

1. INTRODUCTION

1.1 Summary.

One of the tasks of the Community Reference Laboratories (CRL) is to prepare a Bank of Reference Standards (1.4.5). Compounds prepared within the “Bank of Reference Standards” are not commercial available compounds, which are made available to other institutes.

This procedure describes the ampouling and quality control of reference standards. The quality control involved a full spectroscopic characterisation with Mass Spectrometry (MS) frequently combined with Gas Chromatography after derivation (GC-MS). Fourier Infrared Spectroscopy (FTIR), Nuclear Magnetic Resonance Spectroscopy (NMR, ²H or ¹³C) and Ultraviolet Spectroscopy (UV). A detailed report of the quality control of reference standards is in ARO/SOP/429 (1.4.6). The ampoules are also subjected to homogeneity and degradation studies. Datasheets (annex 2) are made containing all relevant spectroscopic information, including results of the degradation study, homogeneity study, chemical name, the synonyms used, the CAS# and molecular weight.

1.2 Field of application.

In order to analyse samples of animal origin, such as serum, plasma, urine, faeces or tissue, analytical reference standards need to be available. Reference standards are the basis for proper identification and quantification. Via CRL the compounds can be supplied in small quantities of known purity and well-determined identity.

1.3 Ordering of the standards.

If available standards are bought at registered companies. Certificates of analyses, quality control data and other information concerning the standard are filed. If compounds are not commercially available they are synthesised at RIVM or acquired through custom synthesis.

1.4 References.

- 1.4.1 Van Rossum, H.J., Zoontjes, P.W. and Schwillens, P.L.W.J., Multi residue analysis; anabolic agents, RIVM Standard Operating Procedure SOP: ARO/113, April 2001 (revision 6).
- 1.4.2 Multi residue method using coupled-column HPLC and GC-MS for the determination of anabolic compounds in samples of urine. RIVM report no. 389002-035, September 1997, H.A. Herbold, S.S. Sterk, R.W. Stephany, L.A. v. Ginkel.
- 1.4.3 Screening method for anabolic steroids in faeces using direct injection/coupled-column HPLC and GCMS. ARO/453, July 2002, H.A. Herbold, (revision 0).
- 1.4.4 6 Monthly Progress report on EC contract mat1 – CT92 - 0020 Bank of Reference Standards period august – dec. 1993 (RIVM contract 573002).
- 1.4.5 Control of reference materials, SOP ARO/429, May 1998, S.S. Sterk, (revision 0).
- 1.4.6 Development of bovine muscle, liver and urine reference materials for zeranol, preparation, homogeneity and stability.
M. O’Keefe, A. Nugent e.a., J. Anal. Chem. (1997) 357: 1029 –1034.

2. MATERIALS

Reference to a company and/or product is for purposes of identification and information only and does not imply approval or recommendation of the company and/or the product by the National Institute of Public Health and Environment (RIVM) to the exclusion of others which might also be suitable.

2.1 Chemicals

- 2.1.1 Ethanol (Baker, 8006)
- 2.1.2 Methanol (Baker, 8045)
- 2.1.3 Nitrogen
- 2.1.4 Heptafluorobutyric Acid Anhydride (HFBA) (Pierce, 63164)
- 2.1.5 N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) (Macherey-Nagel, 70127)
- 2.1.6 Ammonium iodide (Fluka, 09874)
- 2.1.7 Dithioerythritol (Sigma, D-9779)
- 2.1.8 Acetone (Baker, 8001)
- 2.1.9 N,O-bis(trimethylsilyl)trifluoroacetimide + 1% TMCS (BSTFA) (Pierce, 38831).

2.2 Apparatus

Besides standard laboratory glassware and equipment is used:

- 2.2.1 Glass brown flask, 50 ml.
- 2.2.2 Glass brown flask, 15 ml.
- 2.2.3 Vortex mixer MS 1 minishaker (IKA).
- 2.2.4 Repeating pipette 100 µl, total volume 5 ml, #1005 (Hamilton).
- 2.2.5 Glass amber ampoules, 2 ml (Wheaton).
- 2.2.6 Gas burner with H₂- and O₂-gas.
- 2.2.7 Lyophilisor Delta II (Christ).
- 2.2.8 Semi-micro balance RC210P (10⁻⁵), (Sartorius).
- 2.2.9 Injection-vials, Wide Mouth Crimp (Alltech, 98213), with micro inserts (100 µl) (Alltech EK-1022.395).
- 2.2.10 Thermostatic heating block, Thermolyne 16500 Dry Bath, with nitrogen facility 55°C.
- 2.2.11 Automatic pipettes, P100, P200, P1000, P5000 (Gilson).
- 2.2.12 Heater, incubator thermostat adjustable ? 5°C (Mettmert).
- 2.2.13 Parafilm, (American Can Company, Greenwich).
- 2.2.14 GC-MS equipment, (Agilent).

The final analysis of the extracts is performed on a HP 6890 series gaschromatograph equipped with a HP 7673 automatic sampler, a PC with HPChem data acquisition software and a 5973 Mass Spectrometer.

3. ANALYTICAL PROCEDURES.

Flow diagram.

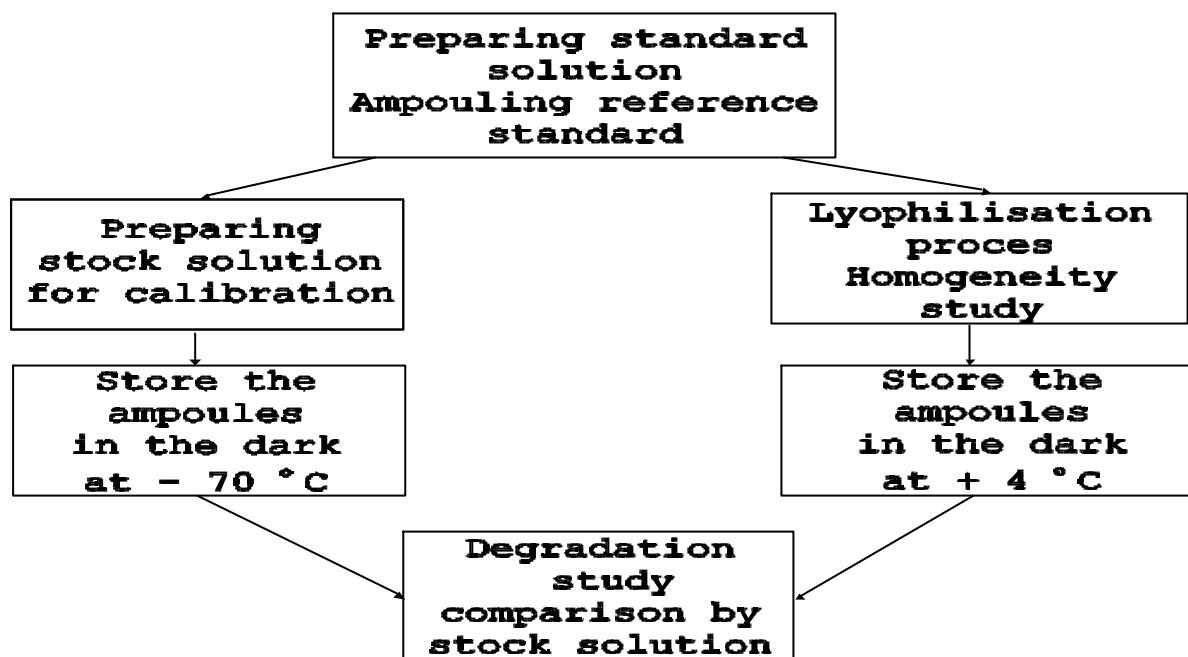


Figure 1

3.1 Preparation of standards.

3.1.1 *Preparing à 1 mg/ml standard solution (stock solution).*

Weigh accurately an amount¹ of source material in a glass brown flask (2.2.1), by using a semi-microbalance (2.2.8). Record the reading.

Add an equal amount of ethanol (x mg source material = x ml ethanol) and homogenise the solution (temp. $20 \pm 2^\circ\text{C}$). Concentration is 1 mg/ml.

3.1.2. *Ampouling 0.10 mg reference standard*

Allow the stock solution (3.1.1) to equilibrate at room temperature ($20 \pm 2^\circ\text{C}$).

Pipet accurately 100 μl (2.2.4) stock solution into each ampoule to be filled.

Evaporate the solvent from the ampoules with a freeze-dryer. Lyophilisation is performed with a Christ Epsilon 2 freeze-dryer. The lyophilisation process is started when the temperature of the material is below the eutectic temperature. For information about the operation of the freeze-dryer, see ARO/SOP 404.

After Lyophilisation, close the ampoules as soon as possible by melting the open tops in a H_2 - O_2 -flame.

Label the ampoules as follows:

Bank of Reference Standards: serial number (Name of Compound) Reference standard EU/CRL: (number)

3.1.3 *Store condition of the ampoules.*

The store condition depends of the degradation process of the analyte. Generally the ampoules are stored in the dark at a temperature of $4 \pm 2^\circ\text{C}$. Each batch of ampoules is subjected to a degradation study which is focussed on general storing condition ($4 \pm 2^\circ\text{C}$) the following degradation study (4.2) in consequence of the period of store.

¹ For indication, weight ± 30 à 35 mg for approximate 300 ampoules.

3.1.4 *Preparing reference standard for calibration curve.*

Accurately fill 10 ampoules with 250 µl stock solution (3.1.1) into each ampoules, close the ampoules (3.1.2) and store them at a temperature of less than – 70°C for reference purpose. Before use: allow the ampoule to warm for at least 30 minutes to room temperature ($20 \pm 2^\circ\text{C}$) and open the ampoule. Prepare a reference solution with concentration 1 ng/µl by transfer quantitatively the contents of the ampoule into a volumetric flask (25 ml) with ethanol until a volume of 25 ml. Dilute 10 times by pipetting 1 ml of the 10 ng/µl solution in a test tube and add 9 ml ethanol p.a., concentration is 1 ng/µl.

3.1.5 *Relevant internal standards.*

The use of internal standards is importance to control the analyses. Prepare a relevant internal standard with a concentration of 1 ng/µl, preferably the deuterated standard according to SOP 401 and/or 113.

3.1.6 *Analysing of the ampoule.*

Reconstitution of Lyophilised ampoules. Allow the ampoules to warm for at least 30 minutes to room temperature ($20 \pm 2^\circ\text{C}$) before opening. Open the ampoules by carefully breaking the neck. Pipet 1 ml ethanol (2.1.1) into the ampoules and close the ampoules with parafilm (2.2.13) vortex for one minute. Place the ampoules in an ultrasonic waterbath for 5 minutes. Allow the ampoules to room temperature ($20 \pm 2^\circ\text{C}$) and prepare a solution containing a concentration of 1 ng/µl, by pipetting 100 µl of the contents of the ampoule into a glass brown flask (2.2.2) add 9.90 ml ethanol. Pipet 100 µl (100 ng) into a derivatisation-vial (2.2.9) and evaporate at 55°C (2.2.10) under a stream of nitrogen (2.1.3) until dryness. GCMS detection is performed after derivatisation with HFBA (2.1.4) or TMS (2.1.5, 2.1.6, 2.1.7):

? Pipet 25 µl of a mixture of Heptafluorobutyric Acid Anhydride:acetone p.a. (1:4) or

? Pipet 20 µl of a mixture of N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA):ammonium iodide:dithioerythritol(1000:2:4, v/w/w).

or

? Pipet 20 µl N,O,-bis(trimethylsilyl)trifluoroacetimide + 1% TMCS (BSTFA).

The selection of the derivatisation technique (HFBA/TMS) it's different for each analyte and depend on the most abundant fragments. For conditions and typical values for GC-MS analyses, see SOP ARO/113 and ARO/401. Place the derivatisation-vial in a heater (2.2.12) at 60°C for 1 hour. Evaporate at 50°C under a stream of nitrogen until dryness and dissolve in iso-octane (or toluene depends analyte) (25 µl), transferred into an injection-vial with micro insert. The vial with insert is capped and ready for GCMS analyses.

Generate a calibration curve by analysing different concentrations of the reference standard (3.1.4). Analyse an amount of the standard containing a concentration of:

0, 2.5, 5, 7.5, 10, 15 ng and an amount of 10 ng of a relevant internal standard (3.1.5). Construct the linear calibration curves with the ratio $[\text{peak area } m/z (\text{analyte})]/[\text{peak area } m/z (\text{internal standard})]$ as dependent variable and the concentration as independent variable.

For quantification the calibration curve must have a coefficient of correlation > 0.98 .

The ions (m/z) need to be characteristic peaks from the analyt and the internal standard.

4. QUALITY CONTROL.

4.1 Homogeneity of the weight and repeating pipetting

In order to check the average value, standard deviation, coefficient of variation (CV) of the weight of the ampouled amount and to control the repeating pipet (2.2.4): randomly weighed 10% of the total batch before and after filling (3.1.2).

Calculate the average weight of the added amount of solution, STD and CV.

Criteria, the average value must correspond with the calculated weight of 0.100 ml ethanol \pm 1% and CV less than 1.0%.

4.2 Repeatability of lyophilisation-process

When the solvent is evaporated the ampoules are weighted again and the solvent losses of the ampoules are calculated. Calculate the average weight of the solvent.

The criteria for lyophilisation is: $A_{AD} - A_{EV} = \bar{X} \cdot 0.5\%$.

A_{AD} = Average weigh after filling ampoules.

A_{EV} = Average weigh after lyophilisation process.

\bar{X} = Average weigh solvent (d^{20} ethanol = 0.789).

0.5% = Inaccuracy of the balance.

d^{20} = Density of ethanol at 20°C.

4.3 Degradation study.

4.3.1 *Influence of the lyophilisation process.*

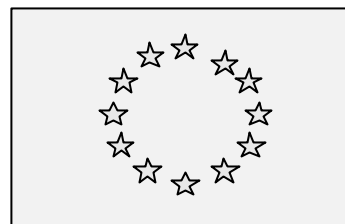
Determine the concentration of the analyte after lyophilisation, to assess the influence of the lyophilisation on the degradation process.

A batch of 10 ampoules must be selected randomly, after lyophilisation. Analyse (3.1.5) the concentration of the analyte in the ampoules and calculate the yield of the analyte after lyophilisation process. Calculate the average value of the added amount and CV.

4.3.2 *Analysing degradation process.*

Determine the concentration of the analyte over time at different temperatures to assess the degradation process. A batch of 4 ampoules are stored for a period of 4 weeks at $-70 \pm 2^\circ\text{C}$, 4 ampoules at $4 \pm 2^\circ\text{C}$, 4 ampoules at $20 \pm 2^\circ\text{C}$ and 4 ampoules at $37 \pm 2^\circ\text{C}$. After 4 weeks the ampoules are analysed (3.1.6). Calculate the amount by a calibration curve (3.1.6) and plot the results (amount versus time), see annex 2 page 11. From these results can be determined the degradation process at the applied storing conditions.

Annex 1.



Datasheet (name compound)

Reference number: EU/CRL....

Date of preparation:

date :

source :

“Bank of Reference Standards”

DRAWING STRUCTURE

FORMULA

Name :
Synonym :
Molecular formula :
CAS # :
Molecular weight :
Indication of purity :

Last update :

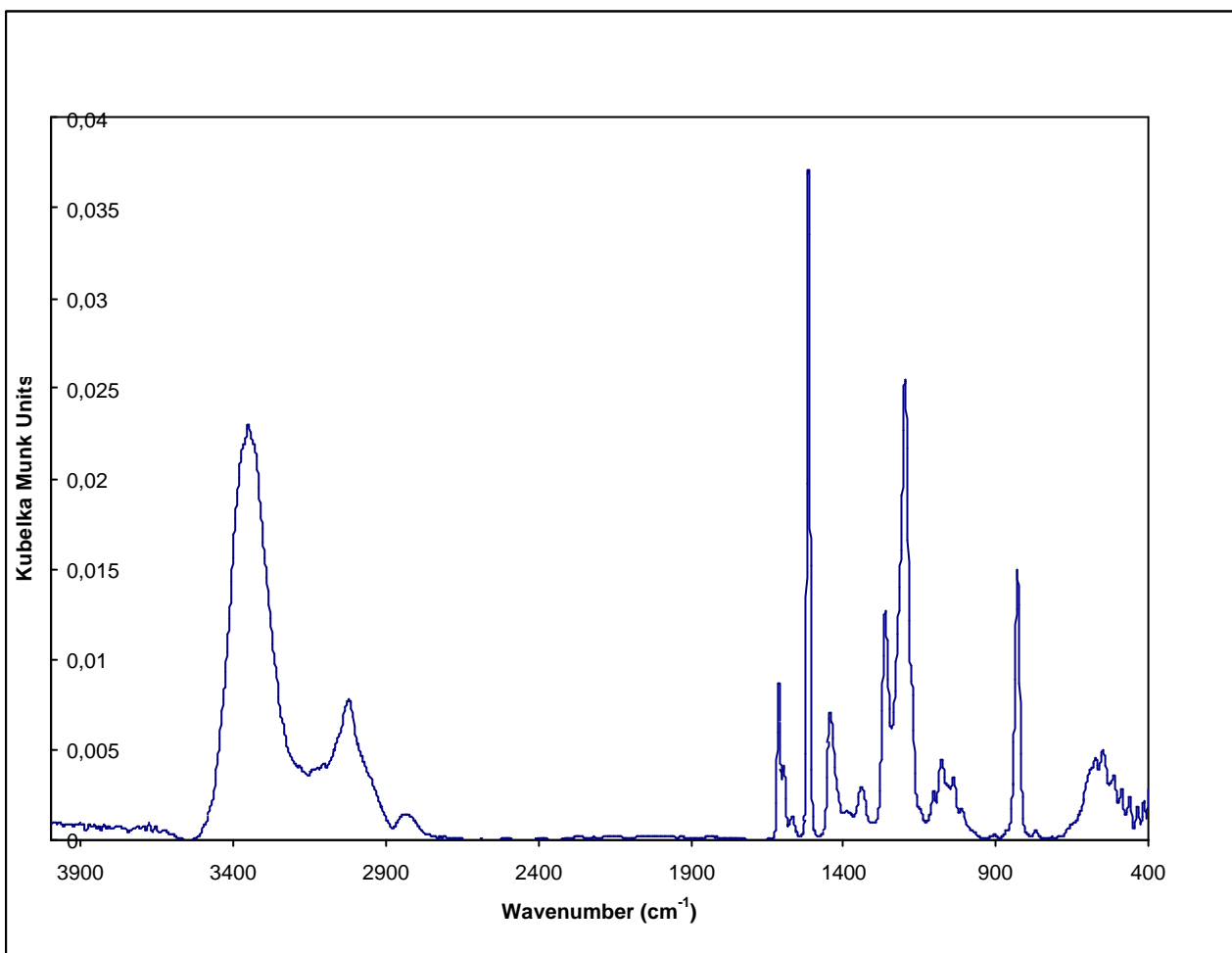
Methods used for characterization

- I** **IR spectroscopy**
- II** **UV spectroscopy**
- III** **Mass spectrometry**
- IV** **¹H-NMR spectrometry**
- V** **Homogeneity and stability obtained with GC-MS**

I IR spectroscopy

Instrument:

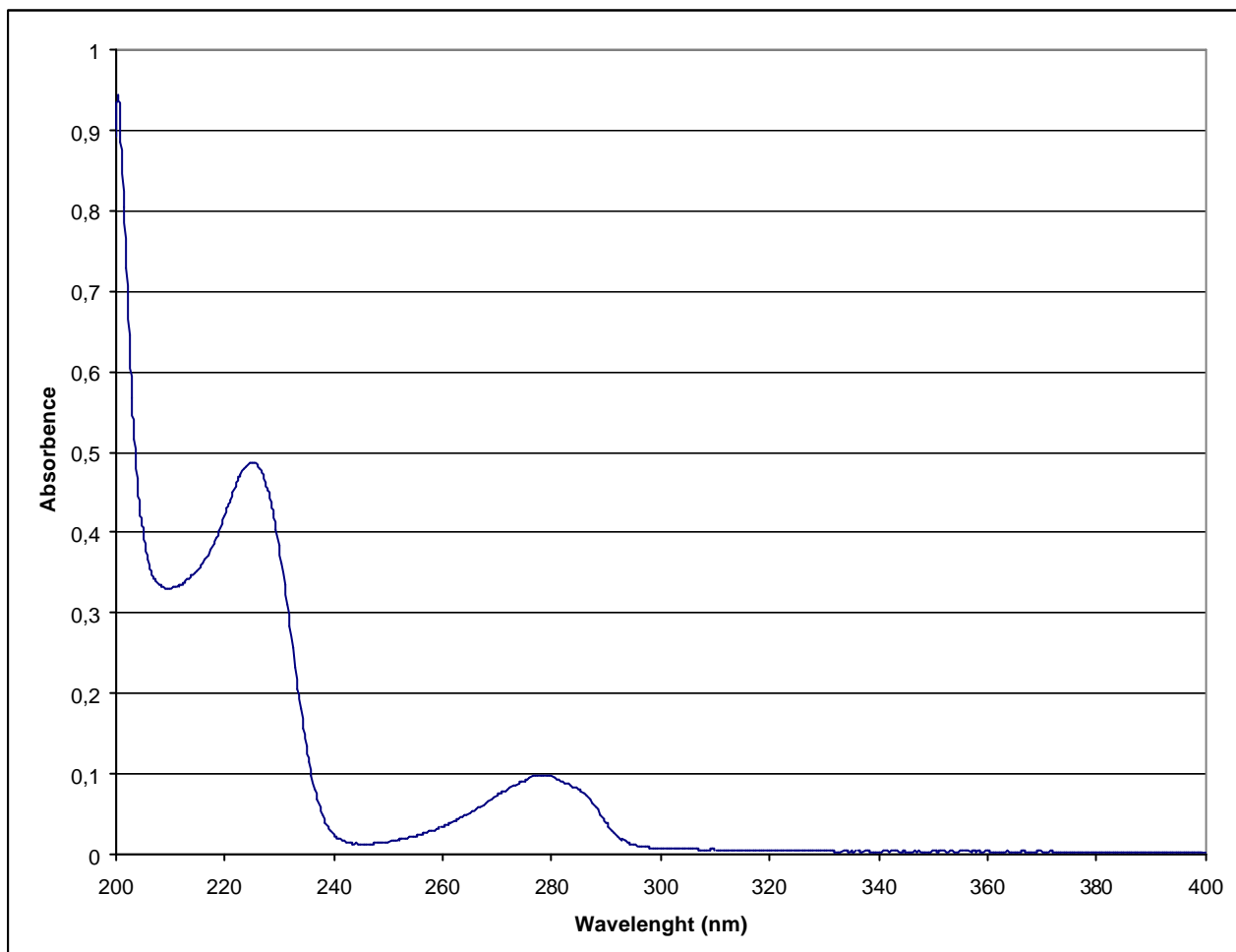
Sampling technique:



II UV spectroscopy

Instrument: UV-Visible Spectrophotometer

Concentration:



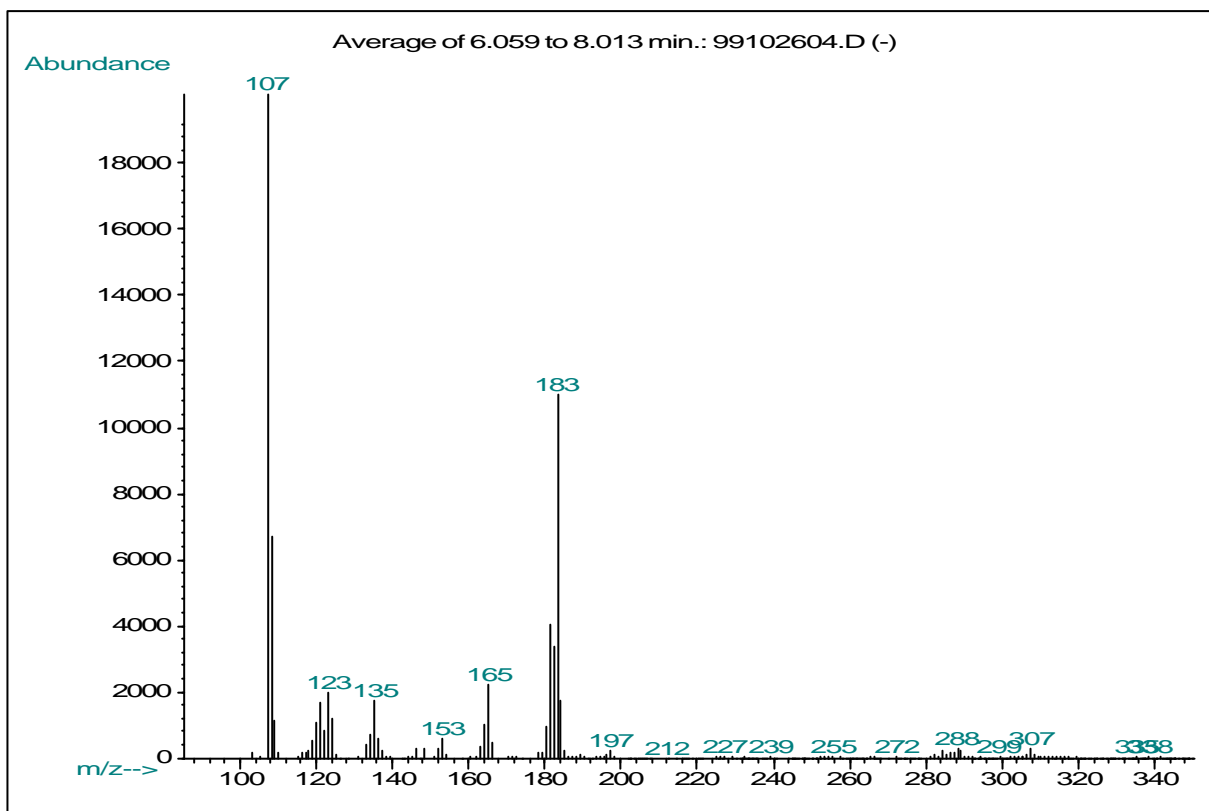
Tabel 1: The UV spectrum

Peak no.	Wavelength (nm)	Absorbance
1		
2		

III Mass spectrometry

Instrument:

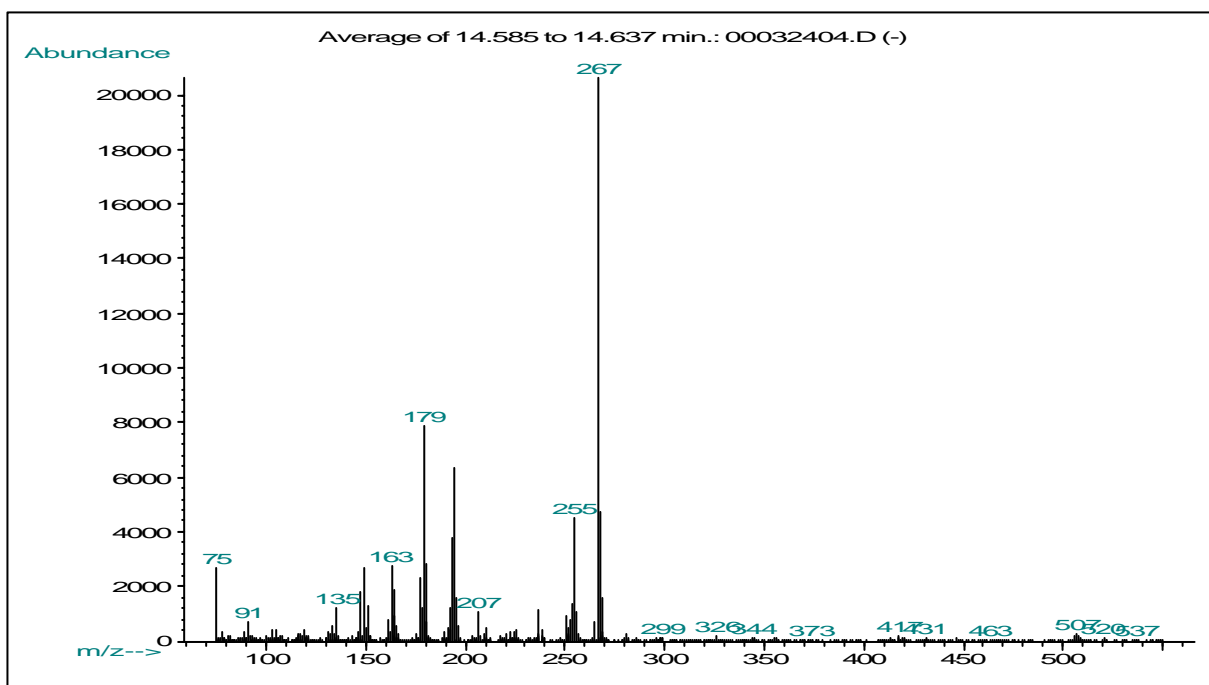
GC-MS spectrum, DIP = direct inlet probe



Instrument:

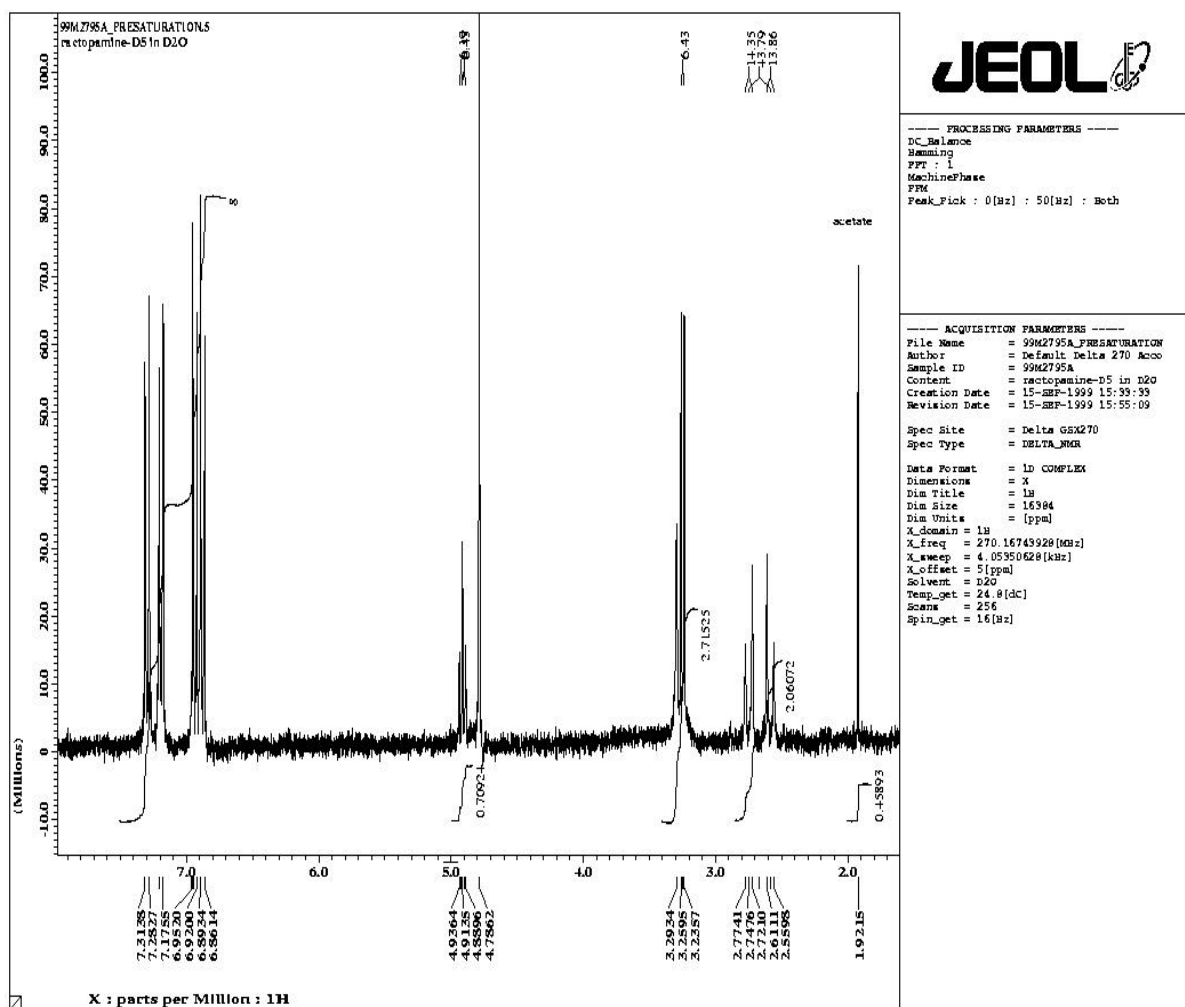
GC-MS spectrum, Full Scan

Mass spectrometry after TMS-derivatisation



IV **¹H-NMR spectrometry**

Instrument:

Solvent: D₂O**V** **Homogeneity and stability obtained with GC-MS**

Temp.	t = 0 months Homogeneity (n = 4) µg (m ± RSD)/ ampoules	t = 1 month (n = 4) µg (m ± RSD)/ ampoules
4°C		
23°C		
37°C		