Project co-financed by the European Regional Development Fund

ARISTOIL

"Reinforcement of Mediterranean olive oil sector competitiveness through development and application of innovative production and quality control methodologies related to olive oil health protecting properties"

PRIORITY AXIS 1: Promoting Mediterranean innovation capacities to develop smart and sustainable growth

OBJECTIVE: 1.1 To increase transnational activity of innovative clusters and networks of key sectors of the MED area

Project website: http://aristoil.interreg-med.eu/

DELIVERABLE Number: 3.3.2
Title of DELIVERABLE: Methodology on Establishing a certification centre

ACTIVITY n. 3.3: Operational Instrument

WP n. 3: STUDYING

PARTNER IN CHARGE: National and Kapodistrian University of Athens, Department of Pharmacognosy and Natural products Chemistry (UoA)

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Confidential ×	*Public* □	

Date: 31 December 2017

Contents

Methodology for establishing a Certification Center

A. NMR technique

According to the method that was optimized as described in Annex 1 the following equipment is required in order to establish a Certification Center using the NMR technique

1. Sample preparation

- Analytical balance KERN ABJ120-4NM with accuracy of 0.0001 g.
- Centrifuge Thermo Electron Corporation-Multifuge 3S/D-37520 Osterode.
- Automatic pipette NICHIRYO NICHIPET EX
- 100 ml round bottom flask
- rotary evaporator (R-114 Buchi).
- Glass dessicator
- Vacuum pump
- $CDCl_3$ (CAS No: 865-49-6) (750 μL)
- 5 mm NMR tubes
- Falcons of 50 ml resistant to cyclohexane Labcon (CT1155).

2. NMR Spectral Analysis

^1H NMR 400 MHz or 600 MHz (Bruker DRX400).

Windows PC with Mesternova or Topspin software for spectra integration

B. LC-MS/MS technique

According to the method that was optimized as described in Annex 2 the following equipment is required in order to establish a Certification Center using the LC-MS/MS technique

1. Sample preparation

-MS2 minishaker from Ika Works (Wilmington, NC, USA) for extraction

-Analysis with: 1200 Series LC system (Agilent Technologies, Waldbronn, Germany) coupled to an Agilent 6460 triple quadrupole (LC–QqQ-MS) detector furnished with an electrospray ionization (ESI) source.

-Column C18 Pursuit XRs Ultra (50×2.0 mm i.d., 2.8 µm particle size) from Varian (Walnut Creek, CA, USA).

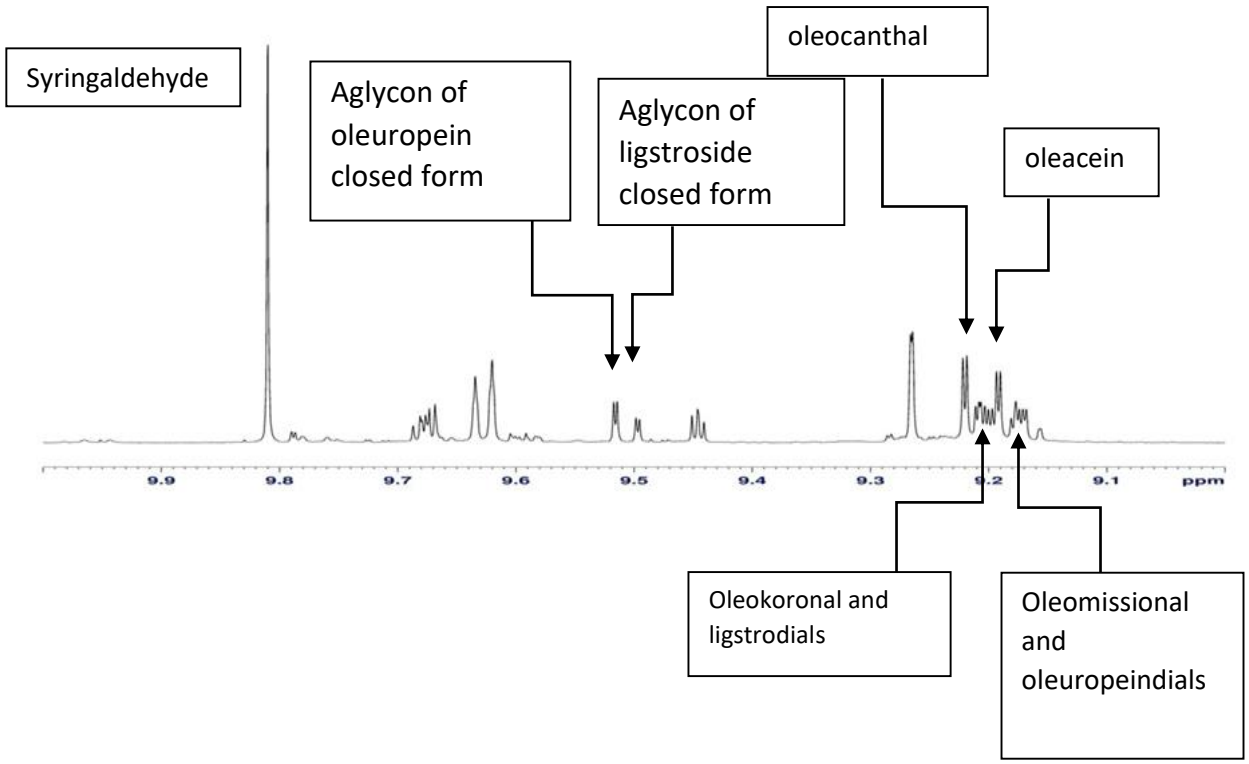
-Millipore Milli-Q water purification system (Bedford, MA, USA) for production of deionized water (18 MΩ•cm).

ANNEX 1

NMR Technique

Quantitative measurement of conjugated phenols (oleocanthal, oleacein, aglycon of oleuropein, aglycon of ligstroside) using H1-NMR

NMR is a technic which succeeds the measurement of concentration in one or more substances of a mixture with high accuracy. The basic principle in which NMR is based is that all the resonant frequencies, produced by a certain nucleus, have amplitude proportional to the concentration of substance and the number of nuclei which create this frequency.



The solvent which we used was CDCl3. Syringaldehyde is used as a standard solution and we integrate all the above peaks compare the integration with that of syringaldehyde.

1. Calibration curve of oleocanthal and oleacein using NMR - method

1st trial

- **Insertion of oleocanthal in olive oil**

60mg oleocanthal was added in olive oil which has zero concentration in phenolic compounds and the mixture was put in ultrasound chamber .

- **Implementation of NMR method for the quantitative measurement of phenolic compounds**

The method was the following:

- ✓ 5g of olive oil was weighed
- ✓ 20ml cyclohexane was added
- ✓ Stir (1min)
- ✓ 25ml acetonitrile was added
- ✓ Stir (1min)
- ✓ Centrifugation for 5min at 4000rpm
- ✓ The phase of acetonitrile was separated
- ✓ 1ml of syringaldehyde was added
- ✓ The phase of acetonitrile was evaporated
- ✓ Acquisition of H^1- NMR spectrum

c(mg/kg)	καθαρότητα(c*0,79)	ολοκλωση_9.62	ολοκλήρωση_9.22	ολοκλ.9.62*1,2
0	0	0	0	0
10	7,9	0,03	0,04	0,036
25	19,75	0,1	0,1	0,12
50	39,5	0,17	0,14	0,204
100	79	0,31	0,34	0,372
250	197,5	0,6	0,65	0,72
350	276,5	1,03	1,13	1,236
500	395	1,48	1,56	1,776
750	592,5	2,48	2,76	2,976
1000	790	3,27	3,54	3,924
1250	987,5	4,08	4,52	4,896
1500	1185	5,38	5,67	6,456

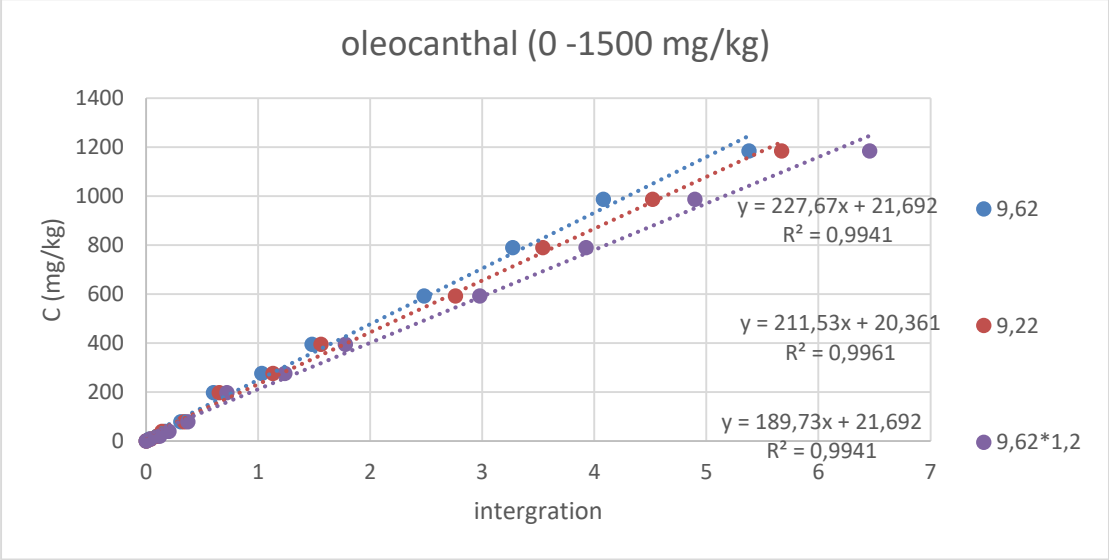


Diagram 19 : Calibration curve of oleocanthal using NMR - method

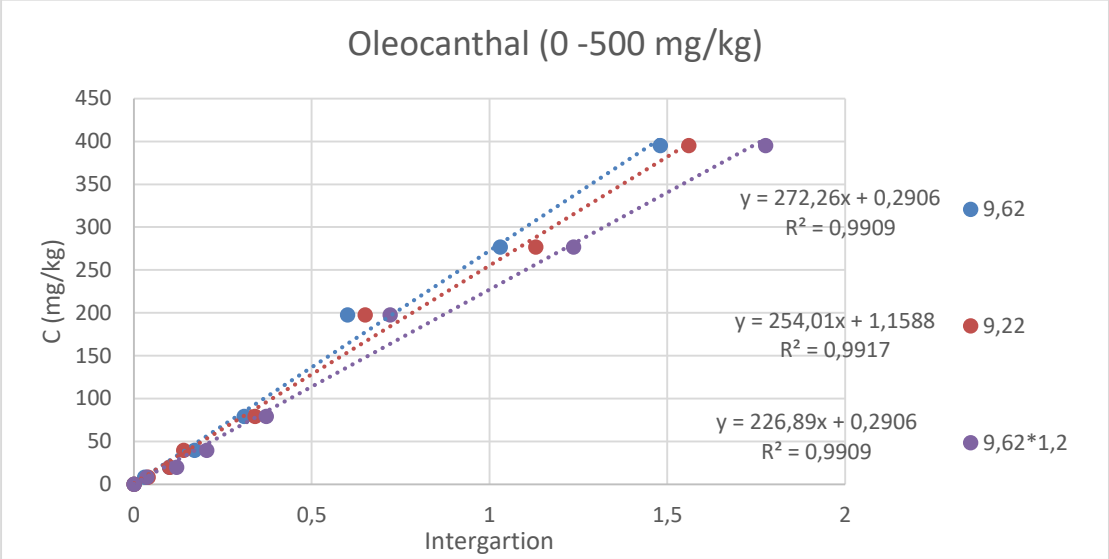


Diagram 20 : Calibration curve of oleocanthal using NMR - method

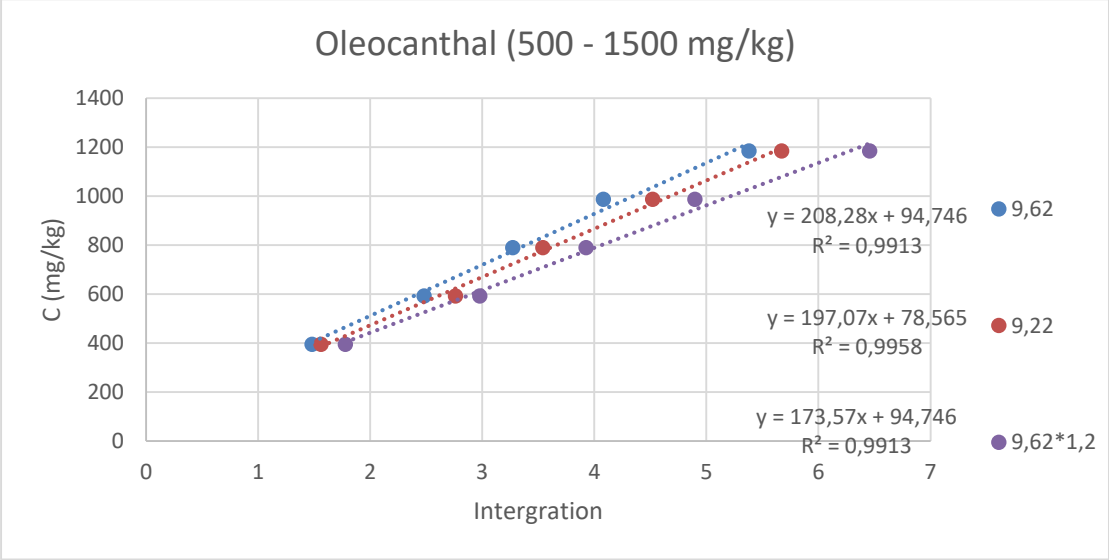


Diagram 21 : Calibration curve of oleocanthal using NMR - method

OLEACEIN

- **Insertion of oleacein in olive oil**

60mg oleacein was added in olive oil which has zero concentration in phenolic compounds and the mixture was put in ultrasound chamber .

- **Implementation of NMR method for the quantitative measurement of phenolic compounds**

The method was the following :

- ✓ 5g of olive oil was weighed
- ✓ 20ml cyclohexane was added
- ✓ Stir (1min)
- ✓ 25ml acetonitrile was added
- ✓ Stir (1min)
- ✓ Centrifugation for 5min in 4000rpm
- ✓ The phase of acetonitrile was separated
- ✓ 1ml of syringaldehyde was added
- ✓ The phase of acetonitrile was evaporated
- ✓ Acquisition of H^1- NMR spectrum

c	καθαρότητα(c*0,83)	ολοκλωση_9.63	λοκλήρωση_9.1	ολοκλ.9.63*1,2
0	0	0	0	0
25	19,75	0,05	0,04	0,06
50	39,5	0,1	0,11	0,12
100	79	0,2	0,28	0,24
250	197,5	0,46	0,67	0,552
350	276,5	0,65	0,92	0,78
500	395	0,92	1,39	1,104
750	592,5	1,53	2,34	1,836
1000	790	2,59	3,54	3,108
1250	987,5	3,29	4,34	3,948
1500	1185	4,06	5,43	4,872

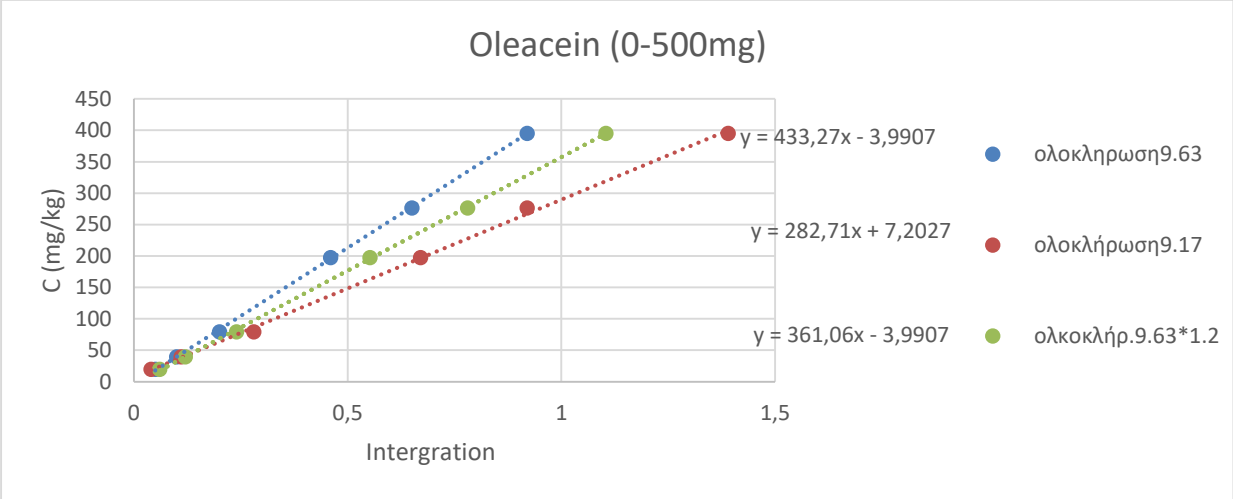


Diagram 22 : Calibration curve of oleacein using NMR - method

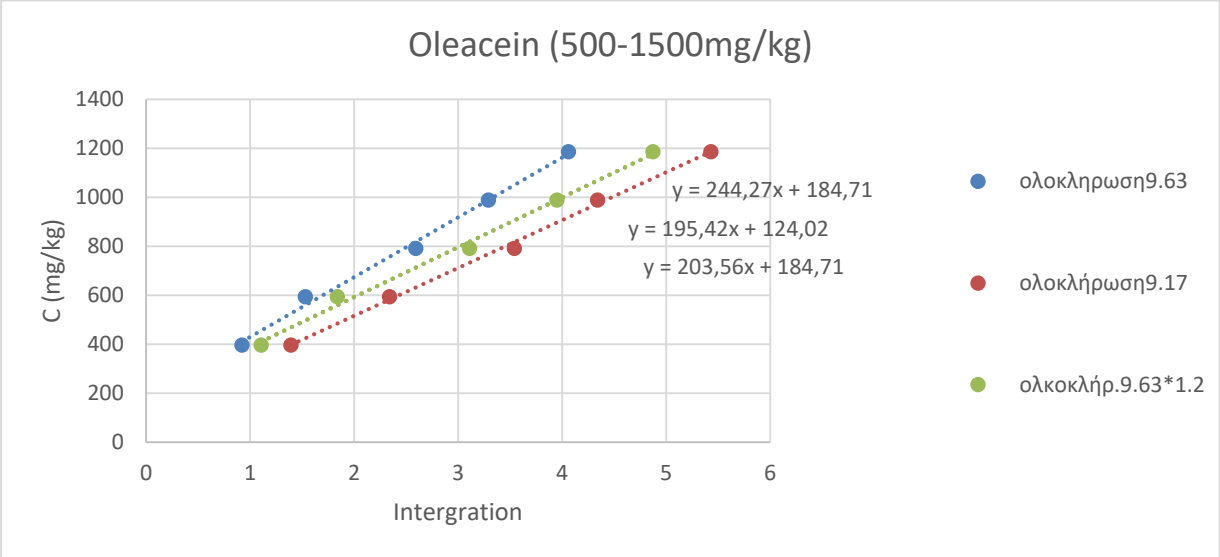


Diagram 23 : Calibration curve of oleacein using NMR - method

2nd Trial

- **Insertion of oleocanthal in olive oil**

60 mg oleocanthal was dissolved in acetonitrile and added in olive oil which contains zero concentration in phenolic compounds and we made olive oil with concentration 1500mg/kg in oleocanthal. Then we mixed this olive oil with zero and we make the following concentrations. (25 , 100, 250, 500 , 750 ,1000 , 1250 and 1500)

We applied the NMR method of quantitative measurement of phenolic compounds.

c(mg/kg)	καθαρότητα(c*0,79)	ολοκλωση_9.62	λοκλήρωση_9.2	ολοκλ.9.62*1,2
0	0	0	0	0
25	19,75	0,05	0,06	0,06
100	79	0,2	0,3	0,24
250	197,5	0,44	0,61	0,52
500	395	1,05	1,41	1,26
750	592,5	1,84	2,25	2,2
1000	790	2,3	2,95	2,76
1250	987,5	2,85	3,48	3,42
1500	1185	3,9	5,17	4,68

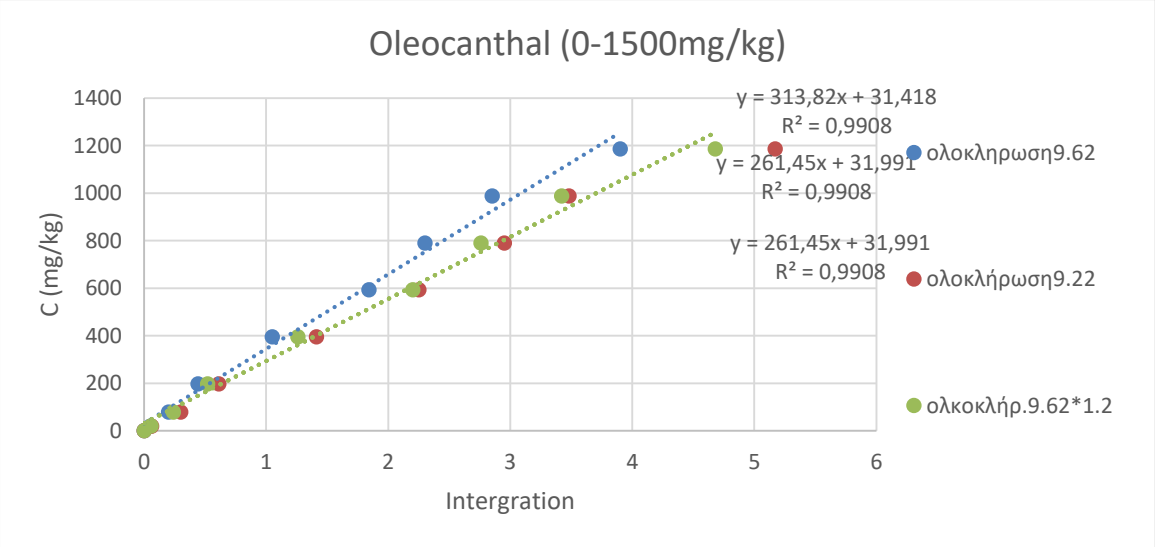


Diagram 24: Calibration curve of oleocanthal using NMR - method

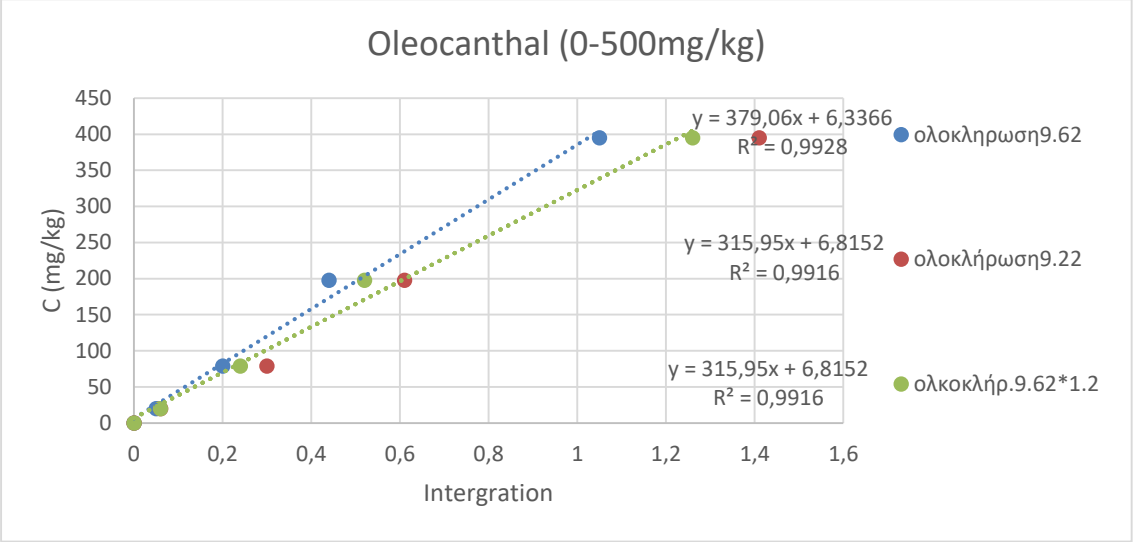


Diagram 25: Calibration curve of oleocanthal using NMR - method

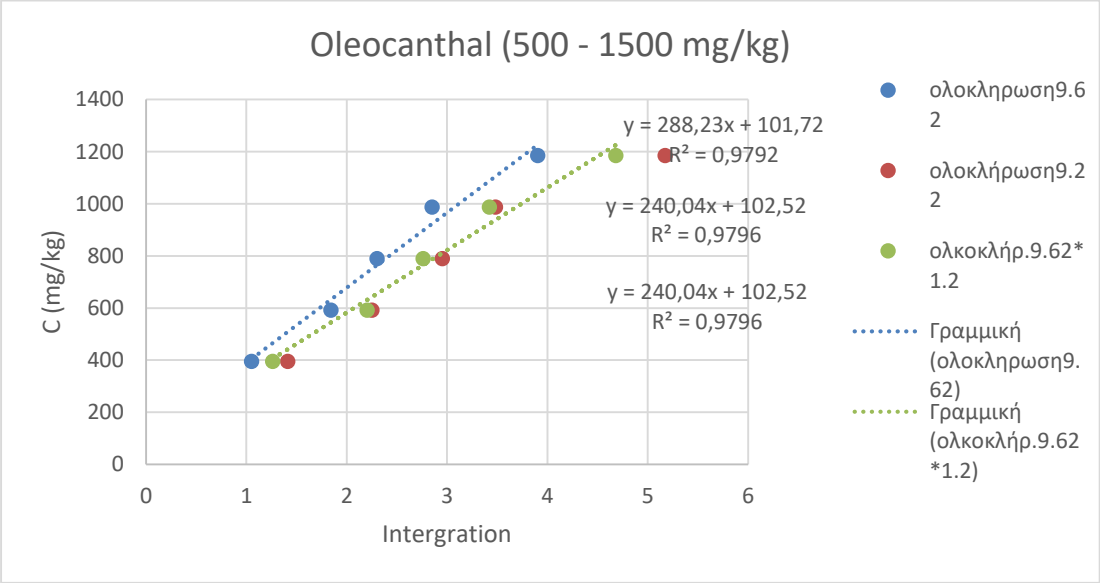


Diagram 26: Calibration curve of oleocanthal using NMR - method

OLEACEIN

- **Insertion of oleacein in olive oil**

60 mg oleacein was dissolved in acetonitrile and added in olive oil which contains zero concentration in phenolic compounds and we made olive oil with concentration 1500mg/kg in oleocanthal. Then we mixed this olive oil with zero and we make the following concentrations. (25 , 100, 250, 500 , 750 ,1000 , 1250 and 1500)

We applied the NMR method of quantitative measurement of phenolic compounds.

c(mg/kg)	καθαρότητα(c*0,83)	ολοκλωση_9.63	ολοκλήρωση_9.17	ολοκλ.9.63*1,2
0	0	0	0	0
50	41,5	0,02	0,04	0,024
100	83	0,1	0,19	0,12
250	207,5	0,89	1,15	1,068
312	258,96	1,06	1,37	1,272
500	415	1,71	2,12	2,052
1000	830	3,45	4,8	4,14
1500	1245	5,75	8,06	6,9

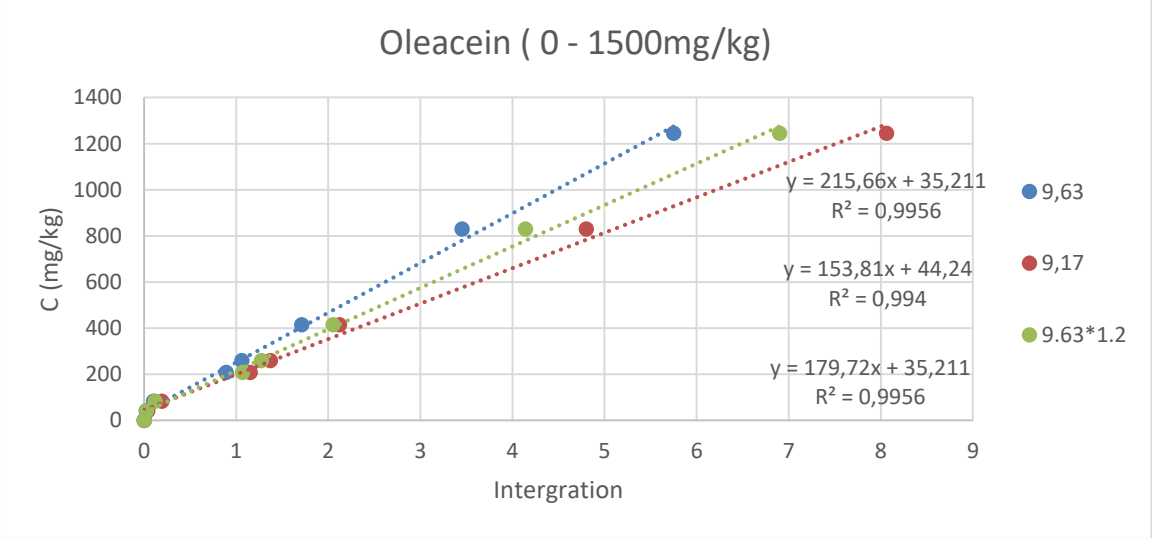


Diagram 27: Calibration curve of oleacein using NMR – method

3rd trial

OLEOCANTHAL

- **Insertion of oleocanthal in olive oil**

40mg oleocanthal was weighed , dissolved in 20ml acetonitrile and appropriate amount of solution was put in 5g of olive oil which had zero concentration of phenolic compounds. (maximum volume of acetonitrile was 150μl)

We applied the NMR method of quantitative measurement of phenolic compounds.

c (mg/kg)	καθαρότητα(c*0,81)	ολοκλωση_9.62	ολοκλήρωση_9.22	ολοκλ.9.62*1,2
0	0	0	0	0
300	243	0,73	0,91	0,876
500	405	1,66	2,01	1,992
750	607,5	2,68	3,32	3,216
1000	810	3,87	4,68	4,644
1250	1012,5	5,05	5,94	6,06
1500	1215	4,97	6,39	5,964

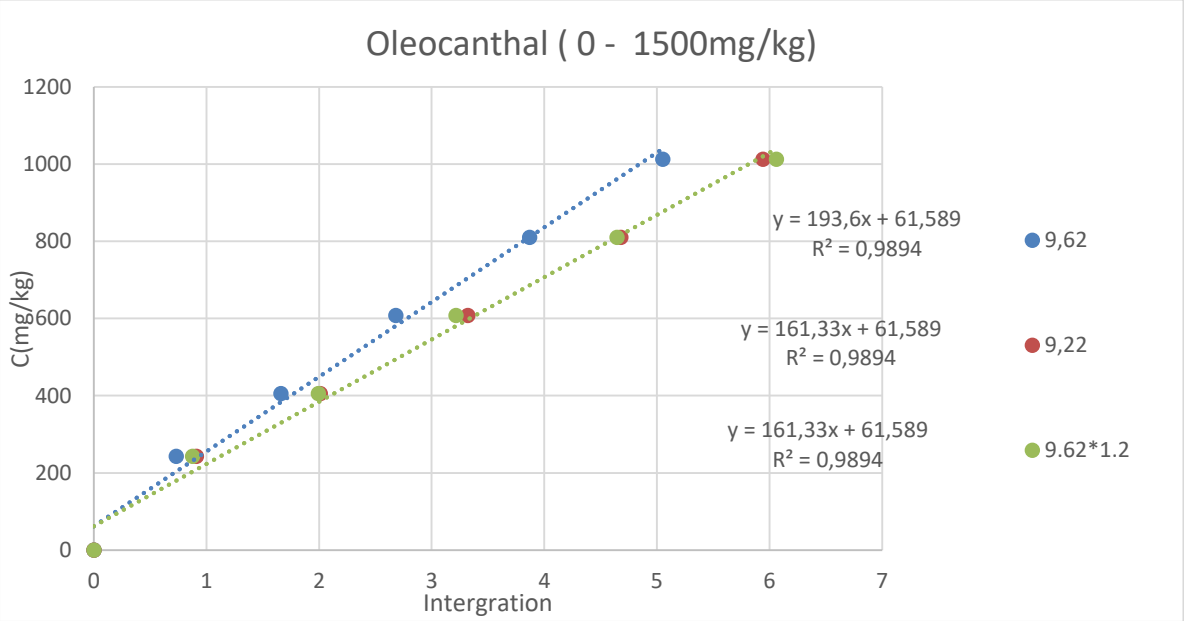


Diagram 28: Calibration curve of oleocanthal using NMR - method

OLEACEIN

- **Insertion of oleacein in olive oil**

40mg oleacein was weighed, dissolved in 20ml acetonitrile and appropriate amount of solution was put in 5g of olive oil which had zero concentration of phenolic compounds. (maximum volume of acetonitrile was 150µl)

We applied the NMR method of quantitative measurement of phenolic compounds.

c (mg/kg)	καθαρότητα(c*0,84)	ολοκλωση_9.63	ολοκλήρωση_9.17	ολοκλ.9.63*1,2
0	0	0	0	0
300	252	0,62	0,91	0,744
500	420	1,39	1,84	1,668
750	630	2,19	2,97	2,628
1000	840	3,16	4,4	3,792
1250	1050	4,16	5,69	4,992
1500	1260	5,46	7,46	6,552

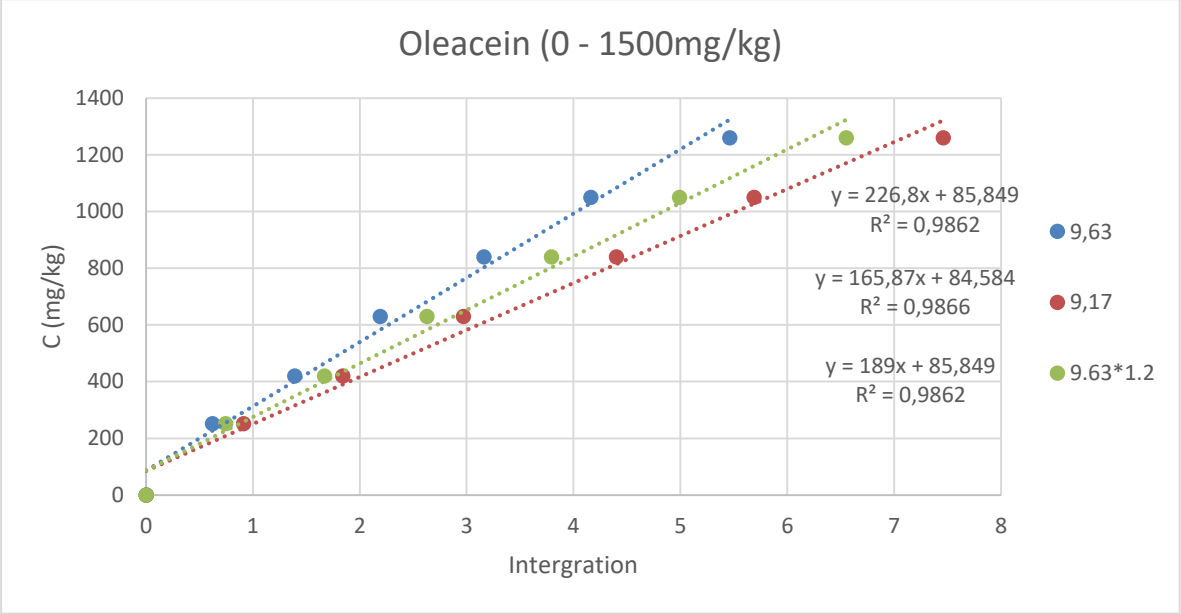


Diagram 29: Calibration curve of oleacein using NMR - method

Final trial

I. Calibration curve of oleacein

40mg oleacein (ikl377) was weighed, dissolved in 20ml acetonitrile and appropriate amount of solution was put in 5g of olive oil (ikl404). The final volume of all samples in acetonitrile was the same. Then we applied the NMR method and all phenolic compounds were measured. The final four points (*) were made by another solution which 40mg of the same substance was weighed and dissolved in 10ml acetonitrile.

C (mg/kg)	C* purity of oleacein (0.83%) (mg/kg)	Concentration of oleacein in 5g of olive oil (mg)	Volume of appropriate amount of oleacein in acetonitrile (µl)
20	16.6	0.1	50
50	41.5	0.25	125
100	83	0.5	250
200	166	1	500
300	249	1.5	750
400	332	2	1000
500	415	2.5	1250
600	498	3	1500
800	664	4	1000*
1000	830	5	1250*
1250	1037.5	6.25	1562*
1500	1245	7.5	1875*

Then we follow the NMR protocol which is the following:

- 5g of the sample was weighed
- 20ml cyclohexane was added
- Stir for 1min
- The appropriate volume of acetonitrile was added and each sample contains 25ml acetonitrile
- Stir for 1min
- The samples were centrifuged for 5min in 4000rpm
- 25ml of acetonitrile was separated with cyclohexane
- 1ml of syringaldehyde was added
- Acetonitrile phase was evaporated
- Receipt 1H-NMR spectrum

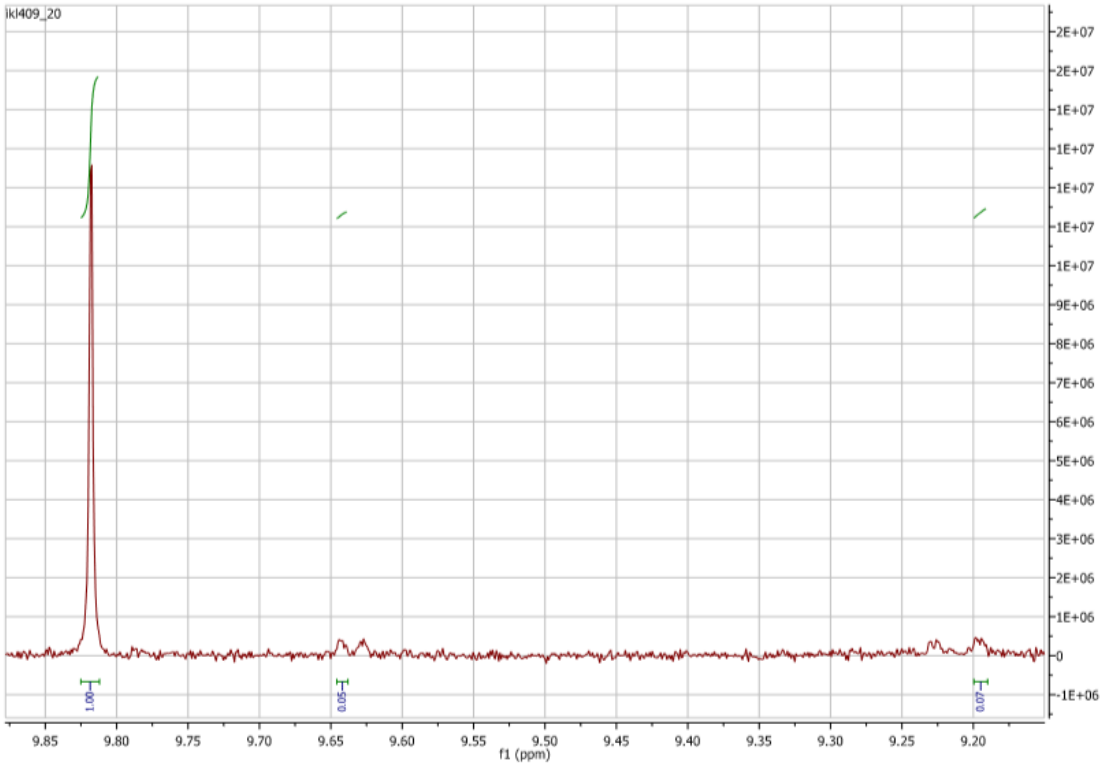


Figure 42: Depiction of the spectrum 1H-NMR of oleacein (20mg/kg)

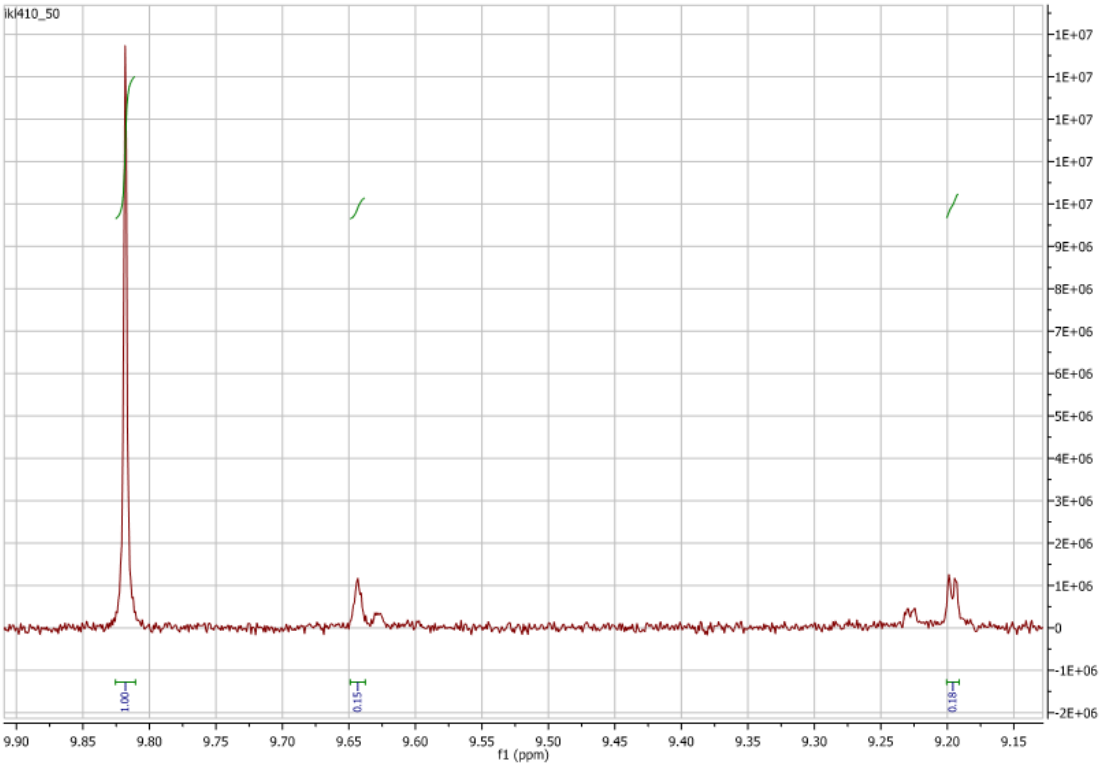


Figure 43: Depiction of the spectrum 1H-NMR of oleacein (50mg/kg)

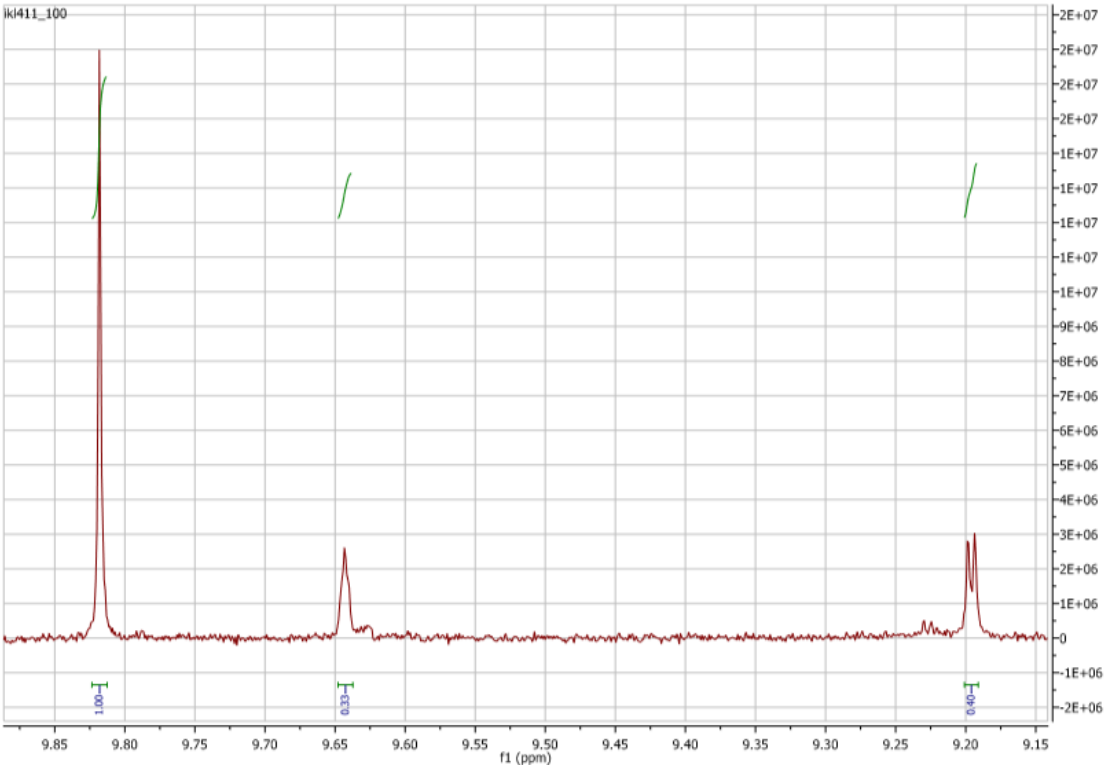


Figure 43: Depiction of the spectrum 1H-NMR of oleacein (100mg/kg)

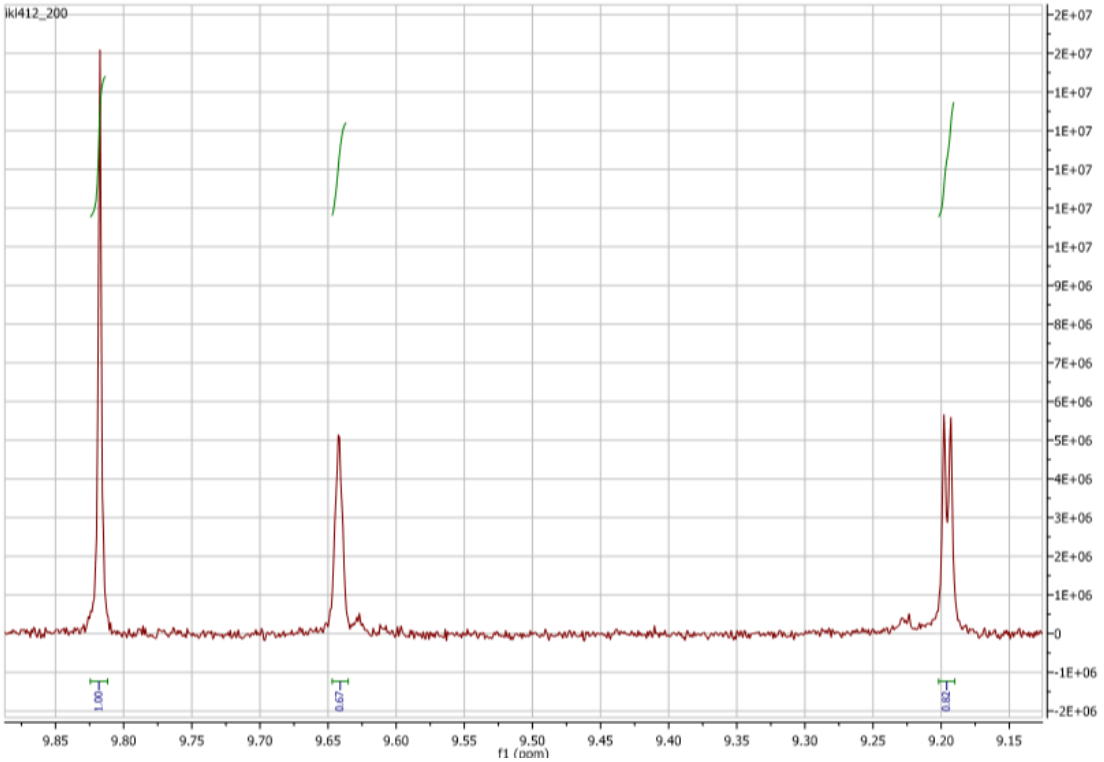


Figure 44: Depiction of the spectrum 1H-NMR of oleacein (200mg/kg)

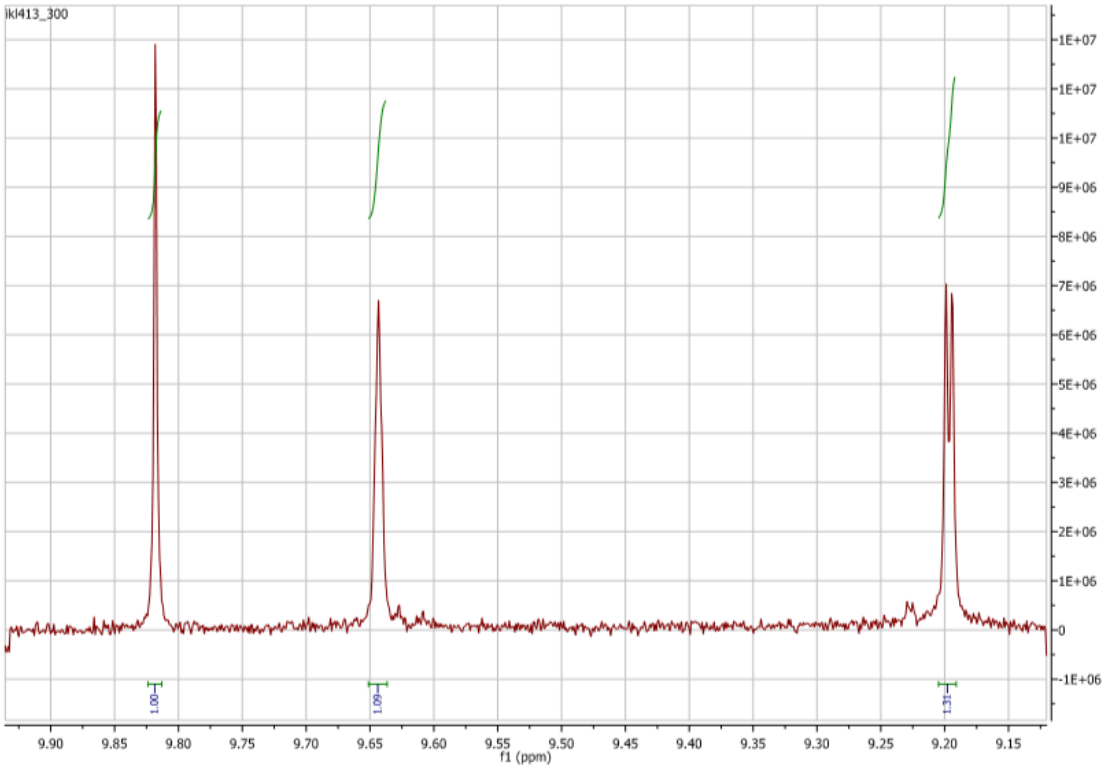


Figure 45: Depiction of the spectrum 1H-NMR of oleacein (300mg/kg)

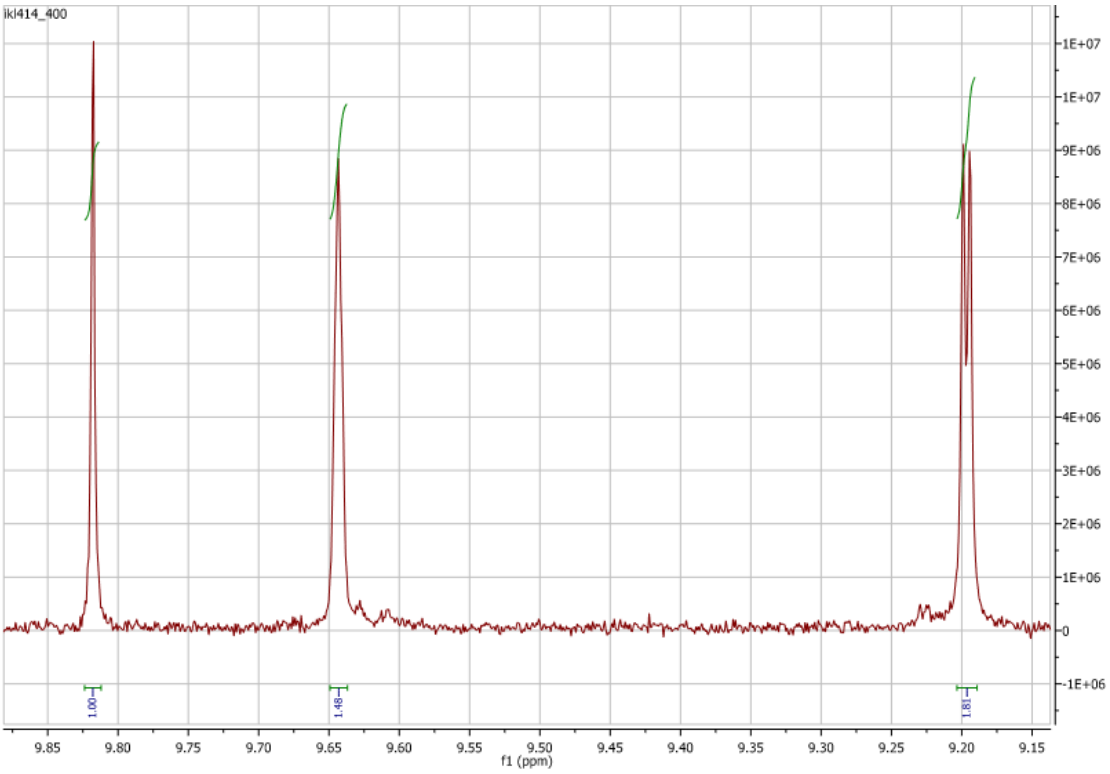


Figure 46: Depiction of the spectrum 1H-NMR of oleacein (400mg/kg)

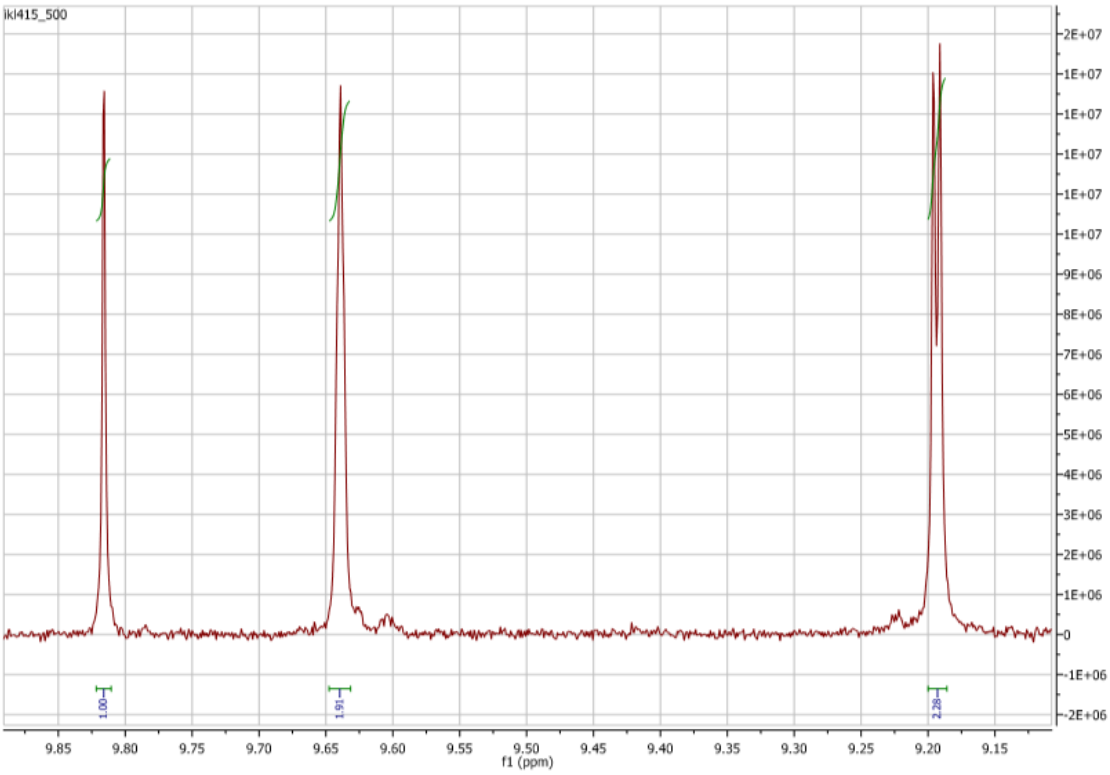


Figure 47: Depiction of the spectrum 1H-NMR of oleacein (500mg/kg)

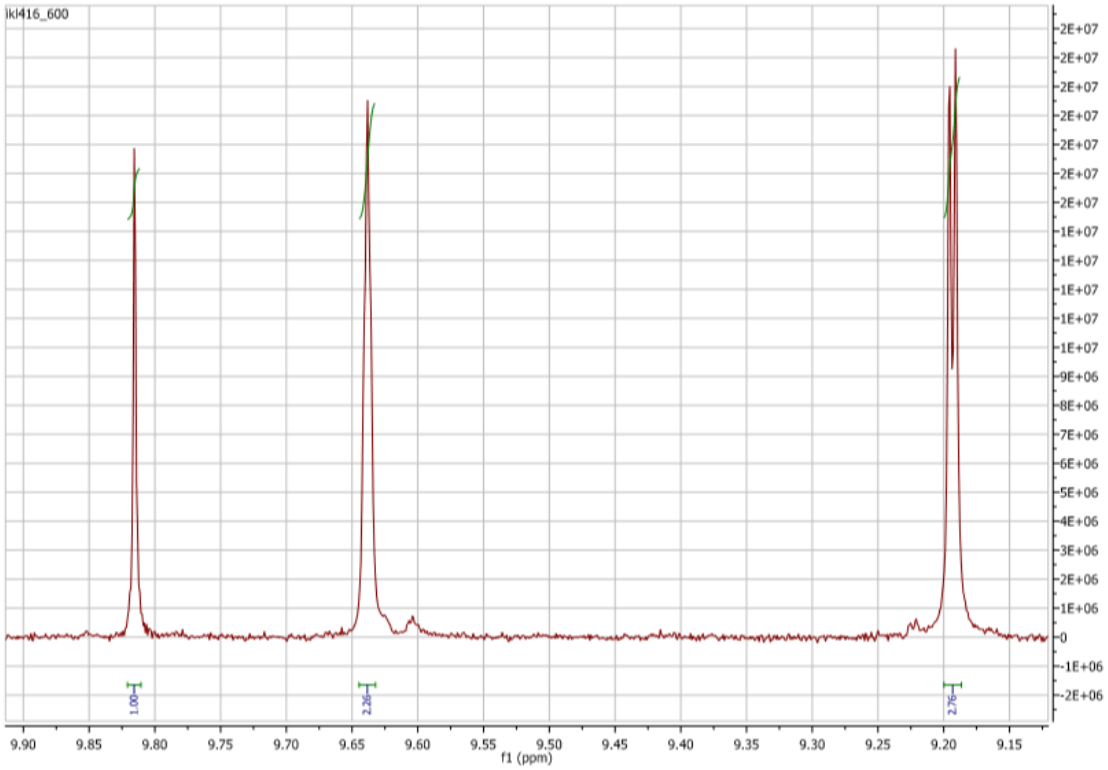


Figure 48: Depiction of the spectrum 1H-NMR of oleacein (600mg/kg)

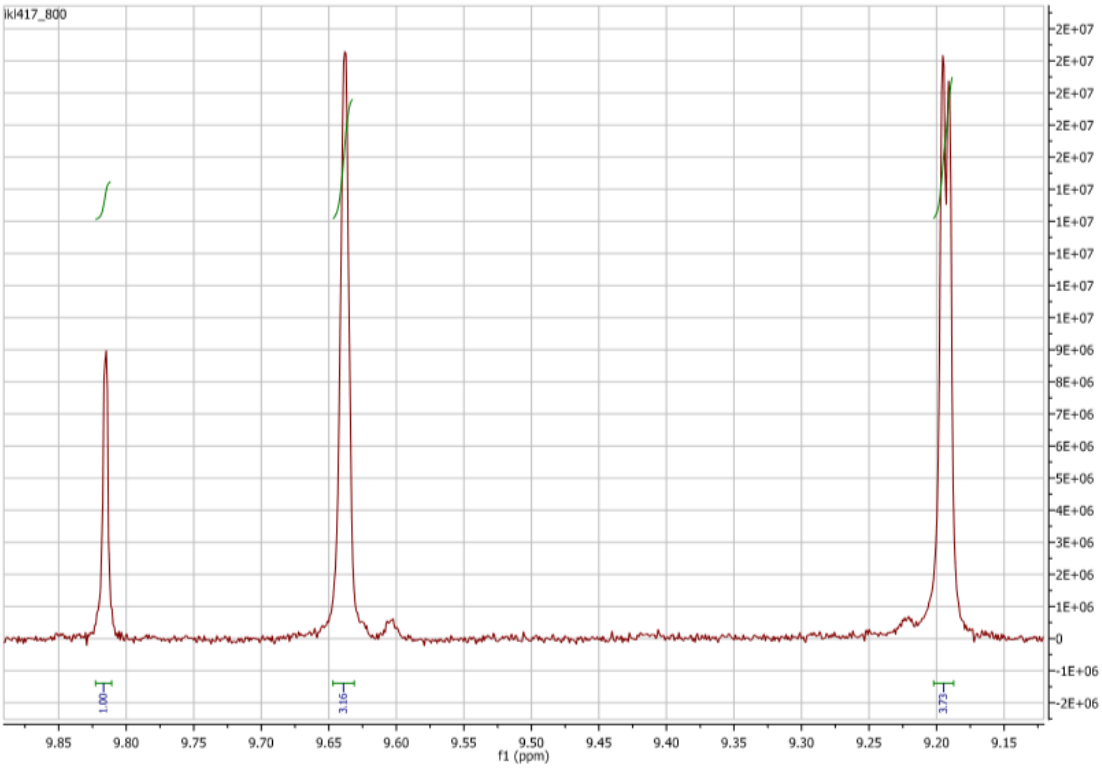


Figure 49: Depiction of the spectrum 1H-NMR of oleacein (800mg/kg)

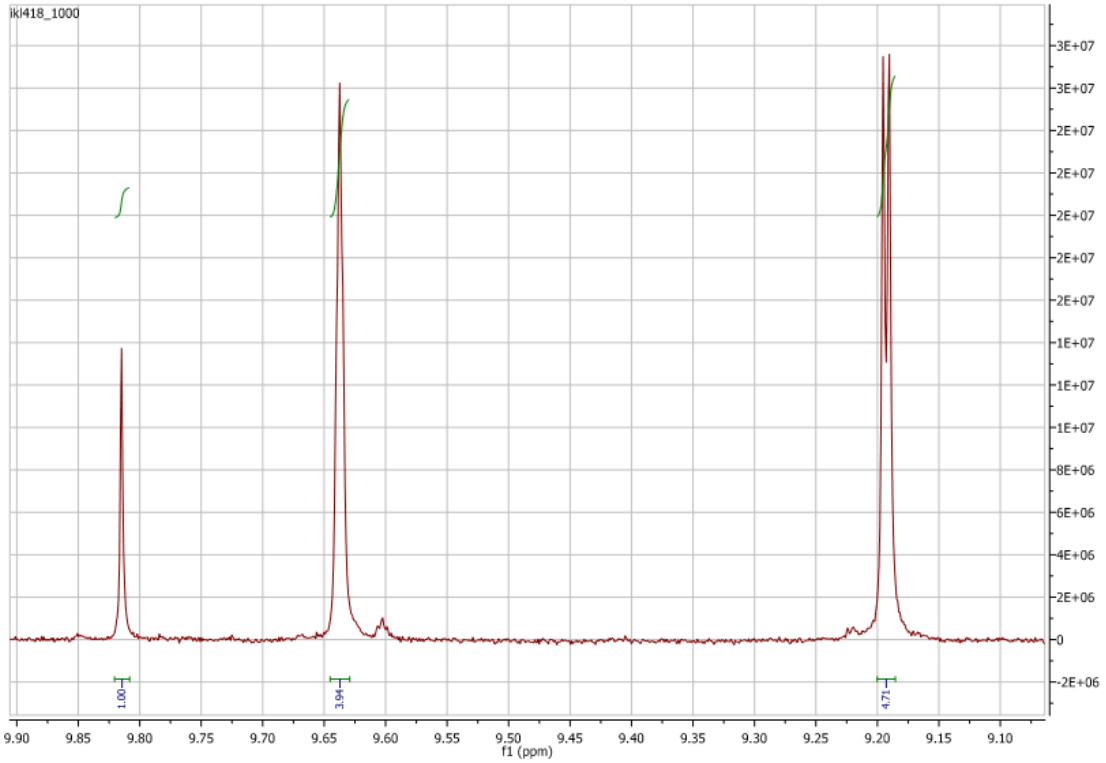


Figure 50: Depiction of the spectrum 1H-NMR of oleacein (1000mg/kg)

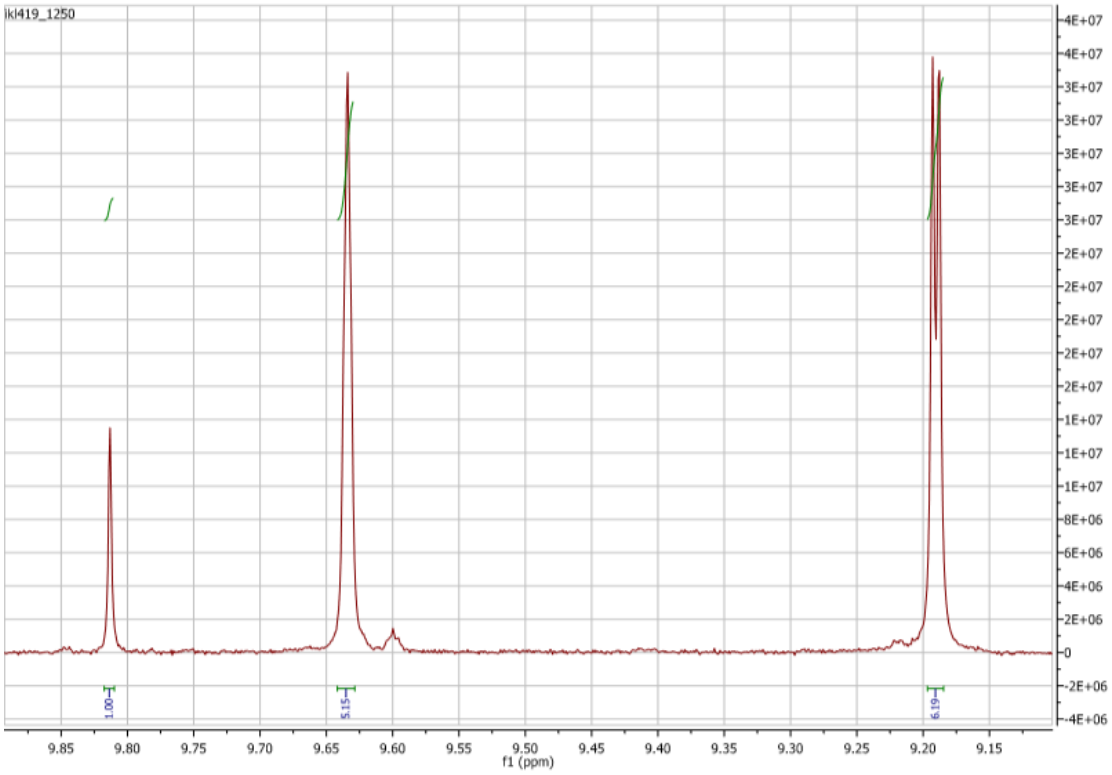


Figure 51: Depiction of the spectrum 1H-NMR of oleacein (1250mg/kg)

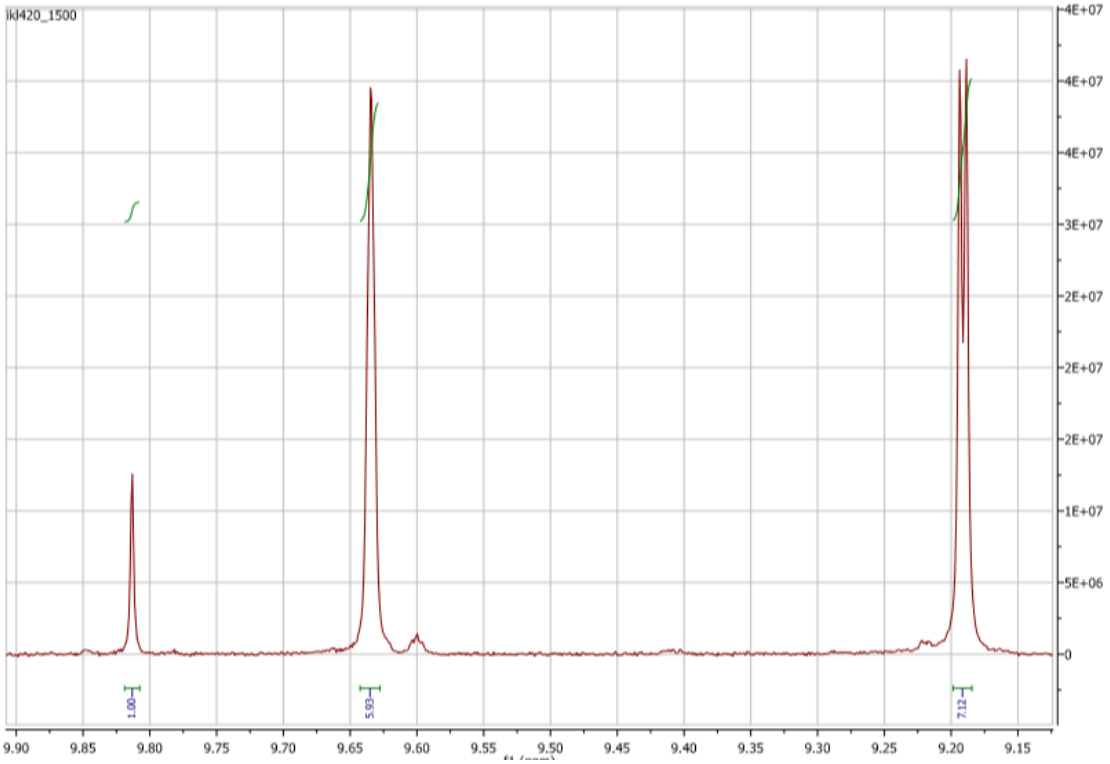


Figure 52: Depiction of the spectrum 1H-NMR of oleacein (1500mg/kg)

C (mg/kg)	C* purity of oleacein (0.83%) (mg/kg)	Integration (9,17ppm)	Integration (9,64ppm)
0	0	0	0
20	16.6	0.07	0.05
50	41.5	0.18	0.15
100	83	0.4	0.33
200	166	0.82	0.67
300	249	1.31	1.09
400	332	1.81	1.49
500	415	2.29	1.91
600	498	2.76	2.26
800	664	3.76	3.16
1000	830	4.71	4.01

1250	1037.5	6.19	5.15
1500	1245	7.12	5.93

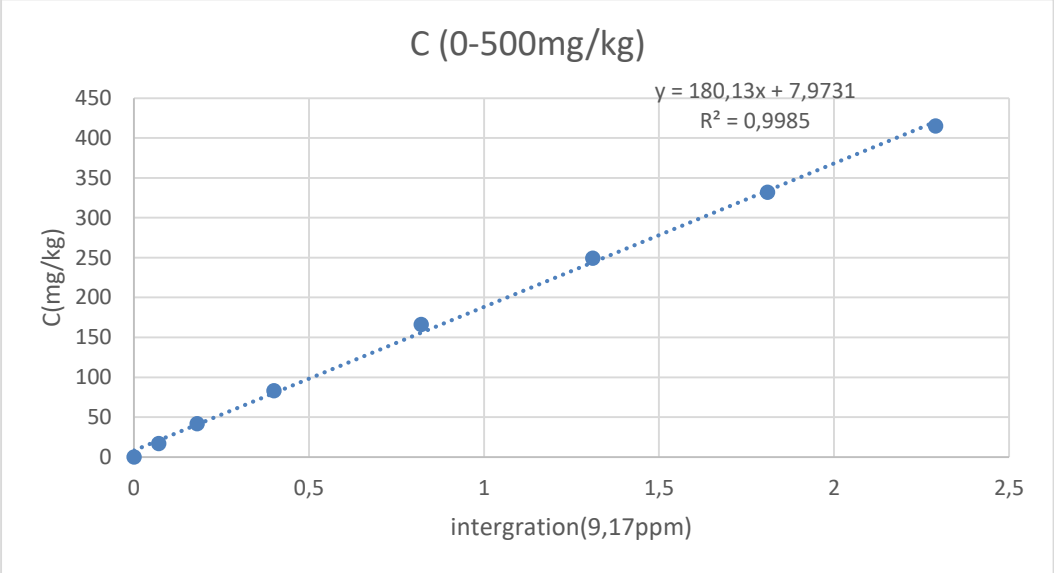


Diagram 30: Calibration curve of oleacein using NMR - method

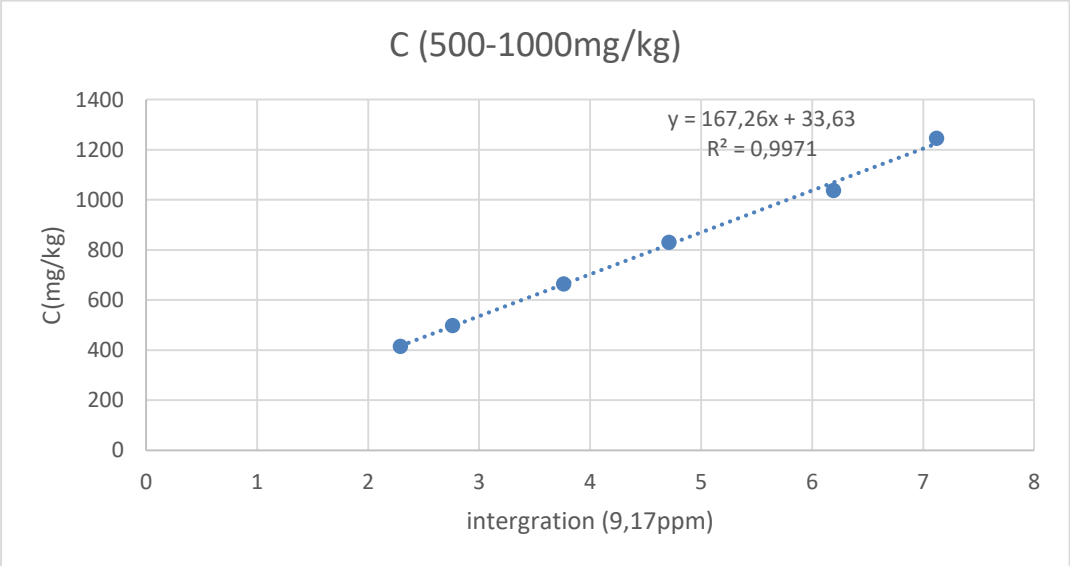


Diagram 31: Calibration curve of oleacein using NMR - method

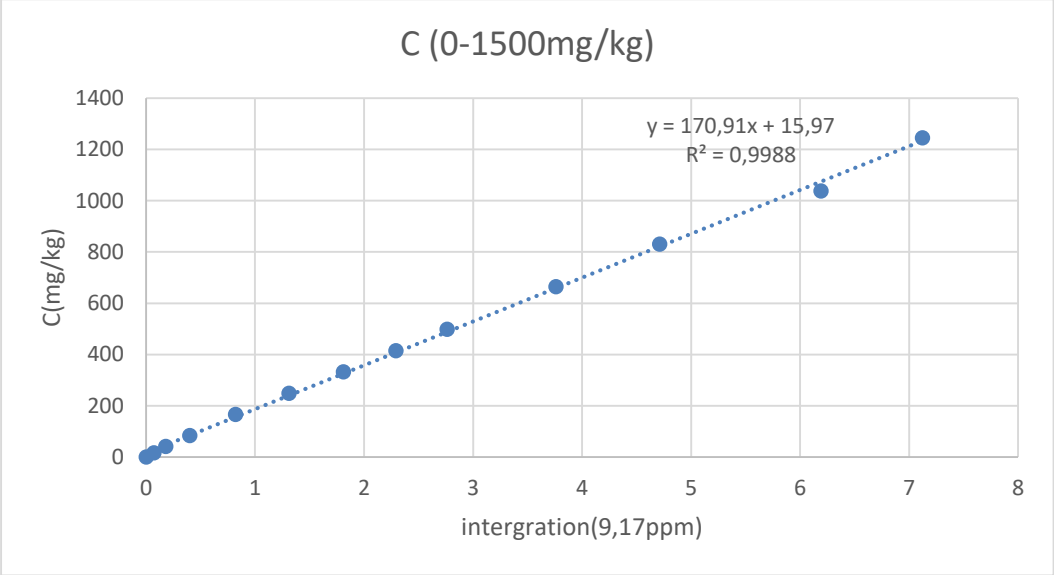


Diagram 32: Calibration curve of oleacein using NMR - method

The final equalization of NMR method in oleacein is the following:

y= 170,91*x + 15,97

Recovery of oleacein

This method was repeated in four concentrations because we wanted to find the recovery of this method. The concentrations for which we applied the method are 100, 500, 1000 and 1500 mg/kg.

C (mg/kg)	C* purity of oleacein (0.83%) (mg/kg)	Integration (9,17ppm)	Integration (9,64ppm)
100	83	0	0
500	415	0	0
1000	830	0	0
1500	1245	0	0

The recovery of oleacein is 100%

II. Calibration curve of oleocanthal

40mg oleocanthal (ikl372) was weighed, was dissolved in 20ml acetonitrile and appropriate amount of solution was put in 5g of olive oil (ikl404). The final volume of all samples in acetonitrile was the same. Then we applied the NMR method and all phenolic compounds were measured. The final four points (*) was made from another solution which 40mg of the same substance was weighed and was dissolved in 10ml acetonitrile.

C (mg/kg)	**C* purity of oleocanthal (0.83%) (mg/kg)**	**Concentration of oleocanthal in 5g of olive oil (mg)**	**Volume of appropriate amount of oleocanthal in acetonitrile (µl)**
20	16.6	0.1	50
50	41.5	0.25	125
100	83	0.5	250
200	166	1	500
300	249	1.5	750
400	332	2	1000
500	415	2.5	1250
600	498	3	1500
800	664	4	1000*
1000	830	5	1250*
1250	1037.5	6.25	1562*
1500	1245	7.5	1875*

Then we follow the NMR protocol and we receive the following spectrum.

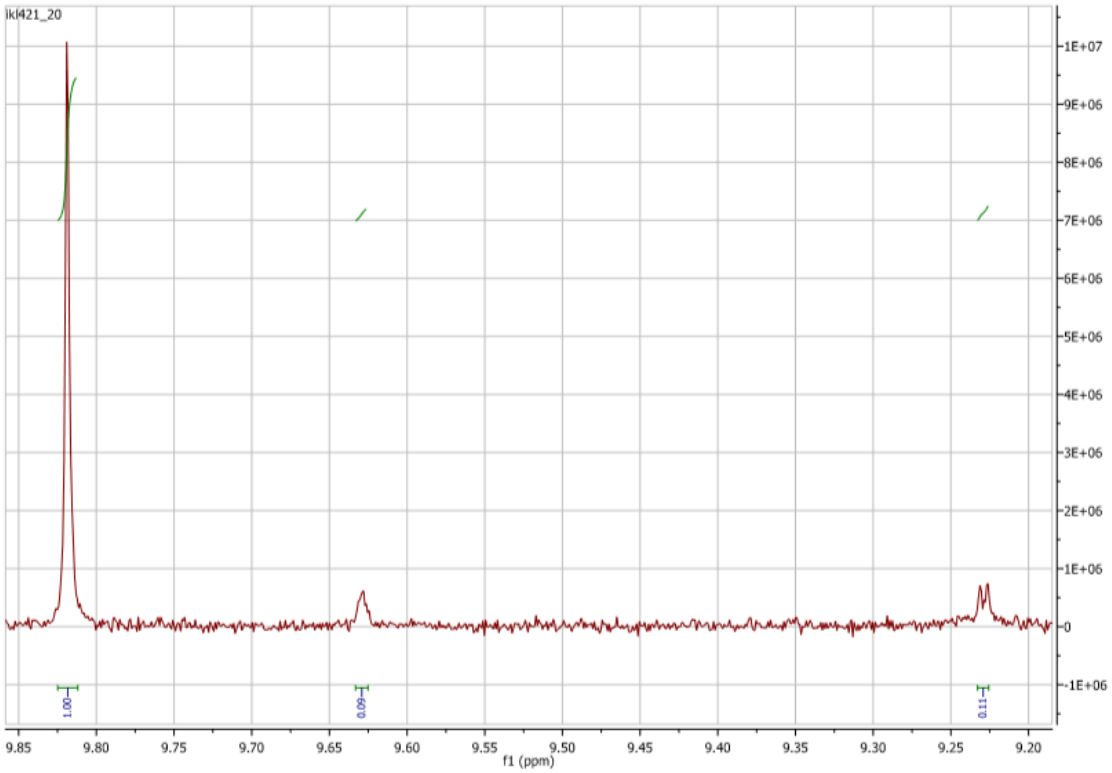


Figure 53: Depiction of the spectrum 1H-NMR of oleocanthal (20mg/kg)

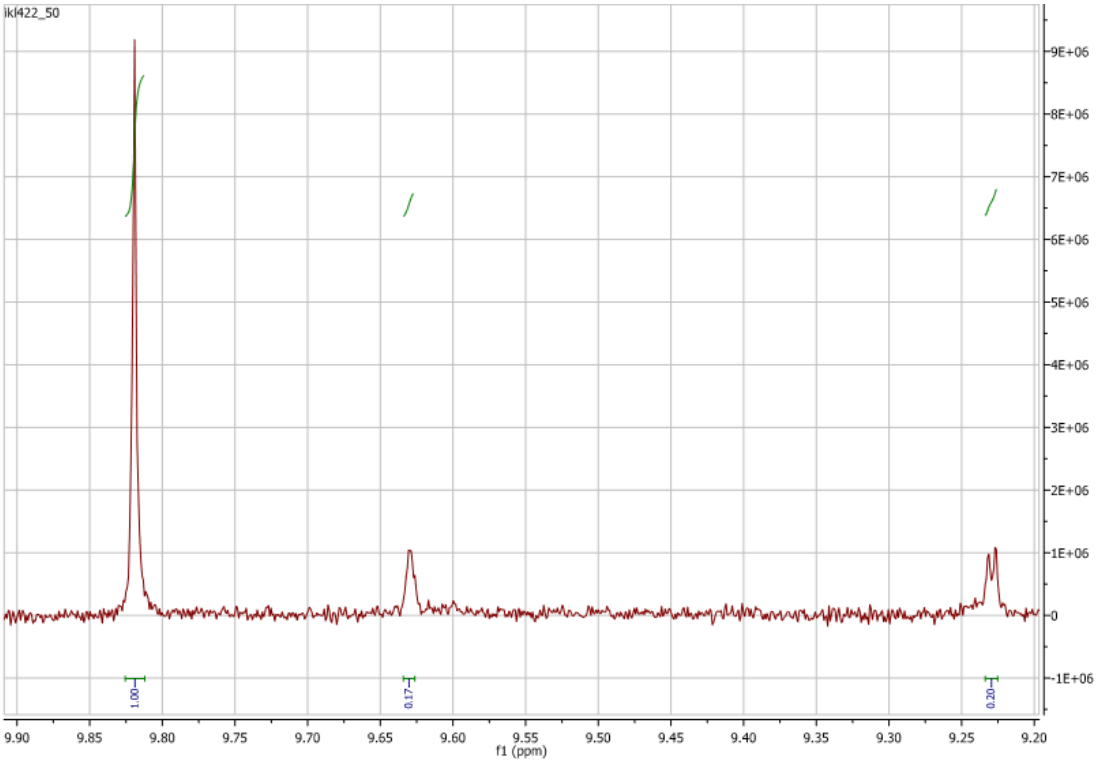


Figure 53: Depiction of the spectrum 1H-NMR of oleocanthal (50mg/kg)

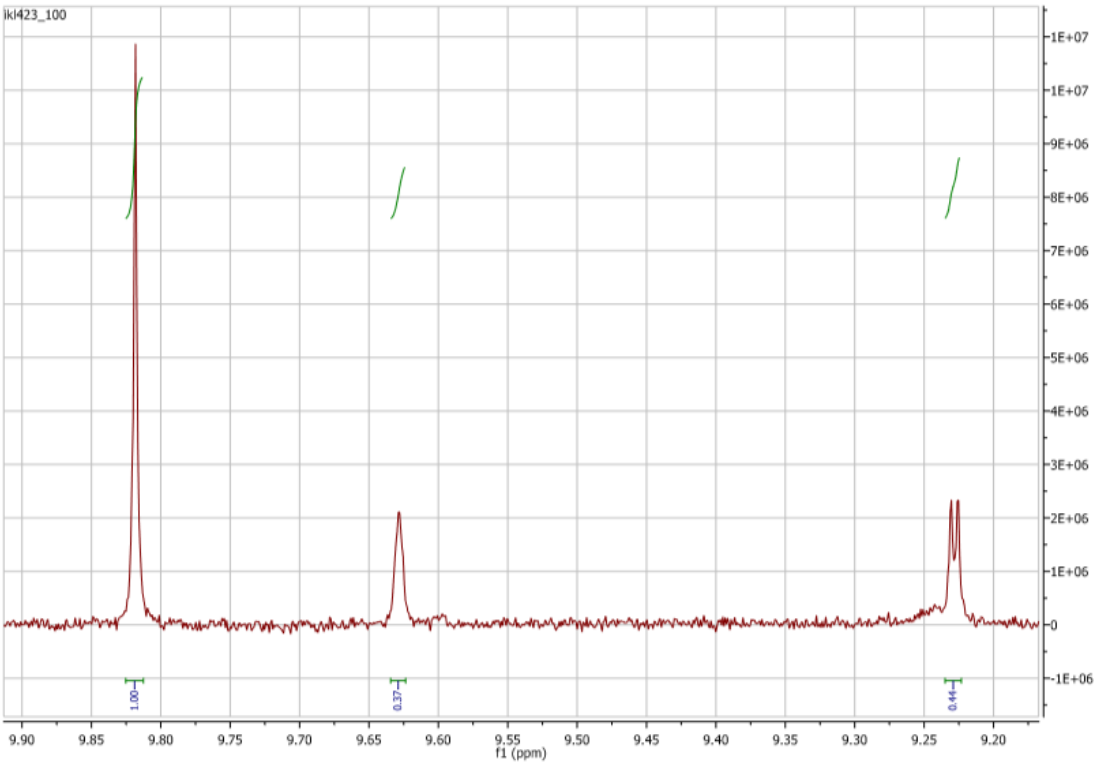


Figure 54: Depiction of the spectrum 1H-NMR of oleocanthal (100mg/kg)

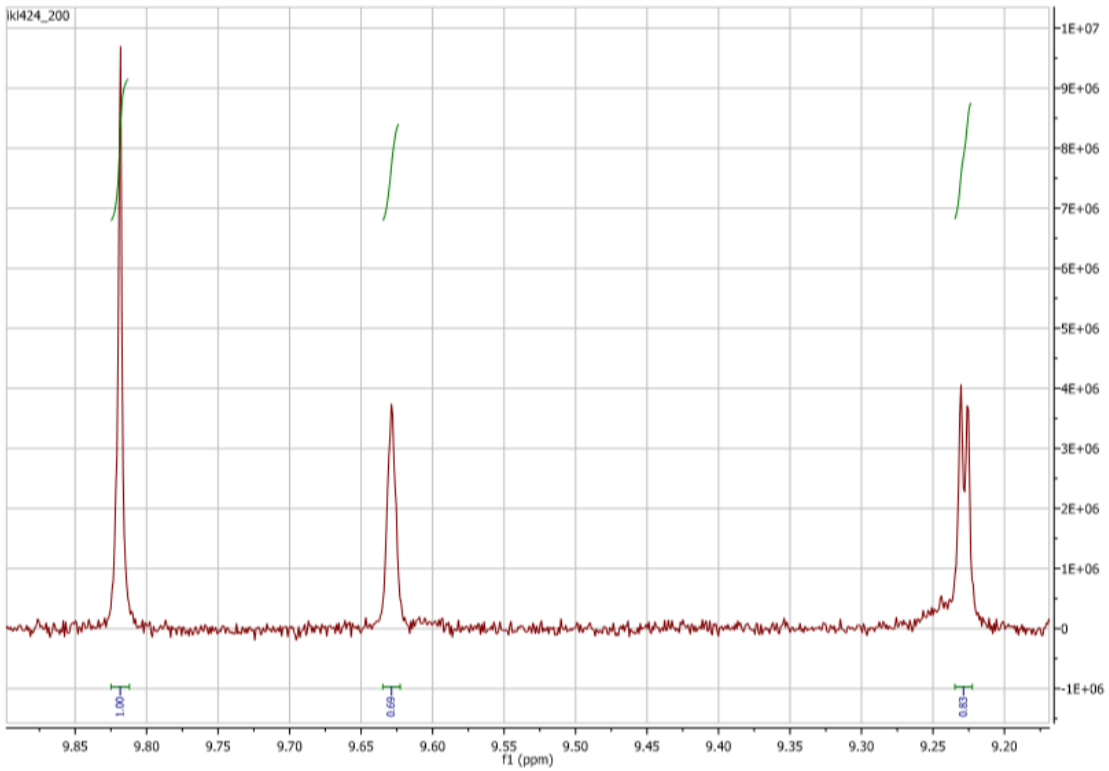


Figure 55: Depiction of the spectrum 1H-NMR of oleocanthal (200mg/kg)

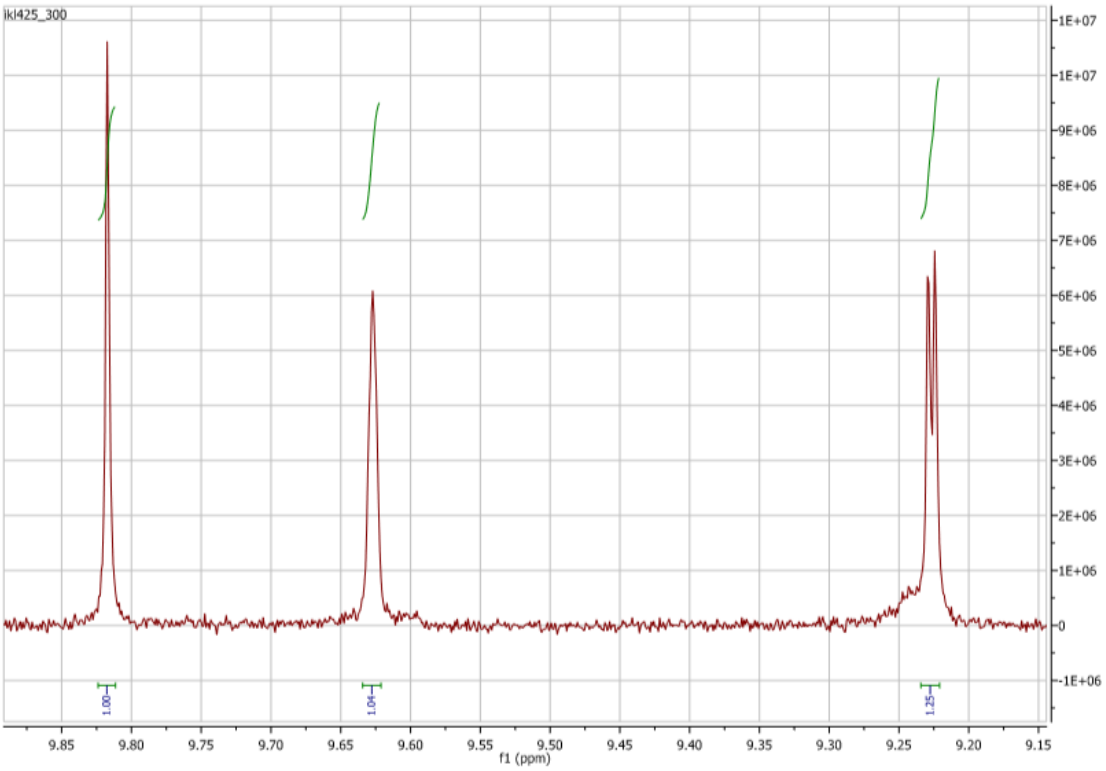


Figure 56: Depiction of the spectrum 1H-NMR of oleocanthal (300mg/kg)

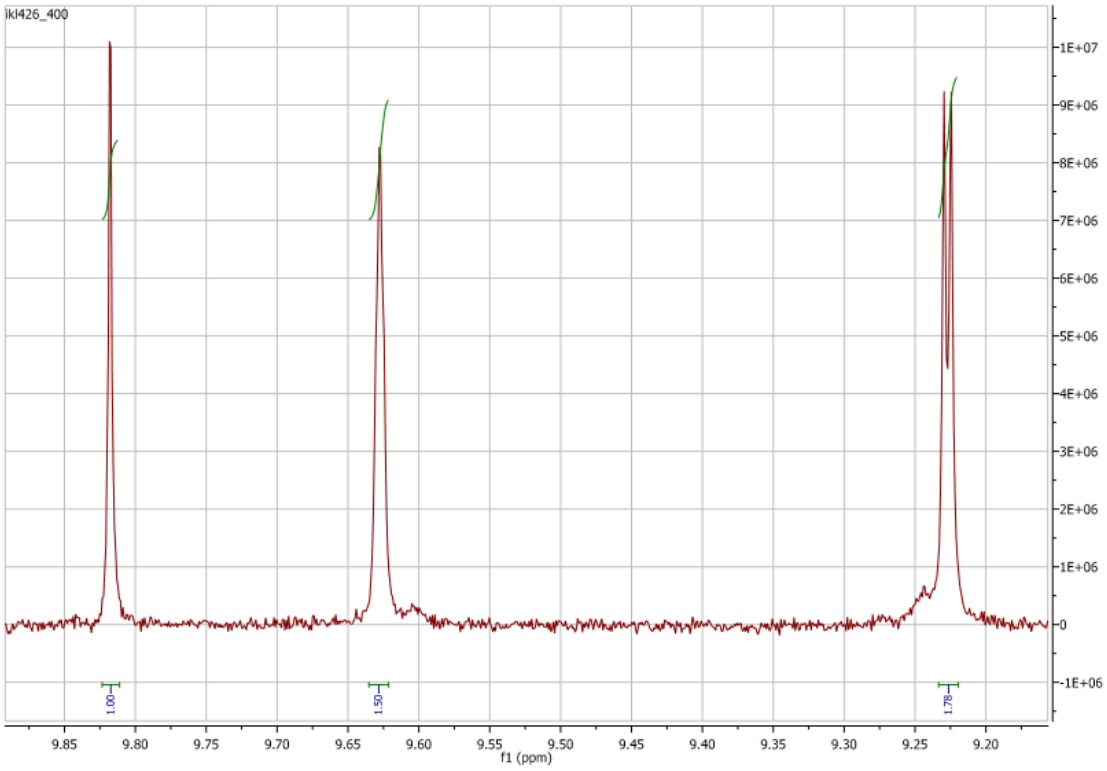


Figure 57: Depiction of the spectrum 1H-NMR of oleocanthal (400mg/kg)

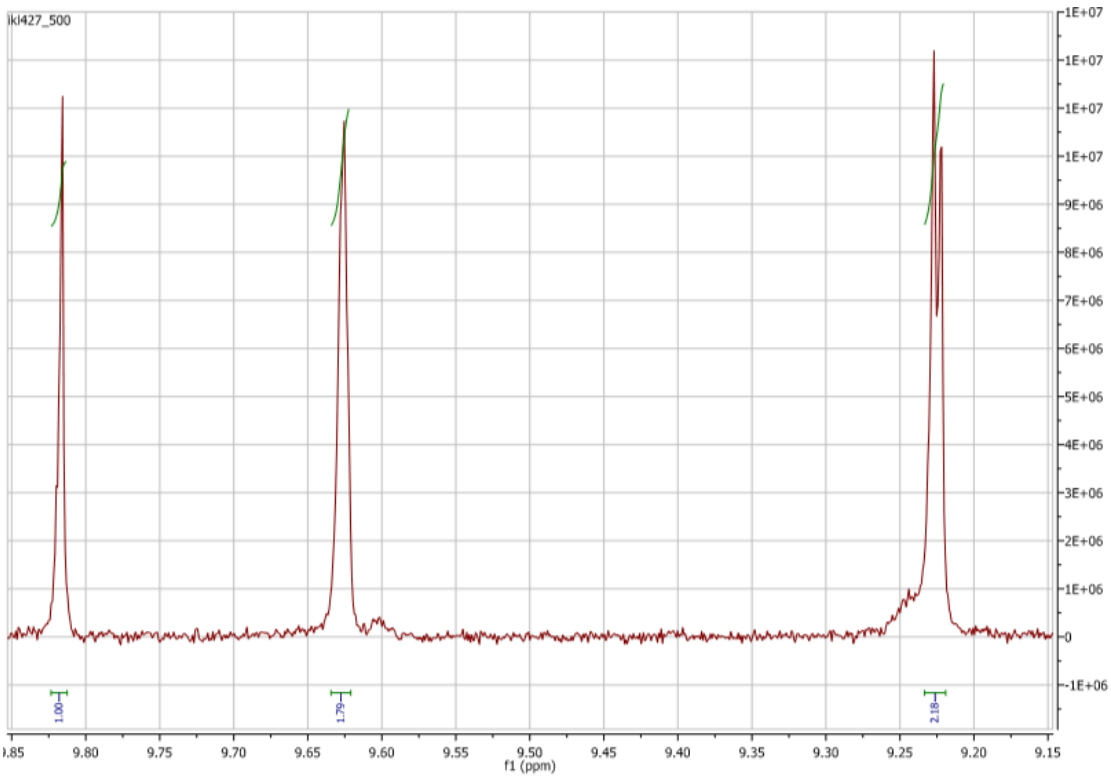


Figure 58: Depiction of the spectrum 1H-NMR of oleocanthal (500mg/kg)

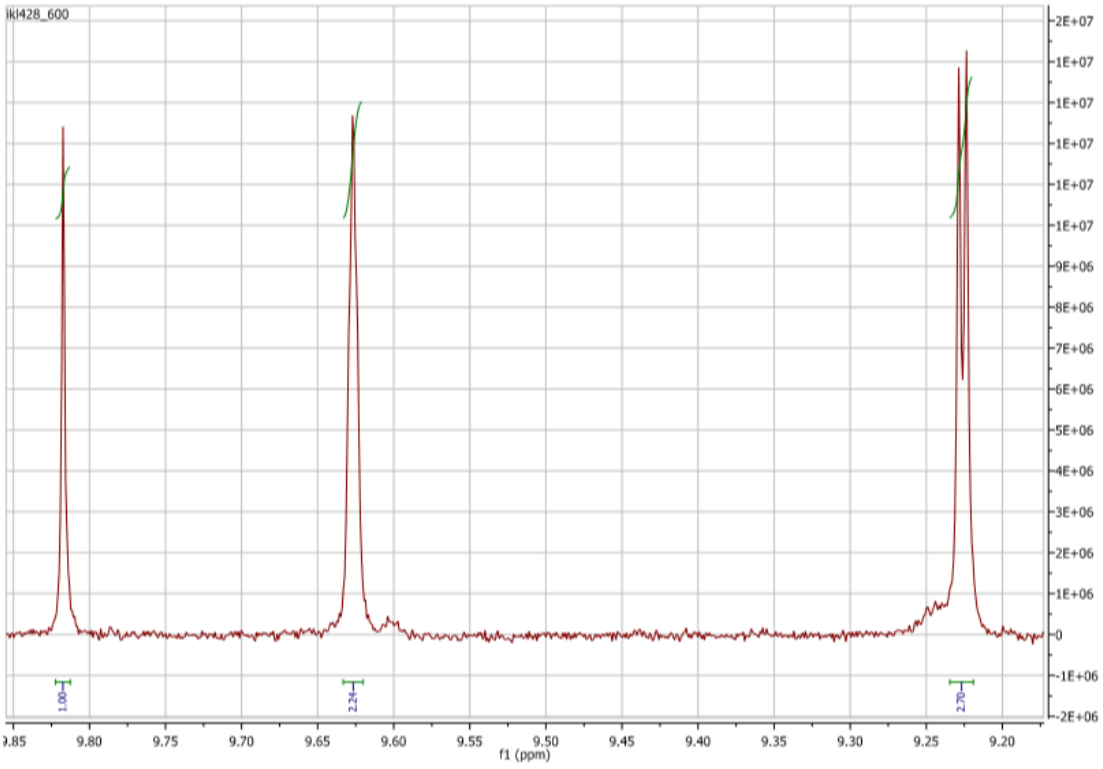


Figure 59: Depiction of the spectrum 1H-NMR of oleocanthal (600mg/kg)

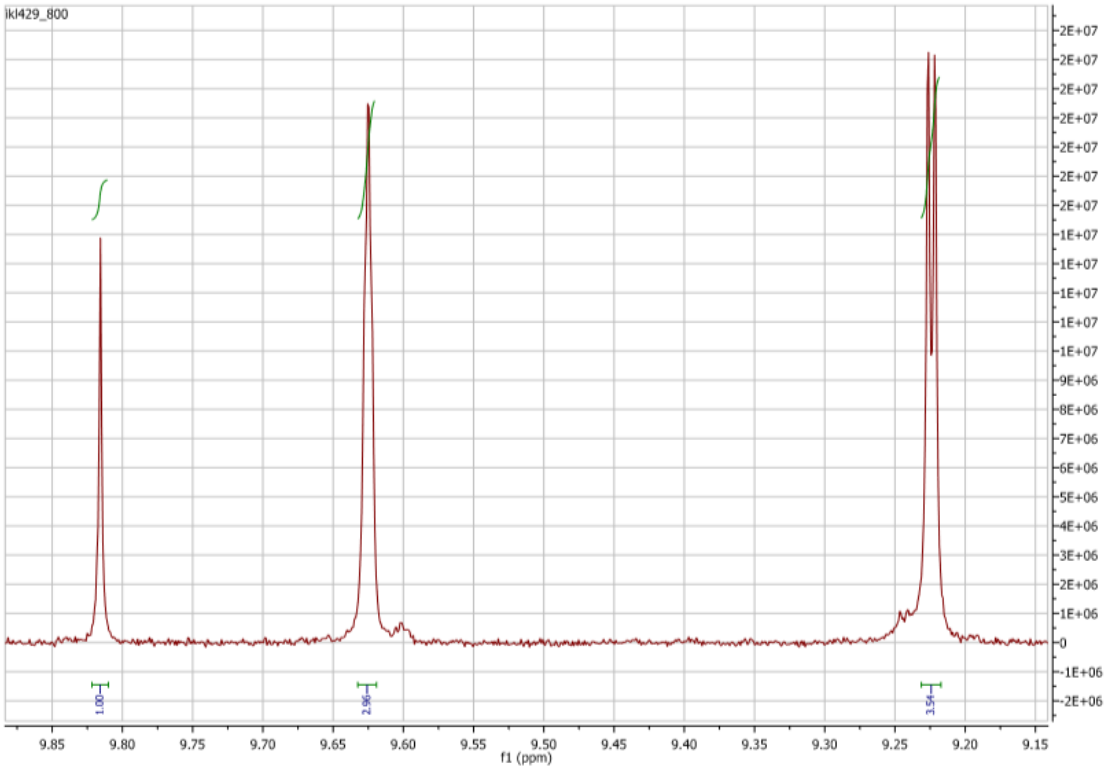


Figure 60: Depiction of the spectrum 1H-NMR of oleocanthal (800mg/kg)

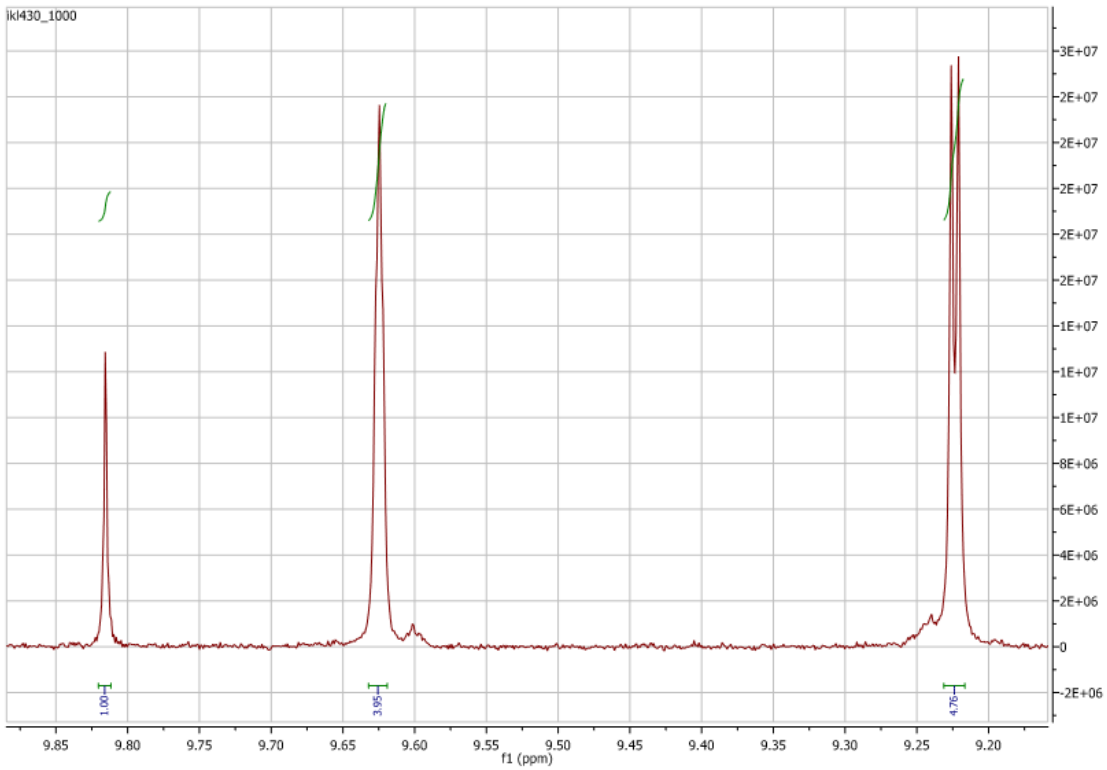


Figure 61: Depiction of the spectrum 1H-NMR of oleocanthal (1000mg/kg)

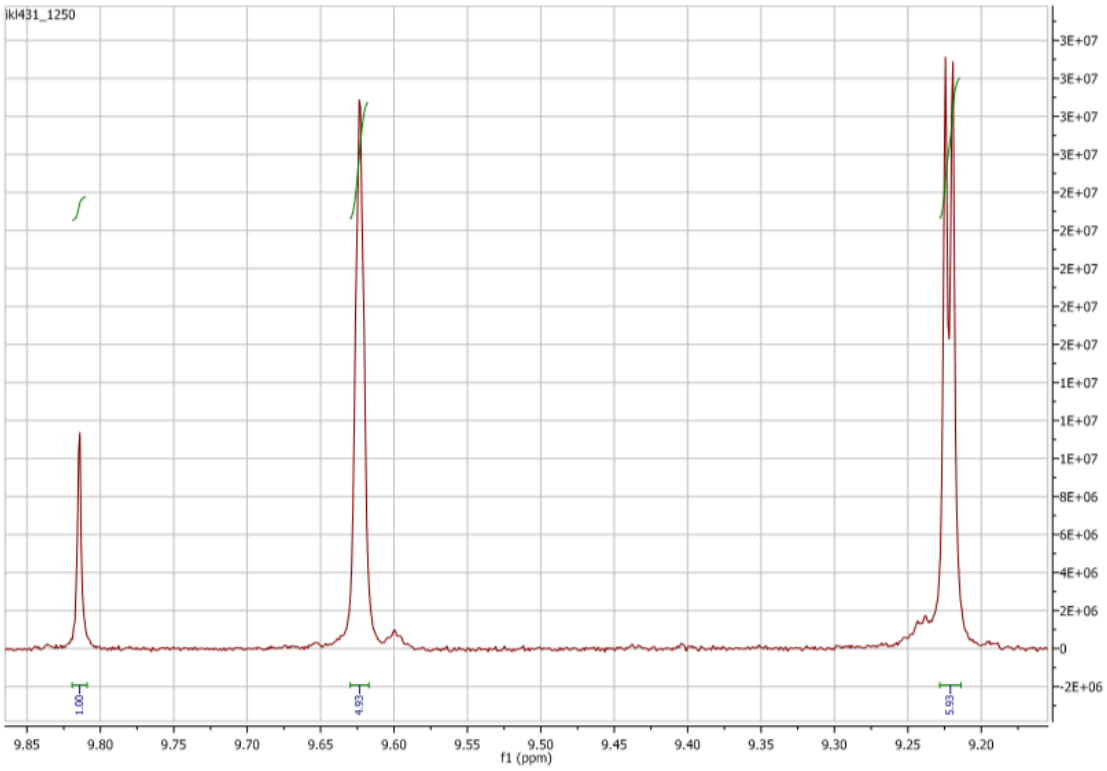


Figure 62: Depiction of the spectrum 1H-NMR of oleocanthal (1250mg/kg)

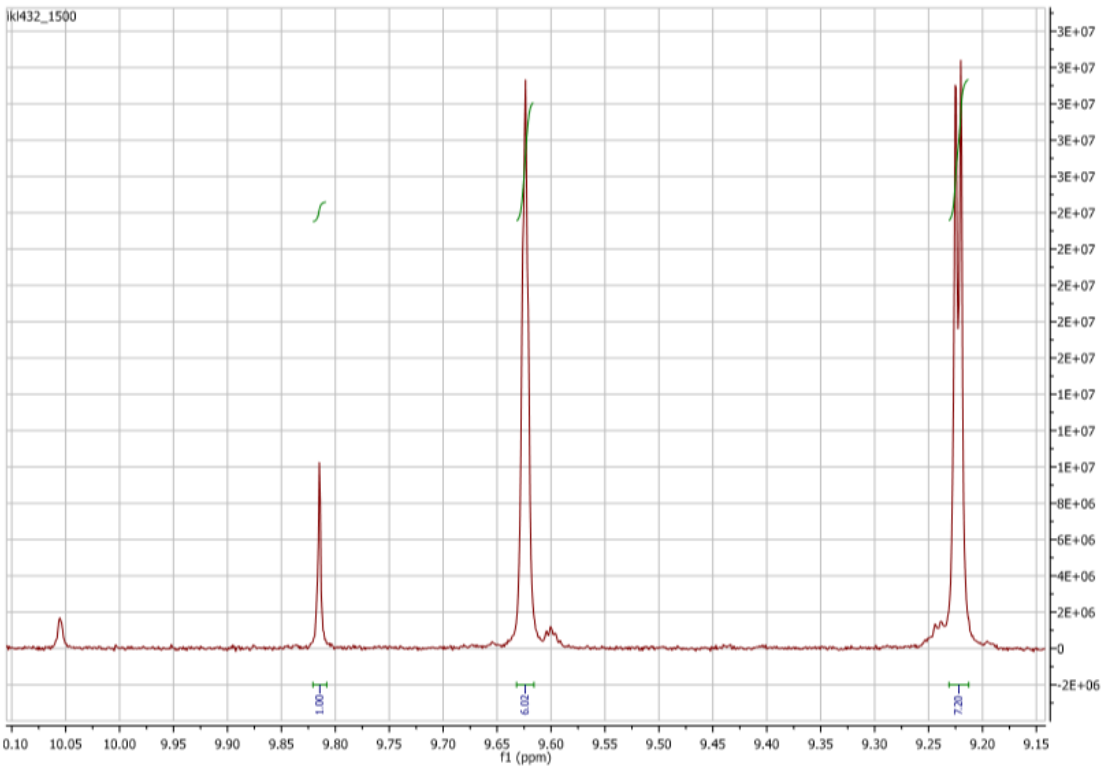


Figure 63: Depiction of the spectrum 1H-NMR of oleocanthal (1500mg/kg)

C (mg/kg)	C* purity of oleocanthal(0.79%) (mg/kg)	Integration (9,22ppm)	Integration (9,63ppm)
0	0	0	0
20	15.8	0.11	0.09
50	39.5	0.2	0.17
100	79	0.44	0.37
200	158	0.83	0.69
300	237	1.25	1.04
400	316	1.78	1.5
500	395	2.18	1.79
600	474	2.7	2.24
800	632	3.54	2.96
1000	790	4.76	3.95
1250	987.5	5.93	4.93
1500	1185	7.2	6.02

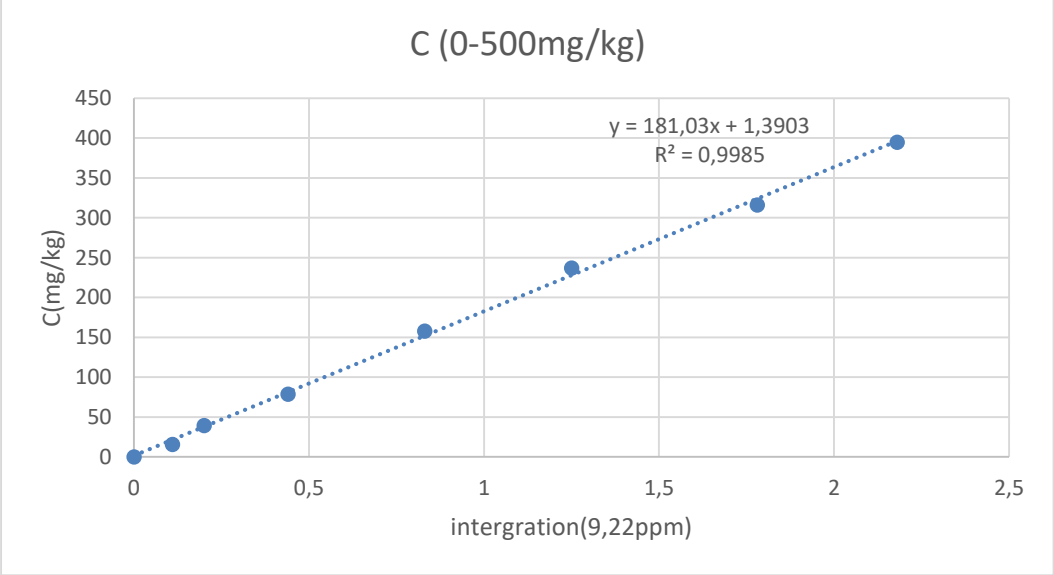


Diagram 33: Calibration curve of oleocanthal using NMR - method

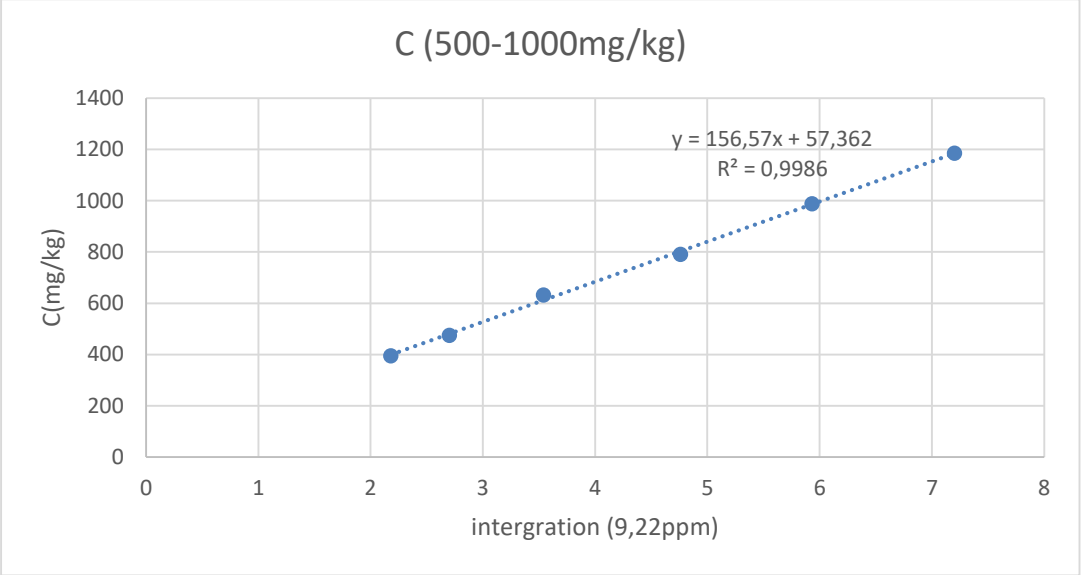


Diagram 34: Calibration curve of oleocanthal using NMR - method

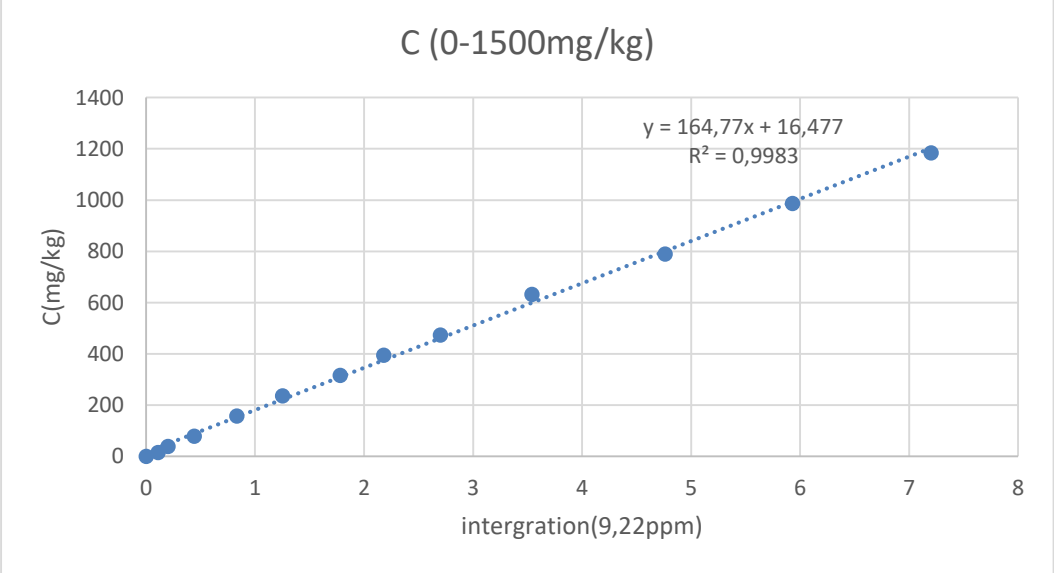


Diagram 35: Calibration curve of oleocanthal using NMR - method

The final equalization of NMR method in oleocanthal is the following:

y= 164,77*x + 16,477

Recovery of oleocanthal

This method was repeated in four points because we want to find the recovery of this method. The points which we apply the method are 100, 500, 1000 and 1500 mg/kg.

C (mg/kg)	C* purity of oleocanthal (0.79%) (mg/kg)	Integration (9,22ppm)	Integration (9,63ppm)
100	83	0	0
500	415	0	0
1000	830	0	0
1500	1245	0	0

The recovery of oleocanthal is 100%

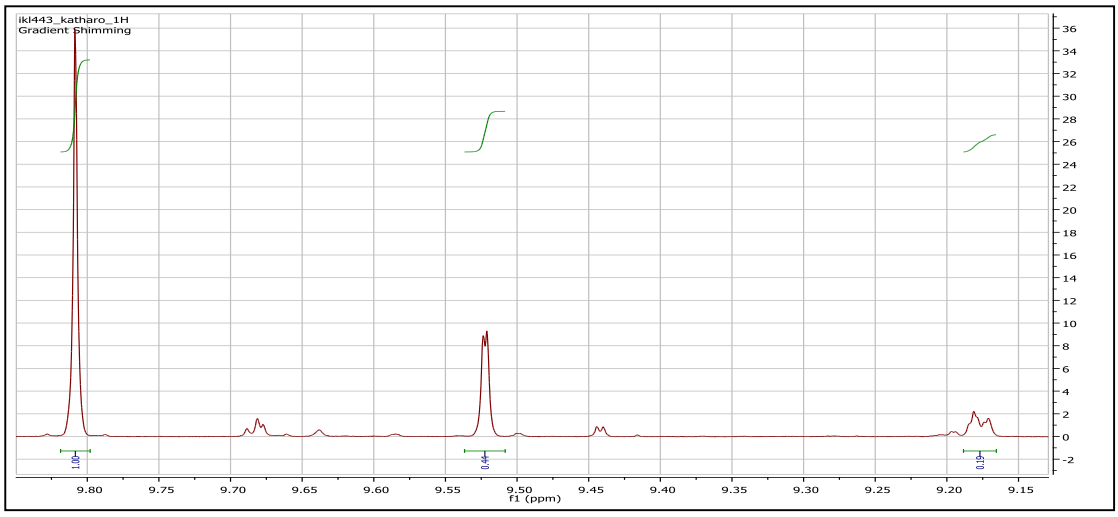
2. Quantification of oleomissional and oleuropein aglycon monoaldehyde closed form

Oleomissional and their two isomers of oleuropeindial are in constant equilibrium with each other and all of them give a signal at 9.17 ppm in NMR. They are conversed to the isomers of closed-type of oleuropein aglycon, which have the same molecular weight too. (Mw=378).

We set up two reference curves (one for oleomissional and another for the closed-type of oleuropein aglycon).

Before the construction of the reference curves, we calculated the percentage of oloeomissional and closed-type of oleuropein aglycon in the mixture, using D_1=10 in NMR parameters. Specifically, 23.7 mg oleomissional were dissolved in 10 mL ACN in a volumetric flask.

By considering that nOLM = nSYR, we took 1.688 mL (4 mg) OLM/ACN and added 1 mL (1.928 mg) from the solution of syr/ACN (44 mg/25 mL) in it. Subsequently, we evaporated the new solution to dryness under vacuum and the residue dissolved in $CDCl_3$. Its ^{1}H-NMR spectrum was received.



44% closed-type oleuropein aglycon

19% oleomissional (open form oleuropein aglycons)

According to the analysis protocol for the phenolic content which we use in our laboratory, we need 5 g of olive oil. We chose an olive oil without phenolic content as blind reference and we added in the isolated oleomissional or closed-type of oleuropein aglycon. We proceeded to set up the reference curve for the calculation of oleomissional and closed-type oleuropein aglycon in olive oils.

In the table below, the concentrations of oleomissional or closed-type of oleuropein aglycon solutions in olive oil and the volumes of solvents (ACN, cHex) which were used, are presented. The mixture of oleomissional and closed-type of oleuropein aglycon dissolved in ACN, it mixed with the suitable quantity of ACN, so that **[V_{ACN} (mL) + $V_{sol\ (OLM/ACN)}$ (μL)= 25 mL]** and it was added in every sample of olive oil so that the desirable concentration of the substances was occurred.

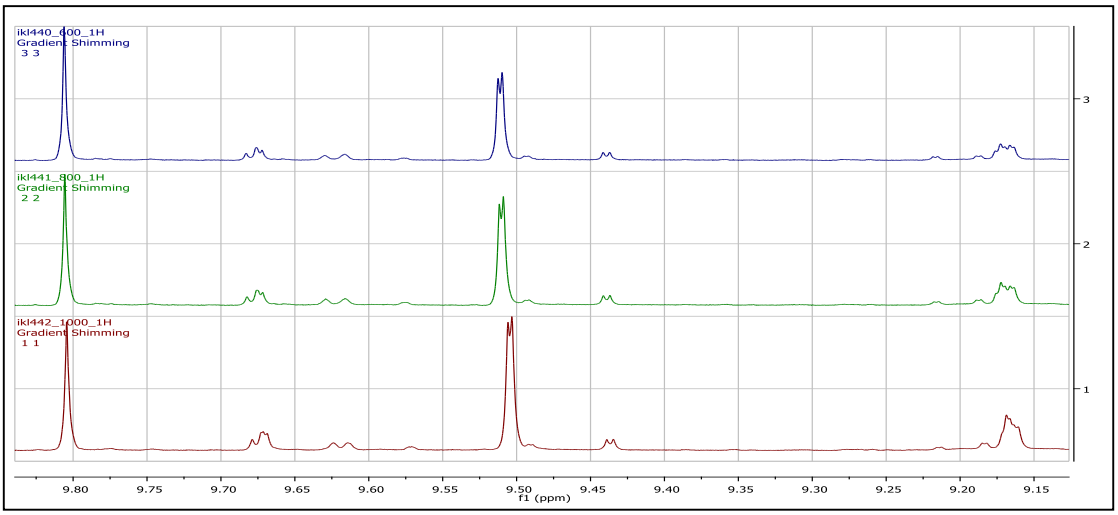
We prepared two solutions:

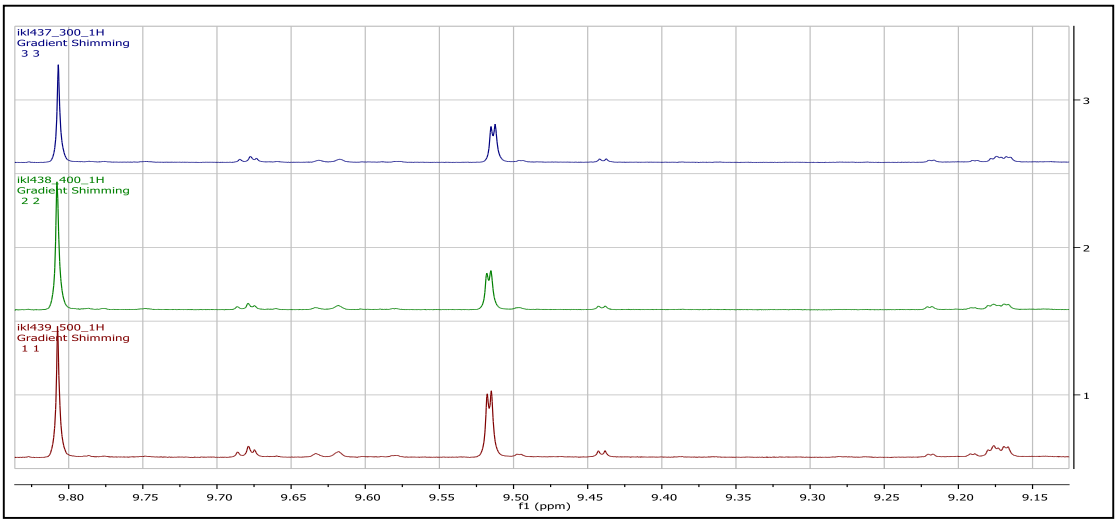
A) 40 mg of the mixture (oleomissional and closed-type of oleuropein aglycon) in 20 mL ACN and

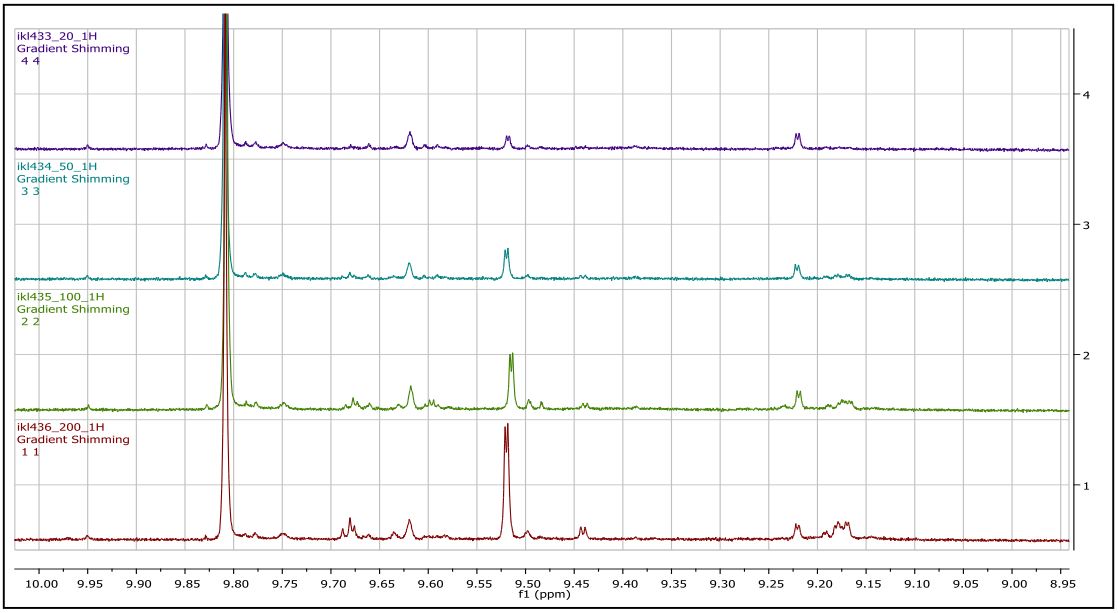
B) 40 mg of the mixture (oleomissional and closed-type of oleuropein aglycon) in 10 mL ACN.

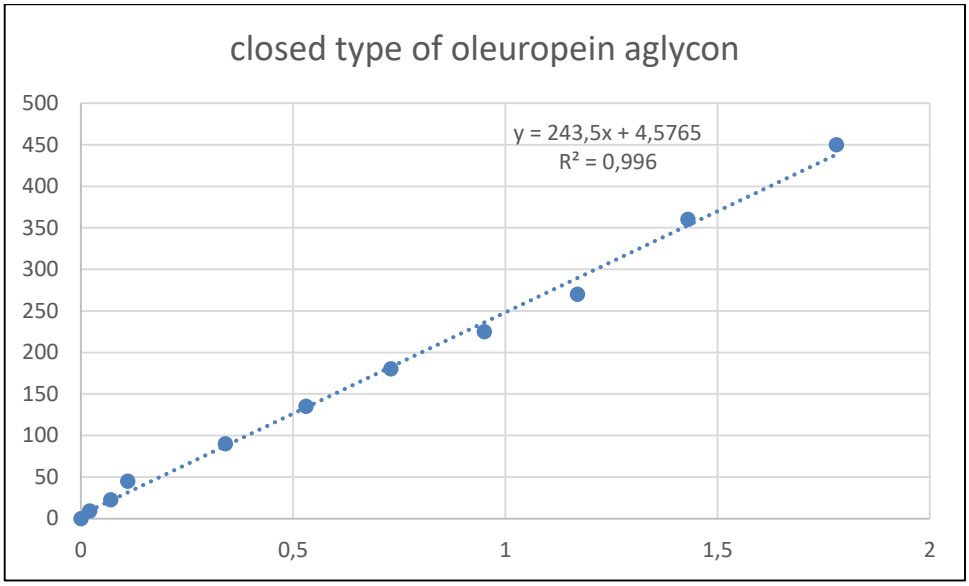
We used the solution A until the creation of sample whose concentration was 600 ppm and the solution B for samples which had 800 and 1000 ppm concentration in our desirable mixture of oleomissional and closed-type of oleuropein aglycon.

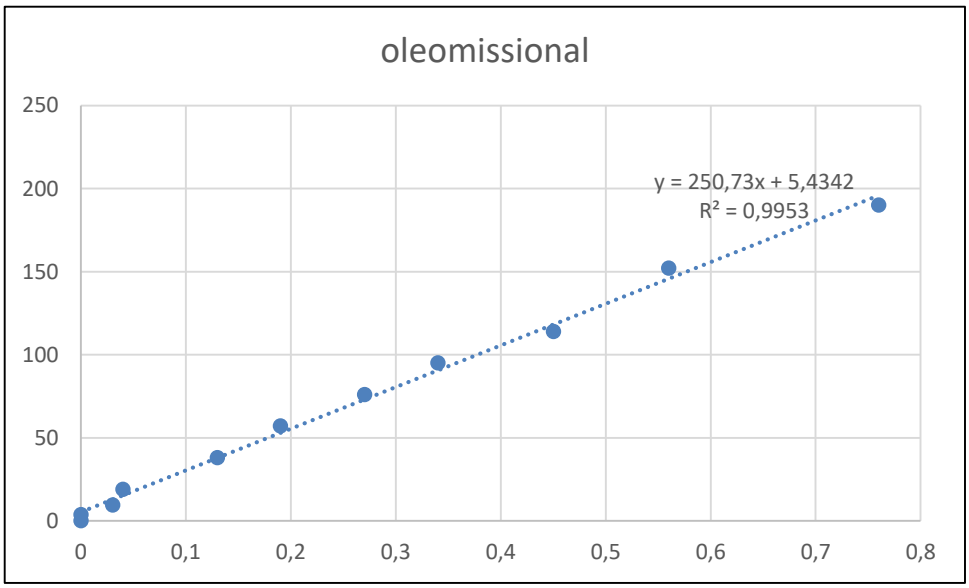
C (mg/kg)	**Spectrum number**	**V_{ACN} (mL)**	**V_{cHex} (mL)**	**V_{sol} (µL) OLM/ACN**
20	Ikl433	24.95	20	50
50	Ikl434	24.875	20	125
100	Ikl435	24.75	20	250
200	Ikl436	24.5	20	500
300	Ikl438	24.25	20	750
400	Ikl437	24	20	1000
500	Ikl439	23.75	20	1250
600	Ikl440	23.5	20	1500
800	Ikl441	24	20	1000
1000	Ikl442	23.75	20	1250


ikl440_600_1H
Gradient Shimming
3 3
ikl441_800_1H
Gradient Shimming
2 2
ikl442_1000_1H
Gradient Shimming
1 1
f1 (ppm)


ikl437_300_1H
Gradient Shimming
3 3
ikl438_400_1H
Gradient Shimming
2 2
ikl439_500_1H
Gradient Shimming
1 1
f1 (ppm)


ikl433_20_1H
Gradient Shimming
4 4
ikl434_50_1H
Gradient Shimming
3 3
ikl435_100_1H
Gradient Shimming
2 2
ikl436_200_1H
Gradient Shimming
1 1
f1 (ppm)


closed type of oleuropein aglycon
y = 243,5x + 4,5765
R² = 0,996
500
450
400
350
300
250
200
150
100
50
0
0
0,5
1
1,5
2


oleomissional
y = 250,73x + 5,4342
R² = 0,9953
250
200
150
100
50
0
0
0,1
0,2
0,3
0,4
0,5
0,6
0,7
0,8

ANNEX 2

Optimization of the LC-MS/MS Method

Evaluation of LC-MS/MS as analytical technique suitable for quantitative analysis of oleocanthal and oleacein in olive oil.

This study was carried out to prove the capability of LC-MS/MS for quantitative determination of oleocanthal and oleacein in olive oil. Several limitations have been described on the chromatographic analysis of secoiridoids in olive oil. This study tested different experimental conditions and finally proved that LC-MS/MS can offer quantitative results in the analysis of both compounds.

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Quantitative Method for Determination of Oleacantal and Oleacein in Virgin Olive Oils by Liquid Chromatography-Tandem Mass Spectrometry

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Abstract

Oleocanthal and oleacein, two key secoiridoid derivatives present in virgin olive oil (VOO), are gaining clinical and nutritional interest thanks to their proved bioactivity; therefore, the determination of both phenols is a growing demanded application to increase the value of VOO. The main problem of previously reported liquid chromatography-based methods for oleocanthal and oleacein measurement is their interaction with water or other polar solvents such as methanol to promote the formation of hemiacetal or acetal derivatives. This interaction can occur during either sample extraction, basically liquid–liquid extraction, and/or chromatographic separation. The aim of this research was to evaluate the suitability of LC–MS/MS for absolute quantitation of oleocanthal and oleacein in VOO. For this purpose, both liquid–liquid extraction and chromatographic separation were studied as potential promoters of acetals and hemiacetals formation from oleocanthal and/or oleacein. The results showed that the use of methanol–water solutions for phenols extraction was not influential on the formation of these artifacts. Acetals and hemiacetals from oleocanthal and/or oleacein were only detected at very low concentrations when methanol gradients under acidic conditions were used for chromatographic separation. With this premise, a protocol based on extraction with acetonitrile and a reverse chromatographic gradient with methanol was established to quantify in absolute terms oleocanthal and oleacein in VOO samples. The resulting protocol was applied to three VOO samples characterized by high, medium, and low levels of these two phenols.

Keywords: Virgin olive oil, Oleocanthal, Oleacein, LC–MS/MS, Hemiacetal, Acetal.

1. Introduction

Virgin olive oil (VOO) contains multiple minor components, such as sterols, volatile compounds, and phenols, among the most important families. Olive oil phenols comprise acids, phenolic alcohols, such as tyrosol (abbreviated as *p*-HPEA) and hydroxytyrosol (3,4-DHPEA), flavonoids, lignans, and secoiridoids

(oleuropein, ligstroside and their derivatives). The bioactive capability of phenols present in VOO is a matter of great interest because of the proved or tentatively described healthy effects attributed to them. Additionally, olive oil phenols are major contributors to the long shelf-life and organoleptical characteristics of VOO [1,2]. Two secoiridoid derivatives should be mentioned in this regard, the dialdehydic forms of decarboxymethyl ligstroside and oleuropein aglycones, also known as oleocanthal (*p*-HPEA-EDA), and oleacein (3,4-DHPEA-EDA), respectively (Supplementary Fig. 1) [3]. These compounds are endowed with antimicrobial, anticancer, and hypoglycemic effects, and are considered key oxidation inhibitors among the main responsible for the antioxidant properties of VOO [4]. It is noteworthy to point out that oleacein has been declared a more potent antioxidant than hydroxytyrosol [4]; furthermore, the interest in these derivatives has been enhanced because of their reported anti-inflammatory properties. Thus, oleocanthal has shown intense anti-inflammatory effects comparable to ibuprofen thanks to its capability to inhibit cyclooxygenases COX-1 and COX-2 but not 15-lipooxygenase [5]. Indeed, some authors have pointed out that oleocanthal is one of the main components responsible for the therapeutic properties of VOO [6]. Recently, oleocanthal has also been proposed as a promising agent to induce selectively cancer cell death via lysosomal membrane permeabilization [7]. Concerning sensory properties, oleocanthal is responsible for the burning pungent sensation of VOO [8].

Due to the relevance of these two secoiridoid derivatives the quantitative analysis of them can provide an added value to VOOs and, therefore, an attractive aim of olive breeding programs. However, their quantitation is a pending goal of the characterization of olive oils due to the lack of both knowledge about them and commercial standards. Several methods have been described for analysis of oleocanthal and oleacein in VOO, mainly based on liquid chromatography (LC) separation followed by UV-Vis or mass spectrometry (MS) detection [5,9,10], and by quantitative NMR [11]. Methanol–water mixtures are commonly used for the extraction of phenols from VOO due to their mid-polar character. Nevertheless, some authors have proposed the use of acetonitrile (ACN) since it provides better extraction efficiency than methanol (MeOH) for isolation of secoiridoids and derivatives such as oleocanthal [12]. Recently, researchers have identified a limitation in the determination of oleocanthal and oleacein by LC-based methods explained by the reaction of these dialdehydic compounds with water or MeOH, both used in the extraction step and in the mobile phases for chromatographic separation. Hence, Karkoula et al. [11] studied the reaction of oleocanthal and oleacein with water, MeOH, ACN, chloroform ($CHCl_3$), dimethyl sulfoxide (DMSO), and their mixtures by NMR using deuterated solvents and monitoring the formation of acetals and hemiacetals (Supplementary Fig. 2). Oleocanthal and oleacein provided NMR spectra that corresponded each to a single molecule only in the case of deuterated chloroform, ACN or DMSO; instead, hemiacetal and acetal derivatives were generated in water or MeOH–water mixtures. No studies dealing with the stability of oleocanthal and oleacein and the formation of hemiacetal and acetal derivatives by LC–MS/MS analysis have so far been

reported. This fact could explain the lack of LC-based methods for quantitative analysis of oleocanthal and oleacein in VOO. In the present research, the two main steps (viz., liquid–liquid extraction and chromatographic analysis) that could potentially interfere in the determination of oleocanthal and oleacein by LC–MS/MS have been studied. After this study, a method for absolute quantitation of oleocanthal and oleacein by LC–MS/MS has been proposed.

2. Materials and methods

2.1. Monovarietal virgin olive oil samples

Three monovarietal olive oils from Arbequina (Córdoba, Spain), Picual (Jaén, Spain), and Lianolia Kerkiras (Corfu, Greece) cultivars obtained in the 2014/2015 season were used in this research. Olive fruits were collected in 2014 at intermediate ripening (when the fruit color is changing from yellowish with reddish spots to reddish) from cultivars located in different places. The selection of these varieties was supported on their content in oleocanthal and oleacein described in previous papers [13,14].

2.2. Reagents

The solvents used for analysis of oleocanthal and oleacein in VOOs were LC–MS grade MeOH, ACN, and *n*-hexane, which were from Scharlab (Barcelona, Spain). LC–MS-grade formic acid, also from Scharlab, was used as ionization agent in the chromatographic mobile phases. Deionized water (18 MΩ•cm) from a Millipore Milli-Q water purification system (Bedford, MA, USA) was used to prepare both the aqueous mobile phase and the hydroalcoholic mixture used as extractant.

Oleocanthal and oleacein (purity>98%) were isolated from a VOO extract prepared using the protocol for extraction, purification and characterization described by Karkoula et al. [11,14]. Standard solutions of both compounds (1 mg/mL) were prepared in pure acetonitrile to preserve their stability.

2.3. Apparatus and instruments

An MS2 minishaker from Ika Works (Wilmington, NC, USA) was used to enhance the transfer of phenols from VOOs to the tested extractants prior to quantitation of oleocanthal and oleacein. Phenolic extracts were analyzed by an 1200 Series LC system (Agilent Technologies, Waldbronn, Germany) coupled to an Agilent 6460 triple quadrupole (LC–QqQ-MS) detector furnished with an electrospray ionization (ESI) source. A confirmatory analysis in accurate mode of the two secoiridoids and the corresponding hemiacetals and acetals was conducted by an 1200 Series LC system coupled to an Agilent 6540 quadrupole-time-of-flight (LC–QTOF-MS) hybrid mass spectrometer with a Dual ESI source for simultaneous spraying of the LC eluent and a reference mass solution enabling continuous calibration of detected *m/z* ratios.

2.4. Extraction of oleocanthal and oleacein from VOOs extracts

Two extraction procedures (both based on shaking VOO solutions in hexane with either ACN or an MeOH–water mixture) were applied to isolate both phenols from VOO samples. Thus, 1 g of VOO was diluted with 1 mL of hexane and shaken for 1 min with either 1 mL of a 60:40 (v/v) MeOH–water mixture or 1 mL of ACN. The hydroalcoholic or ACN phase was separated by centrifugation and the extraction process was repeated to attain quantitative extraction as described by Hrncirik and Fritsche [15]. The resulting phenolic extracts were 1:2 or 1:50 diluted, depending on the content of secoiridoid derivatives, and analyzed by LC–QTOF and LC–QqQ MS/MS.

2.5. LC–QTOF MS/MS confirmatory analysis of oleocanthal, oleacein, and acetal forms in extracts from VOO

Identification of the two olive phenols and the hemiacetal and acetal artifacts was conducted by LC–QTOF MS/MS confirmatory analysis in accurate mode. Analyses were performed by reversed-phase liquid chromatography followed by electrospray ionization (ESI) in negative mode and tandem mass spectrometry (MS/MS) detection. Five µL of extract was injected in triplicate into the LC system for chromatographic separation of the target compounds using a C18 Pursuit XRs Ultra (50×2.0 mm i.d., 2.8 µm particle size) from Varian (Walnut Creek, CA, USA). The column compartment was kept at 30 ºC. Mobile phase A was 0.1% formic acid in water, while phase B was 0.1% formic acid in MeOH. The gradient program, at 0.4 mL/min constant flow rate, was as follows: initially 50% phase A and 50% phase B kept for 0.5 min; from 0.5 to 2 min was from 50 to 20%; from 2 to 4 min, mobile phase A was from 20 to 0% A. This last composition was kept for 1 min. After each analysis, the column was equilibrated for 5 min to the initial conditions and pressure equilibration. The total running time of the analysis was 10 min.

The electrospray ionization source was operated in the negative ionization mode, and the flow rate and temperature of the drying gas (N_2) were 10 L/min and 350 ºC, respectively. The nebulizer pressure was 35 psi, and the voltages of the capillary, skimmer, and octopole radiofrequency were 3250, 65, and 90 V, respectively. The focusing voltage set in the first quadrupole was 90 V. The data were acquired in centroid mode in the extended dynamic range (2 GHz). Full scan with subsequent activation of the three most intense precursor ions per scan (only single or double charged ions were allowed) by tandem mass spectrometry (MS/MS) was carried out at 1 spectrum/s in the *m/z* range 50–1700. Three values for collision energy of (15, 20, and 25 eV) were tested by independent runs to increase the MS/MS information for identification of oleocanthal, oleacein, and their acetal and hemiacetal derivatives. An active exclusion window was programmed after one MS/MS spectrum and released after 0.75 min to avoid repetitive fragmentation of the most intense precursor ions and, in this way, increase the detection coverage. Before the experiments,

the instrument reported mass detection resolution of 25000 full width at half maximum (FWHM) at *m/z* 112.9856 and 45000 FWHM at *m/z* 966.0007. To assure the desired mass accuracy of recorded ions, continuous internal calibration was performed during analyses with the use of signals at *m/z* 119.0363 (proton abstracted purine) and at *m/z* 966.0007 (formate adduct of hexakis(1H,1H,3H-tetrafluoropropoxy)phosphazine). Identification of the compounds and their product ions proceeded by generation of candidate formulae with a mass accuracy limit of 5 ppm.

2.6. LC–QqQ MS/MS analysis of oleocanthal and oleacein in extracts from VOOs

Quantitative analysis was carried out by LC–QqQ MS/MS after identification of both phenols in VOO. The analytical column, mobile phases with the substitution of MeOH as phase B, and gradient program were those used for qualitative analysis by LC–QTOF. The volume of injected extract was also 5 µL. The entire eluate was electrosprayed and monitored by MS/MS in Selected Reaction Monitoring (SRM) mode of selective transitions from precursor to product ions for each analyte. The flow rate and temperature of the drying gas (N_2) were 10 L/min and 300 ºC, respectively. The nebulizer pressure was 50 psi and the capillary voltage 3000 V. The dwell time was set at 200 ms/spec.

2.7. Quantitation of the target compounds

Absolute quantitative analysis was performed by preparing calibration curves using refined olive oil spiked with oleocanthal and oleacein standards. The absence of quantifiable levels of both phenols in the refined oil was checked by direct analysis with the developed method. Nine concentrations from 0.01 µg/mL to 5 µg/mL were injected in triplicate to obtain the calibration curves. The concentrations of oleacein and oleocanthal in the monovarietal VOOs were determined with these models using three replicates per sample. Concerning the acetals and hemiacetals formed during analysis, they were relatively quantified by using the calibration model of the corresponding phenol.

3. Results and discussion

3.1. Determination of oleocanthal and oleacein in VOO

Quantitative analysis of oleocanthal and oleacein in VOOs by LC-based methods suffers from the limitations described by some authors regarding to formation of hemiacetal or acetal derivatives which can interfere in the analysis of these oleopentanedialdehydes [11,16]. Karkoula et al. [11] reported that 96% of the methyl hemicacetals (Supplementary Fig. 2) was generated in MeOH or 1:1 MeOH–water mixture as solvent, while the oleocanthal and/or oleacein monohydrates were detected only when water was used.

According to these results the authors developed a method for direct measurement of oleocanthal and oleacein by ^{1}H NMR without involvement of any potentially reactive solvent.

To confirm the presence of oleocanthal and oleacein in monovarietal VOO samples included in the present study, analysis of the two pure standards by LC–QTOF MS/MS was first programmed using for the separation of the peaks the MeOH chromatographic gradient above mentioned. Oleacein and oleocanthal are characterized by the same dialdehydic structure, the only difference between them being the phenolic moiety, hydroxytyrosol and tyrosol, respectively. Extracted ion chromatograms (EIC) for $[M–H]^-$ ions from standards of oleocanthal with *m/z* 303.1238 and oleacein with *m/z* 319.1181 showed two peaks at 1.45 (Fig. 1a) and 1.00 min (Fig. 1b), respectively, which were clearly identified by MS/MS fragmentation. Fragmentation of the precursor ion *m/z* 303.1238 generated five representative product ions, two of which, detected at *m/z* 137.0608 and *m/z* 119.0505, corresponded to tyrosol and its principal fragment when activated by MS/MS. Two other fragments were detected at *m/z* 139.0767 and *m/z* 123.0445, which were assigned to the dialdehydic moiety, released after separation of the tyrosol, and its main fragment, respectively, as shows Fig. 1a. The fifth ion at *m/z* 59.0135 fit the acetoxy fragment associated to the ester bound. Fig. 1b illustrates the fragmentation of oleacein that led to two main ion products at *m/z* 139.0767 and at *m/z* 59.0135 corresponding to the dialdehydic moiety and the acetoxy fragment released after separation of hydroxytyrosol by analogy to oleocanthal. Besides, one ion at *m/z* 123.0448 was clearly identified as the hydroxytyrosol main fragment, which allowed confirming the identity of oleacein. Fig. 2 shows the EICs corresponding to both phenols provided by analysis of a VOO sample after liquid–liquid extraction with 60:40 (v/v) MeOH–water. The analysis of the hydroalcoholic extract from the VOO sample also allowed detecting the presence of acetals and hemiacetals from oleocanthal and oleacein, which were identified by virtue of the same fragmentation patterns described for their precursors. The dimethyl acetal of oleacein was detected at *m/z* 365.1500, while the analog for oleocanthal was not detected at its *m/z* value at 349.1651. Concerning hemiacetal derivatives, only the methyl hemiacetals were found in the hydroalcoholic extracts from VOO. The oleocanthal and oleacein methyl hemiacetals were found at *m/z* 335.1500 and *m/z* 351.1449, respectively. The MS/MS spectra of acetals and hemiacetals were characterized by the presence of representative fragments of oleocanthal and oleacein at *m/z* 137.0627 and *m/z* 123.0448, respectively. Apart from that, fragments at *m/z* 139.0739 and *m/z* 59.0135, which are also typical from the structure of these secoiridoid derivatives, were detected. Fig. 2 also shows the MS/MS spectra obtained from the methyl hemiacetals from oleocanthal and oleacein and the dimethyl acetal from oleacein. A mass difference in the acetals/hemiacetals MS/MS spectra was observed by loss of 14 Da, which fits the cleavage of the methyl group with the formation of the hydroxyl group.

After confirming the presence of oleocanthal and oleacein in VOO and verifying the formation of hemiacetals and acetals during LC–QTOF MS/MS analysis of hydroalcoholic extracts, an optimization study was designed to develop an MS/MS method based on SRM by LC–QqQ MS/MS. The selection of the SRM transitions and the corresponding acquisition parameters (e.g. the isolation voltage of the first quadrupole and collision energy) were optimized by using phenolic extracts from monovarietal VOOs. The most sensitive transitions from precursors to product ions were used for quantitation of oleocanthal and oleacein, and the corresponding hemiacetals and acetals; whereas secondary transitions were used for confirmatory analysis. A summary of the SRM method is listed in Table 1 that also includes the calibration models, limits of detection and quantitation (LODs and LOQs, respectively), and precision estimated as within-day variability (expressed as percentage of relative standard deviation).

3.2. Influence of sample preparation on the determination of oleocanthal and oleacein

MeOH–water mixtures (the exact composition depending on the target phenols) are frequently used as extractant for isolation of phenolic compounds from VOO. Thus, hydroxytyrosol and tyrosol, with polar character, are better extracted by mixtures with a high concentration of water, while flavonoids and secoiridoids demand for a high proportion of organic solvent. With these premises, the most used extractant composition for isolation of phenols from VOO is 60:40 (v/v) MeOH–water. On the other hand, LC–MS/MS analyses are mainly carried out with reversed-phase gradients from aqueous to methanolic phase under acidic conditions, usually with formic acid, to enhance the ionization of phenols prior to MS detection. Therefore, two potential steps can be involved in the formation of acetals and hemiacetals: extraction and chromatographic separation.

The first study was aimed at knowing the influence of the phenols extraction procedure on the formation of hemiacetals and acetals from oleocanthal and oleacein. For this purpose, MeOH–water extracts were analyzed by LC–QqQ MS/MS in SRM mode to evaluate the formation of derivatives by comparison with extracts obtained with ACN, which does not promote the formation of derivatives from oleocanthal and oleacein. A chromatographic gradient based on ACN was used to minimize the formation of acetals and hemiacetals by LC–MS/MS analysis. Fig. 3 shows the SRM chromatograms obtained by analysis of MeOH–water and ACN extracts from Arbequina and Picual VOOs representing the behavior of the three analyzed monovarietal oils. As can be seen, the formation of acetal and hemiacetal derivatives in the extract was not detected by LC–QqQ MS/MS. The presence of peaks in the extracted ion chromatograms corresponding to the transition 349→137, for monitoring the dimethyl acetal of oleocanthal, is due to the formation of formic acid adducts of oleocanthal. These results allowed deducing that the formation of acetal/hemiacetal artifacts was not influenced by extraction with hydroalcoholic mixtures under these conditions. Additionally, the

quantitative responses led to the conclusion that ACN provided similar extraction efficiency as MeOH–water for phenols in VOOs (data not shown).

Methanolic extracts from VOOs were analyzed again after three months storage at –20 ºC. These analyses allowed discarding the formation of acetals and hemiacetals during the storage period, as shows Supplementary Fig. 3; that is, the reaction did not progress when the extracts are stored at –20 ºC.

3.3. Influence of the chromatographic method on the determination of oleocanthal and oleacein

The influence of the mobile phase on the conversion of oleocanthal and oleacein into hemiacetal and acetal derivatives was also studied. For this purpose, two chromatographic gradients using MeOH and ACN as organic solvents (phase B) were tested for analysis of phenolic extracts obtained with MeOH–water or ACN as extractants. Table 2 shows the relative concentrations, expressed as percentage, as obtained for each compound under the tested experimental conditions. As can be seen, acetals and hemiacetals of oleocanthal and oleacein were only detected at very low concentrations with methanol-based gradients, as also reveals Fig. 4 for ACN extracts. This could be explained by the acidic pH used in the chromatographic separation according to De Nino et al. [17], who found enhanced formation of acetal derivatives in acid media. The formation of methyl hemiacetals was slightly favoured over that of oleocanthal and oleacein monohydrates. In fact, in this work the formation of the monohydrate forms was not observed, which is in agreement with the results obtained by Karkoula et al. [11]. In relative terms, the free form of oleacein constituted 93.9 ± 0.2% of its total concentration in the extracts from VOO samples using MeOH-based chromatographic gradients and ACN extraction, while its methyl hemiacetal represented 5.1 ± 0.3% and the dimethyl acetal derivative was only detected in VOO at 1.0 ± 0.2%. On the other hand, the relative concentration of oleocanthal was estimated around 90.2 ± 1.5% in the ACN extracts from VOOs, while the methyl hemiacetal represented 9.8 ± 1.0% in terms of concentration. However, the dimethyl acetal form of oleocanthal was not detected in any of the extracts from the target monovarietal VOOs. The low conversion rate clearly shows that the use of MeOH gradients in the chromatographic separation should not be discarded since oleocanthal and oleacein could be accurately quantified. In addition, the quantitative response observed for the two phenols was clearly higher in MeOH-based chromatographic gradients than in those using ACN (Supplementary Fig. 4) and the chromatographic resolution was clearly better with the former gradient.

It is worth mentioning that a *t*-test analysis (p-value < 0.05) revealed that no statistically significant differences on the percentages were observed by using MeOH–water or ACN as extractants for the two tested chromatographic methods, as shows Table 2. According to this result, the analysis of phenolic profiles should be carried out after extraction with MeOH–water mixtures due to the variability in the polar character of single phenols. On the other hand, if the determination is targeted at secoiridoids, extraction with ACN

constitutes the best strategy because the interferences from compounds more polar than the target analytes would be avoided.

3.4. Quantitative determination of oleocanthal and oleacein in VOO samples

Once proved that the formation of acetal and hemiacetal derivatives is not kinetically favoured under the experimental conditions described above, quantitative analysis of oleocanthal and oleacein in three VOO samples was planned. For this purpose, the protocol based on phenol extraction with ACN was applied, while the LC–QqQ MS/MS analysis was based on the MeOH gradient due to the ionization efficiency of the target phenols, higher in the MeOH phase than in ACN medium. Absolute quantitation was performed by using the calibration models prepared with oleocanthal and oleacein standards spiked in refined oil (see Table 1). The analyses (n=3) showed that Greek Lianolia Kerkiras VOO contained high concentration of oleocanthal and oleacein (with 537 ± 59 and 392 ± 47 □g/g, respectively). Concerning the two monovarietal VOOs obtained from the two typical Spanish varieties, Picual led to the intermediate levels of oleocanthal with 153 ± 17 □g/g as compared to Arbequina VOO with 67 ± 7 □g/g, while these VOOs provided similar levels of oleacein with 69 ± 8 and 63 ± 7 □g/g for Picual and Arbequina, respectively. It is worth mentioning that a comparison among monovarietal VOOs is not a pursued aim of this research since it is well-known that the concentration of phenols is strongly dependent on several factors, apart from genotype, such as climatic, growing location, fruit ripening, agronomic factors, and mechanical extraction system. As emphasized above, these three monovarietal VOOs were selected according to their content in oleocanthal and oleacein supported on the results cited in the literature.

4. Conclusions

Attending to the results obtained in this study, LC–QqQ MS/MS can be used for quantitative analysis of oleocanthal and oleacein in VOO samples under the conditions described in this research as the conversion of these phenols to acetal and hemiacetal derivatives is very low. The high sensitivity and selectivity levels of SRM makes LC–QqQ MS/MS a competitive technique for analysis of these two phenolic compounds with bioactive properties.

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Figure Legends

Figure 1. Extracted ion chromatograms (EICs) and MS/MS spectra of (a) oleocanthal and (b) oleacein standards by LC–QTOF analysis.

Figure 2. Extracted ion chromatograms (EICs) and MS/MS spectra provided by LC–QTOF analysis of a MeOH–water extract from Picual VOO using the MeOH-based gradient: (a) oleacein and its methyl hemiacetal and dimethyl acetal; (b) oleocanthal and its methyl hemiacetal.

Figure 3. Chromatograms obtained in selected reaction monitoring mode from analysis of MeOH–water and ACN extracts from (a) Arbequina and (b) Picual VOOs using the ACN chromatographic gradient.

Figure 4. Chromatograms obtained in selected reaction monitoring mode from LC–QqQ MS/MS analysis of ACN extracts from (a) Picual and (b) Arbequina monovarietal VOOs using a MeOH-based gradient.

Figure 1

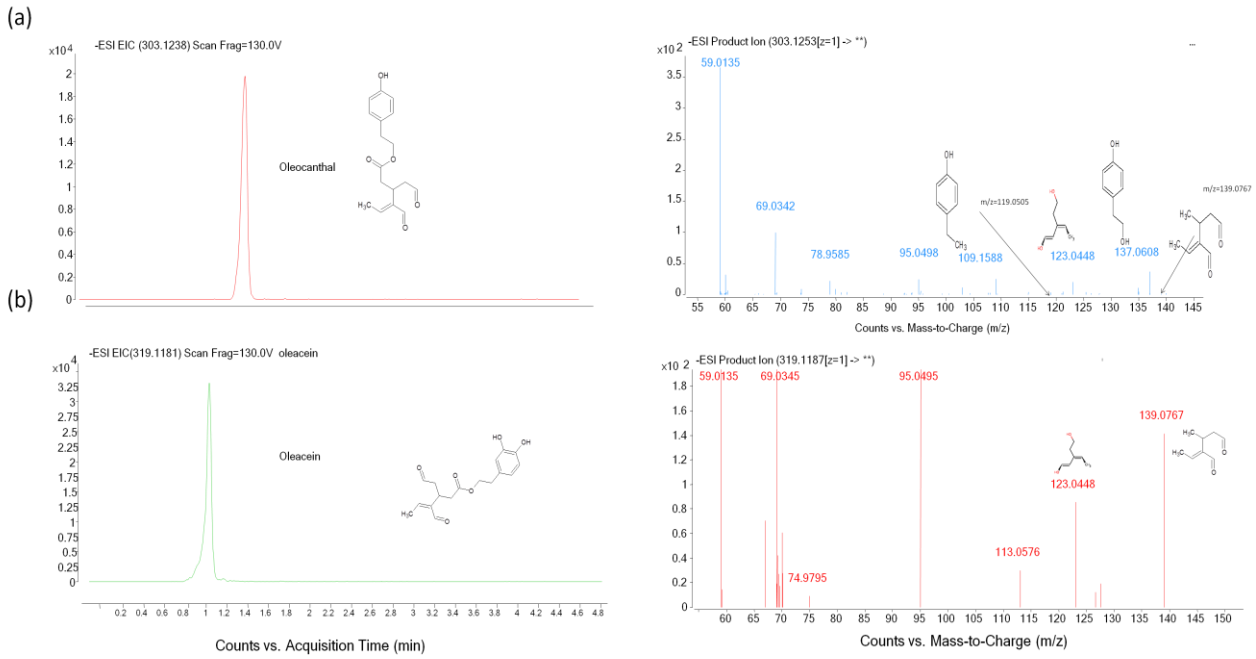
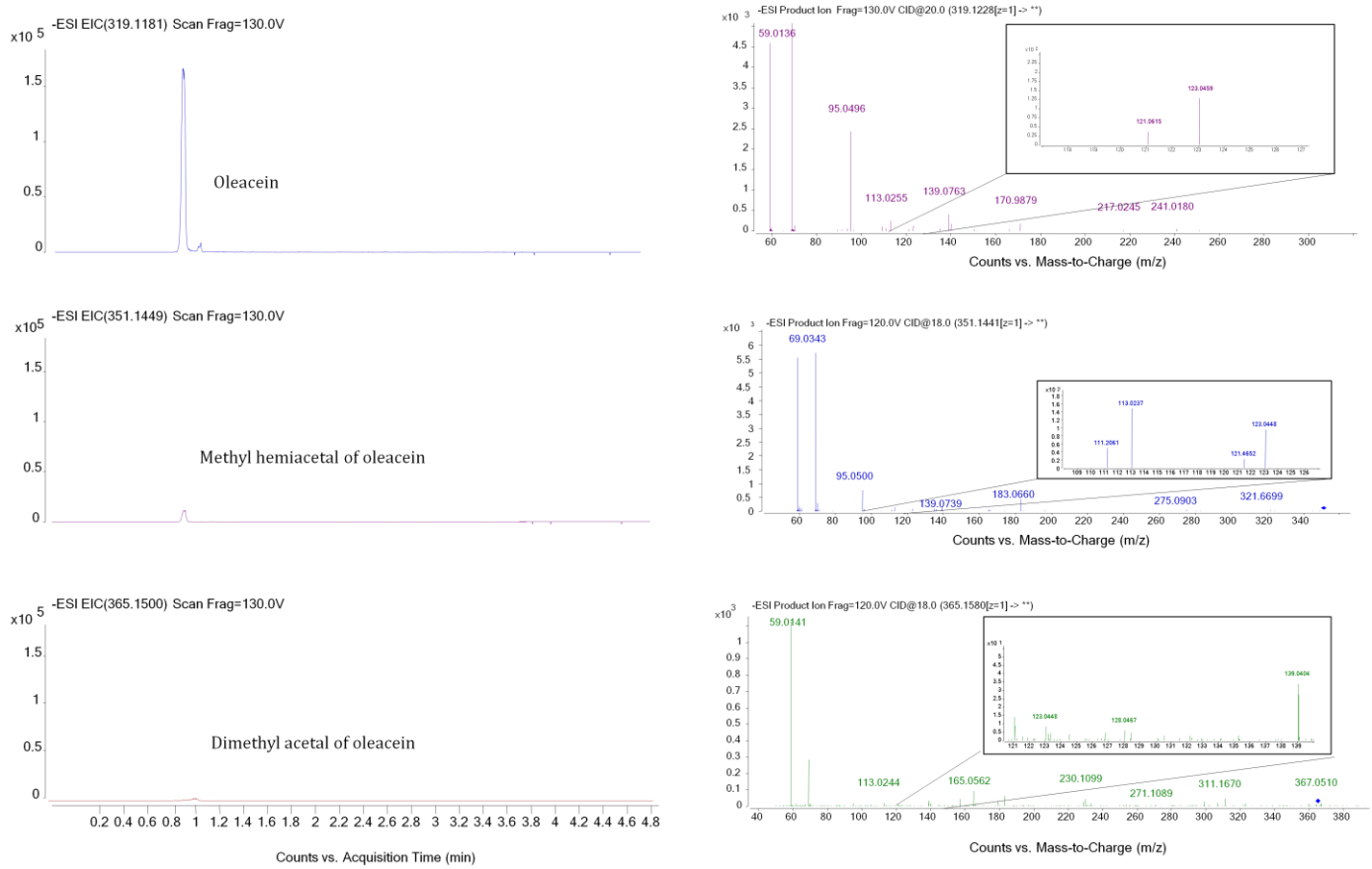

(a)
-ESI EIC (303.1238) Scan Frag=130.0V
Oleocanthal
-ESI Product Ion (303.1253[z=1] -> **)
59.0135
69.0342
78.9585
95.0498
109.1588
123.0448
137.0608
Counts vs. Mass-to-Charge (m/z)
(b)
-ESI EIC(319.1181) Scan Frag=130.0V oleacein
Oleacein
Counts vs. Acquisition Time (min)
-ESI Product Ion (319.1187[z=1] -> **)
59.0135
69.0345
74.9795
95.0495
113.0576
123.0448
139.0767
Counts vs. Mass-to-Charge (m/z)

Figure 2a


-ESI EIC(319.1181) Scan Frag=130.0V
Oleacein
-ESI Product Ion Frag=130.0V CID@20.0 (319.1228[z=1] -> **)
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95.0496
113.0255
139.0763
170.9879
217.0245
241.0180
Counts vs. Mass-to-Charge (m/z)
-ESI EIC(351.1449) Scan Frag=130.0V
Methyl hemiacetal of oleacein
-ESI Product Ion Frag=120.0V CID@18.0 (351.1441[z=1] -> **)
69.0343
95.0500
139.0739
183.0660
275.0903
321.6699
Counts vs. Mass-to-Charge (m/z)
-ESI EIC(365.1500) Scan Frag=130.0V
Dimethyl acetal of oleacein
Counts vs. Acquisition Time (min)
-ESI Product Ion Frag=120.0V CID@18.0 (365.1580[z=1] -> **)
59.0141
113.0244
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230.1099
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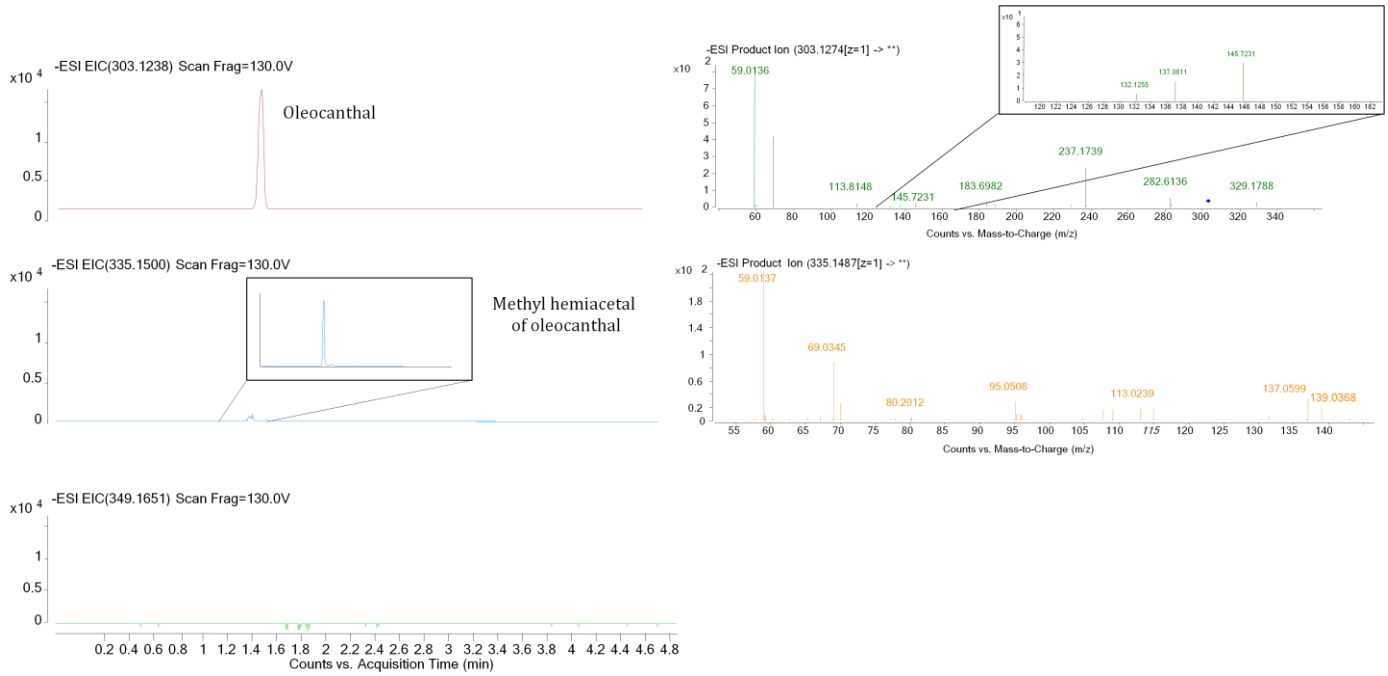
367.0510
Counts vs. Mass-to-Charge (m/z)

Figure 2b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Oleocanthal
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145.7231
183.6982
237.1739
282.6136
329.1788
Counts vs. Mass-to-Charge (m/z)
-ESI EIC(335.1500) Scan Frag=130.0V
Methyl hemiacetal of oleocanthal
-ESI Product Ion (335.1487[z=1] -> **)
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80.2012
95.0508
113.0239
137.0599
139.0368
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Counts vs. Acquisition Time (min)

Figure 3

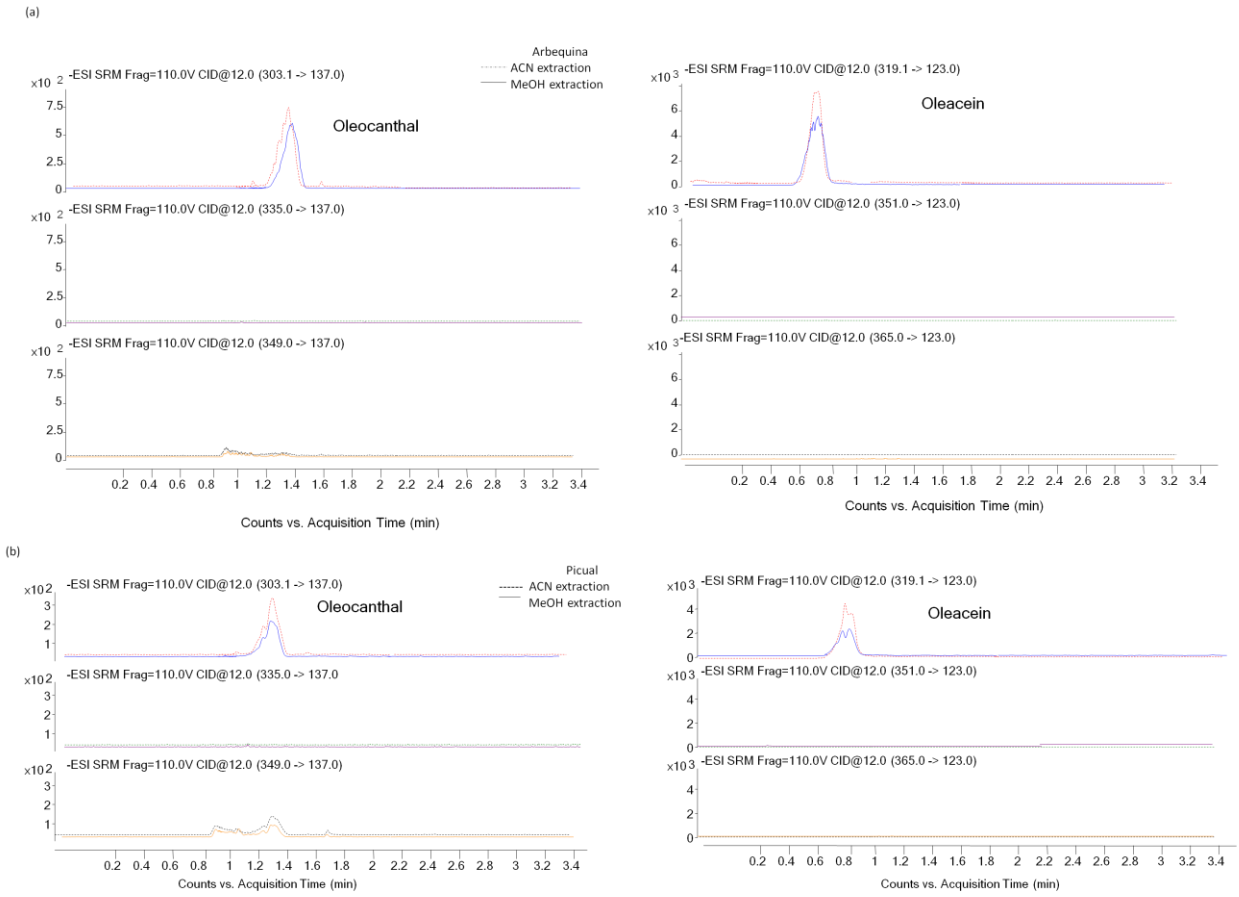

(a)
Arbequina
ACN extraction
MeOH extraction
-ESI SRM Frag=110.0V CID@12.0 (303.1 -> 137.0)
Oleocanthal
-ESI SRM Frag=110.0V CID@12.0 (335.0 -> 137.0)
-ESI SRM Frag=110.0V CID@12.0 (349.0 -> 137.0)
-ESI SRM Frag=110.0V CID@12.0 (319.1 -> 123.0)
Oleacein
-ESI SRM Frag=110.0V CID@12.0 (351.0 -> 123.0)
-ESI SRM Frag=110.0V CID@12.0 (365.0 -> 123.0)
Counts vs. Acquisition Time (min)
(b)
Picual
------ ACN extraction
MeOH extraction
-ESI SRM Frag=110.0V CID@12.0 (303.1 -> 137.0)
Oleocanthal
-ESI SRM Frag=110.0V CID@12.0 (335.0 -> 137.0
-ESI SRM Frag=110.0V CID@12.0 (349.0 -> 137.0)
-ESI SRM Frag=110.0V CID@12.0 (319.1 -> 123.0)
Oleacein
-ESI SRM Frag=110.0V CID@12.0 (351.0 -> 123.0)
-ESI SRM Frag=110.0V CID@12.0 (365.0 -> 123.0)
Counts vs. Acquisition Time (min)

Figure 4a

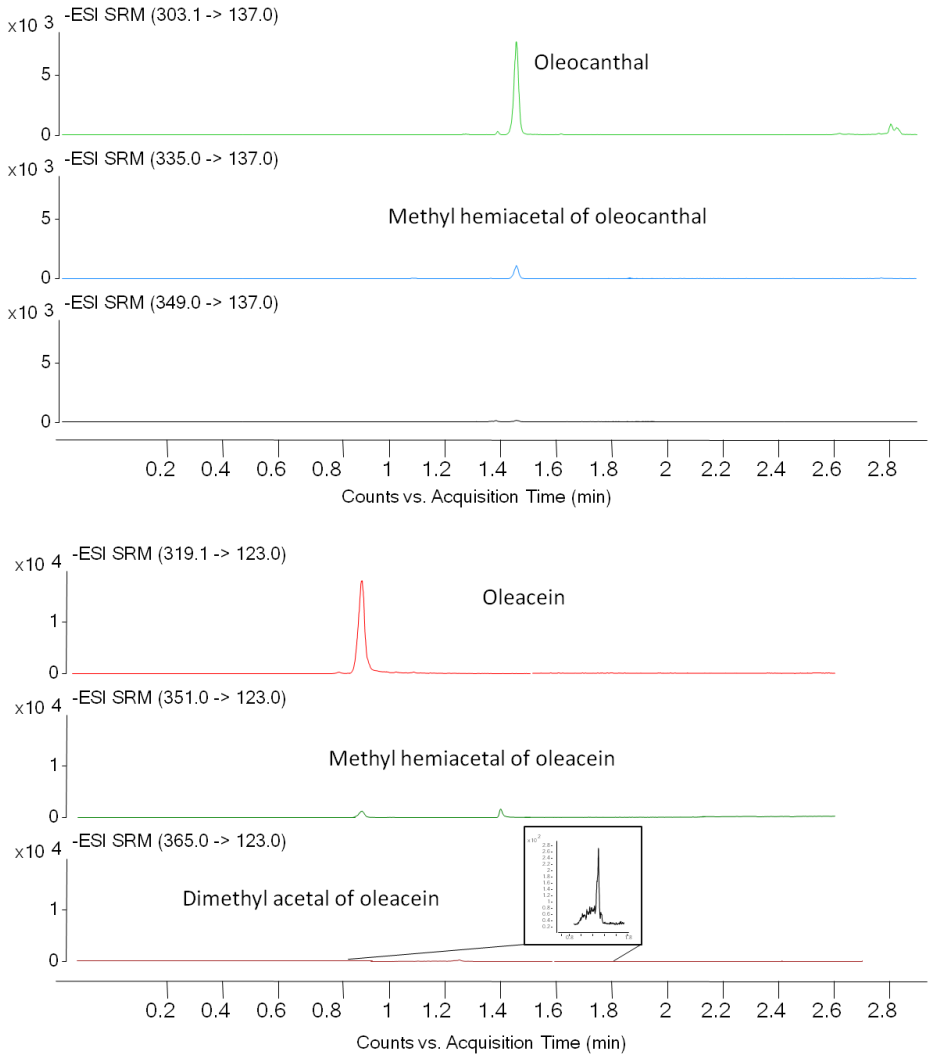

-ESI SRM (303.1 -> 137.0)
Oleocanthal
-ESI SRM (335.0 -> 137.0)
Methyl hemiacetal of oleocanthal
-ESI SRM (349.0 -> 137.0)
Counts vs. Acquisition Time (min)
-ESI SRM (319.1 -> 123.0)
Oleacein
-ESI SRM (351.0 -> 123.0)
Methyl hemiacetal of oleacein
-ESI SRM (365.0 -> 123.0)
Dimethyl acetal of oleacein
Counts vs. Acquisition Time (min)

Figure 4b

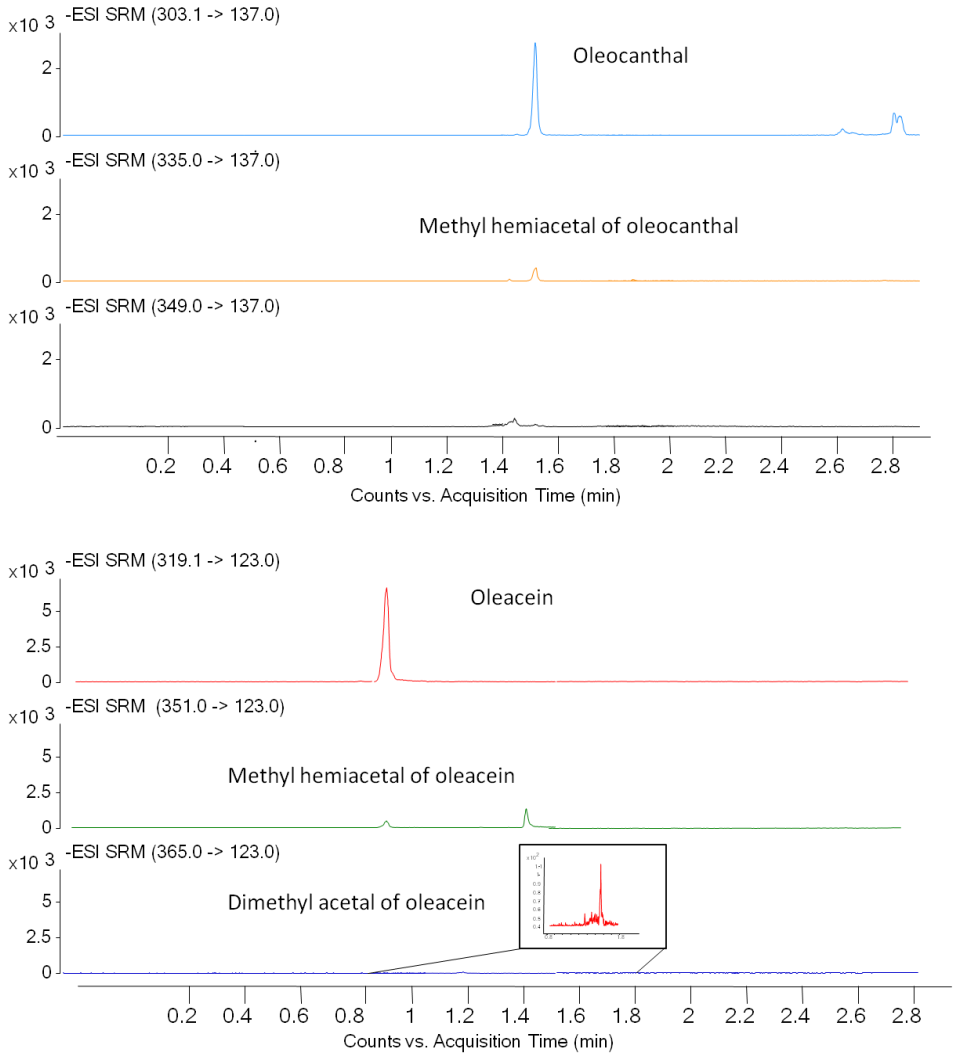

-ESI SRM (303.1 -> 137.0)
Oleocanthal
-ESI SRM (335.0 -> 137.0)
Methyl hemiacetal of oleocanthal
-ESI SRM (349.0 -> 137.0)
Counts vs. Acquisition Time (min)
-ESI SRM (319.1 -> 123.0)
Oleacein
-ESI SRM (351.0 -> 123.0)
Methyl hemiacetal of oleacein
-ESI SRM (365.0 -> 123.0)
Dimethyl acetal of oleacein
Counts vs. Acquisition Time (min)

Table 1.

(A) Optimization of the LC–QqQ MS/MS step for qualitative and quantitative determination of oleacein and oleocanthal.

Compound	**Precursor ion**	**Q1 voltage (V)**	**Collision energy (eV)**	**Quantitative transition (*m/z*)**	**Product ion confirmation (*m/z*)**
Oleacein	319	110	18	319→123	59, 137, 139
Methyl hemiacetal of oleacein	351	110	18	351→123	139, 59
Dimethyl acetal of oleacein	365	110	18	365→123	139, 59
Oleocanthal	303	110	18	303→137	119,139,59
Methyl hemiacetal of oleocanthal	335	110	18	335→137	139, 59
Dimethyl acetal of oleocanthal*	349	110	18	349→137	139,59

*SRM transitions defined by analogy to the acetal derivative of oleacein.

(B) Analytical features of the method for quantitative determination of oleacein and oleocanthal in olive oils by LC–QqQ MS/MS.

Compound	**Calibration model**	**Coefficient of regression (R^2)**	**LOD (µg/mL)**	**LOQ (µg/mL)**	**Within day variability (RSD)**
Oleacein	$y = 5749.8x + 306.7$	0.992	0.002	0.005	11%
Oleocanthal	$y = 2778.4x + 213.5$	0.999	0.004	0.01	10%

Table 2. Relative concentration (expressed as percentage) of oleocanthal, oleacein and their hemiacetals and acetals as an average (n=3) of the target VOOs as a function of the chromatographic method.

	MeOH mobile phase		**ACN mobile phase**	
Compound	**Extraction with MeOH–water**	**Extraction with ACN**	**Extraction with MeOH–water**	**Extraction with ACN**
Oleacein	94.2 ± 0.5	93.9 ± 0.2	100	100
Methyl hemiacetal of oleacein	5.2 ± 0.4	5.1 ± 0.3	0	0
Dimethyl acetal of oleacein	0.6 ± 0.1	1.0 ± 0.2	0	0
Oleocanthal	90.2 ± 1.5	90.2 ± 1.0	100	100
Methyl hemiacetal from oleocanthal	9.8 ± 1.5	9.8 ± 1.0	0	0
Dimethyl acetal of oleocanthal	0	0	0	0

Supplementary Figure Legends

Supplementary Figure 1. Chemical structure of *p*-HPEA-EDA (oleocanthal) and 3,4-DHPEA-EDA (oleacein).

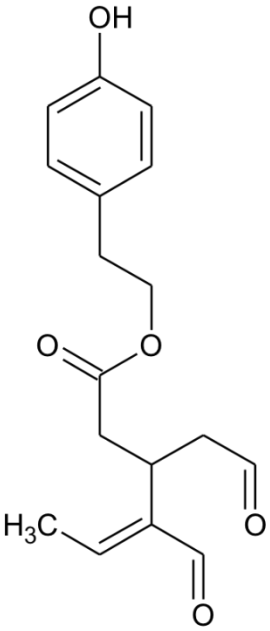
Supplementary Figure 2. Chemical structure of hemiacetals and acetals from oleocanthal and oleacein.

Supplementary Figure 3. Chromatograms obtained in the selected reaction monitoring mode from analysis of MeOH–water extracts stored for three months at –20 ºC from Arbequina (Fig. 3a) and Picual (Fig. 3b) monovarietal VOOs.

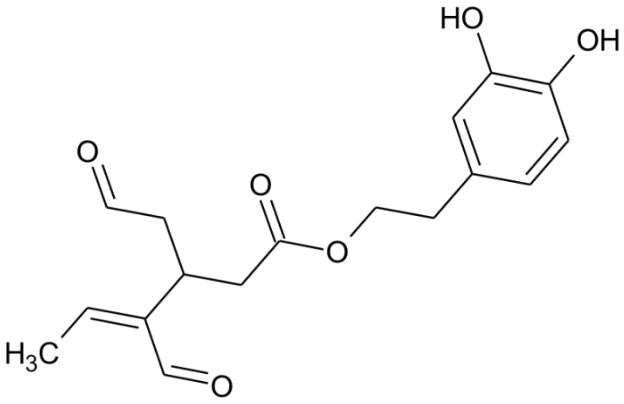
Supplementary Figure 4. Chromatograms obtained in the selected reaction monitoring mode from analysis of oleocanthal and oleacein in ACN extract from Picual VOO with MeOH and ACN chromatographic gradients.

Supplementary Figure 1

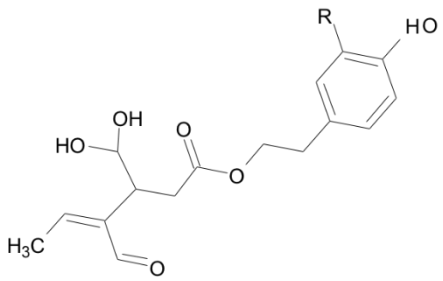
p-HPEA-EDA (Oleocanthal)



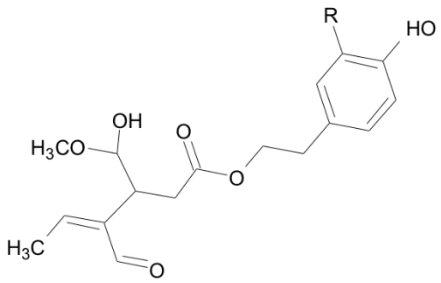
3,4-HPEA-EDA (Oleacein)



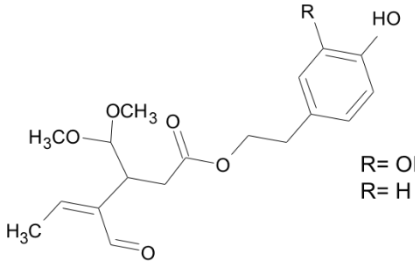
Supplementary Figure 2



R= OH Oleacein monohydrate
R= H Oleocanthal monohydrate

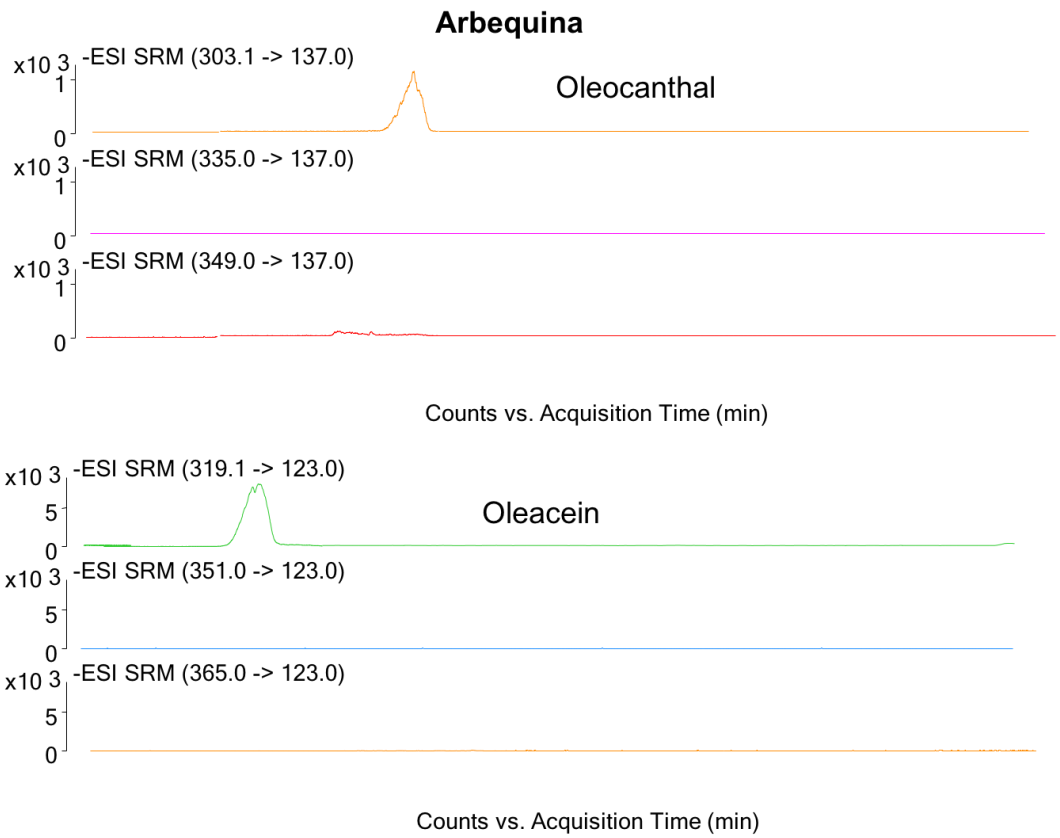


R= OH Methyl hemiacetal of oleacein
R= H Methyl hemiacetal of oleocanthal

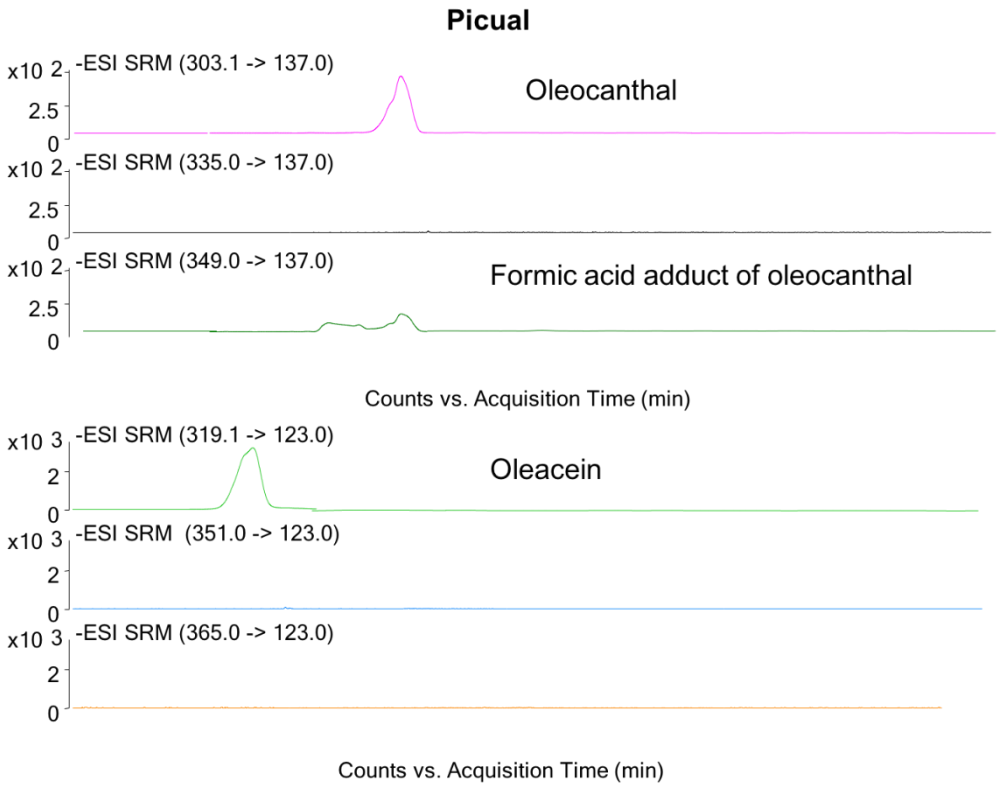


R= OH Dimethylacetal of oleacein
R= H Dimethylacetal of oleocanthal

Supplementary Figure 3 a



Supplementary Figure 3 b



Suplemmentary Figure 4