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RECENT ADVANCES IN DOPING ANALYSIS (2)

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Detection of Mesocarb Metabolite by LC - TS/MS

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There had been two reasons to deal with a modified identification of mesocarb:
- Mesocarb was a very striking substance in our laboratory, because it was included in screening procedure 2a for slightly volatile stimulants and narcotics. In spite of a high dosage, good extraction recovery, liquid chromatographic and detection properties of the metabolite, 50 % of the specimen applied, are lost for the identification of one single substance.
- The high concentration and some problems associated with pyrolysis chromatography suggested to be a good opportunity to check the applicability of LC TS/MS as a confirmation method.

We tried at first to adapt the proposal of Segura et al. (Barcelona laboratory) -to identify the conjugate rather than the free metabolite together with diuretics in one procedure [1] - to our requirements. Two extraction steps had been carried out to separate mesocarb, after using ether at pH 9, the same urine phase was extracted with ethylacetate. It became obvious, that there is a more than 20fold increased recovery of conjugated mesocarb in ethyl acetate after addition of sodium chloride, but a considerable amount of matrix is coextracted. The insertion of an additional extraction step, applying ethyl acetate in combination with the ether extraction of diuretics or with caffeine screening seems to be a useful compromise for sample preparation. The extraction recovery at various pH values has a maximum at alkaline conditions.

A HP 5985 (MS engine) was applied for all thermospray investigations. Instrument parameters are listed below.
Besides the MS source, lens parameters and composition of HPLC mobile phases, the probe temperature (optimized vaporisation temperature) proofed to be of great importance for peak shape and signal intensity. In case of HPLC gradient separation this parameter is difficult to fit into a wide composition range. The unsatisfactory peak shape of the internal standard could be accepted with respect to its high concentration.

Already the first experiences with thermospray mass spectrometry demonstrated an intensive influence of mobile phase composition on chromatographic and spectrometric results. When changing the mobile phase from water + acetonitrile to 0.1M ammonium acetate buffer + acetonitrile, the retention time of the conjugated mesocarb metabolite increases by more then 3 minutes, whereas the times of the similar substances (free hydroxy-mesocarb and internal standard - unchanged mesocarb) remain constant. The fragmentation patterns of the conjugate of metabolised mesocarb are fairly different for all mobile phases observed. Conjugated hydroxymesocarb is totally fragmented in water+acetonitril mobile phase, whereas the MS of free metabolites in phosphate-buffer+acetonitril consist nearly exclusively of the molpeaks. The only appropriate mixture, permitting the detection of an presumptive mole peak, was ammonium acetate buffer + acetonitrile. (Compare figure 1 and 2.)

Fig.1) LC-TS/MS of conjugated hydroxymesocarb, using water+acetonitrile as mobile phase, extracted ion profilings and mass spectrum of the m/e=200-340 scan.

Fig. 2) LC-TS/MS of conjugated hydroxymesocarb, using phosphate buffer+acetonitrile as mobile phase, extracted ion profilings and mass spectrum. The mass spectrum represents a superposition of two scans (m/e=200-340 and m/e=330-550).

Fig. 3) LC-TS/MS of free hydroxymesocarb (after acidic hydrolysis), using water+acetonitrile mobile phase, extracted ion profilings and mass spectrum of a m/e=200-340 scan.

Besides the non specific m/e=204 signal of the base peak, and the m/e=339 fragment that corresponds with the molecular mass of hydroxymesocarb, signals at 419, 477, 494 and 535 were detected. The structures of mesocarb and its main fragments are shown in figure 4. The base peak was previously detected by pyrolysis EI-MS as well as by CI-MS [2] or by particle beam LC-MS as complementary fragment [1].

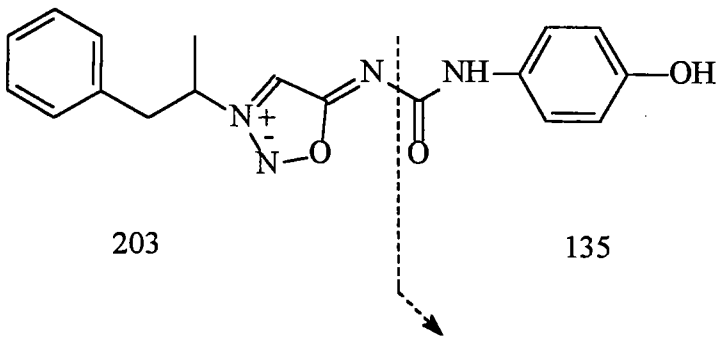


Fig. 4) Molecular structure of mesocarb and its major fragment

These results are in accordance with the structure of a sulphate (molar mass 418) and agree with the results of hydrolysis studies and particle beam MS investigations[1]. The balance of M+1 and M+18 fragments is mainly dependent on mobile phase compositions. Fragments of masses M+n*58+1 or M+n*58+18 are observed preferably after tuning and should represent adducts with monomers of the tuning agent polypropylene glycol.

A selected ion monitoring is proposed, to discriminate the conjugate from free metabolite and parent compound in a screening procedure. (Fig.5-6)
In the first sample - after acidic hydrolysis -, the free metabolite is identified by ions 338 and 204. Both fragments may also be detected in the second sample (extraction as described above) in addition to the M+1, M+18, M+58+1 and M+2*58+1 ions of the sulphate. Low signals at the retention time of the conjugate indicate a certain amount of unchanged sulphate in the hydrolysed sample.

Fig. 5-6: Results of LC-TS/MS -selected ion monitoring screening experiments. A mesocarb control urine was examined with and without hydrolysis.

Summary:

According to the extraction properties, the combination of mesocarb screening with diuretics screening procedure using ethyl acetate as an additional organic solvent is a useful approach to reduce the effort for mesocarb detection.

Thermospray MS proved to be a useful technique to confirm screening results and to obtain additional structural information about unknown compounds. Thus, the hypothetical sulphate conjugation of hydroxymesocarb could be confirmed.

the reproducibility is limited due to the high number of instrumental parameters and to the influence of chromatographic conditions (especially mobile phase composition) on fragmentation pattern.

The information obtained from the TS-mass spectra is comparatively low due to the low amount of fragmentation. Thermospray mass spectra are usually easy to interpret and provide access to the molecular mass of the compound.

Analytical Parameters:

HPLC

mobile phase: water + acetonitril or
0.1M ammonium acetate buffer + acetonitril
flow = 0.7 ml/min
gradient: 5%B at 0 min ---> 65%B at 12 min

column: Hypersil RP 18 (3 µm)

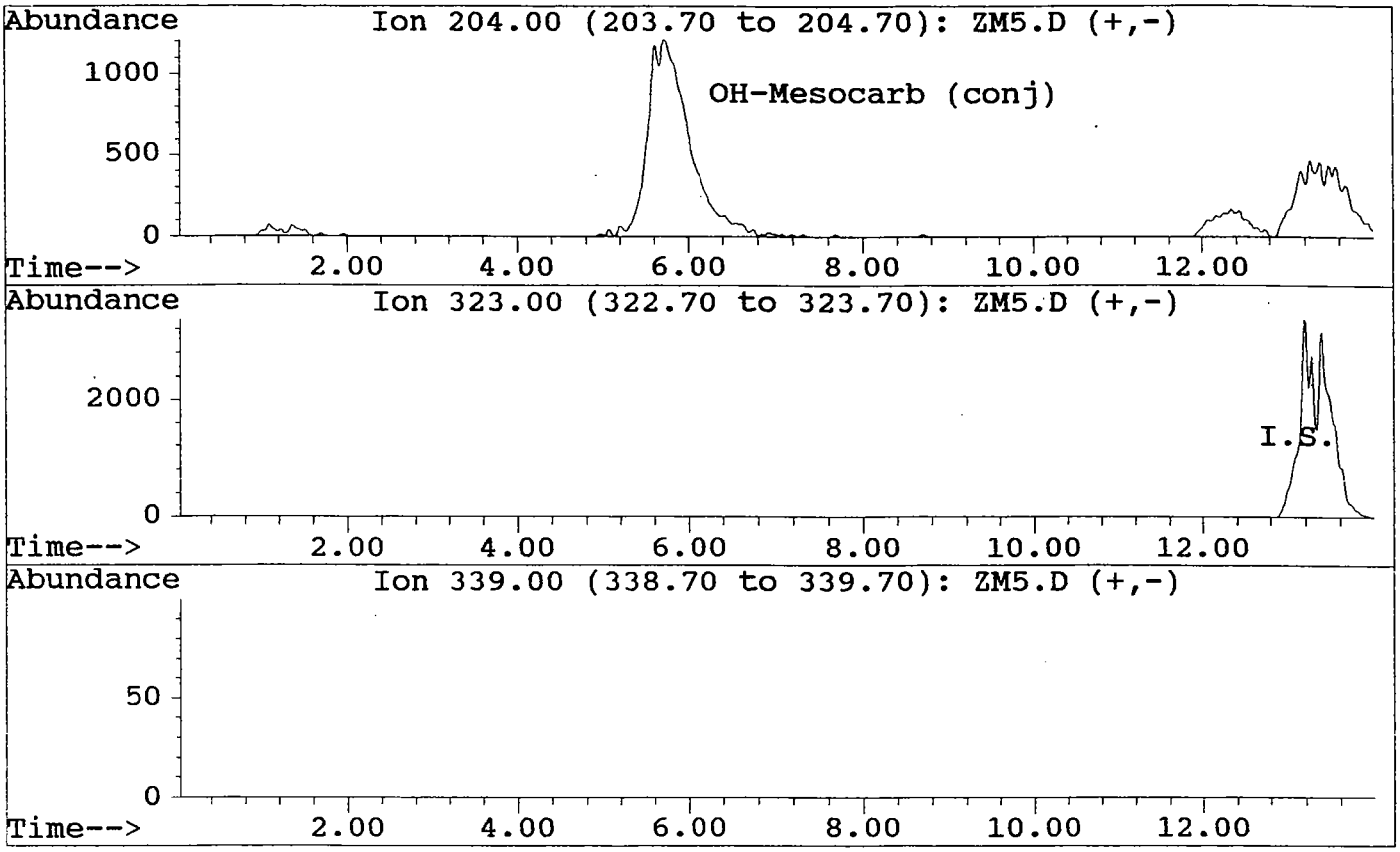
Thermospray: HP 5985 (MS-Engine)

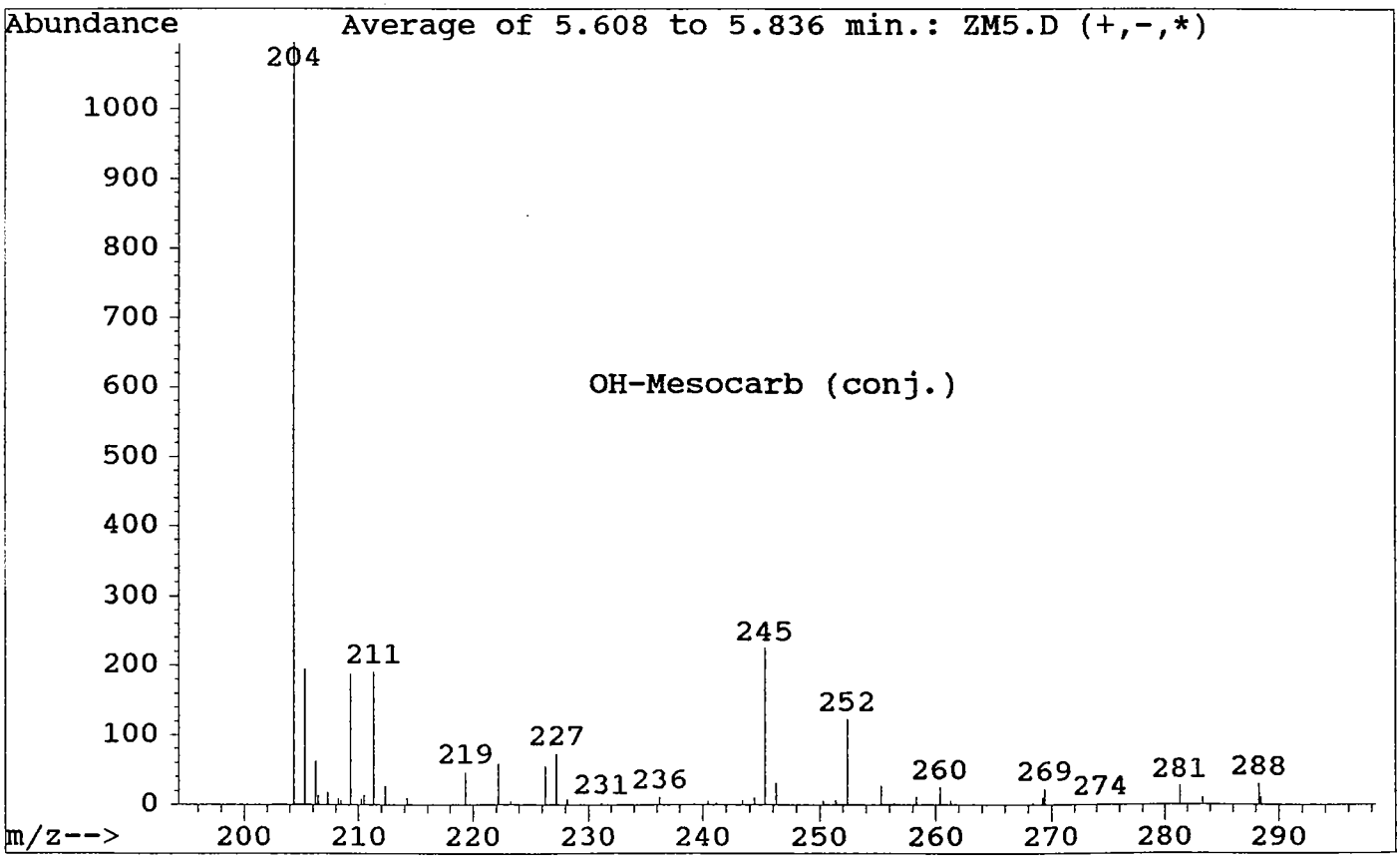
Fillament	On
Discharge El.	Off
Fragmenter	Off
Source Temp.	240°C
Probe Temp.	112--->92°C

References:

[1] Ventura,R., T. Nadal, P.Alcalde and J. Segura. Determination of mesocarb metabolites by high-performance liquid chromatography with UV detection and with mass spectrometry using a particle-beam interface. Journal of Chromatography 647 (1993) 203-210.

[2] Tamas,J.,Polgar,M.,Czira,G. and Vereczkay,L. Mass spectrometric characterization of sydnocarb in Proceedings of the 5th International Symposium on Mass Spectrometry in Biochemistry and Medicine, Rimini (Italy) 1978, 43-52.


Abundance
Ion 204.00 (203.70 to 204.70): ZM5.D (+,-)
1000
500
0
OH-Mesocarb (conj)
Time-->
2.00
4.00
6.00
8.00
10.00
12.00
Abundance
Ion 323.00 (322.70 to 323.70): ZM5.D (+,-)
2000
0
I.S.
Time-->
2.00
4.00
6.00
8.00
10.00
12.00
Abundance
Ion 339.00 (338.70 to 339.70): ZM5.D (+,-)
50
0
Time-->
2.00
4.00
6.00
8.00
10.00
12.00


Abundance
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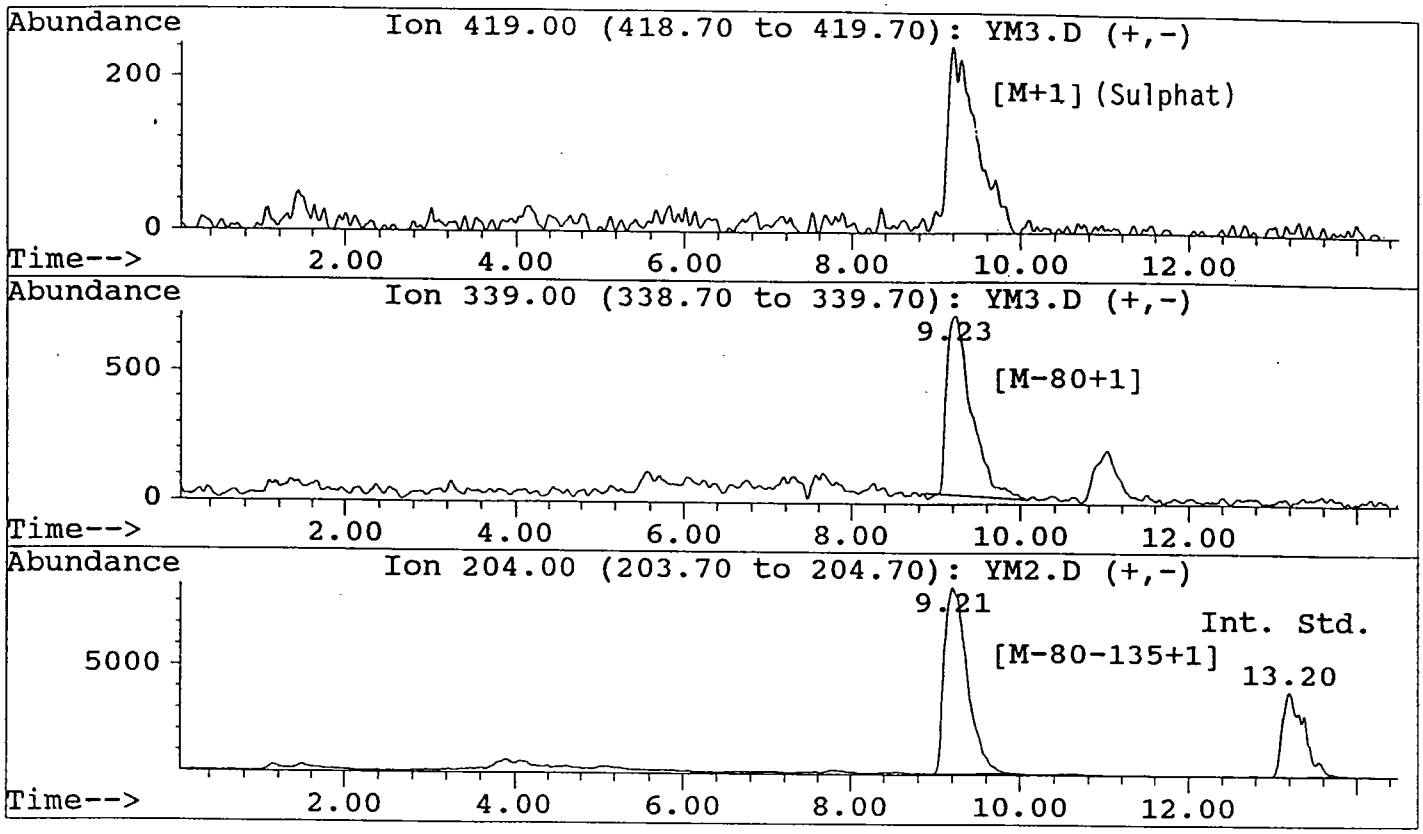
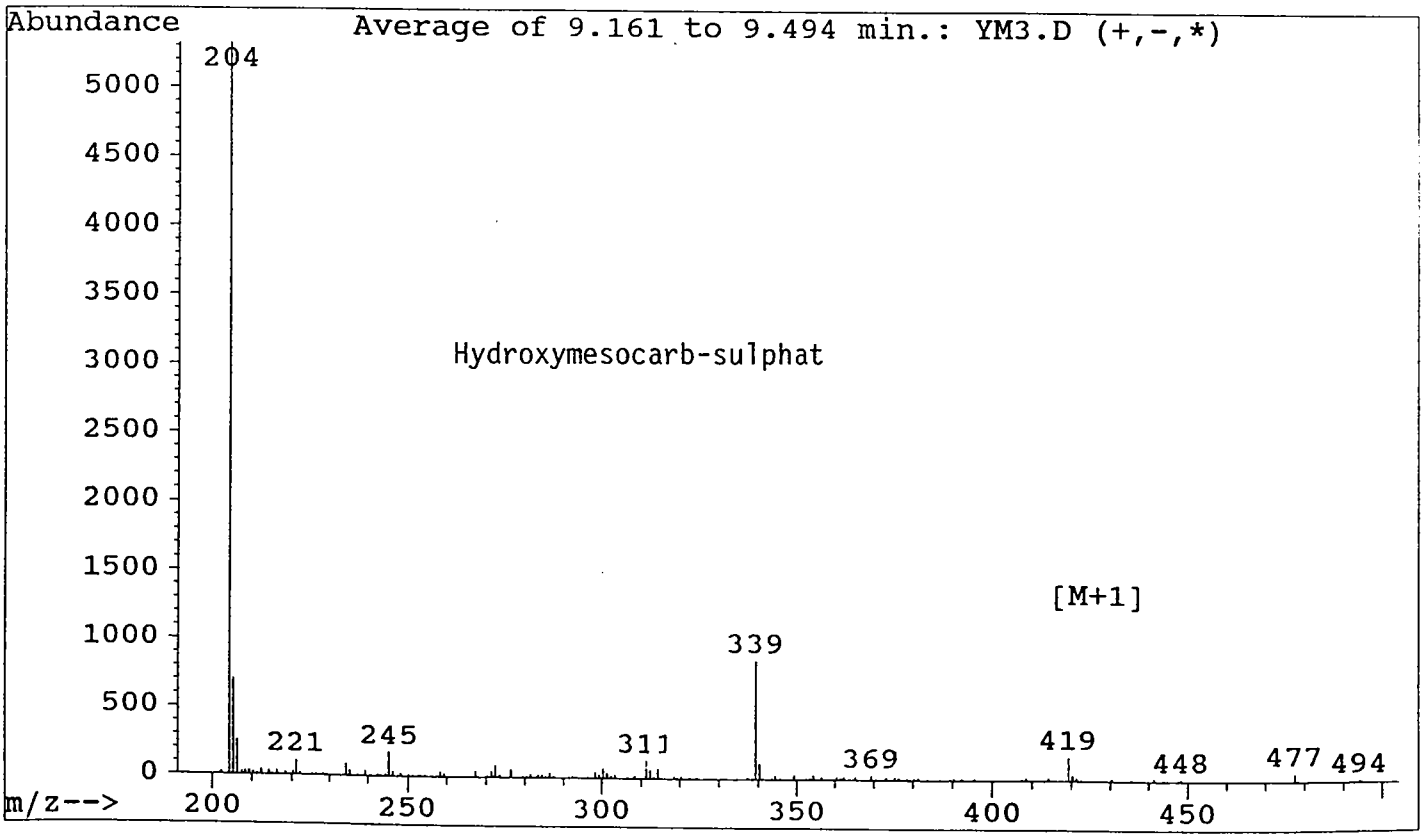
204
1000
900
800
700
600
500
400
300
200
100
0
OH-Mesocarb (conj.)
211
219
227
231
236
245
252
260
269
274
281
288
m/z-->
200
210
220
230
240
250
260
270
280
290


Abundance
Ion 419.00 (418.70 to 419.70): YM3.D (+,-)
200
0
[M+1] (Sulphat)
Time-->
2.00
4.00
6.00
8.00
10.00
12.00
Abundance
Ion 339.00 (338.70 to 339.70): YM3.D (+,-)
9.23
500
0
[M-80+1]
Time-->
2.00
4.00
6.00
8.00
10.00
12.00
Abundance
Ion 204.00 (203.70 to 204.70): YM2.D (+,-)
9.21
Int. Std.
5000
[M-80-135+1]
13.20
Time-->
2.00
4.00
6.00
8.00
10.00
12.00


Abundance
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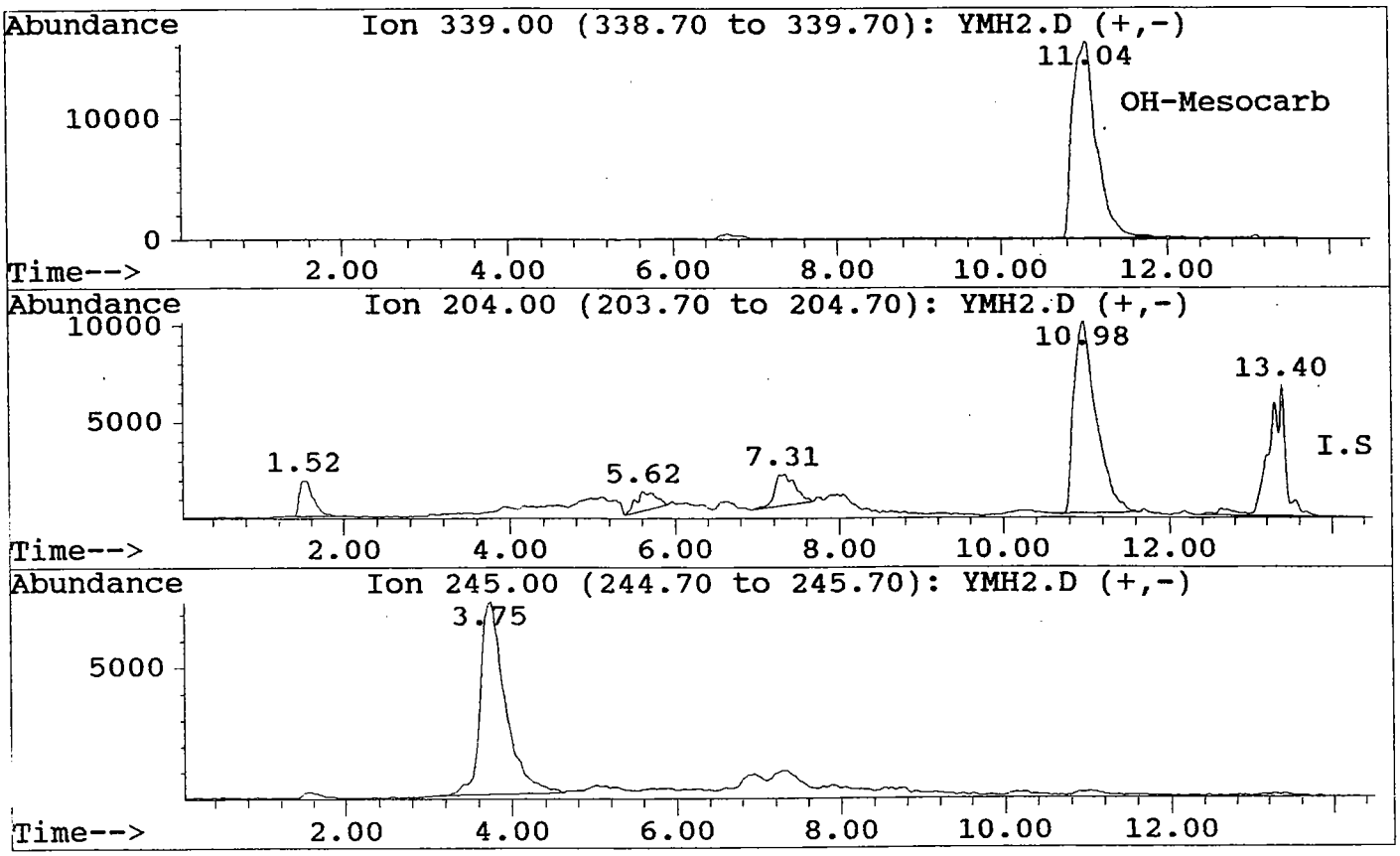
204
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Hydroxymesocarb-sulphat
[M+1]
339
221
245
311
369
419
448
477
494
m/z-->
200
250
300
350
400
450


Abundance
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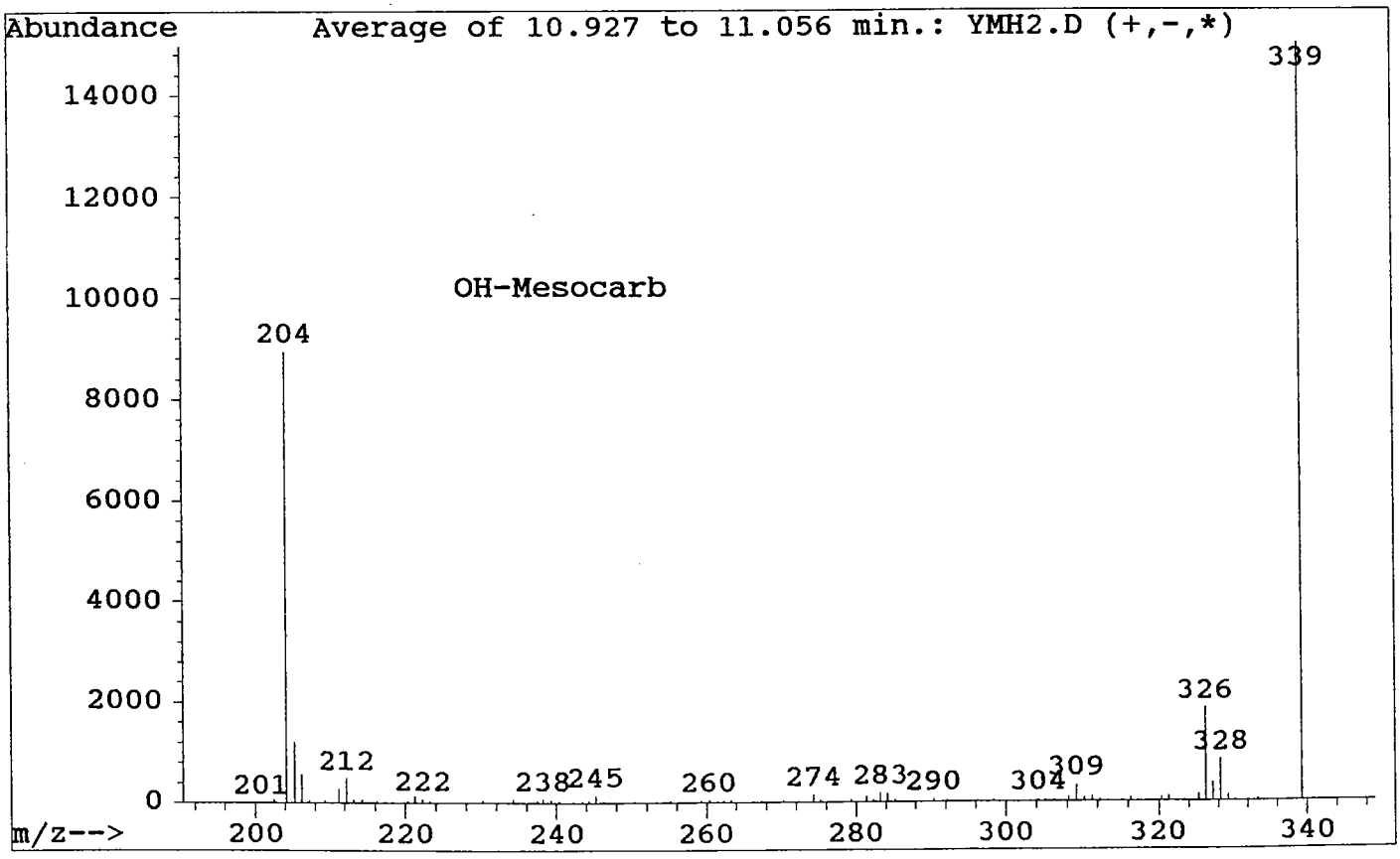
11.04
OH-Mesocarb
10000
0
Time-->
2.00
4.00
6.00
8.00
10.00
12.00
Abundance
Ion 204.00 (203.70 to 204.70): YMH2.D (+,-)
10000
5000
10.98
13.40
I.S
1.52
5.62
7.31
Time-->
Abundance
Ion 245.00 (244.70 to 245.70): YMH2.D (+,-)
3.75
5000
Time-->


Abundance
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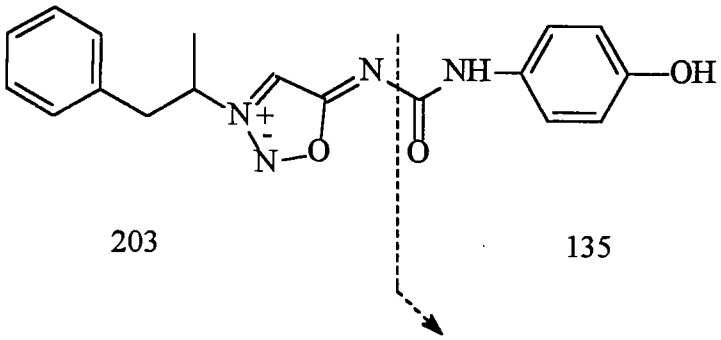
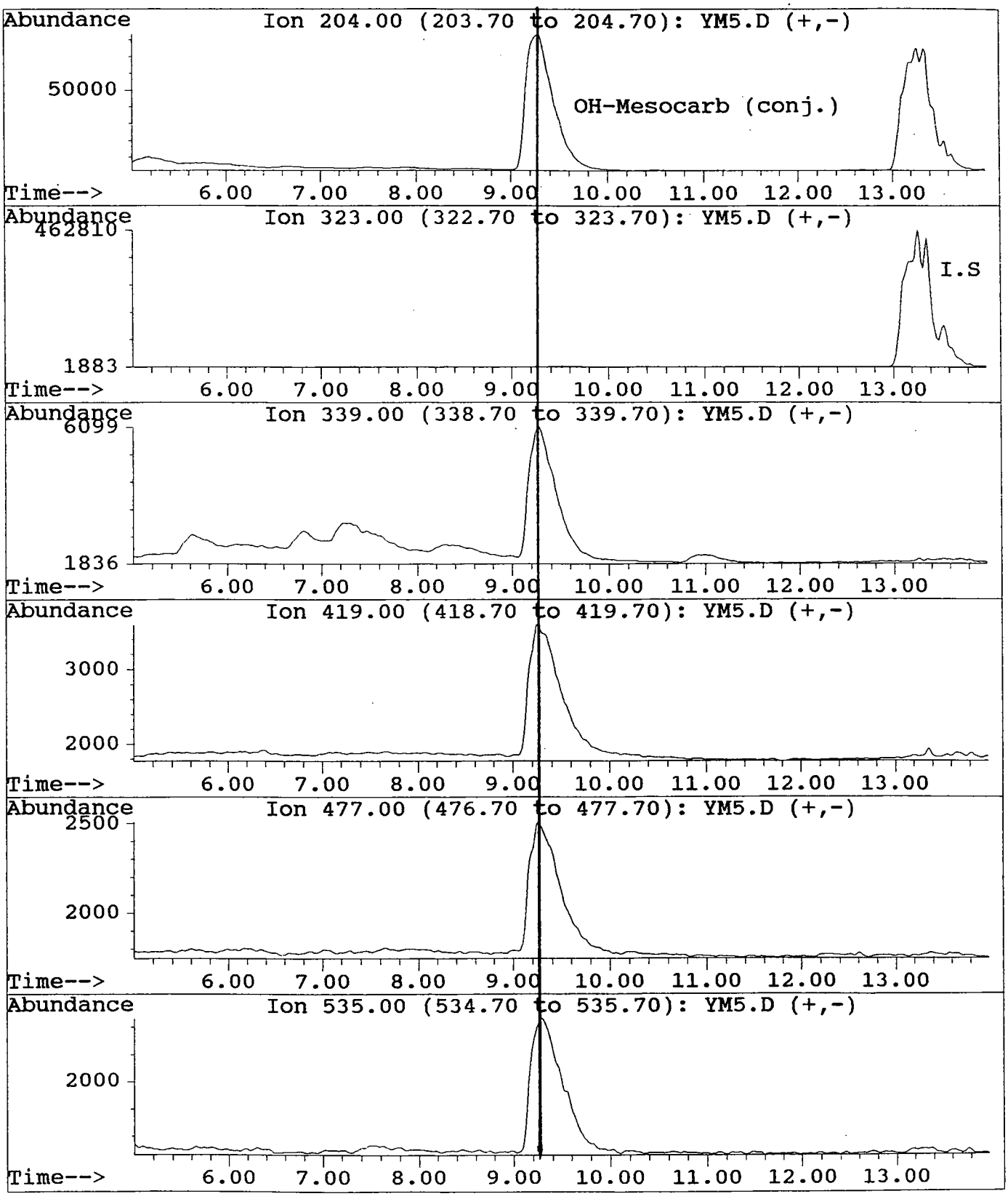
339
14000
12000
10000
OH-Mesocarb
204
8000
6000
4000
2000
326
328
0
201
212
222
238
245
260
274
283
290
304
309
m/z-->
200
220
240
260
280
300
320
340

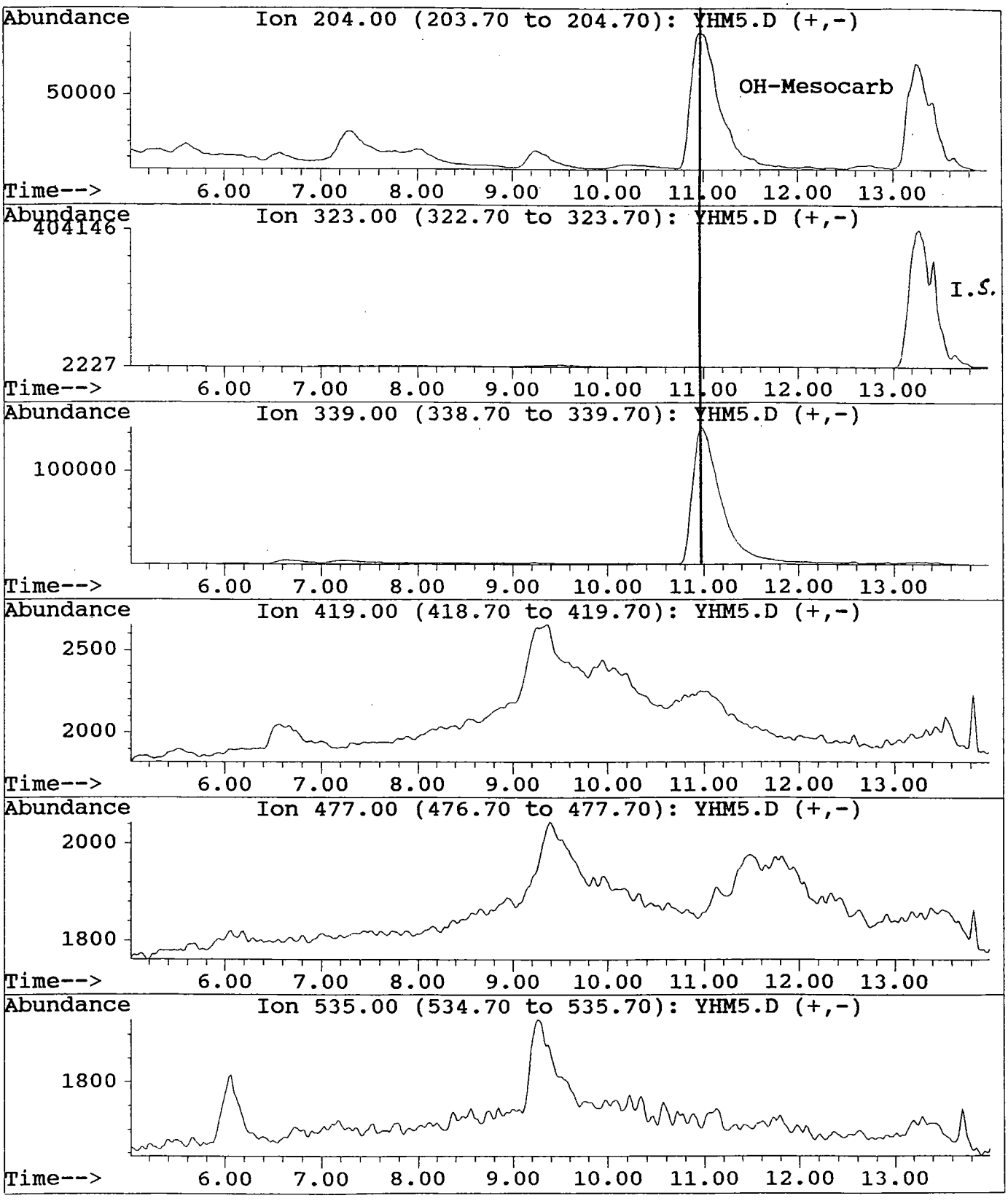


203 135

M=338


Abundance
Ion 204.00 (203.70 to 204.70): YM5.D (+,-)
50000
OH-Mesocarb (conj.)
Time-->
6.00 7.00 8.00 9.00 10.00 11.00 12.00 13.00
Abundance
Ion 323.00 (322.70 to 323.70): YM5.D (+,-)
462810
1883
I.S
Time-->
6.00 7.00 8.00 9.00 10.00 11.00 12.00 13.00
Abundance
Ion 339.00 (338.70 to 339.70): YM5.D (+,-)
6099
1836
Time-->
6.00 7.00 8.00 9.00 10.00 11.00 12.00 13.00
Abundance
Ion 419.00 (418.70 to 419.70): YM5.D (+,-)
3000
2000
Time-->
6.00 7.00 8.00 9.00 10.00 11.00 12.00 13.00
Abundance
Ion 477.00 (476.70 to 477.70): YM5.D (+,-)
2500
2000
Time-->
6.00 7.00 8.00 9.00 10.00 11.00 12.00 13.00
Abundance
Ion 535.00 (534.70 to 535.70): YM5.D (+,-)
2000
Time-->
6.00 7.00 8.00 9.00 10.00 11.00 12.00 13.00

AC


Abundance
Ion 204.00 (203.70 to 204.70): YHM5.D (+,-)
50000
OH-Mesocarb
Time-->
6.00 7.00 8.00 9.00 10.00 11.00 12.00 13.00
Abundance
Ion 323.00 (322.70 to 323.70): YHM5.D (+,-)
404146
2227
I.S.
Time-->
6.00 7.00 8.00 9.00 10.00 11.00 12.00 13.00
Abundance
Ion 339.00 (338.70 to 339.70): YHM5.D (+,-)
100000
Time-->
6.00 7.00 8.00 9.00 10.00 11.00 12.00 13.00
Abundance
Ion 419.00 (418.70 to 419.70): YHM5.D (+,-)
2500
2000
Time-->
6.00 7.00 8.00 9.00 10.00 11.00 12.00 13.00
Abundance
Ion 477.00 (476.70 to 477.70): YHM5.D (+,-)
2000
1800
Time-->
6.00 7.00 8.00 9.00 10.00 11.00 12.00 13.00
Abundance
Ion 535.00 (534.70 to 535.70): YHM5.D (+,-)
1800
Time-->
6.00 7.00 8.00 9.00 10.00 11.00 12.00 13.00