

**Biosynthesis of cannabinoid precursor olivetolic acid in genetically
engineered *Yarrowia lipolytica***

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Supplementary Table 1. Primers used in this study

Primers	Sequence
CsOAC-F	ccgaccagcacttttgcagtactaaccgcaggccgtgaaacaccttattgtcctc
CsOAC-R	ggacaggccatggaactagtcggtaccttatttacgaggggtgtagtcgaaaataag
CsOLS-F	ccgaccagcacttttgcagtactaaccgcagaatcacctccgggcagaagg
CsOLS-R	ggacaggccatggaactagtcggtaccttaataacttaatggggacgcttc
CsOAC-Gly-F	ggaggagggtggtggtggtggtgaggtggcatggccgtgaaacaccttattgtcctc
CsOLS-Gly-R	acggccatgccacctccaccaccaccacctctccataacttaatggggacgcttct
EcFadD-F	ccgaccagcacttttgcagtactaaccgcagaagaaggttggcttaaccgttatcc
EcFadD-R	ggacaggccatggaactagtcggtacctcaggcttattgtccactttgccgcg
EcFadK-F	tccgaccagcacttttgcagtactaaccgcagcatccacaggcccgcattctcg
EcFadK-R	ggacaggccatggaactagtcggtaccttattcaatctcttcacagacatc
ScFAA2-F	tccgaccagcacttttgcagtactaaccgcaggccgctccagattatgcaactaccg
ScFAA2-R	ggacaggccatggaactagtcggtacctaaagcttttctgtcttgactag
ylACC1-F	tccgaccagcacttttgcagtactaaccgcagcagctgcaattgaggacactaacacg
ylACC1-R	ggacaggccatggaactagtcggtacctcacaaccccttgagcagctcagcccc
ylACC1(S667A)-F	agttagacctctgctgacggtggtatcc
ylACC1(S667A)-R	aataccaccgtcagcaagaggtctaactc
ylACC1(S1178A)-F	gtctcgagctgatccgctcctccgacttttc
ylACC1(S1178A)-R	aagtcggagacggcatcagctcgagacac
ylFAA1-F	ccgaccagcacttttgcagtactaaccgcaggtcgatacacaatttctcaaagcc
ylFAA1-R	ggacaggccatggaactagtcggtacctaaagactgctcgtagcactcatcaattc
pTEF-Rvs	ccggatggccagacaagaaca
XPR2-Fw	gtaaatagaaaatctggctttaggtggcaaat
Ku70_DwF	gtccggagcggccgcGCATGCaagtcgacaCTAGGGAGGCACATCTAAACGAATAACG
Ku70_DwR	gttacatcctttatcagacatacctaggAGTGAACGACCAAGACTAAAGGGTG
ku70_UpF	ccctaaatttgatgaaagcctaggCGACTTGATGTTTAGAGGTGCCAGATCC
ku70_UpR	taatgtatgctatacgaagtattTTTCAAAAAGCGGCGGTTCTGTG
ku70_ChkF	GCCAAGTTCTCTTTCCCCTACATG
ku70_ChkR	CTTCAGTAACTGGGCCCACGC
YliC05907-UpF	tggcatccctaaatttgatgaaagcctaggACAGTTCTCTTCTCCTTGTTGAGATATAC
YliC05907-UpR	acttcgtataatgtatgctatacgaagtattGGTGGAGTCATGTGAATTGAGCGCGGTG
YliC05907-DwF	cgtccggagcggccgcGCATGCaagtcgacAGAGTATAGTAATGTATTATTTGCTTAGG
YliC05907-DwR	cctatgtatcctttatcagacatacctaggCCGAACCAAGGAGATGATCAAGTCC
YliC05907-ChkF	ATTGCCGAGATTTCCGCAAAAACCTGAAG
YliC05907-ChkR	AAGAACAAGACCGACAACCTGCCTGTTGG
YliDGA1_DwF	gctagcgagacaataacggaggaGGA AAAACTGCCTGGGTTAGGCAAAT
YliDGA1_DwR	atcagacatagcggccgcTCTCTGATGGCCTGGAGCGAG
YliDGA1_UpF	aatttgatgaaagcggccgcATGCTGCGGGCGGATCCTGG
YliDGA1_UpR	tgtatgctatacgaagtattAGCTTTTGT TTTGTGTGACTTGTCTGT
YliDGA1_ChkF	GTTTATGCATTCTGTTGGACCTTAGTCTG
YliDGA1_ChkR	GTTATCTACCACGATTTTTTGGTTTCTGAGGC

YliDGA2_DwF	gctagcgagacaataacggaggaCATAA CACTCATCAGTAGCCTTTACAGTGAT
YliDGA2_DwR	ccttttatcagacatagcggccgcTTGCTCTTGTAATTCCATAGATAATATATACGAAA
YliDGA2_UpF	aaatttgatgaaaggcggccgcCTTGGGAGTGTATTTGGAAAATGACTTGG
YliDGA2_UpR	tgtatgctatacgaagtatTTTGCGGGCGGTACGGGTACA
YliDGA2_ChkF	CTATCGCCCCAAAGTGTCTTAGCA
YliDGA2_ChkR	GAGATGGCATGCCAACGTTGAC
yIPEX10-F	tccgaccagcacttttgcagtactaaccgcagtggggaagttcacatgcattcgcctgg
yIPEX10-R	ggacaggccatggaactagtcggtaccttactgataggcaacaagttctgctctc
EcLpdA-F	ccgaccagcacttttgcagtactaaccgcagagtactgaaatcaaaactcaggtc
EcLpdA-R	GGACAGGCCATGGA ACTAGTCGGTACCTTACTTCTTCTTCGCTTTCGGGT
EcAceF-F	ccgaccagcacttttgcagtactaaccgcaggctatcgaatcaaagtaccggac
EcAceF-R	GGACAGGCCATGGA ACTAGTCGGTACCTTACATCACCAGACGGCGAATGTC
EcAceE-F	ccgaccagcacttttgcagtactaaccgcagtcagaacgttcccaaatgacgtg
EcAceE-R	ggacaggccatggaactagtcggtaccttacgccagacgcggttaactt
EcLplA-F	ccgaccagcacttttgcagtactaaccgcagtcacattacgcctgctcatctc
EcLplA-R	GGACAGGCCATGGA ACTAGTCGGTACCCTACCTTACAGCCCCCGCCATCCATG
yI MAE1-F	ccgaccagcacttttgcagtactaaccgcagttacgactacgaacctgcgacc
yI MAE1-R	GGACAGGCCATGGA ACTAGTCGGTACCCTAGTCGTAATCCCGCACATGGATG
yI ANT1-F	tccgaccagcacttttgcagtactaaccgcaggcagctatttcaaagactatgttc
yI ANT1-R	ggacaggccatggaactagtcggtaccttacccttgatcaaggtggggccttcatg

Supplementary Table 2. Plasmids used in this study

Plasmids	Characteristics
pYLXP'	pYaliA1 vector backbone with leucine marker and Ampicillin resistance gene
pYLXP'-CsOLS	pYLXP' containing gene CsOLS
pYLXP'-CsOAC	pYLXP' containing gene CsOAC
pYLXP'-CsOLS-CsOAC	pYLXP' containing gene CsOLS and CsOAC
pYLXP'-CsOLS-Gly-CsOAC	pYLXP' containing gene CsOLS and CsOAC with Glycine linker
pYLXP'-CsOAC-Gly-CsOLS	pYLXP' containing gene CsOAC and CsOLS with Glycine linker
pYLXP'-CsAAE1	pYLXP' containing gene CsAAE1
pYLXP'-CsAAE3	pYLXP' containing gene CsAAE3
pYLXP'-EcFadD	pYLXP' containing gene EcFadD
pYLXP'-EcFadK	pYLXP' containing gene EcFadK
pYLXP'-ScFAA2	pYLXP' containing gene ScFAA2
pYLXP'-ylFAA1	pYLXP' containing gene ylFAA1
pYLXP'-PpLvaE	pYLXP' containing gene PpLvaE
pYLXP'-CsOLS-CsOAC-CsAAE1	pYLXP' containing gene CsOLS, CsOAC and CsAAE1
pYLXP'-CsOLS-CsOAC-CsAAE3	pYLXP' containing gene CsOLS, CsOAC and CsAAE3
pYLXP'-CsOLS-CsOAC-EcFadD	pYLXP' containing gene CsOLS, CsOAC and EcFadD
pYLXP'-CsOLS-CsOAC-EcFadK	pYLXP' containing gene CsOLS, CsOAC and EcFadK
pYLXP'-CsOLS-CsOAC-ScFAA2	pYLXP' containing gene CsOLS, CsOAC and ScFAA2
pYLXP'-CsOLS-CsOAC-ylFAA1	pYLXP' containing gene CsOLS, CsOAC and ylFAA1
pYLXP'-CsOLS-CsOAC-PpLvaE	pYLXP' containing gene CsOLS, CsOAC and PpLvaE
pYLXP'-ylACC1	pYLXP' containing gene ylACC1
pYLXP'-ylACC1 ^{S667A}	pYLXP' containing gene ylACC1 ^{S667A}
pYLXP'-ylACC1 ^{S667A,S1178A}	pYLXP' containing gene ylACC1 ^{S667A,S1178A}
pYLXP'-CsOLS-CsOAC-PpLvaE-ylACC1	pYLXP' containing gene CsOLS, CsOAC, PpLvaE and ylACC1
pYLXP'-CsOLS-CsOAC-PpLvaE-ylACC1 ^{S667A,S1178A}	pYLXP' containing gene CsOLS, CsOAC, PpLvaE and ylACC1 ^{S667A,S1178A}
pYLXP'-ylPEX10	pYLXP' containing gene ylPEX10
pYLXP'-SeACS ^{L641P}	pYLXP' containing gene SeACS ^{L641P}
pYLXP'-EcPDH-EcLpIA	pYLXP' containing gene EcPDH and EcLpIA
pYLXP'-ylPEX10-SeACS ^{L641P} -EcPDH-EcLpIA	pYLXP' containing gene ylPEX10, SeACS ^{L641P} , EcPDH and EcLpIA
pYLXP'-ylMAE1-ylANT1-McMAE2	pYLXP' containing gene ylMAE1, ylANT1 and McMAE2
pYLXP'-ylPEX10-SeACS ^{L641P} -EcPDH-EcLpIA-ylMAE1-ylANT1-McMAE2	pYLXP' containing gene ylPEX10, SeACS ^{L641P} , EcPDH, EcLpIA, ylMAE1, ylANT1 and McMAE2
pYLXP'-loxP-ura	pYLXP' containing the loxP-URA-loxP cassette
pYLXP'-loxP-hygr	pYLXP' containing the loxP-hygr-loxP cassette
pYLXP'-Cre	pYLXP' containing gene Cre
pURLA	<i>Ku70</i> site integration plasmid
pURLB	<i>YALIOC05907g</i> site integration plasmid

pYLXP'-loxP-ura-yIDGA2

pYLXP'-loxP-hygr-yIDGA1

pURLA-CsOLS-CsOAC-PpLvaE-yIACC1

pURLB-CsOLS-CsOAC-PpLvaE-yIACC1

pYLXP'-loxP-ura containing gene yIDGA2 deletion cassette

pYLXP'-loxP-hygr containing gene yIDGA1 deletion cassette

pURLA containing gene CsOLS, CsOAC, PpLvaE and yIACC1

pURLB containing gene CsOLS, CsOAC, PpLvaE and yIACC1

NOTE: Cs, *Cannabis sativa*; Ec, *Escherichia coli*; Sc, *Saccharomyces cerevisiae*; yl, *Yarrowia lipolytica*; Pp, *Pseudomonas putida* KT2440; Se, *Salmonella enterica*; Mc, *Mucor circinelloides*.

Supplementary Table 3. Strains used in this study

Strains	Characteristics
po1g	Wild-type strain W29 (ATCC20460) derivative; W29 Δ matA Δ xpr2-332 Δ xpr-2 Δ leu2-270 pBR platform
po1f	po1g derivative; Further deletion of gene <i>ura</i> ; po1g Δ ura3
po1fk	po1f derivative; Further deletion of gene <i>ku70</i> ; po1f Δ ku70::loxP
YL100	po1fk with the empty plasmid pYLXP'
YL101	po1fk with plasmid pYLXP'-CsOLS-CsOAC
YL102	po1fk with plasmid pYLXP'-CsOLS-Gly-CsOAC
YL103	po1fk with plasmid pYLXP'-CsOAC-Gly-CsOLS
YL104	po1fk with plasmid pYLXP'-CsOLS-CsOAC-CsAAE1
YL105	po1fk with plasmid pYLXP'-CsOLS-CsOAC-CsAAE3
YL106	po1fk with plasmid pYLXP'-CsOLS-CsOAC-EcFadD
YL107	po1fk with plasmid pYLXP'-CsOLS-CsOAC-EcFadK
YL108	po1fk with plasmid pYLXP'-CsOLS-CsOAC-ScFAA2
YL109	po1fk with plasmid pYLXP'-CsOLS-CsOAC-yIFAA1
YL110	po1fk with plasmid pYLXP'-CsOLS-CsOAC-PpLvaE
YL111	po1fk derivative; Further deletion of genes <i>yIDGA2</i> ; po1fk Δ yIDGA2::loxP
YL112	YL111 derivative; Further deletion of genes <i>yIDGA1</i> ; po1fk Δ yIDGA2 Δ yIDGA1::loxP
YL113	YL111 with plasmid pYLXP'-CsOLS-CsOAC-PpLvaE
YL114	YL112 with plasmid pYLXP'-CsOLS-CsOAC-PpLvaE
YL115	po1fk with plasmid pYLXP'-CsOLS-CsOAC-PpLvaE-yIACC1
YL116	po1fk with plasmid pYLXP'-CsOLS-CsOAC-PpLvaE-yIACC1 ^{S667A,S1178A} po1fk derivative; Further integration of genes <i>CsOLS-CsOAC-PpLvaE-yIACC1</i> at <i>YALI0C05907g</i> site;
YL117	po1fk <i>CsOLSCsOACPPpLvaEylACC1::loxP</i> po1fk derivative; Further integration of genes <i>CsOLS-CsOAC-PpLvaE-yIACC1</i> at <i>ku70</i> site; po1fk
YL118	<i>CsOLSCsOACPPpLvaEylACC1::loxP</i> po1fk derivative; Further integration of genes <i>CsOLS-CsOAC-PpLvaE-yIACC1</i> at pBR platform; po1fk
YL119	<i>CsOLSCsOACPPpLvaEylACC1::Leu</i>
YL120	YL117 with the empty plasmid pYLXP'
YL121	YL118 with the empty plasmid pYLXP'
YL122	YL118 with plasmid pYLXP'-yIPEX10
YL123	YL118 with plasmid pYLXP'-SeACS ^{L641P}
YL124	YL118 with plasmid pYLXP'-EcPDH-EcLpIA
YL125	YL118 with plasmid pYLXP'-yIPEX10-SeACS ^{L641P} -EcPDH-EcLpIA
YL126	YL118 with plasmid pYLXP'-yIIMAE1-yIANT1-McMAE2
YL127	YL118 with plasmid pYLXP'-yIPEX10-SeACS ^{L641P} -EcPDH-EcLpIA-yIIMAE1-yIANT1-McMAE2 YL118 derivative; Further integration of genes
YL128	yIPEX10-SeACS ^{L641P} -EcPDH-EcLpIA-yIIMAE1-yIANT1-McMAE2 at pBR platform; YL118 yIPEX10SeACS ^{L641P} ECPDHEcLpIAyIIMAE1yIANT1McMAE2::Leu

Supplementary Note 1: Codon-Optimized gene sequences

>CsOLS

atgaatcacctccgggcagaaggccccgcatccgtcctggctatcggactgcaaatcccgaaaacatccttccaagacgagttccccgattat
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>CsOAC

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>CsAAE1

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>CsAAE3

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>PpLvaE

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Olivetolic Acid by LC-MS/MS



For: Jingbo Ma (CBEE)
January 7, 2020

Report prepared by: M. LaCourse

Report reviewed by: Joshua Wilhide

Date: January 7, 2020

Date:

Objective & Solution Preparation

- The goal of this analysis is to utilize LC-MS/MS to determine the olivetolic acid concentration in 3 samples
- Sample and standard preparation:
 - Samples were analyzed as provided by transferring to autosampler vials
 - Sample 3 was non-homogenous and thus vortexed thoroughly before analysis but still separated quickly
 - A standard curve was created in methanol from the provided 10 ppm stock following the scheme below:

C1 (ppb)	V1 (uL)	C2 (ppb)	V2 (uL)	Diluent (uL)
10,000	150	1000	1500	1350
10,000	50	500	1000	950
1,000	250	250	1000	750
1,000	100	100	1000	900
1,000	50	50	1000	950
1,000	10	10	1000	990

Instrumentation & Method

Instrumentation:

- Qsight LX50:
 - PerkinElmer UHPLC Precision Sampling Module
 - Perkin Elmer UHPLC Solvent Delivery Module
 - PerkinElmer UHPLC Column Temperature Module
- Perkin Elmer QSight 210 Mass Spectrometer
- Column: Agilent Zorbax Eclipse XDB-C18 2.1 x 50 mm, 1.8 μ m (P/N 981757-902)

HPLC Method: Olivetolic Acid V1

- Injection Volume: 10.0 μ L
- Column Temperature: 40°C
- Run time: 5.00 min
- Flow: 0.3 mL/min
- Isocratic: (40:60 water:methanol)

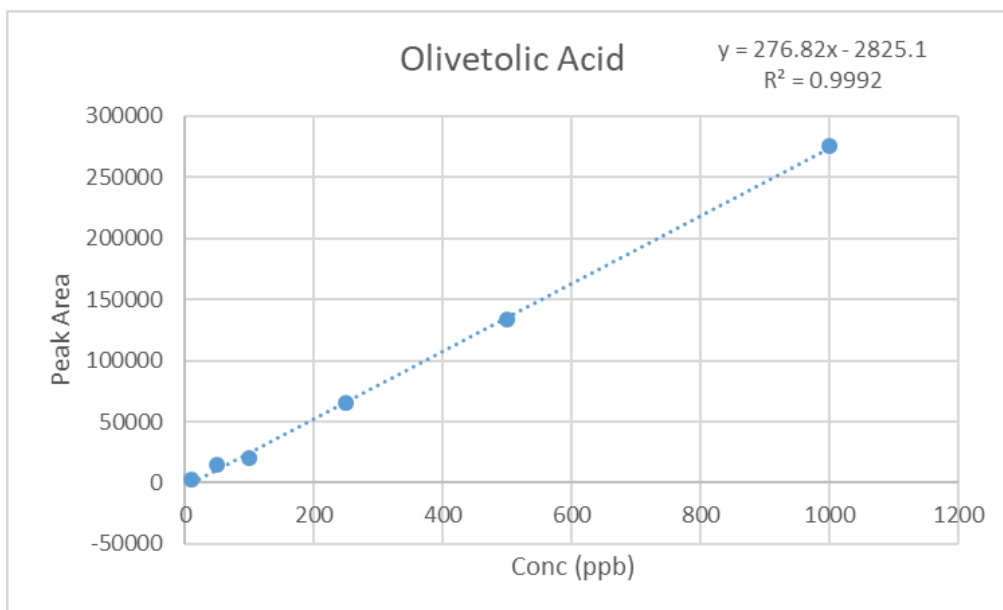
MS Method: Olivetolic Acid V1

- Drying Gas: 80.0
- HSID Temperature (°C): 320.0
- Nebulizer Gas 1: 100.0
- ElectroSpray V1 Pos: -3000.0
- Source 1 Temperature (°C): 200.0

Olivetolic Acid (negative mode)

- Q1 Mass: 223.5
- Q2 Mass: 180
- Dwell Time: 795 ms
- Resolution (Q1:Q2): Unit_Unit
- CE: 22, EV:-30, CCL2: 20

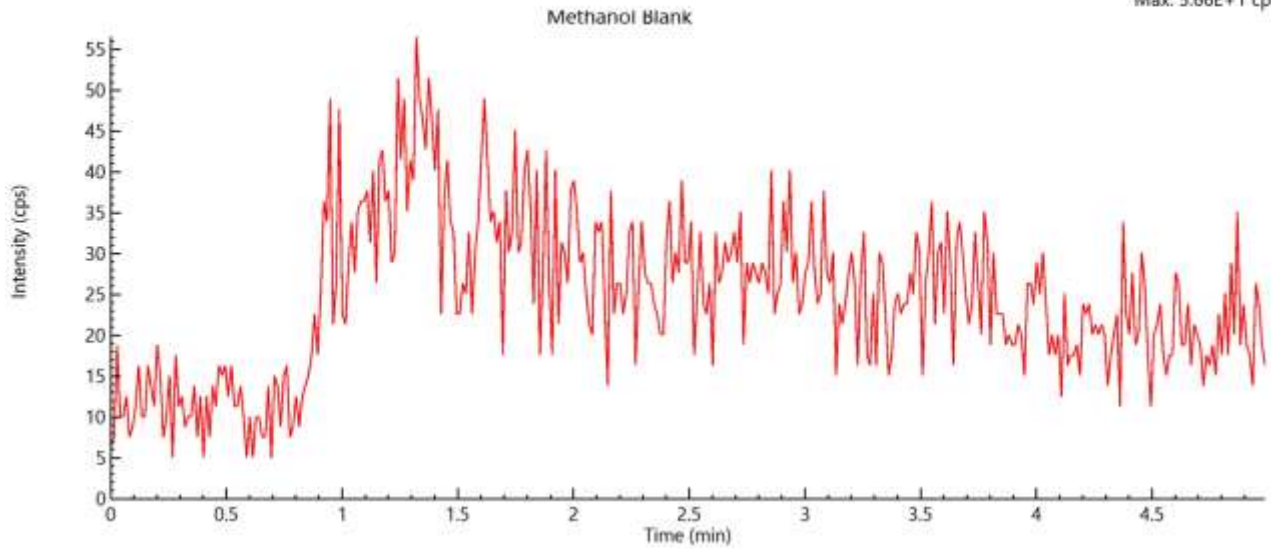
Calibration Curve



Methanol Blank

2020-01-07: Methanol Blank_112841
EIC -MRM 223.50/180.00 (1 pair) EV: -30 V CC: 22 V Exp "Experiment 1" OlivetolicAcid

Number of Scans: 375
Max: 5.66E+1 cps

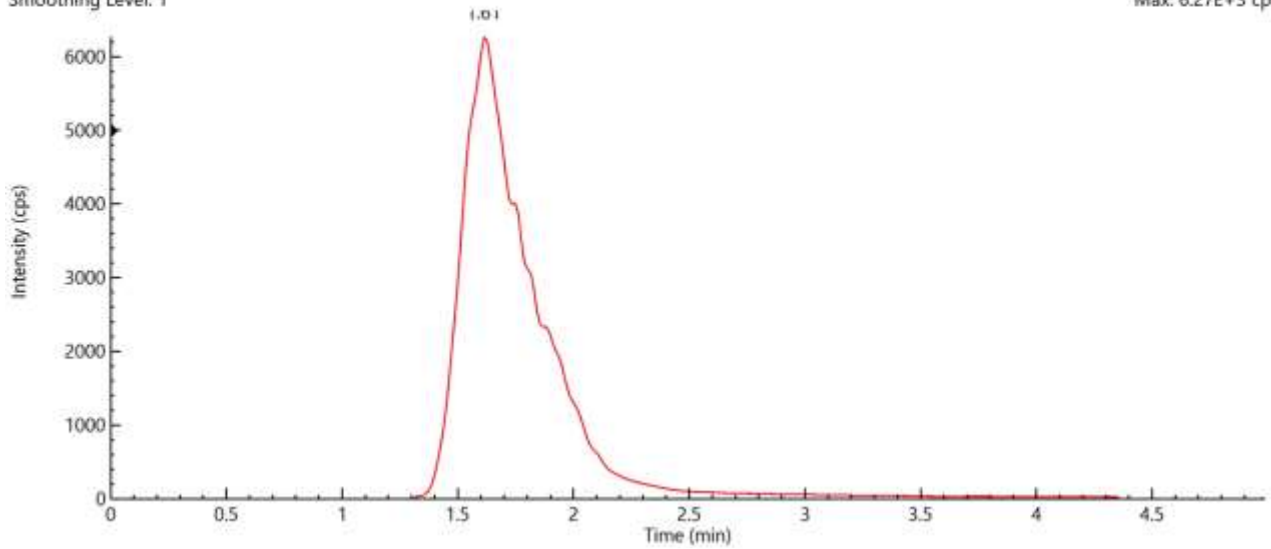


500 ppb Olivetolic Acid Standard

2020-01-07: 500 ppb Olivetolic Acid Standard_111728
EIC -MRM 223.50/180.00 (1 pair) EV: -30 V CC: 22 V Exp "Experiment 1" OlivetolicAcid

Number of Scans: 375
Max: 6.27E+3 cps

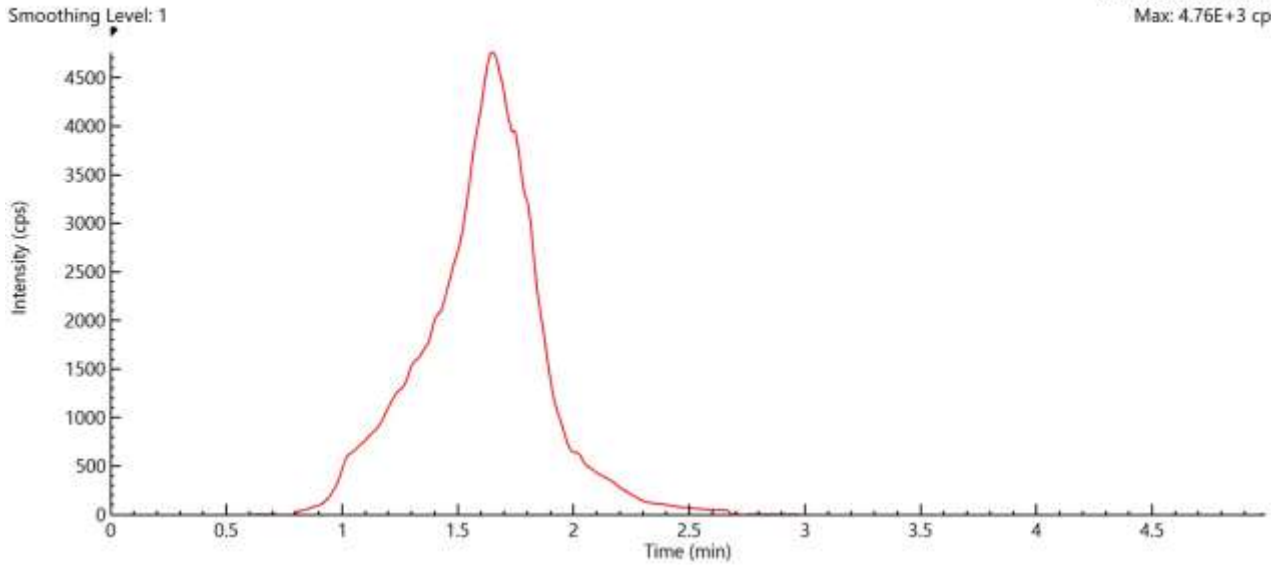
Smoothing Level: 1



Sample 1

2020-01-07: Sample 1_113417
EIC -MRM 223.50/180.00 (1 pair) EV: -30 V CC: 22 V Exp "Experiment 1" OlivetolicAcid

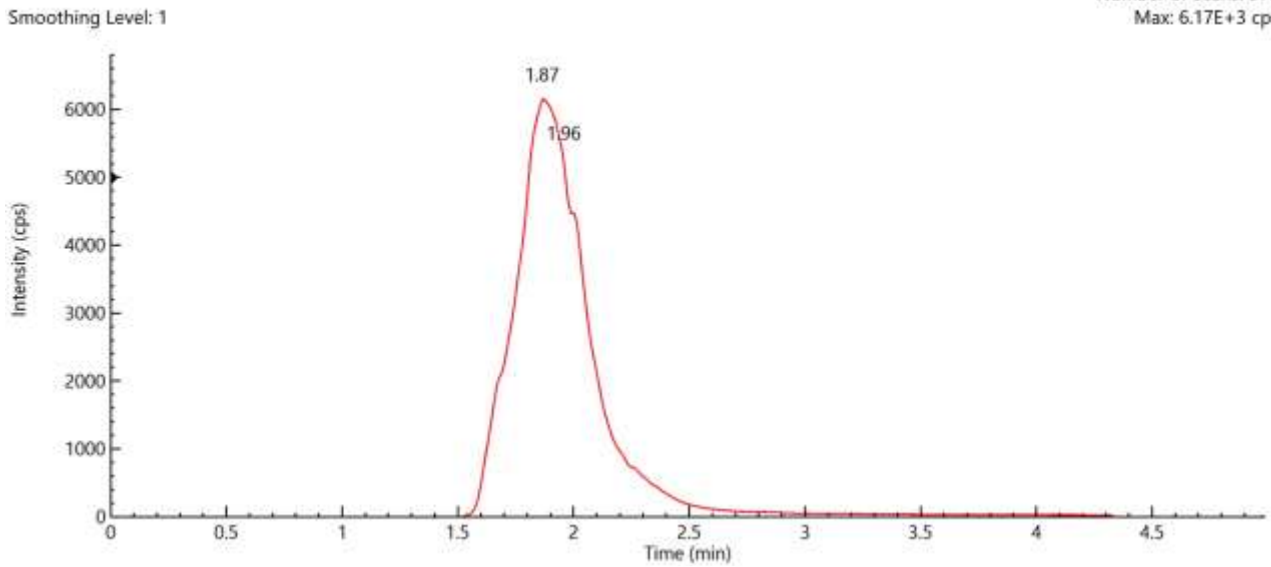
Number of Scans: 375
Max: 4.76E+3 cps



Sample 2

2020-01-07: Sample 2_115104
EIC -MRM 223.50/180.00 (1 pair) EV: -30 V CC: 22 V Exp "Experiment 1" OlivetolicAcid

Number of Scans: 375
Max: 6.17E+3 cps

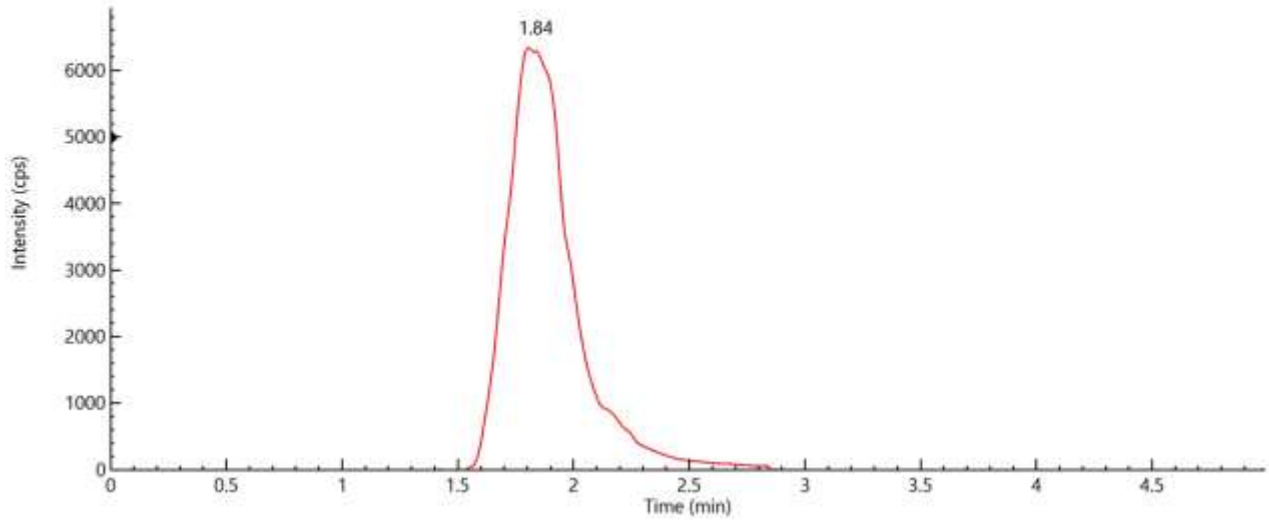


Sample 3

2020-01-07: Sample 3_120752
EIC -MRM 223.50/180.00 (1 pair) EV: -30 V CC: 22 V Exp "Experiment 1" OlivetolicAcid

Number of Scans: 375
Max: 6.34E+3 cps

Smoothing Level: 1



Results

- See corresponding spreadsheet for all raw values and calculations
- Determined concentrations:
 - Sample 1: 649 ppb
 - Sample 2: 533 ppb
 - Sample 3: 467 ppb

Olivetolic Acid by MS/MS



For: Jingbo Ma (CBEE)
January 14, 2020

Report prepared by: M. LaCourse

Report reviewed by: Joshua Wilhide

Date: January 14, 2020

Date:

Instrumentation & Method

Instrumentation:

- Perkin Elmer QSight 210 Mass Spectrometer

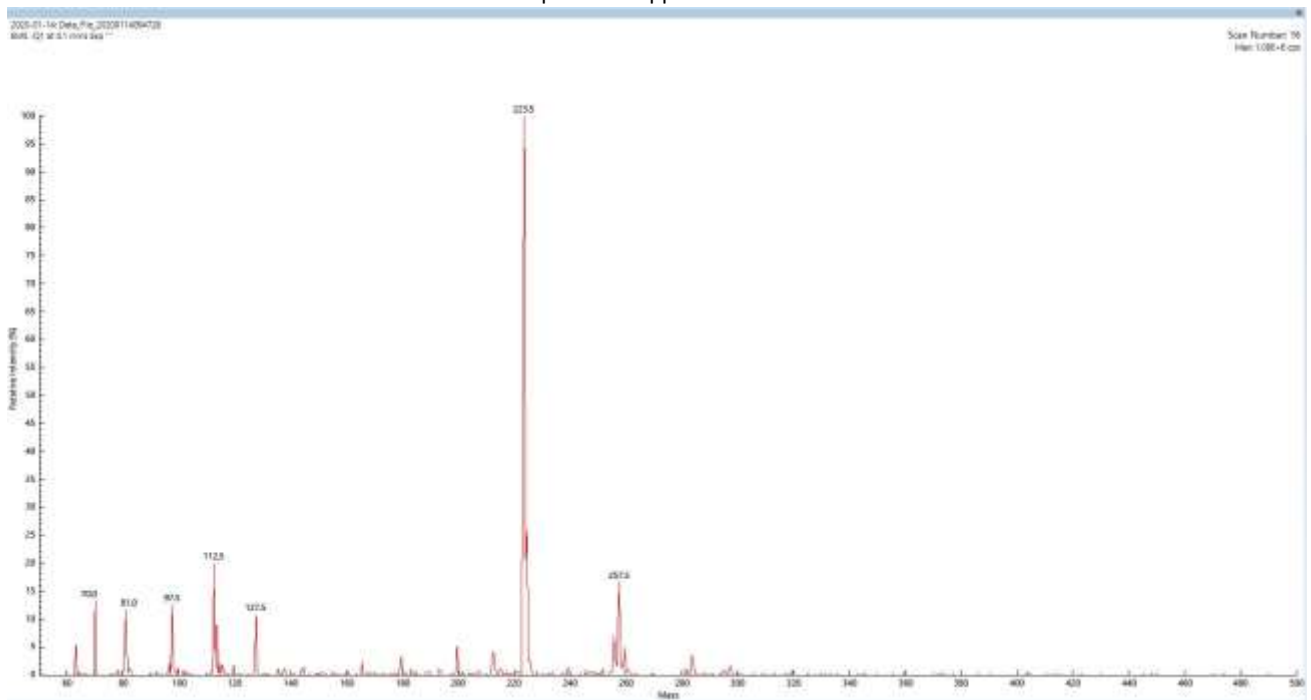
MS Method:

- Drying Gas: 80.0
- HSID Temperature (°C): 320.0
- Nebulizer Gas 1: 100.0
- ElectroSpray V1 Pos: -3000.0
- Source 1 Temperature (°C): 0.0

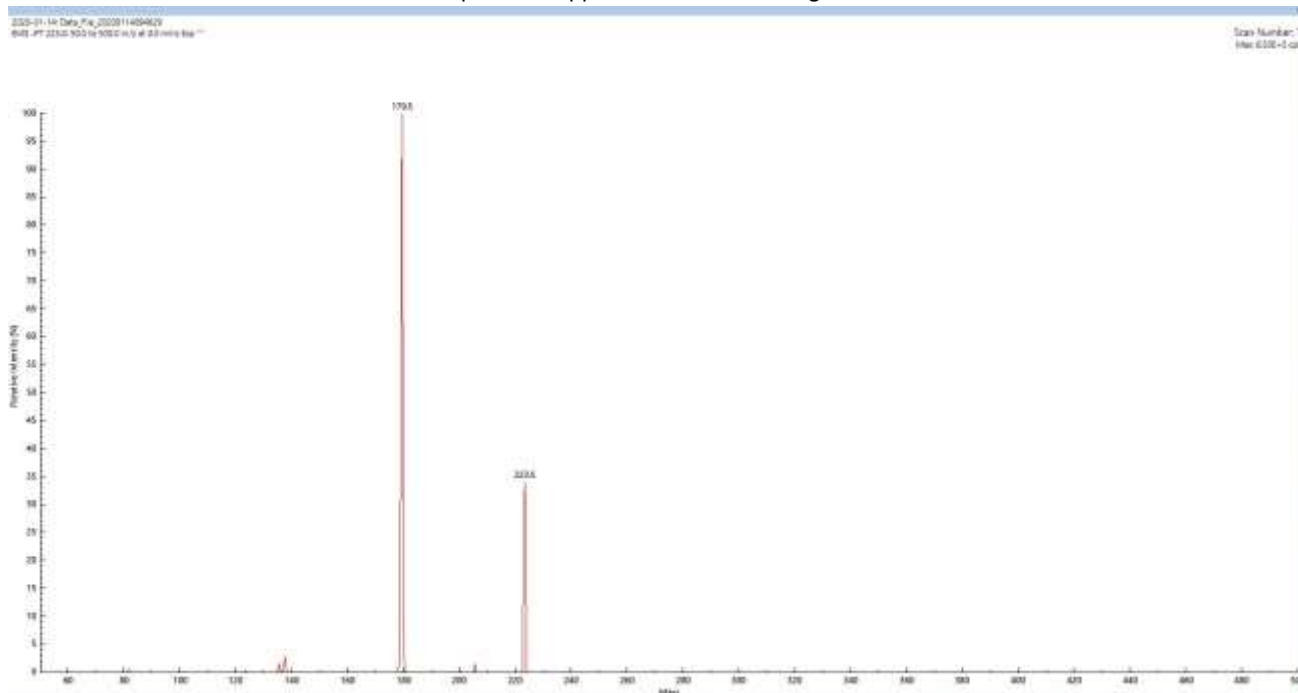
Olivetolic Acid (negative mode)

- CE: 22
- Fragmentation:
 - EV:- 30
 - CCL2: 20

Mass Spectrum 1 ppm Olivetolic Acid



Mass Spectrum 1 ppm Olivetolic Acid - Fragmentation



Olivetolic Acid by High Resolution Mass Spectrometry (HRMS)



For: Jingbo Ma (CBEE)
January 15, 2020

Report prepared by: M. LaCourse

Report reviewed by: Joshua Wilhide

Date: January 15, 2020

Date:

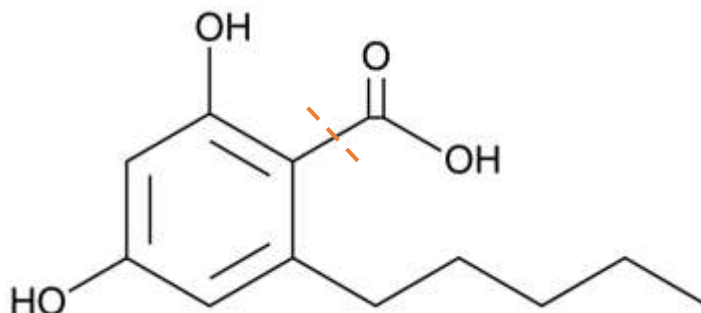
Instrumentation & Method

Instrumentation:

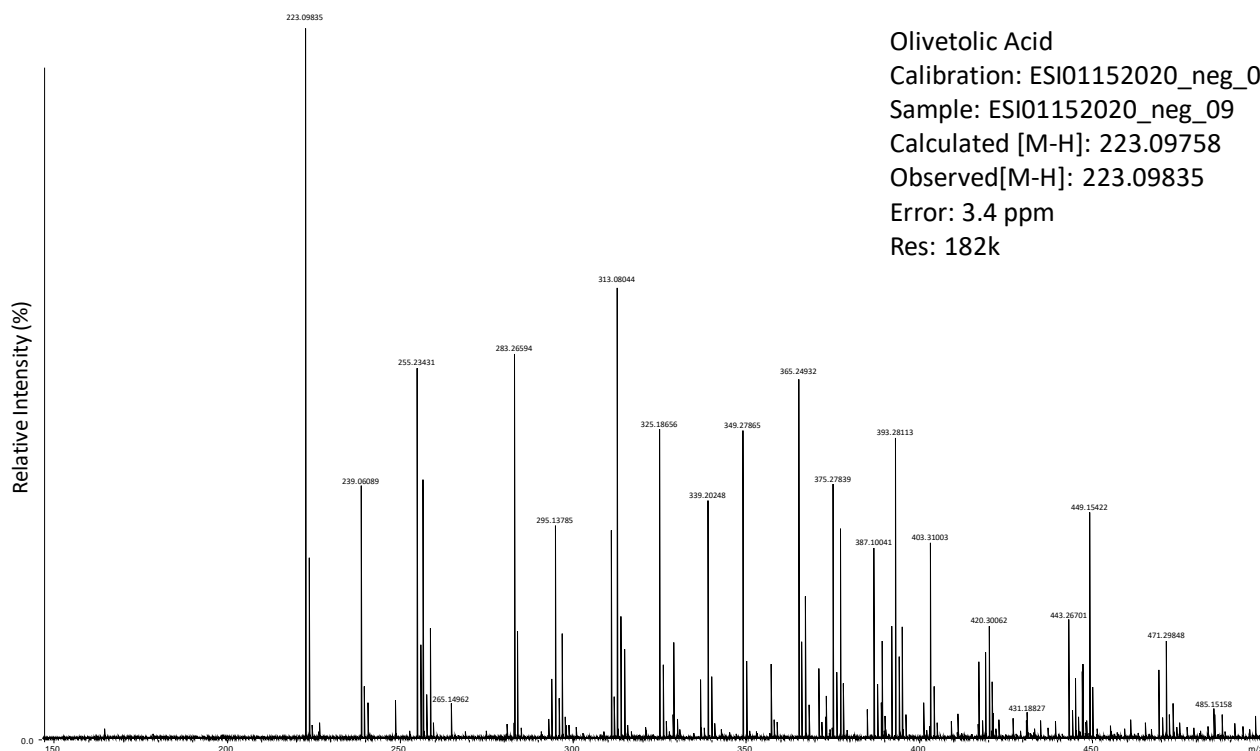
- Bruker 12T solariX FT-ICR-MS

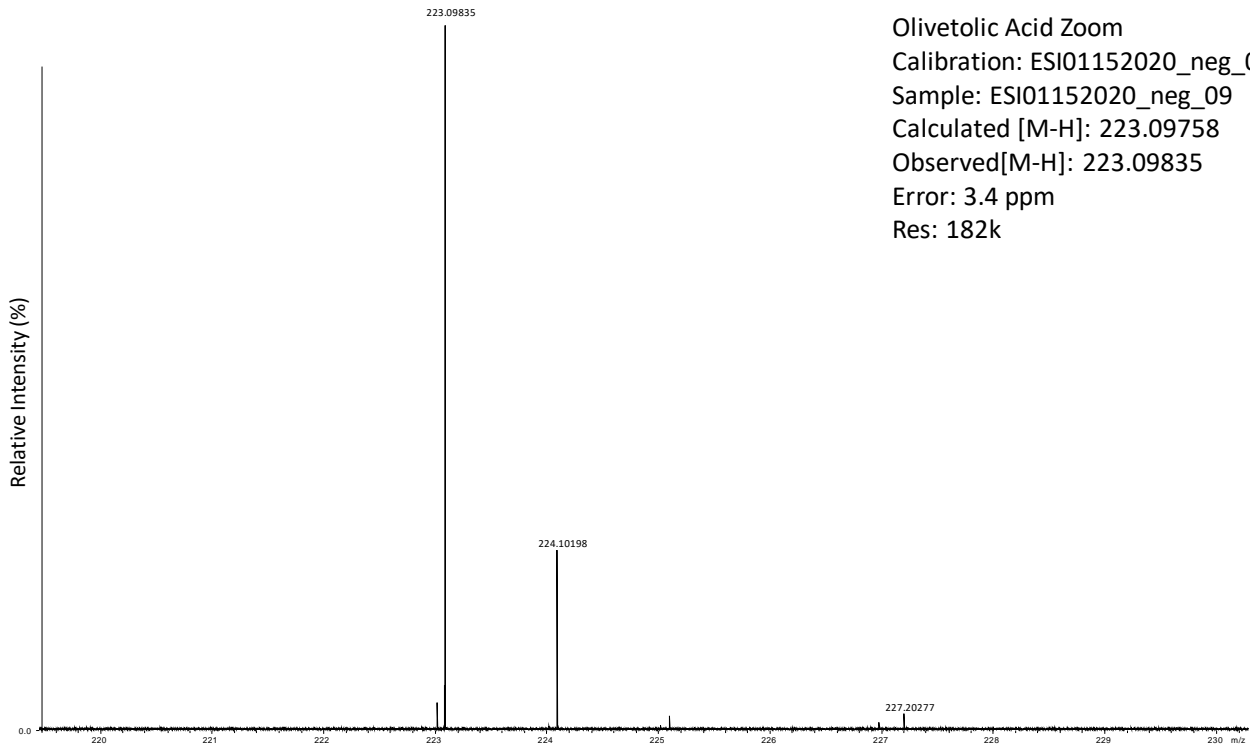
MS Method:

- Negative mode ESI ionization
- Fragmentation with collision energy 12V

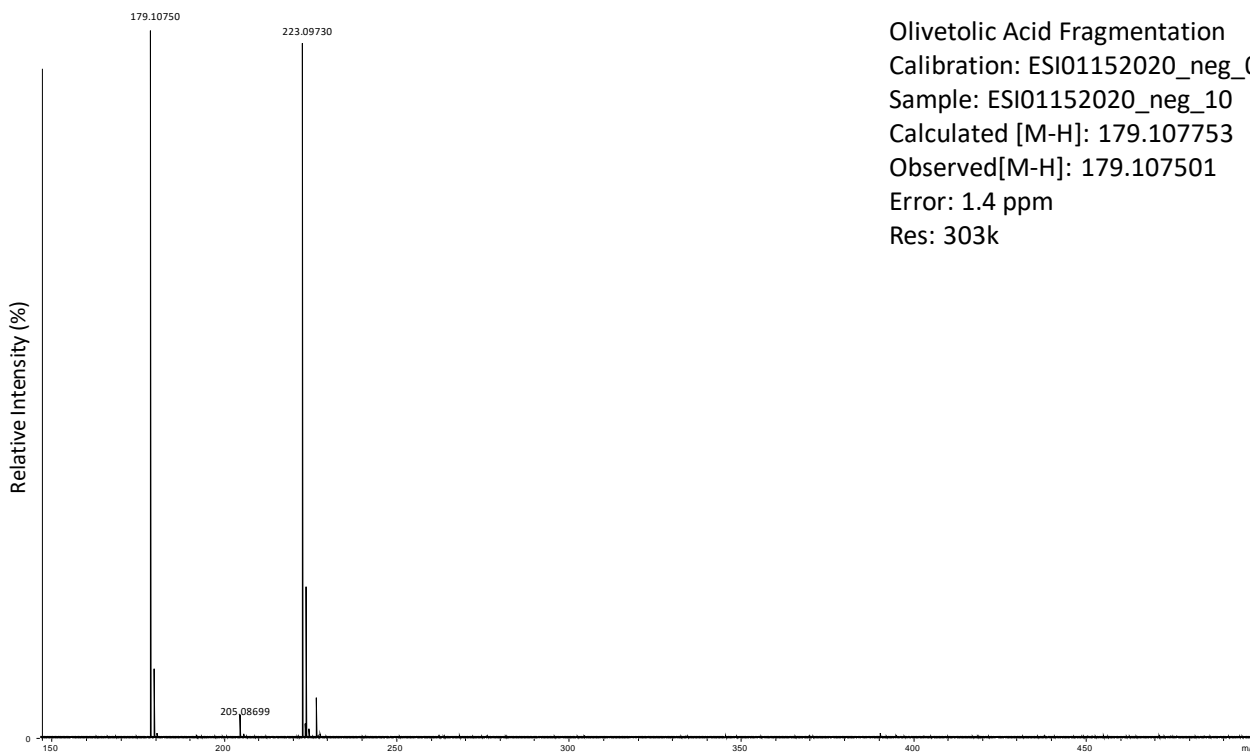


Fragmentation





Olivetolic Acid Zoom
Calibration: ESI01152020_neg_08
Sample: ESI01152020_neg_09
Calculated [M-H]: 223.09758
Observed[M-H]: 223.09835
Error: 3.4 ppm
Res: 182k



Olivetolic Acid Fragmentation
Calibration: ESI01152020_neg_08
Sample: ESI01152020_neg_10
Calculated [M-H]: 179.107753
Observed[M-H]: 179.107501
Error: 1.4 ppm
Res: 303k

