



QUANTIFICATION OF ALKALOIDS IN
CORDIA:
Cordia lutea L.

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1. Plant Material.

Cordia lutea L. (Boraginaceae) was identified by Blga. Felicia Díaz Jarama (Universidad Nacional de la Amazonia Peruana) and provided to us by Dr. José González Cabanillas on July 22, 2014 (Sabell Peru, SAC; 2.2 kg of powdered plant; DHL # 5619428566). Plant material is kept in cold (5 °C) in a sealed plastic bag.

2. Extractions, Separation and Purification.

(See Flow Diagram in page 4)

2.1. Soxhlet extraction.[1]

In a 5 L Soxhlet apparatus powdered plant (1110 g) was extracted with hexane (3 L, 3 h). Hexane extract was concentrated to dryness under vacuum to give an orange syrup (approx. 25 g, sample named **HEX**). Soxhlet extraction of the defatted plant was then carried out with methanol (4 L, 7 h). Methanol extract gave negative Mayer's Reagent test. Methanol was

removed under vacuum to give a brownish syrup named crude methanolic extract (CME, 310 g).

2.1.1. Standard acid-base treatment. The crude methanolic extract (CME, 100 g) was treated with a solution of HCl in water (5% w/v, 1.5 L) under stirring for 4 h. After this period, solid was filtered off and acidic solution was basified with 30% aqueous NH_3 . Then, the basic mixture was extracted with chloroform (3 x 100 mL). Organic extract was washed with water, dried over anhydrous MgSO_4 , filtered and concentrated under vacuum to dryness to give a yellowish syrup named extract AB (0.15 g). The Mayer's Reagent test of this sample was also negative. Sample AB was analyzed by ^1H NMR and GC-MS.

2.1.2 Acid-base treatment and Zn dust reduction.[2] The crude methanolic extract (CME, 78 g) was treated with a solution of H_2SO_4 in water (2 M, 0.5 L) and stirred overnight with excess of Zn dust. After filtration, the acidic solution was extracted with chloroform (3 x 150 mL). The aqueous solution was basified with 30% aqueous NH_3 (pH > 9). Then, the basic mixture was extracted with chloroform (3 x 100 mL). Organic extract was washed with water, dried over anhydrous MgSO_4 , filtered and concentrated under vacuum to dryness to give a yellowish syrup named extract ZN (0.2 g). The Mayer's Reagent test of this sample was negative. Sample ZN was analyzed by ^1H NMR and GC-MS.

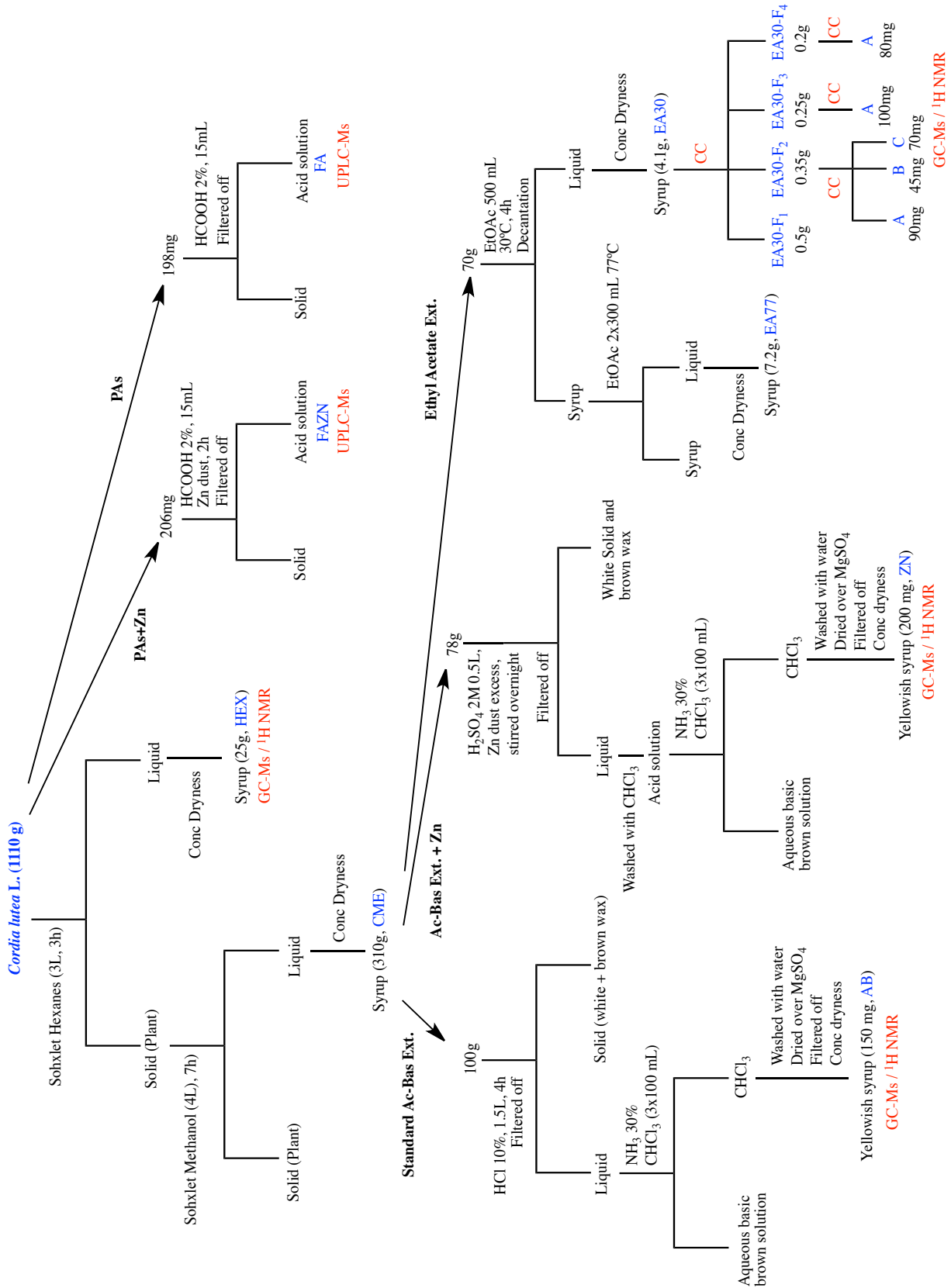
2.1.3. Ethyl acetate extraction. In order to study the flavonoids and related compounds [3], the crude methanolic extract (CME, 70 g) was treated with ethyl acetate (500 mL, 4 h, 30 °C). The organic layer was decanted and the solvent was removed under vacuum to give the 30 °C crude acetate extract, named as EA30 (4.1 g). The oily residue was then extracted with ethyl acetate (2 x 300 mL) under reflux to give the EA77 crude acetate extract (7.2 g). Samples EA30 and EA77 were analyzed by ^1H NMR and MS. EA30 was chromatographed in SiO_2 eluting with mixtures of CHCl_3 :MeOH and fractions EA30-F1, EA30-F2, EA30-F3 and EA30-F4 were obtained. Purification of these fraction was carried out by flash column chromatography.

2.2. Direct pyrrolizidine alkaloid UPLC-MS analysis.[4]

2.2.1. Formic acid extraction and zinc reduction. *Cordia lutea* ground plant material (206 mg) was extracted with 2% formic acid (15 mL). Excess of zinc dust was added to the formic acid solution and shaken for 2 h to reduce the PAs *N*-oxides. If during shaking no visible hydrogen bubbles were formed, additional zinc was added. Heliotrine (100 µg/mL in methanol) was added as an internal standard to a concentration of 1 µg/mL. An aliquot of the extract (FAZN) was diluted with water and injected in the UPLC-MS system. Pyrrolizidine alkaloids were not detected.

2.2.2. Formic acid extraction. *Cordia lutea* ground plant material (198 mg) was extracted with 2% formic acid (15 mL). The plant extract solution was shaken for 1 h. Solid plant material was removed by filtration. Heliotrine (100 µg/mL in methanol) was added as an internal standard to a concentration of 1 µg/mL. An aliquot of the extract (FA) was diluted with water and injected in the UPLC-MS system. Pyrrolizidine alkaloids were not detected.

Flow Diagram of extraction, separation and purification of *Cordia lutea*.



3. Analytical Methods.

- TLC analyses were performed on silica gel 60 F 254 plates
- Separation of extracts and purification of compounds was carried out by flash column chromatography on silica-gel (40-63 μm) eluting with mixtures of CHCl_3 :MeOH, CH_2Cl_2 :MeOH in ascending methanol percentage.
- ^1H and ^{13}C NMR spectra were recorded with a 400 MHz ARX 400 Bruker spectrometer by using the residual solvent peak in CDCl_3 (δ_{H} 7.24 ppm for ^1H and δ_{C} 77.0 ppm for ^{13}C).
- Mass spectrometry was carried out in a Waters Quattro microGC instrument by using the GC injection mode in an Agilent 7890A chromatograph and electron ionization (EI) mode or in a Waters Synap G2 mass spectrometry system coupled to an UPLC Acquity H Class.

4. Results.

- Analytical samples of the extracts were studied by GC-MS and UPLC-MS in order to detect the presence of pyrrolizidine alkaloids.[5-8]
- **HEX**, **AB** and **ZN** extracts were studied by GC-MS (see gas chromatograms in appendix) and pyrrolizidines were not detected.
- **FAZN** and **FA** extracts were studied by UPLC-MS. Alkaloids were not detected. Two intense picks at the UPLC chromatogram at MW 678 *amu* and 452 *amu* were observed.
- The alkaloid content in this plant, if any, it is very little. Mayer's Reagent tests were negative in all cases and fractions corresponding to alkaloids (**AB** and **ZN**) were very small by weight.
- ^1H NMR spectrum of **HEX** fraction shows the presence of fatty acids as main

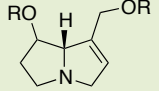
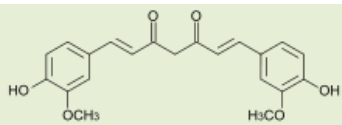
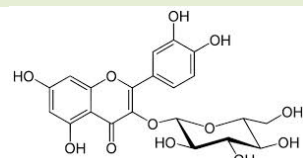
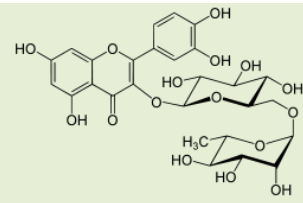
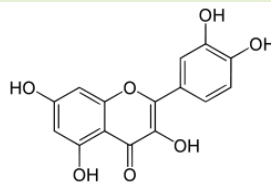
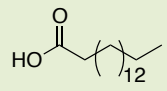
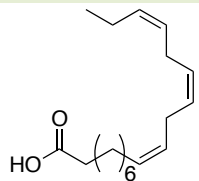
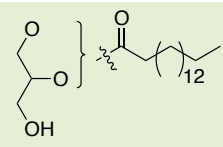
compounds.

- In any of the mass analyses, compounds of MW of 597, 613 or 629 *amu* mentioned on the A+ chemical characterization and assigned to acetogenins were not detected.
- Extractions with ethyl acetate (EA₃₀ and EA₇₇ fractions) were carried out to study the non-alkaloidal content of this plant. Composition of both fractions is very similar (¹H NMR).
- Separation of fraction EA₃₀ on silica gel column chromatography afforded rutin, quercetin, linolenic acid, hexadecanoic acid and hexadecanoic acid glyceryl ester. From fraction EA₃₀-F₂ were isolated quercetin, hexadecanoic acid, linolenic acid and hexadecanoic acid glyceryl ester, from fraction EA₃₀-F₃ was isolated rutin, and finally from fraction EA₃₀-F₄ was isolated a compound still unidentified (EA₃₀-F₄-A). ¹H NMR of this compound suggests a flavonoid glycosil eter structure.
- Isolated compounds were characterized by MS, ¹³C NMR and ¹H NMR.
- Quercetin in *Cordia lutea* should be present only as glycosyl eter and not as isolated flavonoid. UPLC analysis confirms this point since there is a coincidence in the R_t for the mass pics (see appendix, UPLC data).

5. Conclusions.

- Pyrrolizidine alkaloids have not been detected in any of the extracts of this plant (see included certification).
- Alkaloids were not detected.
- Main active components of *Cordia lutea* L. are flavonoids.
- Main chemical composition in *Cordia lutea* L. is shown in Table I.

Table I. Chemical composition of *Cordia lutea* L.

Compound	Fraction	MW (amu)	% of CME	Structure
Pyrrolizidine Alkaloids	Non-Detected	--	--	
Other Alkaloids	Non-Detected	--	--	Nitrogen derivatives
Curcumin	Non-Detected	--	--	
Isoquercetin	Non-Detected	--	--	
Rutin	EA30-F3-A	610	0.14	
Quercetin	EA30-F2-C	302	0.10	
Hexadecanoic Acid (Palmitic acid)	HEX fraction and EA30-F2-A	256	0.13	
Linolenic Acid	HEX fraction and EA30-F2-A	278	0.13	
Hexadecanoic Acid Mono Glycerol Ester	EA30-F2-B	312	0.06	
Flavonoid Derivative (?)	EA30-F4-A	(?)	0.11	(?)



APPENDIX

ANALYTICAL DATA



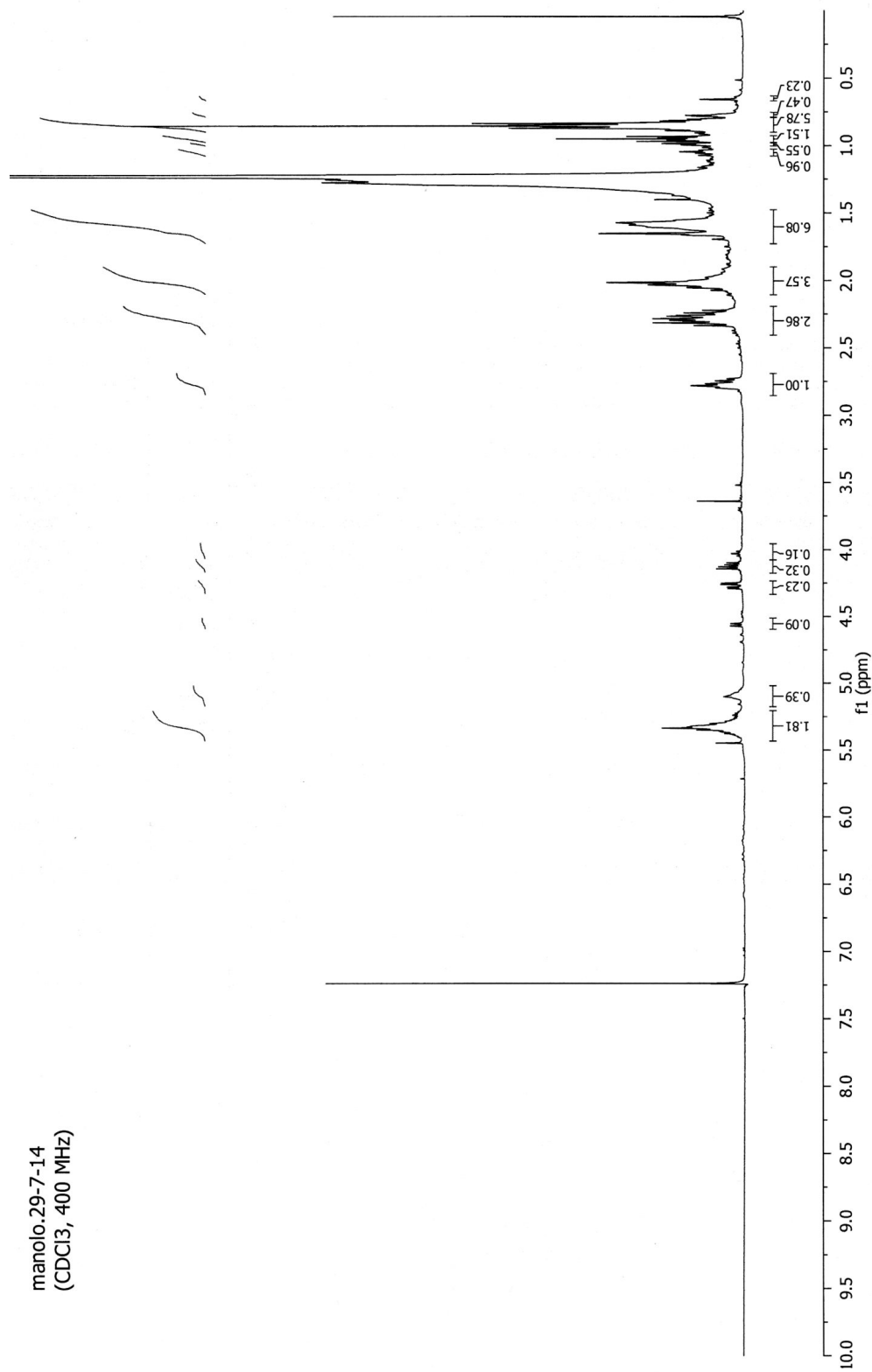
Cordia lutea Plant:





HEX sample.

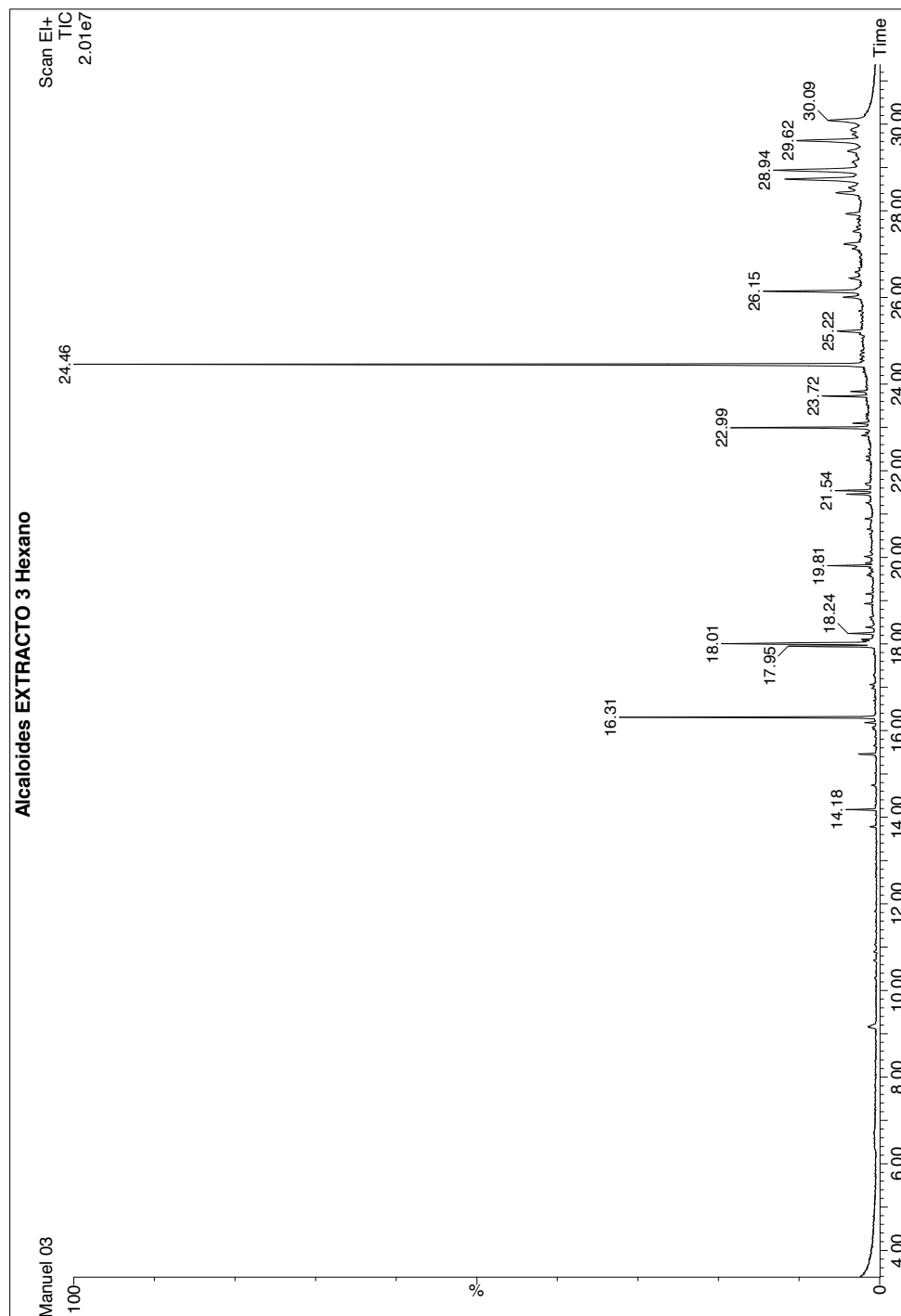
$^1\text{H NMR CDCl}_3$





HEX sample.

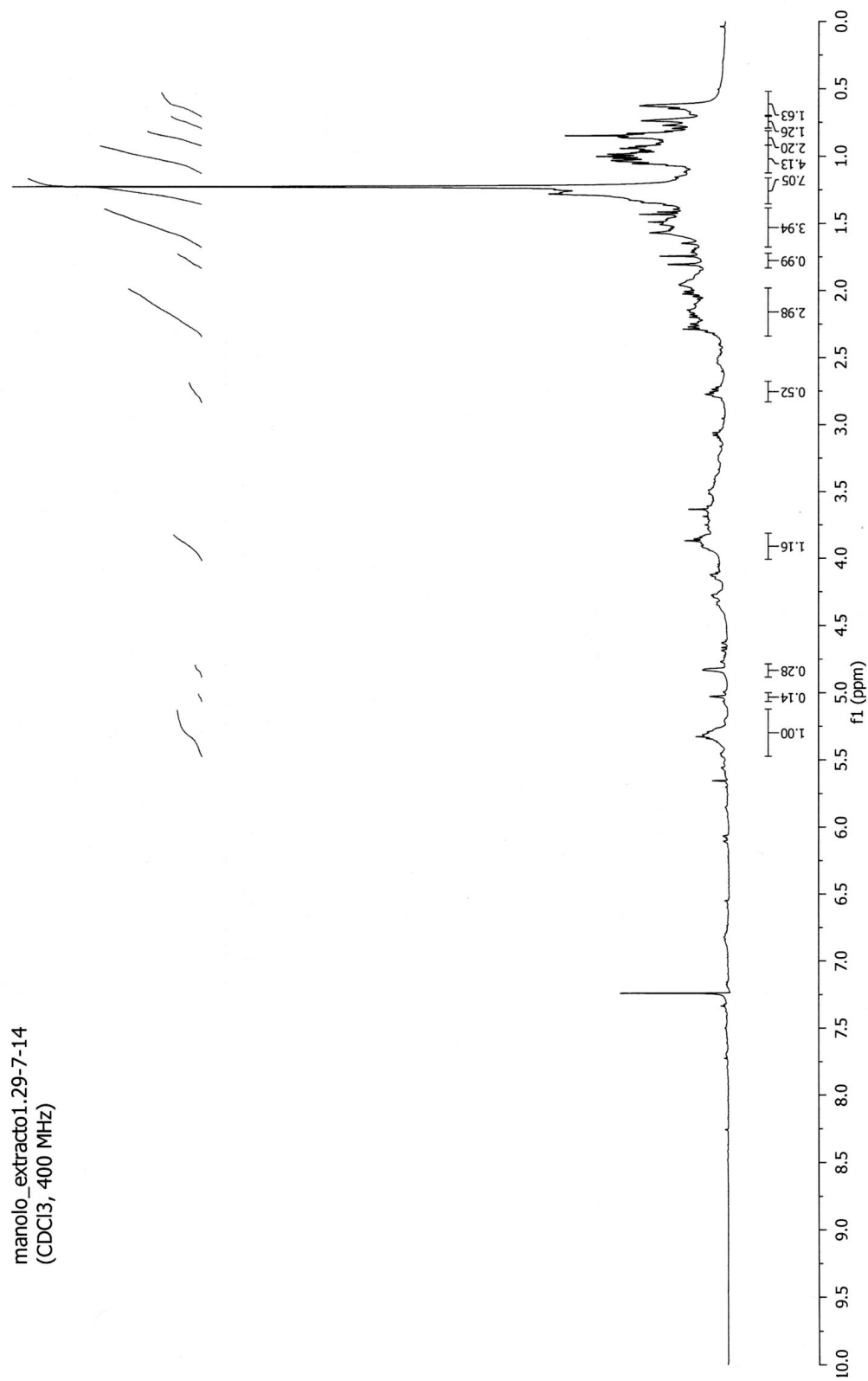
Gas Chromatography





AB sample.

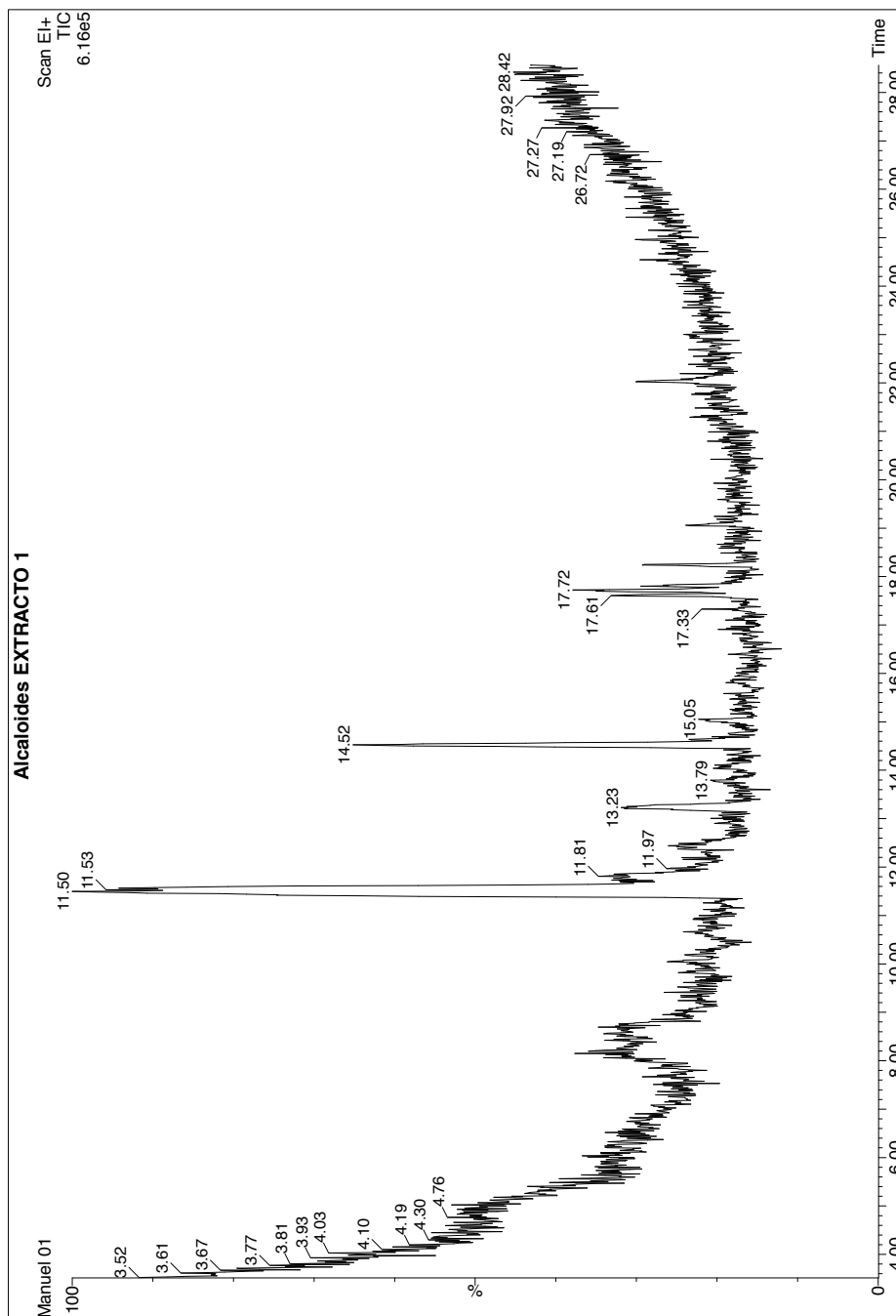
¹H NMR





AB sample.

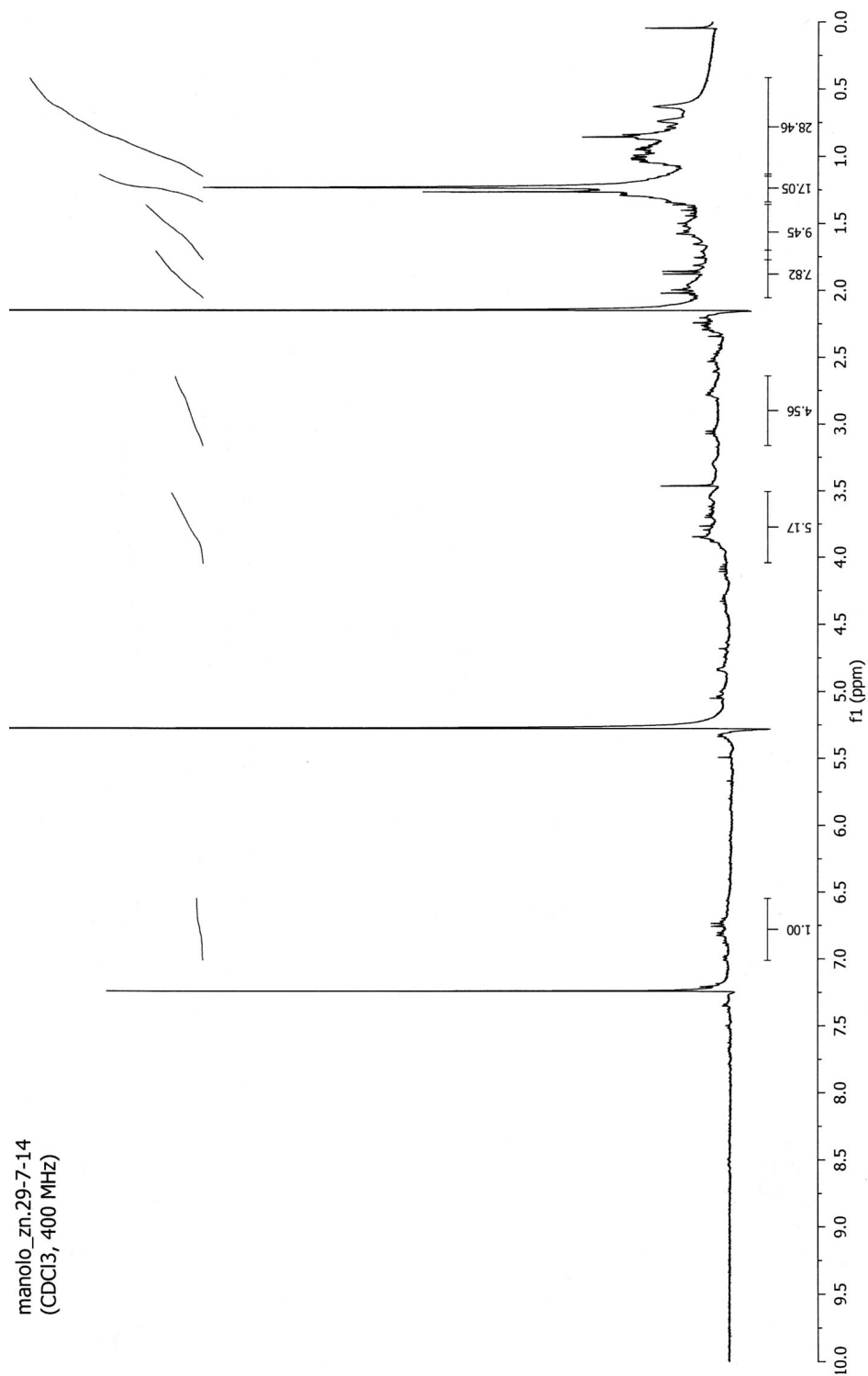
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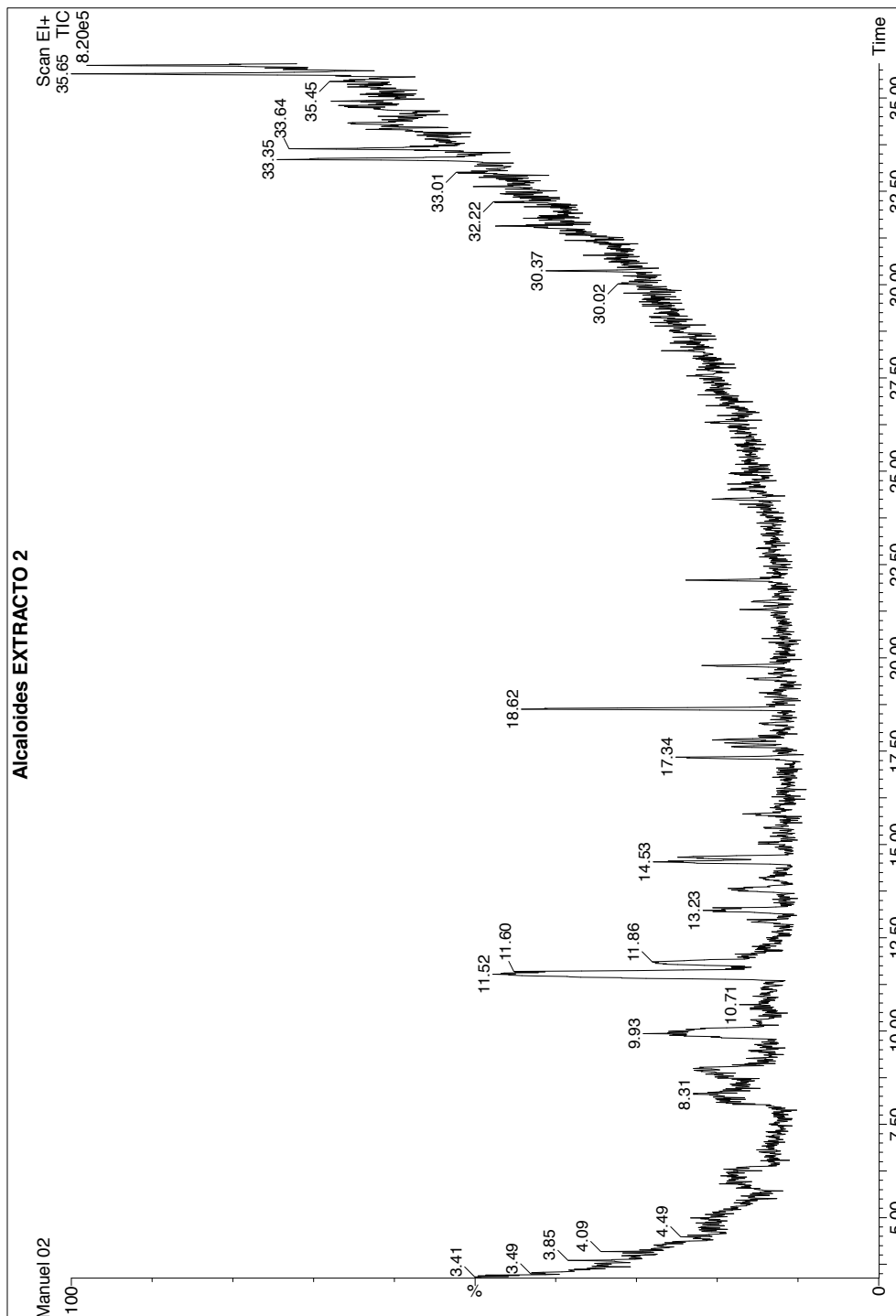
ZN sample.

¹H NMR



ZN sample.

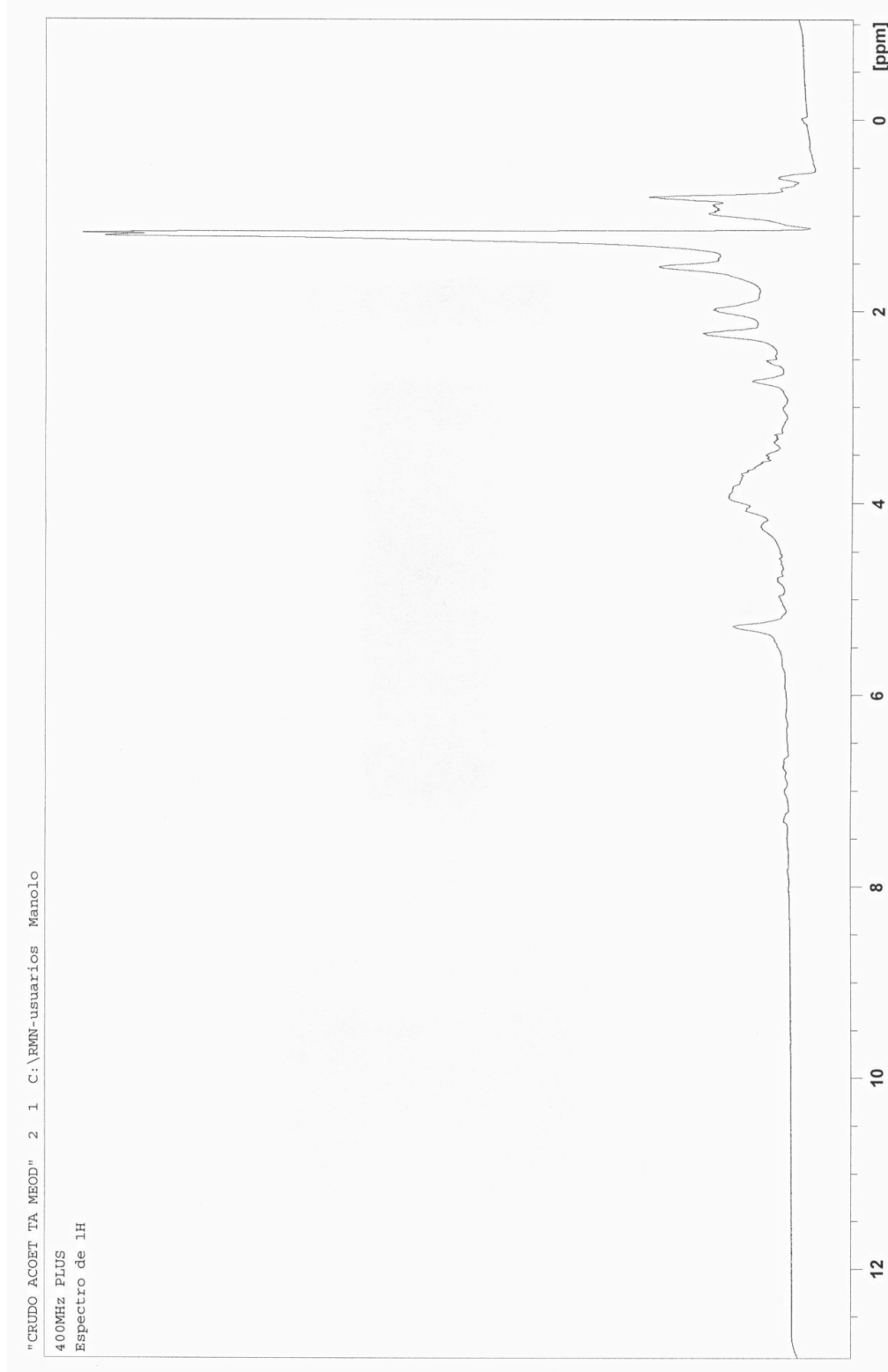
Gas Chromatography





EA₃₀ sample.

¹H NMR CDCl₃:MeOD





EA77 sample.

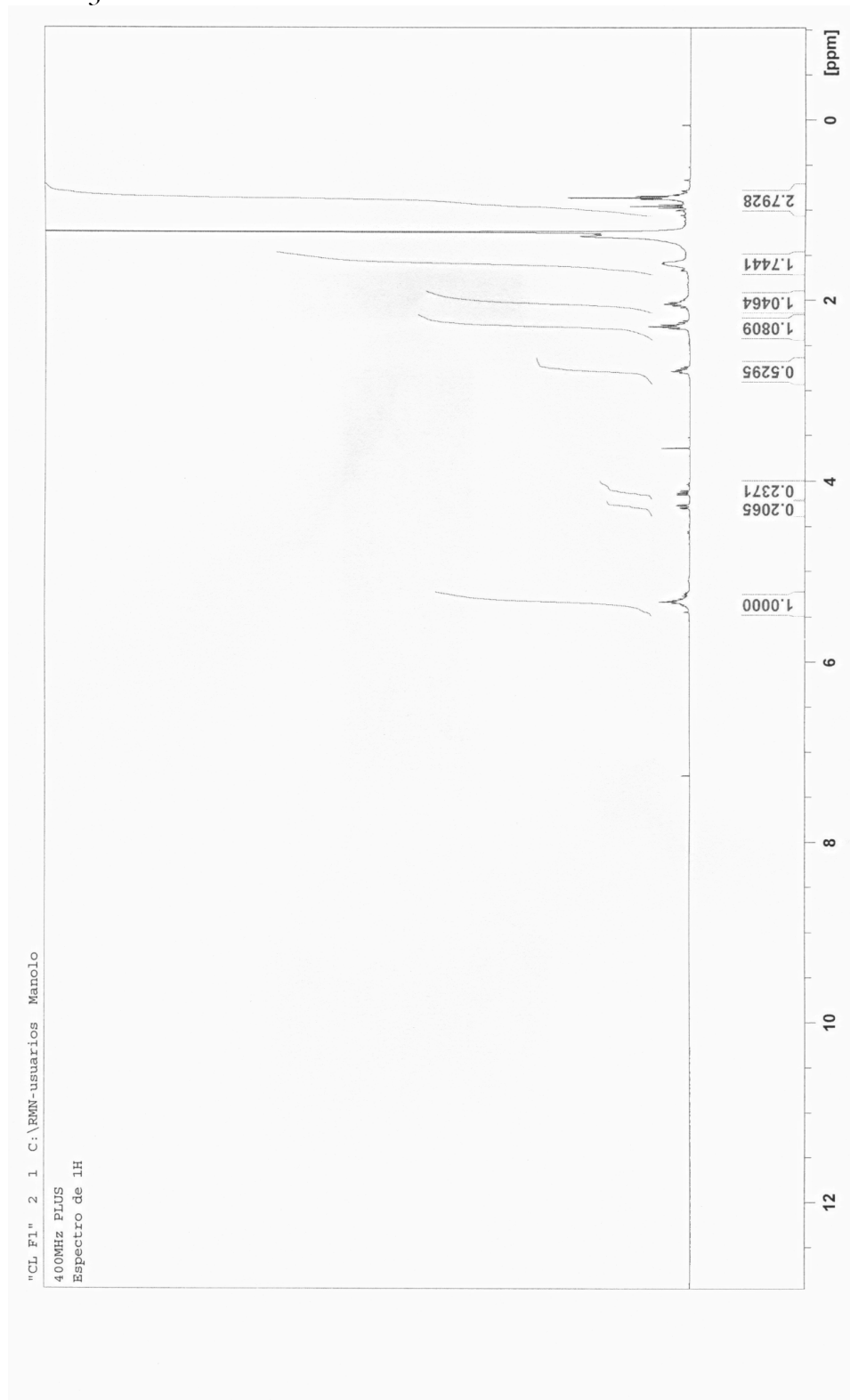
¹H NMR





EA30-F1 sample.

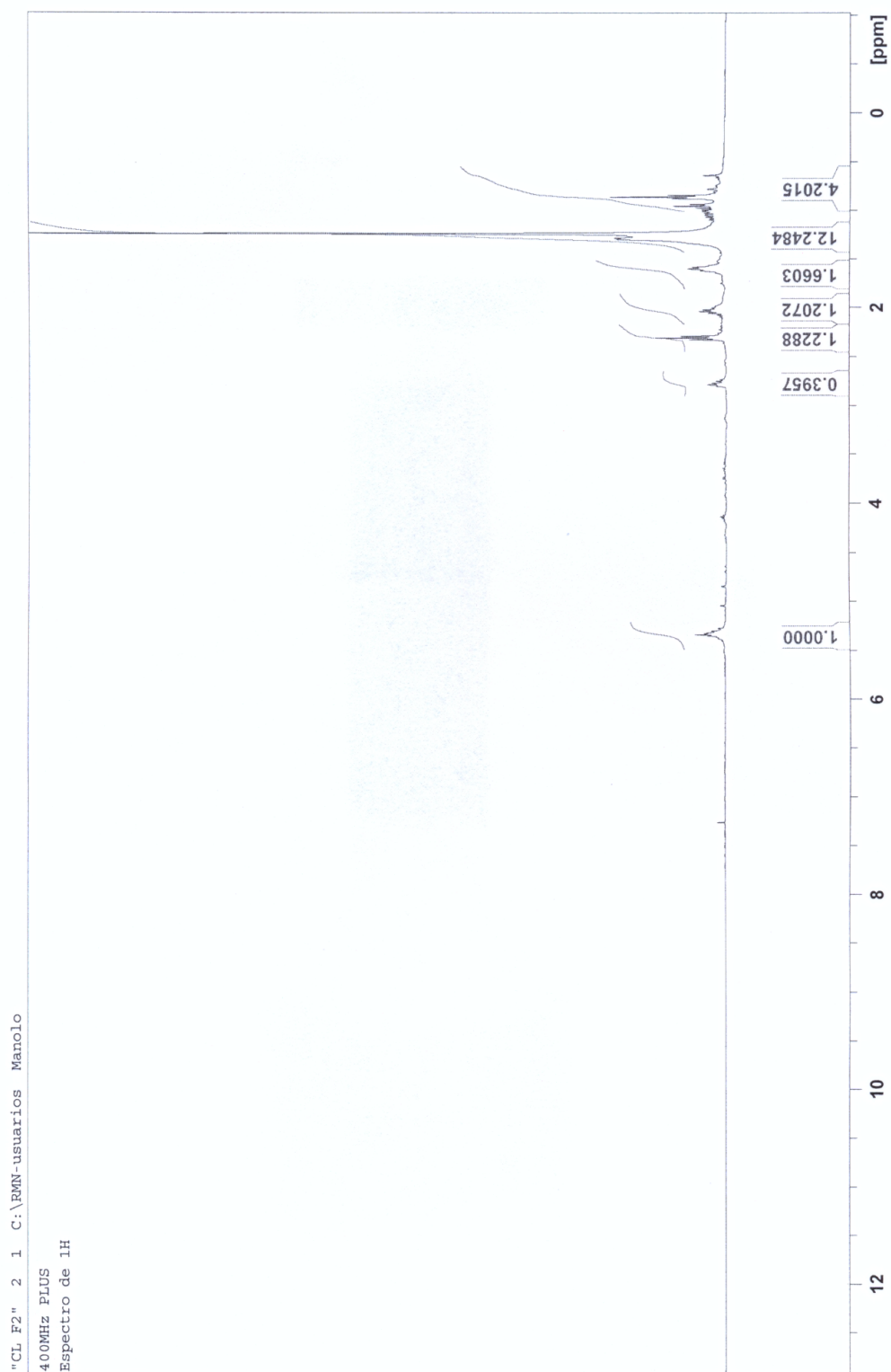
^1H NMR CDCl_3





EA₃₀-F₂ sample.

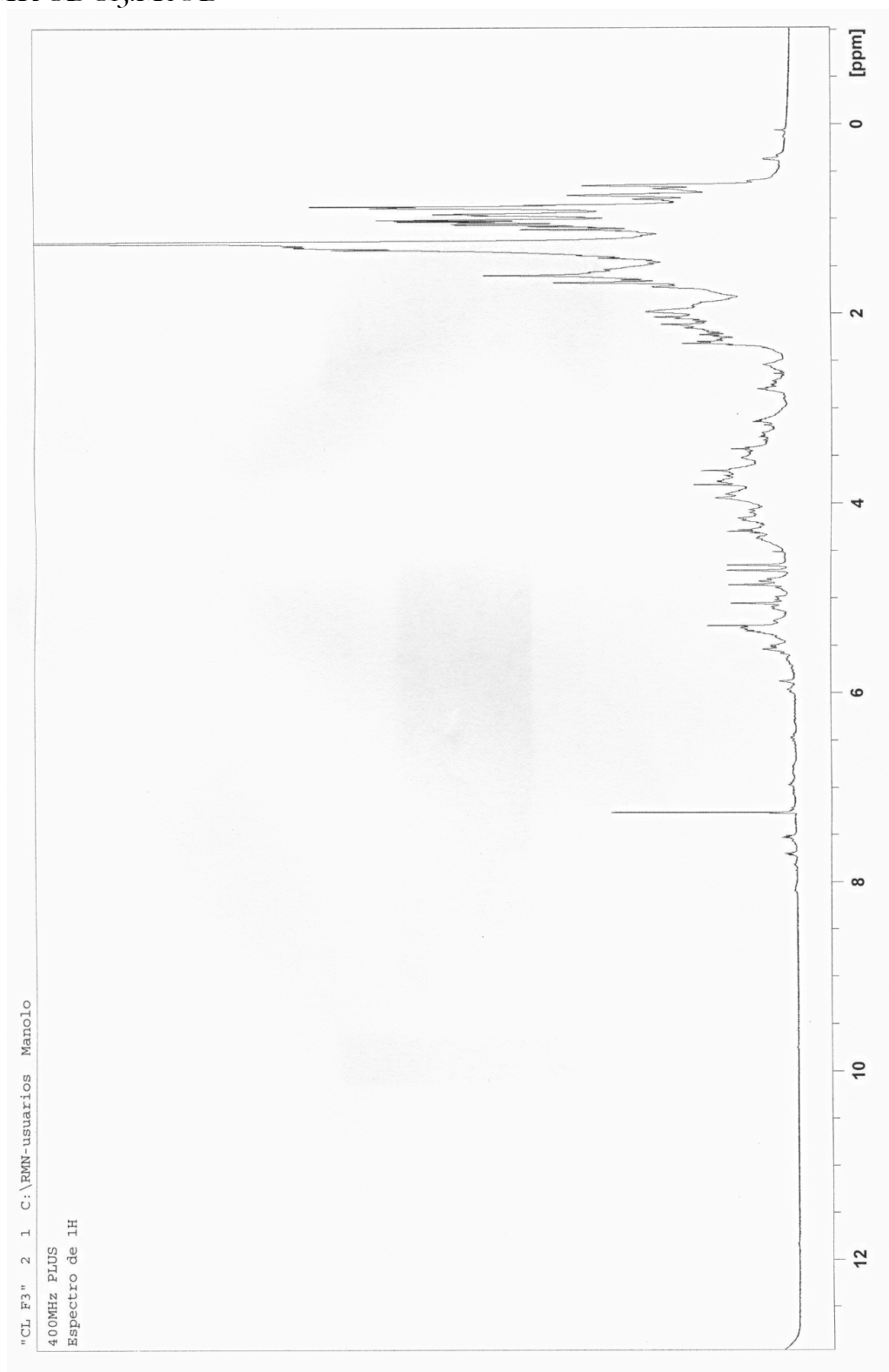
¹H NMR CDCl₃





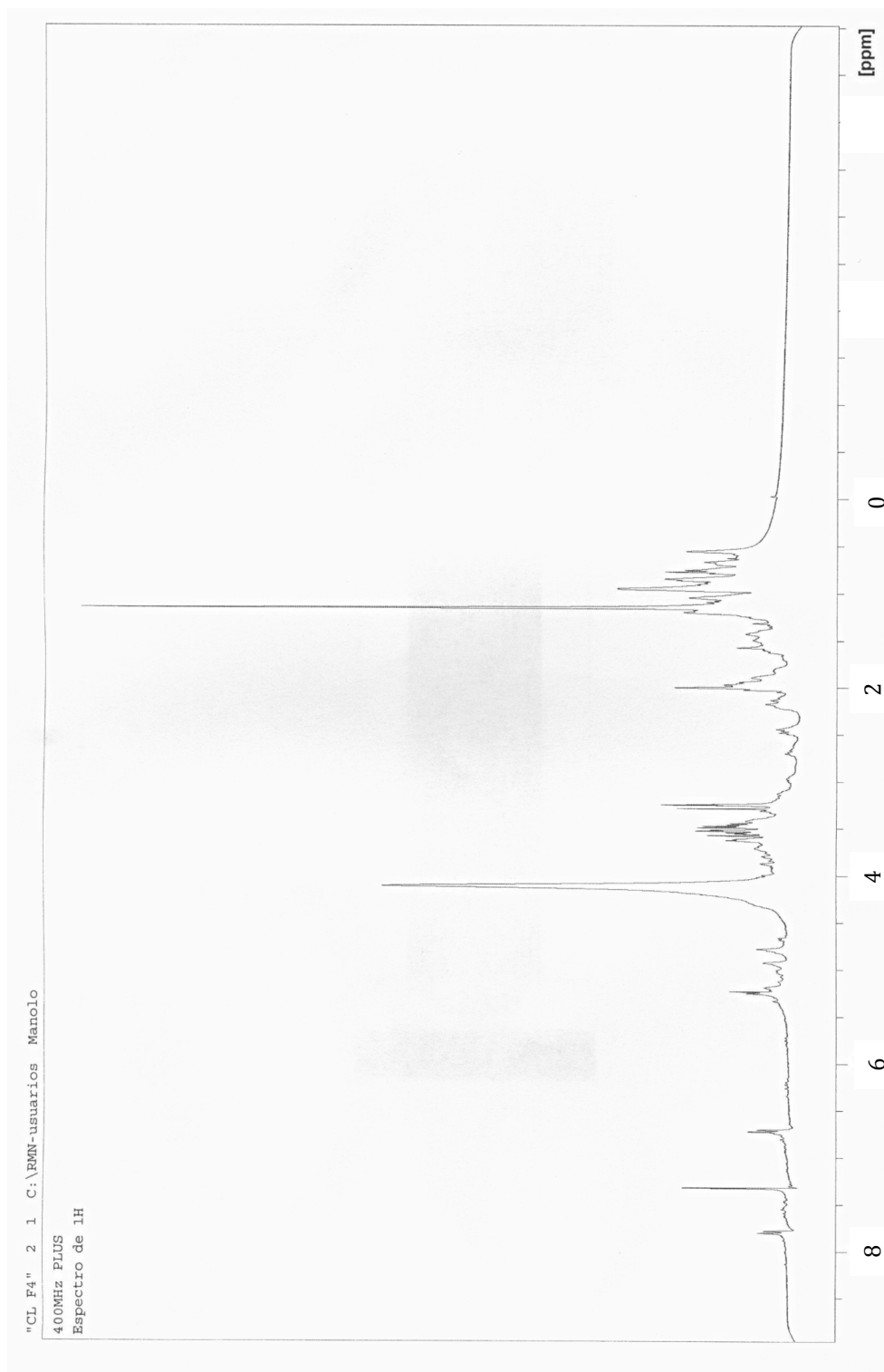
EA₃₀-F₃ sample.

¹H NMR CDCl₃:MeOD



EA30-F4 sample.

^1H NMR CDCl_3 :MeOD

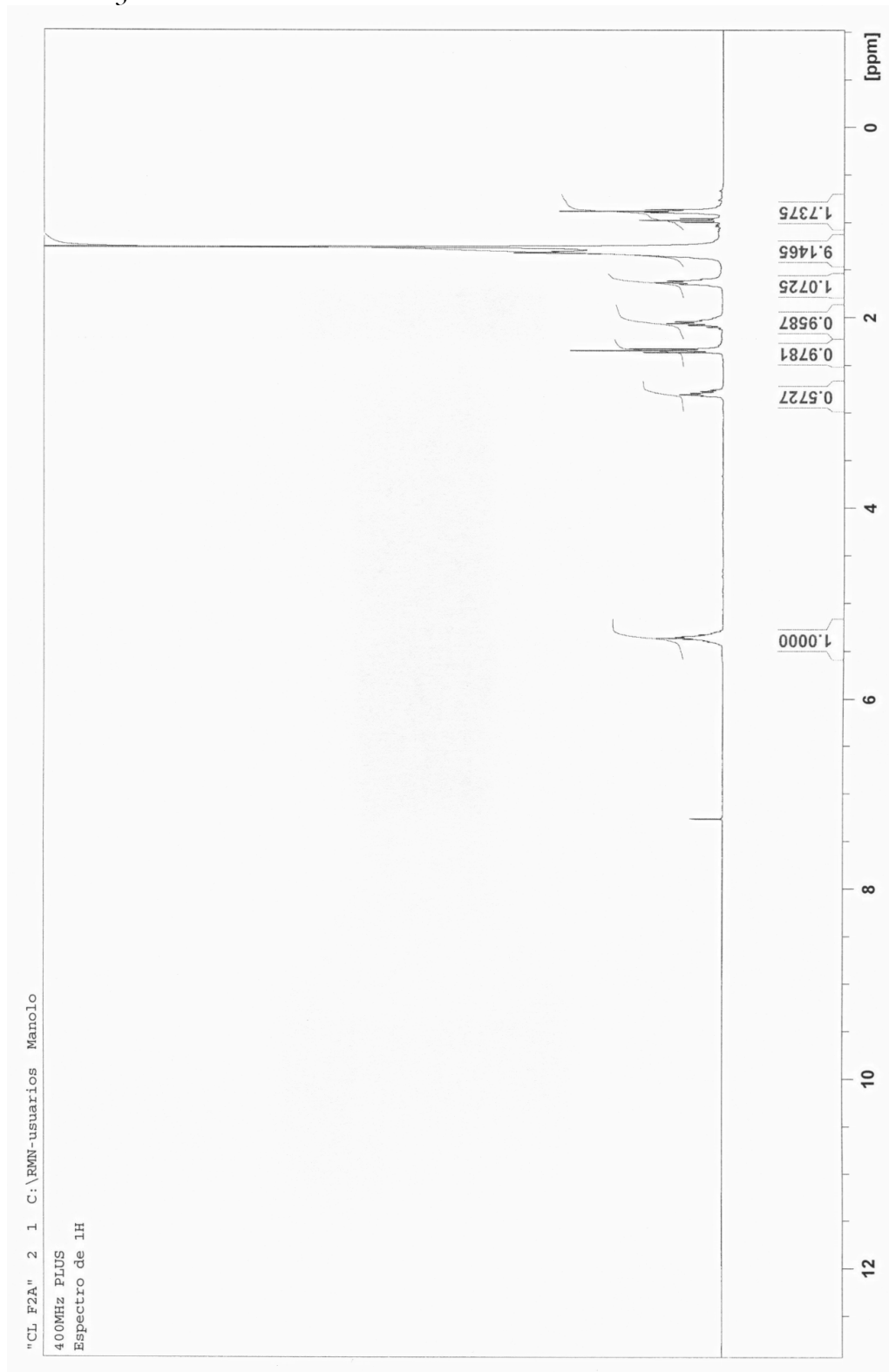




EA₃₀-F₂-A.

No UV absorption.

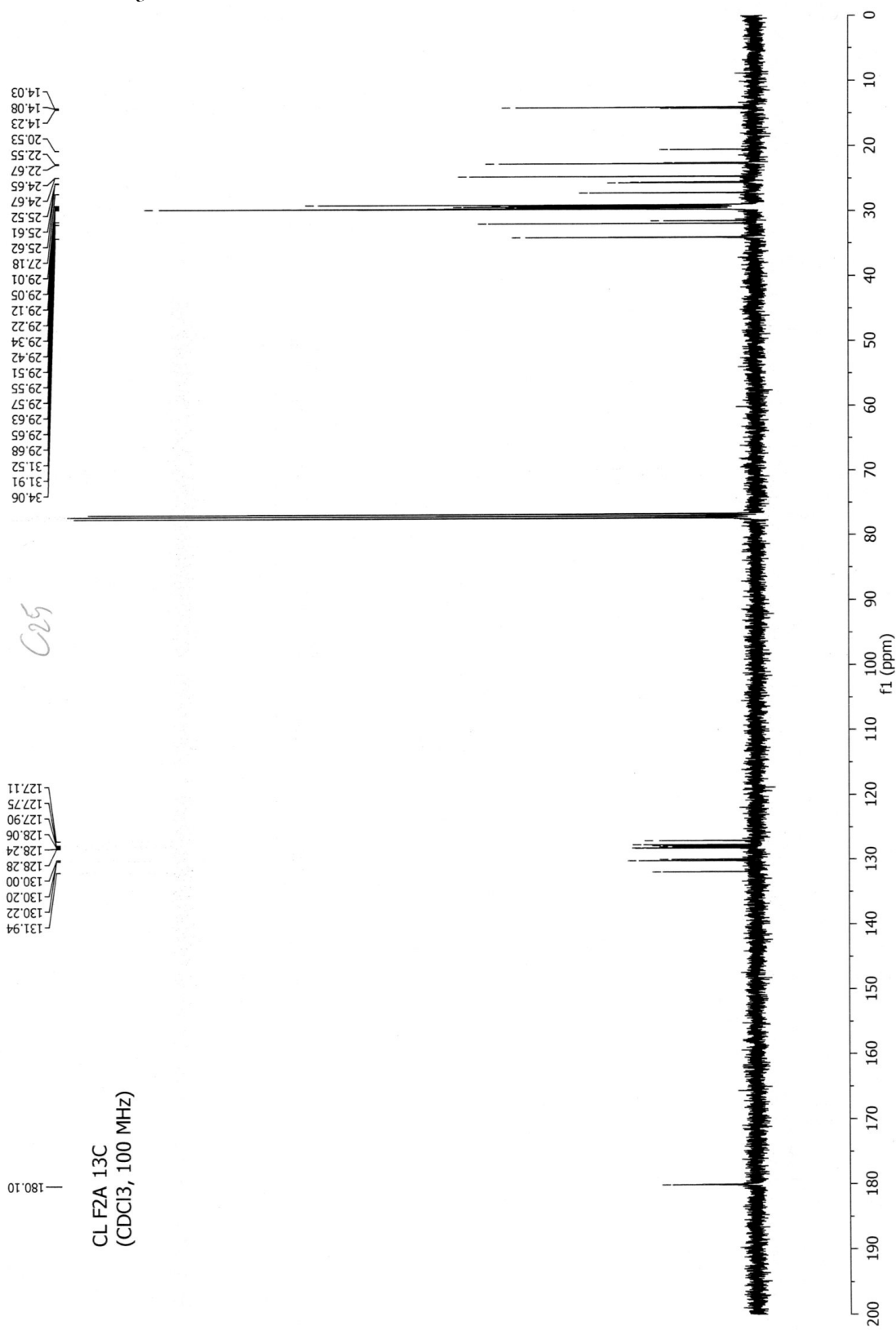
¹H NMR CDCl₃





EA₃₀-F₂-A.

¹³C NMR CDCl₃

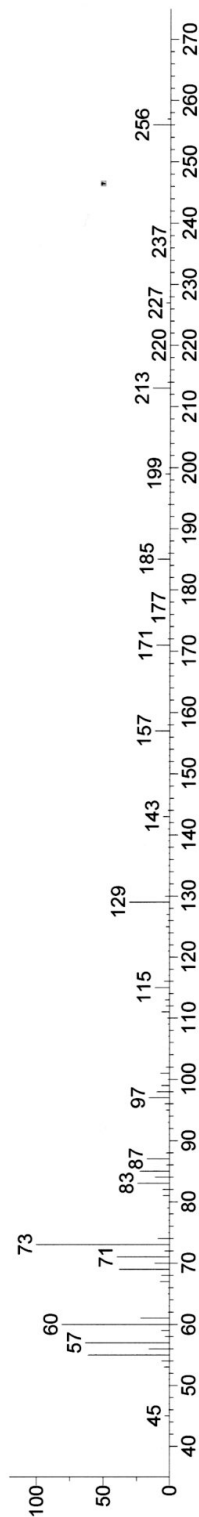


EA30-F2-A.

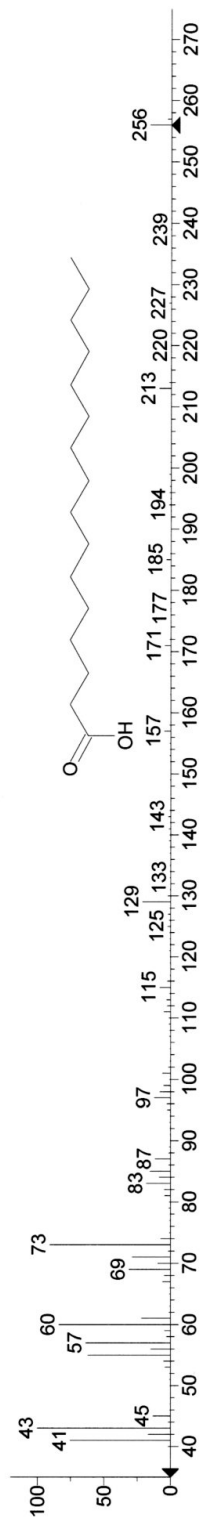
Mass spectra Rt = 15.83

** Search Report Page 1 of 1 **

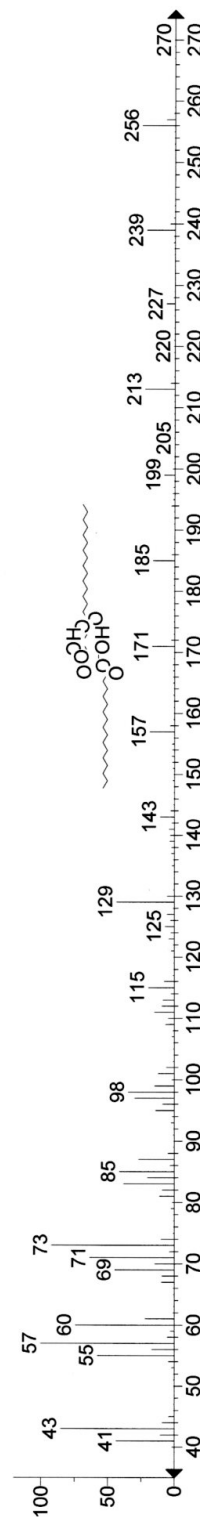
Unknown: Malaga 01 801 (15.835)
Compound in Library Factor = -138



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C₁₆H₃₂O₂; MF: 896; RMF: 897; Prob 66.1%; CAS: 57-10-3; Lib: mainlib; ID: 8479.



Hit 2 : L-(+)-Ascorbic acid 2,6-dihexadecanoate
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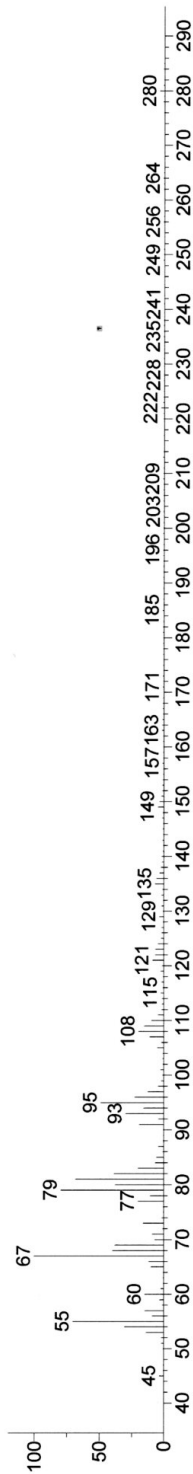


EA30-F2-A.

Mass spectra Rt = 15.52

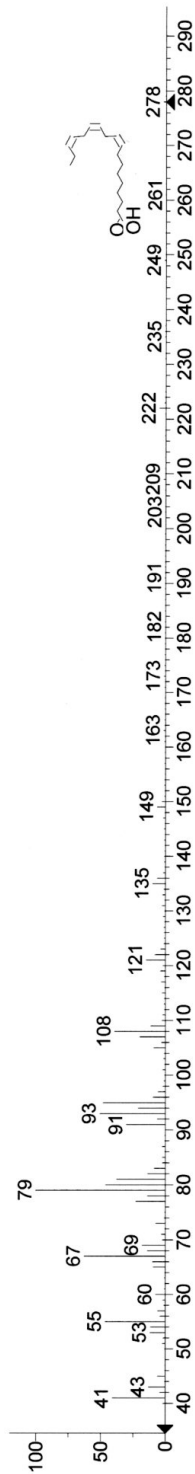
** Search Report Page 1 of 1 **

Unknown: Malaga 01 915 (17.519)
Compound in Library Factor = -258

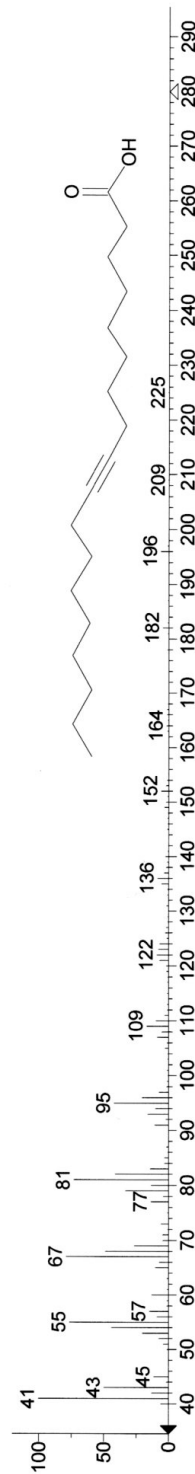


Hit 1 : 9,12,15-Octadecatrienoic acid, (Z,Z,Z)-
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(linolenic acid)



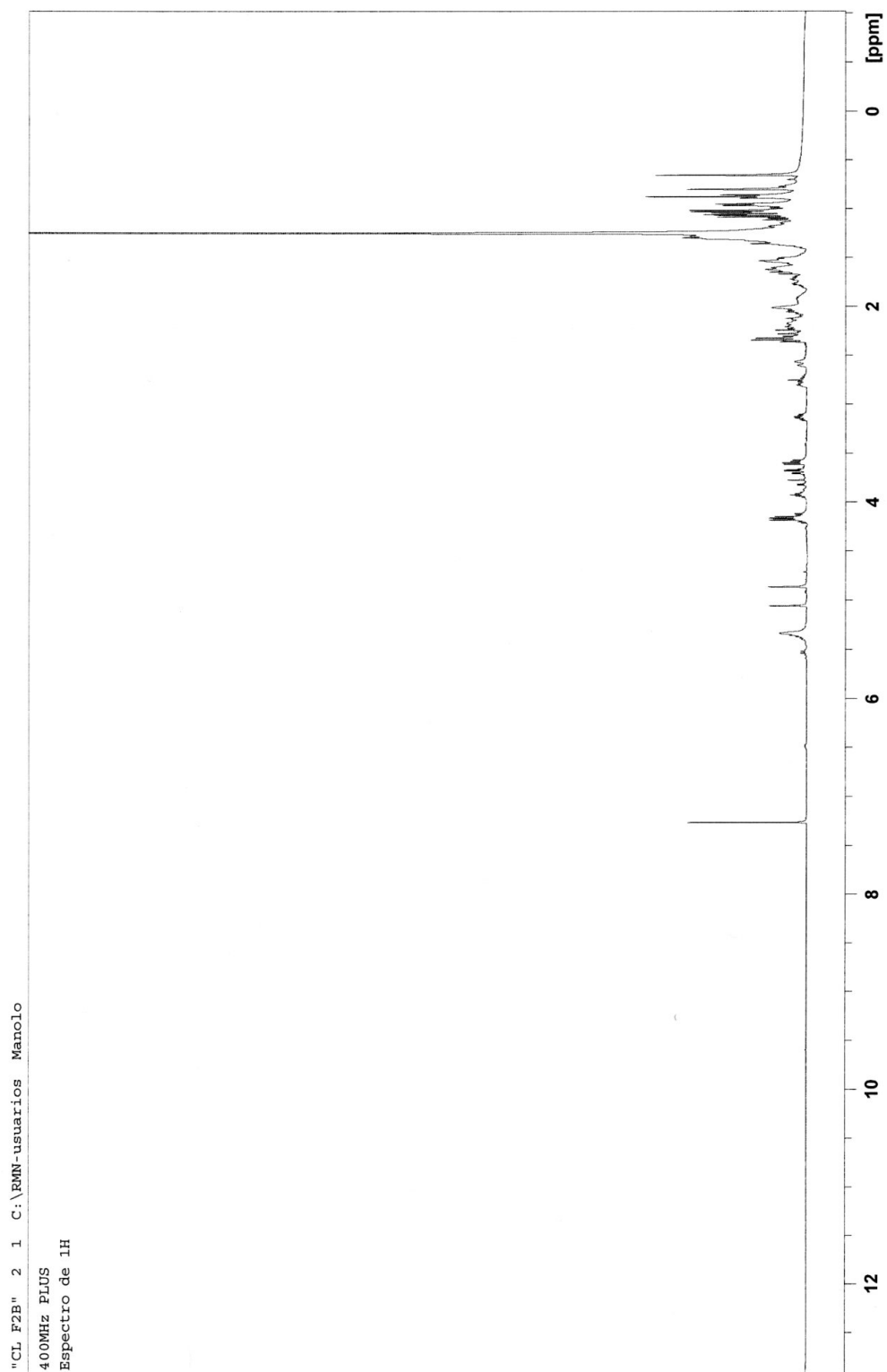
Hit 2 : 9-Octadecynoic acid
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EA30-F2-B.

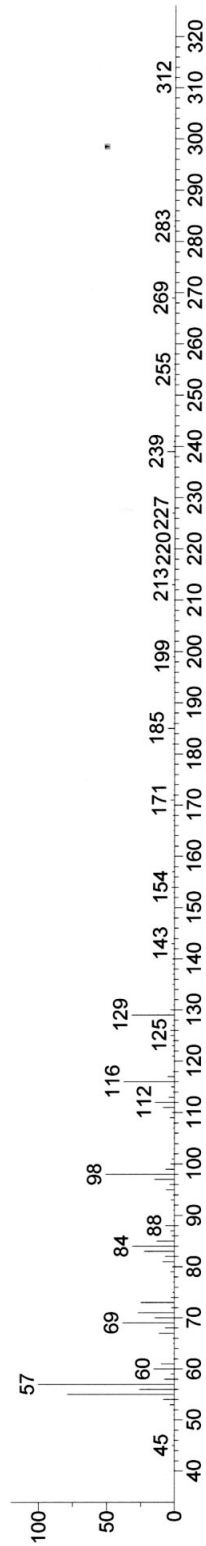
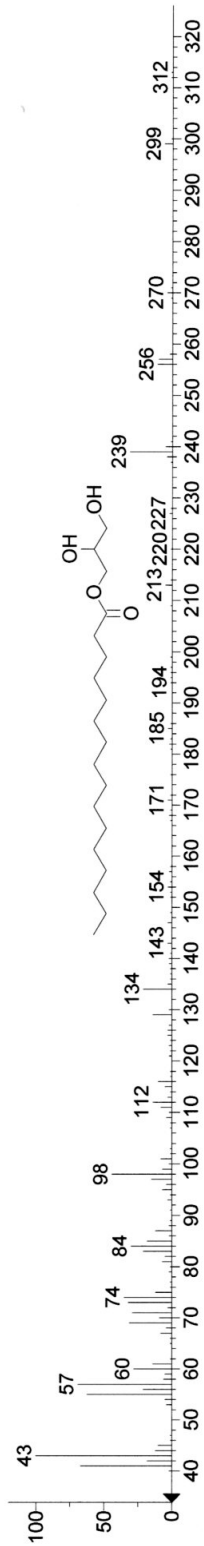
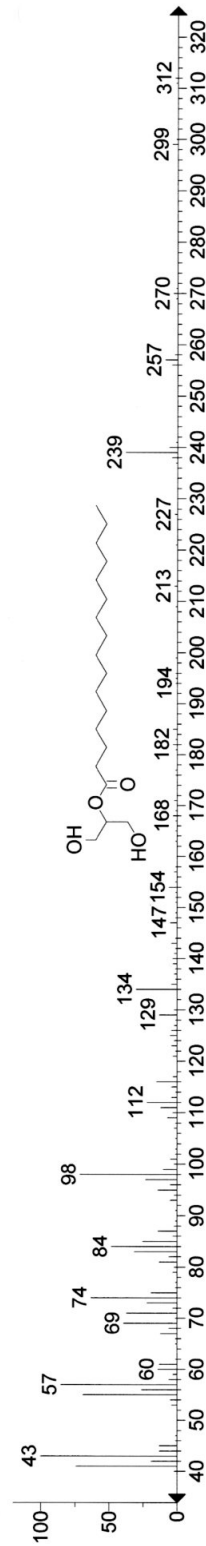
^1H NMR CDCl_3



EA₃₀-F₂-B.

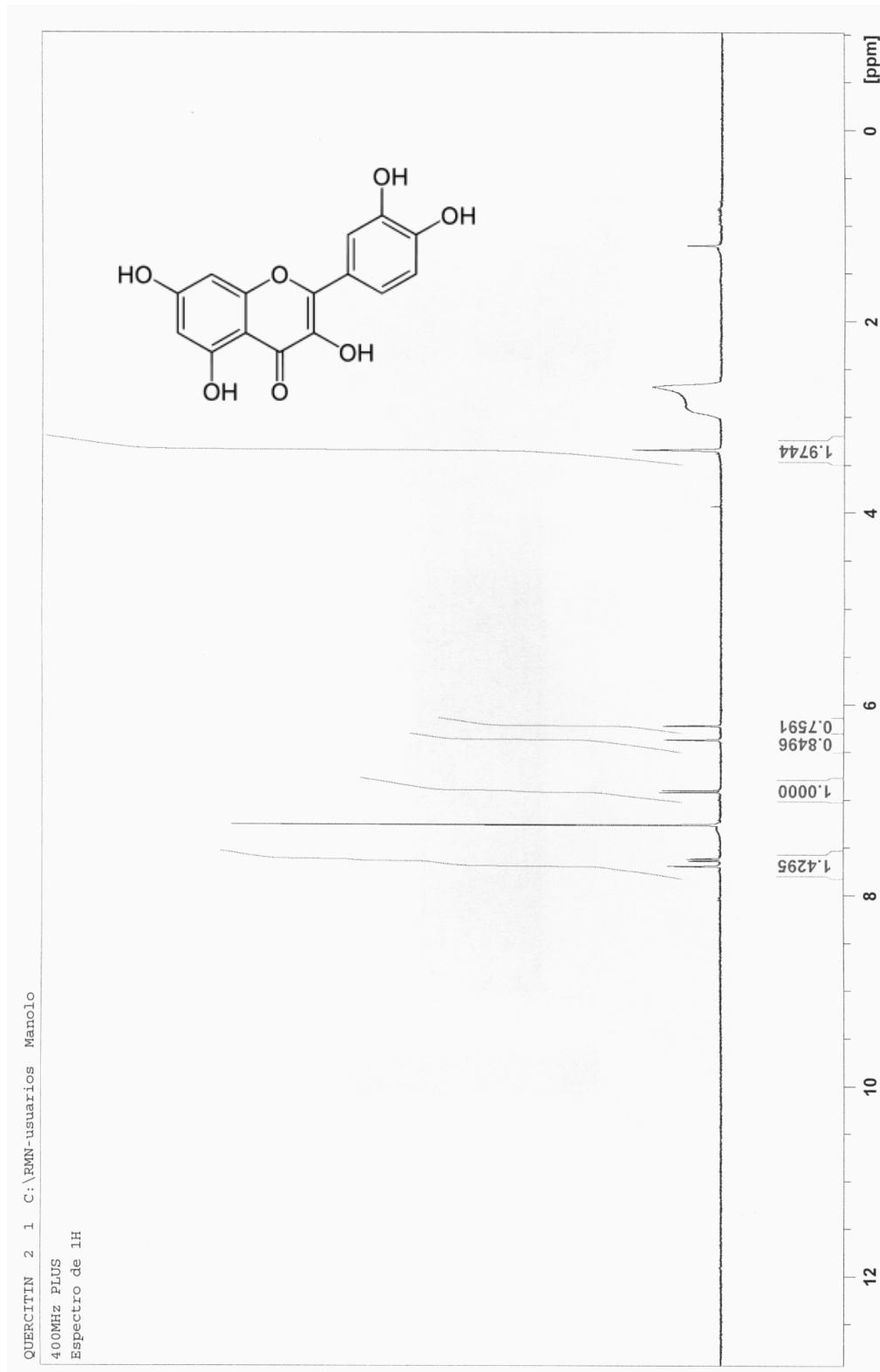
Mass Spectra

** Search Report Page 1 of 1 **

Unknown: Malaga 02 999 (18.748)
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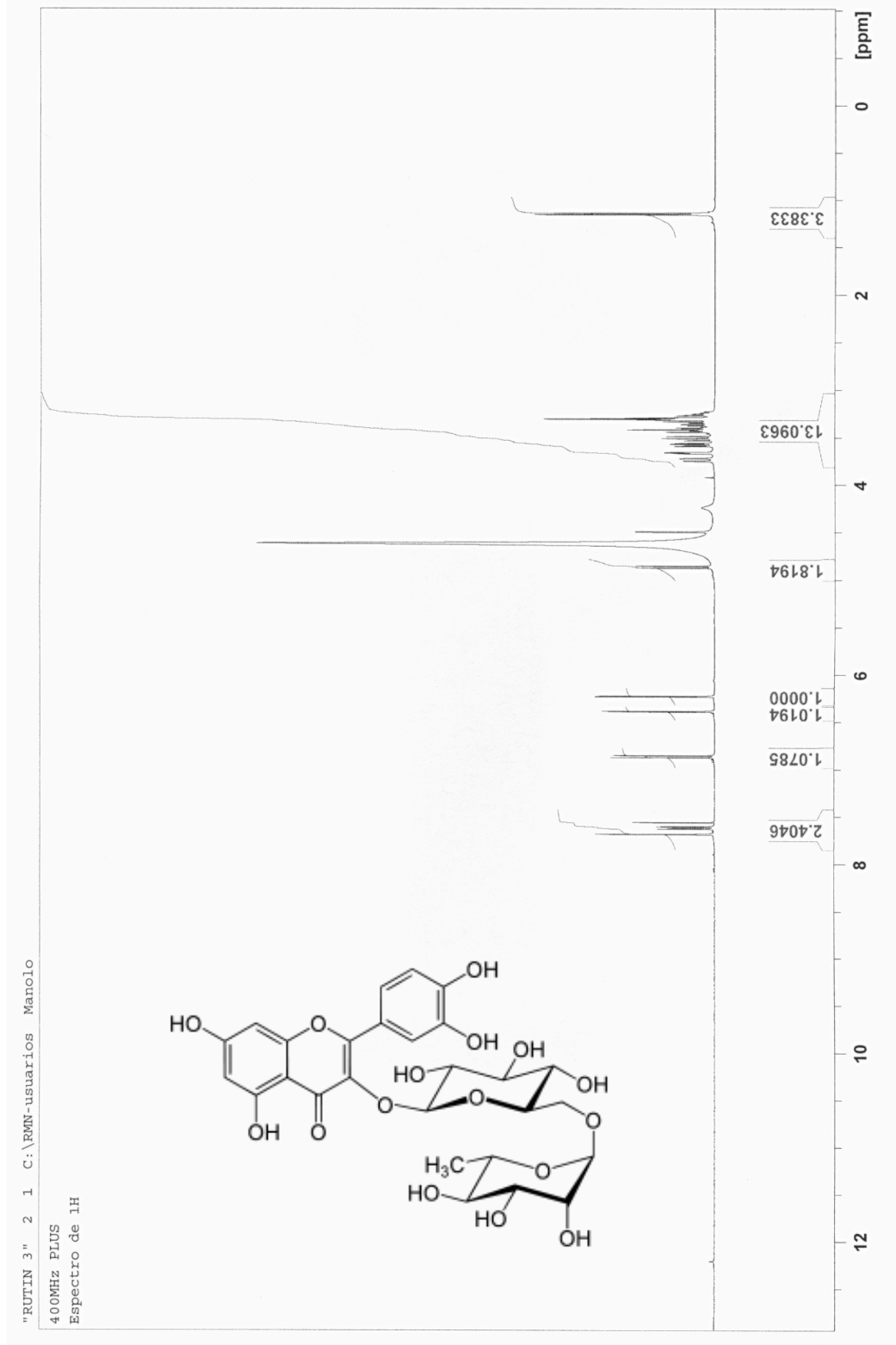
EA₃₀-F₂-C (Quercetin).

¹H NMR CDCl₃:MeOD



EA30-F3-A (Rutin).

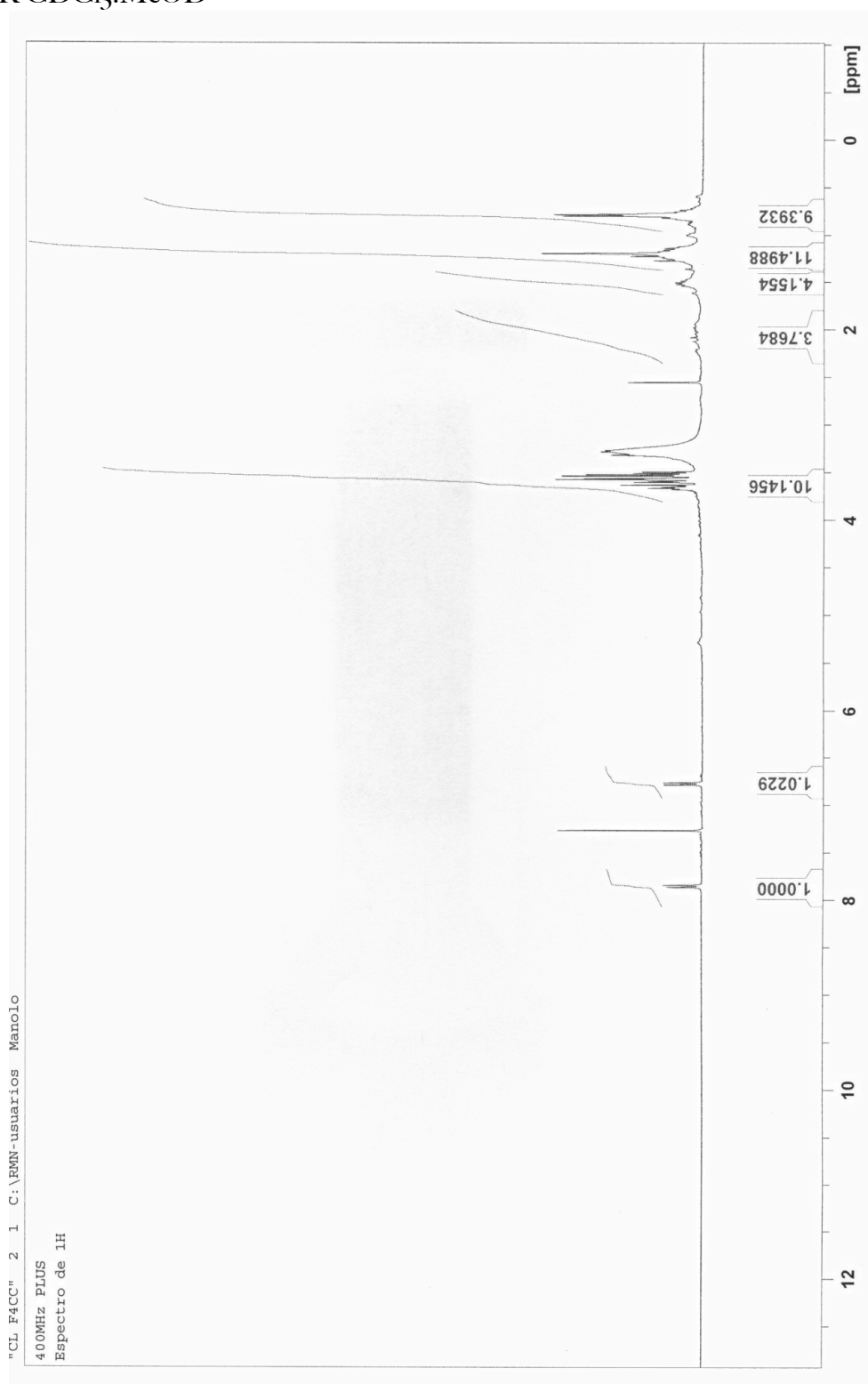
¹H NMR MeOD





EA₃₀-F₄-A.

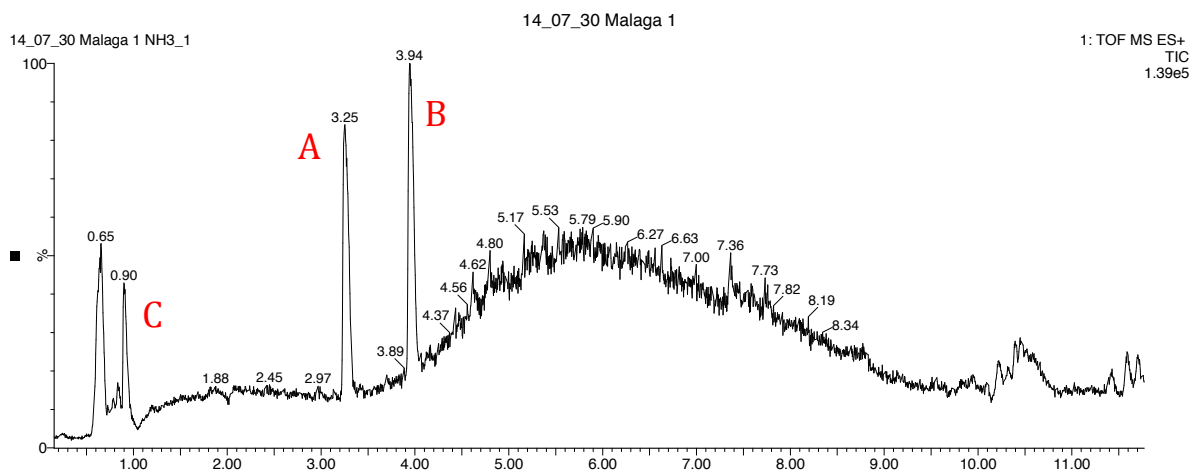
¹H NMR CDCl₃:MeOD



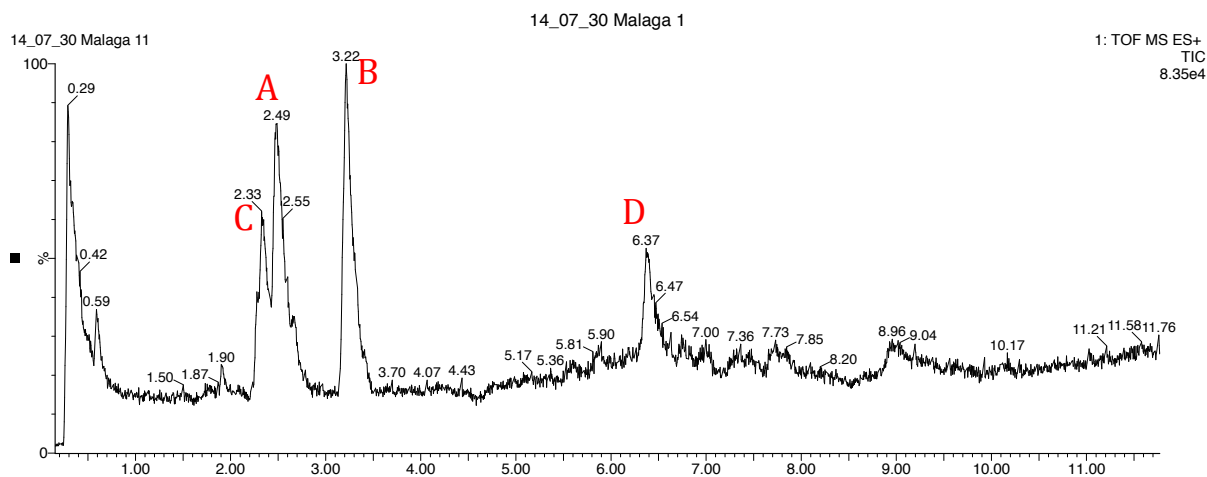
FAZN sample.

LC-MS/MS analyses.

Basic eluent (NH₃).



Acid eluent



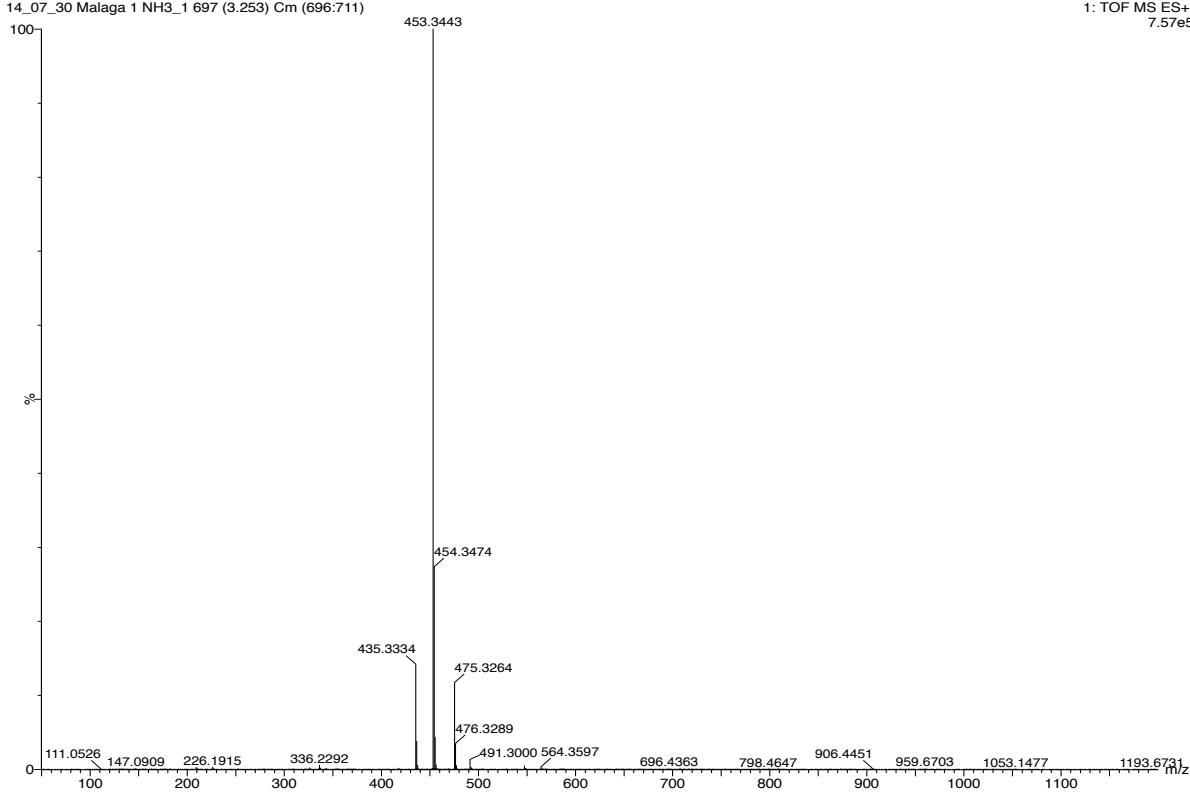


Compound A. Mass 452 uma

14_07_30 Malaga 1

14_07_30 Malaga 1 NH3_1 697 (3.253) Cm (696:711)

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7.57e5

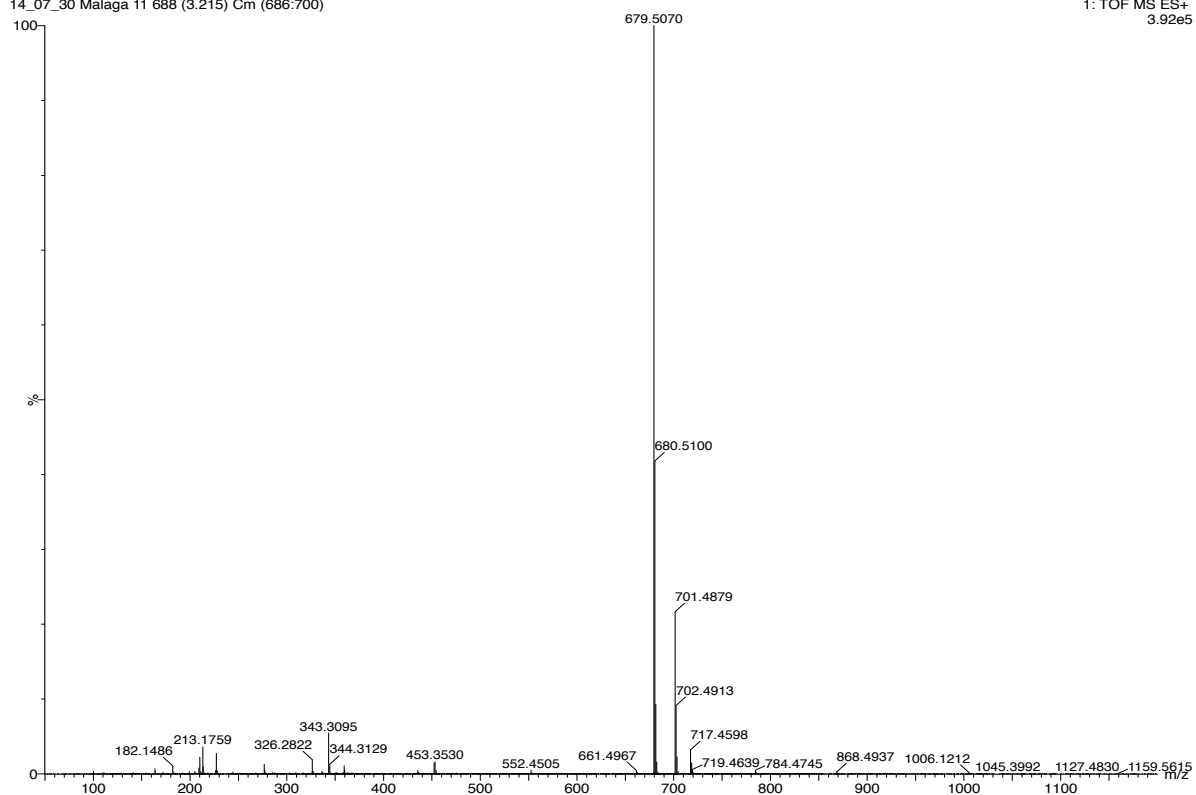


Compound B. Mass 678 uma

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14_07_30 Malaga 11 688 (3.215) Cm (686:700)

1: TOF MS ES+
3.92e5



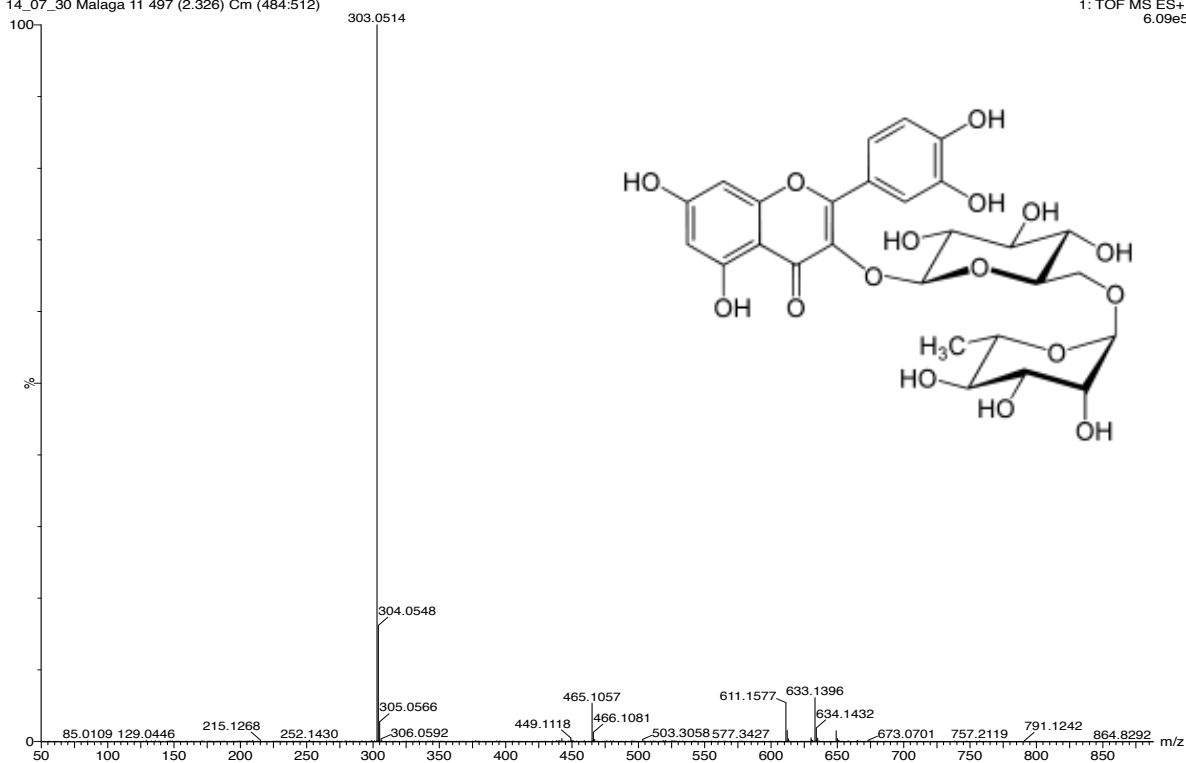


Compound C. Mass 610 uma. RUTIN

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6.09e5

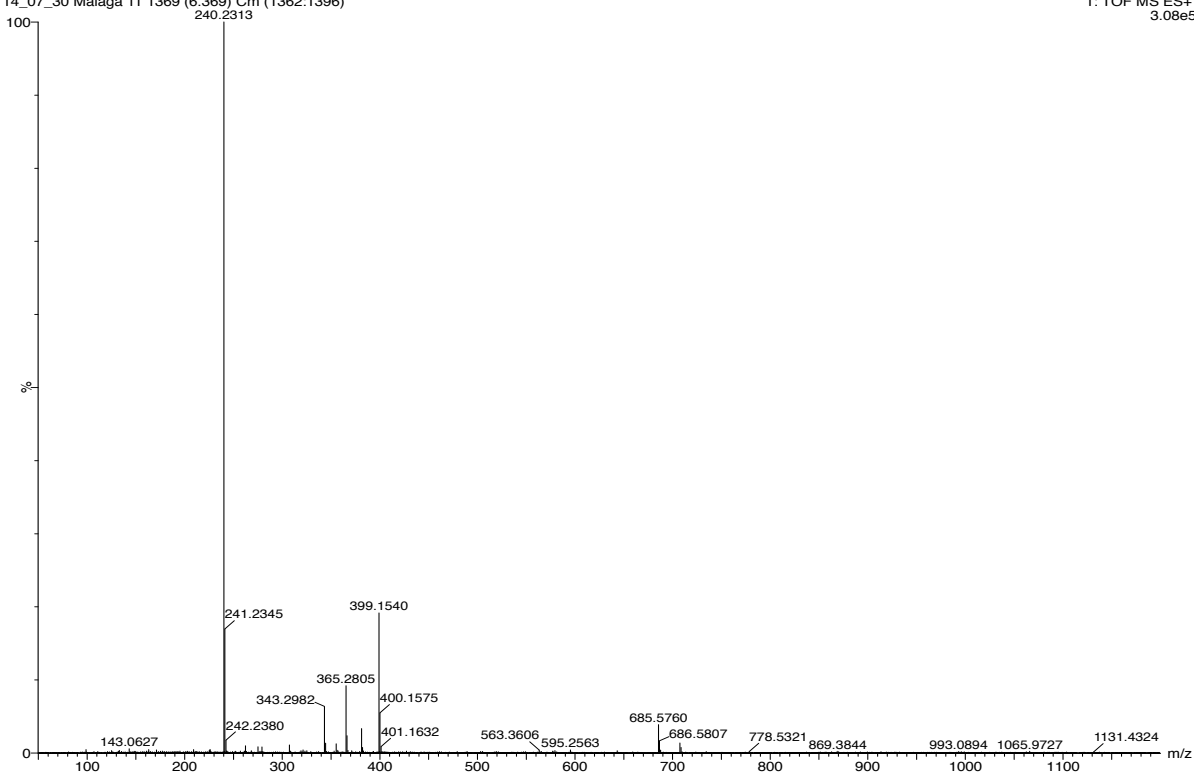


Compound D.

14_07_30 Malaga 1

14_07_30 Malaga 11 1369 (6.369) Cm (1362:1396)

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3.08e5

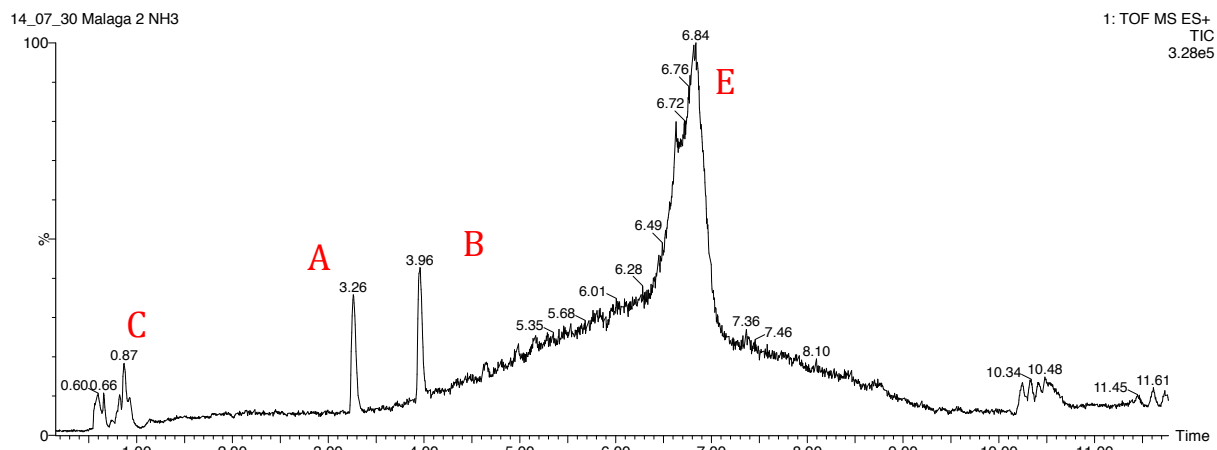




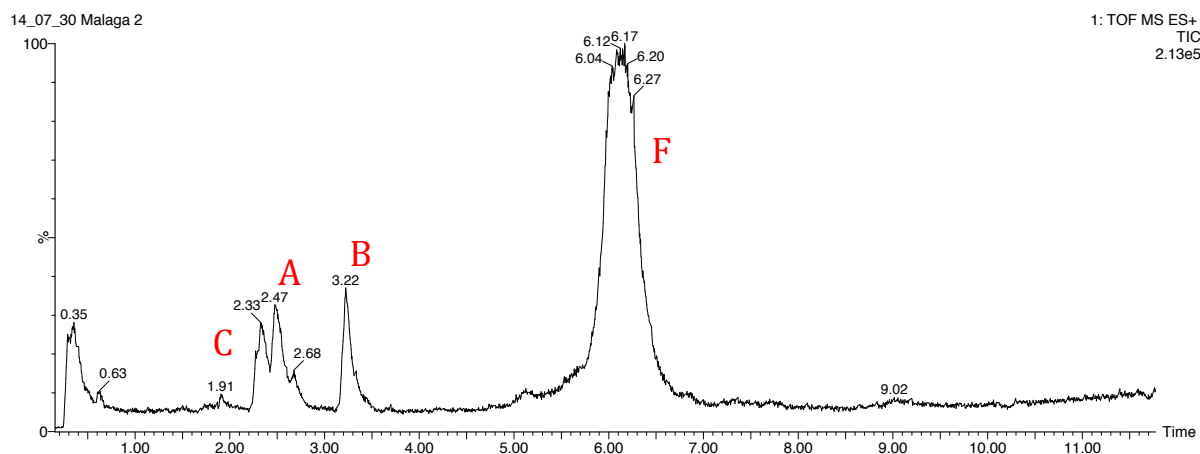
FA sample.

LC-MS/MS analyses.

Basic eluent (NH₃).



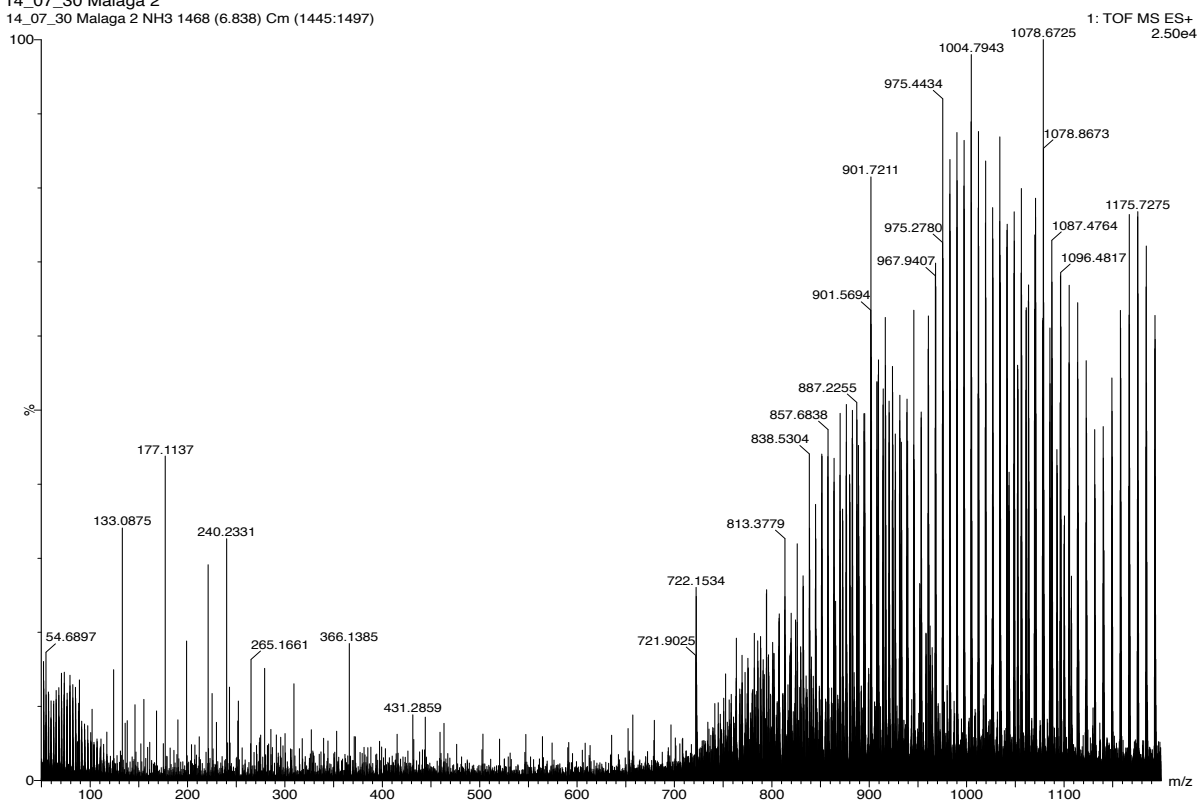
Acid eluent





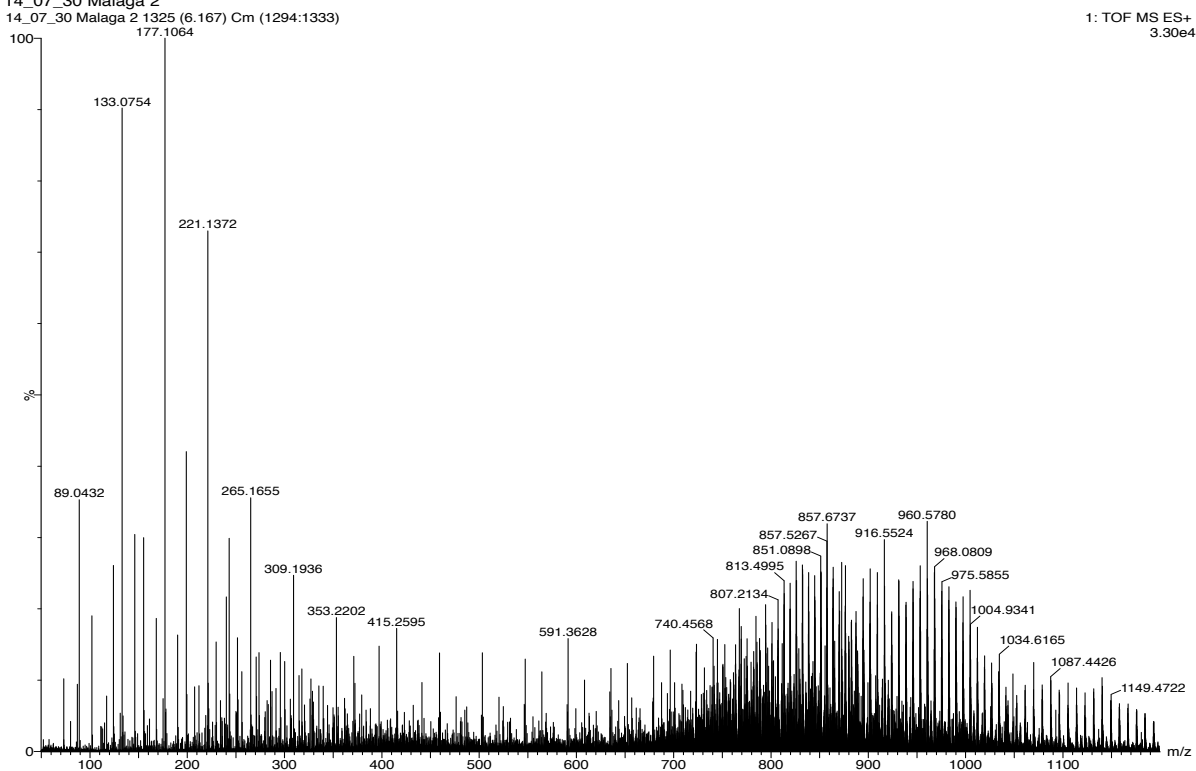
Compound E.

14_07_30 Malaga 2
14_07_30 Malaga 2 NH3 1468 (6.838) Cm (1445:1497)



Compound F.

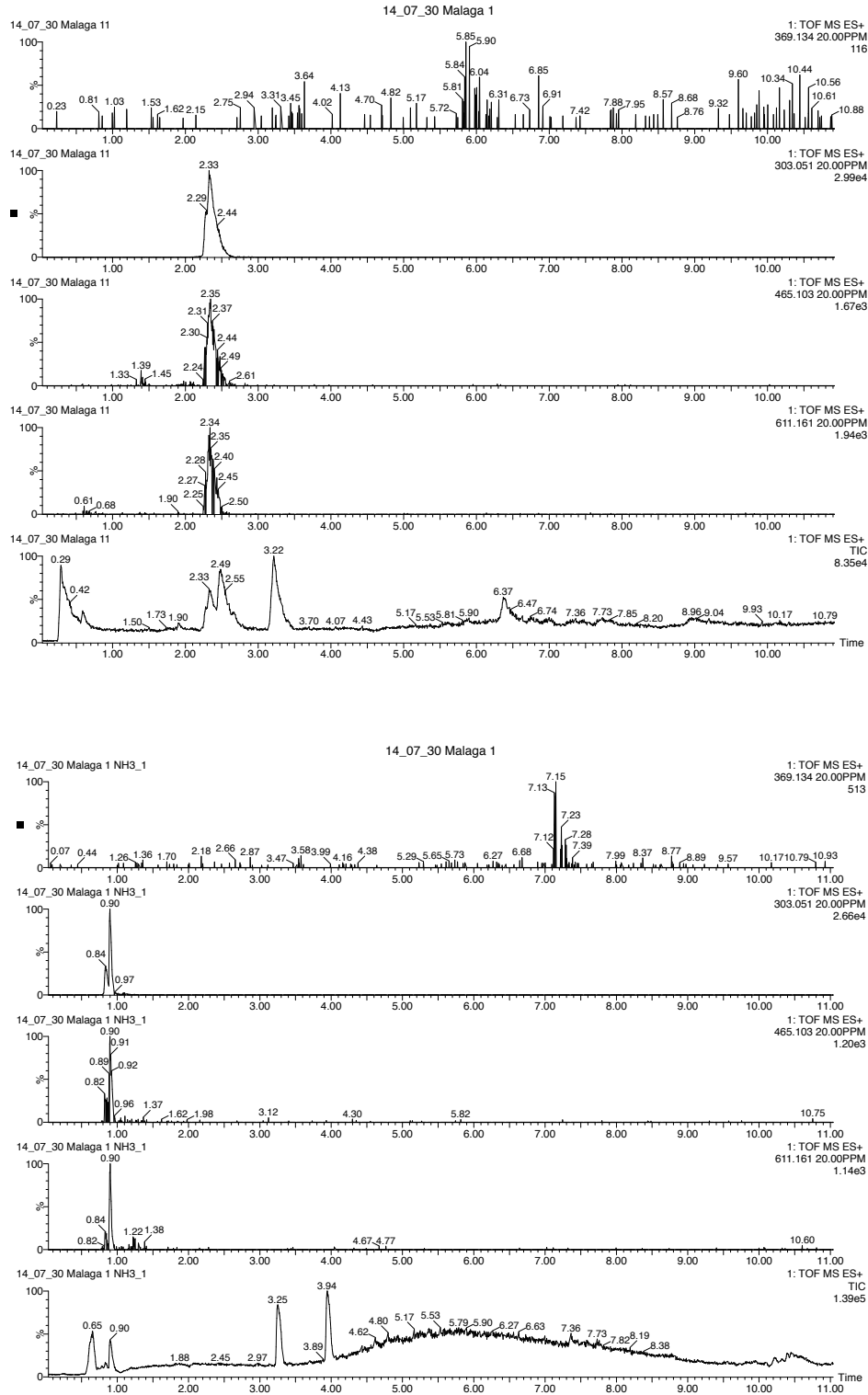
14_07_30 Malaga 2
14_07_30 Malaga 2 1325 (6.167) Cm (1294:1333)





FAZN sample.

LC-MS/MS analyses (acid eluent above, basic eluent below)



6. Bibliography.

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Certificate of Analysis

Description: *Cordia lutea* L. (Boraginaceae)
Identification: Blga. Felicia Díaz Jarama (Univ. Nal. Amazonia Peruana)
Sender: Dr. José Gonzalo Cabanillas Coral, Sabell Perú, SAC
Date of reception: July 22, 2014
Batch number: 1 (2.2 kg of powdered plant)
Batch date: April, 30, 2014

To whom it may concern

This is to certify the absence of pyrrolizidine alkaloids (PAs) in the plant *Cordia lutea* L. Analyses were carried out by the acid-base standard method for isolation of alkaloids, Zn dust reduction and UPLC-Mass spectrometry. The absence of PAs in *C. lutea* suggests non-toxicological risk due to this kind of alkaloids.

September 17, 2014

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