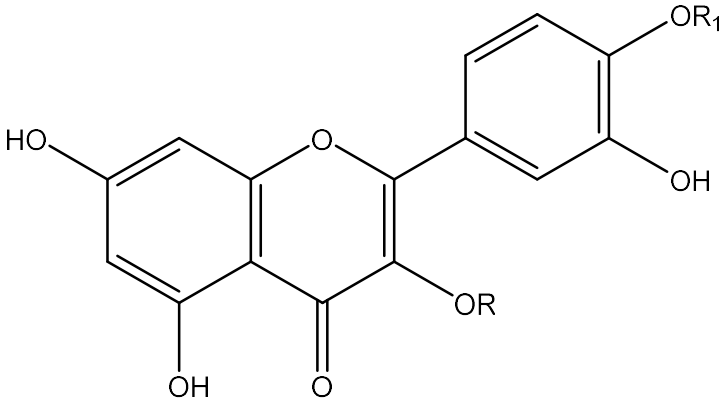
Because peaks C and D could only be detected after infection, we considered them as markers of fungal and bacterial infections in *Allium cepa* L. (Table 5-1).

Peak	Retention time	Properties	Appearance
A	10.2 min	High and wide	Healthy and infected bulbs
B	15.5 min	Medium to high and wide	Healthy and infected bulbs
C	25.0 min	High and sharp	Infected bulbs
D	30.0 min	Medium and sharp	Infected bulbs

Table 5-1 The remarked peaks in healthy and infected onion extracts using HPLC

It was interesting to know the chemicals which were represented by the peaks A, B, C, and D. So, the extracts were analyzed using HPLC-MS system.

Despite that peaks A and B appeared in healthy and infected bulbs extracts, peak B was bigger in infected bulbs, whereas peak A was smaller. Peak A represented quercetin-4′-*O*-glucoside, and peak B represented the free quercetin (Figure 5-1).



Compound	R	R_1
Quercetin	H	H
Quercetin-4′-*O*-glucoside	H	glu
Quercetin-3,4′-*O*-diglucoside	glu	glu

Figure 5-1 The main quercetin forms in onions [72]

Allium cepa has the highest content of quercetin in fruits and vegetables [73][74][25][75]. Quercetin monoglucoside and quercetin diglucoside account for about 80% of the total flavonoids in onions [76].

Quercetin amounts in onions vary according to the bulb's color and type; Yellow bulbs have been found to have higher levels of quercetin than red bulbs. Pink and white bulbs have the lowest concentrations [77]–[79]. Quercetin and the other flavonols are distributed mostly in the outer layers of onion bulbs [62], [80]. Quercetin can exist in onions mainly in three predominant forms: quercetin-3,4′-*O*-diglucoside, quercetin-4′-*O*-glucoside and free quercetin aglycone [72], [78] although several forms of the monoglucoside and diglucoside conjugates have been reported [72][81].

The presence of quercetin and its glycosides in different concentrations according to the type and layers can explain the appearance of the peaks A and B in healthy bulbs. It can also clarify the detection of peak B in all bulbs infected by *Botrytis aclada* and *Erwinia carotovora*, when we focused to extract infected parts in the outer layers. On the other hand, with infected bulbs by *Fusarium* species, we focused on the inner layers and peak B was relatively small.

By extraction with distillation, peaks A and B did not appear in the chromatograms (Table 5-2).

Peaks	**Normal extraction**	**Distillation**
A	√	-
B	√	-
C	√	√
D	√	√

Table 5-2 The remarked peaks using normal and distillation methods.

Boiling onions transfers quercetin and its glycosides into the water [82][83]. They are not volatile and won't appear in the extracted steam. So, the distillation method was used to extract infected onions and collect only the volatile content.

Microbial bioconversion of flavonols and flavonoids has been previously investigated [84]. It relies on glycosylation, hydroxylation, and *O*-methylation or *O*-demethylation of the parent compounds (Figure 5-2) [85].

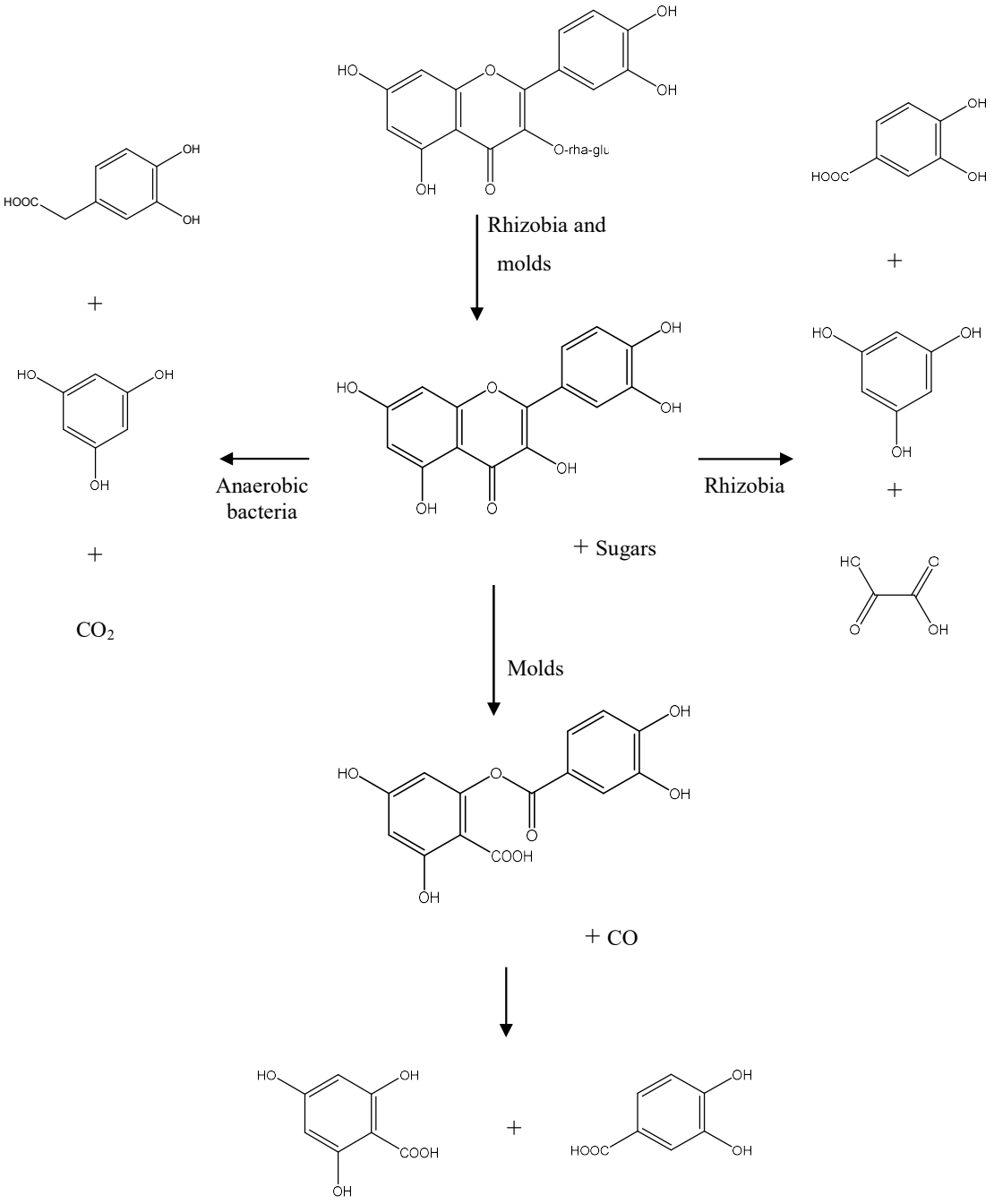


Figure 5-2 Summary of flavonol microbial catabolism [84]

As shown in Figure 5-2, the first step of quercetin glycosides bioconversion is removing the sugar part and producing the free aglycone. It is well known that various molds and bacteria can utilize rutin or other flavonoids as the source of carbon and energy by releasing a glycosidase [85][84]. That can explain the increase in the peak B after fungal and bacterial infection and the decrease in peak A.

While peaks A and B appeared in healthy and infected onion extracts, peaks C and D were noticed only in infected ones. In our methods, these two peaks are very clear and significant markers distinguishing the chromatograms of infected onion bulbs.

HPLC-MS, HRMS, UV, IR, and NMR spectrums confirmed the structure of 2-hexyl-5-methyl-3(2H)-furanone for peak C, and 2-octyl-5-methyl-3(2H)-furanone for peak D. Most of the previous researches listed these two markers as normal volatile substances in *Allium cepa* and its oil [12], [66], [86]–[88]. Some previous articles considered them as markers of authenticity for onion oil [63], [68], [87]. They named 2-hexyl-5-methyl-3(2H)-furanone and 2-octyl-5-Methyl-3(2H)-furanone as onion furanones [67] or norcepanone (peak C) and cepanone (peak D) [64].

By testing control and infected bulbs using GC-MS, Lie *et al*. found 2-hexyl-5-methyl-3(2H)-furanone in healthy and infected bulbs, while 2-octyl-5-methyl-3(2H)-furanone was recorded only in infected ones by *Burkholderia cepacia* [47].

Another study isolated 1,3-dion-5-octyl-cyclopentane (tsibulin 1d) and 1,3-dion-5-hexyl-cyclopentane (tsibulin 2d) from infected bulbs by *Botrytis cinerea* using HPLC [45]. It seems that they found the same markers, peaks C and D, but with different interpretation for the structure.

In our work, the structures of 2-hexyl-5-methyl-3(2H)-furanone for the peak C, and 2-octyl-5-methyl-3(2H)-furanone for the peak D were proved by all the possible analytical methods. These two markers could be found only in the infected samples. Because onion bulbs can be infected without any clear symptoms[48], the two markers have been considered as normal components of onion volatiles in the previous studies. Extracting huge amounts of healthy onions in former researches can lead to finding 3(2H)-furanones as normal substances when some onions were slightly infected without clear symptoms.

5.2 3(2H)-furanones in plants

As mentioned before, the two markers of bacterial and fungal infection in *Allium cepa* L. according to our results are 2-hexyl-5-methyl-3(2H)-furanone and 2-octyl-5-methyl-3(2H)-furanone. The main goal of our project was identifying the markers. It was also interesting to figure out the role of these substances in the plant, especially after infection.

In literature, some other 3(2H)-furanones have been well investigated in many vegetables and fruits. The most well-known 3(2H)-furanone is 4-hydroxy-2,5-dimethyl-3(2H)-furanone (Furaneol, HDMF) (Figure 5-3). It is an important chemical aroma because of its low odor thresholds and attractive flavor properties in jellies, jams, ice creams, sweets, beverages and alcoholic drinks [89]. HDMF was reported in 1960 as a product of Milliard reaction (non-enzymatic browning) [90]. This reaction occurs between amino acids and reducing sugars using heat [91], [92]. Synthetic HDMF is used in the food industry as an additive exhibiting caramel odor [93].

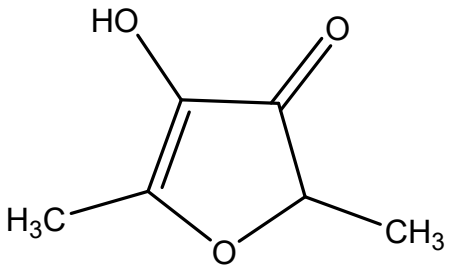


Figure 5-3 Chemical structure of 4-hydroxy-2,5-dimethyl-3(2H)-furanone

In 1965, HDMF and its metabolites were discovered in pineapples and in many other fresh fruits like tomato [94], kiwi [95], raspberry [96], [97], lychee [98], strawberry [99], and snake fruit [100]. It was reported that besides the chemical formation of HDMF, plants and microorganisms could biosynthesize it [101]–[104]. However, the particular pathway of this formation is still unknown although it has been intensively studied especially in strawberry [105]. Some radiotracer studies have identified the natural precursors of HDMF and its derivatives as carbohydrates [106].

Some studies have shown that HDMF has anti-carcinogenic, antimicrobial, anti-cell adhesion, and anti-oxidative activities [105], [107]–[110].

Ascorbic acid is another very important and well-known furanone in vegetables and fruit chemistry. It could also be considered as 3(2H)-furanone when it is deprotonated [111] (Figure 5-4). It is regarded as an essential antioxidant in the human body (Vitamin C) and involved in several physiological functions in the living organisms [112].

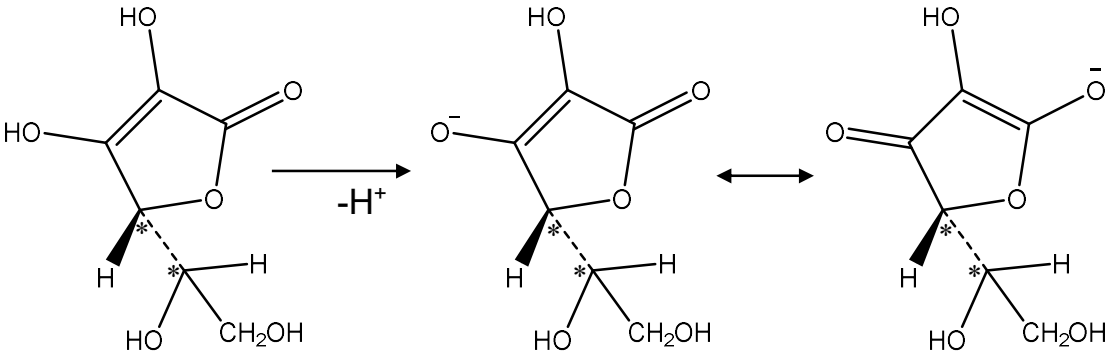
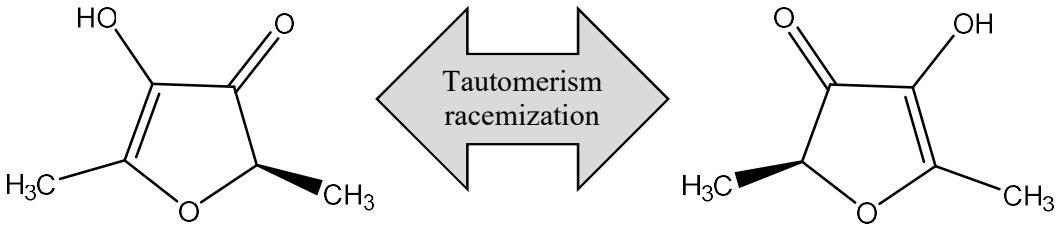


Figure 5-4 Deprotonating ascorbic acid [111]

5.3 Stereochemistry of 3(2H)-furanones

In this research, we could elucidate the structure of two infections markers. However, it was not possible to get complete information about their stereochemistry by the used analytical devices. Some articles discussed the stereochemical properties of 3(2H)-furanones.

The stereochemical studies of HDMF have been investigated by vibrational circular dichroism especially for its keto-enol tautomerism racemization (Figure 5-5) [113], [114]. It has been found that (*R*)-(+) isomer represents an intensive burnt caramel odor.



(*R*)-(+)-HDMF (*S*)-(-)-HDMF

Figure 5-5 Keto-enol tautomerism racemization in HDMF [113]

Ascorbic acid molecule has two asymmetric carbon atoms, C4 and C5. Therefore, there are three stereoisomers in addition to L-ascorbic acid itself (Figure 5-6). The three other isomers have very slight or no antiscorbutic activity [112].

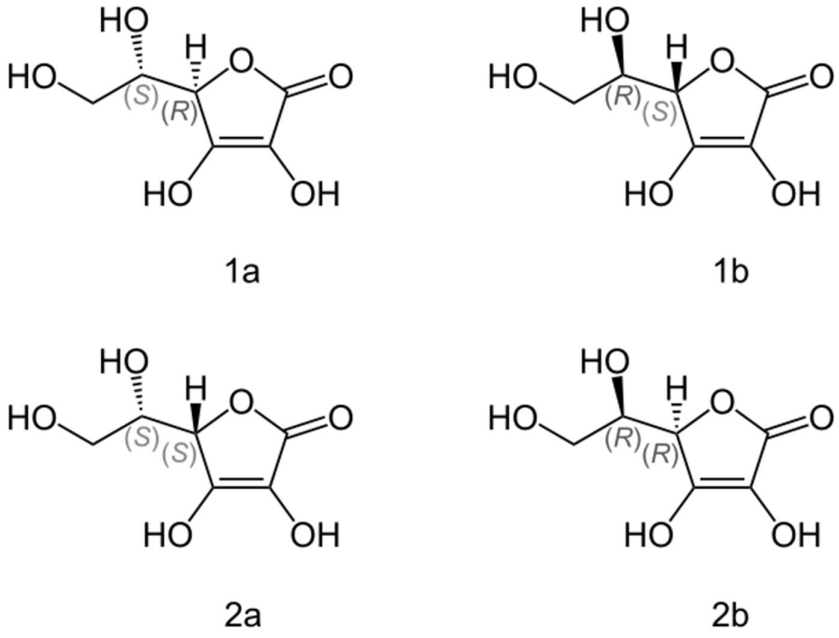
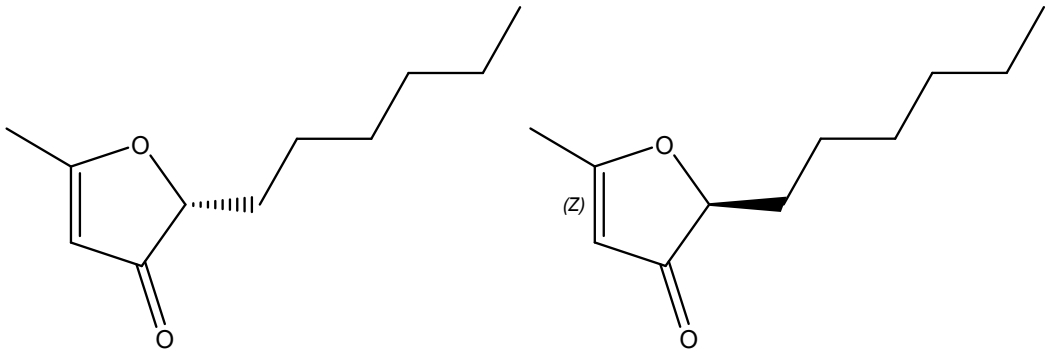


Figure 5-6 Ascorbic acid Stereo isomers: L-ascorbic acid (1a); D-ascorbic acid (1b); L-isoascorbic acid (2a); D-isoascorbic acid (2b)

The two investigated markers in our project have only one asymmetric center C2. That leads to two stereo isomers of each marker (Figure 5-7).



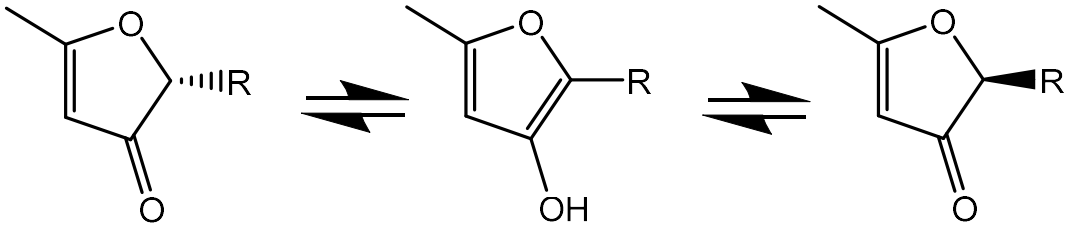
(*R*)-2-hexyl-5-methylfuran-3(2 *H*)-one (*S*)-2-hexyl-5-methylfuran-3(2 *H*)-one

Figure 5-7 The two possible stereo isomers of 2-Hexyl-5-methyl-3(2H)-furanone

Our data could not answer if we have (*R*) or (*S*) 3(2H)-furanones. Enantiomers cannot be distinguished easily in an achiral medium using NMR spectrums since the resonances of

enantiotopic nuclei are isochronous [115]. The absence of available reference substances could not help even to compare them with the natural compounds. Unfortunately, no previous studies could be found for very similar molecules. Investigating the stereochemistry of these substances is an interesting approach for further studies. The determination of the enantiomeric purity, in this case, using NMR requires the use of a chiral auxiliary in order to convert the mixture of enantiomers into a diastereoisomeric mixture [115].

We suggested that (*R*) or (*S*) 3(2H)-furanones could also exhibit keto-enol tautomerism racemization (Figure 5-8). The furanone molecule could change into furanol for seconds to reproduce the other furanone enantiomer.



(*R*) 3(2H)-furanone 2-R,3-hydroxy,5-methylfuranol (*S*) 3(2H)-furanone

Figure 5-8 The possible keto-enol tautomerism racemization in the markers

The minor peaks in ^{1}H and ^{13}C NMR spectrums might prove the short presence of the furanol structure. A small triplet peak at 2.29 ppm was shown in ^{1}H NMR spectrum (Figure 8-43), which could represent the protons at position 7 in the furanol. In ^{13}C NMR spectrum (Figure 8-44), two tiny peaks at 167 and 179 ppm could represent the carbons 5 and 3 respectively in the furanol. According to this suggestion, the conversion between (*R*) and (*S*) isomers in 3(2H)-furanones could happen spontanusely through keto-enol tautomerism to form racemic mixtures.

5.4 Bioactivity tests of the infected bulb extracts

Several defense strategies have been observed in plants to stop invading pathogens. These strategies may include physical defense like cuticle structure, various thorns and hairs [116]. Sometimes, plants show a chemical defense strategy. These chemicals can be constitutive or inducible defense [117]. Constitutive defense components are found in

healthy plants, for example, cysteine sulfoxides in *Alliums*. However, in some conditions, this constitutive defense is not enough to protect the plant against pathogens [118], [119]. So, the plant may induce new chemicals when an infection starts. These new chemicals can have antibacterial or antifungal activities, or they act like alarming phytochemicals to enhance the production of protective chemicals by the surrounding tissues or plants [48].

In the bioactivity tests, we tried to see if the new metabolites in infected onions can act as phytoalexins against bulb diseases. Antifungal and antitumor tests were ongoing using other *Allium* species in our research group. So, simple tests were done only to figure out the potential efficacy.

A preliminary antifungal test was done to evaluate the sensitivity of the fungi to the new compounds produced by the onion's bulb after infection including 2-hexyl-5-methyl-3(2H)-furanone and 2-octyl-5-methyl-3(2H)-furanone. The results were not promising. The extract of infected bulbs showed weak or no antifungal activity compared to the healthy onion extract against fungal microorganisms.

The efficacy of onion extracts and onion oil against different fungal species has been already tested and proved in the literature [120]. The slight efficacy of the infected onion extract can be related to the effective substances from the healthy part in extracted bulbs.

Our experiment aimed to evaluate the efficacy of the new substances which can be active phytoalexins. The negative preliminary results prevented further investigations in this direction.

In the antitumor activity test, ethyl acetate extracts obtained from infected and healthy onions were applied using the same protocol of similar tests done by our group [121]. The results also didn't show better activity in the infected onion extracts. That means the new components including 3(2H)-furanones in the infected bulbs do not have antitumor activity. The effective concentration (more than 0.27 mg/ml) were too high for the applied extracts; these concentrations can cause cytotoxicity for both normal and cancer cells. In literature, other natural and synthetic furanones have been tested in vitro against six cancer cell lines [122]. They found that furanones have a very week anticancer efficacy. The preliminary results and literature data were not motivating to continue with antitumor tests.

According to our experiments, the extracts of infected bulbs which include 3(2H)-furanones have neither antifungal nor antitumor activity. That raised new questions about the origin and the aim of furanone production in infected onions.

5.5 Furanones and quorum sensing

Quorum sensing (QS) is the mechanism which is used by bacteria to perceive the density of surrounding bacterial populations and to respond coordinately to this information by regulating several genes [109], [123]. In Gram-negative bacteria, Quorum sensing usually includes the production of acyl homoserine lactones (AHL) as the signal [109] (Figure 5-9).

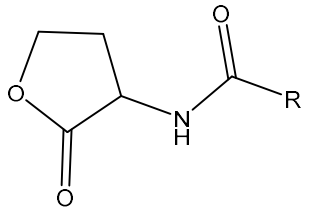


Figure 5-9 Acyl-homoserine lactones (AHL)

Antibacterial efficacy does not mean only killing the bacteria or inhibiting their growth. A new way to control certain bacterial infections suggests that the bacterial quorum-sensing regulates bacterial virulence and the formation of biofilms [124], [125].

Halogenated furanones, isolated from the red algae *Delisea pulchra*, showed promising quorum sensing inhibitory (QSI) compounds [126]. Furanones from *Delisea pulchre* (Figure 5-10) were tested to inhibit the expression of the QS-controlled gene in *P. aeruginosa* by displacing the acyl homoserine lactones (AHL) signals from the cognate receptor proteins [125], [127].

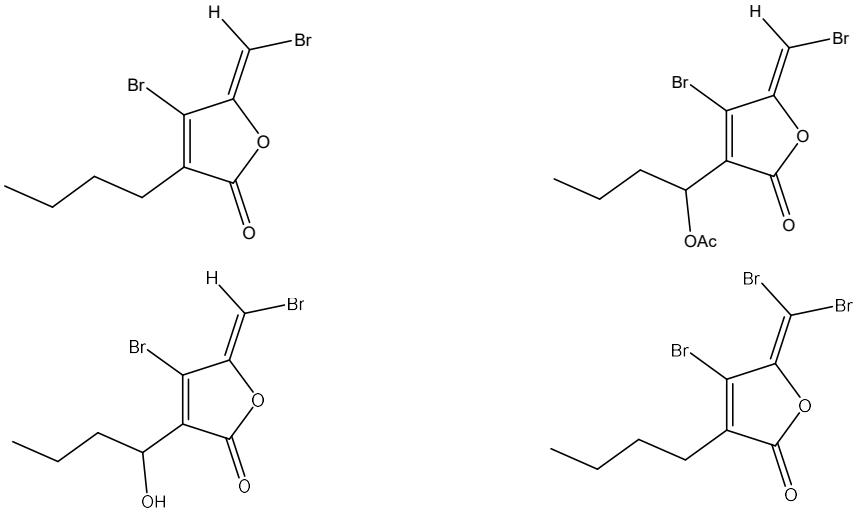


Figure 5-10 Natural brominated furanone compounds isolated from *D. pulchra* [124]

After that, a variety of furanones have been synthesized to enhance QSI activity against *Erwinia carotovora*, *Vibrio fischeri*, *Serratia liquefaciens*, *P. aeruginosa*, and *Chromobacterium violaceum* [108], [125], [128]–[130] (Figure 5-11).

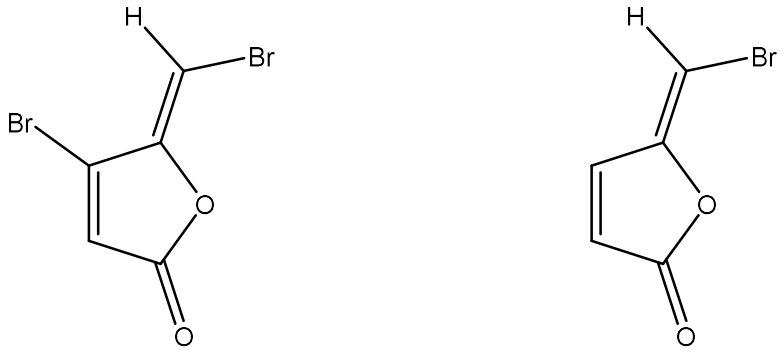


Figure 5-11 Synthetic quorum-sensing (QS) inhibitors [124]

HDMF could reduce the biofilm production by *Pseudomonas aeruginosa*. It has also affected the virulence factor expression significantly. These findings indicate that HDMF has a good potential as an inhibitor of quorum sensing [109].

Ascorbic acid has also been studied as a quorum-sensing analog to control *Clostridium perfringens* growth, sporulation, and enterotoxin production. The results were positive and promising as well [131].

According to these data, it is proposed that furanones play a biological role in the signaling mechanisms between the individual organisms. The chemical properties of furanone structure are ideal for this purpose. Several furanones can be water or/and fat soluble, or volatile depending on their substituents on the central ring [132].
The halogenated furanones are toxic to treat bacterial infections in human and animals because they are highly reactive [124], [133], [134]. Finding effective and safe QSI compounds could provide a good strategy to control the QS-regulated pathogenesis and the biofilm forming.

Our two furanones in this project, 2-hexyl-5-methyl-3(2H)-furanone and 2-octyl-5-methyl-3(2H)-furanone, can be very promising as natural therapeutic agents to control virulence and biofilm. Testing their activity in this field is a very interesting subject for further studies.

5.6 3(2H)-Furanones concentrations in infected *Allium cepa* L.

To achieve a quantitative analysis, dimethyl dihydrofuranone was used as internal and external standard. The results showed that 3(2H)-furanones occur in concentrations not more than 30 ppm related to the fresh onions. 2-Hexyl-5-methyl-3(2H)-furanone concentration was in the most of the samples higher than 2-octyl-5-methyl-3(2H)-furanone. That can be the actual situation, but also 2-octyl-5-methyl-3(2H)-furanone can tend more to be lost during extraction process because of its high volatility. By using GC-MS, El-wakil *et al*. found that 2-hexyl-5-methyl-3(2H)-furanone consists 0.28% of the volatile compounds in white onion oil. 2-Octyl-5-methyl-3(2H)-furanone was not found in this study [65].

In our results, we have also tried to find a relation between the concentration and the time of infection. However, the exact concentration seems to depend on the grade of infection, and that was varied according to the type of infection and *Allium cepa* L. cultivar. We could also see different grades of infection among the onions from the same batch, the same infection, and at the same time of infection. Similar variation in the total volatile content between infected samples has been found in studies using GC analysis [47].

5.7 3(2H)-Furanones in some *Allium* species

In order to know if they also have 2-hexyl-5-methyl-3(2H)-furanone and 2-octyl-5-methyl-3(2H)-furanone as infection markers, seven *Alliums; A. altaicum* Pall.*, A. pskemense* B. Fedt.*, A. cornutum* Clementi ex Vis.*, A. bastard, A. fistulosum* L.*, A. porrum* L. *, and A. sativum* L. were infected by *Fusarium oxysporum* and tested on the development of the two volatile chemical markers after infection. The investigated markers appeared in all infected *Alliums* species except *Allium sativum* L. In literature, it has been reported that the essential oils of *Allium fistulosum* L. and *Allium porrum* L. contain onion furanones [67], [135] as a part of their volatile profile without referring to the presence of infection. In our results, the two furanones could not be detected in healthy bulbs.

5.8 IMS and GC analysis of the markers

The two marker substances were tested using GC and IMS devices. Despite we could find the retention times and IMS patterns for the isolated substances, it was difficult to distinguish them easily in the full infected extract spectrums within the current methods.

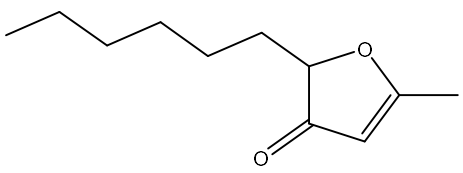
Onion has many volatile substances, especially sulfur compounds [7], which could be located in high concentrations confusing furanones peaks. By using GC device, other infection markers could be found in previous studies[46]–[48], which means that some other marker volatile substances could also be detected in infected onion bulbs.

Most of the unique substances detected after infection using GC-MS were in very low abundance [46], [47]. Some of them could be products of degraded substances by using high temperatures in the used methods. Dipropyl disulfide, methyl propyl disulfide and 2,5-dimethyl-thiophene were produced relatively in high abdunance. The presence of these substances in onion volatiles have been already reported [136]–[138] without presence of infection. The thiosulfinates and polysulfides are products of the alliinase mediated reactions in onion [7], [30]. Infections could cause cell rupture and aliinase release. Disulfides and trisulfides have been implicated in defense mechanisms and shown to be active against some pathogens [139]. Their normal production might be increased after infection to protect the undamaged tissues.

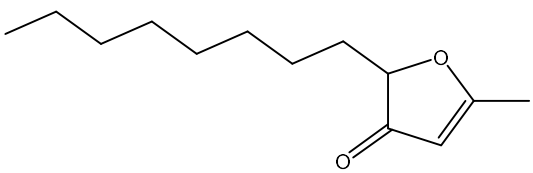
In the current work, we focused on the markers using HPLC systems. That helped to avoid using high temperatures, which can affect the results. Liquid chromatography also allowed to isolate pure markers for further analyses. Accumulated results using HPLC, GC and IMS techniques can provide valuable data to detect early stages of infection by all pathogen. Therefore, another project in our group had started to investigate the markers using GC and IMS systems.

6. SUMMARY

The genus *Allium* covers more than 750 species. *Allium cepa* L. (common onion) and *Allium sativum* L. (garlic) are considered as the most common and vital *Allium* species. Onion bulbs can usually be stored over several months. However, some bacteria and fungi infect the stored *Allium* bulbs and spoil them. In this project, the pattern of volatile compounds in healthy and infected *Allium cepa* L. bulbs were investigated using HPLC to find volatile infection markers. The spectrums were similar in four different fungal and one bacterial infection (*Fusarium oxysporum*, *Fusarium proliferatum, Penicillium sp., Botrytis aclada,* and *Erwinia cartovora)*. In bulbs, which were infected, two highly volatile compounds, namely 2-hexyl-5-methyl-3(2H)-furanone and 2-octyl-5-methyl-3(2H)-furanone (Figure 6-1), could be identified and structure has been elucidated by NMR, IR, and MS.



2-Hexyl-5-methyl-3(2H)-furanone



2-octyl-5-Methyl-3(2H)-furanone

Figure 6-1 The markers of onion infection

The concentrations of 2-hexyl-5-methyl-3(2H)-furanone and 2-octyl-5-methyl-3(2H)-furanone varied according to the grade of infection, type of infection, and onion cultivar. However, they ranged between 0.1-30 mg/kg.

A. sativum L.*, A. altaicum* Pall.*, A. . pskemense* B. Fedt.*, A. cornutum* Clementi ex Vis.*, A. fistulosum* L.*,* and *A. porrum* L. were also infected by *Fusarium oxysporum* and tested on the development of the markers. The investigated markers appeared in all infected *Allium* species except *Allium sativum* L. So, they can be considered as infection markers in many *Allium* species as well as cultivars.

The two furanones did not exhibit antifungal or antitumor activity, but they might mimic the quorum sensing signals of the pathogens, which could be a very promising approach as natural agents to control virulence and biofilm.

This research provided many answers for questions about the infection markers in *Allium cepa* L., their structure, concentration, presence in other *Alliums* and different analytical spectrums. Anyway, more questions appeared about their stereochemistry and bioactivity, which can be subjects for further projects. Some related approaches, like synthesis, GC and IMS applications, has been already started by colleagues in our research groups. The accumulated results are supposed to provide an effective early stage detection of onion infection.

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8. APPENDIX

8.1 HPLC spectrums

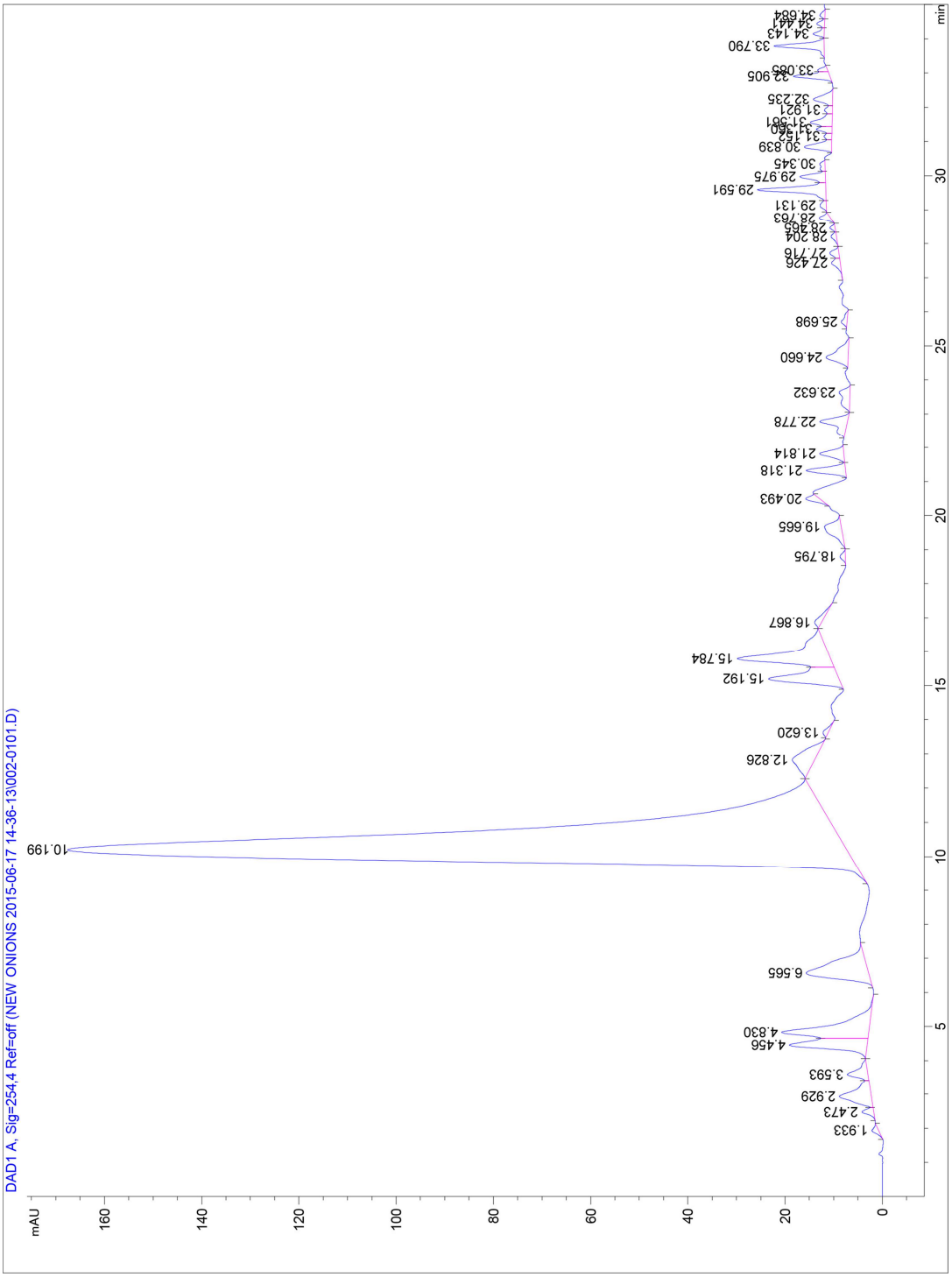

Figure 8-1 Healthy *Allium cepa* L. bulb's extract spectrum

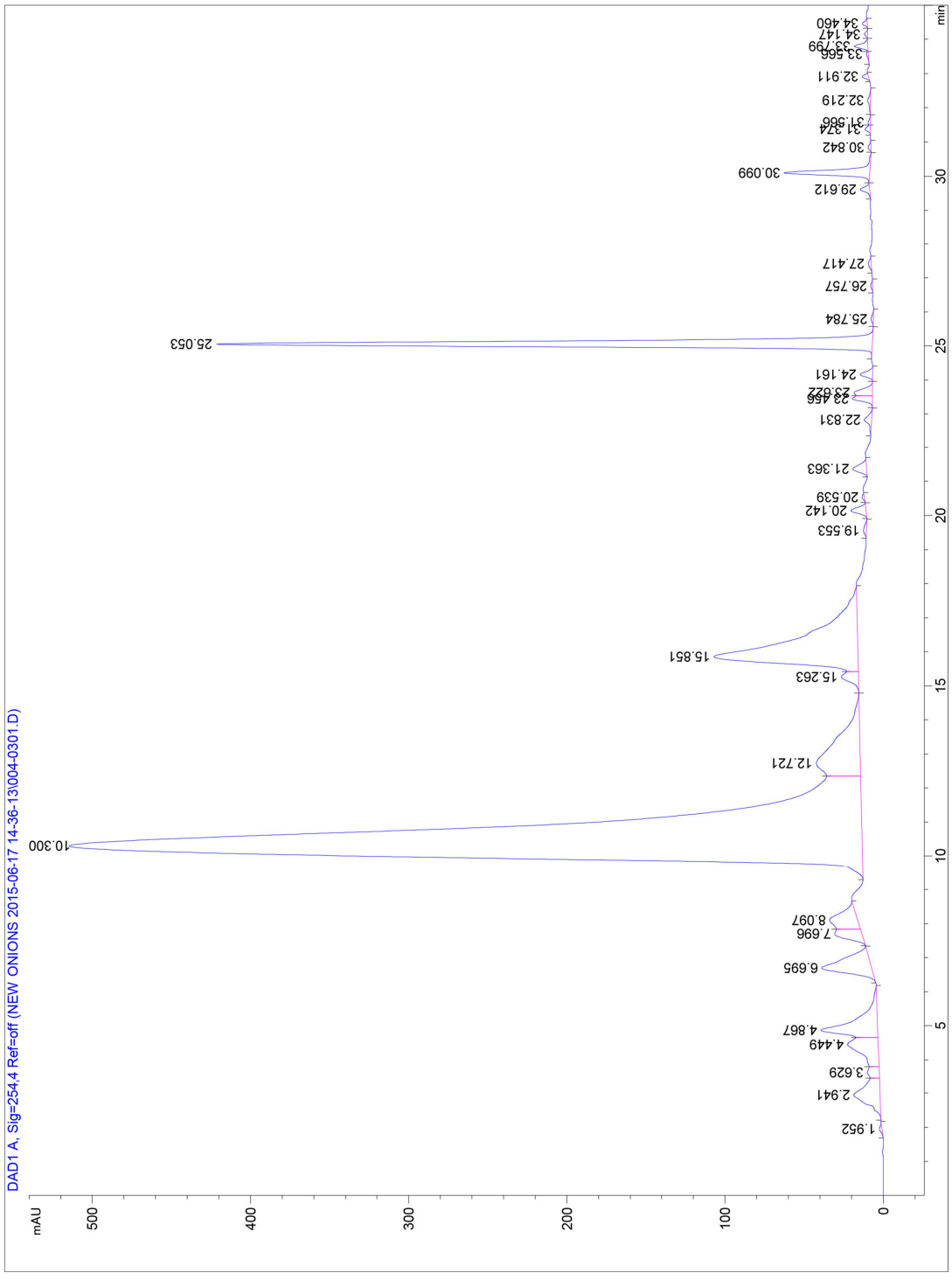

Figure 8-2 Infected *Allium cepa* L. bulb's extract spectrum (*Fusarium oxysporum)*

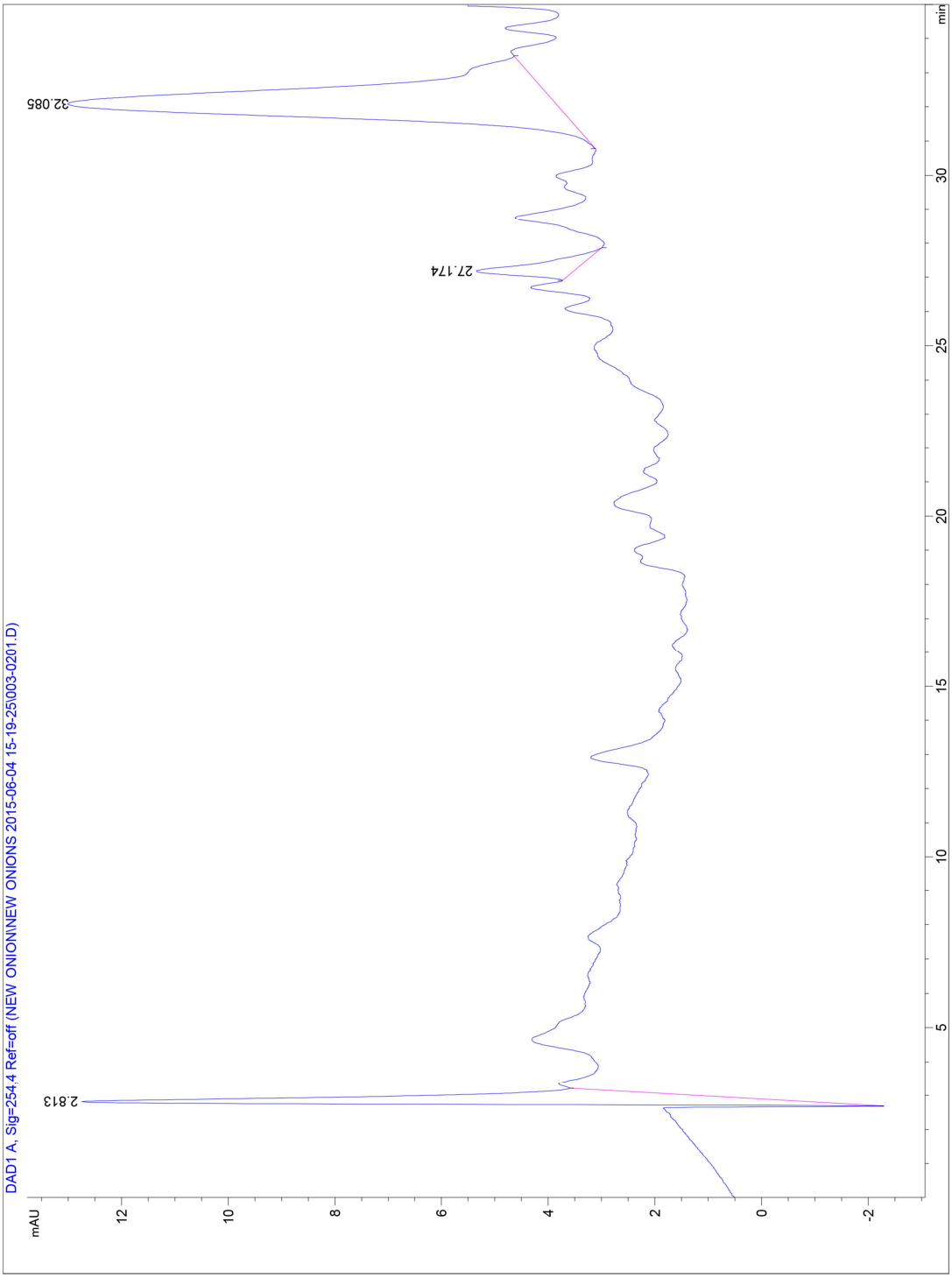

Figure 8-3 Pure *Fusarium oxysporum* extract spectrum

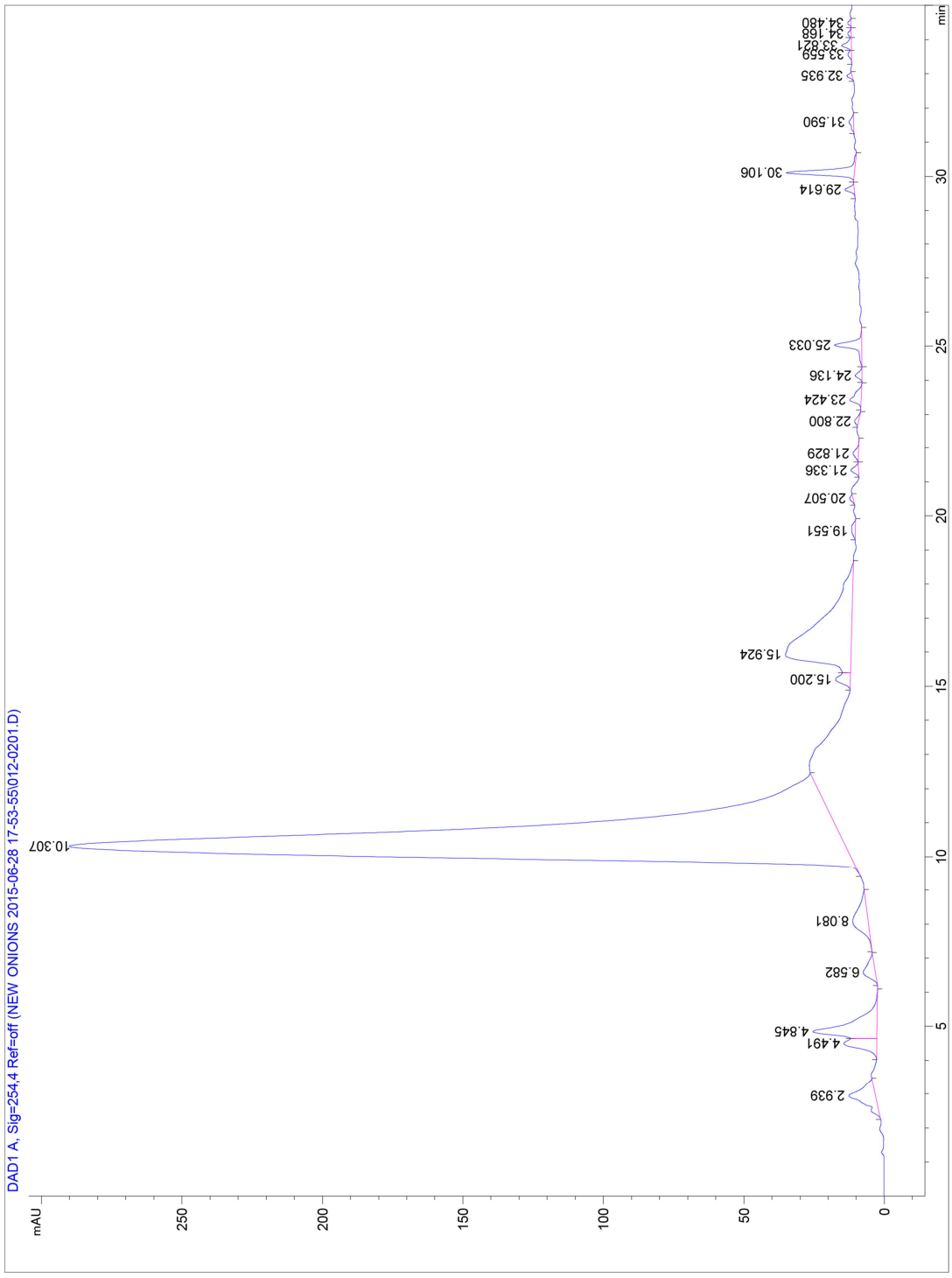

Figure 8-4 Infected *Allium cepa* L. bulb's extract spectrum with alliinase inhibition

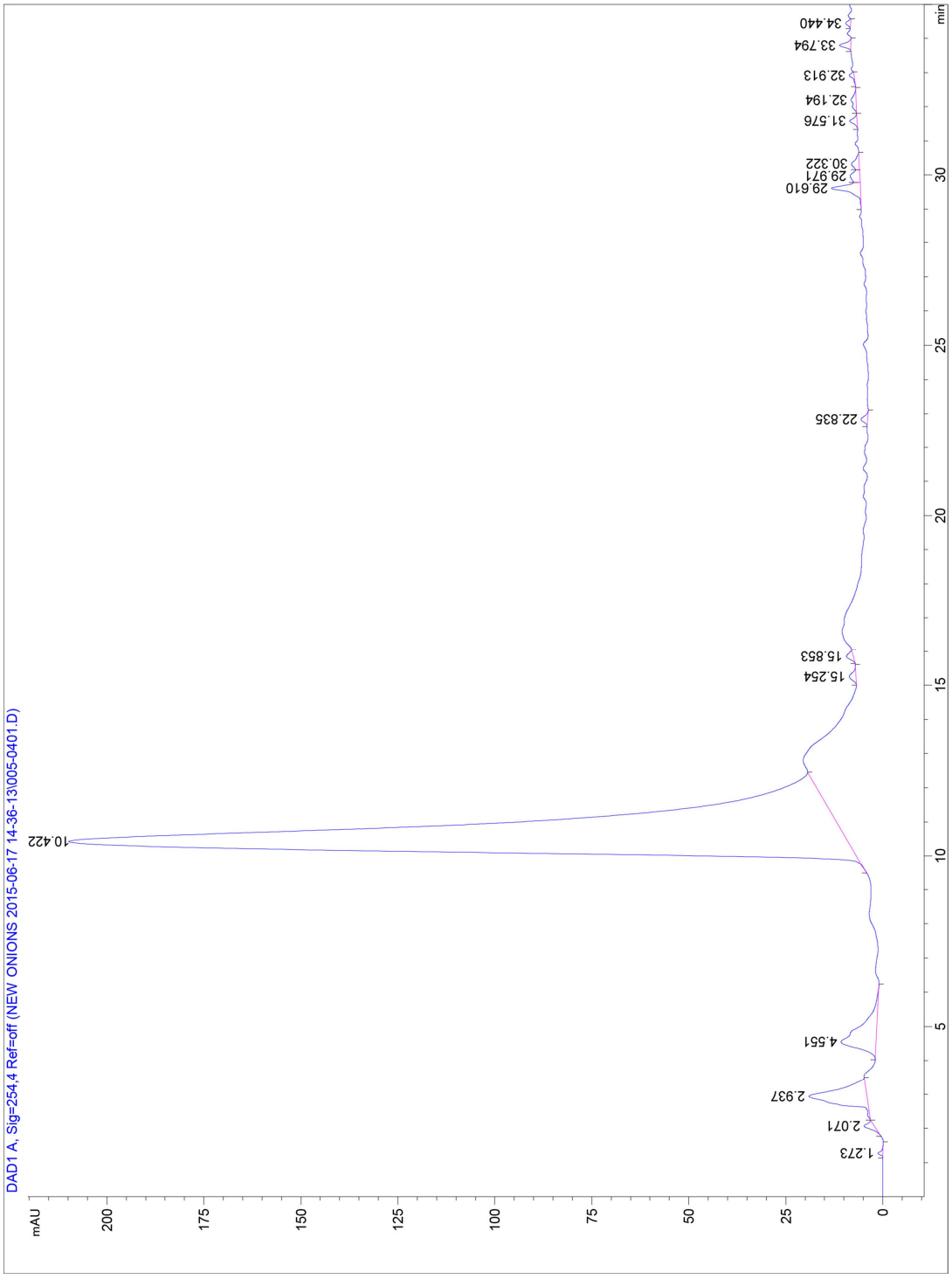

Figure 8-5 Spectrum of extracted healthy part of an infected *Allium cepa* L. bulb *Fusarium oxysporum*

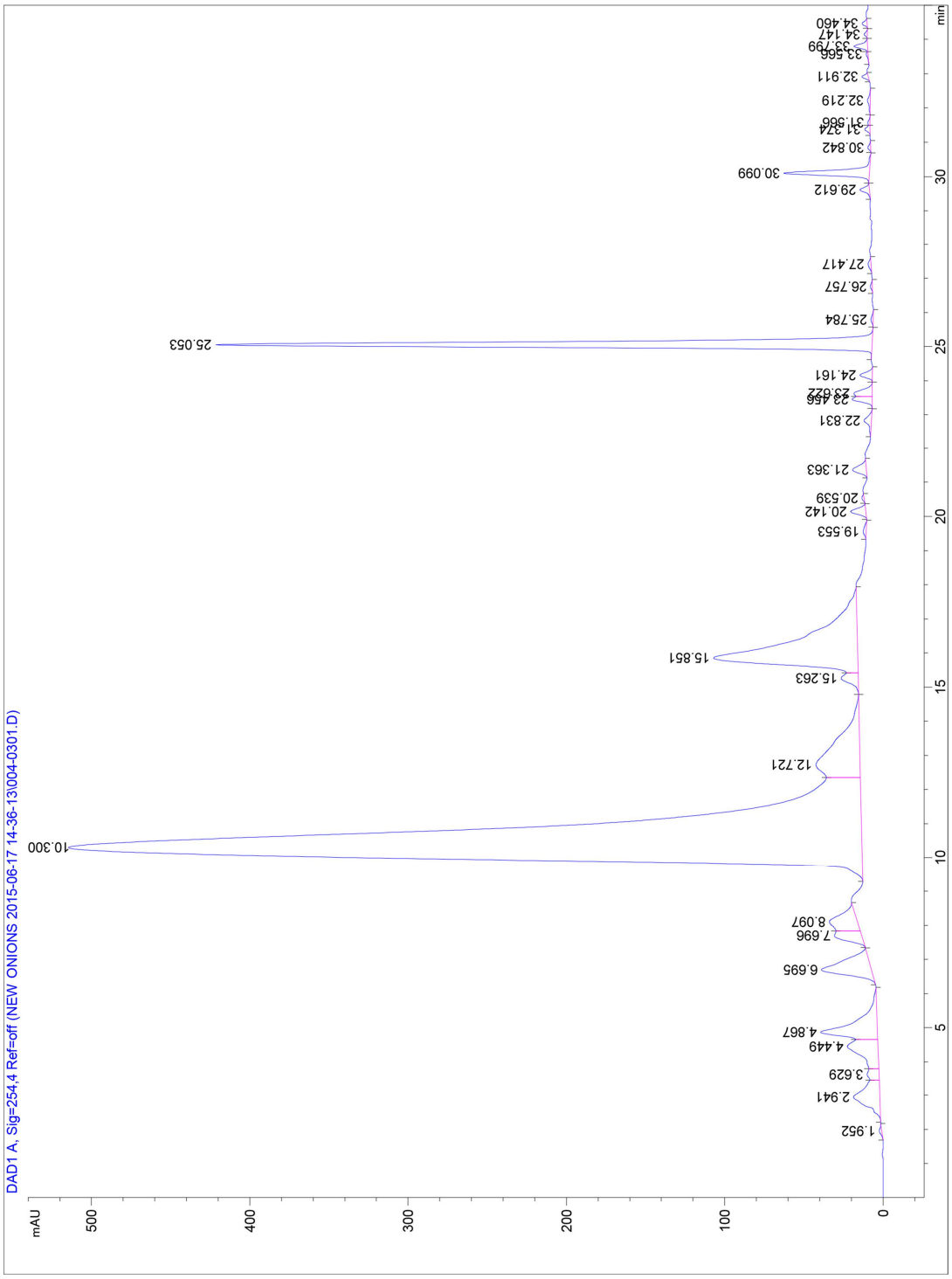

Figure 8-6 Spectrum of extracted infected part of an infected *Allium cepa* L. bulb *Fusarium oxysporum*

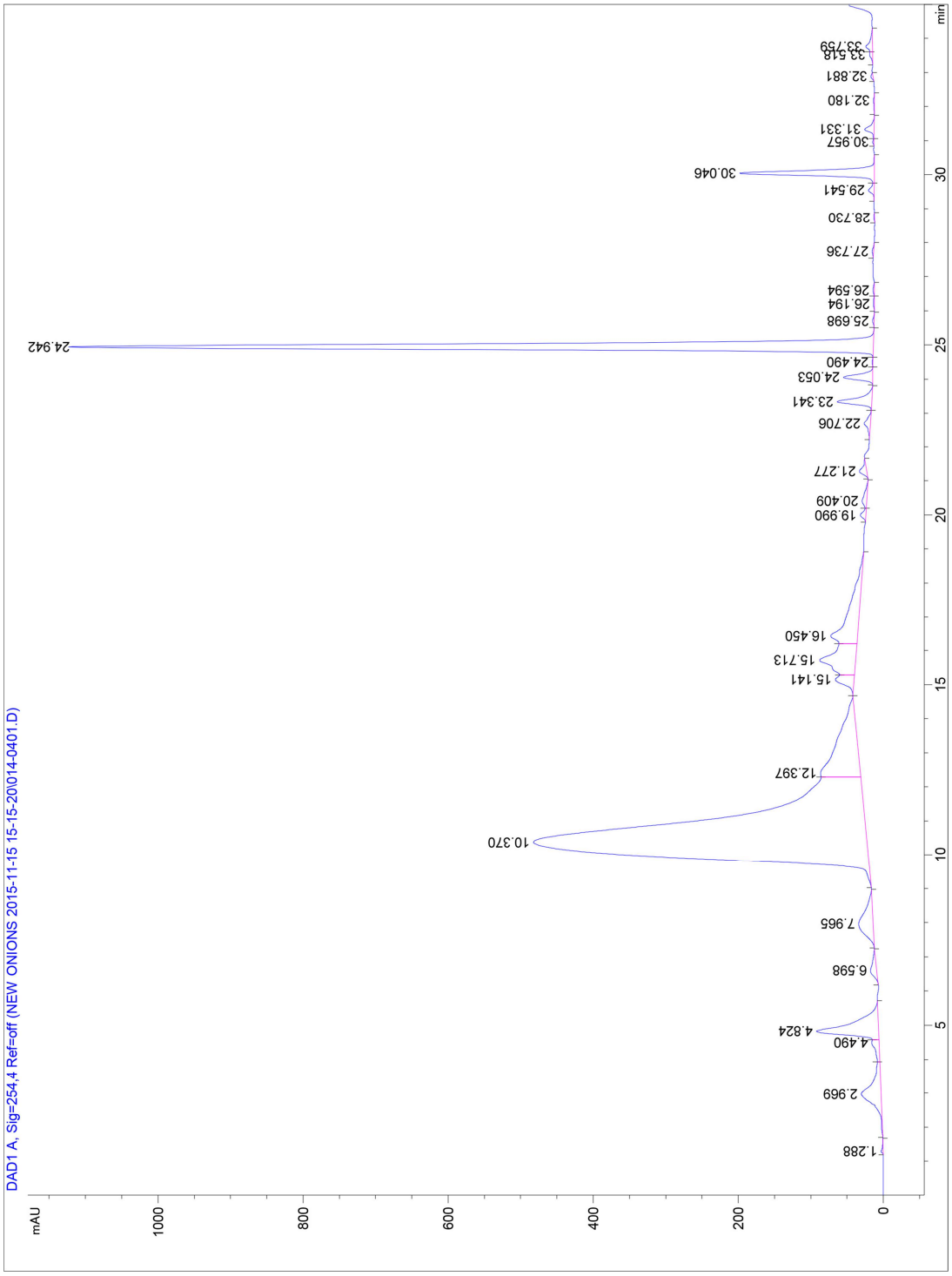

Figure 8-7 Infected *Allium cepa* L. bulb's extract spectrum (*Fusarium proliferatum)*

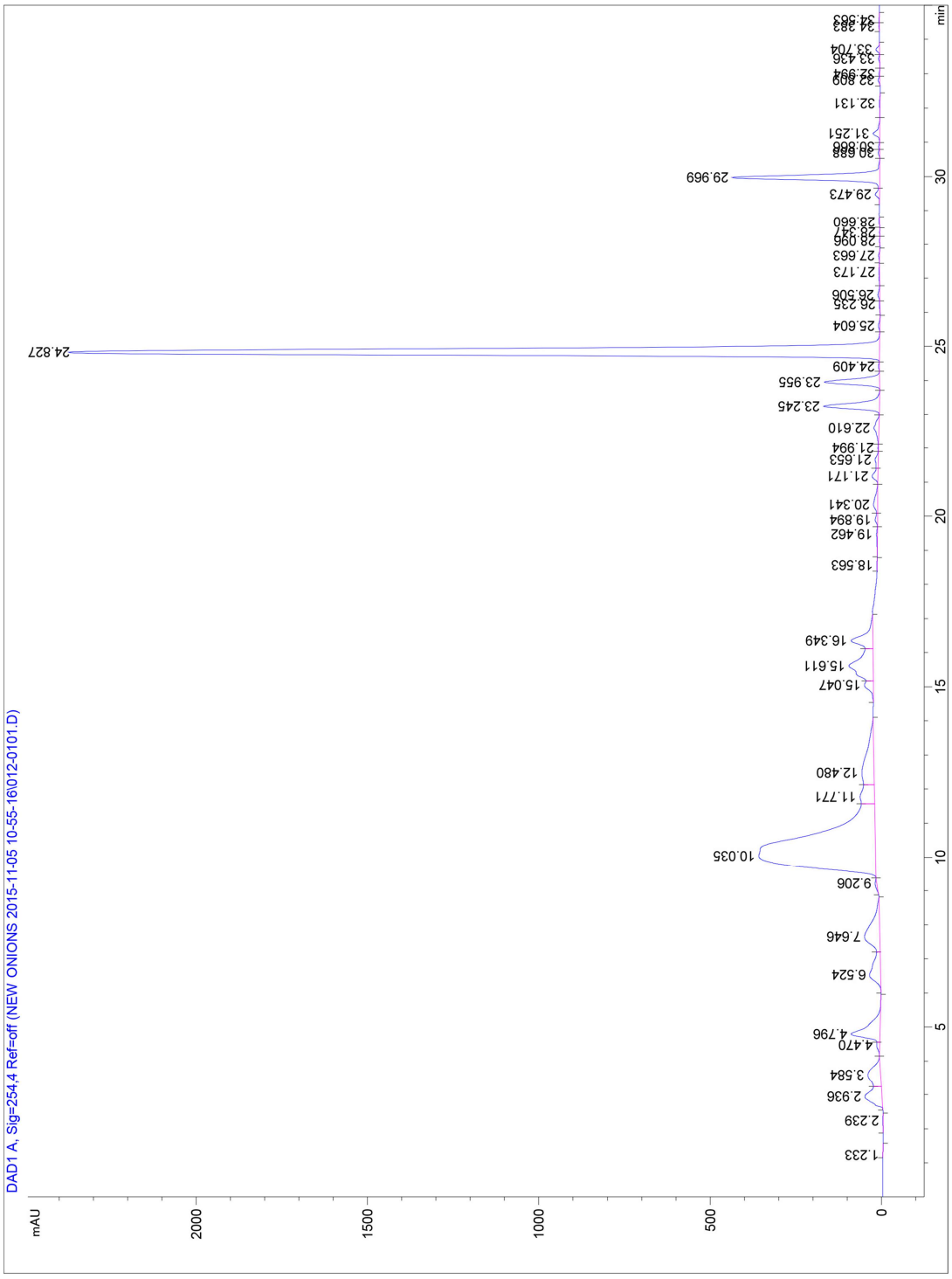

Figure 8-8 Infected *Allium cepa* L. bulb's extract spectrum (*Penicillium sp)*

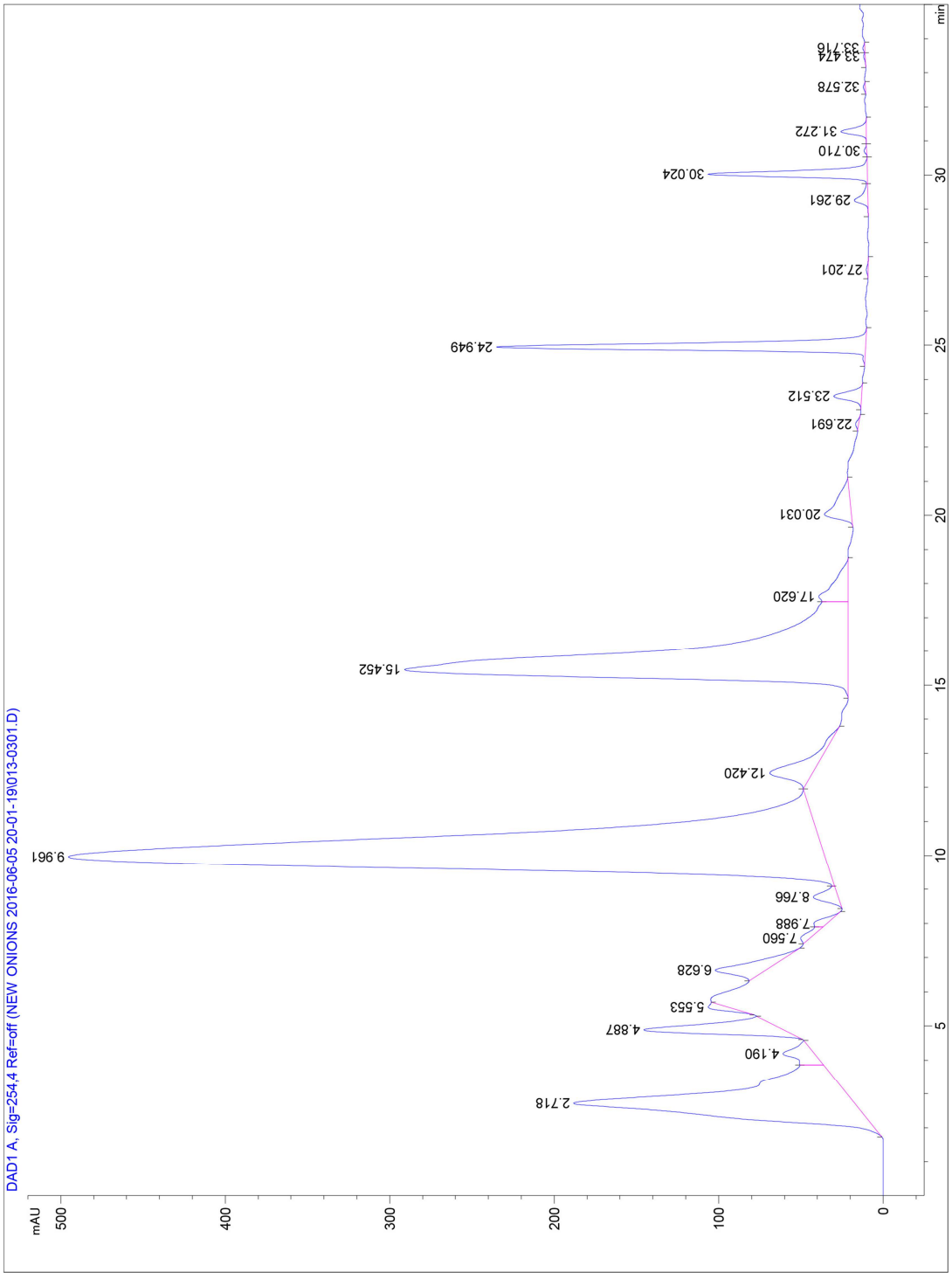

Figure 8-9 Infected *Allium cepa* L. bulb's extract spectrum (*Botrytis aclada)*

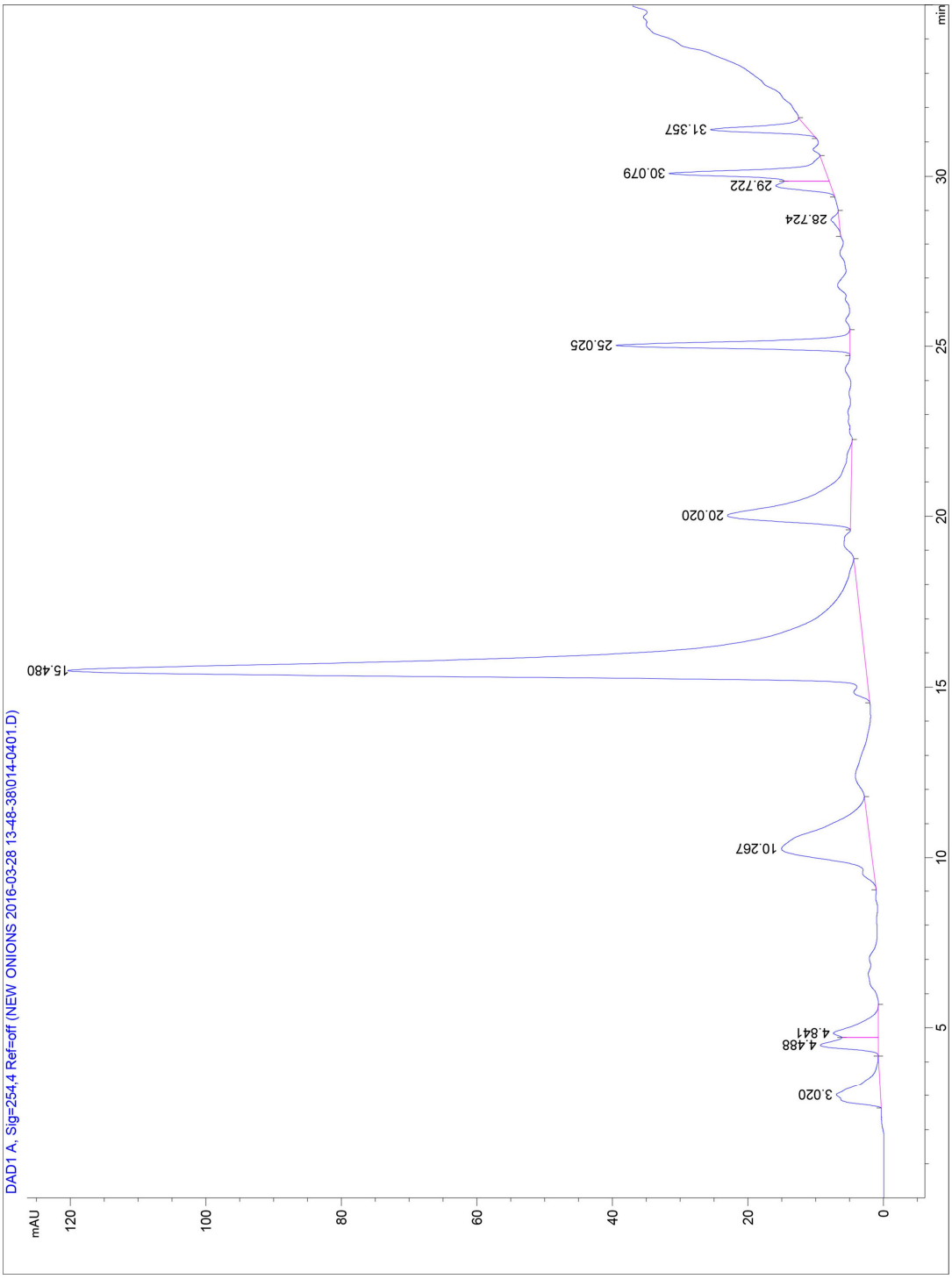

Figure 8-10 Infected *Allium cepa* L. bulb's extract spectrum (*Erwinia carotovora)*

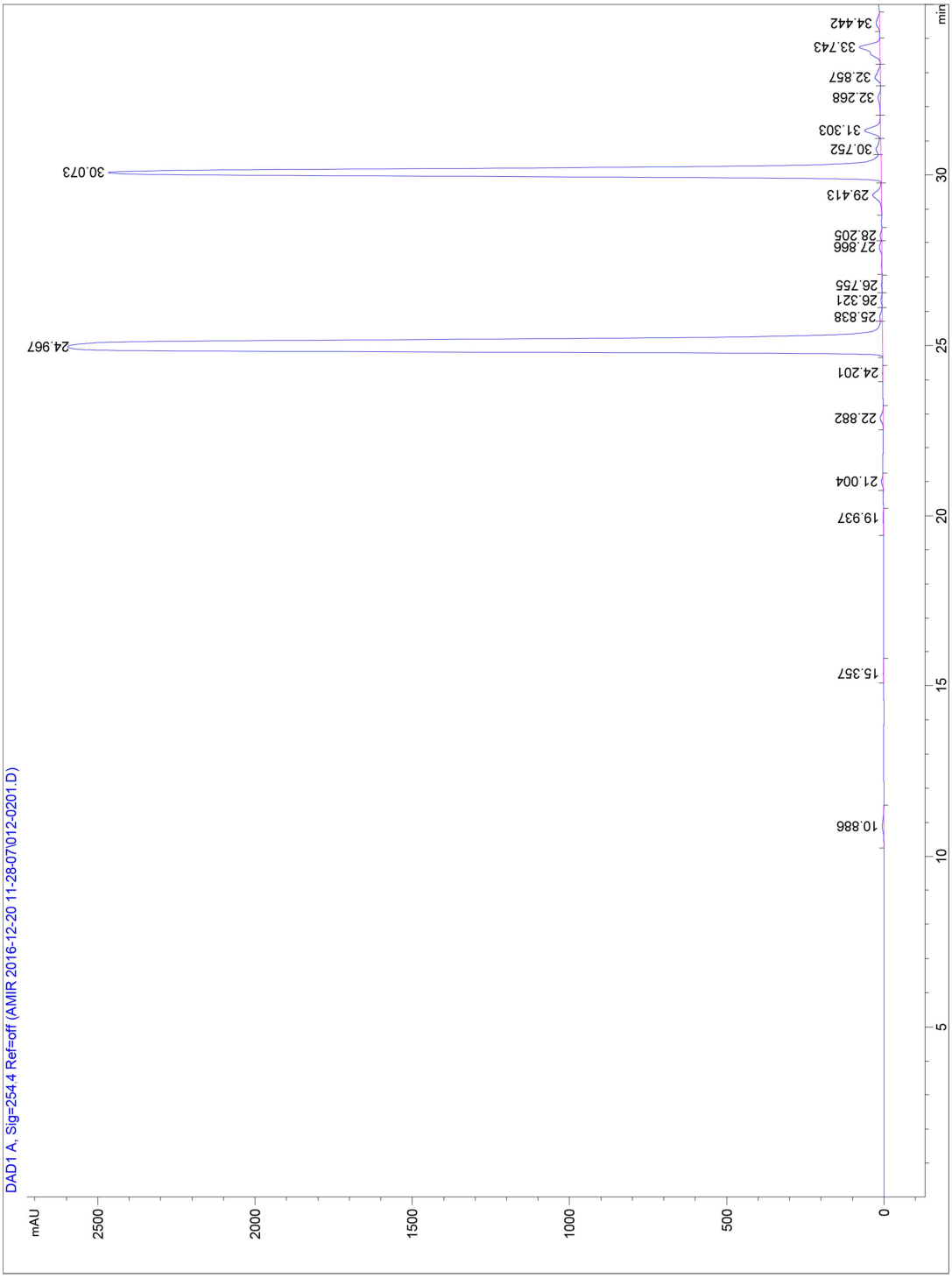

Figure 8-11 Infected bulbs extracted by distillation

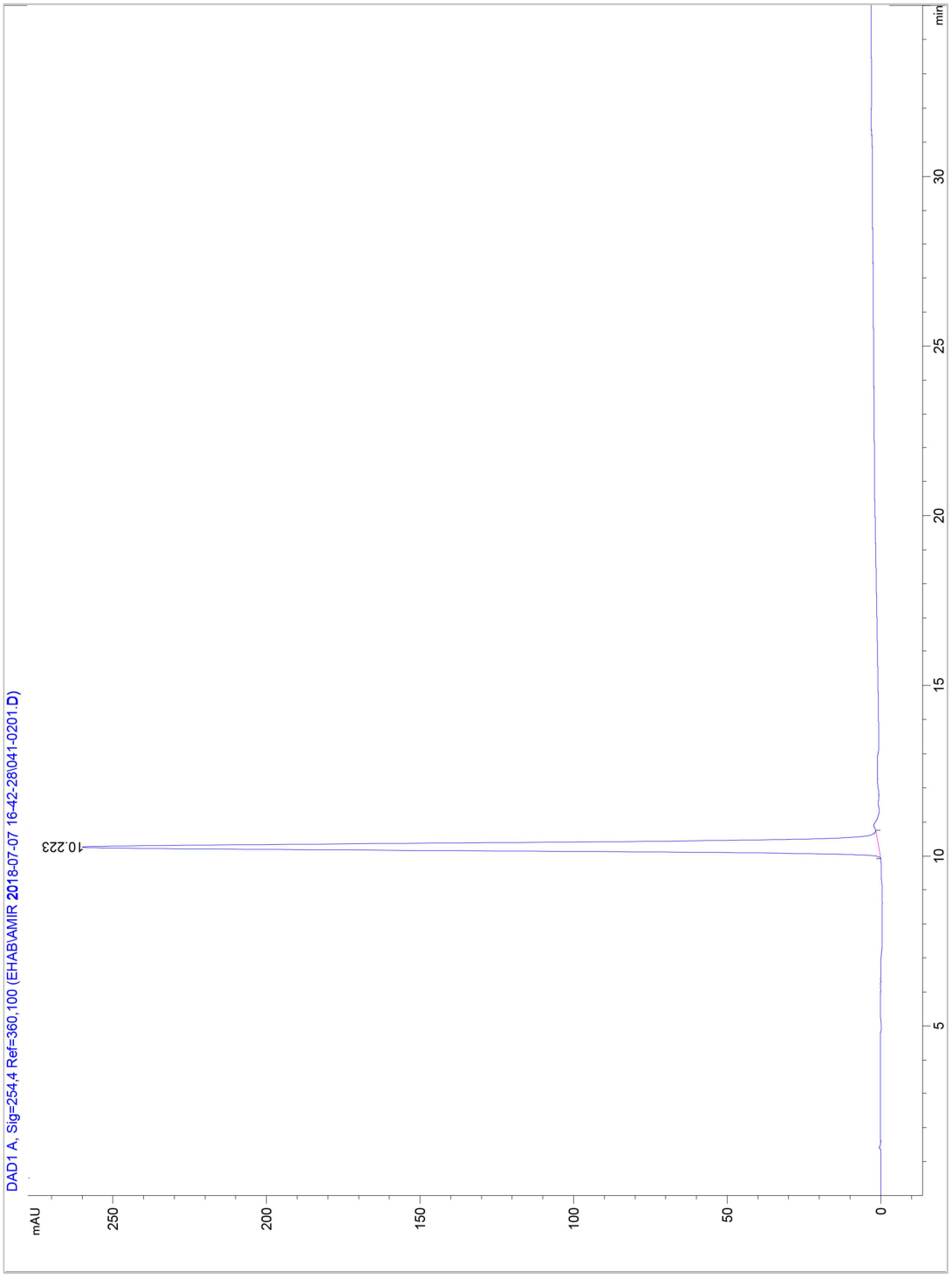

Figure 8-12 Quercetin-4'-O-glucoside in HPLC

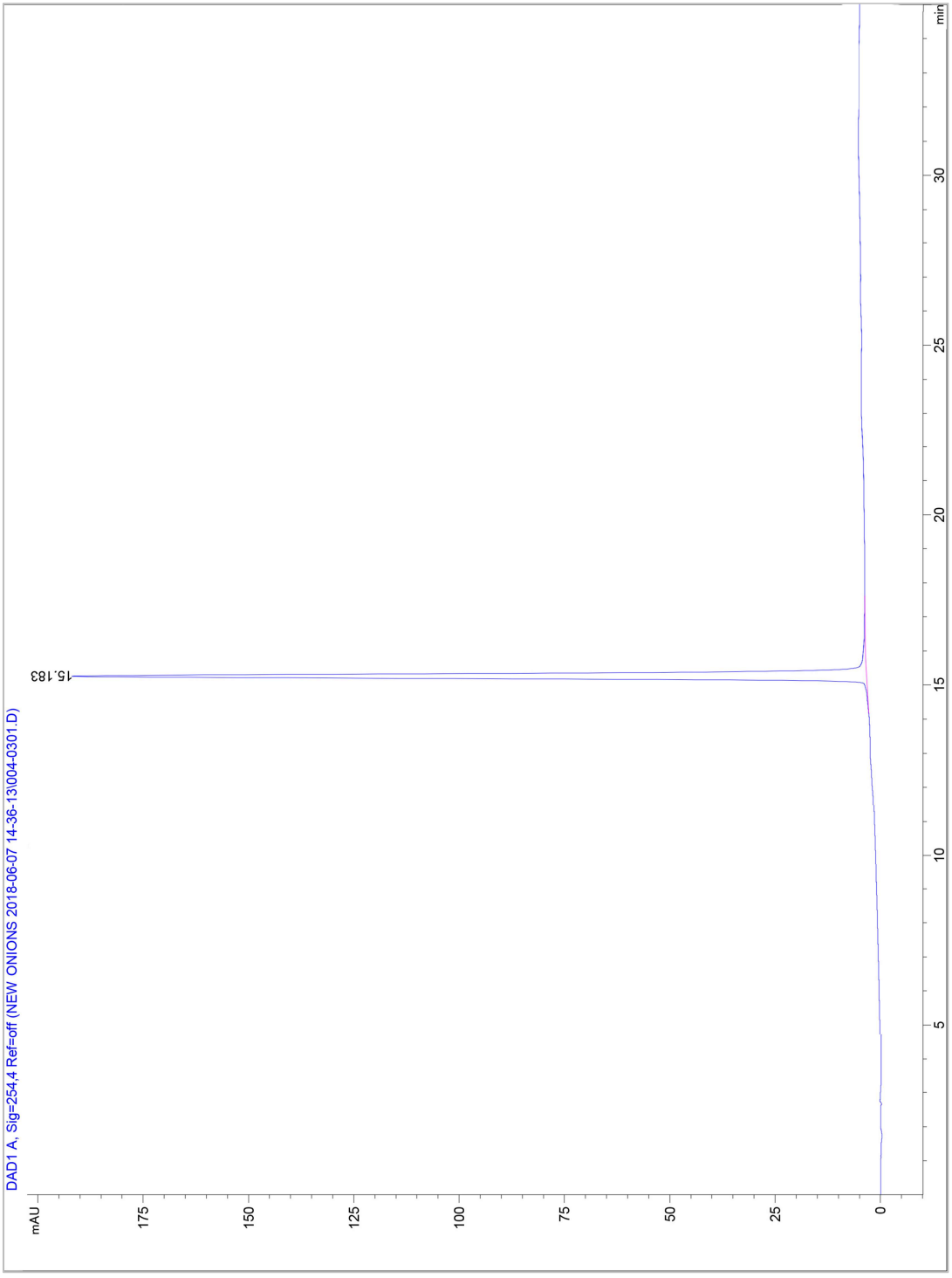

Figure 8-13 Quercetin in HPLC

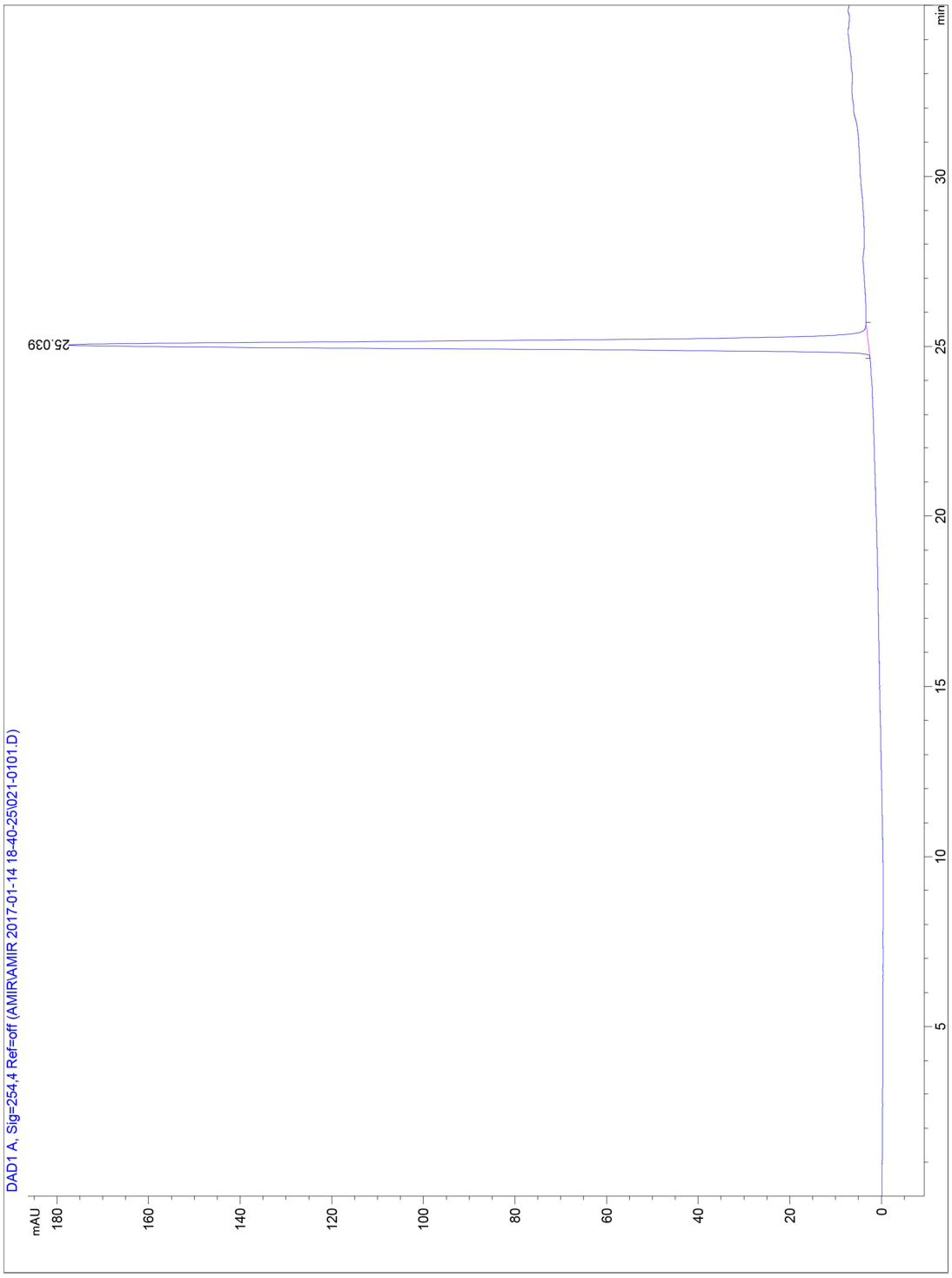

Figure 8-14 Peak C after preparative fractionation

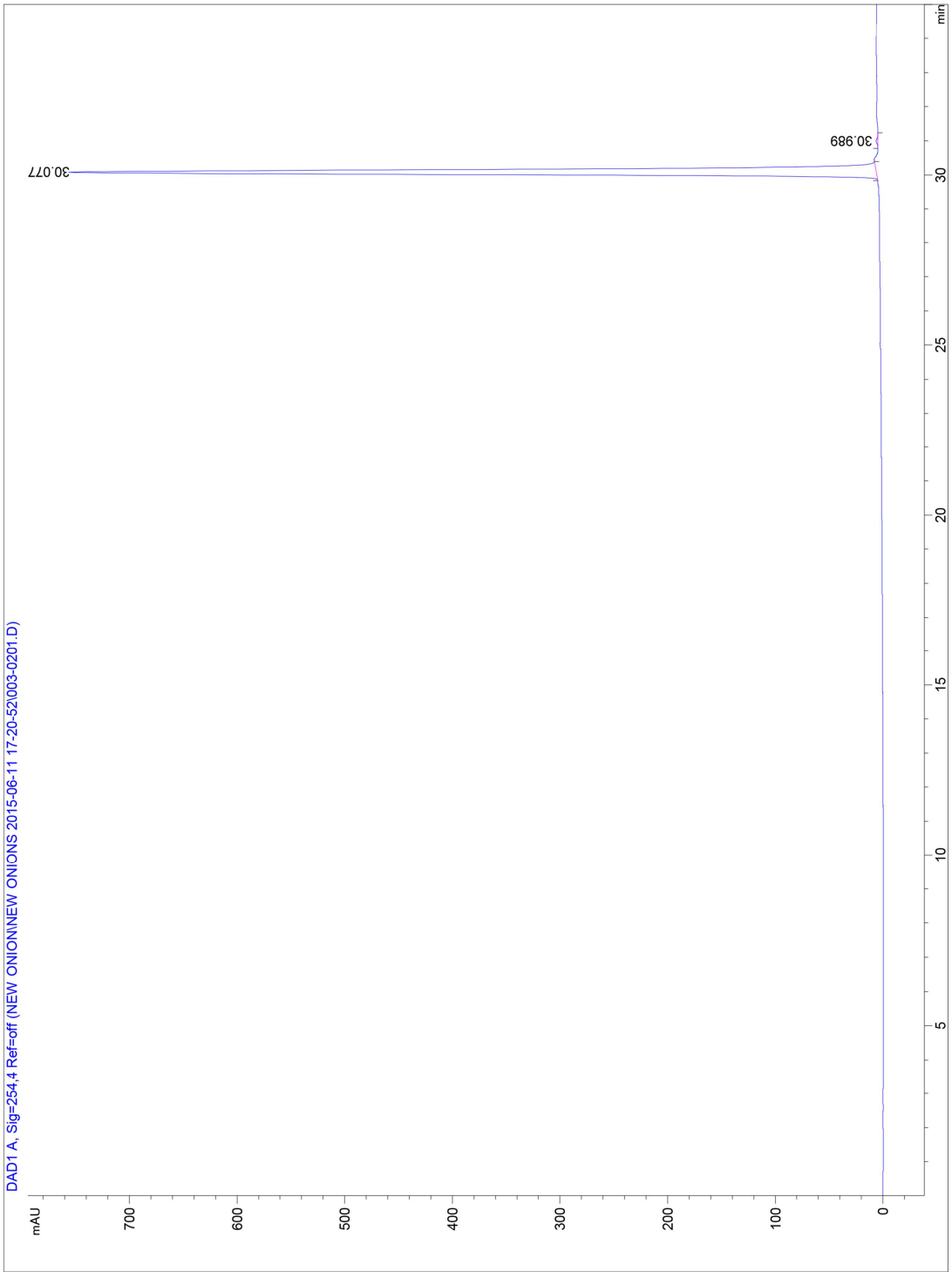

Figure 8-15 Peak D after preparative fractionation

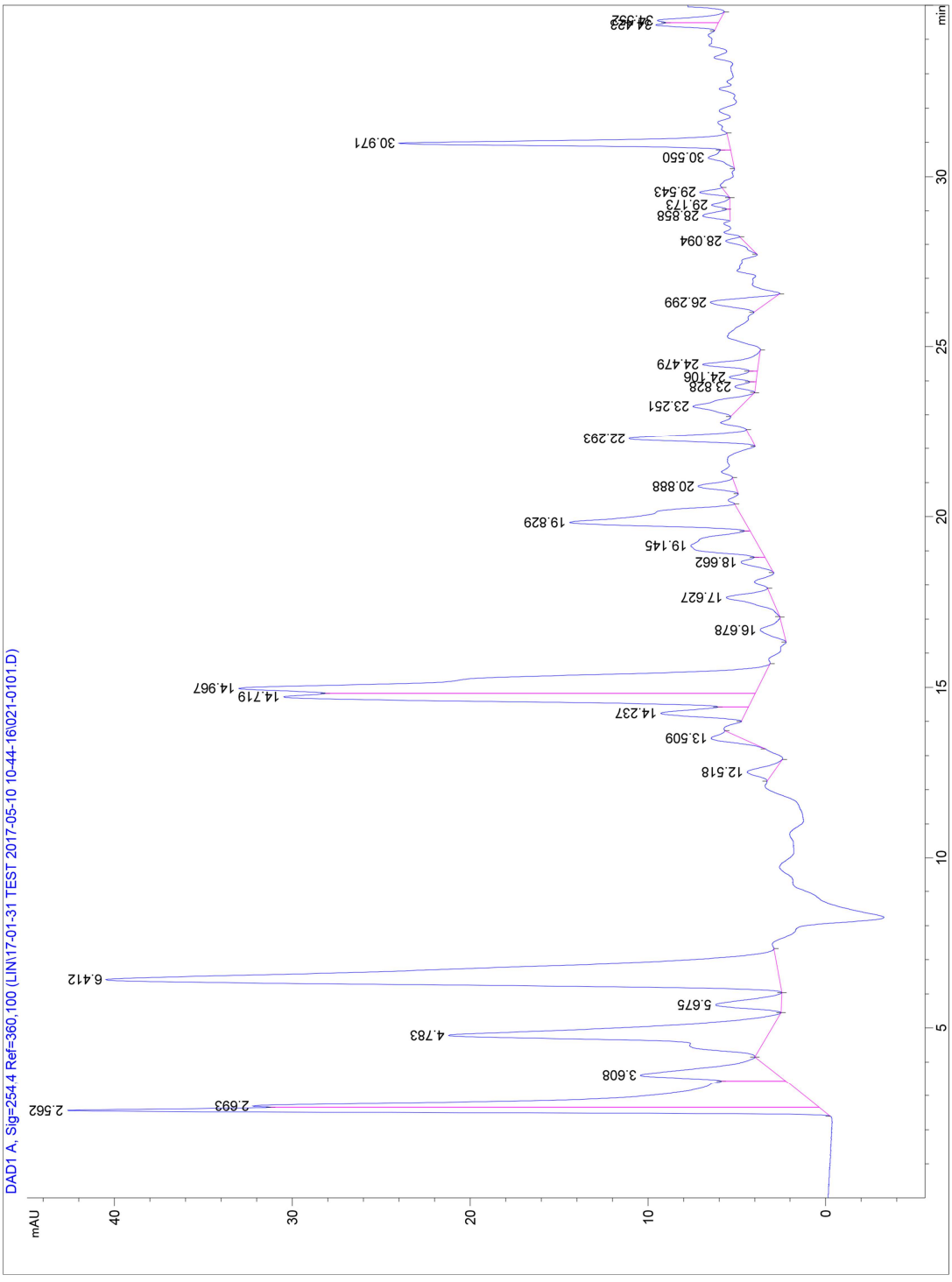

Figure 8-16 Healthy *Allium altaicum* Pall. bulb's extract spectrum

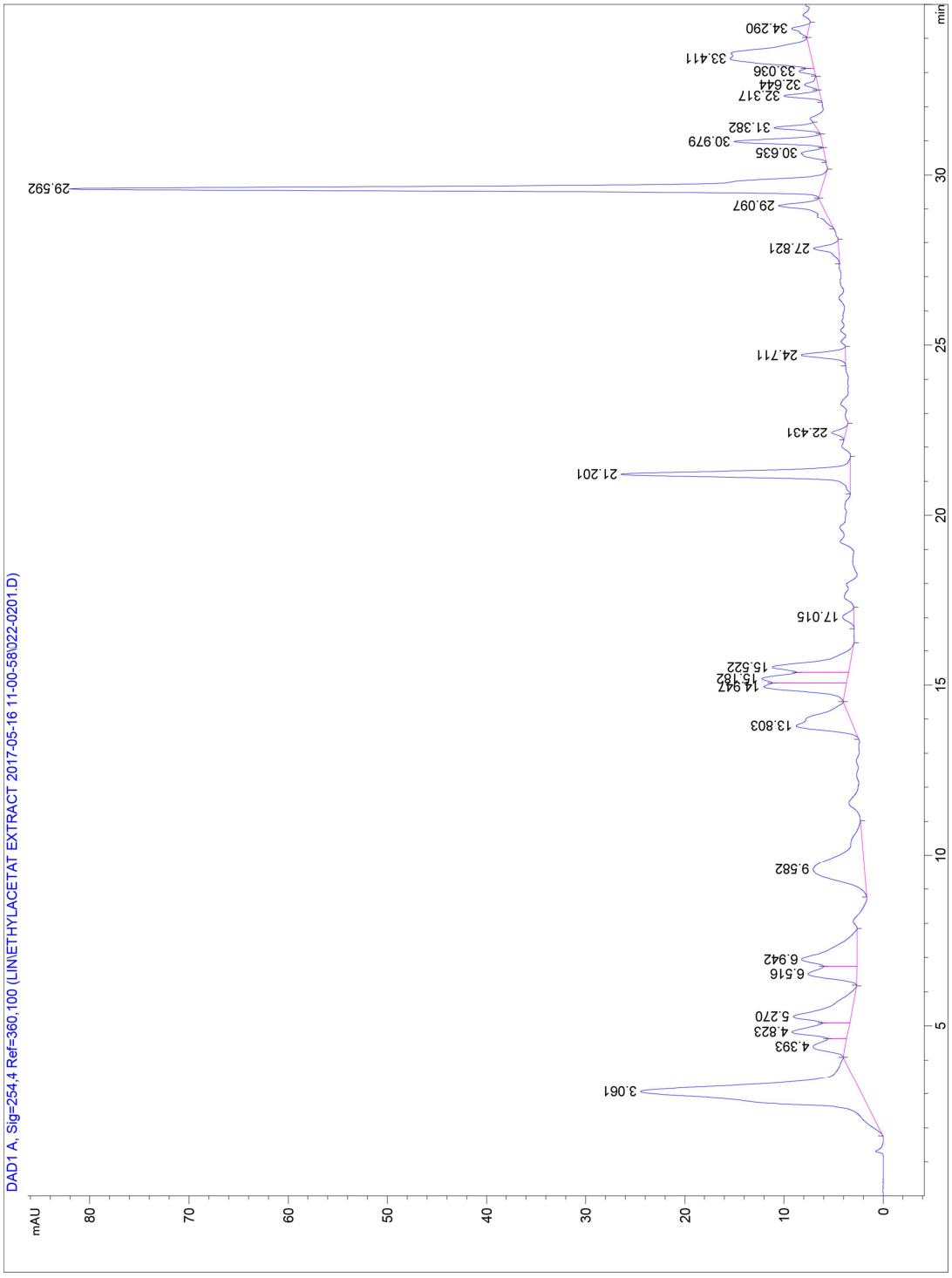

Figure 8-17 Infected *Allium altaicum* Pall. bulb's extract spectrum

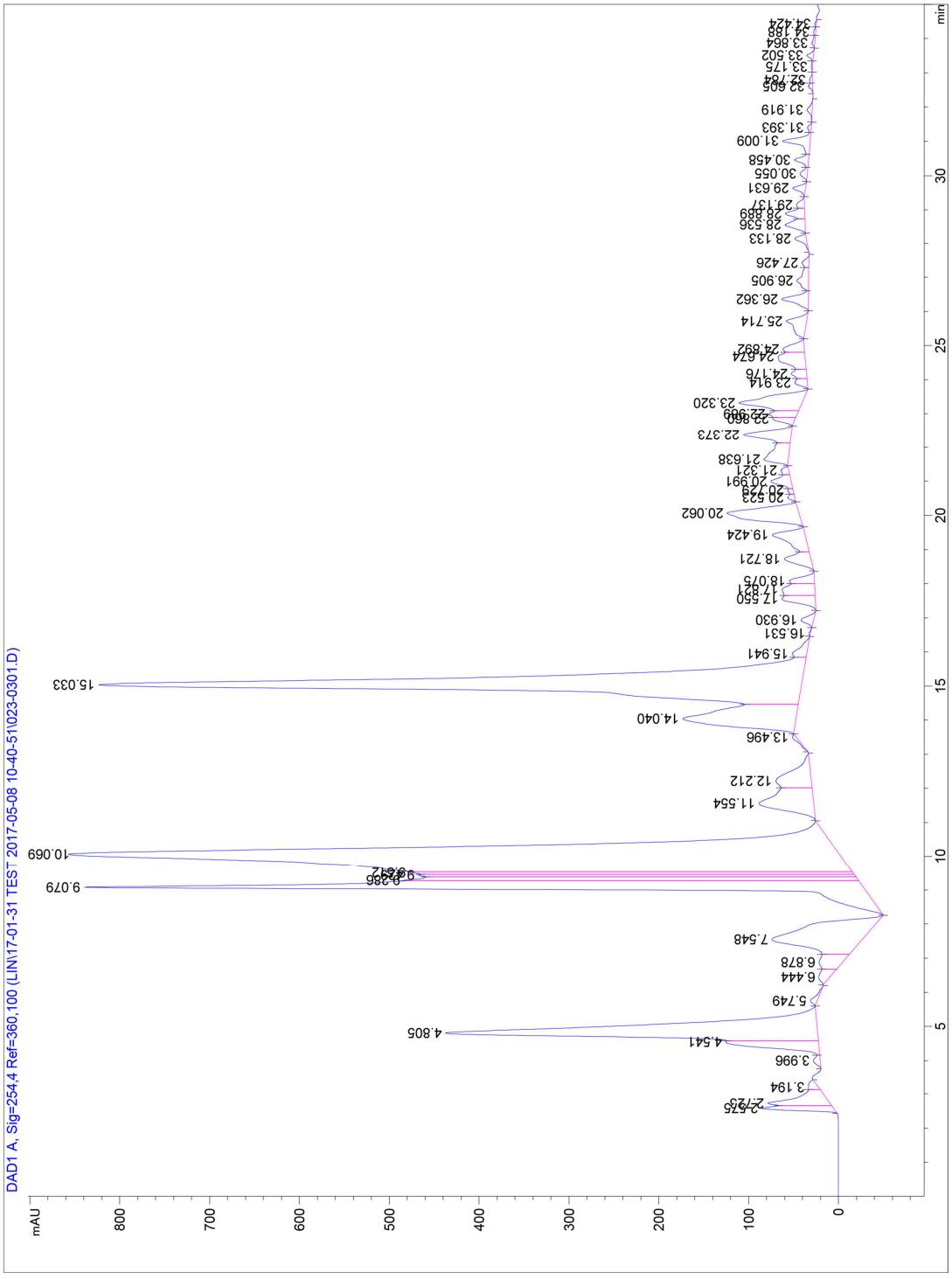

Figure 8-18 Healthy *Allium pskemense* B. Fedt. bulb's extract spectrum

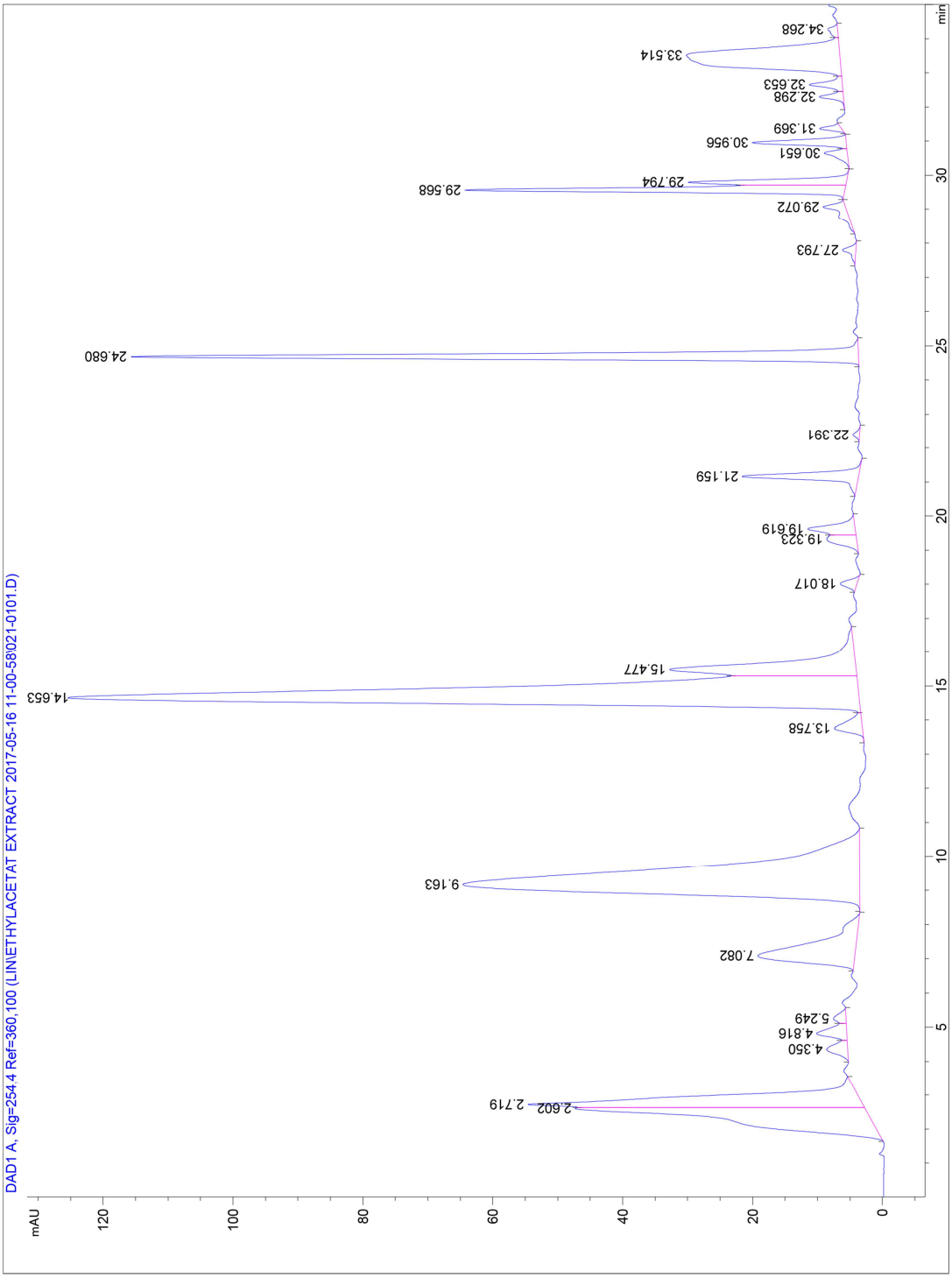

Figure 8-19 Infected *Allium pskemense* B. Fedt. bulb's extract spectrum

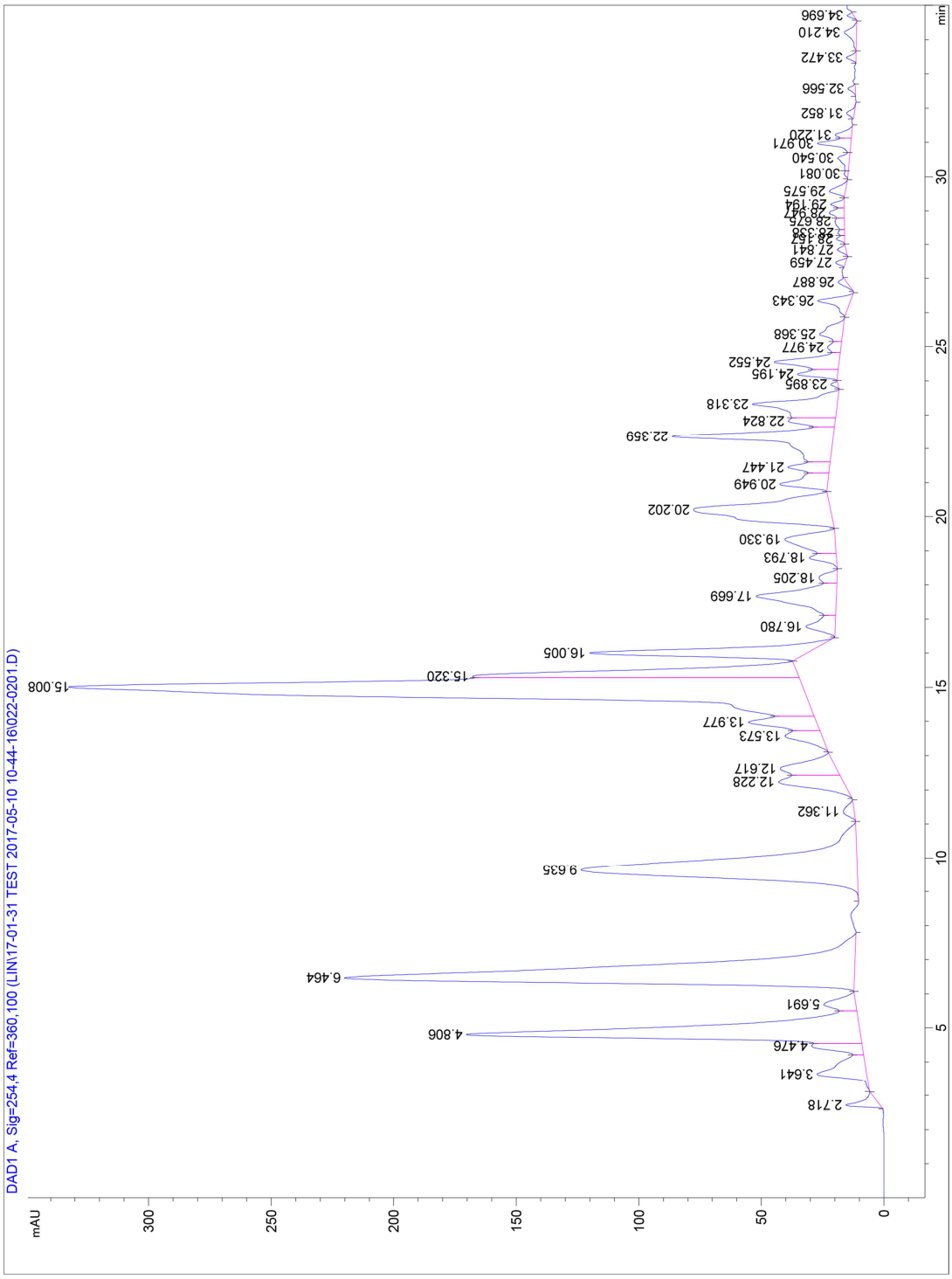

Figure 8-20 Healthy *Allium cornutum* Clementi ex Vis. bulb's extract spectrum

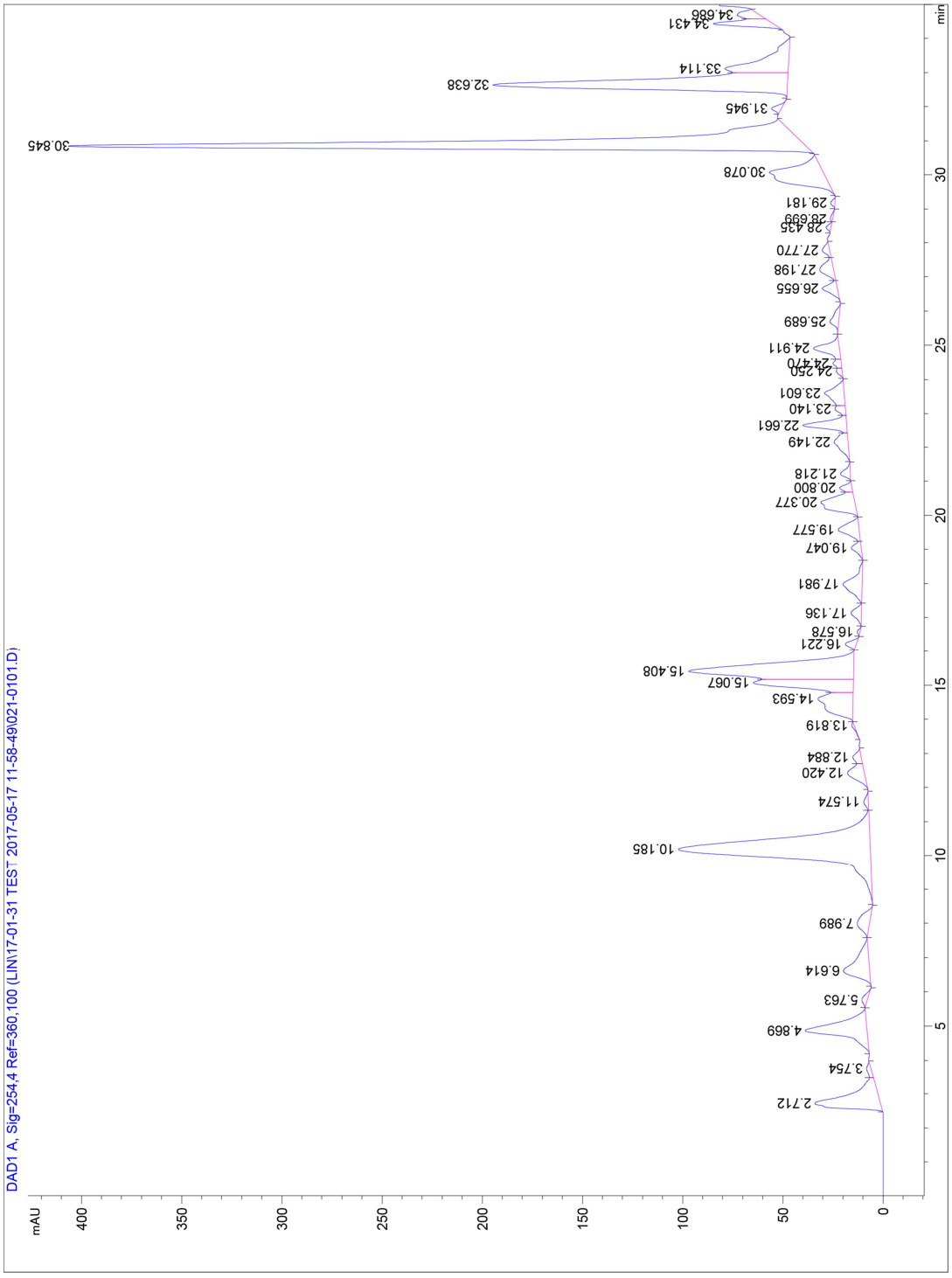

Figure 8-21 Infected *Allium cornutum* Clementi ex Vis. bulb's extract spectrum

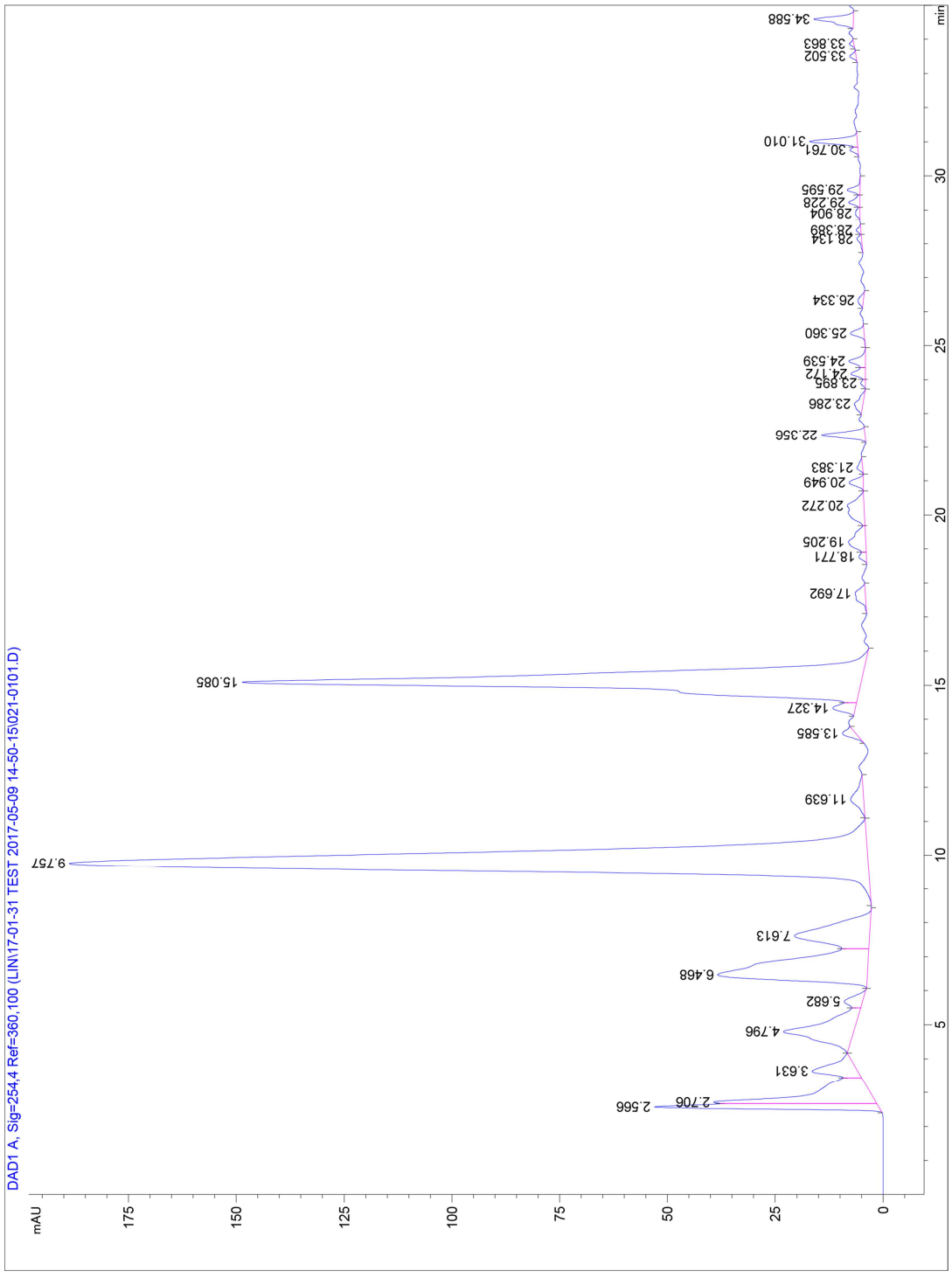

Figure 8-22 Healthy *Allium bastard* bulb's extract spectrum

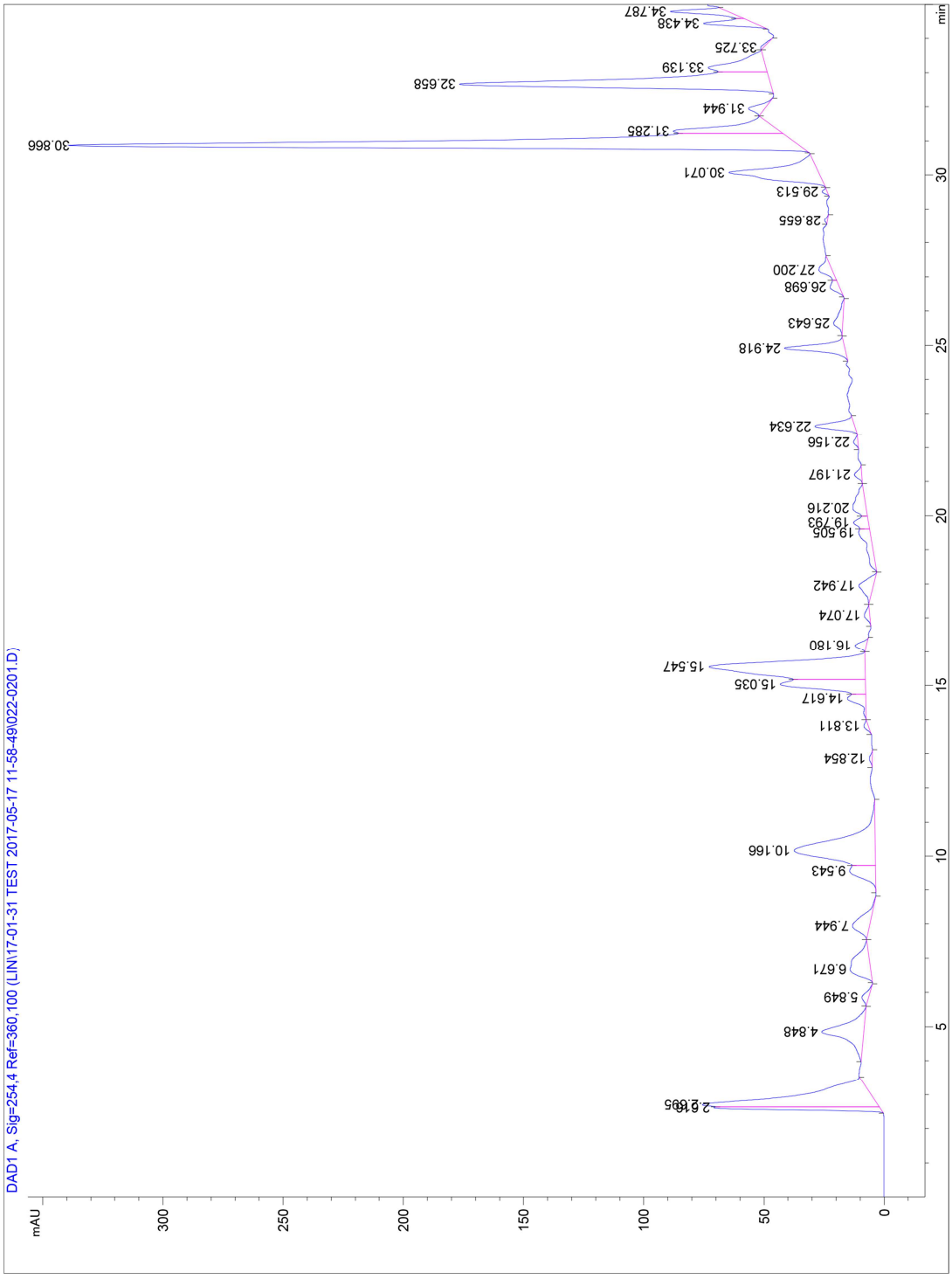

Figure 8-23 Infected *Allium bastard* bulb's extract spectrum

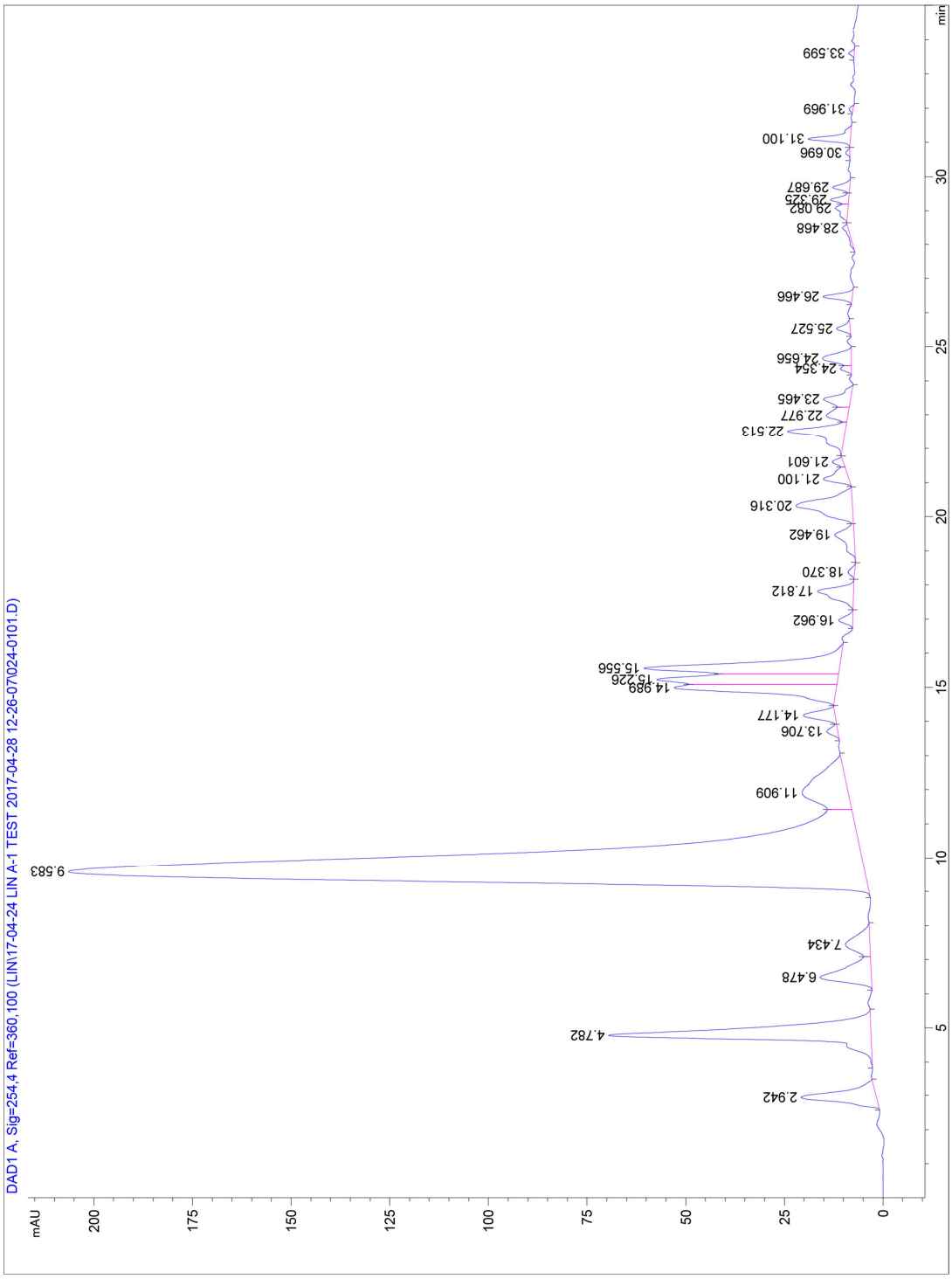

Figure 8-24 Healthy *Allium fistulosum* L. bulb's extract spectrum

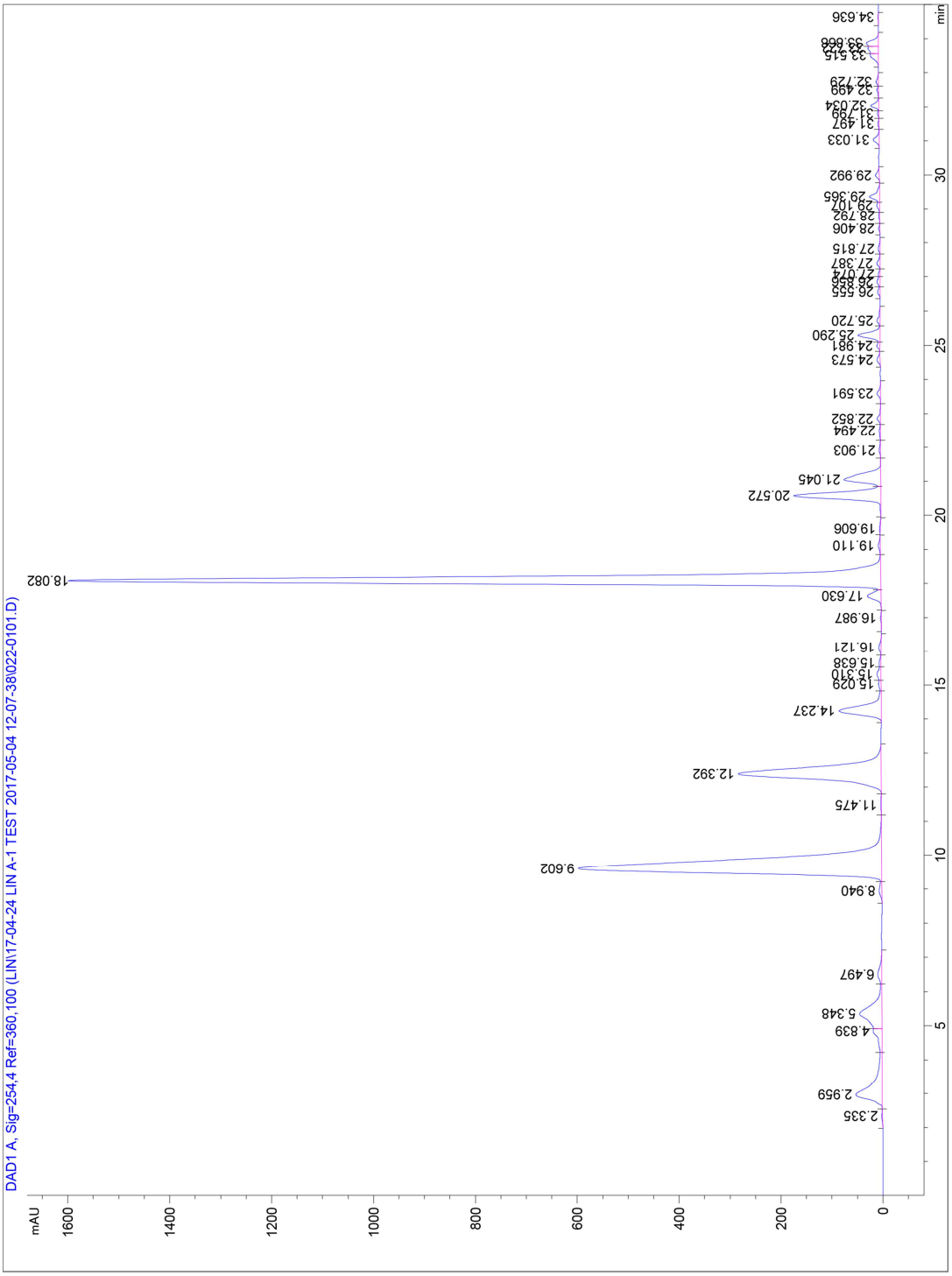

Figure 8-25 Infected *Allium fistulosum* L. bulb's extract spectrum

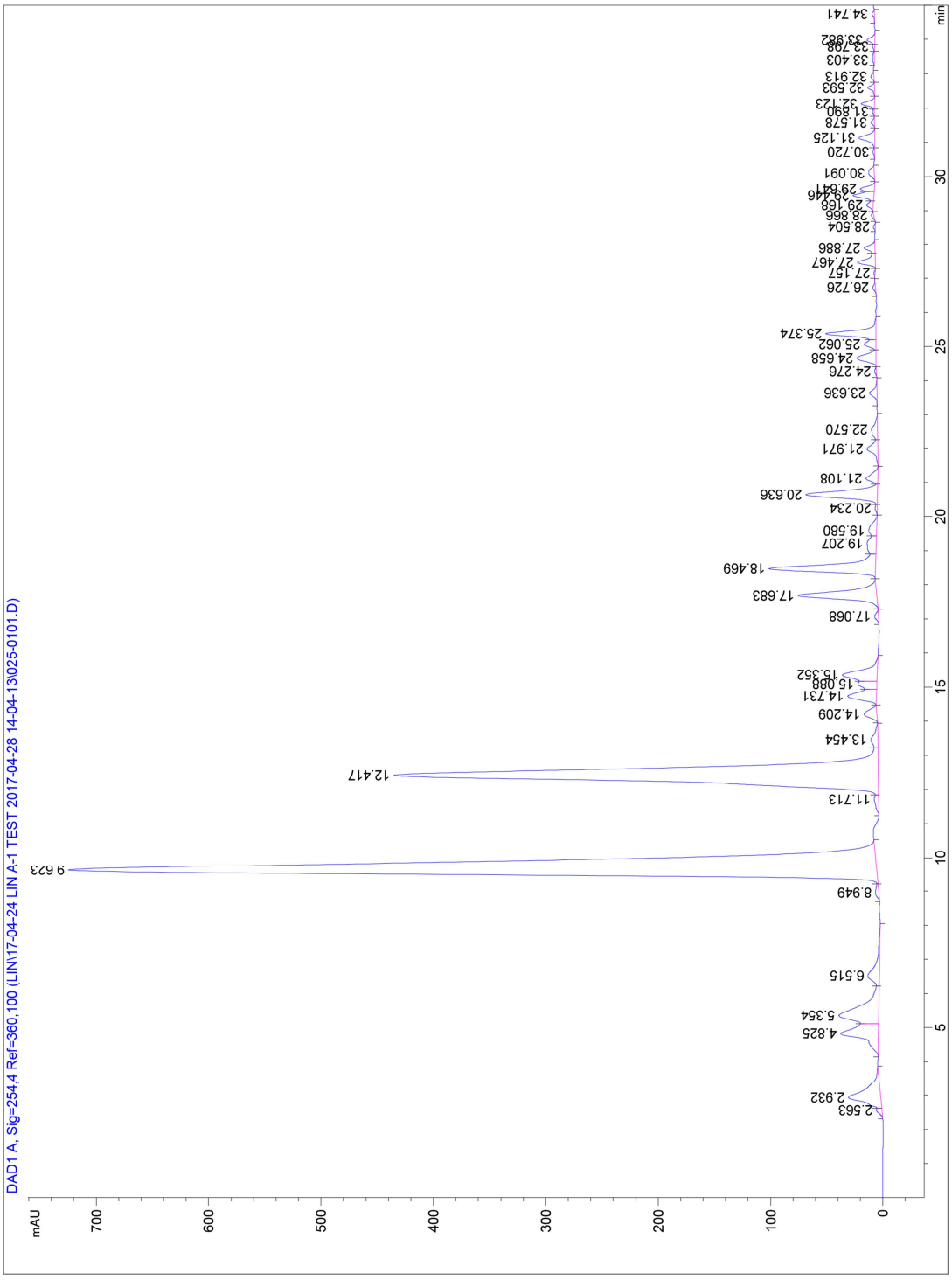

Figure 8-26 Healthy *Allium porrum* L. bulb's extract spectrum

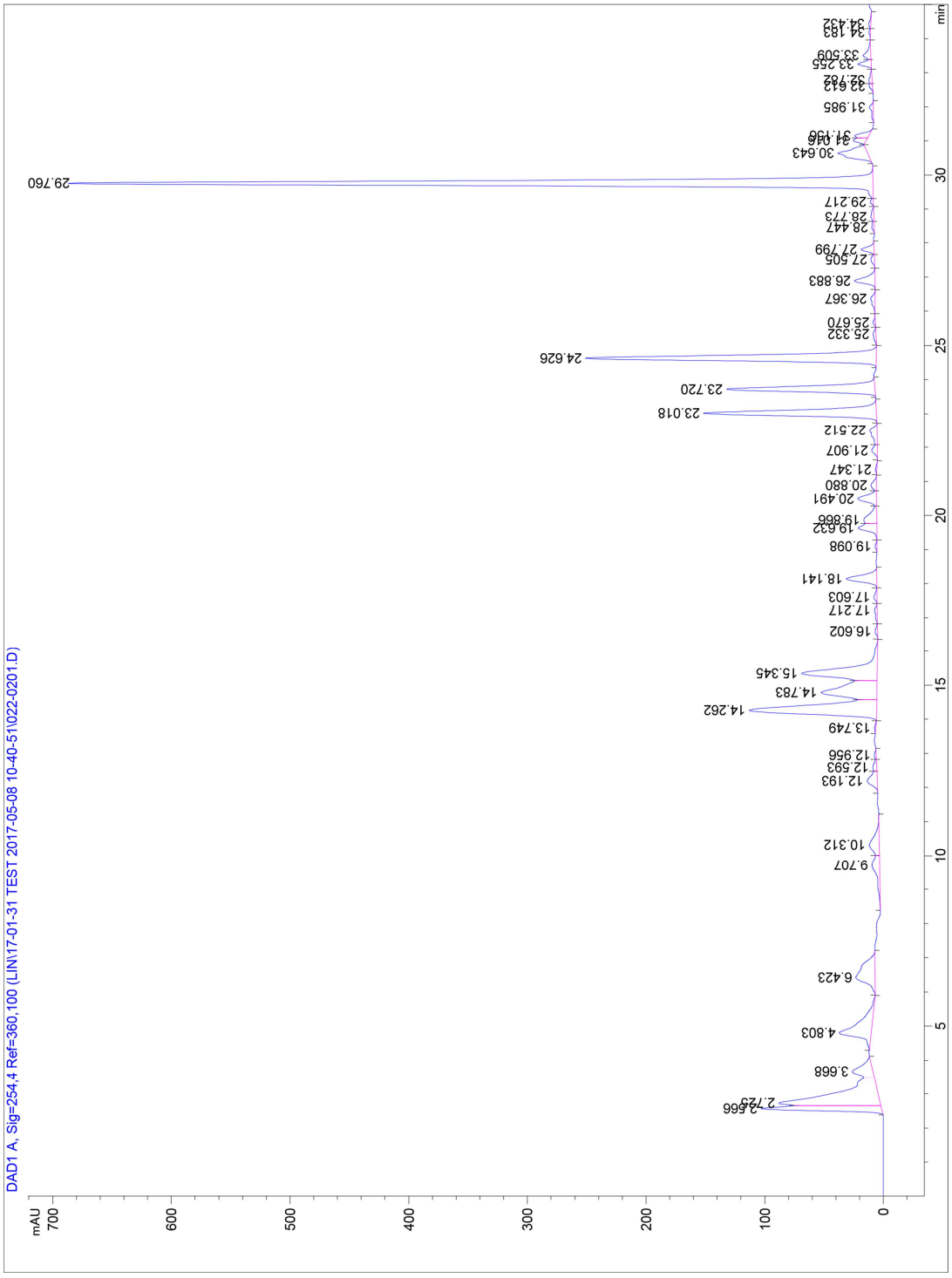

Figure 8-27 Infected *Allium porrum* L. bulb's extract spectrum

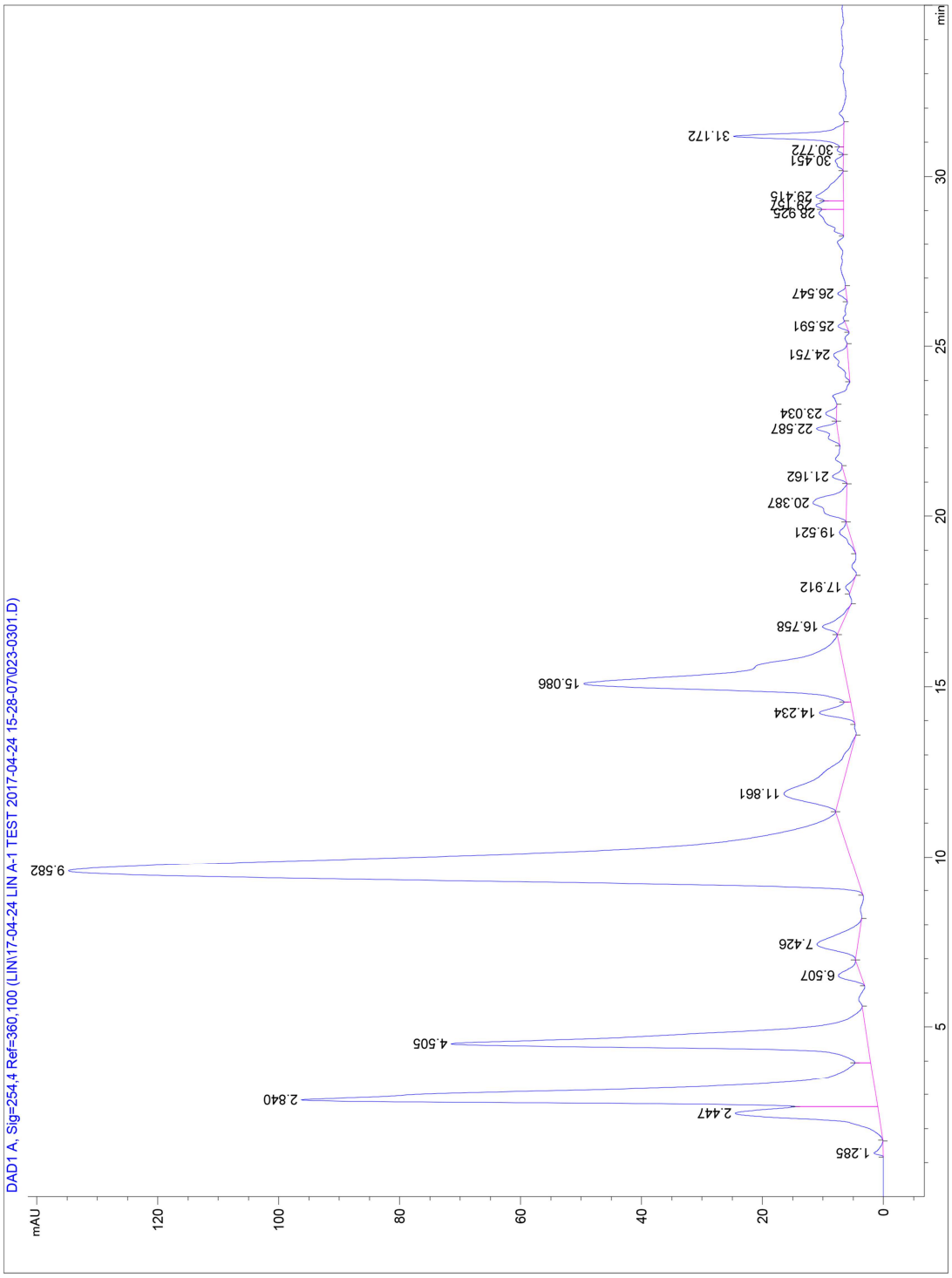

Figure 8-28 Healthy *Allium sativum* L. bulb's extract spectrum

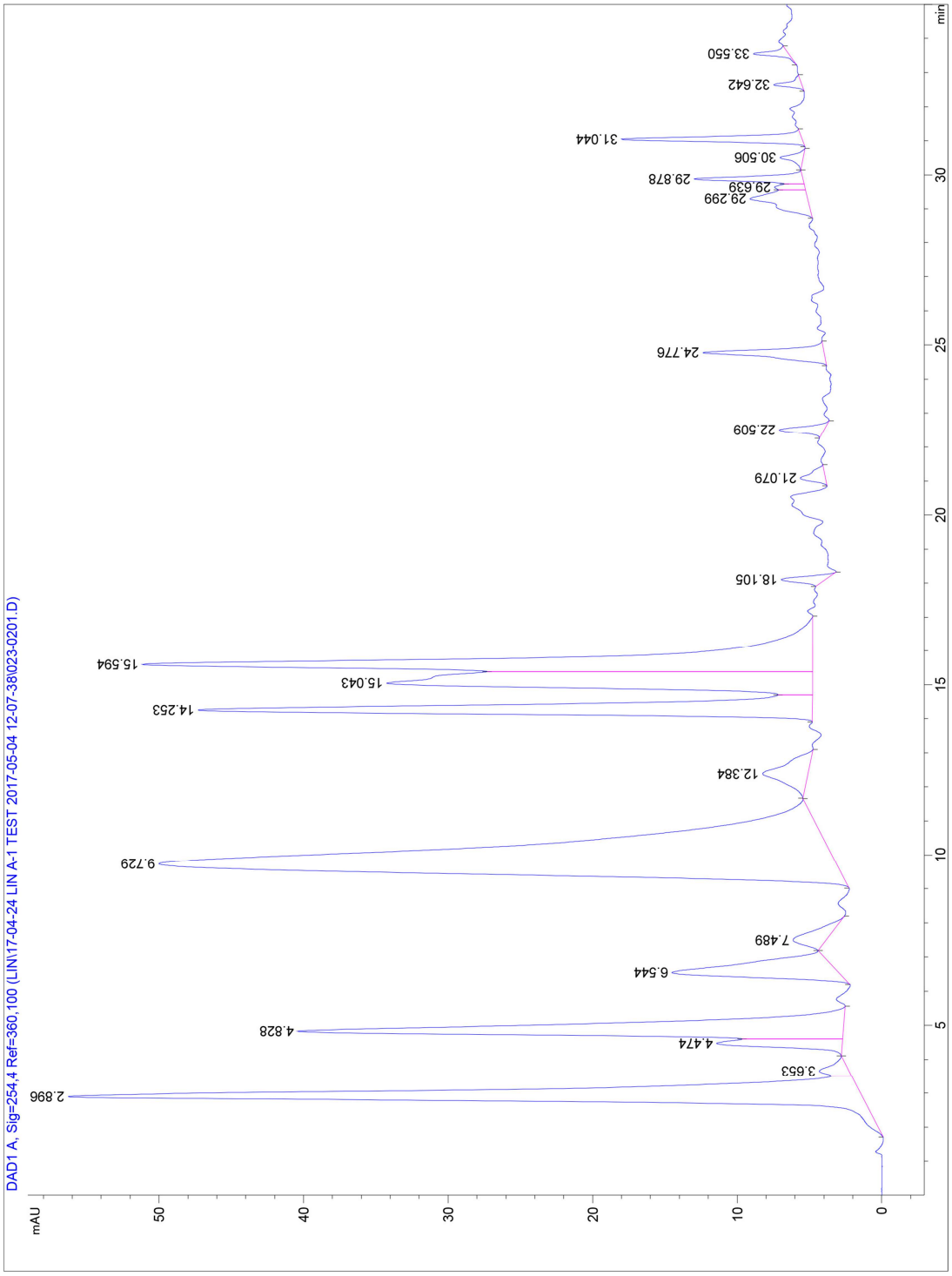

Figure 8-29 Infected *Allium sativum* L. bulb's extract spectrum

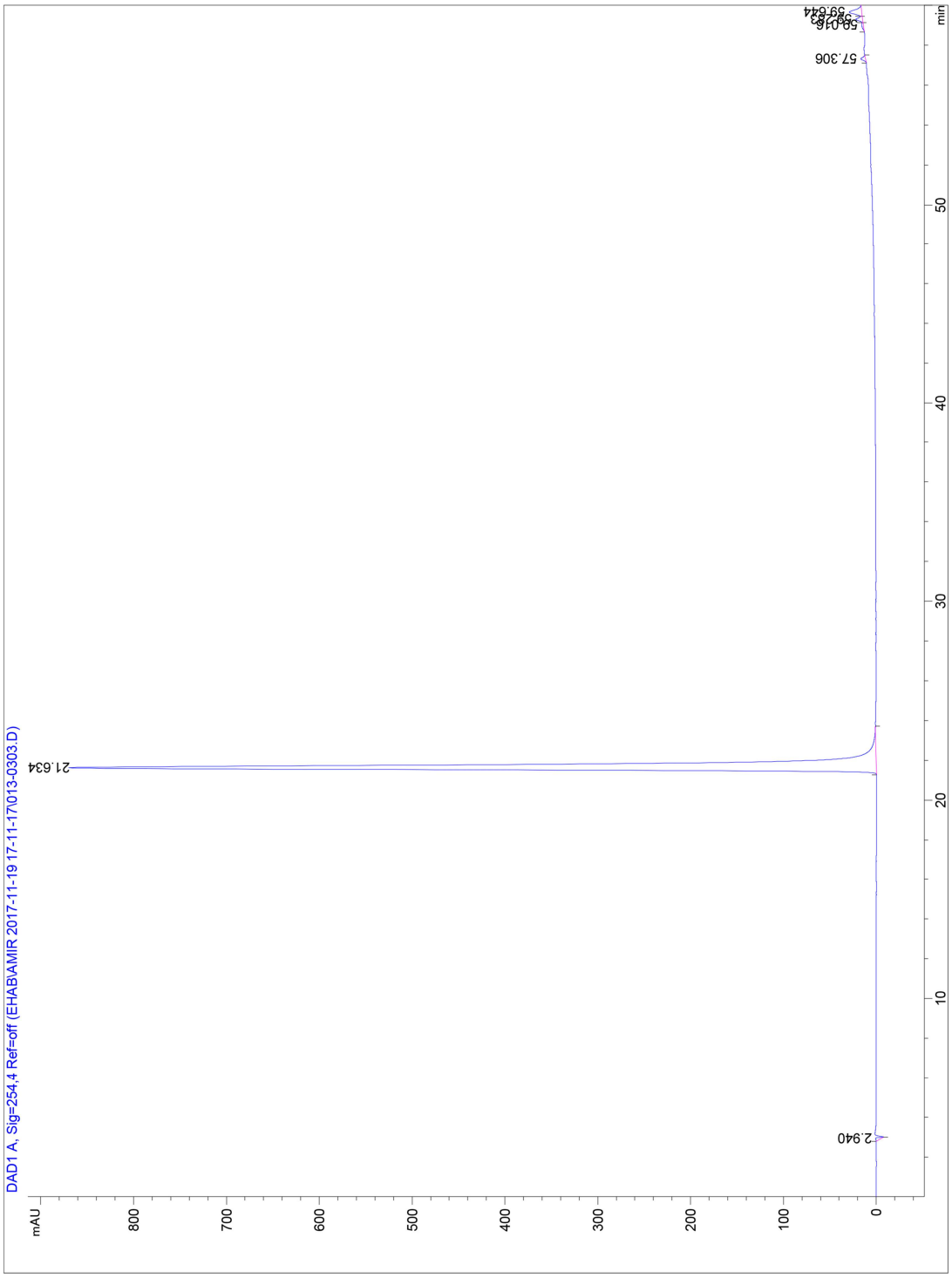

Figure 8-30 Spectrum of dimethyl dihydrofuranone solution in Agilent HPLC

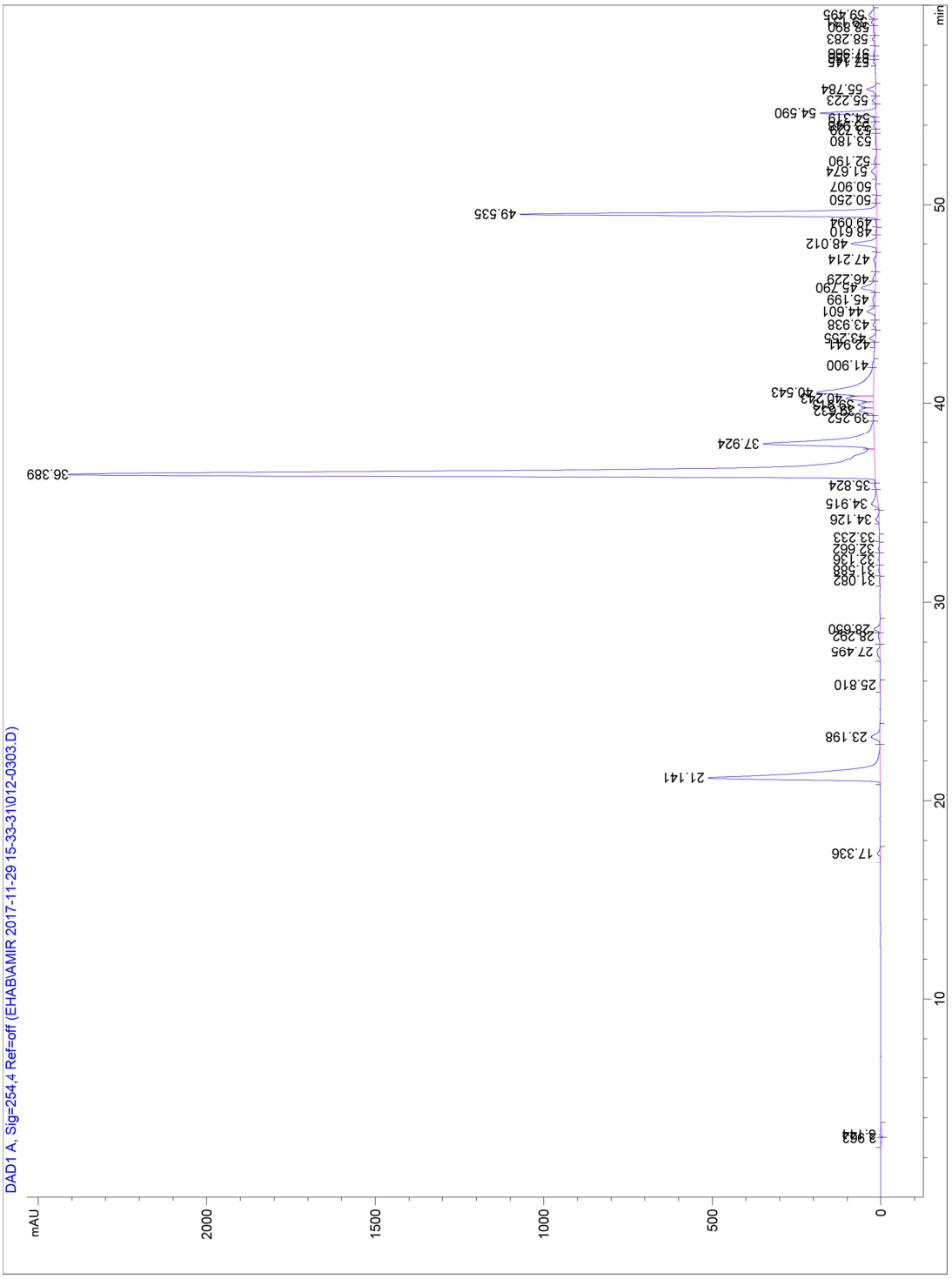

Figure 8-31 Spectrum of infected extract and DMDHF as an internal standard

8.2 Mass spectrums

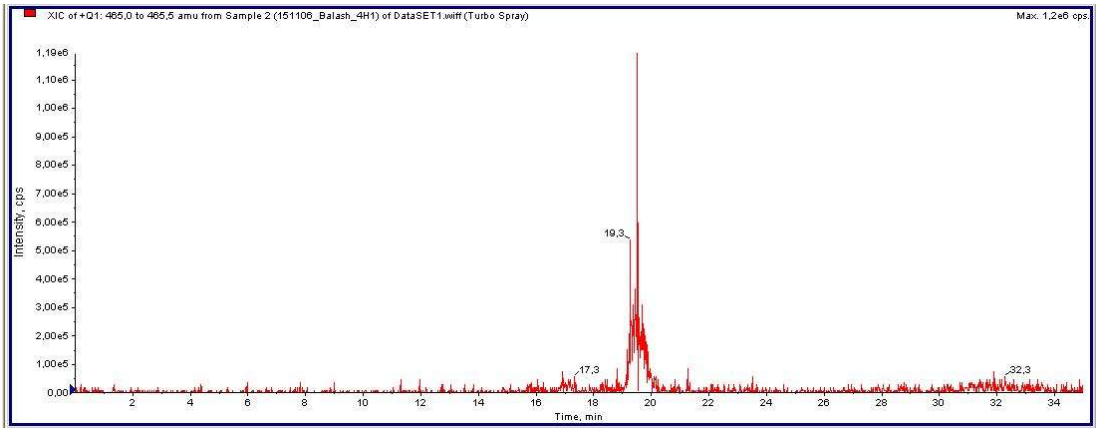

Figure 8-32 The XIC of m/z = 465 $[M+H]^+$ for peak A using HPLC/MS

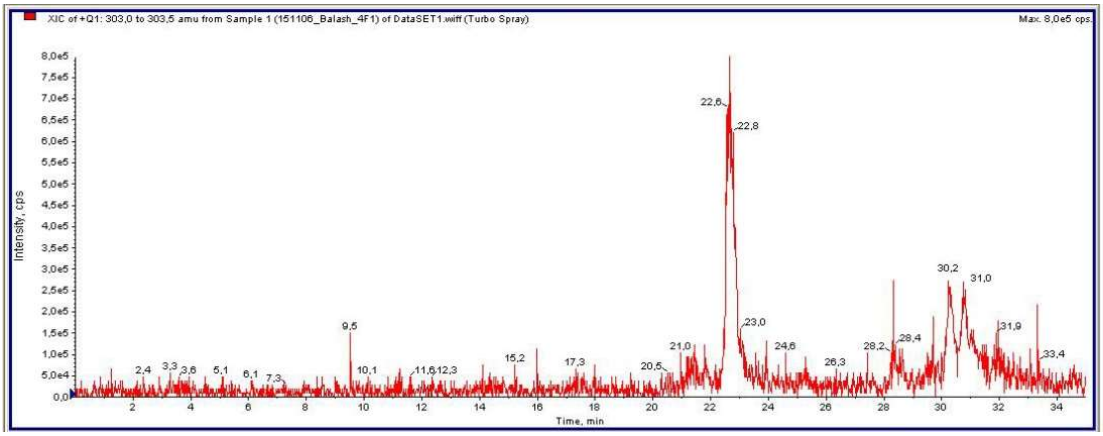

Figure 8-33 The XIC of m/z = 303 $[M+H]^+$ for peak B using HPLC/MS

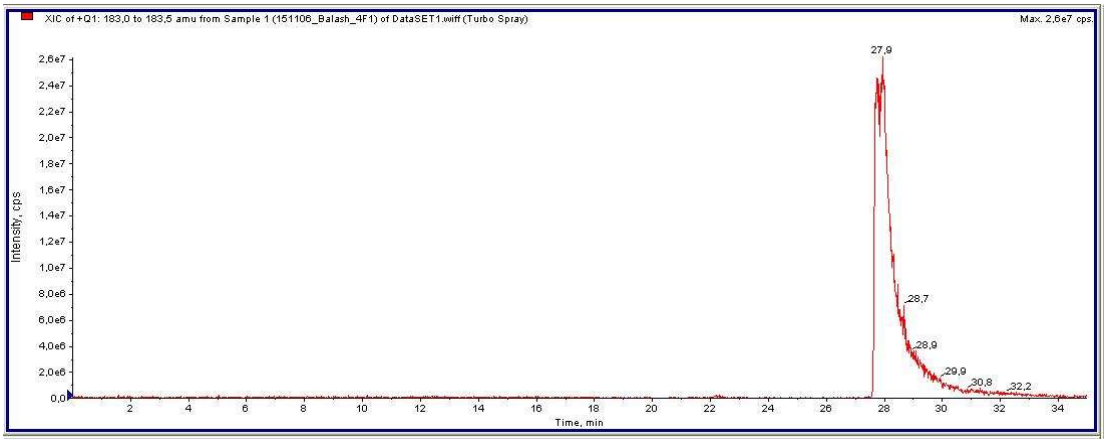

Figure 8-34 The XIC of m/z = 183 $[M+H]^+$ for peak C using HPLC/MS

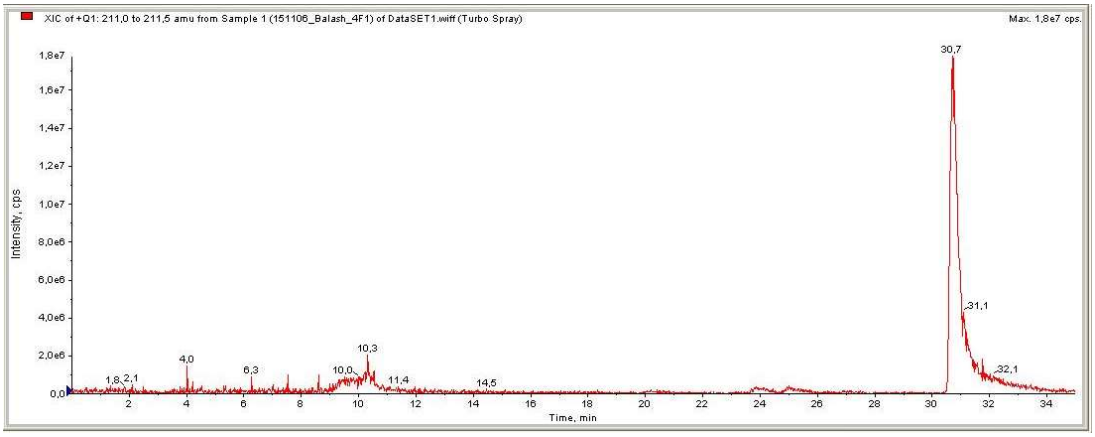

Figure 8-35 The XIC of m/z = 211 $[M+H]^+$ for peak D using HPLC/MS

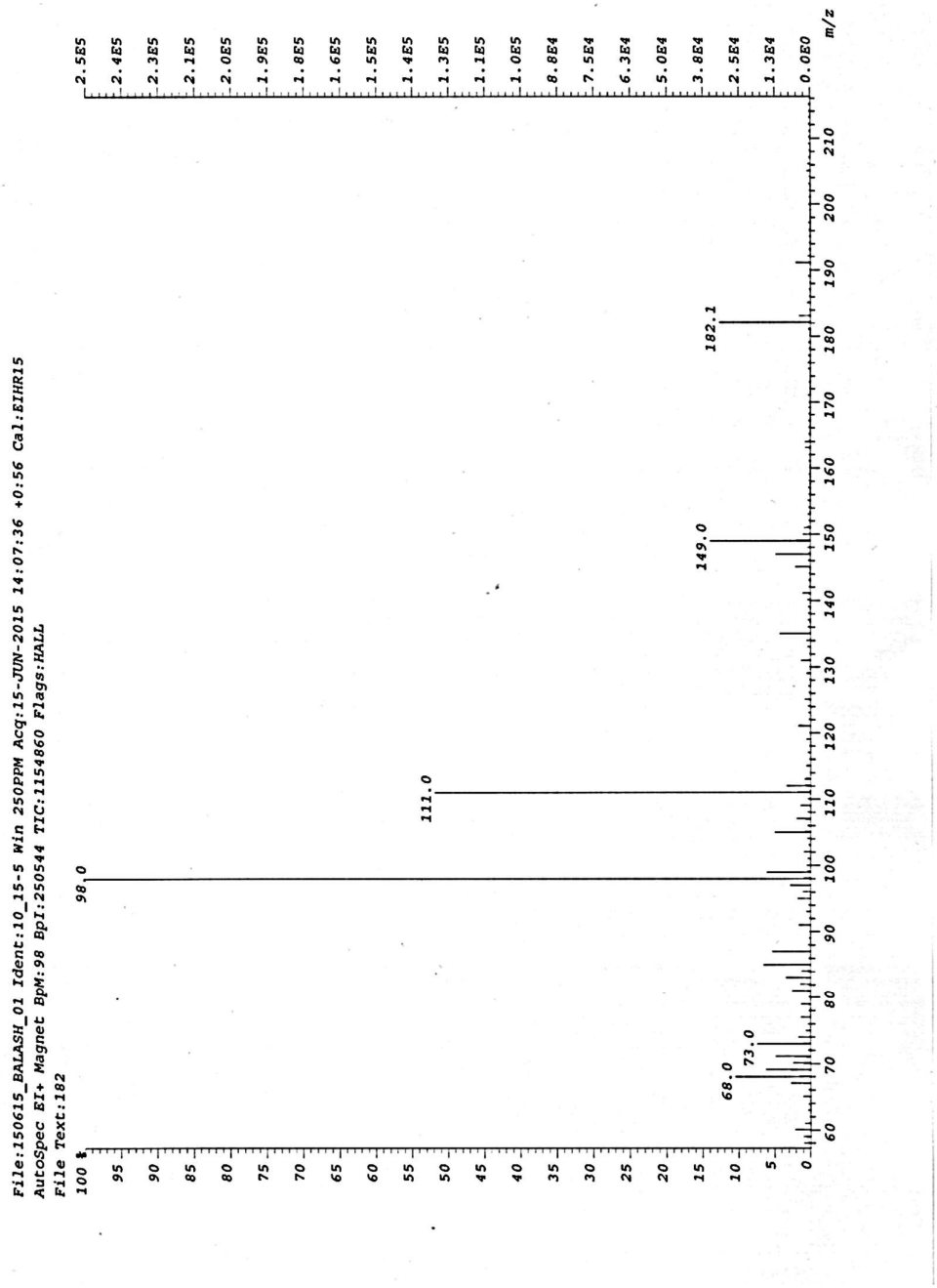

Figure 8-36 The positive electron ionization for peak C

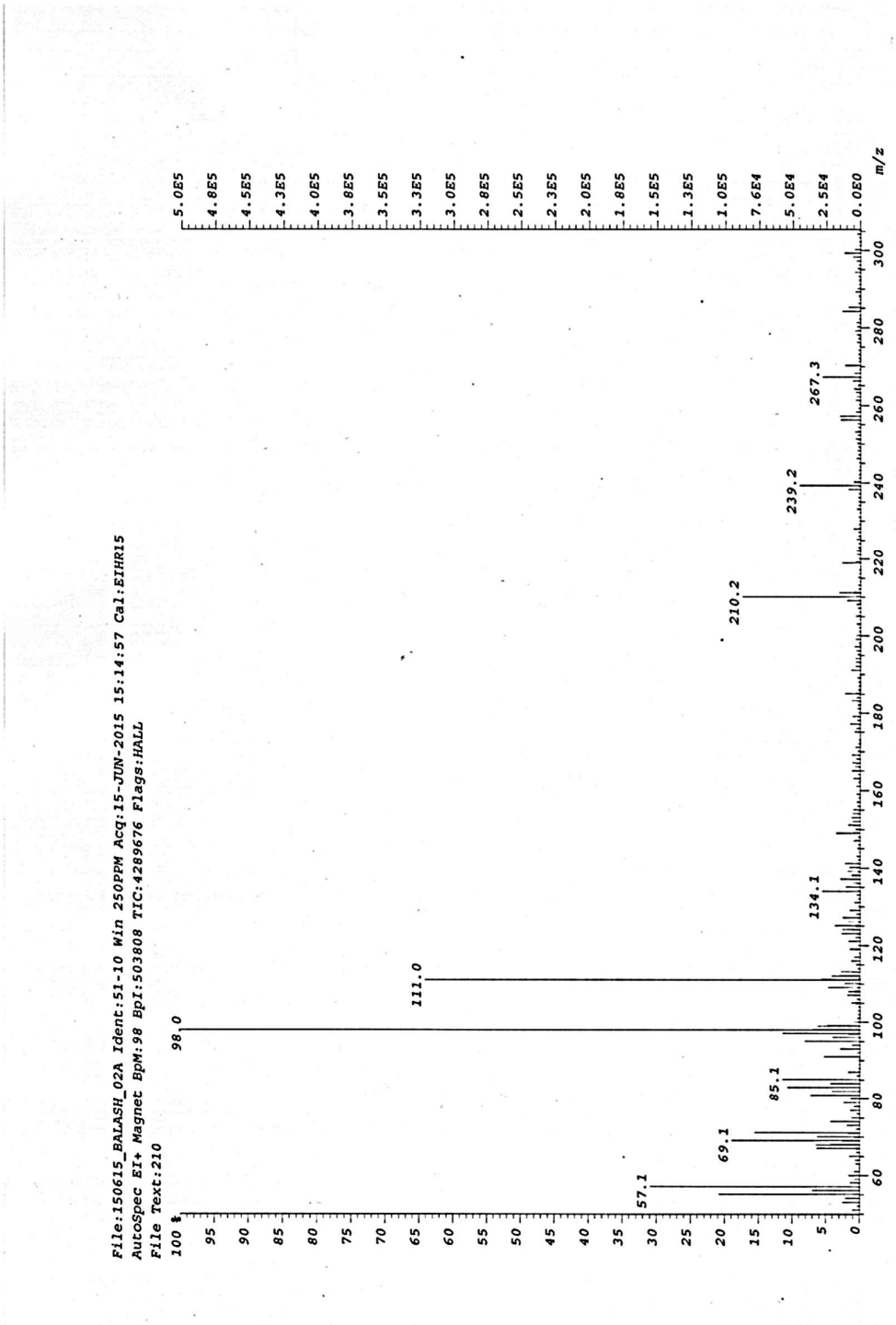

Figure 8-37 The positive electron ionization for peak D

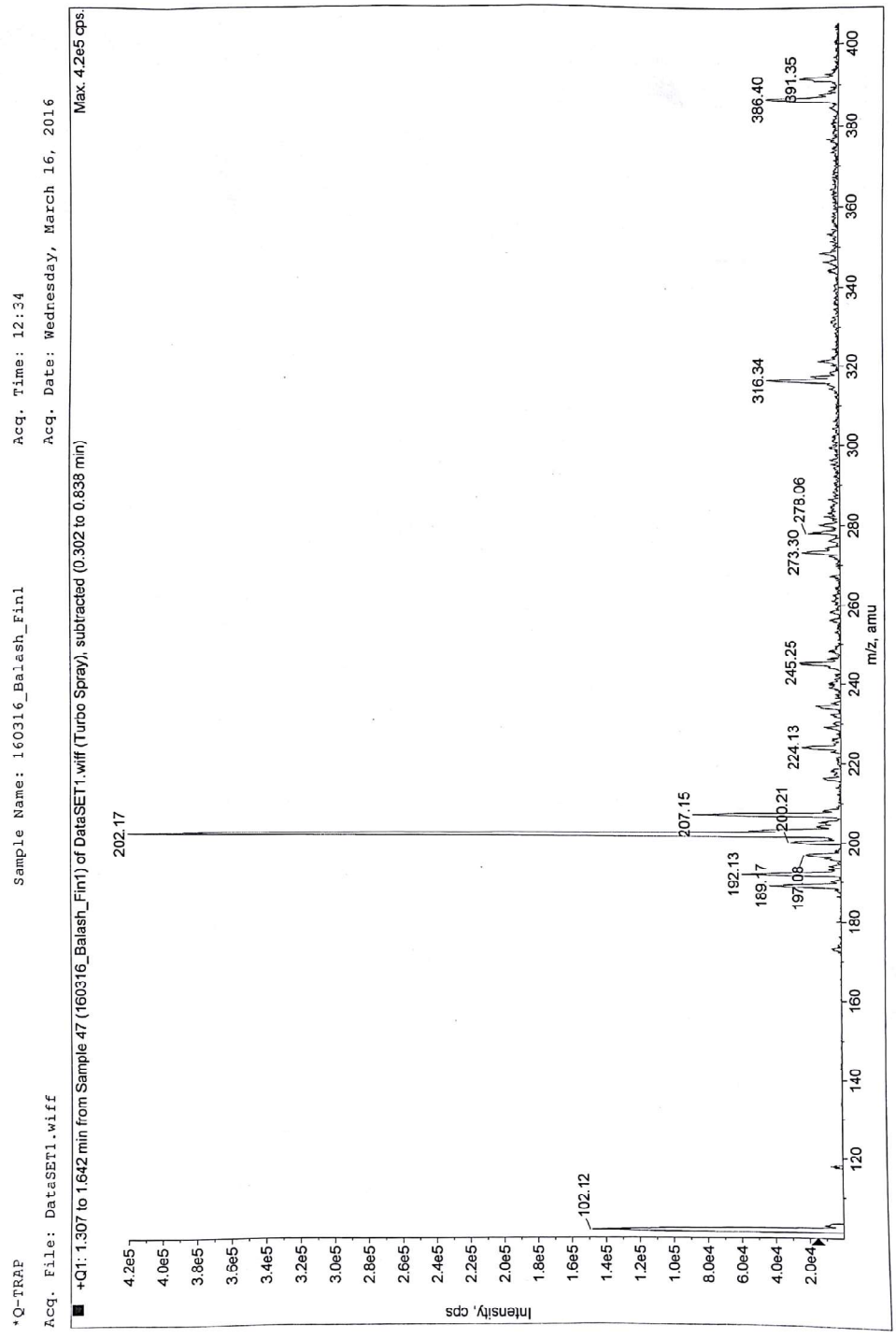

Figure 8-38 The Electrospray ionization of the synthesis products

8.3 IR spectrums

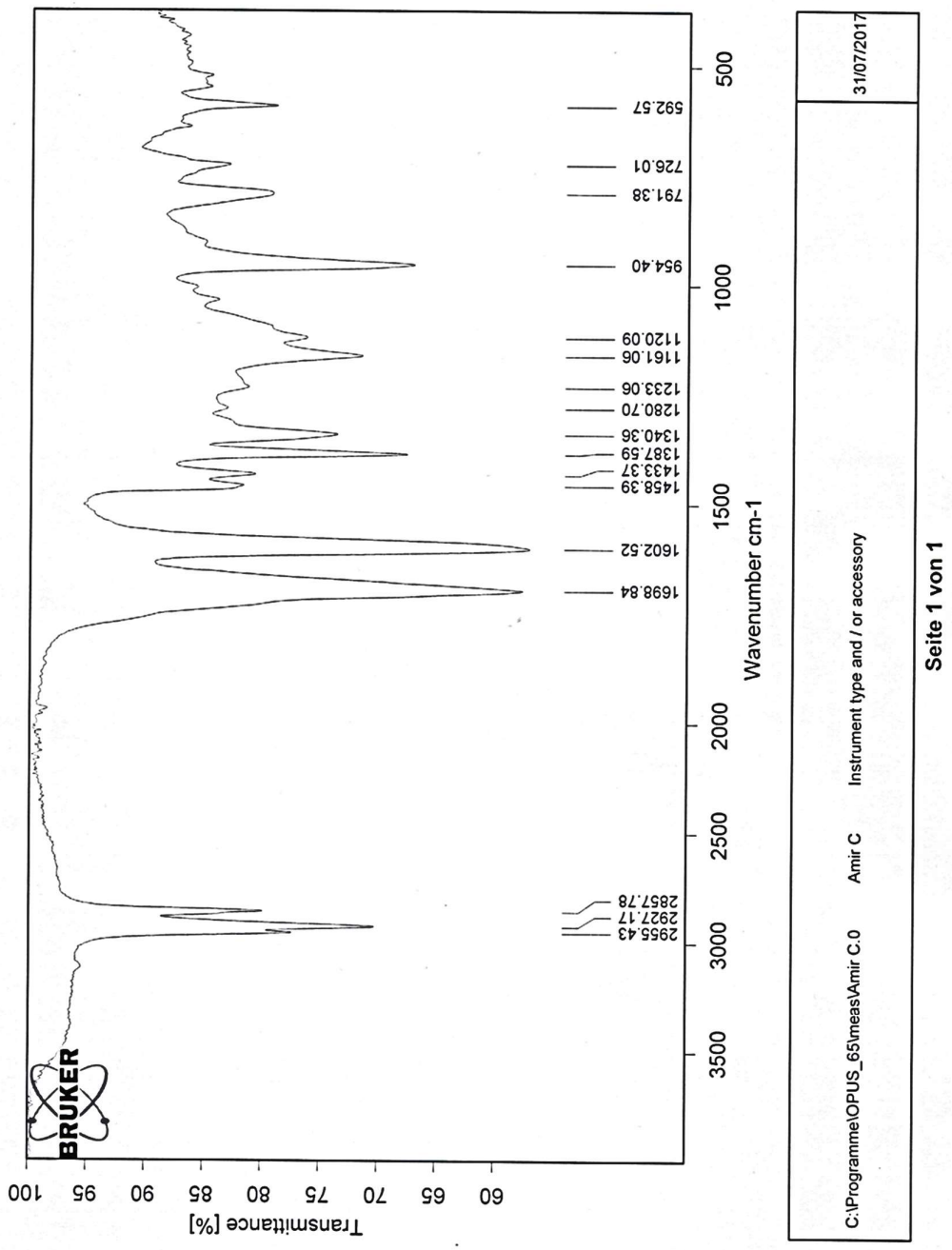

Figure 8-39 IR spectrum for peak D

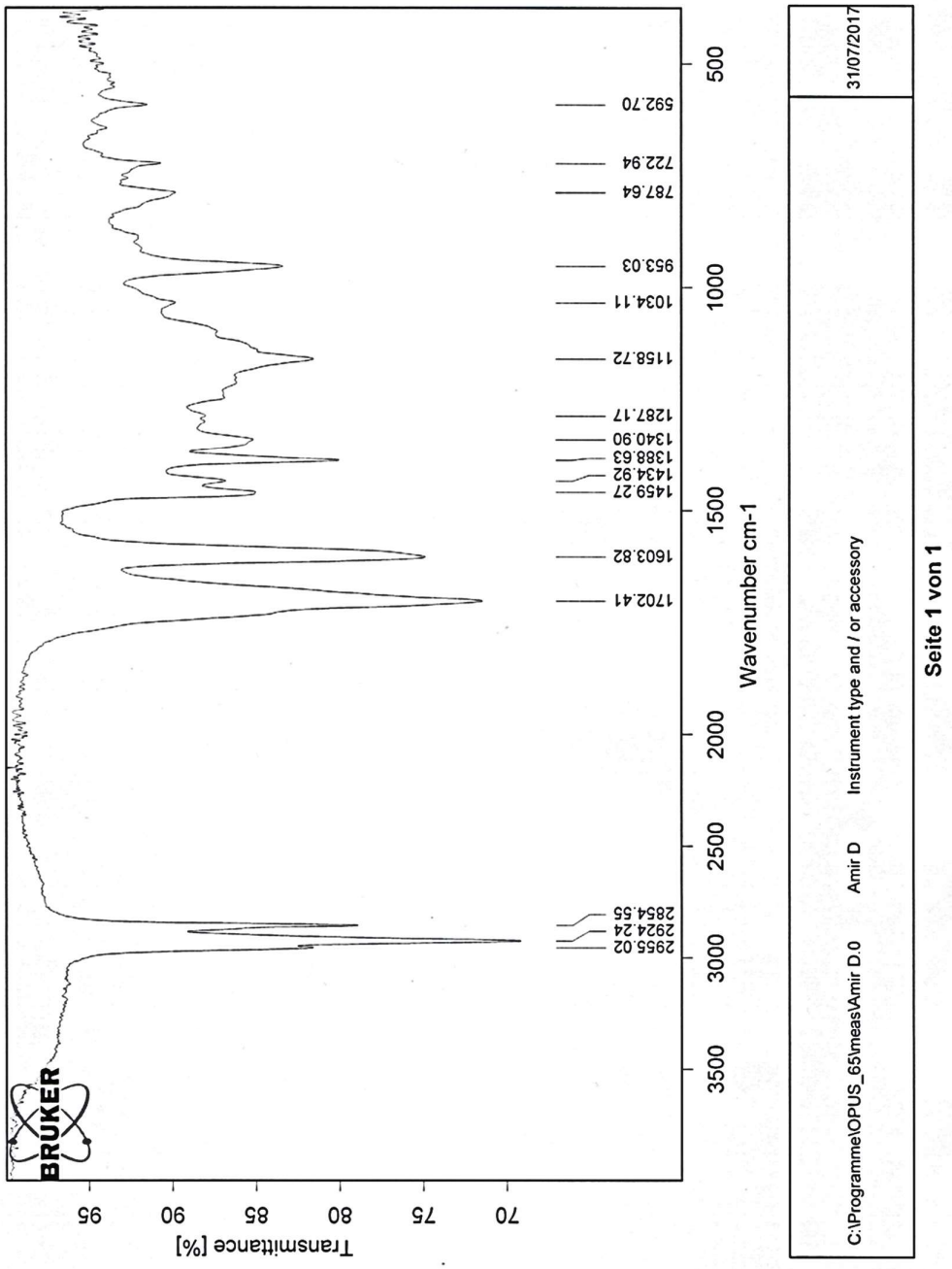

Figure 8-40 IR spectrum for peak D

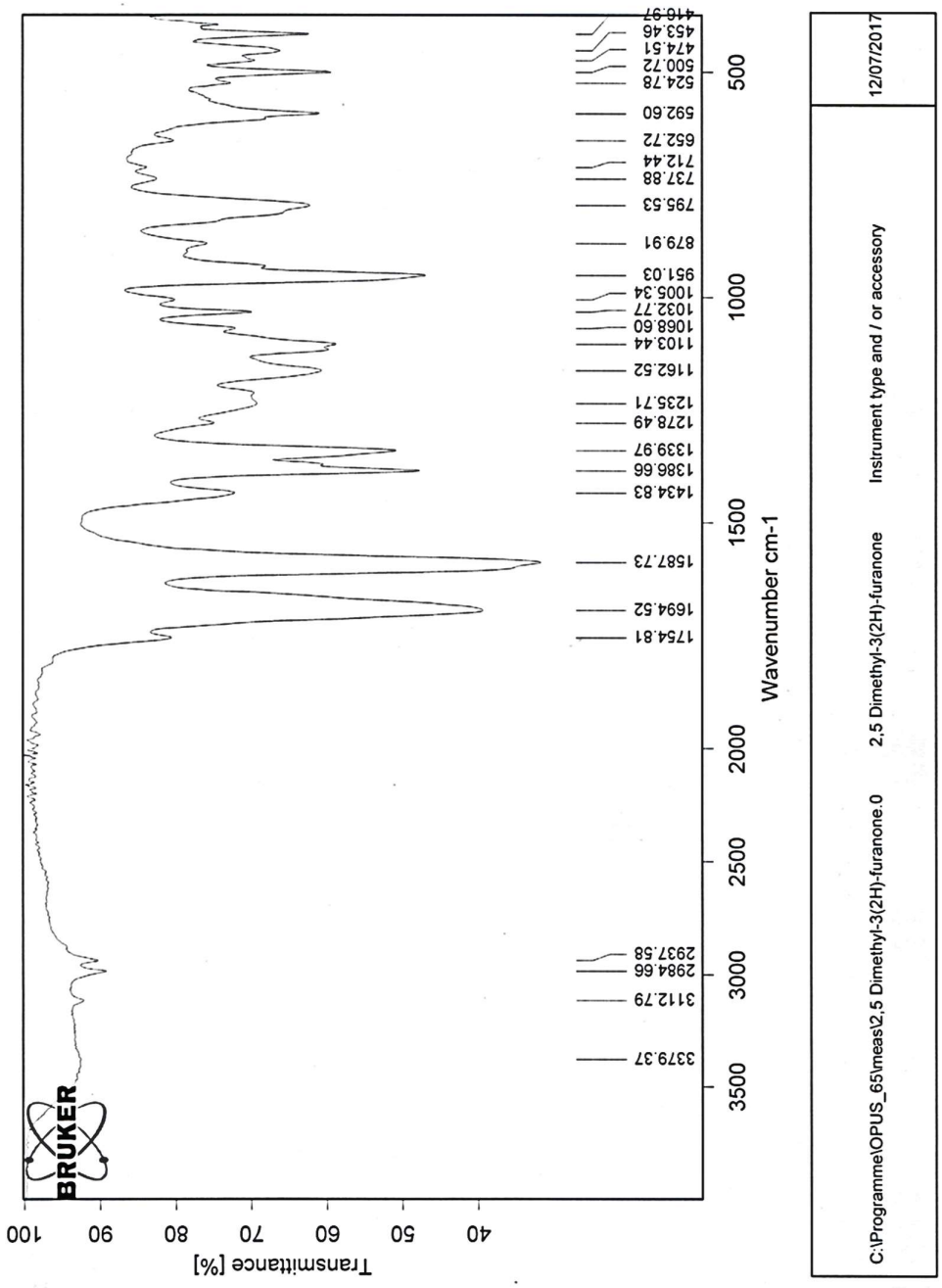

Figure 8-41 IR spectrum for dimethyl dihydrofuranone

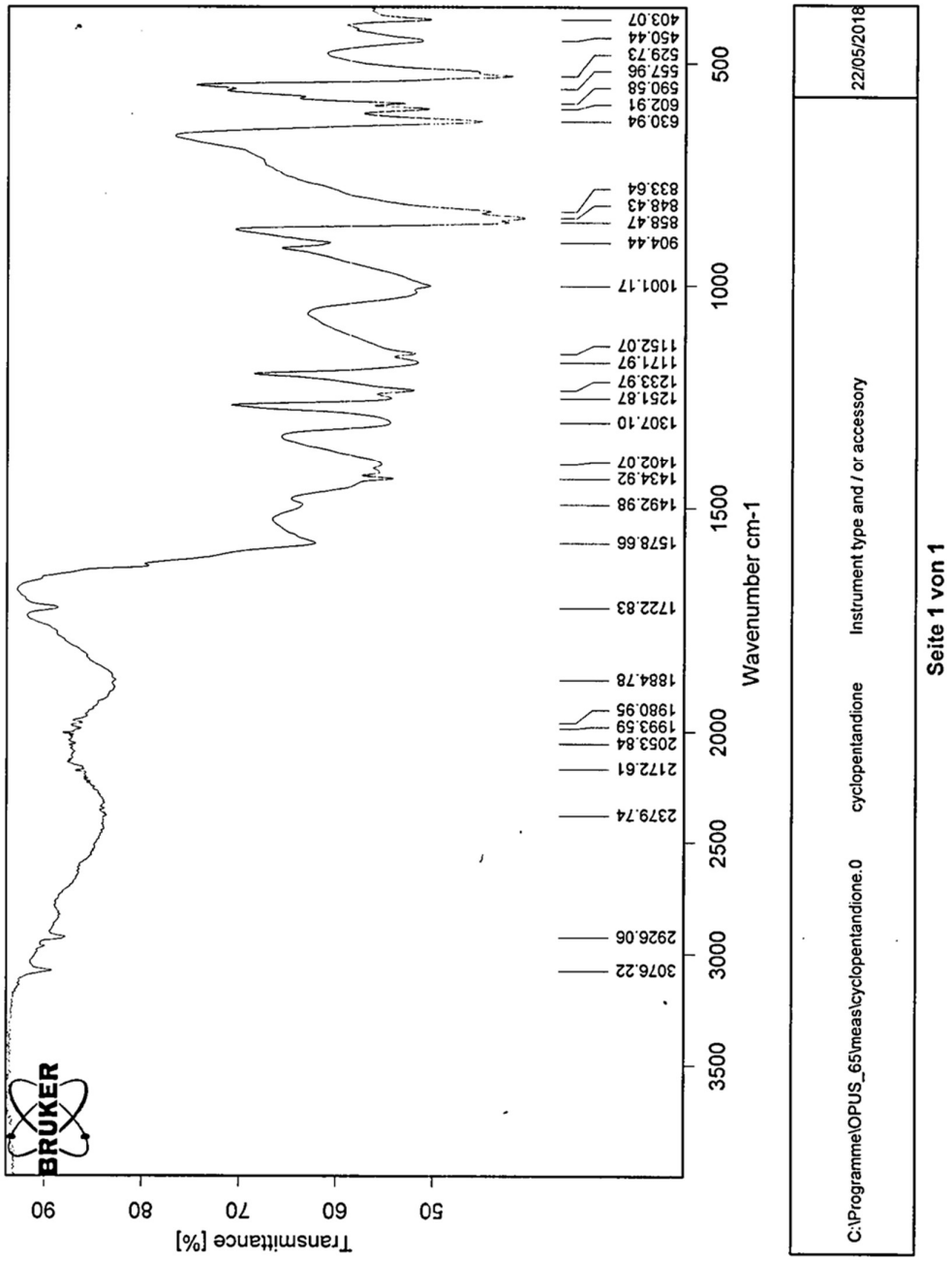

Figure 8-42 IR spectrum for 1,3-cyclopentanedione

8.4 NMR spectrums

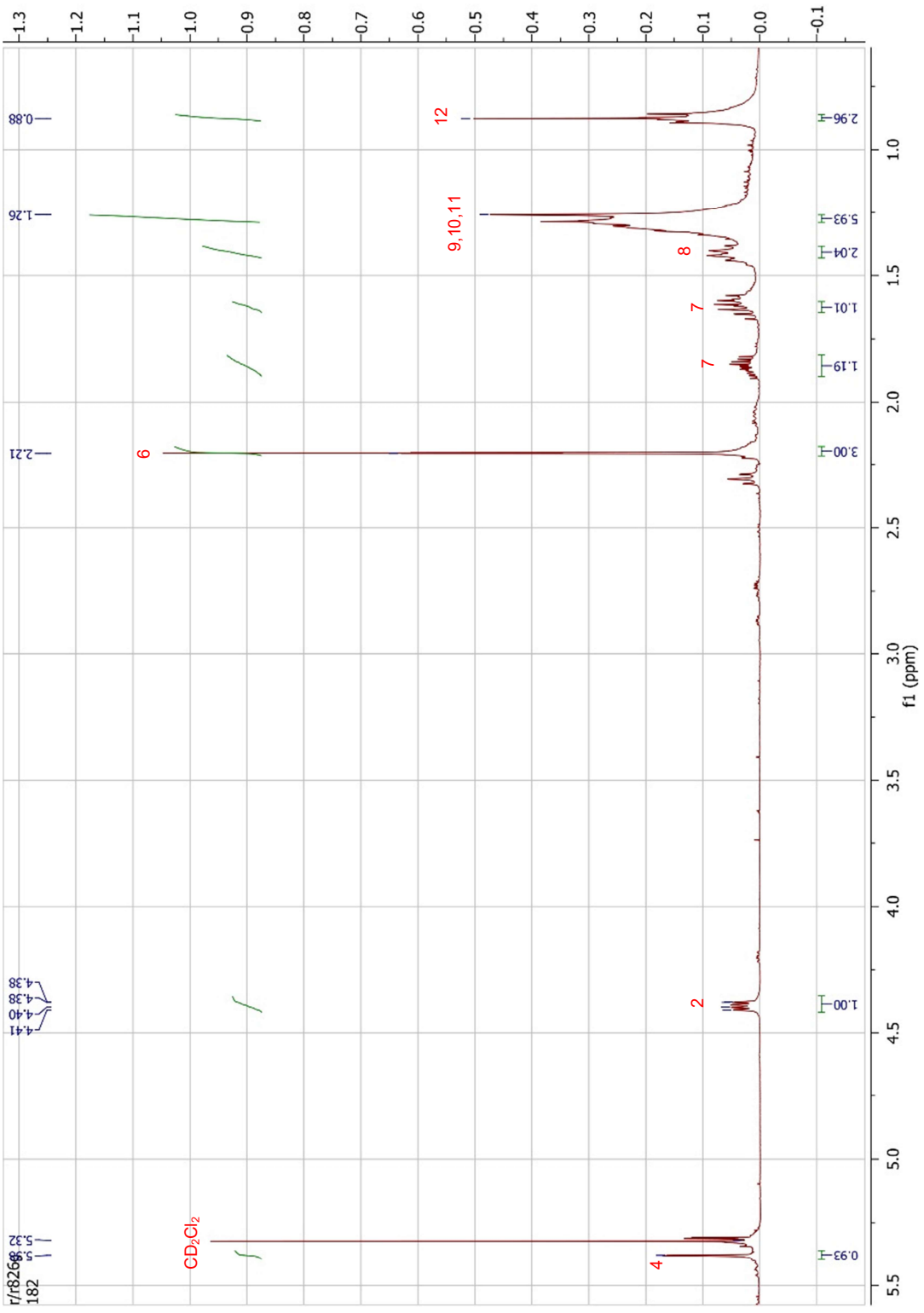

Figure 8-43 Proton ^{1}H NMR for peak C

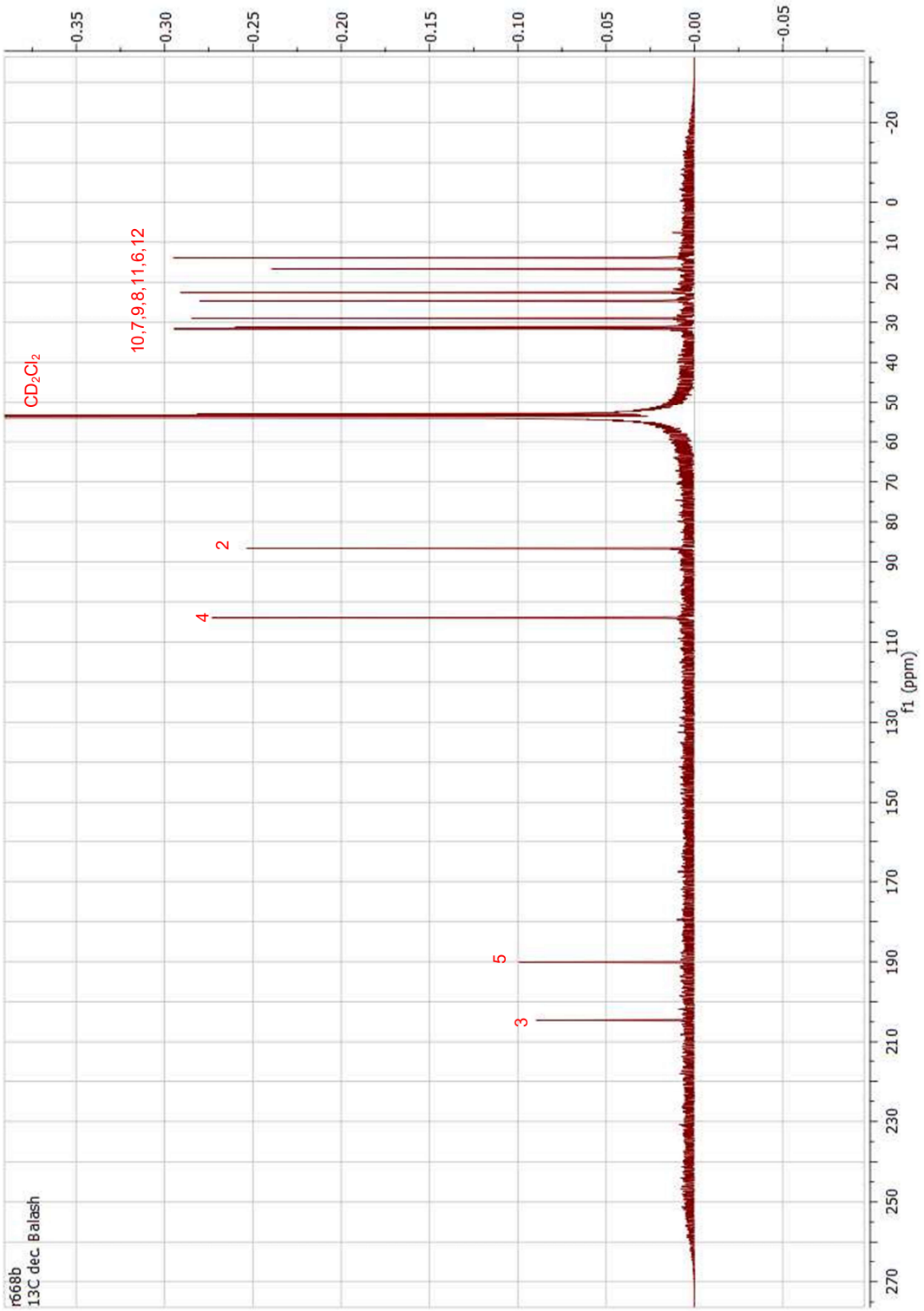

Figure 8-44 Carbon ^{13}C NMR for peak C

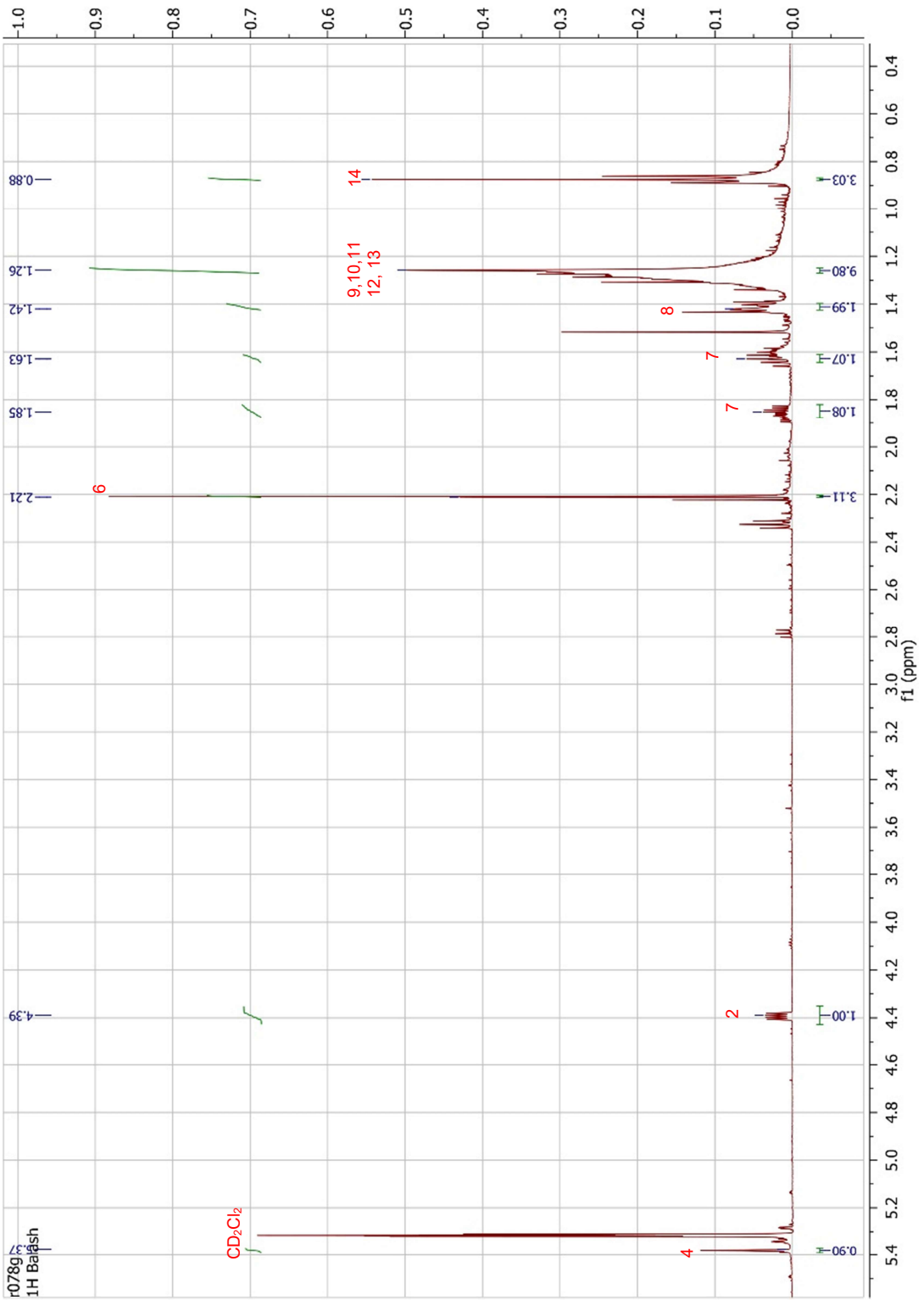

Figure 8-45 Proton ^{1}H NMR for peak D

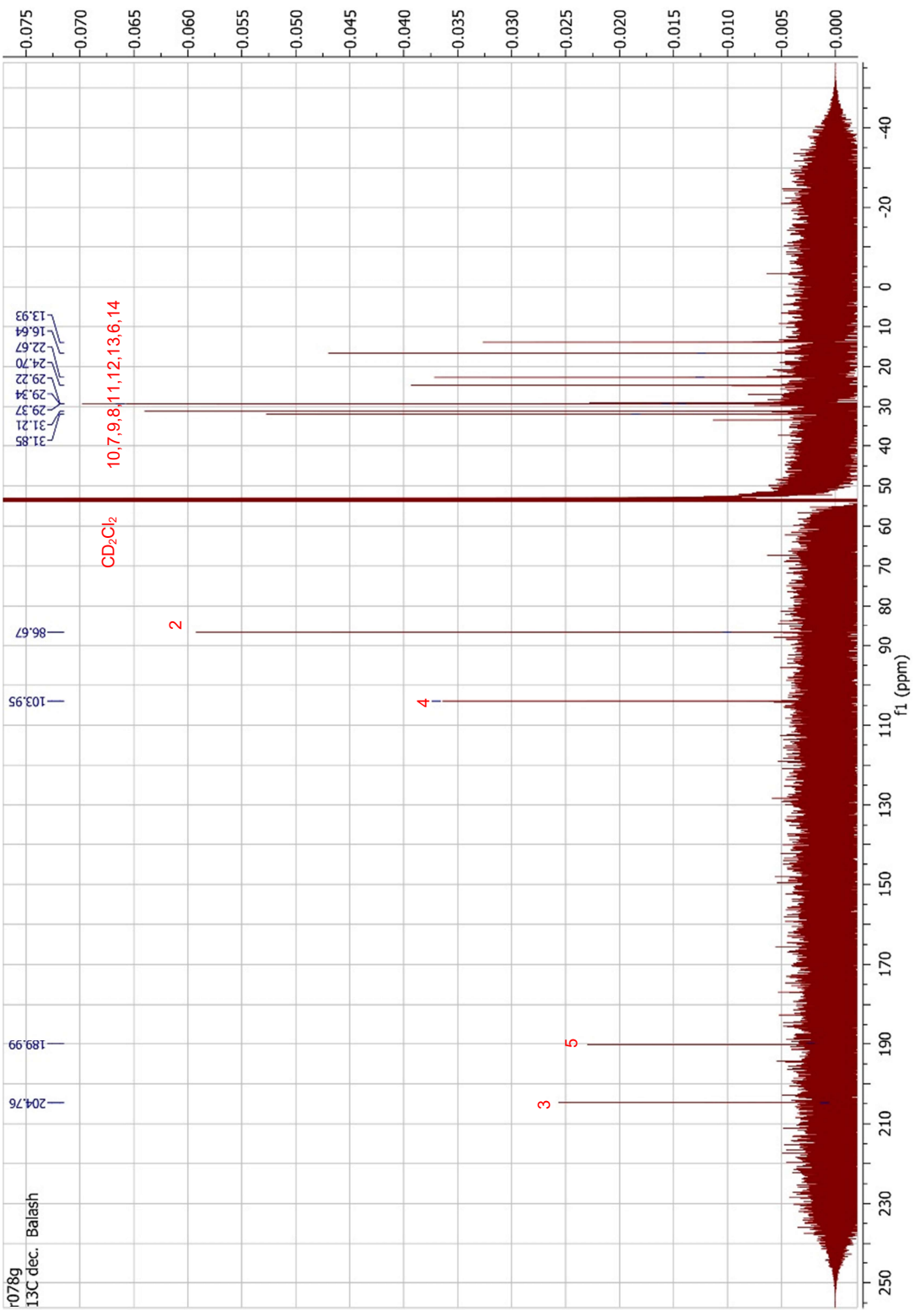

Figure 8-46 Carbon ^{13}C NMR for peak D

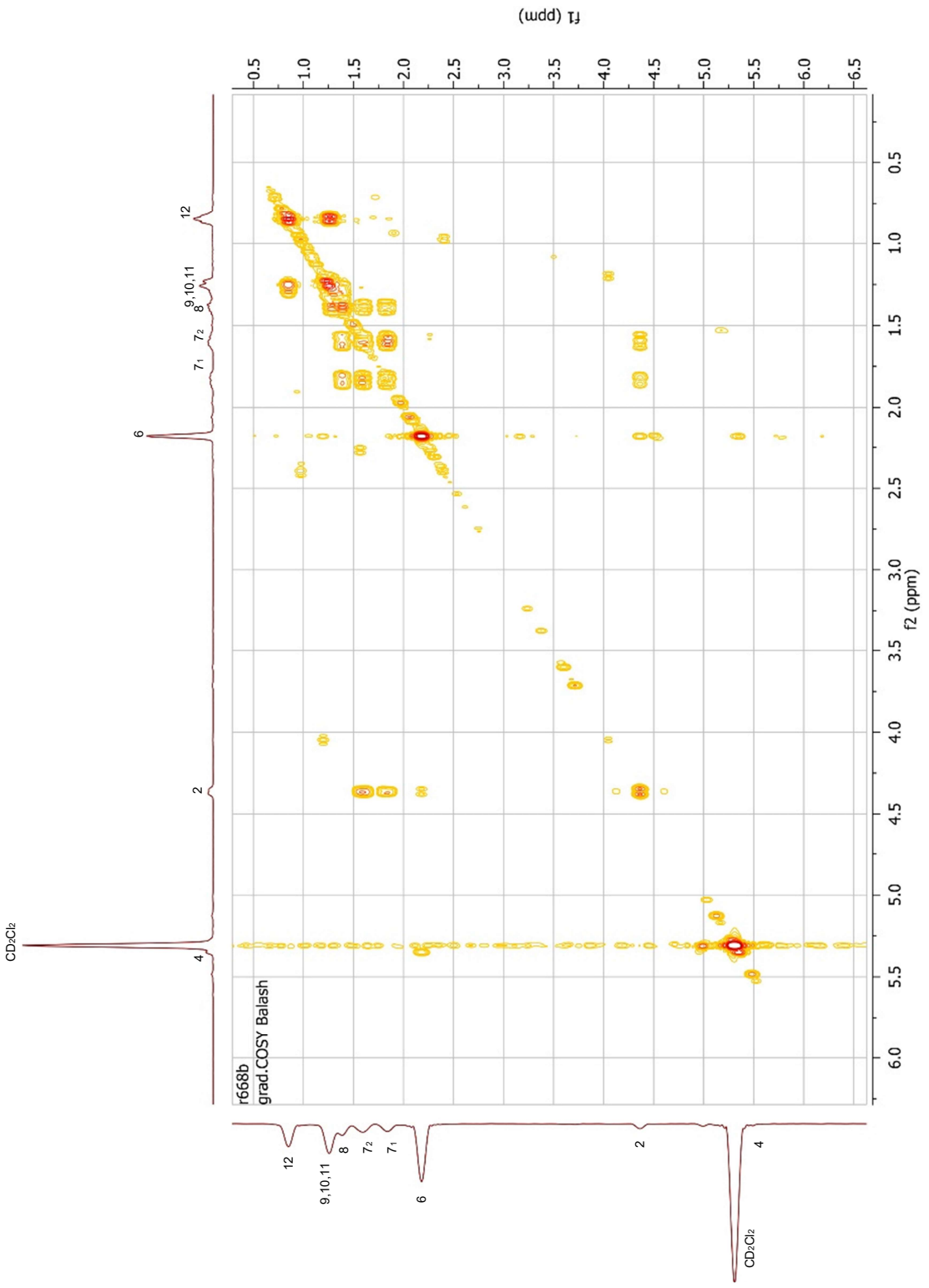

Figure 8-47 COSY NMR spectrum for peak C

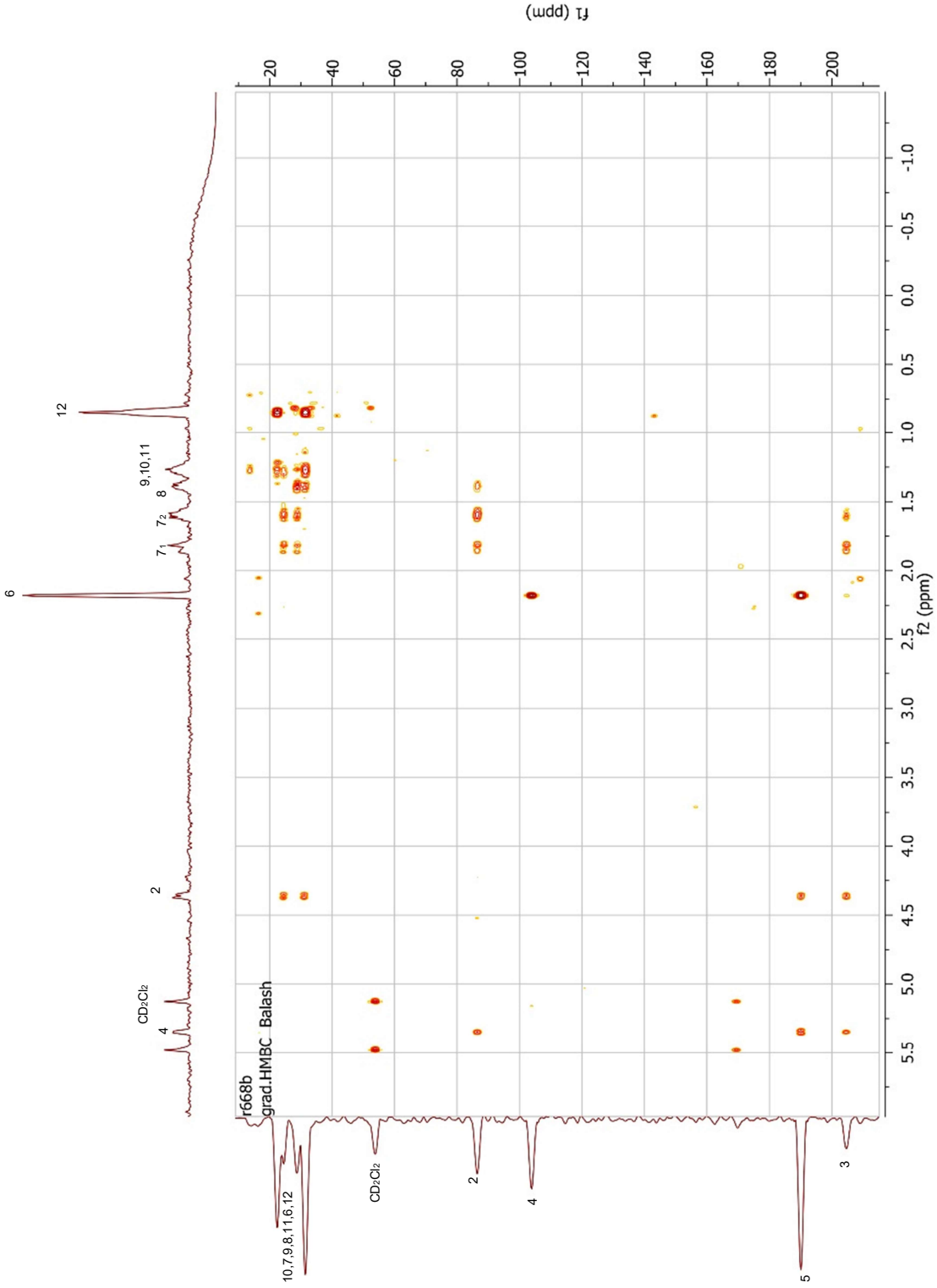

Figure 8-48 HMBC NMR spectrum for peak C

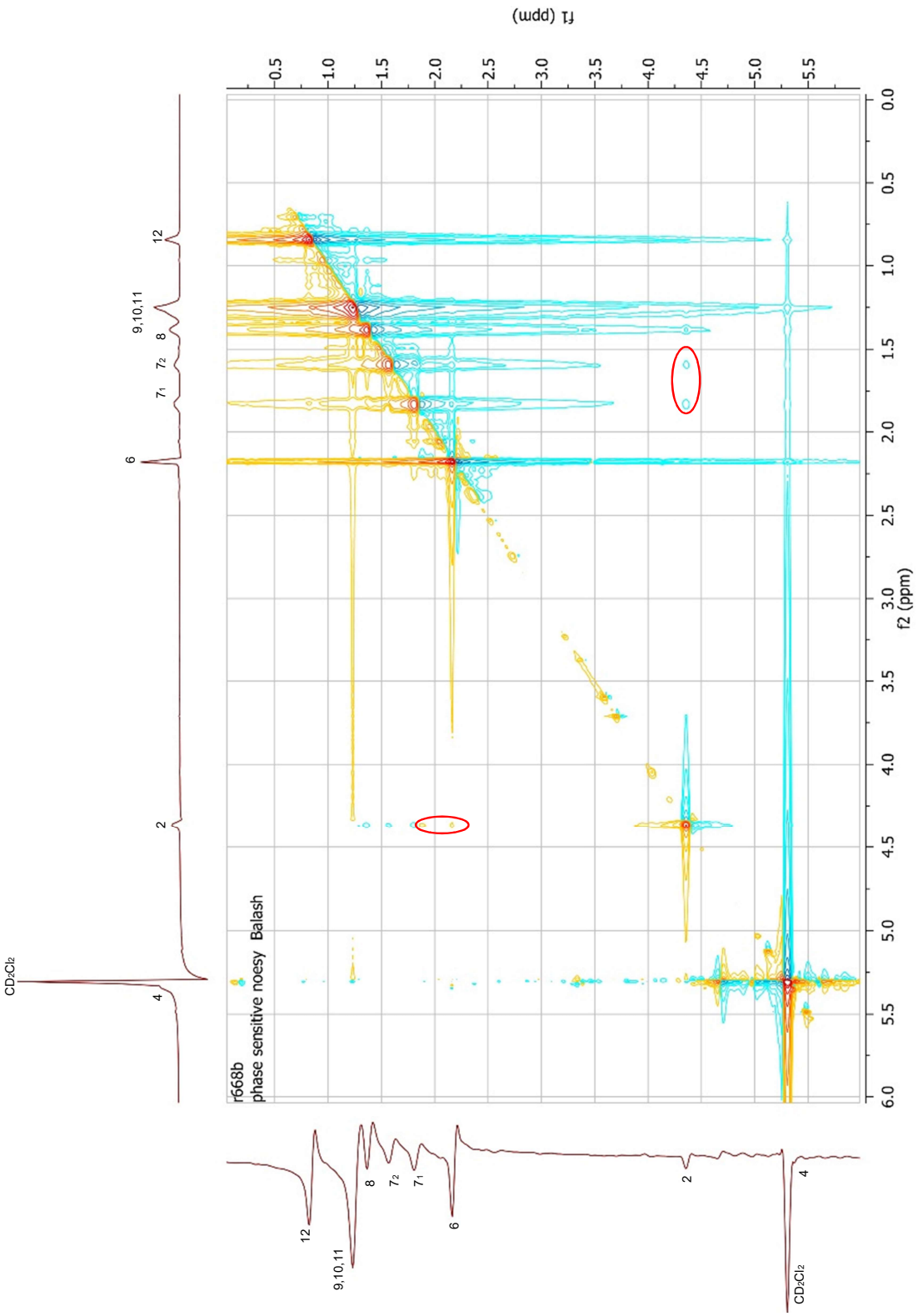

Figure 8-49 NOESY NMR spectrum for peak C

Erklärung

Ich versichere, dass ich meine Dissertation:

„**Finding the Markers of Fungal and Bacterial Infections in *Allium cepa* L.**"

selbständig ohne unerlaubte Hilfe angefertigt und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen bedient habe. Alle vollständig oder sinngemäß übernommenen sind Zitate als solche gekennzeichnet.

Die Dissertation wurde in der jetzigen oder einer ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

Marburg, den 12.09.2018

Amir Balash

List of publications

Poster with abstract

1. Amir Balash, Michael Keusgen:

   Marker Compounds of Microbial Infection in *Allium cepa*

   Abstract 65th GA Annual Congress (4th September, 2017), 157.

2. Amir Balash, Michael Keusgen:

   Detection of infection markers in some *Allium* species

   Abstract 66th GA Annual Congress (27th August, 2018), 133.

3. Amir Balash, Michael Keusgen:

   Quantitative analysis of 3(2H)-furanones in infected *Allium cepa*

   Abstract 66th GA Annual Congress (28th August, 2018), 110.

Lebenslauf

Name	Balash
Vorname	Amir
Staatsangehörigkeit	Syrer
Geburtsdatum	29.01.1984
Geburtsort, -land	Aleppo, Syrien

Ausbildung

09.1989 – 06.1995	Grundschule, Aleppo, Syrien
09.1995 – 06.1998	Weiterführenden, Aleppo, Syrien
09.1998 – 06.2001	Gymnasium, Aleppo, Syrien
09.2001 – 07.2006	Abschluss: Bachelor of Pharmacy and Pharmacologic Chemistry, Aleppo University, Syrien
09.2007 – 07.2010	Abschluss: Master of Biomedical Engineering, Aleppo University, Syrien
03.2009 – 02.2011	Abschluss: Master of Business Administration, Die syrische virtuelle Universität, Syrien
06.2014 – Heute	Ph.D., Naturwissenschaften, Fachbereich Pharmazie, Philipps-Universität Marburg, Deutschland

Berufliche Laufbahn

10.2007 – 07.2009	Medizinische Vertreter, Barakat Pharmaceutical Industries, Aleppo, Syrien
08.2009 – 12.2012	Produktmanager, Barakat Pharmaceutical Industries, Aleppo, Syrien
01.2013 – 05.2014	Einkaufsleiter, Barakat Pharmaceutical Industries, Aleppo, Syrien
10.2015 – 09.2018	Wissenschaftliche Mitarbeiter, Fachbereich Pharmazie, Philipps-Universität Marburg, Deutschland