Reprint from

RECENT ADVANCES IN DOPING ANALYSIS (5)

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Sport und Buch Strauß, Köln, 1998

R. DE LA TORRE, X. DE LA TORRE, A. ARTOLA, S. CORCIONE, S. DICENTA, J. SEGURA:
Hydrolyses Procedures in Sample Preparation. Esterase Activity Associated to Helix Pomatia Glucuronidase Preparations
In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) Recent advances in doping analysis (5). Sport und Buch Strauß, Köln, (1998) 103-121

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High sensitivity analysis of anabolic agents. Some alternative instrumental possibilities.

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Introduction

The need for longer retrospectivity in the analysis of anabolic agents has been the main reason for the demand of lower detection limits in the screening for those substances in routine doping control.

High resolution mass spectrometers have proven to offer better sensitivity than the conventional single quadrupole benchtop mass spectrometers and have become the reference for high sensitivity measurements (1). Long-term excreted metabolites are tried with the new instrumental methods to extend the retrospective period for detection.

However, it is worth having in mind that other techniques have been used with success in the bioanalytical field (2-4) such as tandem mass spectrometry (MS/MS) in connection with either low or high resolution instrumentation. On the other hand, new instruments are being developed that open new perspectives (5,6). New generation single quadrupole mass spectrometers offer better sensitivities and ion trap instruments with MS/MS capabilities open a complete new world in terms of selectivity and sensitivity with an easy-to-use, lower cost instrumentation.

The aim of the present work is showing the use of the new GC/MS "GCQ" based on an ion trap mass detector with external ion source in the analysis of quality control samples at the low concentrations required by the IOC as well to extend its use to a general screening procedure covering most of the anabolic agents currently monitored.

Material and Methods

Reference compounds were obtained either from Sigma (Saint Louis, USA) or from the Cologne laboratory (Institute of Biochemistry. German Sports University, Cologne).

Samples were extracted following our routine screening procedure for the combined fraction of the anabolic steroids (7) starting with 2.5 ml of urine and reconstituting the final extract in 50 µl of derivatisation reagent. Methyltestosterone is used as internal standard at a final concentration of 10 ng/ml in urine.

GC conditions:

- Injector:
  Volume : 1 µl
  Split : 1:10
  Temp : 280°C
  Pressure : 80 kPa (constant)
- Carrier : He, 0.86 ml/min (at 180°C)
- Column : hp ULTRA-1, 17 m, 0.2 mm, 0.11 µm
- Oven : 185°C (0) – 5°C/min – 240°C (0) – 20°C/min – 310°C (2)

MS conditions:

Method 1: Restricted screening (only for the detection of a few compounds)

- Mode: SRM → scan daughter ion spectrum
- Notch width: 1

START TIME	Parent ion	C.E.	SUBSTANCES MONITORED
2.00	335	1	Clenbuterol
4.00	358	0.85	Methandienone-Met4 *(18-nor-17,17-dimethyl-5β-androsta-1,13-dien-3α-ol)*
6.00	405	1	Nandrolone-Met1 *(19-nor-androsterone)*
6.62	358	1	Methandienone-Met3 *(epimethendiol)*
7.00	405	1	Nandrolone-Met2 *(19-nor-etiocholanolone)*
7.75	435	1.2	Methyltestosterone-Met1,2 *(17α-methyl-5(α,β)-androstandiol*
10.00	446	1	Methyltestosterone PC (ISTD)
13.00	545	2	Stanozolol-met1 *(3'-hydroxy-stanozolol)*

Method 2: Extended screening

- Mode: SRM → selected daughter ions

START TIME	Parent ion	Notch width	C.E.	Daughter ions	SUBSTANCES MONITORED
2.00	335	1	1	227, 300, 335	Clenbuterol
3.75	358	2	0.85	253, 268, 343	Methandienone-met4
6.00	405	2	1	225, 315, 405	Nandrolone-met1
6.55	432	2	1.1	194, 206, 342	Boldenone-met1
	358	2	1	268, 301, 343	Methandienone-Met3
6.80	405	2	1	225, 315, 405	Nandrolone-Met2
	433	2	1	253, 343, 433	Dromostanolone-met1
8.00	435	2	1	255, 345, 435	Methyltestosterone-Met1,2
	447	4	1.5	250-254 340-344 430-434	Methenolone-met1 Mesterolone-met1
9.39	421	2	0.8	241, 331, 421	Norethandrolone-met1
	451	2	1.1	325, 361, 451	Clostebol-met1
10.00	446	2	1	301, 356, 446	Methyltestosterone PC (ISTD)
11.65	517	2	1.5	228-230 336-338 516-518	Methandienone-met1
13.00	545	2	2	455, 545	Stanozolol-met1
	560	2	0.8	455, 560 471-473	Stanozolol-met3

Results

When changing from a standard quadrupole (e.g. hp5973) to an ion trap detector (in this case the GCQ instrument), the first thing that must be highlighted is the difference in mass spectra. GCQ gives spectra with a considerable positive discrimination towards high masses and/or very poor at low masses, especially below m/z 100. As an example, Figure 1 shows the spectra obtained using both instruments for 17α-methyl-5β-androstan-3α,17β-diol (methyltestosterone metabolite) as its bis-O-TMS derivative. Nevertheless, heavier ions are normally cleaner for chromatographic monitoring and higher abundance will also benefit further MS/MS developments. Then, although we first need to establish the new library of spectra for the substances of interest, the results obtained are "better" for high sensitivity detection.

Depending on the vendor and the instrument model, each ion trap allows the user to play with different parameters affecting the sensitivity in the attempt to optimise the detection conditions. GCQ shows a very simple software with just a few parameters that can be configured as part of the MS method. Of this parameters is the "mass range" to be detected. According to the instrument we have three different working modes: SCAN, SIM and SRM (or MS/MS).

- In scan mode, the mass range is just the scan range (e.g. m/z 70-700) to be monitored.
- In SIM mode we can specify the "width" of the mass to be detected. With the intention to get the most specific and clean detection one may want to specify a very narrow "width" for each ion to monitored. How the system interpret this width deserves a comment since it is very important for the results. A log file generated together with the data file can be checked to see how the system interpreted the parameters.
  - · When choosing **width 2**, the system understands it as m/z±0.5 (e.g. m/z=300 width=2 means: 299.5 – 300.5)
  - · When choosing **width 1**, following the same example the log file will show 300-300 which has no direct explanation.
- In SRM mode a so called "notch width" can be chosen for the parent ion.

When going through all this modes of operation from scan to SIM and to SRM an increase in sensitivity is noticeable (S/N can increase more than 10 times depending on the cases). But

more relevant is the difference between choosing notch with 2 or 1 as shown in figure 2 for the case of the internal standard (methyltestosterone bis-O-TMS). When notch width 1 is chosen, the noise is drastically reduced so that the S/N ratio increases. This is one of the key features in getting the best selectivity and response out of this kind of instrument.

A quality control sample was sent by the Cologne laboratory to all accredited laboratorioes in order to check and compare the performance of the different approaches to reach the desired "high sensitivity". This sample coded QC960415 had the composition shown in Table 1.

Table 1. Composition of the quality control sample QC960415

SUBSTANCE	Abbrev.	CONC. (ng/ml)	PRECURSOR
Clenbuterol	CLE	1	Clenbuterol
19-Nor-androsterone	NAN-met1	2	Nandrolone
17β-Methyl-5β-androst-1-en-3α,17α-diol	MED-met3	3	Methandienone
17α-Methyl-5β-androstan-3α,17β-diol	MET-met2	2	Methyltestosterone
3'-Hydroxy-stanozolol	STA-met1	3	Stanozolol

The method used is described under material and methods as "Method 1". Following this procedure, the results obtained for the analysis of the quality control sample in a batch containing a blank urine and a spiked urine at 10 ng/ml of the same compounds were as shown in figures 3 to 5.

Results show very good sensitivity for the compounds analysed at the low detection limits required. Using the same analytical procedure we also analysed the "accreditation samples" sent to the Atlanta Olympic Laboratory. Sample composition is shown in table 2.

Table 2. Composition of the 4 accreditation samples sent to the Atlanta Olympic Laboratory.

SAMPLE CODE	SUBSTANCE	Abbrev.	CONC. (ng/ml)	PRECURSOR
QCA1	3'-hydroxy-stanozolol	STA-met1	4	Stanozolol
	4β-hydroxy-stanozolol	STA-met3	4	
	17α-Methyl-5α-androstan-3α,17β-diol	MET-met1	3	Methyltestosterone
	17α-Methyl-5β-androstan-3α,17β-diol	MET-met2	6	
QCA2	17β-Methyl-5β-androst-1-en-3α,17α-diol	MED-met3	3	Methandienone
	18-nor-17,17-dimethyl-5β-androsta-1,13-dien-3α-ol	MED-met4	3	
QCA3	Clenbuterol	CLE	2	Clenbuterol
	19-nor-androsterone	NAN-met1	4	Nandrolone
	19-nor-etiocholanolone	NAN-met2	2	
BLANK	Absence of any forbidden substance	----	--	------

Results obtained are shown in figures 6 to 9. All substances present were correctly identified, except for the methandienone-met4 which apparently was not correctly monitored.

Results obtained suggested the possibility to extend the original reduced high sensitivity screening to other compounds in an attempt to use the ion trap system for general screening purposes. Using "Method 2" it was possible to detect an extended series of anabolic agents as can be seen in figure 10.

Discussion

For many years, single quadrupole mass filters have been used as GC detectors for the screening of anabolic steroids given the sensitivity and selectivity of such systems. Furthermore, their versatility to work in SIM mode allow monitoring hundreds of different ions.

Nevertheless, new requirements regarding detection limits made traditional methods obsolete and new instruments have entered the doping control arena. High resolution instruments have been successfully used to increase sensitivity (1). Nevertheless, this kind of instruments have some drawbacks when used for screening purposes. They are very expensive and, although it can be achieved, they are not very amenable to the detection of many different ions in just one

run. Ion trap instruments may be a future solution of this problem. On one hand they are cheaper, provide the flexibility to monitor plenty of different ions and have the sensitivity and selectivity (especially in MS/MS mode) to arrive and surpass the detection limits required.

Although other aspects must be taken into account like stability, reproducibility, etc. at present, and most probably in the future, this kind of instruments may become the tool for a highly sensitive screening procedure.

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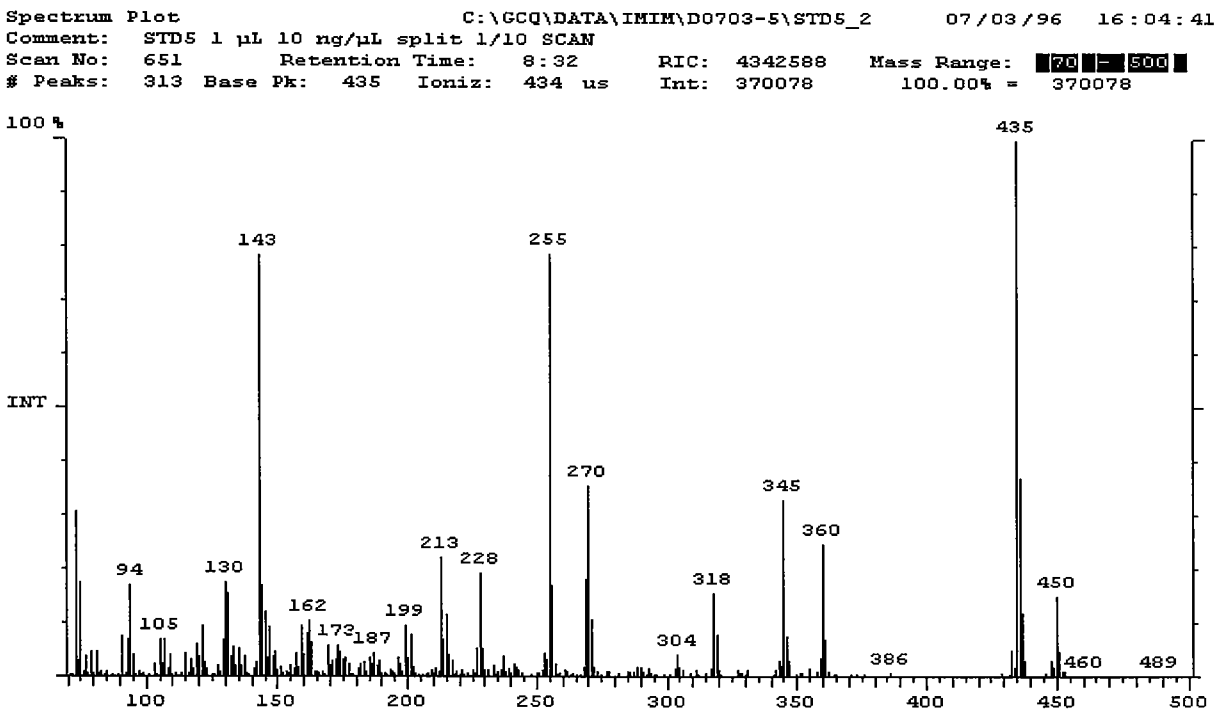
Figure 1. Full scan mass spectra of 17α-methyl-5β-androstan-3α,17β-diol (methyltestosterone metabolite) as its bis-O-TMS derivative obtained using an in trap detector (GCQ) or a single quadrupole mass selective detector (hp5973).

ION TRAP (GCQ)



QUADRUPOLE (hp 5973)

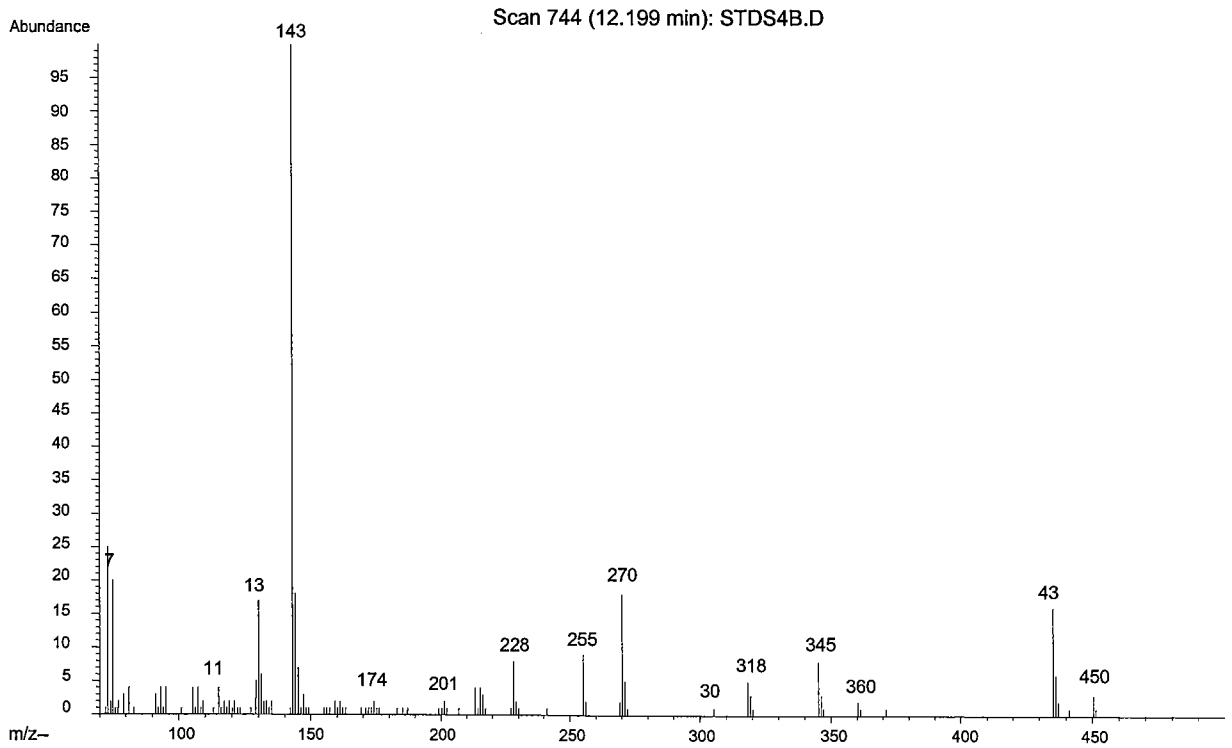


Figure 2. Comparison of the results obtained for teh analysis of Methyltestosterone bis-O-TMS in SRM mode when choosing notch width 2 or 1.

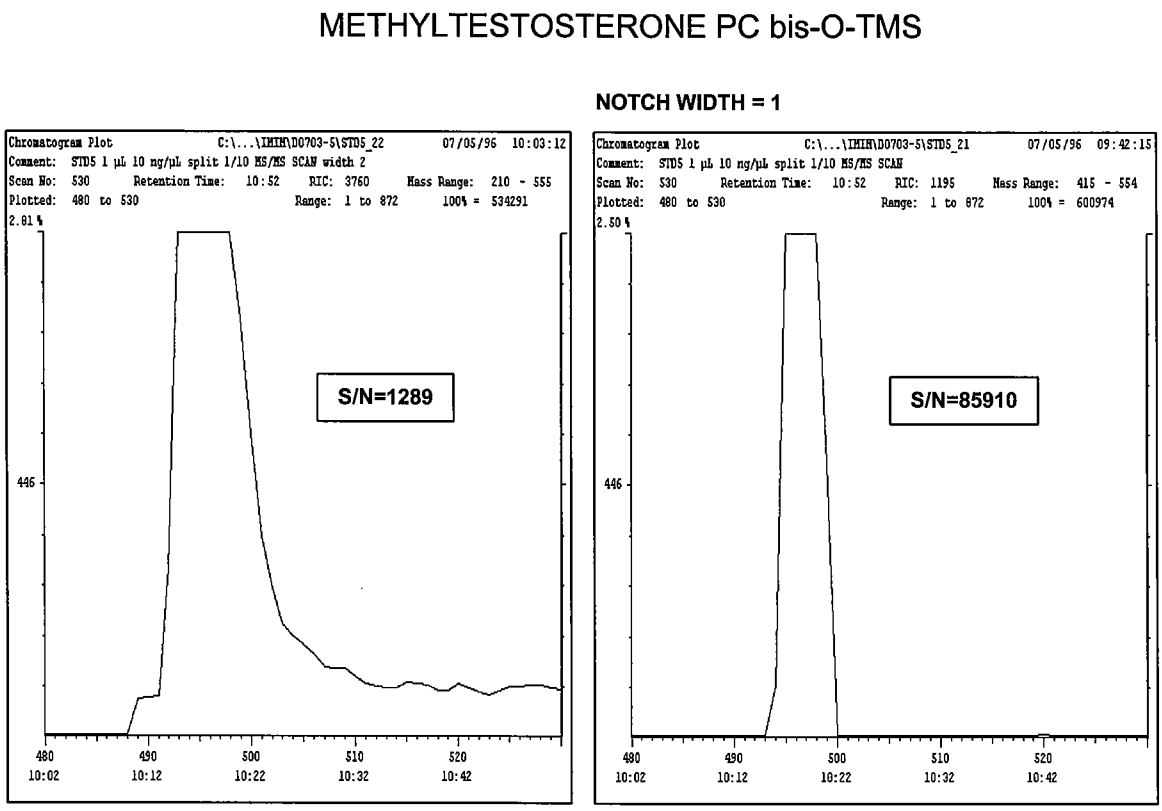


Figure 3. Results obtained for the analysis of the quality control sample QC960415. Analysis of clenbuterol and nandrolone metabolites.

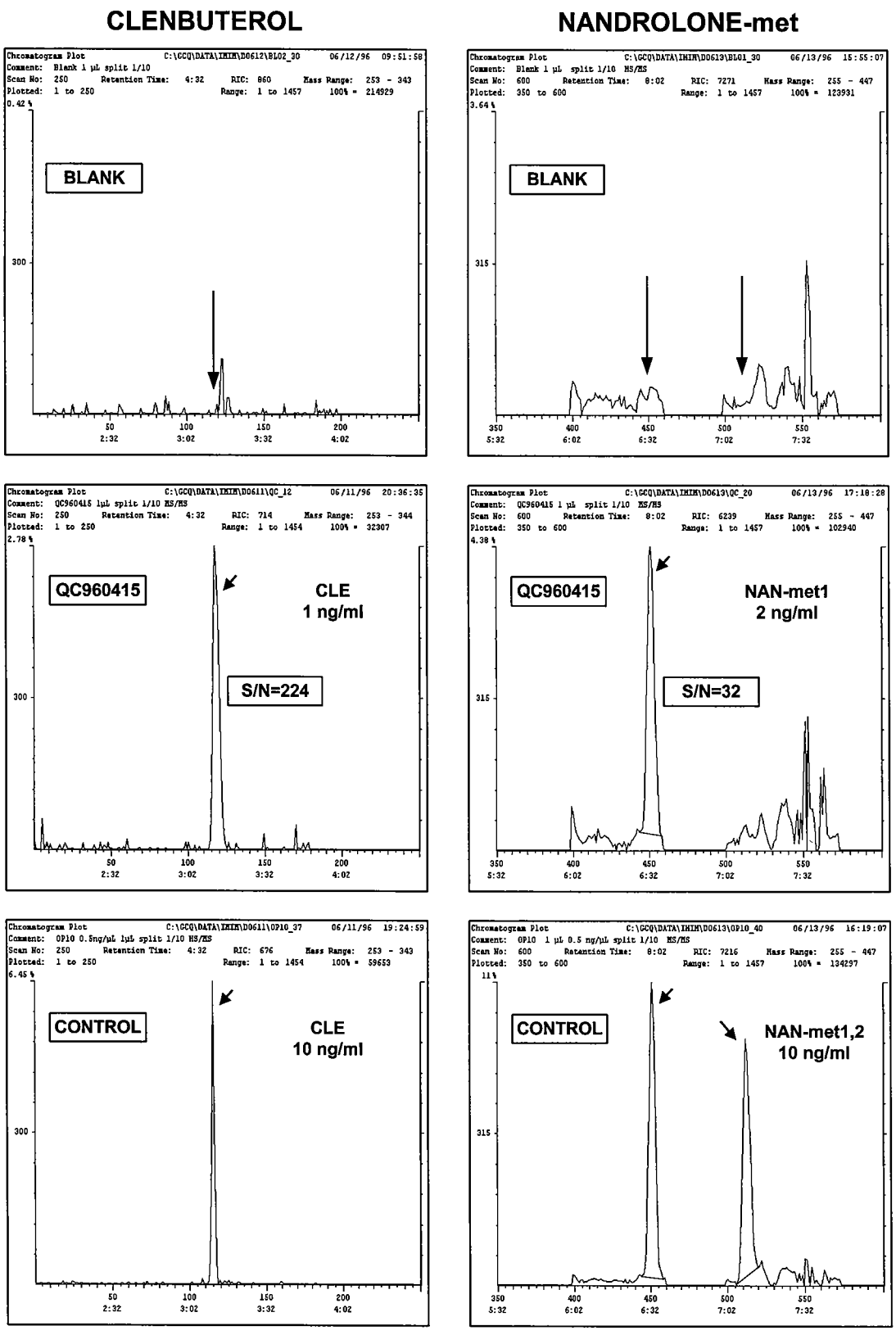


Figure 4. Results obtained for the analysis of the quality control sample QC960415. Analysis of epimethendiol and methyltestosterone metabolites.

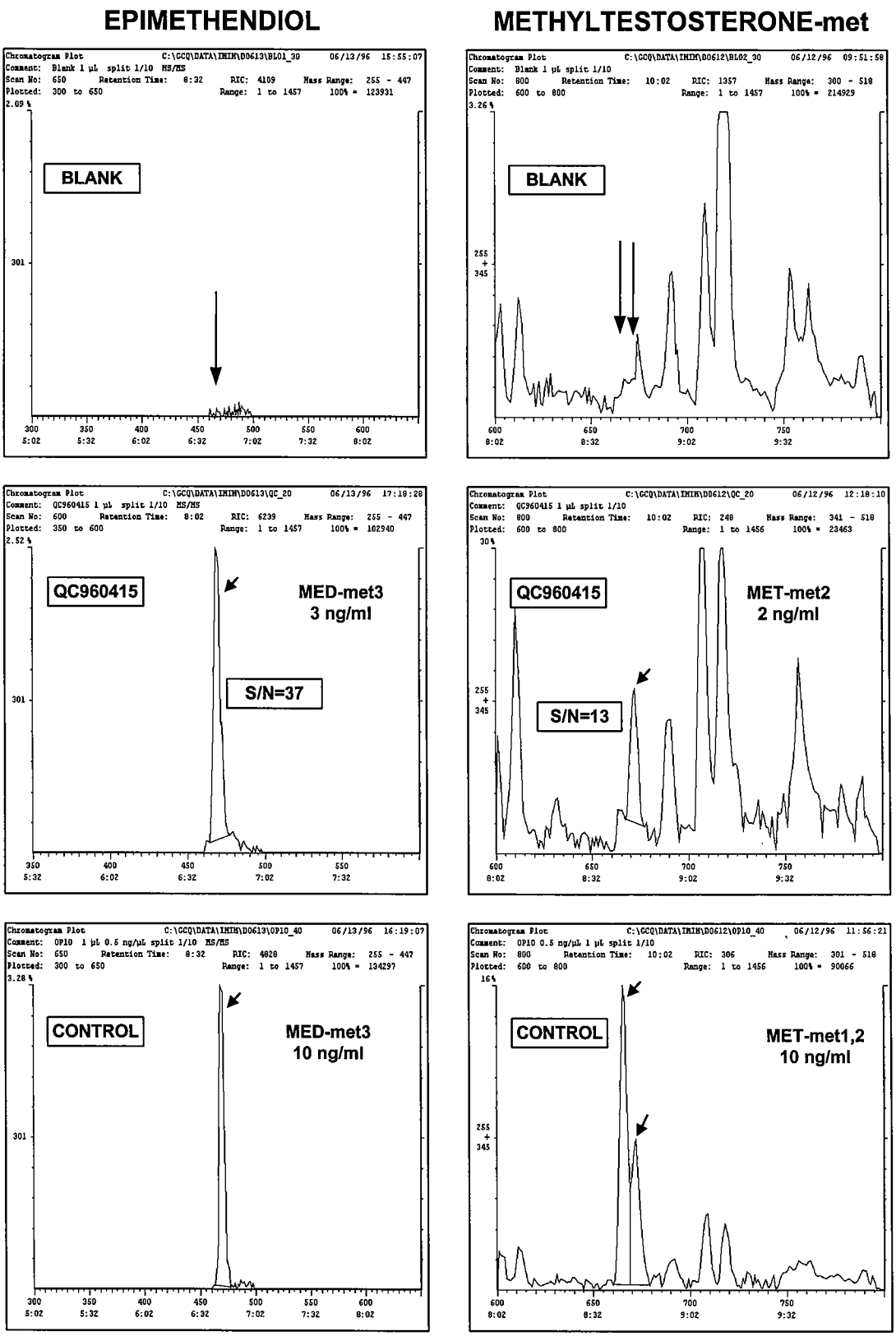


Figure 5. Results obtained for the analysis of the quality control sample QC960415. Analysis of 3'-hydroxy-stanozolol.

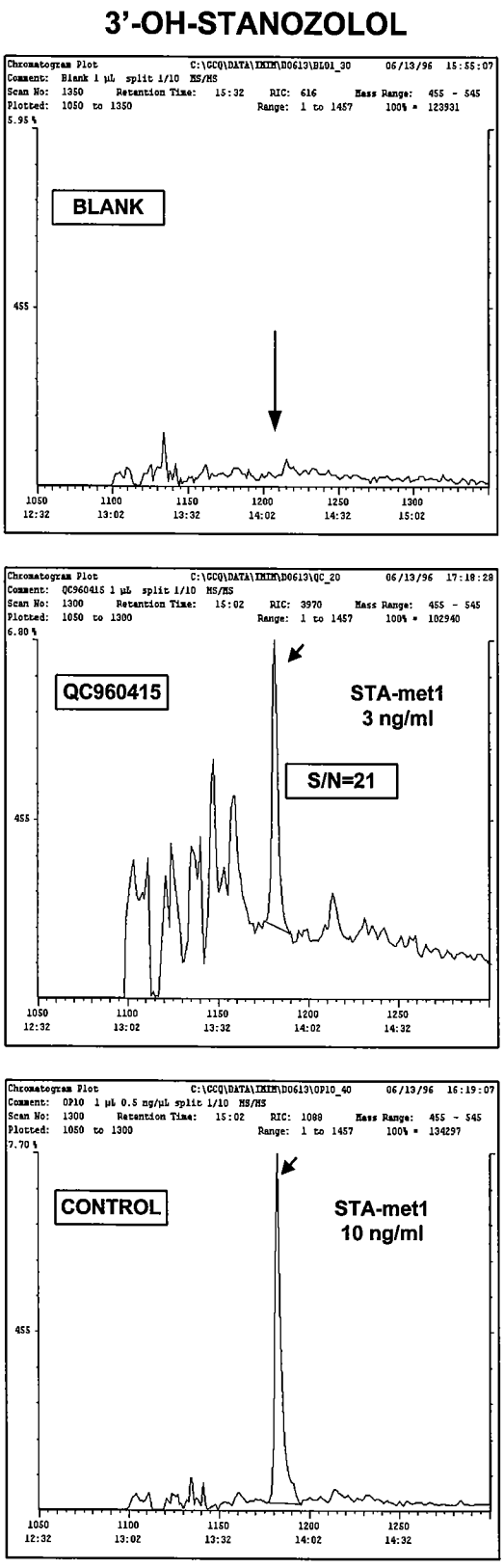


Figure 6. Results obtained for the analysis of the accreditation samples sent to the Atlanta Olympic Laboratory. Sample QCA1 (positive to stanozolol and methyltestosterone).

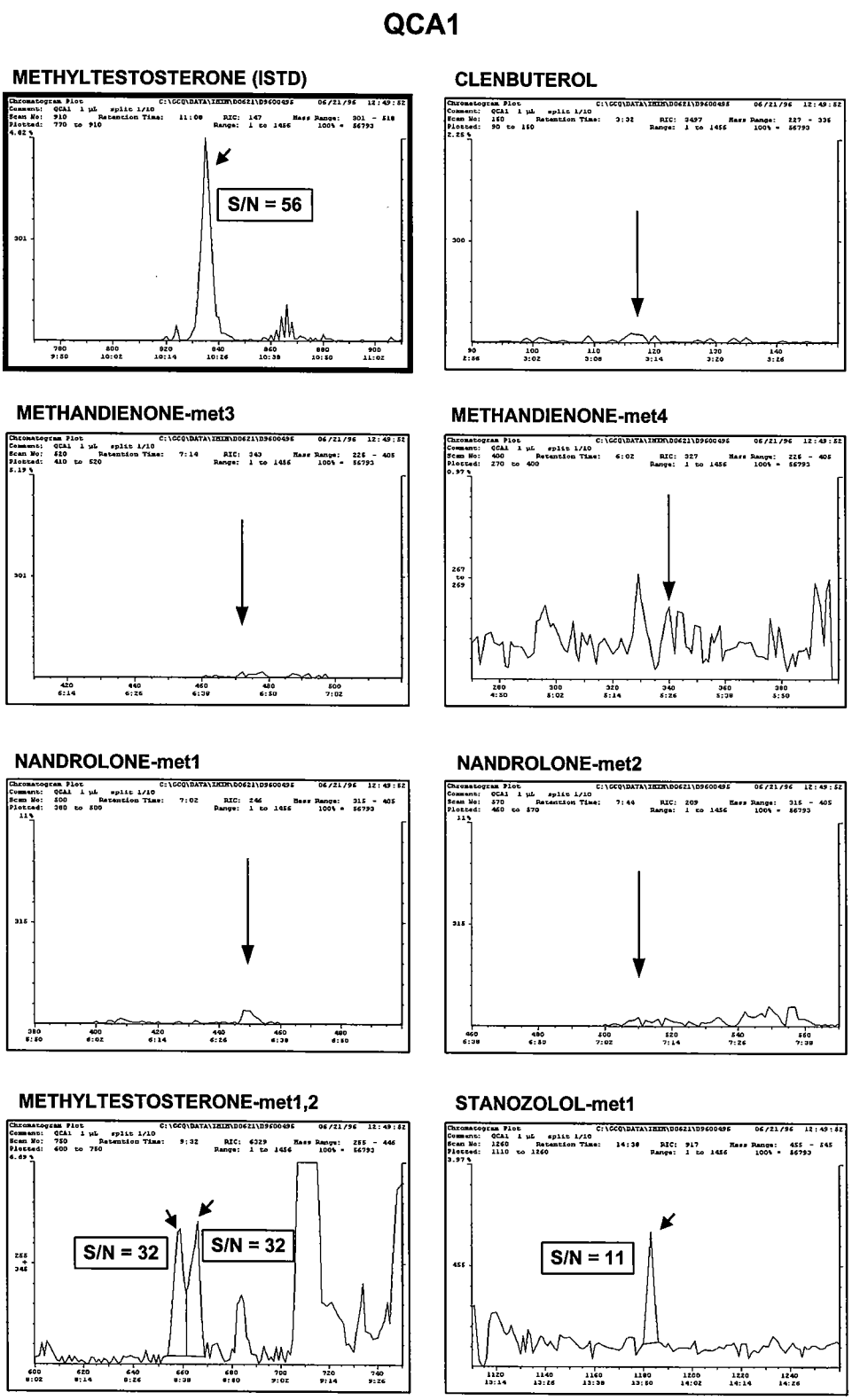


Figure 7. Results obtained for the analysis of the accreditation samples sent to the Atlanta Olympic Laboratory. Sample QCA2 (positive to methandienone).

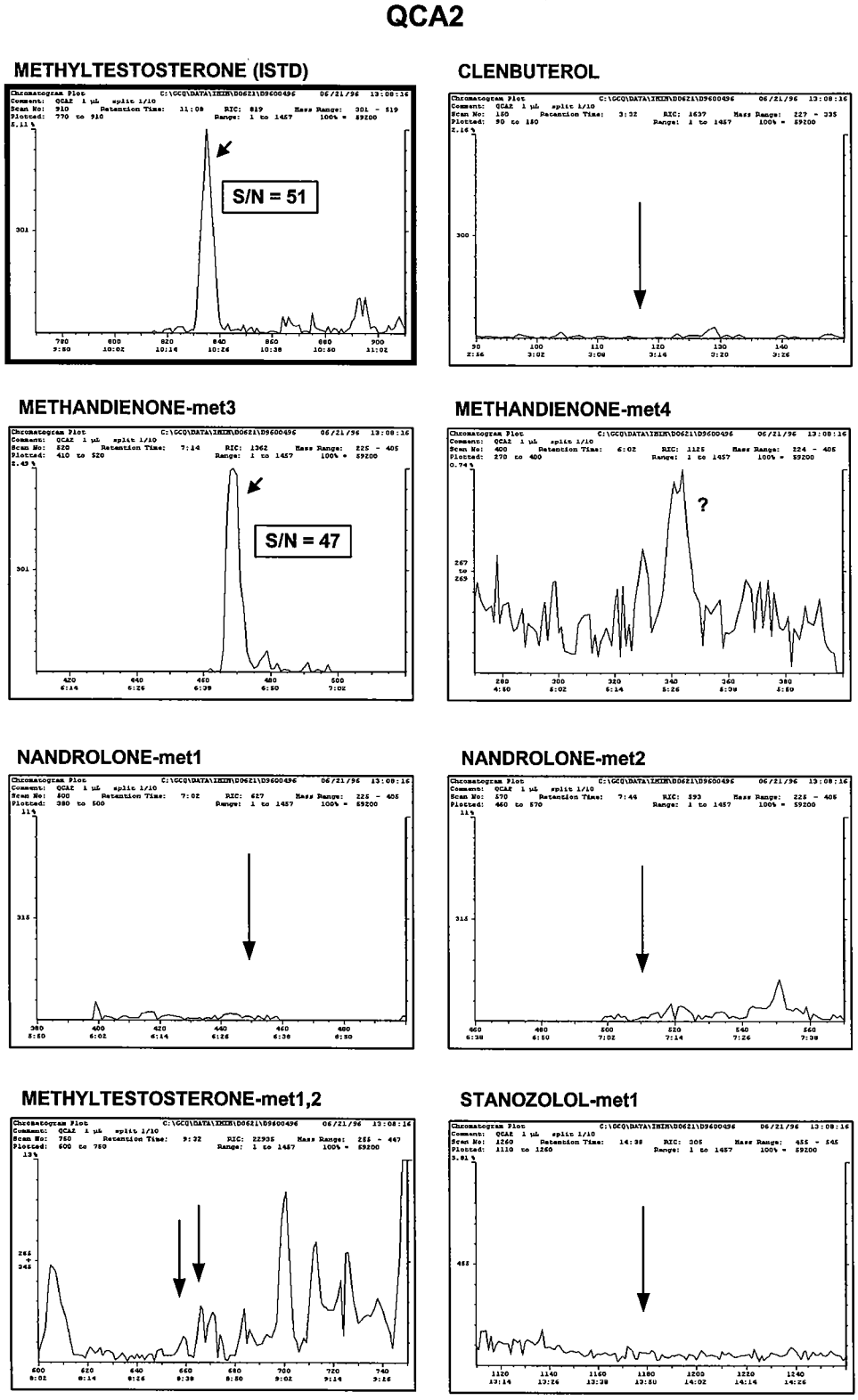


Figure 8. Results obtained for the analysis of the accreditation samples sent to the Atlanta Olympic Laboratory. Sample QCA3 (positive to clenbuterol and nandrolone).

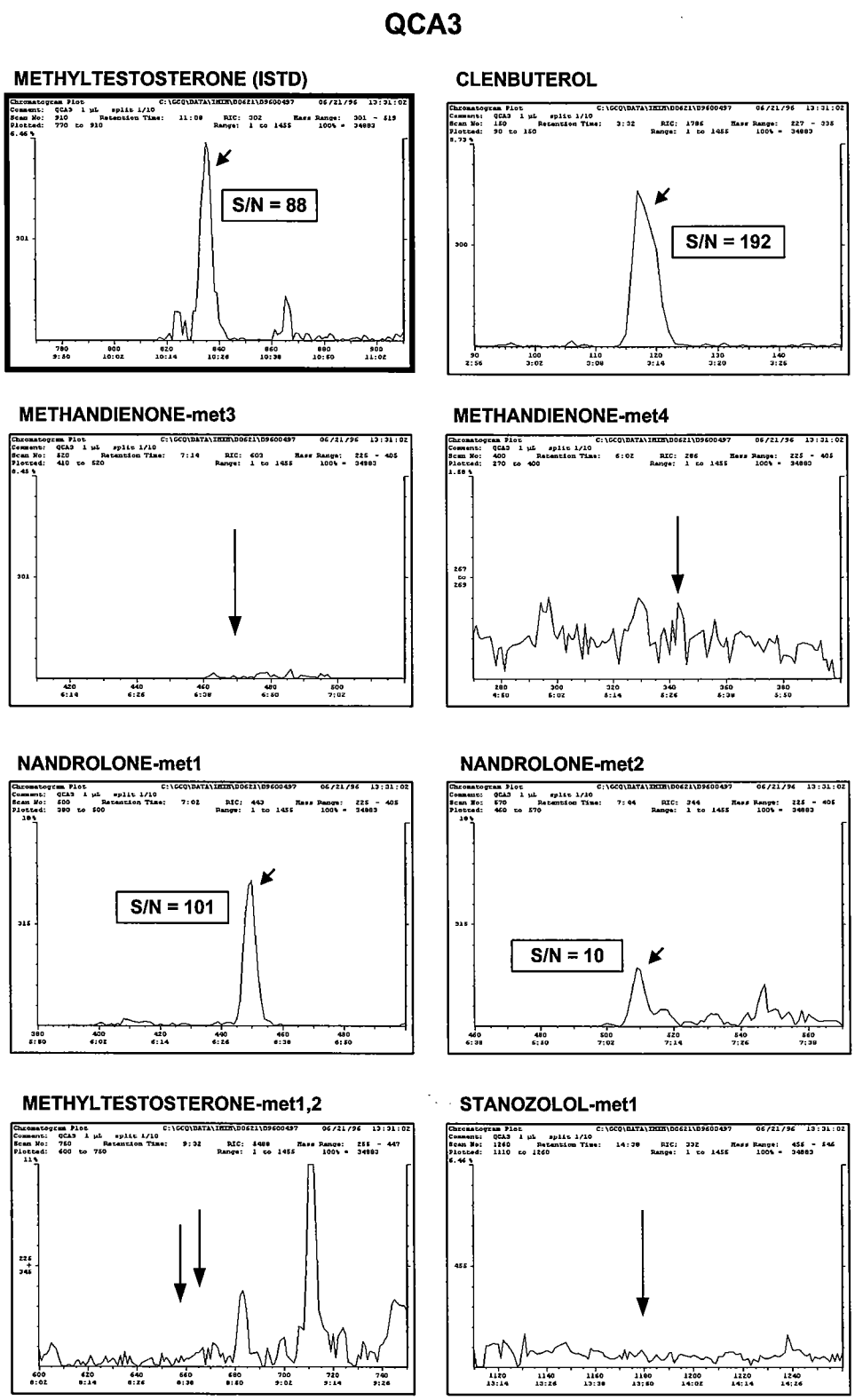


Figure 9. Results obtained for the analysis of the accreditation samples sent to the Atlanta Olympic Laboratory. Sample "BLANK" (negative urine).

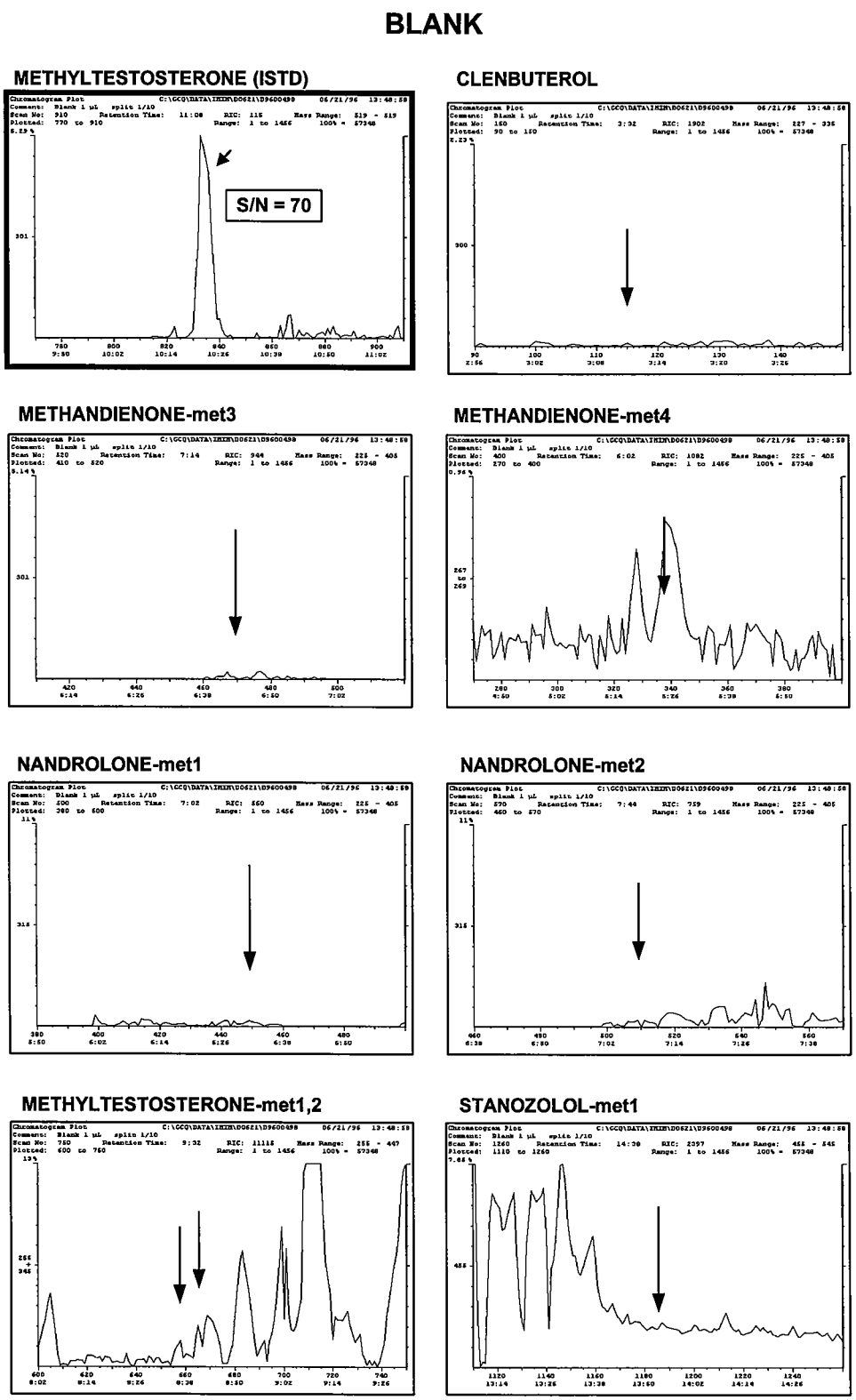


Figure 10. Results obtained for the analysis of a control urine spiked with different anabolic steroids at a concentration of 5 ng/ml each.