

Method of confirmation by GC-MS

INTRODUCTION

In the early 80 's, a series of studies was begun on the possibility of determining substances of abuse in keratinous array and then use hair analysis as a means of finding the previous use of these substances. Once embedded in the hair, by the systemic circulation and by the contribution of sweat and sebum, substances of abuse remain impounded in the keratinous array in a stable way over time as a function of their lipophilic, molecular weight, the pKa and the steric encumbrance. The analysis of substances of abuse in keratinous array is an ideal addendum to the analysis of blood or urine as it provides information regarding an earlier time than that covered by other biological matrices and refers to a longer time period (one or more months) as a function of the length of the hair.

Considering an average growth of 1 cm per month, you can learn the history of the consumption by analyzing segmented hair per cm or fractions of cm. It is also possible to highlight external contamination by washing solvents by comparing them with the results obtained by digested hair. The search for substances of abuse in hair can be used to test use, abuse and misuse over time to determine intensity and its history and then provide analytical data with medico-legal value. Determination in hair may be required in the following cases: deaths related to the use of substances of abuse, assessment of ineligibility for driving, criminal liability, reliance custody of infants, prenatal exposure to substances of abuse and finally as confirmation of sporadic consumption or a dependency in workers.

Immunochemical tests can be used as initial methods to analyse in a short time a large number of samples in a standardized, efficient and economic way and exclude samples that are negative, i.e. samples that did not contain the substance or class of substances, or those in which the concentration is below a threshold value (cut-off).

Confirmation analyses are defined as those that serve to verify false positive due to the non-specificity of screening tests. In the case of keratinous array, because there are no real screening tests we cannot talk about real "confirmatory analysis". The "Society of Hair Testing" (SOHT) recommends the use of methodologies coupled to chromatographic separative disclosure in mass spectrometry for the determination of substances of abuse in keratinous matrix for medical-legal purposes and/or clinical trials.

This method allows to to determine the delta-9-THC prior extraction from keratinous matrix (hair) followed by liquid-liquid extraction (to remove the interfering presence of complex matrix) and determination in Gas chromatography/mass spectrometry (GC/MS).



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N° 005	Delta-9-THC in hair	09/2024
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TECHNICAL FEATURES

Principle of the Method :

This method determines **delta-9-THC** after hydrolysis of the keratin matrix, SPE extraction and GC-MS in reading.

The method was validated according to the criteria established at the international level by the FDA and by the ICH.

Recovery : >90 %

Sensitivity (LLOD) : 0,008 ng/mg

Minimum Concentration analyzable (LLOQ) : 0,025 ng/mg

Linearity : 0,025 – 2.500 ng/mg

Accuracy intra serie (relative error %):

Ci	Cs
0,1 ng/mg	1 ng/mg
9,0%	7,5%

Accuracy inter serie (relative error %):

Ci	Cs
0,5 ng/mg	5 ng/mg
9,9%	9,0%

Reproducibility intra serie (variation coefficient %):

C LLOQ	Cm	Cs
0,05 ng/mg	0,5 ng/mg	5 ng/mg
10,5%	7,5%	4,2%

Reproducibility inter serie (variation coefficient %):

C LLOQ	Cm	Cs
0,05 ng/mg	0,5 ng/mg	5 ng/mg
8,7%	9,5%	9,9%

Coefficient of correlation R2 + Dev. std: 0,9948 ± 0,0018

Cut-off Value:

delta-9-THC:

0.1 ng/mg

Ref. Gazzetta Ufficiale n°236 – 8 ott 2008

<u>Components of the kit (50 test) :</u>	All the reagents are ready to use and stable 3 years at 2 – 8 °C.
Reagent A – Diluting Solution, 1 x 120 ml	
Reagent B – Test Solution, 1 x 500 µl	<u>See Warnings</u>
Reagent C – Internal Standard Solution, 1 x 1 ml	<u>See Warnings</u>
Reagent D – Hair Wash Solution, 1 x 250 ml	
Reagent E – Extraction Solution, 1 x 250 ml	
Reagent F - Blank Hair, 1 x 2000 mg	
Reagent G – Chemical Standard Sol., 2 x 250 µl	Code GC53016 (Packed separately – see data sheets)
Reagent H – Digestion Solution, 1 x 50 ml	
Reagent M – Derivatization Sol., 3 x 1 ml (3 ampullas of 1 ml)	<u>See Warnings - For the sampling of Reagent M, the cap sealing requires the use of 50 µl Hamilton syringe Cod. ZRE24533</u>
<u>Minimum Instrumental equipment required:</u>	GC-MS Instrument Operational Computer
<u>Optional Equipment:</u>	Autosampler
<u>Hair collection procedure:</u>	Recommended length: 5 cm, starting from the scalp. Is cut a strand, not torn (the bulb has no purpose at the end of these investigations) in the region of the vertex back of the head, of at least 200 mg which is divided into 2 aliquots of similar weight (A and B) of each of which is fixed to the proximal end. These aliquots are placed in separate containers non-transparent bearing caps airtight and sealed with tape irremovable and stored at room temperature. The cut of the hair must be performed with clean scissors with a small amount of alcohol and dry well.
<u>Pubic hair collection procedure:</u>	It's need to cut 200 mg of hair from the pubic region; the hairs collected are divided into two aliquots of similar weight (A and B).

PREANALITICAL PROCEDURE

STEP 1 : Pipette in a glass tube with teflon cap :

- 50 µl of **Reagent B – Test Solution**

Conservation **Reagent B**: After opening the vial, withdrawn the necessary volume, close it with the reagent is not used and store a- 20 ° C.

STEP 2 : Evaporate to dryness with the aid of a stream of helium or nitrogen (in this step the temperature must be < 40 °C)

STEP 3 : Add 40 µl of **Reagent M - Derivatization Solution** using the appropriate syringe Hamilton of 50 µl (code ZRE24533)

Seal the tube with the cap

Vortex for 20 sec.

STEP 4 : Incubate at 75 °C for 30 minutes

Cooling at Room Temperature

Open glass tube

STEP 5 : Transferring the sample vials with reduction volume autosampler

NOTE: Before starting the analytical session is advisable to inject 2 ml of this freshly prepared solution in the gas chromatograph to identify the retention time and mass spectrum of all basic substances that must be similar to those shown in Figures below. If the test was successful, proceed to assay. If not, check the functionality of the analytical system.

ANALYTICAL PROCEDURE :

A) PREPARATION of unknown samples

STEP 1 : Wash hair intact inside a glass tube of 10 ml with 2 ml of **Reagent D – Hair Wash Solution**

Seal the tube with the cap

Vortex for 1 minute

Centrifuge at 3.500 rpm for 5 minutes

Open glass tube

STEP 2 : Evaporate to dryness the solvent with the aid of a light stream of helium or nitrogen paying attention to remain in the hair / fur on the bottom of the tube. Alternatively remove with a clean forceps with Acetone (not supplied in the kit), the hair / fur from the tube and place on paper towels until completely dry. In this last case the blotter paper should be changed for each sample.

Repeat steps 1 and 2 for two consecutive

STEP 3 : Cut your hair, washed and dried, in segments of 1-2 cm with scissors cleaned with Acetone (not supplied in the kit)

STEP 4 : Weigh an aliquot of sample, between 30 and 50 mg depending on the availability of the sample, in a Pyrex glass tube with screw cap clean and previously named.

B) PREPARATION of CALIBRATORS ON 5 LEVELS

Preparation of the Standard Chemical for enrichment after:

Prepare 3 glass tubes, call them A, B, C and dilute **Reagent G – Chemical Standard** with **Reagent A – Diluting Solution** as reported in tab. below:

Tubes	Reagent G – Chemical Std	Reagent A – Diluting Sol.
A	10 µl	9990 µl
B	10 µl	990 µl
C	20 µl	180 µl

Close the tubes – Vortex for 1 minute

Prepare 5 glass tubes of 10 ml called C0-C1-C2-C3-C4.

Add at each tube (C0-C1-C2-C3-C4) the standard chemical just prepared (A, B, C) referring to the table below:

Calibrator C0	Calibrator C1	Calibrator C2	Calibrator C3	Calibrator C4
100 µl Reagent A – Diluting Sol.	100 µl of A	200 µl of A	200 µl of B	100 µl of C

Evaporate to dryness with the aid of a stream of helium or nitrogen (in this step the temperature must be < 40 °C).

Add to each tube of the calibrators 50 mg of **Reagent F – Blank Hair** (supplied in kit).

At this point Calibrators of 5 points are ready and the concentrations to be used for the calibration curve will be:

Analyte	Calibrator C0	Calibrator C1	Calibrator C2	Calibrator C3	Calibrator C4
Delta-9-THC	0 ng/mg	0.05 ng/mg	0.1 ng/mg	1 ng/mg	5 ng/mg

C) PREPARATION OF INTERNAL STANDARDS

Reagent C - Internal standard Sol supplied in the kit should be diluted 1:10 with water before each use, in the necessary amount for the entire assay.

Since, for each sample and for each Calibrator / Controls are required 20ul of Internal Standard diluted, calculate the volume of reagent needed for each run and at the same time dilute the reagent.

Eg. for an analytic session with 20 unknown samples and 5 calibrators are required 500 µl of each Standard Internal dilution (**diluted Reagent C**). Therefore you fetch 50 µl of **Reagent C - Internal standard Sol** and is diluted respectively with 450 µl of H₂O in a glass tube. Vortex for at least 10 sec. The solutions are now ready for use.

The **Reagent C - Internal standard Sol** undiluted and not used are closed again tightly and kept to the residual quantity in the freezer at -20°C.

D) DIGESTION OF KERATINIC MATRIX

STEP 5 : In each tube previously prepared (phase A and phase B) dispense:

	Calibrator C0 (prepared in STEP B)	Calibrator C1 (prepared in STEP B)	Calibrator C2 (prepared in STEP B)	Calibrator C3 (prepared in STEP B)	Calibrator C4 (prepared in STEP B)	Controls	Samples (prepared in STEP A)
Reagent C diluted - (Internal Std Sol. diluted as shown in STEP C)	20 ul	20 ul	20 ul	20 ul	20 ul	20 ul	20 ul
Reagent H – Digestion Sol.	1000 µl	1000 µl	1000 µl	1000 µl	1000 µl	1000 µl	1000 µl

Seal the tubes with the cap - without invert gently mix the vial for 10 seconds

STEP 6 : Centrifuge 5 minutes at 10.000 rpm

STEP 7 : Incubate at 90 °C for 1 hour

Cooling at Room Temperature

STEP 8 : Transfer the digested into new glass tubes.

E) EXTRACTION OF DELTA-9-THC

STEP 9 : Open the glass vials prepared in STEP D

Add 5 ml of **Reagent E – Extraction Sol.** at each Calibrator, Samples and Control prepared before

Seal the tubes with the cap - mix the vial for 10 seconds on stirrer

Vortex for 30 sec

Centrifuge at 3.500 rpm for 15 min

Transfer 4 – 4,5 ml of supernatant in glass tubes called C0, C1, C2, C3, C4, Sample 1, Sample 2, etc. named THC

F) DERIVATIZATION OF DELTA-9-THC

Evaporate all the tubes prepared in STEP E to dryness with the aid of a stream of helium or nitrogen (in this step the temperature must be < 40 °C).

Derivatization: Add to each tube 40 µl of **Reagent M – Derivatization Solution** using the appropriate syringe Hamilton of 50 µl (code ZRE24533)

Seal the tubes with the cap

Vortex for 30 sec.

Incubate at 75°C for 30 min

Cooling at Room Temperature

Open the glass tube

Injection: Transfer the derivatized in the vial with reducing volume and inject 2 µl in the gas chromatograph.

GENERAL CONSIDERATIONS about QUANTITATIVE CALCULATION OF UNKNOWN SAMPLES

The calculation of the unknown samples is performed as a function of the equation obtained for the calibration curve where the abscissa axis shows the concentration in ng/mg and along the ordinate the ratio between the areas of the analyte/internal standard. To calculate the concentration in ng/mg hair in the case of unknown samples, please note that once you have the value of concentration on the line, it should be multiplied by the mg of hair that has built the calibration curve (20 mg) and divided by the actual weight of the sample under examination (eg. 10 mg if it is weighed amount of this hair).

DELTA-9-THC in hair - Warnings

REAGENT B : TEST SOLUTION

Delta-9-THC	About 10.000 ng/ml
Storage: Once you open the vial, withdrawn the necessary volume, close it with the reagent used and stored at -20°C.	

REAGENT G : CHEMICAL STANDARD

Delta-9-THC	25.000 ng/ml
Storage: Once you open the vial, withdrawn the necessary volume, close it with the reagent used and stored at -20°C.	

REAGENT M : DERIVATIZATION SOLUTION

Once opened ampoule transfer all the content, 1 ml, in a glass vial. Close cap tightly and store at Room Temperature.

REAGENT C : INTERNAL STANDARD SOLUTION

Delta-8-THC	250.000 ng/ml
Storage: at – 20 °C	

DETECTOR DI MASSA CONDITIONS (4000) : (these conditions may be varied depending on the instrument used)

• Massa Range 280 – 420 m/z • Massa Temperature: 180 °C; • Transfer Line Temperature: 270 °C; • Manifold Temperature: 80 °C • Filament on: 3 minutes
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GAS-CHROMATOGRAPH CONDITIONS : (these conditions may be varied depending on the instrument used)

• Column Restek Rxi-5Sil 30 m x 0,25 mm, 0,25 µm (after conditioning according to the indications provided by the manufacturer) • Injector Temperature 280 °C • Split Ratio: ON split 20 0 min: OFF 1 min: ON split 100 3 min: ON split 20 NB: The split ratio can be varied depending on the instrument used • Heat Schedule: 120 °C x 2,42 minutes 120-250°C at 40 °C/min 250-300°C at 15 °C/min 300x 2,0 minutes (run 11 minutes) • Helium Gas Flow 1 ml/min
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COLUMN Restek Rxi-5Sil CONDITIONING

Follow the manufacturer's instructions.

COLUMN Restek Rxi-5SiI CLEANING

If the analytical performance suffer significant changes (e.g. Low sensitivity or presence of interfering in chromatograms), you must make the cleaning of the column as reported in accordance with the indications of the manufacturer.

AUTOSAMPLER WASH

Wash the syringe before each injection with ACETONE.

ACCESSORIES AND CONSUMABLES

CODE	DESCRIPTION	PACKAGING
GC53016	Calibrator in hair for delta-9-THC	1 Pc
GC53019	Control in hair for delta-9-THC – Levels 1 and 2	1 Pc
S29057U	Standard glass vial of 2 ml with screw cap	1 x 100 Pcs
S29046U	Caps for Standard glass vials of 2 ml with screw cap	1 x 100 Pcs
S24717	Insert 100 ul for vials of 2 ml	1 x 100 Pcs
Z1636/26	Pyrex Tubes of 10 ml with SWL caps	1 x 40 Pcs
S13623	Restek Rxi-5SILms (30 m x 0,25 mm – 0,25 um) Column	1 Pc
ZRE24533	Hamilton Syringe of 50 ul	1 Pc

Delta-9-THC IN HAIR

(Reference Chromatograms/Spectra)

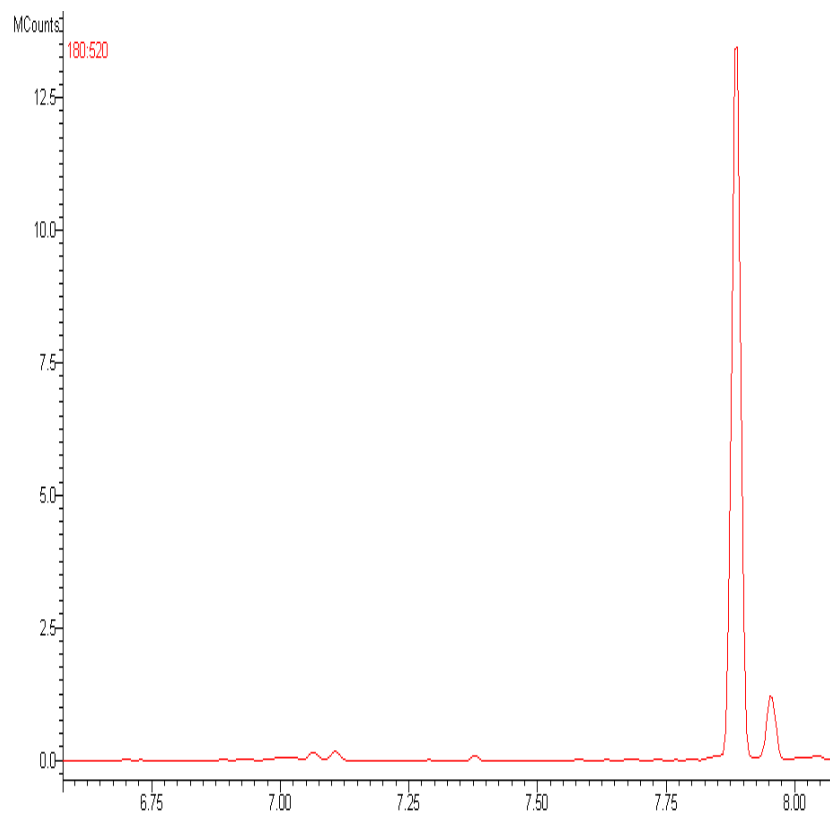


Fig. 1 :	Test Solution of delta-9-THC
	R.T. 7.96 delta-9-THC

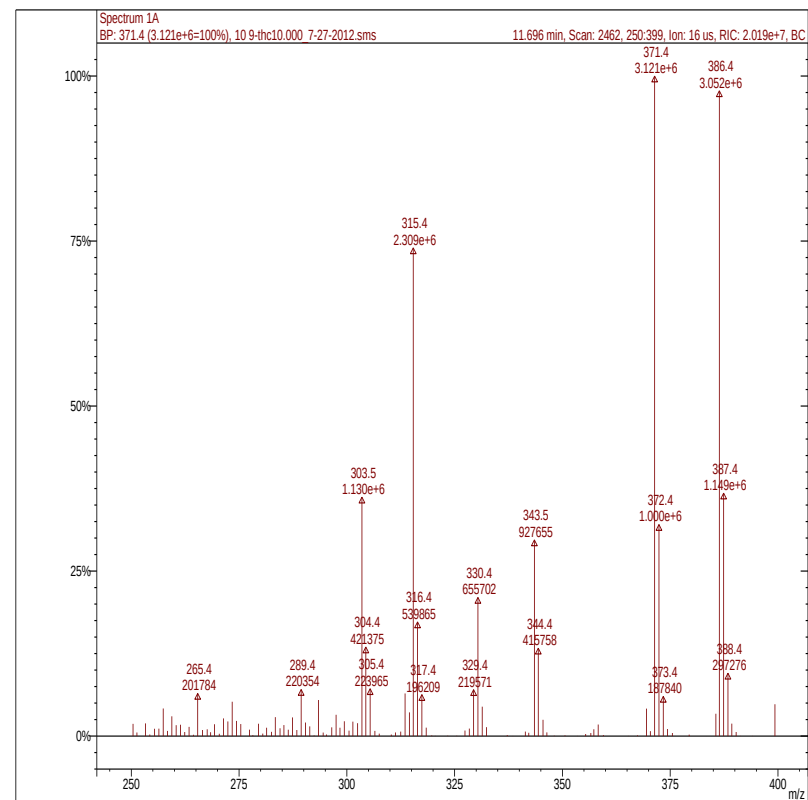


Fig. 2 :	Massa Spectrum of delta-9-THC
	MOLECULAR IONS: 303-371-386

Delta-9-THC IN HAIR

(Reference Chromatograms/Spectra)

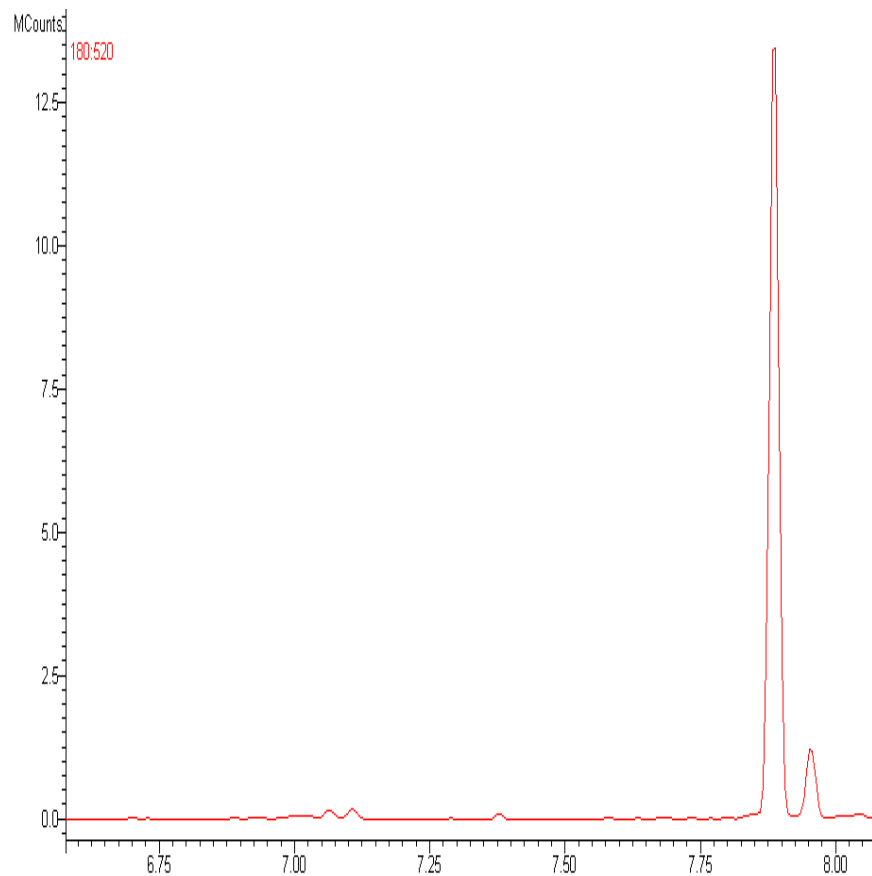


Fig. 3 :	Internal Standard Solution
	R.T. 7.88 Internal Standard

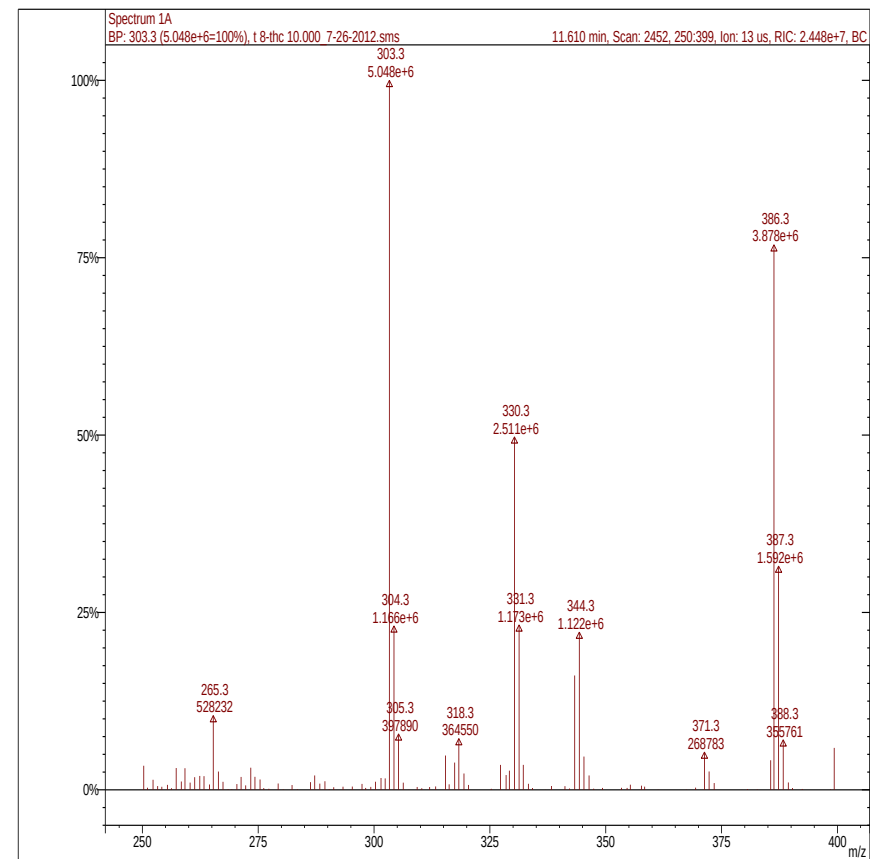


Fig. 4 :	Massa Spectrum of Internal Standard
	MOLECULAR IONS: 303-330-386