

Reprint from

RECENT ADVANCES  
IN DOPING ANALYSIS  
(3)

M. Donike  
H. Geyer  
A. Gotzmann  
U. Mareck-Engelke  
(Editors)

Sport und Buch Strauß, Köln, 1996

---

D. Thieme, J. Grosse, R. Lang and R.K. Mueller:  
Application of High-Resolution-MS and Tandem-MS to the Identification of Anabolic Agents  
In: M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) Recent advances in doping  
analysis (3). Sport und Buch Strauß, Köln, (1996) 285-297

## Application of High-Resolution-MS and Tandem-MS to the identification of anabolic agents

### Introduction

Several examples on the application of high resolution mass spectrometry or tandem mass spectrometry in doping analysis have been given recently, for instance by D. DeBoer [1], S. Horning [2], X. de la Torre [3] and G. Debruyckere [4]. Aiming to an evaluation of the efficiency of different mass spectrometric techniques to enhance the analytical performance, a comparison of both methods is made.

A reliable comparison of different analytical techniques is always difficult, because very often the quality of the various instruments used, the experiences and preferences of the operators and a commercial background influence the results of such investigations more than the actual analytical problem. In our case, the compatibility of the analytical results is obvious due to the application of a hybrid instrument combining a high resolution sector field MS with a quadrupole, which applies the same chromatograph and ion source and identical detectors. Therefore the differences in the chromatograms are assumed to reflect the different detection capability of high resolution and tandem MS techniques, respectively.

### High Resolution Mass Spectrometry

In high resolution mass spectrometry, the reduction of measurement of masses to narrow ranges permits a discrimination of background signals from substance fragments of interest. The desirable resolution for a measurement is governed by the mass difference between signal and noise. This difference is usually unknown, as the background substances are unidentified. Therefore the effect of an increase of the mass resolution can be predicted only with some difficulty. The example figured in fig. 1 illustrates this problem. This example is mainly hypothetical, as for instance the referring clenbuterol signal is of low abundance and most of the substances listed do not coelute in GC. Nevertheless there are some very similar problems, like the disturbance of norandrosterone by coeluting vitamin E metabolite.

The substances to be distinguished by mass spectrometry are

clenbuterol-bis-TMS	[M]	=420.159,
vitamin E Metabolite-bis-TMS	[M-2]	=420.215,
nandrolone-bis-TMS	[M+2]	=420.273 and
norandrosterone-bis-TMS	[M]	=420.288.

Figure 1 shows the overlay of these signals of equal abundances and demonstrates the various separation requirements. At 2500, resolution no separation is possible; 10 000 enables the identification of clenbuterol, vitamin E and nandrolone, and at 20 000 resolution it is even possible to distinguish between the M+2 isotope peaks of nandrolone and norandrosterone.

A remaining disadvantage of HRMS is the narrow mass range which can be detected at a high rate. The magnet scan rate of sector field instruments decreases with increasing magnet size (ultimate resolution). Therefore, different masses are set up by decrease of acceleration voltage, and the maximum mass range is about half of a decade.

## Tandem MS

An examination of fragmentation processes can be carried out by a combination of two mass spectrometers. In a first stage, a specific ion is isolated by mass spectrometry, which undergoes a defined fragmentation in a collision cell. All characteristic fragments corresponding to this parent ion are analysed in a second mass spectrometer. Application of this technique enables several analytical modifications, i.e.

- examination of all fragments corresponding to a given precursor ion (daughter scan),
- search for all parent ions which could produce a given daughter ion (parent scan) and
- observation of the loss of a specific fragment (neutral loss).

All versions can be carried out in a continuous (full scan) as well as in a discontinuous mode (focussing on selected fragmentations only). A main problem consists in the choice of appropriate fragmentations, which are both abundant and specific. In the framework of analysis of anabolic steroids there are typical fragmentations like M-CH<sub>3</sub>, M-TMS, or formation of typical ring fragments, which are quite intense but less specific. Therefore general recommendations cannot be given, and the design of the tandem MS analytical method is mainly empirical.

Several examples from the routine screening analysis for conjugated anabolic substances (clenbuterol, nandrolone, metenolone, Fig 2) are chosen to demonstrate typical advantages and problems of both techniques.

### Clenbuterol

Due to the relevance of low concentrations (for instance 5ng/ml in the control urine figured above (2)), the separation from the nonspecific chemical noise is difficult. In case of substances like clenbuterol, which contain heteroatoms causing a high mass defect, even a slight increase of mass resolution is fairly effective. As reported previously by several authors, the detection limit can be decreased considerably by application of HRMS. In fig. 3, an increase of peak to noise ratio by a factor of thousand could be obtained by an increase of resolution from 1000 to 10 000.

### Norandrosterone

There is a wellknown interference of norandrosterone with a minor fragment of the vitamin E metabolite (figure 4). Although this problem may be overcome with conventional methods (n-pentane extraction or examination of the major vitamin E signal at 422), it is an interesting example because both interfering species are known. In such a case, the required resolution to separate both substances (fig. (1) ) can be calculated easily:

$$R = m/\Delta m = 420/(420.288 - 420.215) \approx 6000$$

Complete separation of both species is given at any resolution higher than 6000. The example in figure (5) shows the detection of both metabolites (norandrosterone 4ng/ml,

noretiocholanolone 8ng/ml) by HRMS. There is no interference between the traces for nandrolone and vitamin E metabolites.

The same sample examined by MS/MS is shown below (fig. 6). At least three different fragmentations provide a useful signal for identification of low concentrations of nandrolone metabolites.

### **Metenolone**

The identification of metenolone and its metabolite by examination of the molecular ion at  $m/e=446$  is -depending on the performance of gas chromatography- influenced by some endogeneous substances. Application of high resolution mass spectrometry at (resolution of 10000 and 30000) could not improve the peak to noise ratio (fig 7). The application of tandem MS demonstrates the flexibility of this technique (fig. 8). Some ions out of a variety of possible fragments may be scanned at high scanning rates, and for each of the substances several specific fragmentations exist. Compared to the single-stage experiments (upper two boxes of figure 8), a considerable increase of peak to noise ratio is evident.

### **Summary**

Aiming to an improvement of the detection of anabolic steroids, a comparison of high resolution and tandem MS is carried out. The variety of similar endogeneous and synthetic steroids with similar structure and chemical properties (including derivatisation, chromatographic properties and fragmentation) requires specific identification criteria.

If the interfering substances have chemical compositions considerably different from substances of interest (especially for those containing heteroatoms like clenbuterol or clostebol), application of high resolution mass spectrometry improves detection limits tremendously.

The main advantage of an application of tandem MS consists in its high flexibility. Many fragmentations can be combined and tested. Without detailed knowledge about the chemical structure of interfering substances, an increased peak to noise ratio may be obtained very often.

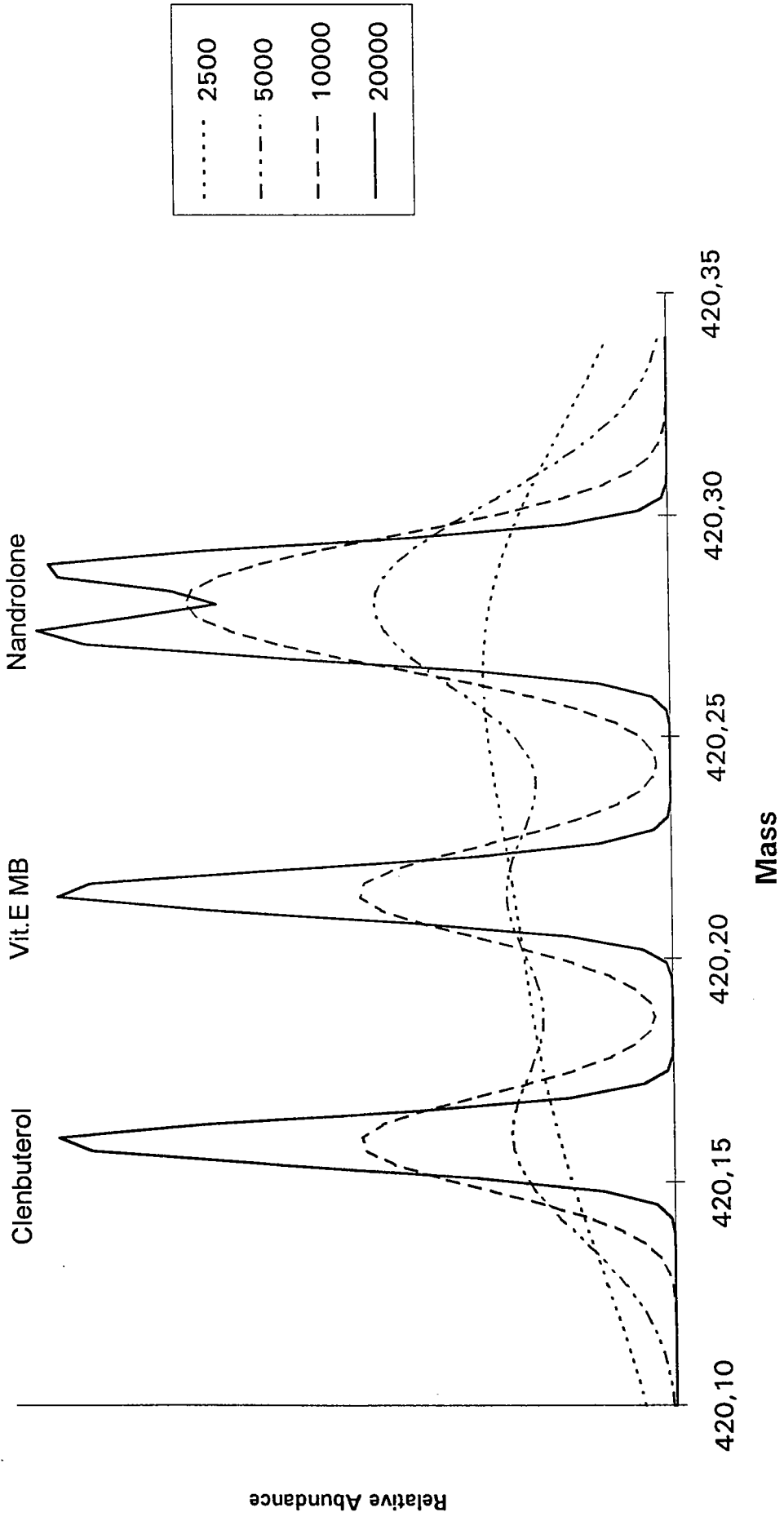
Combinations of both techniques are possible, but a reduction of flexibility and an increasing effort to fit the high number of technical parameters may cause problems.

## References

- [1] DeBoer, D., Bensink, S.N., Borggreve, A.R., VanOoijen R.D., Maes, R.A.A. Profiling 19-Norsteroids III GC/MS/MS Analysis of 19-Norsteroids during Pregnancy *10th Cologne Workshop on Dope Analysis 1992 - Proceedings* Eds M. Donicke, H. Geyer, A. Gotzmann, U. Marek-Engelke, S. Rauth, Sport und Buch Strauß Edition Sport Köln (1993) pp 121-122.
- [2] Horning S., and Donike M. High Resolution GC/MS *Recent Advances In Doping Analysis 11th Cologne Workshop on Dope Analysis 1993 - Proceedings* Eds M. Donicke, H. Geyer, A. Gotzmann, U. Marek-Engelke, Sport und Buch Strauß Edition Sport Köln (1994) pp 155-161.
- [3] De la Torre X., Segura, J., Poletini, A. Detection of Testosterone Esters in Human Plasma by GC/MS and GC/MS/MS *Recent Advances In Doping Analysis 12th Cologne Workshop on Dope Analysis 1994 - Proceedings* Eds M. Donicke, H. Geyer, A. Gotzmann, U. Marek-Engelke, Sport und Buch Strauß Edition Sport Köln (1995) pp 59-71.
- [4] Debruyckere, G., de Sagher, R., Van Peteghem, C. Detection of Interferences in Urinary Anabolic Steroid Analysis *Recent Advances In Doping Analysis 11th Cologne Workshop on Dope Analysis 1993 - Proceedings* Eds M. Donicke, H. Geyer, A. Gotzmann, U. Marek-Engelke, Sport und Buch Strauß Edition Sport Köln (1994) pp 173-184.

## Figures

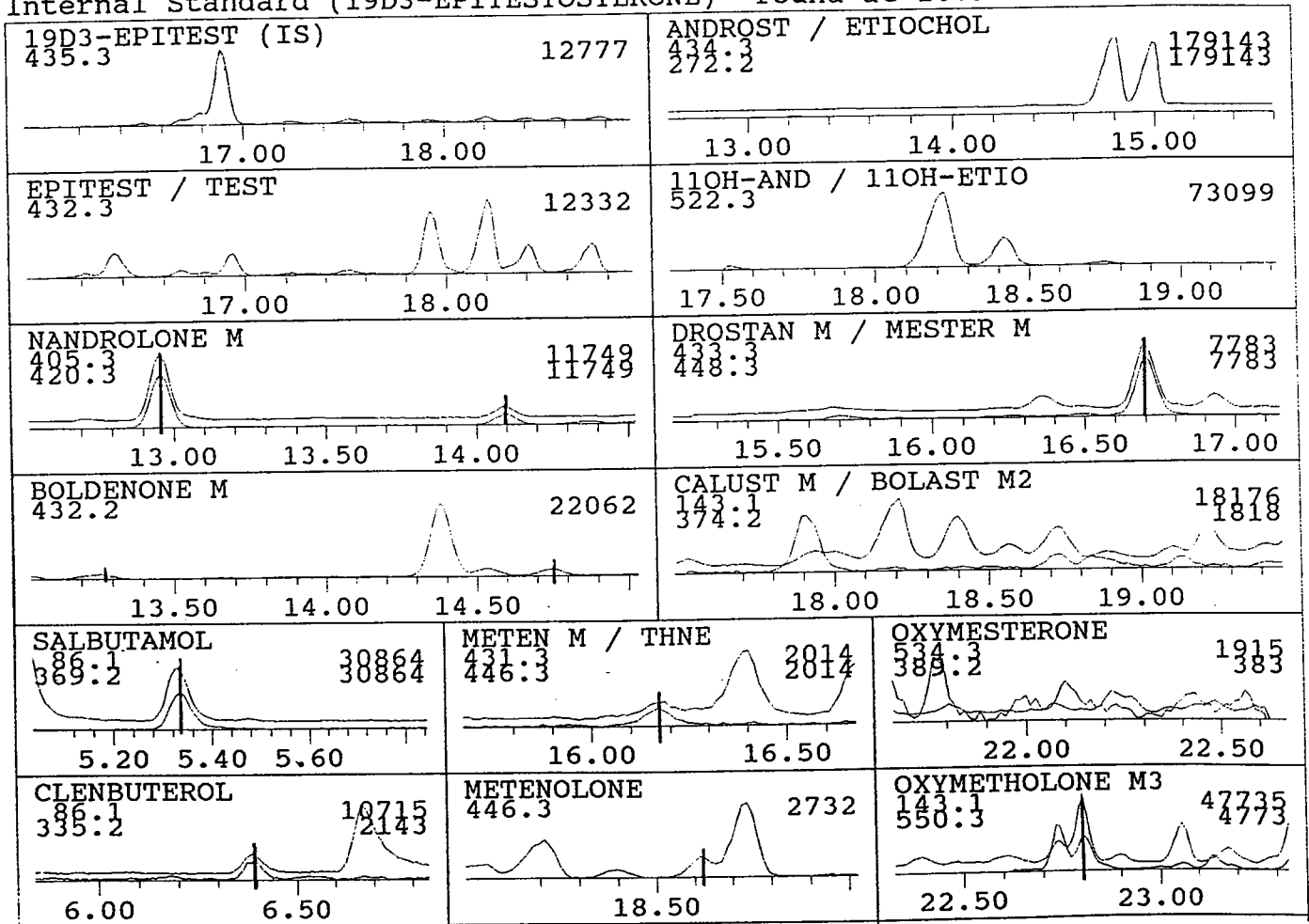
1. Resolution requirements for a separation of various substances by high resolution MS. A high resolution mass spectrometer is assumed to produce gaussian shaped peaks. A different level of separation is obtained, if a hypothetical mixture of 4 substances is identified at different resolutions.
2. Extract from a screening 4b analytical report (conjugated steroids) carried out at a HP 5970 benchtop instrument. Identification of the substances of interest (labelled 1-3) is possible but noise is considerably high. The extracts injected corresponds to some of those, applied in the following examples (figures 3, 7-8)
3. Identification of clenbuterol by HR/MS. An increase of resolution from 1000 to 10 000 results in an improvement of peak to noise ratio by a factor 1000.
4. Norandrosterone detection is disturbed by the presence of a coeluting vitamin E metabolite. Due to the high dosages of vitamin E, a interference is observed in spite of different molecular weights of the concerning bis-TMS derivatives.
5. The selected ion recording plots of norandrosterone (bis-TMS derivative, molecular ion at 420.288) and of the vitamin E (bis-TMS derivative of the metabolite, minor [M-2] fragment at 420.215) do not influence each other at a resolutions of at least 6000.
6. Undisturbed identification of norandrosterone and noretiocholanolone by MS/MS. The detection of the transitions M-15, M-15-90 and ring fragmentation enables an identification of both metabolites at urine concentrations of about 4 and 8ng/ml, respectively.
7. Peak to noise ratio for the identification of the metenolone metabolite cannot be improved by application of HR/MS at resolutions of 1000, 10 000 and 30 000.
8. Comparison of "single stage" (upper two windows) and tandem MS recordings, measured in the same chromatographic run. Selected ion measurements of molecular ions and of the [M-15] signal of metenolone (at 22:21) and its metabolite (20:52) are interfered by background signals, whereas the multiple reaction measurement of 4 typical fragmentations exhibits abundant and specific signals.



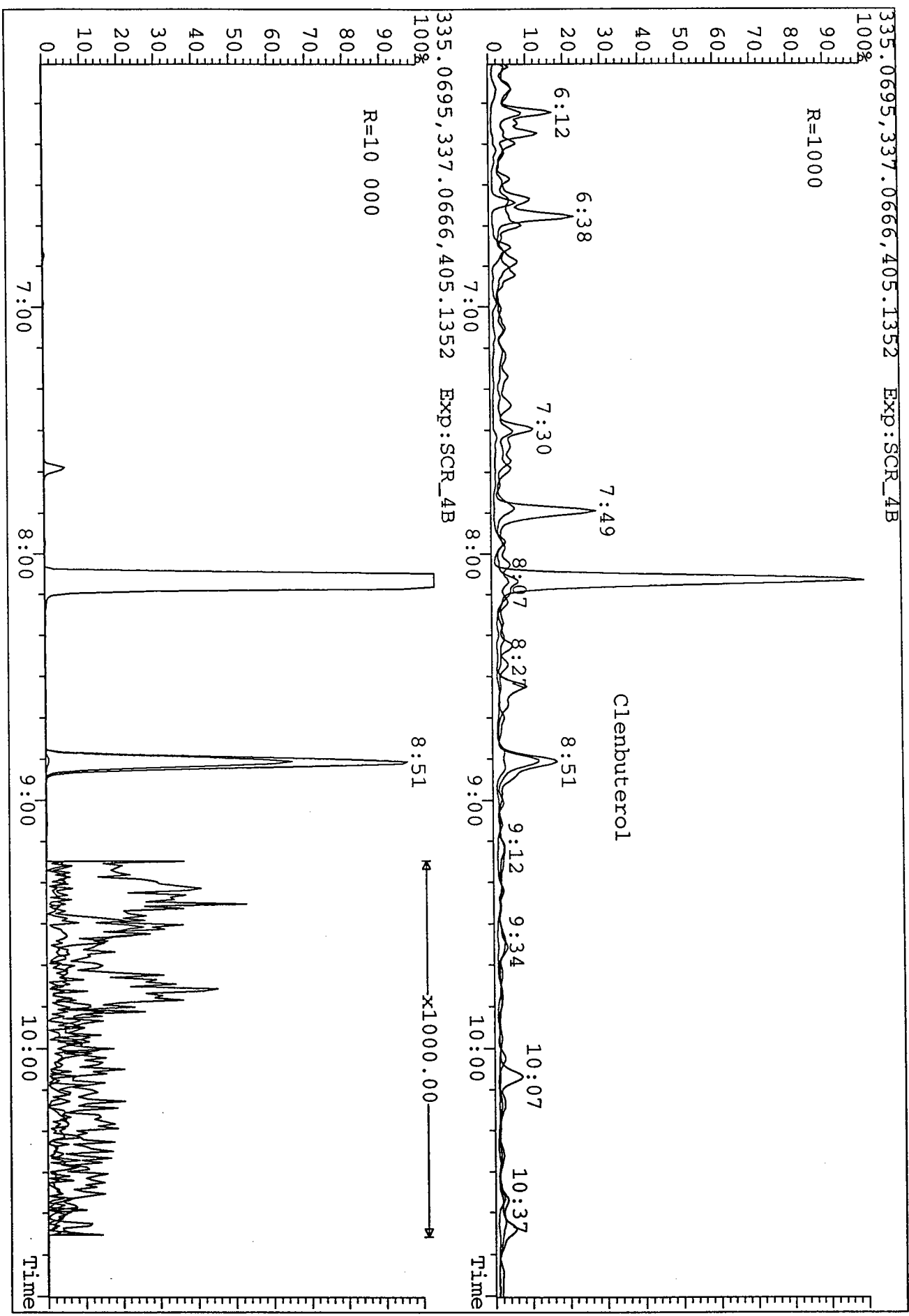
2)

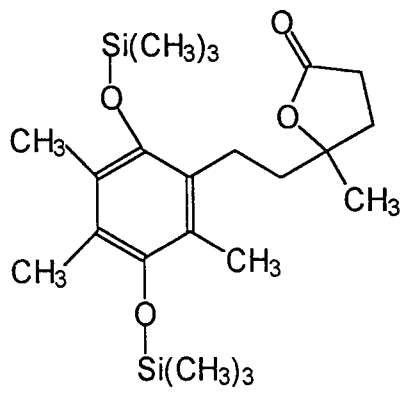
Sample: MKU

Internal Standard (19D3-EPITESTOSTERONE) found at 16.90 min.

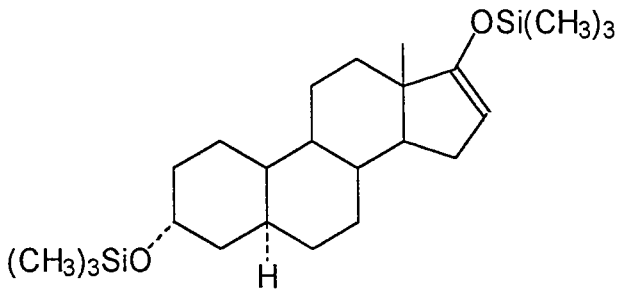






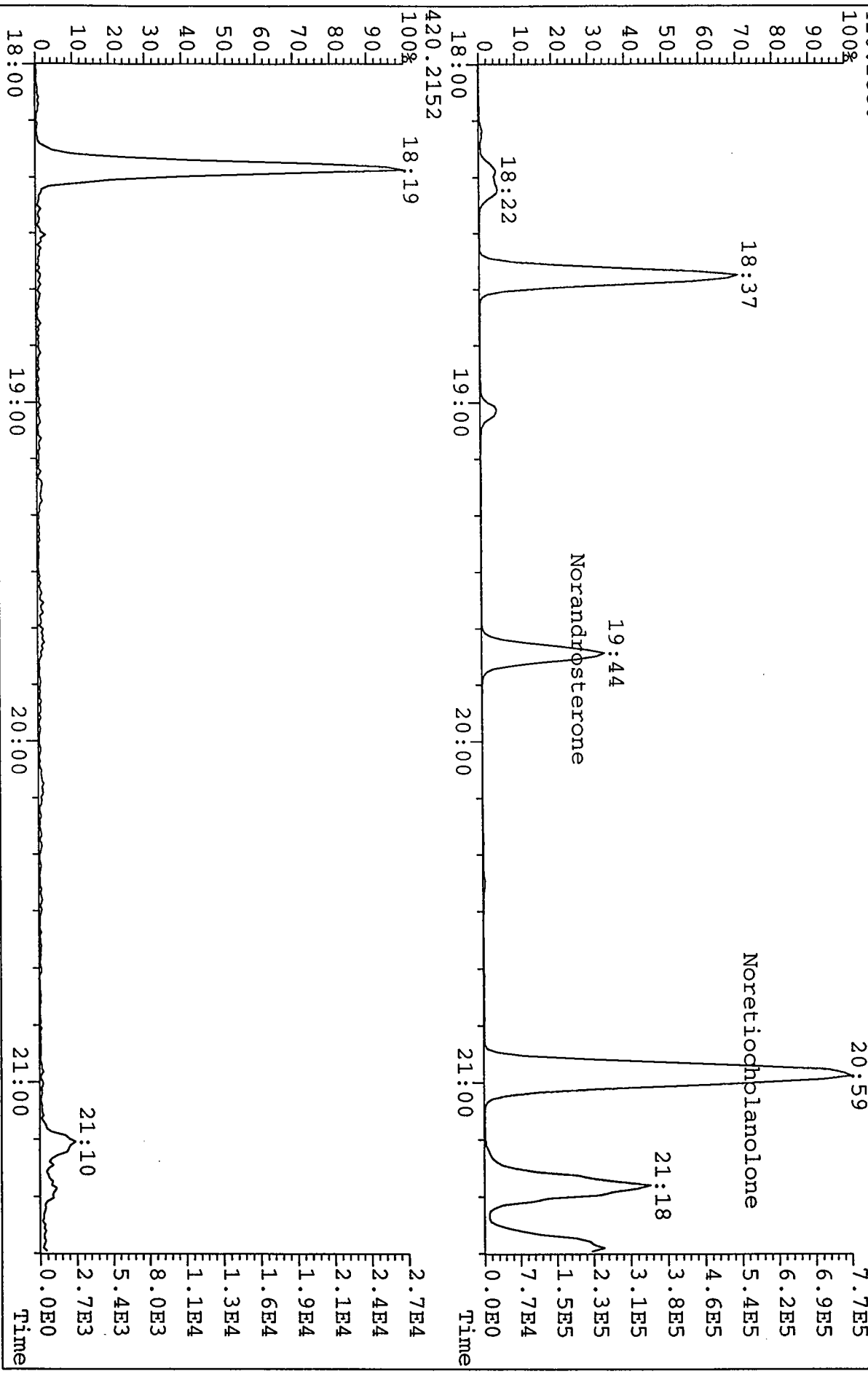


Vitamine-E Metabolite (Bis-TMS-Derivative)



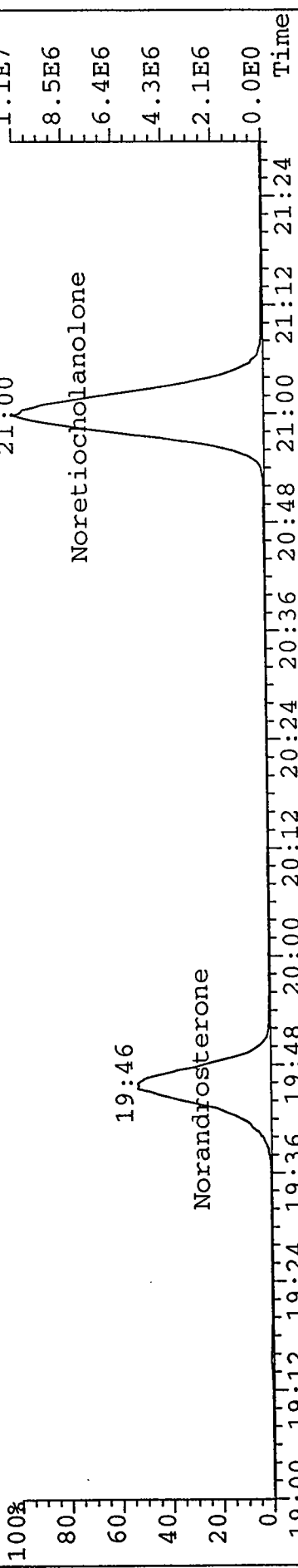
Norandrosterone (Bis-TMS-Derivative)

File: B\_PROBE\_1239\_2 #1-1031 Acq: 22-JUL-1994 17:41:02 GC EI+ Voltage SIR Autospec-UltimaQ  
 Sample#1 Text: WK 1239/B Exp: NANDRO\_HR\_SIR

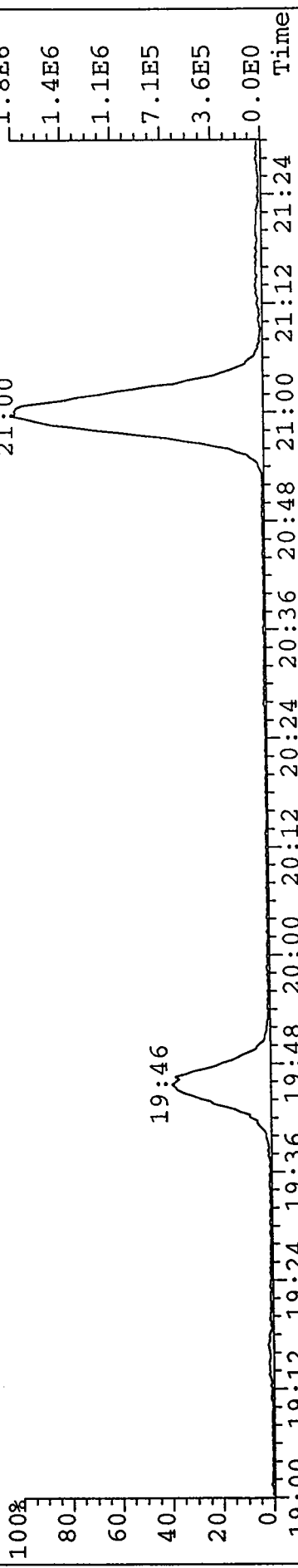


File: B\_PROBE\_1239\_2 #1-1031 Acq: 22-JUL-1994 17:41:02 GC EI+ Voltage SIR Autospec-UltimaQ  
Sample#1 Text: WK 1239/B  
Exp: NANDRO\_HR\_SIR

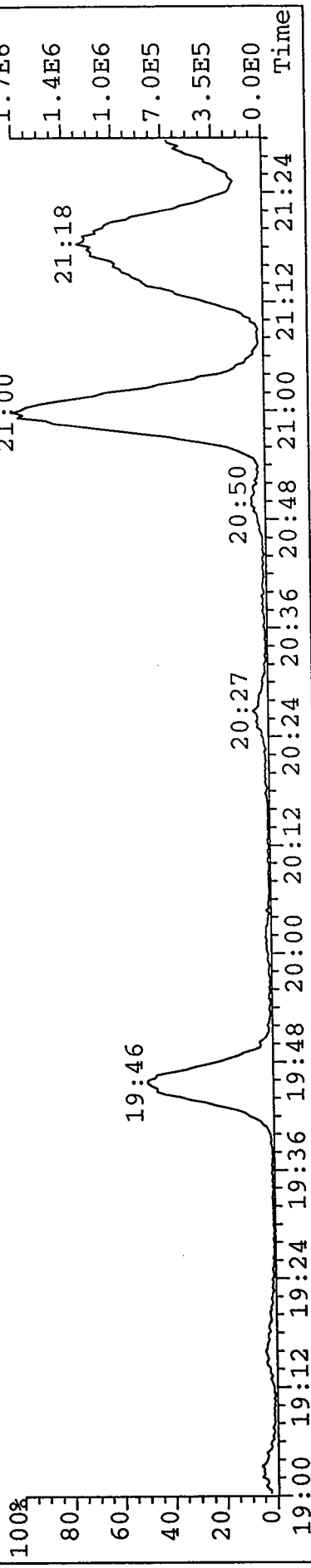
420.2900 -> 405.0000 @155.0 S:4

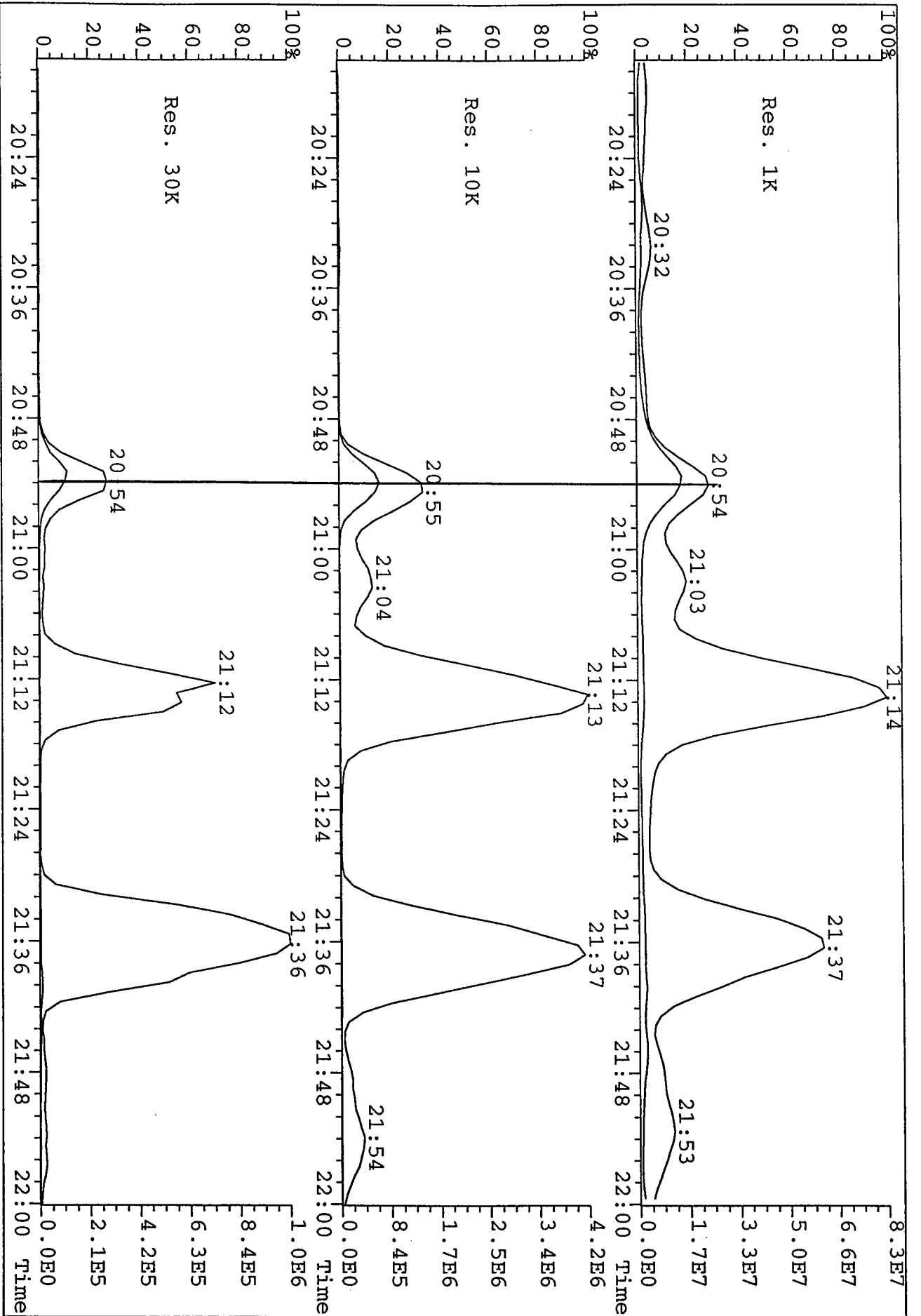


420.2900 -> 315.0000 @155.0 S:4



420.2900 -> 169.0000 @155.0 S:4





File:FE23\_58\_PAO\_1K3Q #1-1310 Acq:23-FEB-1995 19:58:35 GC EI+ QMR>  
 Sample#1 File Text:Methodentest SCR\_4B Text:MKU1 >>  
 446.3000->446.3000 @26.0 SMO(2,5)

