EURL-MP-report_003

Biological synthesis and semi-preparative purification of zearalenone and α-zearalenol conjugates

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Project: 1287362201-EURLMP 1.4.1a M MYCO ZENcon-EURLMP2018

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Suggested Citation: Biological synthesis and purification of zearalenone and α-zearalenol conjugates, EURL-MP-report_003, 2018, EURL mycotoxins and plant toxins, RIKILT Wageningen University & Research.

Abstract

This report describes the results of the experiments carried out to synthesise (using fungi) and to purify produced α-zearalenol conjugates – sulfates and glucosides.

Preliminary experiments showed that the fungus *Cunninghamella elegans* is able to conjugate zearalenone, zearalanone and β-zearalenol to the corresponding glucosides and sulfates. Bioconversion, both conjugate class and amount, of the corresponding substrates to glucosides or sulfates depended on the nutrients present in the growth media. Incubation with potato dextrose broth (PDB) showed the potential to produce corresponding sulfates, incubation with Czapek-Dox (sulfate free) broth showed capacity to produce corresponding glucosides.

Cunninghamella elegans was inoculated in PDB or Czapek-Dox broth, respectively, spiked with 1 mg of α-zearalenol and incubated for two weeks at 27°C under continuous shaking. Samples were centrifuged. Target compounds were pre-purified and pre-concentrated from the supernatant by Strata™-X – solid phase extraction (SPE) column. The eluents were concentrated by evaporation under the nitrogen stream. Compounds in the resulting concentrated solutions were identified using LC-MS/MS and HPLC-UV and further purified by semi-preparative HPLC.

Incubation of *C. elegans* in PDB medium showed ~85% bioconversion of α-zearalenol (1 mg) to its corresponding α-zearalenol-14-sulfate (~1 mg).

Incubation in Czapek-Dox broth showed bioconversion of α-zearalenol to α-zearalenol-14-glucoside, α-zearalenol-16-glucoside and to zearalenone, zearalenone-14-glucoside and zearalenone-16-glucoside. The latter situation resulted in a low bioconversion rate, in the range 0.12-1.5% for each individual conjugate, and below 5% (sum of all mentioned conjugates) of the possible total theoretical conversion. The low yields of compounds from Czapek-Dox broth might also indicate possible error in preparation of spiking experiments.

The method developed for biological synthesis of α-zearalenol-14-sulfate, α-zearalenol-14-glucoside, α-zearalenol-16-glucoside, zearalenone-14-glucoside and zearalenone-16-glucoside using *C. elegans*, followed by pre-purification and pre-concentration by Strata™ X SPE column and final purification by semi-preparative HPLC were developed and can be used in the future for larger scale synthesis of the mentioned above compounds in order to obtain qualitative analytical standards.

Abbreviations of listed mycotoxins

ZEN	Zearalenone
ZEN14G	Zearalenone-14-glucoside
ZEN16G	Zearalenone-16-glucoside
ZEN14S	Zearalenone-14-sulfate
ZEN14,16diG	Zearalenone-14,16-di-glucoside
ZEN-S	Zearalenone glucoside(s)
αZEL	α-Zearalenol
αZEL14G	α-Zearalenol-14-glucoside
αZEL16G	α-Zearalenol-16-glucoside
αZEL-G	α-Zearalenol-glucoside(s)
αZEL14S	α-Zearalenol-14-sulfate
αZEL-S	α-Zearalenol-sulfate(s)
βZEL	β-Zearalenol
βZEL14G	β-Zearalenol-14-glucoside
βZEL16G	β-Zearalenol-16-glucoside
βZEL14S	β-Zearalenol-14-sulfate
βZEL-S	β-Zearalenol-sulfate(s)
βZEL-G	β-Zearalenol-glucoside(s)
ZAN	Zearalanone
ZAL	Zearalanol
αZAL	α-Zearalanol
βZAL	β-Zearalanol
HPLC	High performance liquid chromatography
LC-MS/MS	Liquid chromatography – tandem mass spectrometry
LC-HR-MS	Liquid chromatography – high resolution mass spectrometry
PAD	Photodiode array detector
UV	Ultraviolet (detection/detector)
NMR	Nuclear magnetic resonance

Contents

Introduction

Zearalenone (ZEN) and zearalenol (ZEL) are produced by *Fusarium* species which infect cereal crops during the field period of the cereal crop. Plants can conjugate the mycotoxins to glucosides or sulfates and store the conjugates in the seeds. ZEN conjugates are easily hydrolysed back to ZEN in the gastrointestinal tract after consumption, thus increasing exposure to ZEN.

ZEN, is an estrogenic mycotoxin produced by fungi of the genus *Fusarium.* It is a β-resorcyclic acid lactone, see figure 1. ZEN can be modified (conjugated) in plants, fungi and animals by phase I and phase II metabolism. Conjugated forms of ZEN occurring in food and feed include its reduced phase I metabolites, i.e. α-zearalenol and β-zearalenol (αZEL and βZEL), α-zearalanol and β-zearalanol (αZAL and βZAL), zearalanone (ZAN) and its phase II derivatives, such as those conjugated with glucose, sulfate and glucuronic acid. ZEN and its modified forms differ considerably in their estrogenic activity. Based on their 'uterotrophic activity' assessed in rodents, ZEN and its modified forms are ranked as follows: αZEL > αZAL > ZEN > ZAN > βZAL > βZEL. αZEL and those glucosides and sulfates are about 60 times more estrogenic, than ZEN or those glucosides or sulfates (EFSA 2016). αZAL, one of the phase I metabolites of ZEN, is used as a growth promoter in non-European Union (EU) countries under the name of Zeranol. It is banned in Europe, and therefore, it is included in official control plans (EFSA 2017).

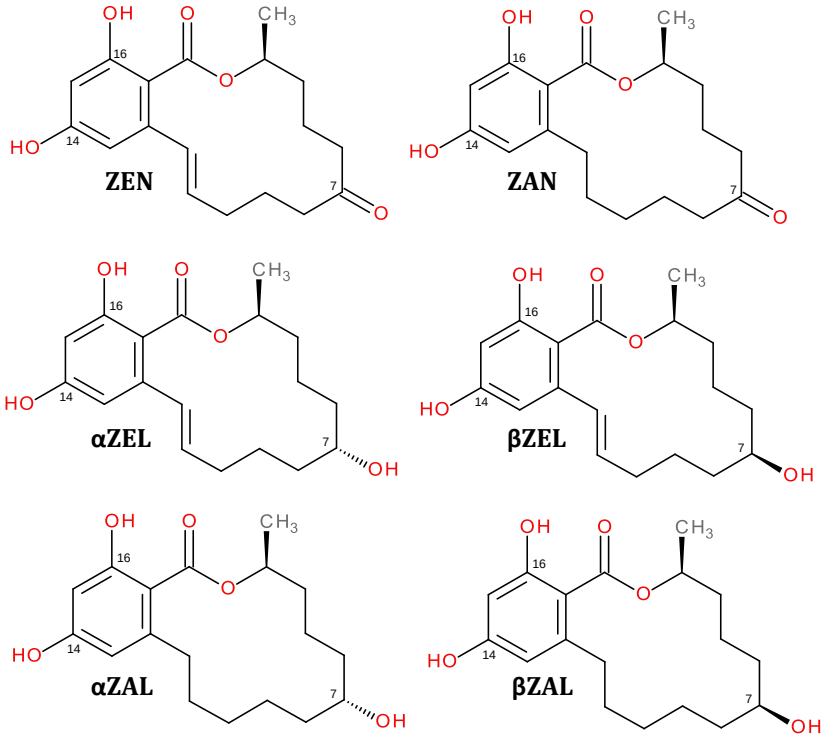


Figure 1. Chemical structures of ZEN, ZAN,α-/β-ZEL and α-/β-ZAL

As mentioned above, infected plants and other fungi can metabolise ZEN into conjugated (formerly known as masked) forms: glucosides and sulfates (figures 2 and 3) (Brodehl, Moller et al. 2014). These conjugates are mostly not toxic, however, human and animal gut microbiota are capable of hydrolysing them back to the estrogenic ZEN, and the reduced α- and βZEL thus increasing exposure (Berthiller, Crews et al. 2013). It is therefore not enough to test for ZEN and structurally related compounds such as αZEL and αZAL etc.; insight must be gained on the

occurrence of conjugated forms (sulfates and glucosides) of ZEN, αZEL and βZEL as these increase exposure to the toxic compounds once digested.

The CONTAM Panel noted that there is a need for more data on the occurrence of conjugated forms of ZEN in food/feed. This means that there is a need of analytical standards and reference materials for the development of properly validated and sensitive routine analytical methods for ZEN conjugated forms in the feed and food commodities and especially highly sensitive methods to identify the most potent form, namely αZEL.

Analytical methods for ZEN and its forms in food/feed are developed. However, while methods reported in the scientific literature are widely based on the more sensitive liquid chromatography–tandem mass spectrometry (LC–MS/MS), most of the routine analyses are still performed by liquid chromatography-fluorescence detection (LC-FLD) or liquid chromatography-ultraviolet detection (LC-UV). Of note, analytical standards for ZEN conjugates and reference materials for phase I and phase II conjugates are mostly not commercially available.

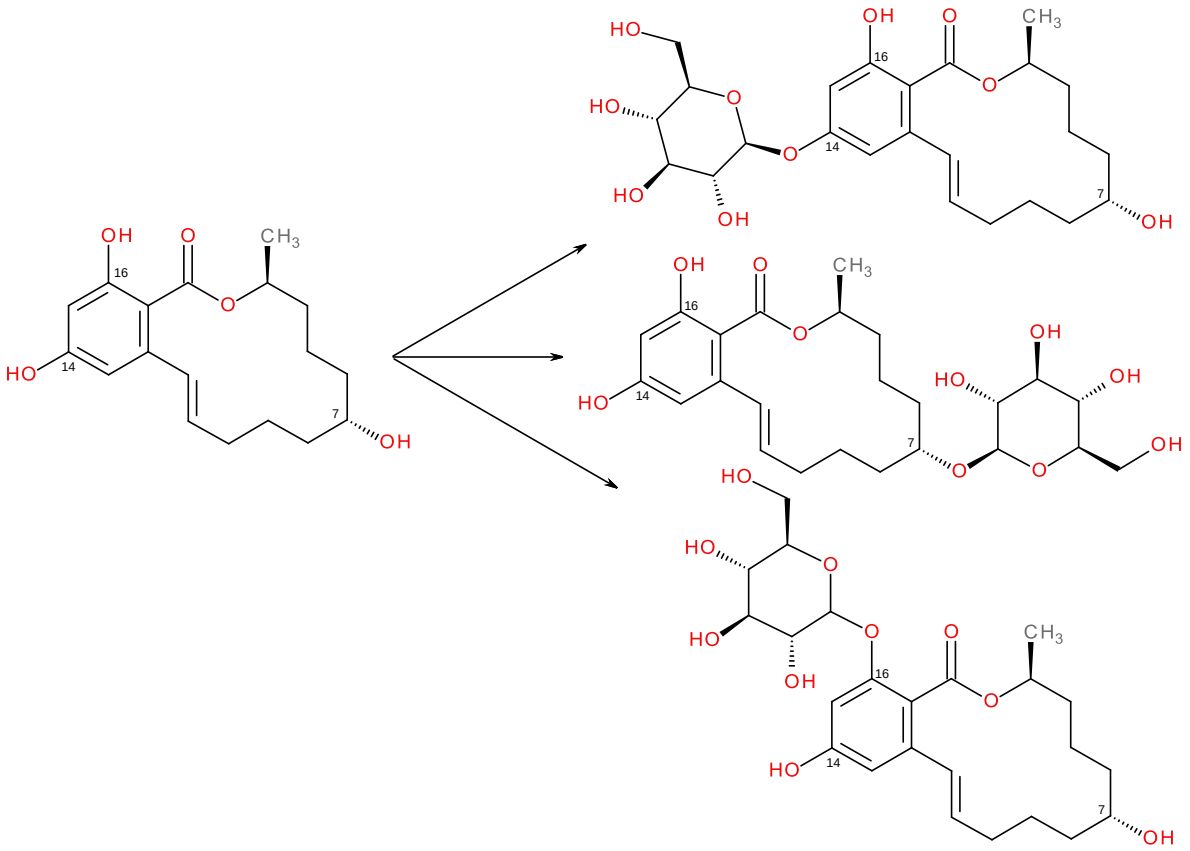


Figure 2. αZEL-(-7-/-14-/-16-)-glucosides expected to be formed via bio-conjugation of αZEL by *C. elegans*

Research performed at RIKILT (Wageningen University & Research) proved that *Cunninghamella elegans* can metabolise ZEN to ZEN-14-glucoside (ZEN14G), ZEN-16-glucoside (ZEN16G) and ZEN-14-sulfate (ZEN14S). Obtained conjugates were isolated and separated by preparative HPLC and for some of them structures were confirmed by NMR. Therefore, it was assumed that

previously developed method for biosynthesis of ZEN conjugates by *C. elegans* could be used for synthesis of αZEL conjugates (see figures 2 and 3).

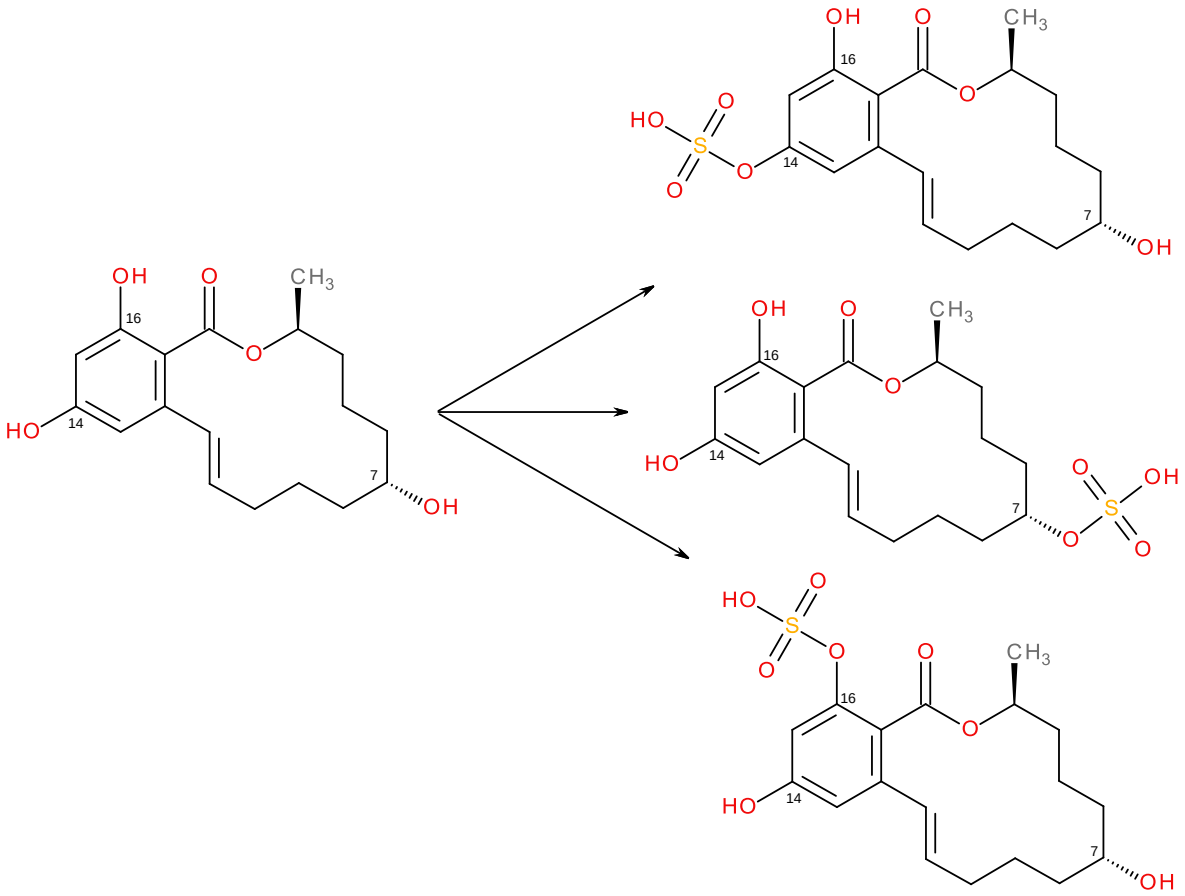


Figure 3. αZEL-(-7-/-14-/-16-)-sulfates expected to be formed via bio-conjugation of αZEL by *C. elegans*

This report describes the results of the experiments carried out to biologically synthesise αZEL conjugates (sulfates and glucosides) and to purify the compounds by using semi-preparative HPLC.

1. Materials and methods

1.1 Preliminary experiments

Preliminary experiments carried out at RIKILT (Wageningen University & Research) showed that out of three tested fungi – *Cunninghamella elegans*, *Cunninghamella echinulata* and *Rhizopus arrhizus*, only *C. elegans* (*Cunninghamella echinulata var. elegans*) is able to perform bio-conjugation of zearalenone, zearalanone and βZEL to those corresponding glucosides and sulfates. Structures of obtained conjugates were identified by high resolution mass spectrometry and NMR. Bioconversion of the corresponding substrates to glucosides or sulfates, depends on the nutrients in the growth medium. Incubation with potato dextrose broth (PDB) showed capacity to produce corresponding substrate sulfates, and incubation with Czapek-Dox broth (sulfate free) medium showed capacity to produce corresponding substrate glucosides. It was assumed that incubation of *C. elegans* spiked with αZEL in mentioned media could give results similar to obtained with βZEL.

1.2 Growth media and preparation of filtrate

Fungal media were prepared using the following conditions:

1. Incubation of *C. elegans* in potato dextrose broth (PDB) for production of αZEL sulfates
2. Incubation of *C. elegans* in Czapek-Dox broth (sulfate free) for production of αZEL glucosides

Incubation in PDB

C. elegans (ATCC® 9245™) was grown in 400 mL (2 L Erlenmeyer flasks) of potato dextrose broth (PDB) (Sigma-Aldrich) for three days at 27°C, on a horizontal shaking incubator at 200 rmp. After three days the broth was spiked with 1 mg of αZEL (Fermentek) standard solution dissolved in 1 mL of methanol. The broth was further incubated for two weeks at the same conditions. After two weeks, the broth (~350 mL) was transferred to seven 50 mL PP Greiner tubes, centrifuged at 3000 rmp and placed in a freezer at -20°C and stored until the purification by HPLC.

Incubation with Czapek-Dox broth

C. elegans (ATCC® 9245™) was grown in 400 mL (2 L Erlenmeyer flasks) of Czapek-Dox broth (home-made) for three days at 27°C, on a horizontal shaking incubator at 200 rmp. After three days the broth was spiked with 1 mg of αZEL (Fermentek) standard solution dissolved in 1 mL methanol The broth was further incubated for two weeks at the same conditions. After two weeks the resulting solution (~350 mL) was transferred to seven 50 mL PP Greiner tubes, centrifuged at 3000 rmp and placed in a freezer at -20°C and stored until the purification by HPLC.

1.3 Pre-concentration of samples using Strata™-X SPE

Approximately 350 mL of each broth was obtained (seven 50 mL PP tubes) which contained water soluble compounds, including salts and sugars, and fungal structures. This required pre-cleaning and concentration before purification of the target compounds by preparative HPLC.

Based on chemical properties of analytes solid phase extraction (SPE) was chosen as possible pre-purification and pre-concentration step. For this purpose a Strata™-X SPE column (Phenomenex, Torrance, CA, USA) was chosen. Strata™-X SPE column contains polymeric sorbent (styrene divinylbenzene) which, depending on condition steps, is able to bind neutral, acidic or basic small molecules.

For assessing the capability of the Strata™ X SPE column to bind ZEN and αZEL and their glucosides and sulfates, the following standard solutions and mixtures were prepared:

1. Zearalenone-14-glucoside (50 ng/mL) (IFA Tulln)
2. Zearalenone-16-glucoside (50 ng/mL) (IFA Tulln)

3. α-Zearalenol-14-glucoside (10 ng/mL) (IFA Tulln)
4. β-Zearalenol-14-glucoside (10 ng/mL) (IFA Tulln)
5. Zearalenone-14-sulfate (~1.3 µg/mL) (standard solution obtained from BAM (~2 mg/mL); also contained ZEN, ZEN-14&16-glucosides and βZEL)
6. Mixed standard solution containing approximately 100 ng/mL of each of the following compounds: ZEN-14-glucoside, ZEN-16-glucoside, αZEL-14-glucoside, βZEL-14-glucoside; and approximately 3000 ng/mL of ZEN-14-sulfate
7. PDB, 10x diluted, containing approximately 285 ng/mL of αZEL (before bio-conjugation)
8. Czapek-Dox broth, 10x diluted, containing approximately 285 ng/mL of αZEL (before bio-conjugation)
9. Mix of zearalenone and α-/β-zearalenol (10 ng/mL each) (mix of Biopure standard solutions)

Mixed standard solution as well as diluted PDB and Czapek-Dox broth (3 mL each) were each splitted in two parts (1.5 mL each). One part of each solution was used for direct injection into LC-MS/MS system (used for comparison and quantification), the other part was used to test capability of Strata™-X SPE column to retain the compounds mentioned above. This step was crucial in order to pre-purify and pre-concentrate fungal media solutions. It would also give additional information on applicability of this kind of SPE columns for sample preparation in future method development for food/feed or bio-liquid samples.

Strata™-X SPE procedure (Strata™-X column 200 mg):

1. Wash with 2 volumes of MeOH
2. Condition with 2 volumes of H_2O
3. Pass mixed standard solution or (PDB or Czapek-Dox) broths through the column (fraction 1)
4. Wash column with 2 volumes of H_2O (fraction 2)
5. Elute analytes with 1.5 mL of MeOH (fraction 3)

For step 3, 1.5 mL of mixed standard solution prepared in MeOH, was diluted with 8.5 mL of water prior to passing through the column. So, total organic content was ~15%. Fractions 1 and 2 were combined and called F1. Fractions F1 and 3 were analysed by LC-MS/MS in order to see if Strata™-X SPE column is capable to retain the mentioned mycotoxin conjugates. Recovery of the tested compounds was calculated from the prepared solutions, which were not passed through the SPE column.

1.4 LC-MS/MS screening of broths for target conjugates

For screening of the both broths, each set of seven frozen 50 PP tubes was thawed at room temperature. The contents of the seven PP tubes of each set of broth (7 tubes, 50 mL each; ~350 mL in total) were mixed in a 500 mL glass bottle to ensure homogeneity of total sample. The mixed solution of each set was transferred back to seven 50 mL PP tubes and centrifuged at 3600 rmp. An aliquot of 200 µL was transferred from each tube into an Eppendorf centrifugation vial (1.5 mL) and centrifuged for 10 minutes at 10 000 rmp. An aliquot of 100 µL of the supernatant was transferred into HPLC vial, diluted with 900 µL of milli-Q water and injected into the LC-MS/MS system. Since 1 mg of αZEL was spiked to 400 mL of each media and after incubation for two weeks only ~350 mL was left, theoretical amount of unconjugated αZEL could be around 2.857 µg/mL (if conjugation would not occur). The LC-MS/MS method described in RIKILT SOP-A-1280 was used for screening of both broths. Twelve zearalenone and related compounds were included: zearalenone (ZEN), zearalenone-14-glucoside (ZEN14G), zearalenone-16-glucoside (ZEN16G), zearalenone-14,16-di-glucoside (ZEN14,16diG),

zearalenone-14-sulfate (ZEN14S), α-zearalenol (αZEL), α-zearalenol-14-glucoside (αZEL14G), β-zearalenol (βZEL), β-zearalenol-14-glucoside (βZEL14G), α-zearalanol (αZAL), β-zearalanol (βZAL). In addition to the mentioned compounds, three theoretical MRM transitions for screening of α-zearalenol-sulfates (αZEL-S) were added.

MS/MS fragmentation settings (MRM transitions)

No	Q1 (m/z)	Q3 (m/z)	Rt (min)	Analyte_MRM transition No	DP (V)	EP (V)	CE (V)	CXP (V)
1	319.3	160	6.1	βZEL_1	-110	-10	-41	-13
2	319.3	130	6.1	βZEL_2	-110	-10	-47	-20
3	317.1	175	6.6	ZEN_1	-175	-10	-32	-15
4	317.1	131.1	6.6	ZEN_2	-175	-10	-36	-11
5	319.2	160	6.7	αZEL_1	-110	-10	-41	-13
6	319.2	130	6.7	αZEL_2	-110	-10	-47	-20
7	478.9	317	3.9	ZEN14G_1	-105	-10	-26	-27
8	478.9	273	3.9	ZEN14G_2	-105	-10	-42	-19
9	479	317	5	ZEN16G_1	-90	-10	-30	-25
10	479	273	5	ZEN16G_2	-90	-10	-44	-21
11	397.2	316.9	4.5	ZEN14S_1	-55	-10	-30	-21
12	397.2	175	4.5	ZEN14S_2	-55	-10	-46	-11
13	481	319	5.1	αZEL-G_1	-130	-10	-14	-23
14	481	275	5.1	αZEL-G_2	-130	-10	-44	-23
15	481.1	319	4.3	βZEL-G_1	-130	-10	-14	-23
16	481.1	275	4.3	βZEL-G_2	-130	-10	-44	-23
17	319.1	275.15	6.4	ZAN_1	-60	-10	-30	-10
18	319.1	205.15	6.4	ZAN_2	-60	-10	-30	-10
19	321.1	277.15	6.3	αZAL_1	-60	-10	-30	-10
20	321.1	259.15	6.3	αZAL_2	-60	-10	-30	-10
21	321.15	277.15	5.7	βZAL_1	-60	-10	-30	-10
22	321.15	259.15	5.7	βZAL_2	-60	-10	-30	-10
23	687.1	316.9	3.1	ZEN14,16diG_1	-25	-10	-48	-23
24	687.1	479.1	3.1	ZEN14,16diG_2	-25	-10	-24	-31
25	335.2	185.1	6.6	13C18-ZEN	-175	-10	-32	-15
26	326.2	162.1	6.7	αZEL-d7	-110	-10	-41	-13
27	326.2	162.1	6.7	βZEL-d7	-110	-10	-41	-13
28	325.15	281.15	6.4	ZAN-d6	-60	-10	-30	-10
29	326.2	282.2	6.3	αZAL-d5	-60	-10	-30	-10
30	326.2	282.2	6.3	βZAL-d5	-60	-10	-30	-10
31	399.1	399.1	5.9	αZEL-S_1	-30	-10	-30	-21
32	399.1	319.2	5.9	αZEL-S_2	-55	-10	-30	-21
33	399.1	130.0	5.9	αZEL-S_3	-55	-10	-46	-11

LC-MS/MS system used: Shimadzu UPLC equipped with an autosampler, two gradient pumps and a precise temperature control oven equipped with Acquity HSS T3, 1.8 µm, 2.1 x 100 mm UPLC column and connected to Applied Biosystems QTRAP 5500 triple-quad mass spectrometer.

MS/MS detection conditions:

Parameter	Settings
Ionisation mode	ESI negative
Scan type	MRM
Scheduled MRM	No
Dwell time	5 ms
Resolution Q1	unit
Resolution Q3	unit
Settling time	0 msec
MR pause	5 msec
Curtain gas (CUR)	40
Collision gas (CAD)	Medium
Ion Spray Voltage (IS)	-4500 V
Temperature (TEM)	400 °C
Ion Source Gas 1 (GS1)	50
Ion Source Gas 2 (GS2)	50

LC conditions:

Injector Temperature: 10°C
Colum T: 40°C
Injection volume: 5 µL
Flow: 0.4 mL/min
Mobile phase A: 2 mM ammonium formate/0.05% formic acid in water
Mobile phase B: 2 mM ammonium formate/0.05% formic acid in 95% acetonitrile

Gradient:

Time (min)	%A	%B
0.0	100	0
1.0	100	0
7.0	0	100
9.0	0	100
9.5	100	0
12.0	100	0

1.5 Analytical and semi-preparative HPLC

1.5.1 Sample clean-up and concentration for preparative HPLC

Seven 50 mL PP tubes of PDB and seven of Czapek-Dox broth were thawed to reach room temperature. They were vortex mixed and centrifuged for 15 minutes at 3600 rmp. Seven Strata™-X SPE columns (Strata™-XL 500 mg) for PDB broth and seven Strata™-X columns for Czapek-Dox broth were placed on a vacuum manifold and the procedure described in section 1.3 was applied with one modification, analytes were eluted with 2+2 mL of MeOH.
Fractions of steps 3 and 4 were collected separately and stored in the freezer to preserve any compounds that were not retained. Both volumes of elution fractions of step 5 were collected, combined, mixed and evaporated in a water bath under the nitrogen stream at 32°C to dryness.

The residue was dissolved in 6 mL MeOH. Final volumes of approximately 6 mL (solvent 100% MeOH) of pre-purified and pre-concentrated PDB and Czapek-Dox samples were further used for analytical and preparative HPLC-PDA method development.

1.5.2 Analytical and semi-preparative HPLC-PAD parameters

The following HPLC columns for analytical and semi-preparative method development were used:

1. Waters Atlantis T3 4.6*250 mm, 5 µm (analytical HPLC column)
2. Waters Atlantis OBD T3 10*250 mm, 5 µm (semi-preparative HPLC column)

In order to be able to purify the target analytes from the pre-cleaned broths, the analytical HPLC method was first developed using small quantities of pre-cleaned and concentrated broth, followed by further semi-preparative method development. Parameters of developed methods are shown below.

HPLC conditions for analytical and semi-preparative methods:
Injector Temperature: ambient
Colum T: 35°C (analytical); 37°C (semi-preparative)
Injection volume: 10-50 µL (analytical); 100-250 µL (semi-preparative)
Flow: 1.0 mL/min (analytical); 3 mL/min (semi-preparative)
Mobile phase A: 2 mM ammonium formate/0.05% formic acid in water
Mobile phase B: 2 mM ammonium formate/0.05% formic acid in 95% acetonitrile

Gradient (final analytical gradient = initial semi-preparative gradient):

Time (min)	%A	%B
0.0	70	30
3.0	70	30
27.0	55	45
30.0	37.5	62.5
31.0	0	100
34.0	0	100
35.0	70	30
40.0	70	30

Final gradient of semi-preparative method for αZEL14S purification from PDB, fraction collector was set to collect fraction containing αZEL14S between 28 and 32 min:

Time (min)	%A	%B
0.0	70	30
3.0	70	30
30.5	66	34
33.0	60	40
35.0	0	100
38.0	0	100
39.0	70	30
43.0	70	30

Final gradient of semi-preparative method for αZEL- & ZEN-glucosides purification from Czapek-Dox broth:

Time (min)	%A	%B
0.0	75	25
3.0	75	25
37.0	60	40
38.0	0	100
41.0	0	100
42.0	75	25
45.0	75	25

Fraction collector was set to collect fractions containing target compounds based on retention time frames: αZEL16G (13.6-15 min); ZEN16G (21.6-23 min); αZEL14G (25.5-27) min & ZEN14G (33-34.5 min).

1.6 Analytical HPLC-PAD parameters for purity tests of purified compounds

To check purity and quantify of purified compounds, available standard solutions and advanced HPLC gradient was used. Purified fractions of each compound were properly mixed and sub samples to test purified compounds for purity by HPLC-PAD were taken.

1.6.1 Analytical HPLC-PAD method parameters

The following HPLC-PAD method was used in order to estimated purity and quantity of purified compounds.

Injector Temperature: ambient
Colum T: 35°C
Injection volume: 10-50 µL
Flow: 1.0 mL/min
Mobile phase A: 2 mM ammonium formate/0.05% formic acid in water
Mobile phase B: 2 mM ammonium formate/0.05% formic acid in 95% acetonitrile
PAD detector set at: full scan from 210-400 nm; wavelengths for monitoring: 235 and 254 nm
HPLC column: Waters Atlantis T3 4.6*250 mm, 5 µm

Final gradient for purity tests:

Time (min)	%A	%B
0.0	75	25
3.0	75	25
30.0	55	45
40.0	25	75
50.0	0	100
55.0	0	100
56.0	75	25
60.0	75	25

Wavelength of 254 nm was used for quantification.

1.6.2 Standard solutions for purity tests and quantification of purified compounds

The following standard solutions were used for quantification:

1. ZEN (Biopure) 10 µg/mL
2. αZEL (Biopure) 10 µg/mL
3. ZEN14G (Aokin) 10 µg/mL
4. ZEN16G (IFA Tulln) 50 µg/mL
5. αZEL14G (IFA Tulln) 10 µg/mL
6. ZEN14S (Aokin) 100 µg/mL

For purity tests and quantification, all standard solutions were diluted to 1 µg/mL, ZEN14S was diluted to 10 µg/mL, and injected separately into HPLC-PAD system. Obtained peak areas and UV spectra were used for identification, quantification and purity estimation of the purified compounds. Since analytical standards for reliable quantification of all purified compounds were not available it was possible to directly quantify only: ZEN, ZEN-14&16-glucosides, ZEN14S, αZEL and αZEL14G. For estimation of purified compounds where analytical standards for quantification were not available compounds with similar UV spectra were used for estimation of quantity. For instance ZEN or αZEL conjugated at a position 14 have similar spectra, where λ_{max} differs by 1-3 nm; at the same time if ZEN or αZEL are conjugated at a position 16 they have different spectra to 14-position conjugates, but are similar to each other (again, λ_{max} differs by 1-3 nm only). Thus, to estimate an amount of αZEL14S in the final purified solution, an analytical standard of ZEN14S obtained from Aokin was used. To estimate an amount of αZEL16G in a purified solution an analytical standard of ZEN16G obtained from IFA Tulln was used.

2. Results and Discussion

2.1 Pre-cleaning and pre-concentration

As mentioned in section 1.3, mixed standard solution containing approximately 100 ng/mL of each of the following compounds: ZEN14G, ZEN16G, αZEL14G, βZEL14G; and approximately 3000 ng/mL of ZEN14S, was passed through the Strata™-X column. In addition, PDB and Czapek-Dox broths (each diluted 10 times) containing approximately 285 ng/mL of αZEL (before bio-conjugation) were separately passed through the same type (Strata™-X) columns. Strata™-X columns showed high capability to retain the selected analytes. Recoveries in the range 95-109% were obtained for mixed standard solution and 68-87% for target compounds in both broths (Table 1).

Table 1. Summary of recovery results from Strata™-X column

Compound Name	Recovery for mixed st. solution (%)	Recovery for PDB (%)	Recovery for Czapek-Dox broth (%)	Mean estimated recovery (%)
ZEN	95	n.a.	77	86
ZEN14G	101	n.a.	68	85
ZEN16G	110	n.a.	69	89
ZEN14S	97	**75***	n.a.	86
αZEL	n.a.	**64***	87	76
αZEL14G	103	n.a.	85	94
βZEL14G	109	n.a.	n.a.	109
αZEL14S	n.a.	70	n.a.	70
βZEL	n.a.	n.a.	**129***	129

n.a. – not applicable = not tested; * – concentration in the sample was too low to quantify it correctly, so shown value is indicative.

Due to the limited amounts of selected compounds, available experiments were based on a single experiment only. If final conclusions on recovery must be draw, it is necessary to perform separate experiments with at least 6 replicates per tested level.

2.2 Results of PDB and Czapek-Dox broth screening and purification

LC-MS/MS screening of diluted PDB and further HPLC-PAD analysis, showed that αZEL was almost fully converted to αZEL14S (≥90 % of total areas of αZEL14S and ZEN14S) and partially to ZEN14S (≤10 % of total areas) by *C. elegeans*. Traces of αZEL were detected (≤1%) but no traces of αZEL (-7- or -16-)-sulfates were found. This proves that most likely position 14 in most cases is preferable for bio-conjugation. The presence of ZEN14S could explain why no position 7 sulfates were detected. Most likely αZEL has been reduced to ZEN, so position 7 becomes unavailable for sulfatation. Not clear if it happens before or after sulfatation of position 14. To fully proof this assumption additional large scale experiments are necessary in order to obtain lager amounts of compounds and perform full NMR, high resolution mass spectrometry and elemental composition etc. analysis. Due to limited time and complexity of the matrix (even after pre-purification) it was decided to purify only αZEL14S as a major metabolite from biosynthetic mixture.

LC-MS/MS screening of diluted Czapek-Dox broth and further HPLC-PAD analysis, showed that αZEL was partially converted to αZEL-14&16-glucosides, ZEN and ZEN-14&16-glucosides, also traces of βZEL were detected. αZEL-7-glucoside was not detected, which again supports the assumption made above. See appendixes 1-4 for LC-MS/MS, LC-HR-MS and HPLC-PAD representative chromatograms and spectra.

2.3 Quantification of purified compounds

Table 2 shows estimated total amounts of selected and purified compounds during LC-MS/MS and Strata™-X screening and after purification by preparative HPLC.

Table 2. Estimated amounts of selected and purified compounds after LC-MS/MS screening and after purification

Compound name	Estimated total amount in PDB from LC-MS/MS screening (µg)	Estimated total amount in PDB from LC-MS/MS screening after Strata X (µg)	HPLC purified PDB, final amount (µg)	Estimated total amount in Czapek-Dox from LC-MS/MS screening (µg)	Estimated total amount in Czapek-Dox from LC-MS/MS screening after Strata X (µg)	HPLC purified Czapek-Dox, final amount (µg)
βZEL	-	-	-	0.6	0.8	-
ZEN	-	-	-	7.5	5.8	-
αZEL	0.9	0.5	-	82.9	71.9	-
ZEN14G	-	-	-	34.6	23.4	22.9
ZEN16G	-	-	-	8.7	6.0	6.3
ZEN14S	142	107	-	-	-	-
αZEL-G (total)	-	-	-	20.6	17.4	11.3
αZEL14G	-	-	-	-	-	9.5
αZEL16G	-	-	-	-	-	1.8
αZEL14S	1120	788	1064	-	-	-

Cunninghamella elegans showed great potential of biosynthesis of α-zearalenol conjugates. Its incubation in spiked potato dextrose broth showed potential to produce corresponding α-zearalenol-14-sulfate (yield ~85%) and even some zearalenone-14-sulfate. Incubation in Czapek-Dox (sulfate free) broth showed capacity to partially convert α-zearalenol to zearalenone and produce corresponding 14 and 16 glucosides of both compounds. Bioconversion of α-zearalenol to its glucosides in Czapek-Dox broth was less than 5% of the theoretical possible amount (total sum of all mentioned conjugates). Bioconversion of individual conjugates was in the range 0.12-1.5% of the theoretically possible conversion.

See appendices 1-4 for representative chromatograms, spectra and estimation of amounts of purified compounds.

All purified products were obtained in 2 mM ammonium formate buffer, that contained approximately 35% of acetonitrile and 0.05% of formic acid. Each product was splitted in a 50 mL PP tubes, tightly closed and stored in the freezer at -20°C. Total volumes and concentrations of purified products are shown in the table 3 below.

Table 3. Total volumes and concentrations of purified products

Compound name	Total volume, mL	Estimated concentration, µg/mL ± 10%	No of 50 mL PP tubes in a freezer
ZEN14G	55 ± 5	0.42	2
ZEN16G	55 ± 5	0.11	2
αZEL14G	55 ± 5	0.17	2
αZEL16G	55 ± 5	0.06	2
αZEL14S	258 ± 5	4.13	6

Due to limited amounts of available standards and time, estimation of concentrations was performed on a basis of single-point calibration from prepared and injected standards (see 1.6.2 for details).

3. Conclusions

Cunninghamella elegans showed great potential of biosynthesis of α-zearalenol conjugates. Its incubation in potato dextrose broth spiked with α-zearalenol showed potential to produce corresponding α-zearalenol-14-sulfate (yield ~85%) and even some zearalenone-14-sulfate. Incubation with Czapek-Dox (sulfate free) broth showed capacity to partially convert α-zearalenol to zearalenone and produce corresponding 14 and 16 glucosides of both compounds however, at less than 5% of the theoretical possible amount.

Methods for biological synthesis of α-zearalenol-14-sulfate, α-zearalenol glucosides, as well as zearalenone glucosides and sulfates were developed.

Methods for pre-purification, pre-concentration and final purification by semi-preparative HPLC were developed. Obtained purified standard solutions of α-zearalenol-14-sulfate (~1mg), α-zearalenol-14-glucoside (~9.5 µg), α-zearalenol-16-glucoside (~1.8 µg), zearalenone-14-glucoside (~22.9 µg) and zearalenone-16-glucoside (~6.3 µg) may be used for qualification purposes in LC-MS/MS.

4. Recommendations

Since yield of obtained glucosides from Czapek-Dox broth was low, as well as the quantity of left over αZEL (which was not the case in earlier experiments with other similar compounds, including βZEL), there is a reason to assume that more attention must be payed to spiking. Therefore, experiments with Czapek-Dox media needs to be repeated.

References

Berthiller, F., C. Crews, C. Dall'Asta, S. De Saeger, G. Haesaert, P. Karlovsky, I. P. Oswald, W. Seefelder, G. Speijers and J. Stroka (2013). "Masked mycotoxins: A review." Molecular Nutrition & Food Research **57**(1): 165-186.

Brodehl, A., A. Moller, H. J. Kunte, M. Koch and R. Maul (2014). "Biotransformation of the mycotoxin zearalenone by fungi of the genera *Rhizopus* and *Aspergillus*." FEMS Microbiology Letters **359**(1): 124-130.

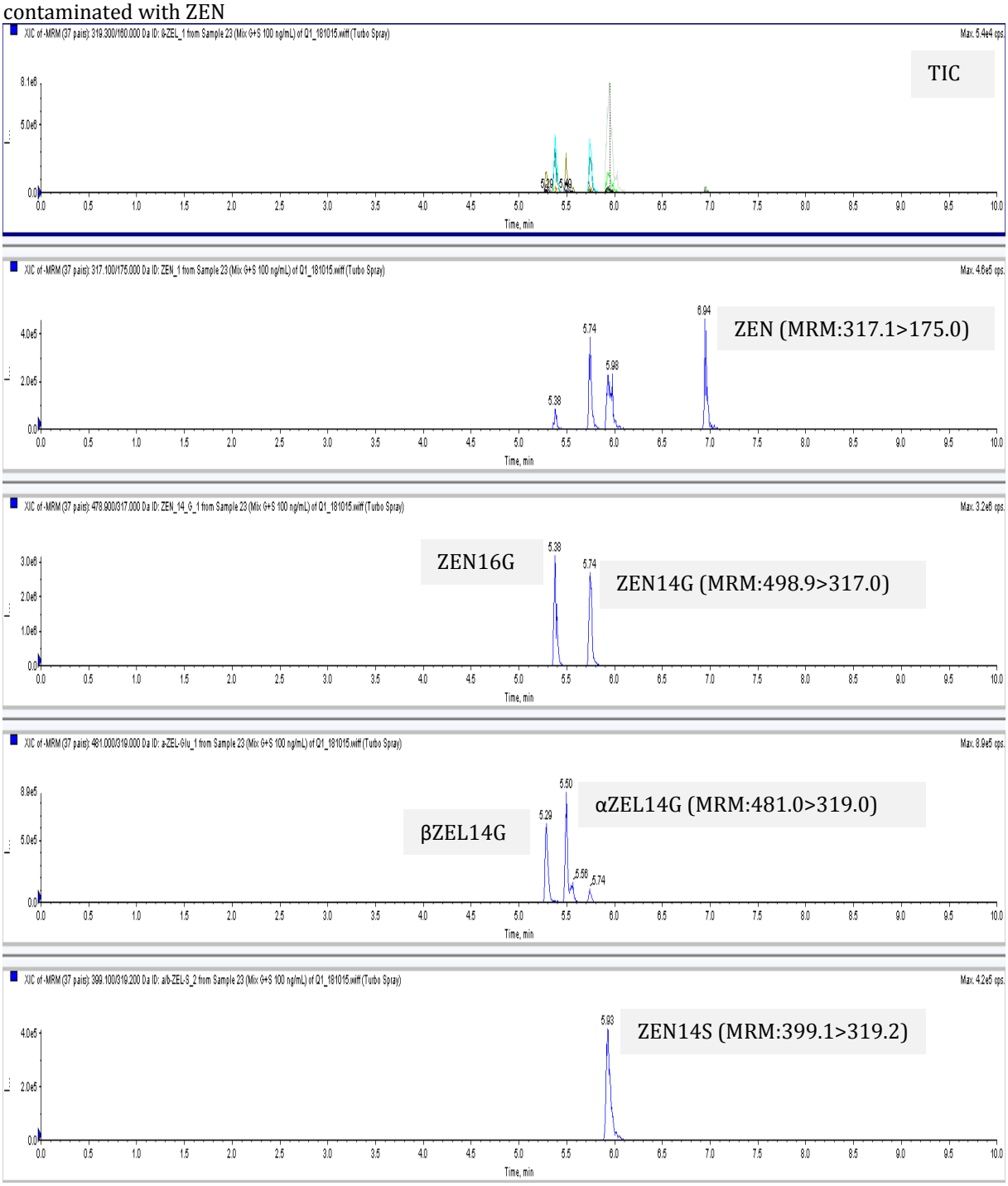
EFSA (2016). "Appropriateness to set a group health-based guidance value for zearalenone and its modified forms. EFSA Panel on Contaminants in the Food Chain." EFSA Journal 2017;14(4):4425: p. 46.

EFSA (2017). "Risks for animal health related to the presence of zearalenone and its modified forms in feed." EFSA Journal 2017;15(7):4851: pp. 123.

Appendix 1

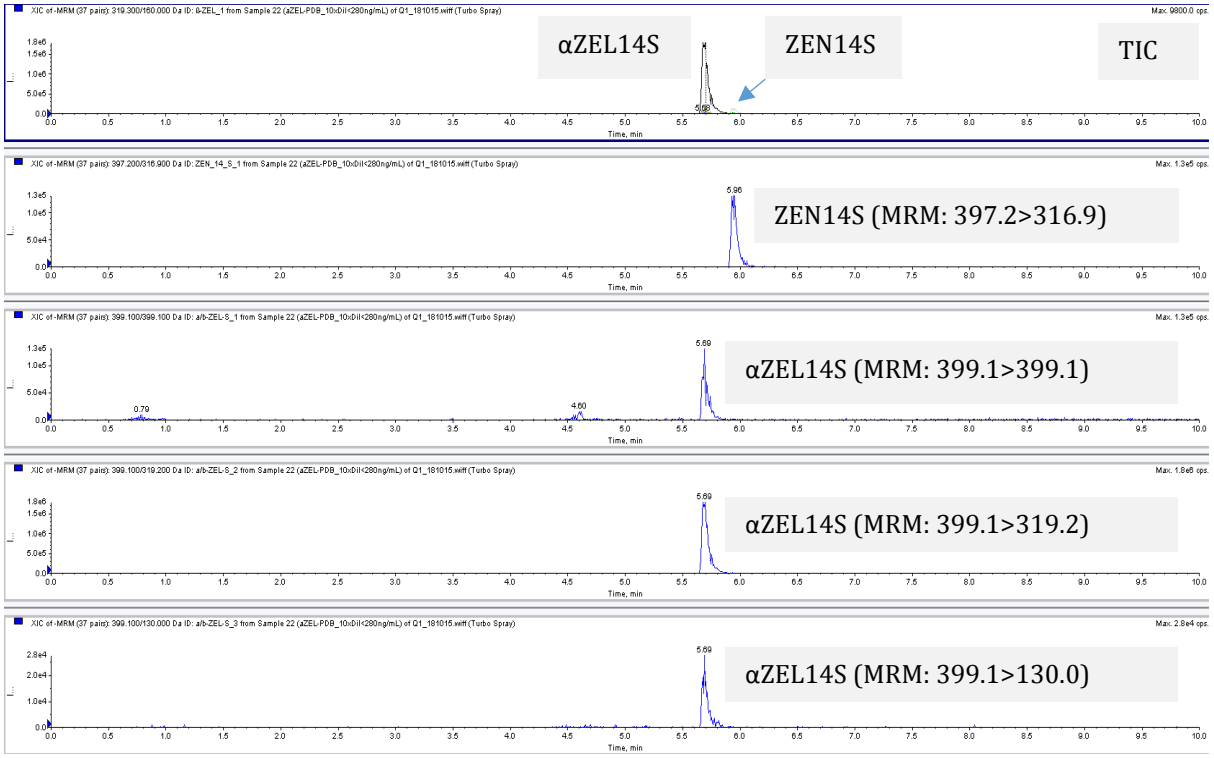
Chromatograms of LC-MS screening

LC-MS/MS total ion chromatogram (TIC) & selected MRM chromatograms of Mixed standard solution containing approximately 100 ng/mL of each of the following compounds: ZEN-14-glucoside, ZEN-16-glucoside, α-ZEL-14-glucoside, β-ZEL-14-glucoside; and approximately 3000 ng/mL of ZEN-14-sulfate; contaminated with ZEN

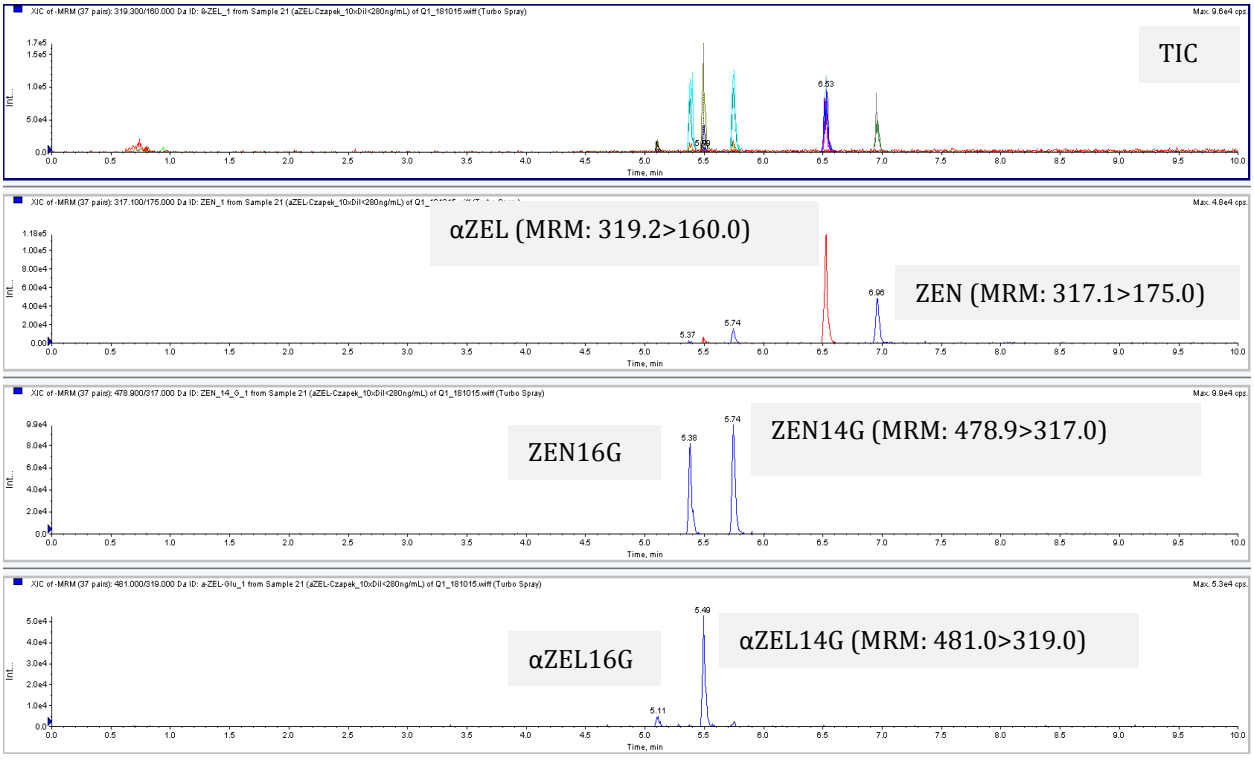


Appendix 1 continued

LC-MS/MS TIC & selected MRM chromatograms of 10x diluted PDB media

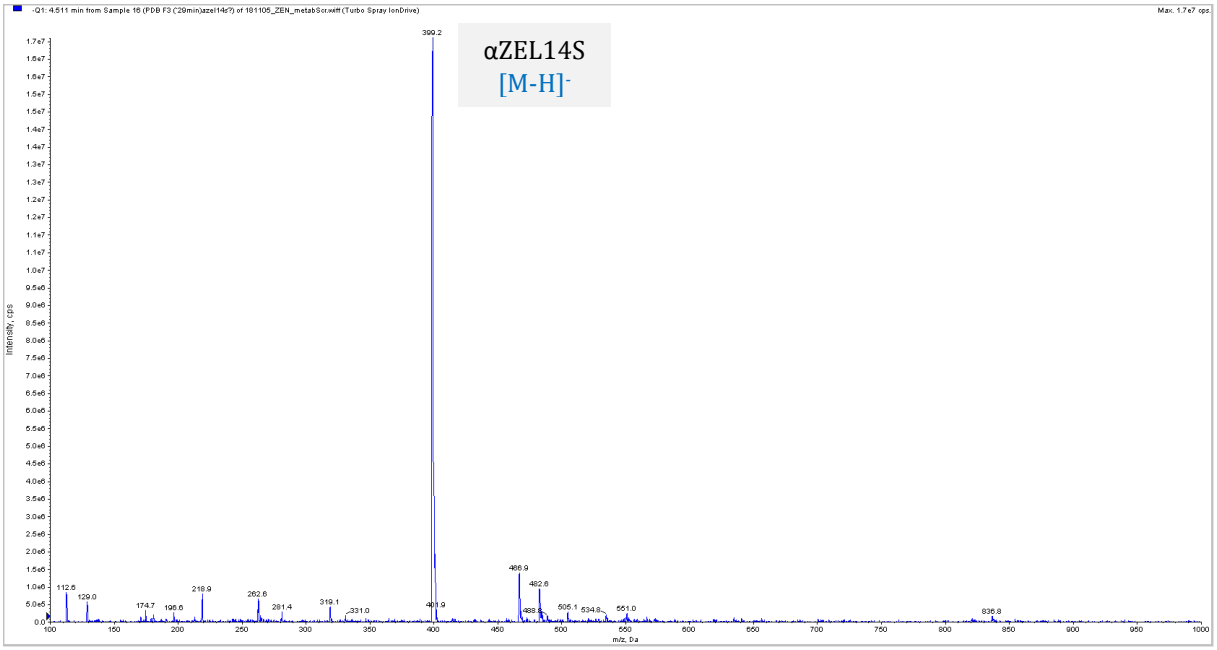


LC-MS/MS TIC & selected MRM chromatograms of 10x diluted Czapek-Dox media

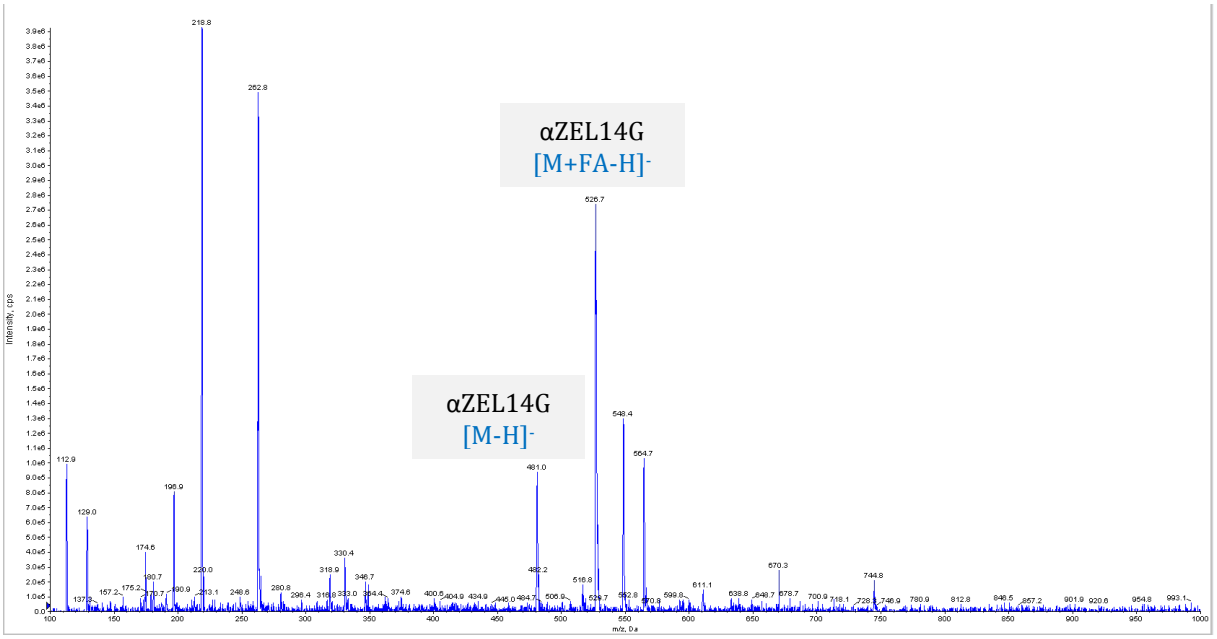


Appendix 1 continued

Example of full scan spectrum of **αZEL14S** (MW 400.12)

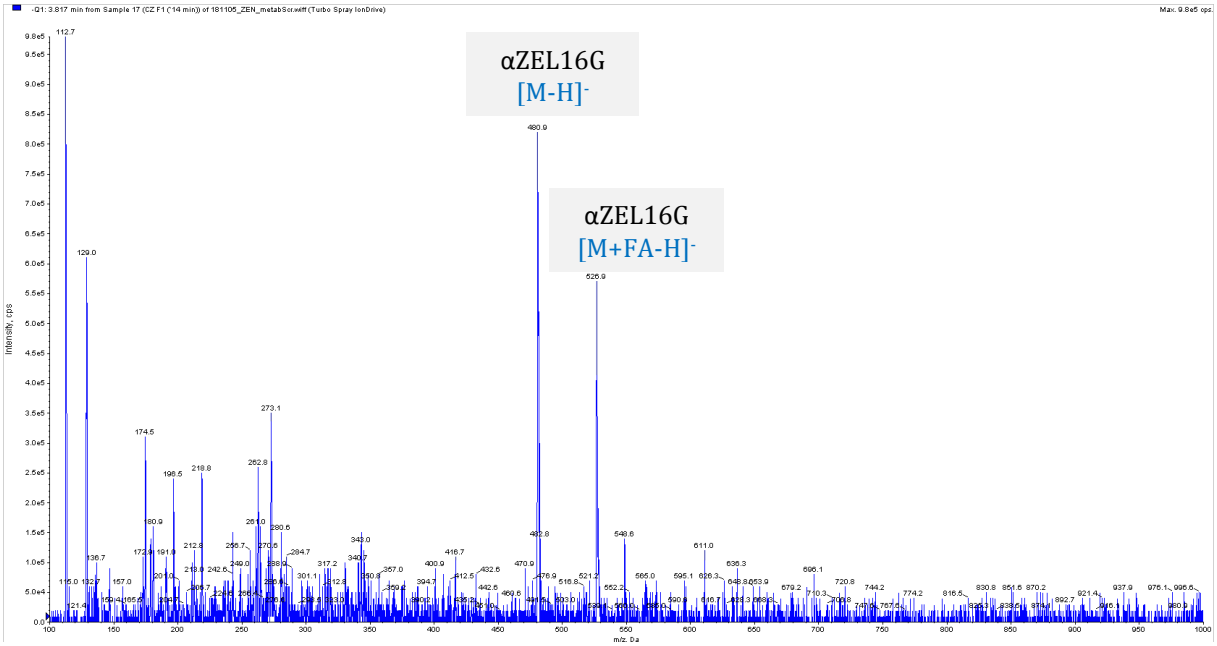


Example of full scan spectra of **αZEL14G** (MW 482.16)

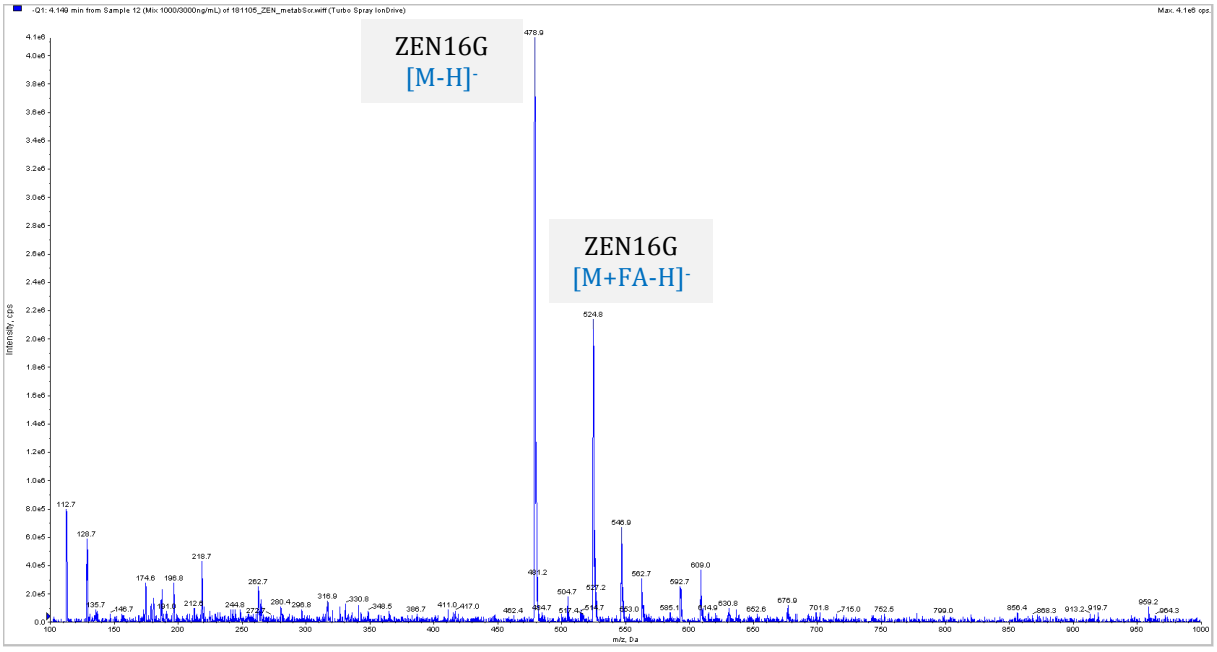


Appendix 1 continued

Example of full scan spectrum of **αZEL16G** (MW 482.16)



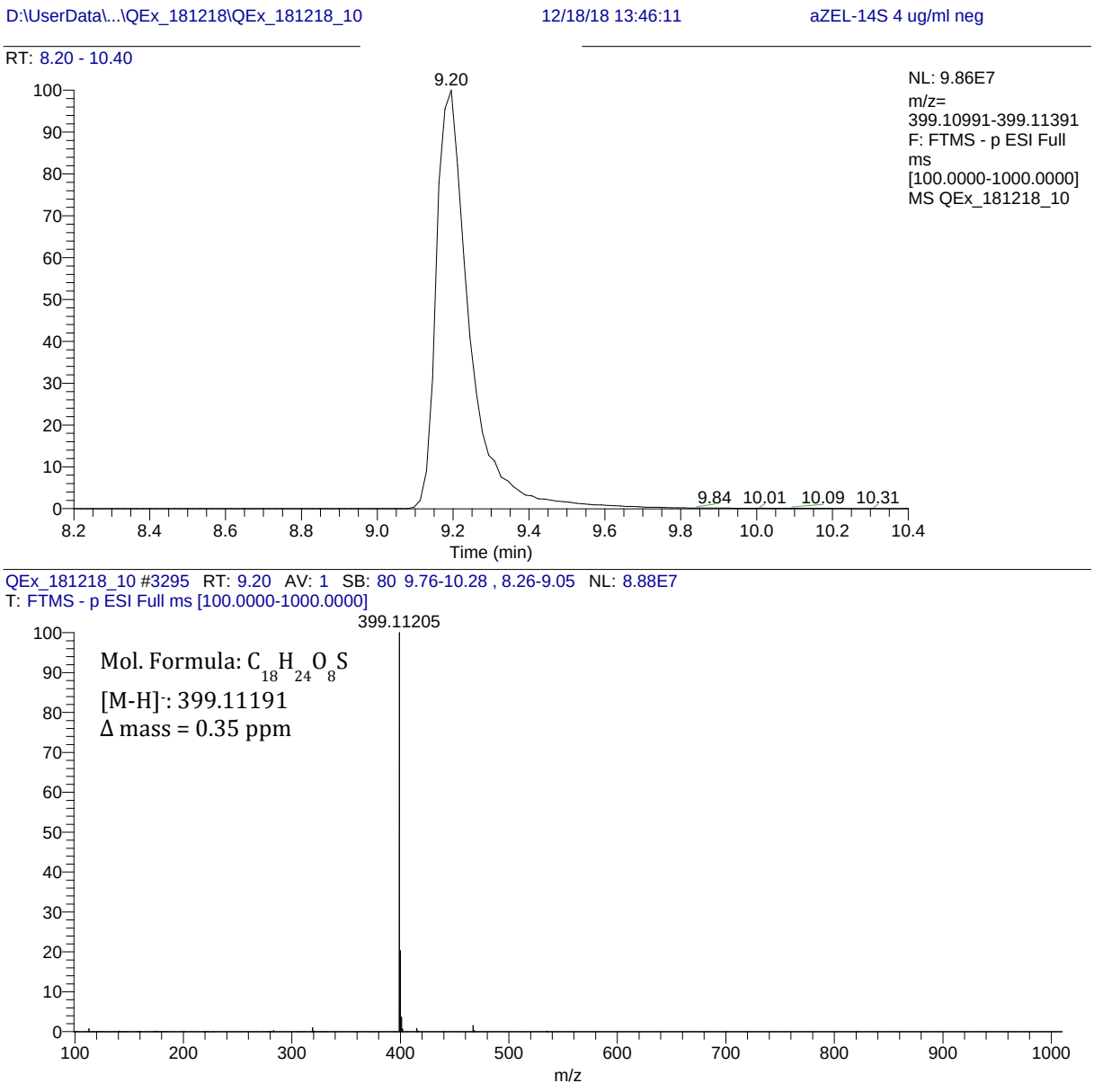
Example of full scan spectra of **ZEN16G** (MW 480.20)



Appendix 2

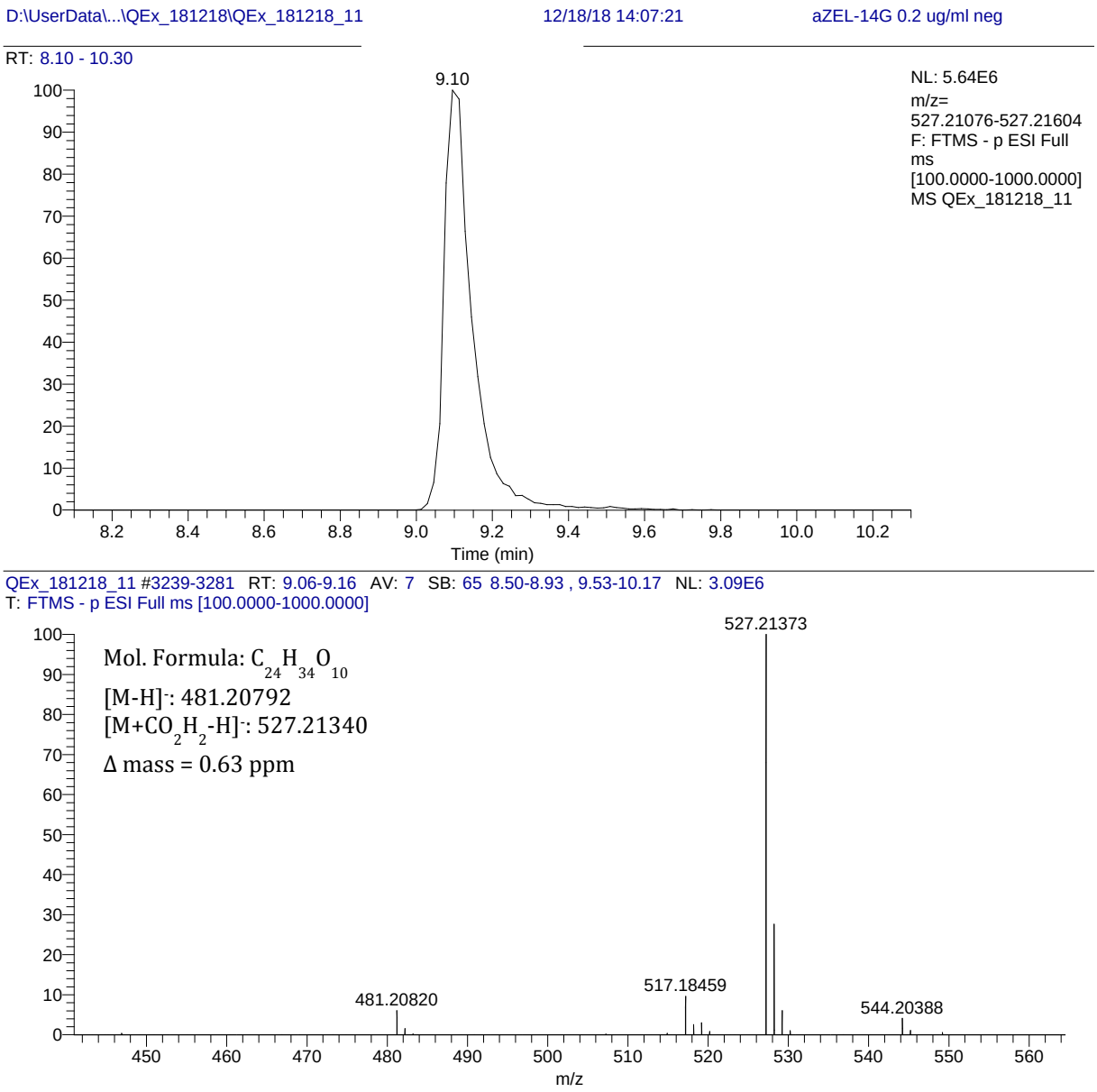
High resolution MS (HR-MS) spectra of purified conjugates

HR-MS spectrum of purified **αZEL14S**



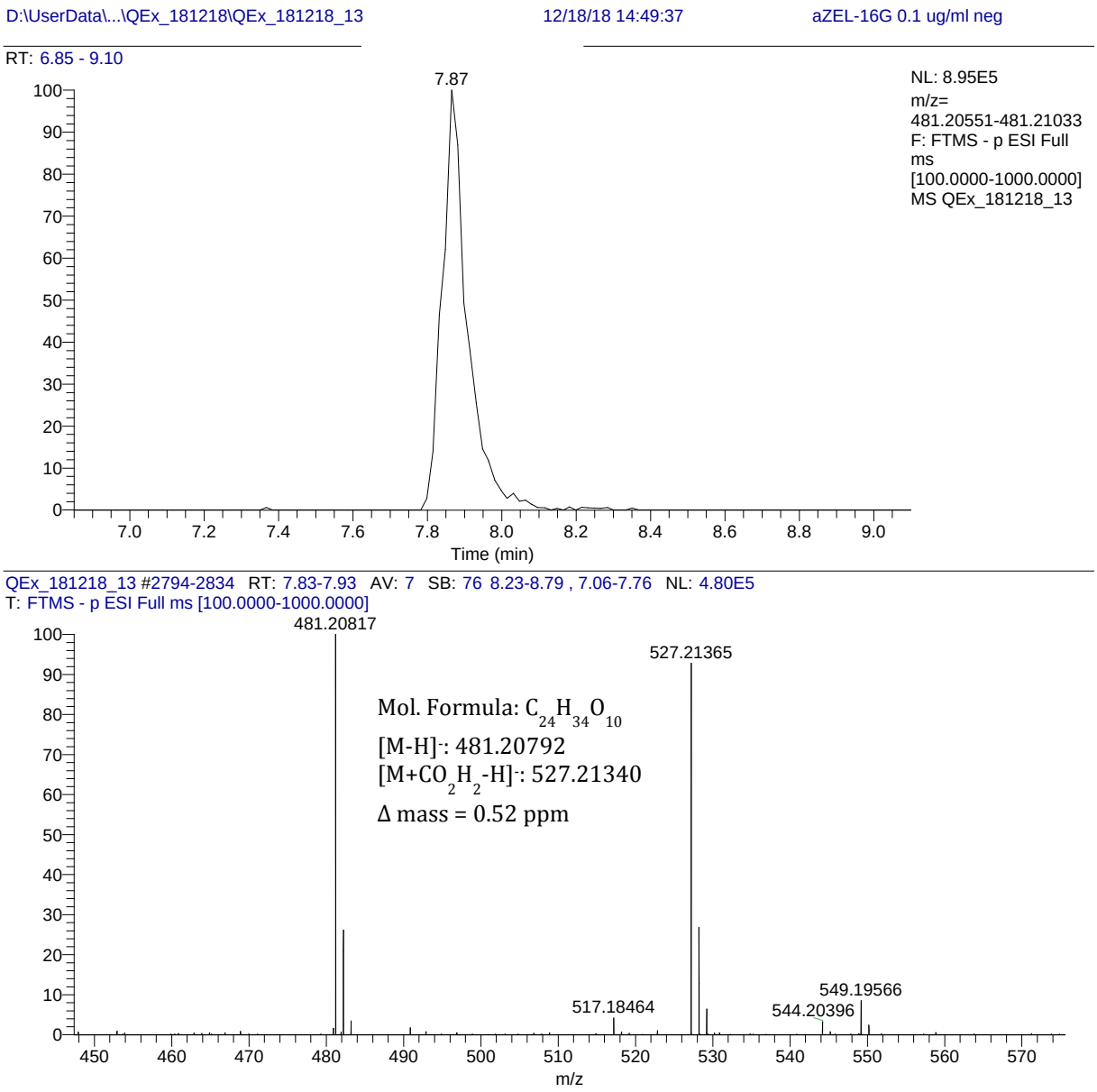
Appendix 2 continued

HR-MS spectrum of purified **αZEL14G**



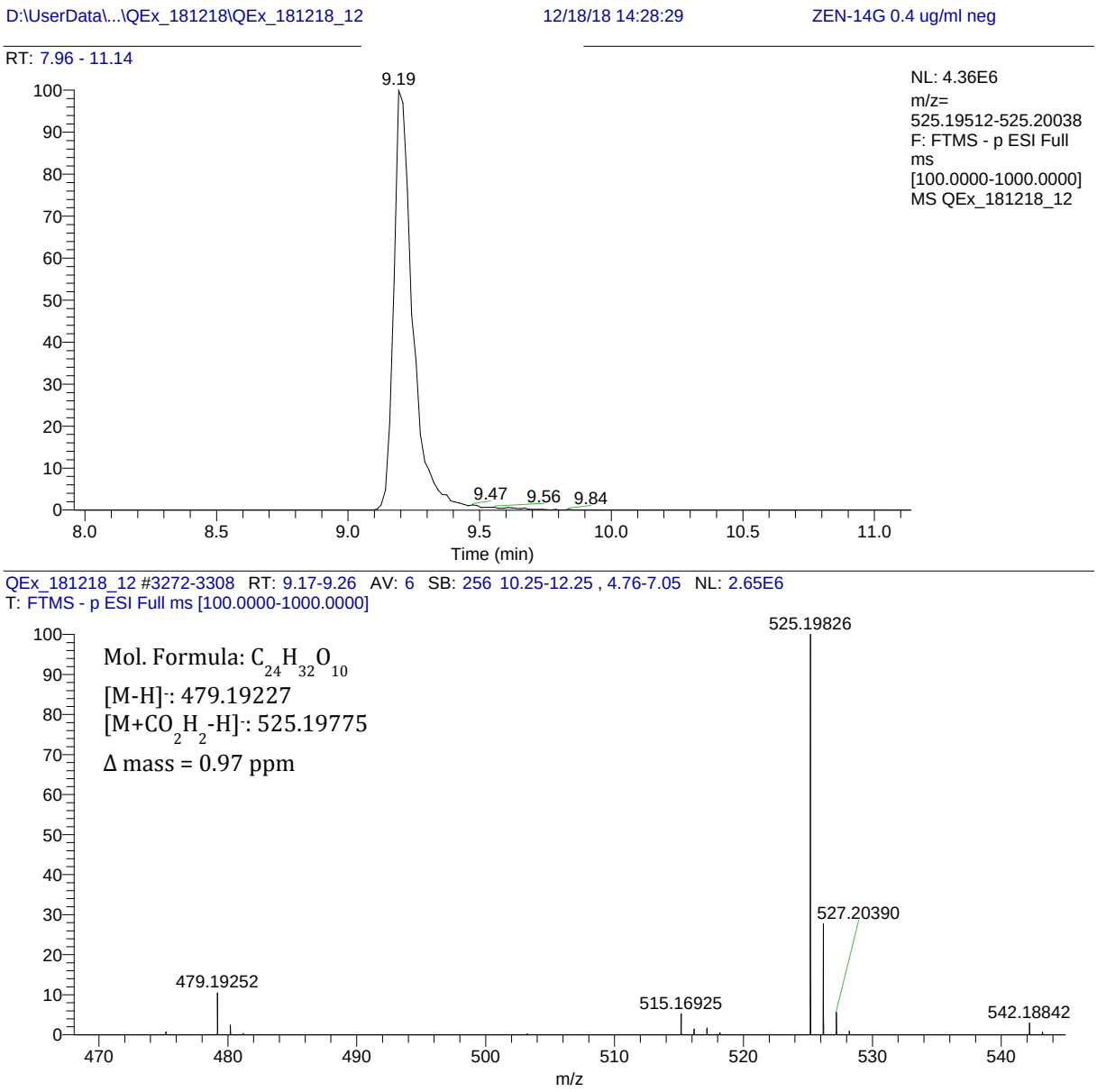
Appendix 2 continued

HR-MS spectrum of purified **αZEL16G**



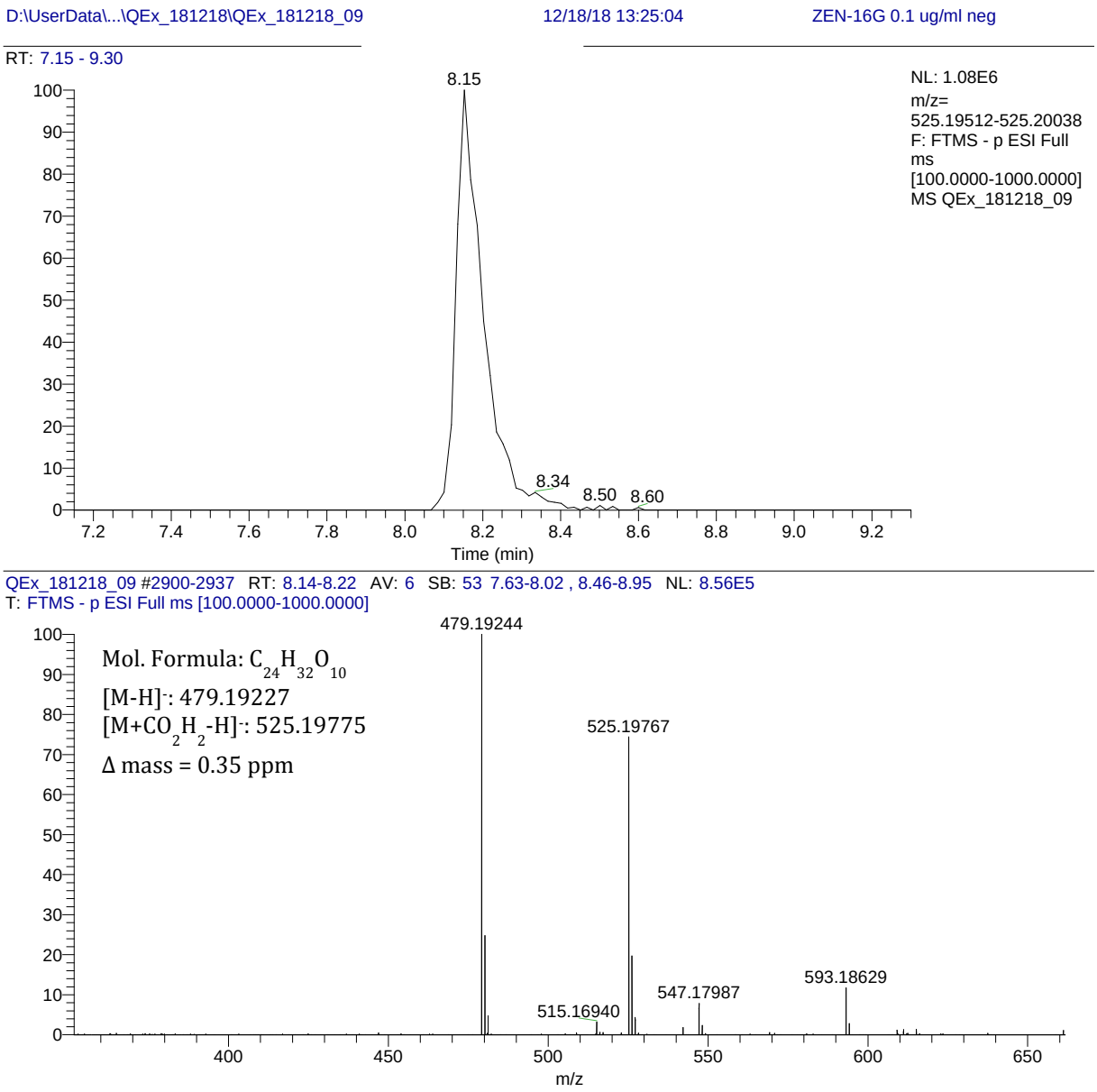
Appendix 2 continued

HR-MS spectrum of purified **ZEN14G**



Appendix 2 continued

HR-MS spectrum of purified **ZEN16G**

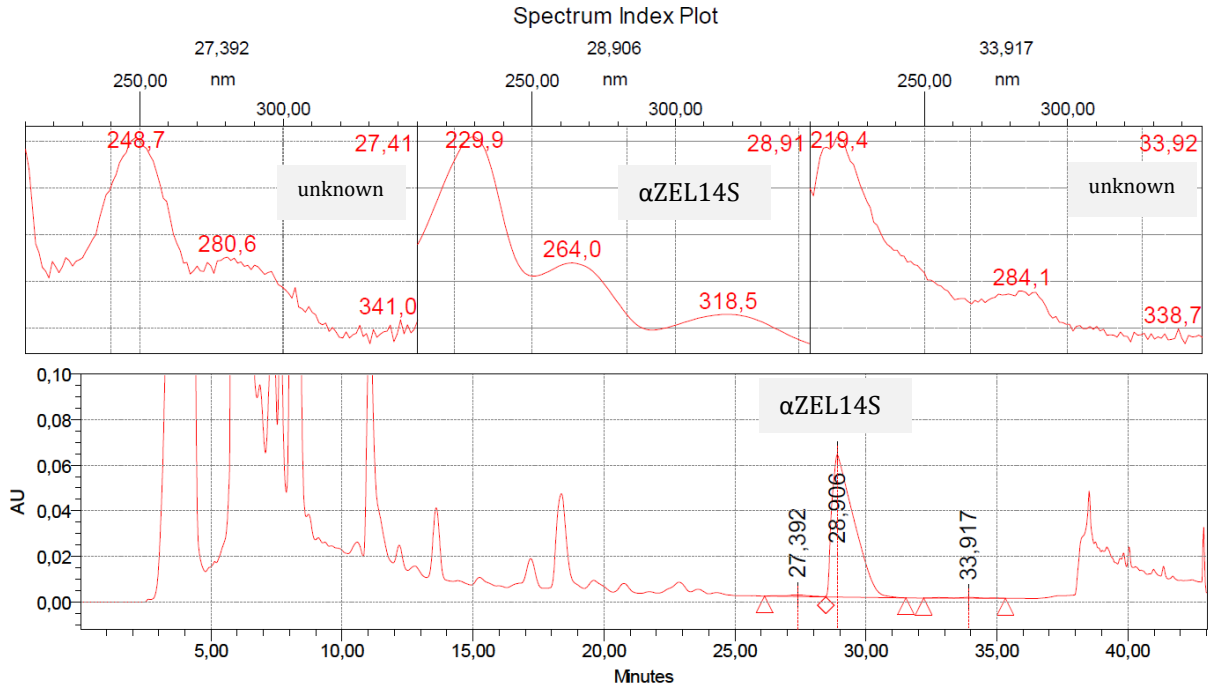


Samples were analysed on a Q-Exactive Plus mass spectrometer (Thermo Scientific, Bremen, Germany) coupled to an Ultimate 3000 LC system (Thermo). An Atlantis T3 column, 3x100 mm, 3 µm particles (Waters, Millford, MA, USA) was used . Chromatography was performed using eluents containing water and methanol/water 95:5, both containing 2 mM ammonium formate and 20 µl formic acid per litre. Flow rate was 0.3 ml/min, injection volume 5 µl and the column temperature was 40°C. Measurements were performed in both positive (data no shown) and negative modes at a resolving power of 70000 (@ m/z 200).

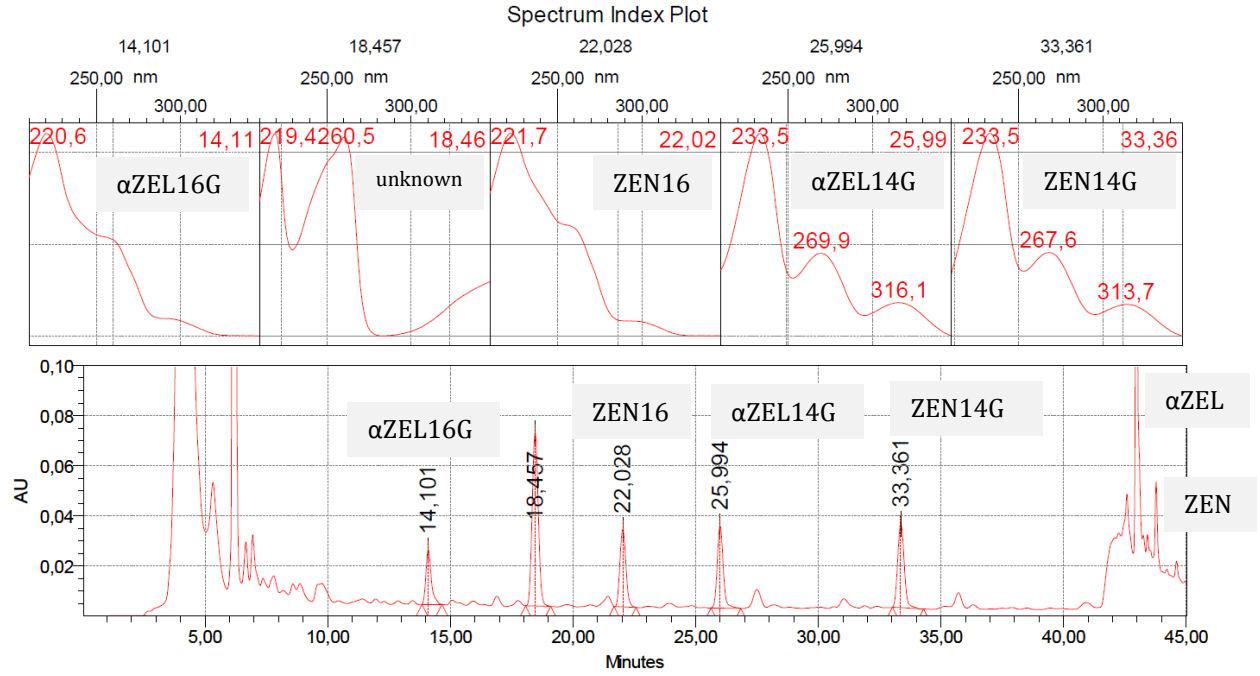
Appendix 3

Some representative HPLC-PAD chromatograms

Representative chromatograms and PAD spectrum of purification of PDB media



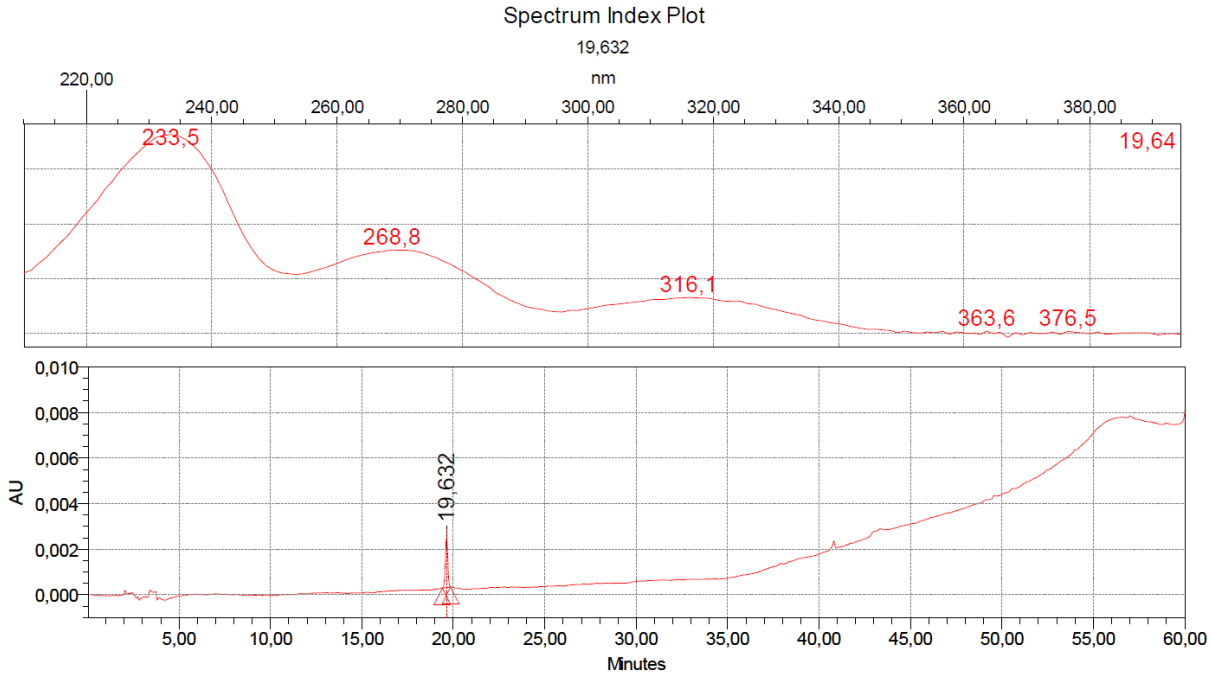
Representative chromatograms and PAD spectrum of purification of Czapek-Dox media



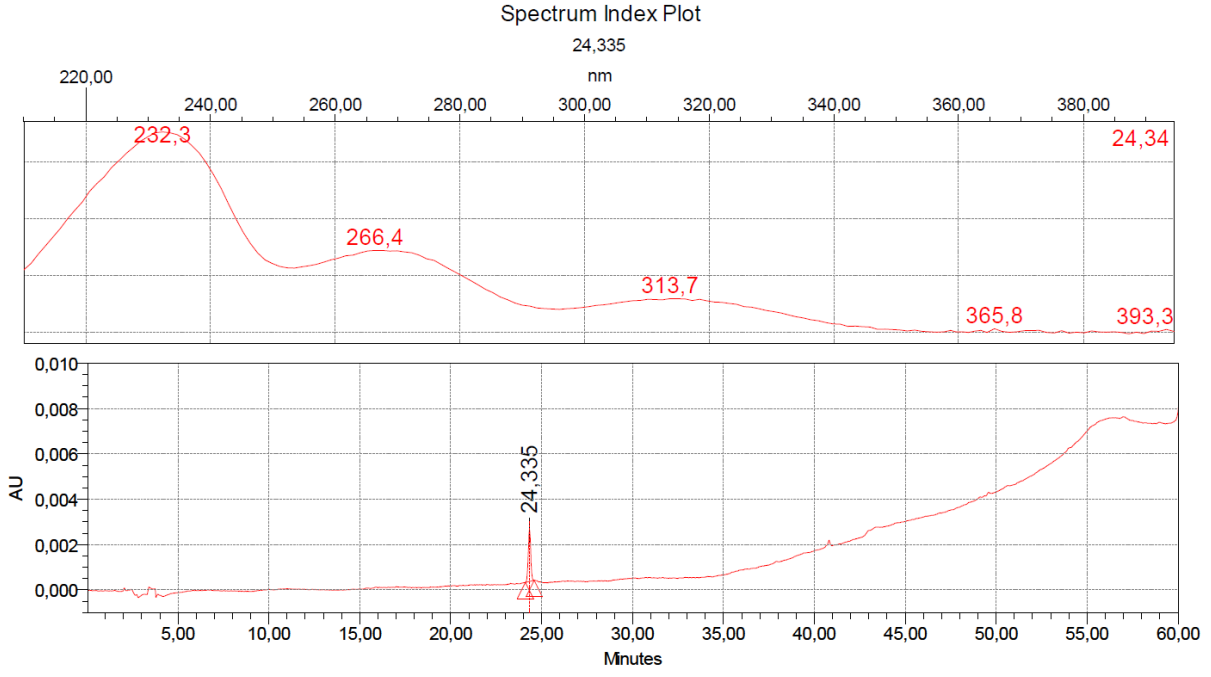
Appendix 3 continued

Some representative HPLC-PAD chromatograms of purified products

Representative chromatogram and PAD spectrum of purified **αZEL14G** from Czapek-Dox media

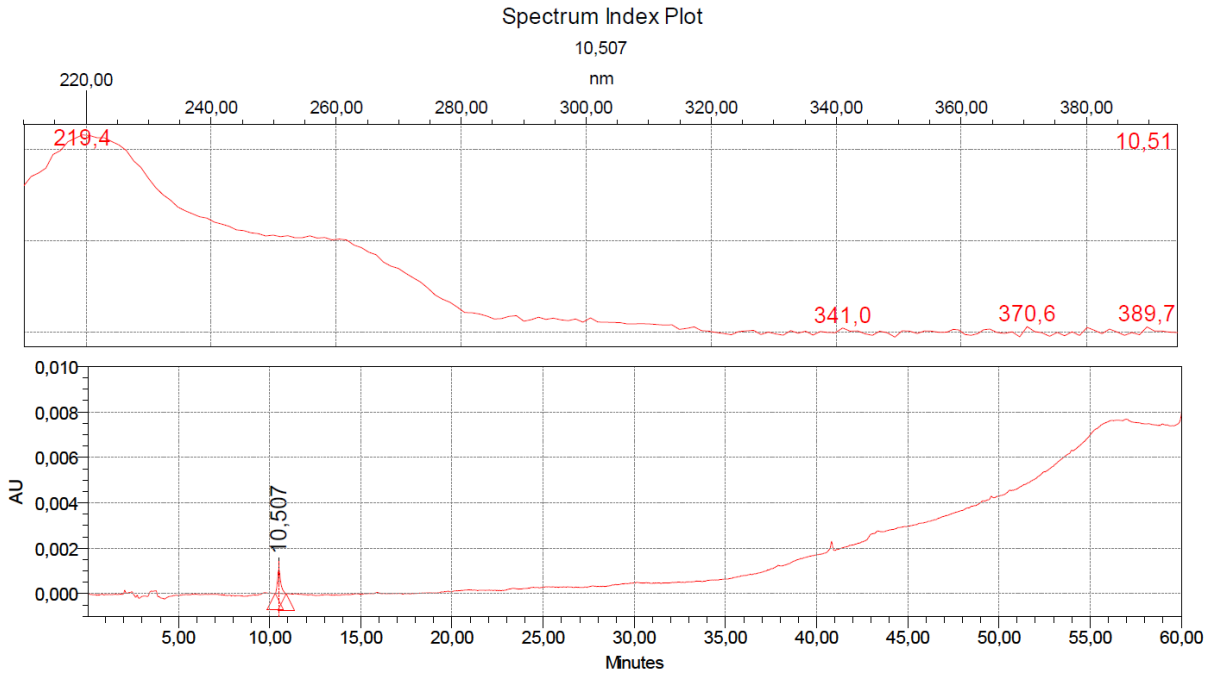


Representative chromatogram and PAD spectrum of purified **ZEN14G** from Czapek-Dox media

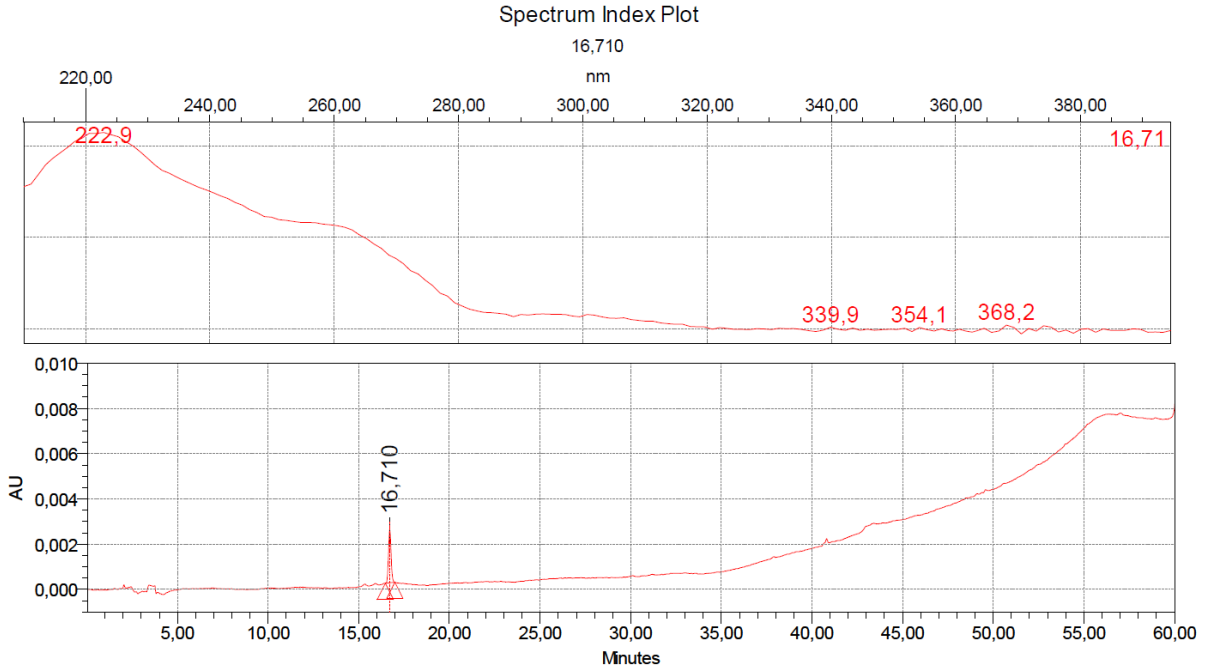


Appendix 3 continued

Representative chromatogram and PAD spectrum of purified **αZEL16G** from Czapek-Dox media

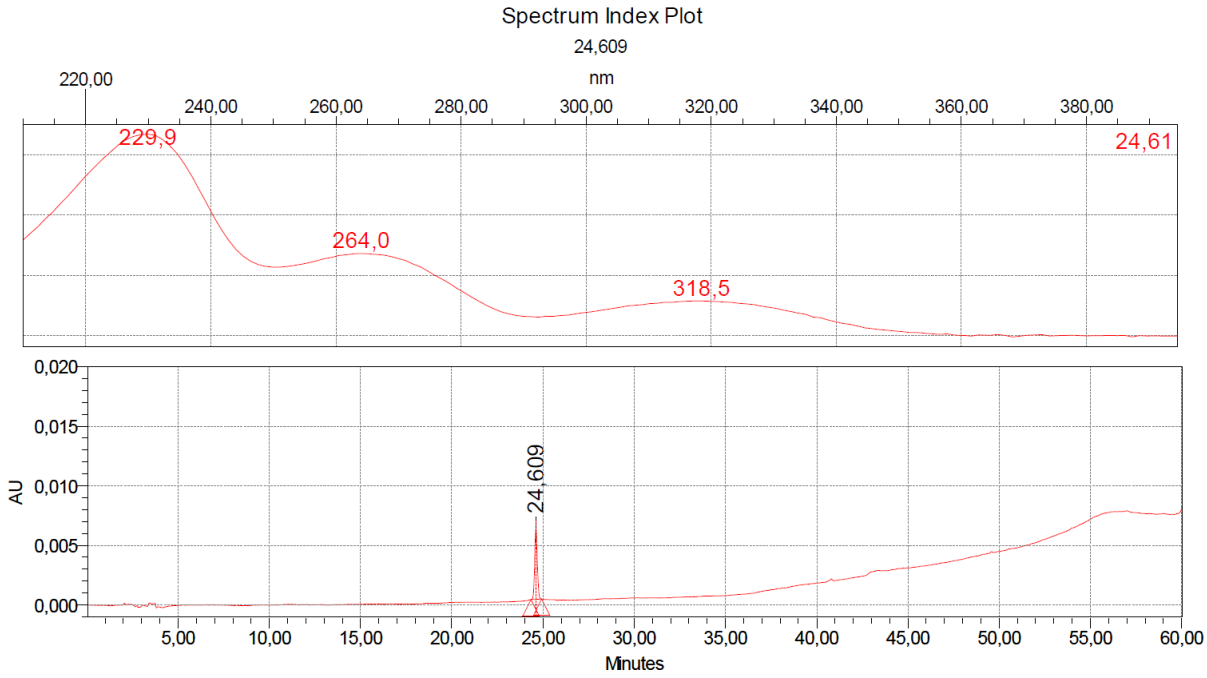


Representative chromatogram and PAD spectrum of purified **ZEN16G** from Czapek-Dox media



Appendix 3 continued

Representative chromatogram and PAD spectrum of purified **αZEL14S** from PDB media



HPLC system used:

Waters 717 Plus autosampler

Waters 996 Photo Array Detector (PAD)

Waters 515 HPLC pumps

Waters temperature control module connected to Waters column heater module

Foxy Jr. fraction collector (Teledyne ISCO)

Waters Empower-2 software for data processing

Appendix 4

Calculations related to estimation of amounts of purified αZEL and ZEN conjugates

Compound name	Peak area from HPLC-PAD at 254 nm	Reference compound used for quantitation	Peak area of reference compound from HPLC-PAD at 254 nm	Concentration of reference compound, µg/mL	Estimated concentration of purified compound in purified solution, µg/mL	Estimated concentration corrected by used coefficient in purified solution, µg/mL	**Total estimated amount, µg**
αZEL14S	**60013**	**ZEN14S**	**65363**	**10**	**9.181**	**4.131**	**1064***
αZEL14G	20375	αZEL14G	118363	1	0.172	-	**9.46**
αZEL16G	**13234**	**ZEN16G**	**206097**	1	**0.064**	**0.032**	**1.77****
ZEN16G	23562	ZEN16G	206097	1	0.114	-	**6.29**
ZEN14G	22149	ZEN14G	53268	1	0.415	-	**22.86**

*Intensity of peak area of 1 µg/mL of ZEN14G injected in HPLC-PAD and measured at 254 nm is 53268 and intensity of 1 µg/mL of αZEL14G is 118363, which is 2.222 times higher. It was assumed that intensity differences in corresponding sulfates could be similar. Therefore, the coefficient 2.222 was used for estimation of concentrations of αZEL14S: estimated concentration of αZEL14S calculated on the basis of peak are of ZEN14S which was divided by 2.222. This assumption could be also proved by obtained concentration. If calculated concentration is not corrected by dividing by 2.222, than estimated amount is 2364 µg, which is impossible because media was spiked with 1000 µg of αZEL and if theoretical conversation is 100%, than end amount would be 1249 µg.

**It was assumed that differences in intensities of the same concentration of αZEL16G and ZEN16G could be similar to ones αZEL14G and ZEN14G, therefore, the same coefficient as above was used.

Theoretical and practical (estimated) yields of synthesised and purified compounds

Compound name	MW, g/M	Spiked amount, µg	Spiked amount, $mM*10^{-3}$	Expected compound	MW, g/M	Theoretical amount of expected compound, µg	Practically obtained (estimated) amount, µg	**Yield, %**
αZEL	320.16	1000	3.1234	αZEL14S	400.12	1249	1064	**85.2**
αZEL	320.16	1000	3.1234	αZEL14G	482.16	1506	9.46	**0.63**
αZEL	320.16	1000	3.1234	αZEL16G	482.16	1506	1.77	**0.12**
αZEL	320.16	1000	3.1234	ZEN14G	480.20	1450	22.86	**1.58**
αZEL	320.16	1000	3.1234	ZEN16G	480.20	1450	6.29	**0.42**