Analysis of Anabolic Agents in Urine by LC / MS / MS

Application

Forensics / Doping Control

Authors

Michael Zumwalt
Agilent Technologies, Inc.
Englewood, CO
USA

John Hughes
Agilent Technologies, Inc.
Pleasanton, CA
USA

Matthew Slawson, Chad Borges, and Dennis Crouch
Sports Medicine Research and Testing Laboratory
University of Utah
Salt Lake City, UT
USA

Abstract

The use of the Agilent 1200 Series liquid chromatography (LC) system coupled to the 6410 Triple Quadrupole Mass Spectrometer (QQQ) by way of the G1948B electrospray ionization (ESI) source is demonstrated in the analysis of anabolic substances in urine. The high degree of sensitivity of the QQQ instrument allows for excellent quantitation and linearity for meeting Minimum Required Performance Levels (MRPLs) for each compound as specified by the World Anti-Doping Agency (WADA). For increased chromatographic resolution and speed, a 2.1 mm id C18 column with a 3.5-µm particle size is employed. The seven compounds, including a designated internal standard, all elute within 10 minutes at a flow rate of 0.4 mL / min.

Introduction

The use of anabolic substances for performance enhancement in sports seems to be a ubiquitous topic of discussion. While their use is a growing problem in high school and collegiate athletics, their use at the professional level is an ongoing controversy. It is therefore the mandate of agencies like WADA to ensure that fair competition is being maintained by monitoring the possible use of banned substances like anabolic compounds.

Traditionally, doping control analysis for anabolic substances, including steroids, in urine includes screening by derivatization and GC/MS [1], followed by confirmation of the presumptive positive using high-resolution magnetic sector GC/MS in EI mode [2]. The high purchase and operational costs of high-resolution magnetic sector instruments make alternative techniques like LC/MS attractive for confirming the presence of the banned compounds.

More than 40 anabolic substances are currently targeted in doping control analysis, many of which are not easily analyzed using GC/MS but are amenable to LC/MS. The analysis of some of these compounds is very challenging, as they must be detected and confirmed at MRPLs of 2 ng/mL or lower in urine.

This work describes the results of using the Agilent LC/QQQ instrument for detection and confirmation of a number of anabolic substances at the

WADA MRPL or, more specifically, covering the 1/2 × – 10 × MRPL range. The anabolic compounds analyzed in this work are listed in Table 1 along with their MRPLs.

Some previous work [3] used the TOF to analyze these compounds and found that accurate mass could be used for both screening and confirmation. However, the QQQ is more specific with MS/MS, increasing the confidence in confirmation and quantitating compounds of interest.

Table 1. Minimum Required Performance Levels (ng / mL of urine)

Compound	MRPL
Clenbuterol	2
19-norandrosterone	1
4β-OH-stanozolol	10
Tetrahydrogestrinone (THG)	10
Methyl testosterone metabolite (MeTest metabolite) or 17α-methyl-5β-androstane-3α,17β-diol	2
Epimetendiol	2
Methyl testosterone – Internal Standard	NA

In this study all compounds are steroids except for clenbuterol.

The structures of the compounds analyzed in this work are shown in Figure 1. Based on the results of work presented elsewhere [4], a derivatizing agent is used on these samples, but only reacts with 19-norandrosterone to improve sensitivity. The derivatizing agent, known as Girard's Reagent P (Sigma Aldrich, St. Louis, MO), reacts with ketone groups to form a quaternary amine, which is more easily ionized by ESI.

Experimental

Sample Preparation

The anabolic agents and their metabolites are purchased from Sigma Aldrich (St. Louis, MO), Steraloids (Newport, RI), and the National Measurement Institute (Sydney, Australia). Girard's Reagant P (GRP) is purchased from Sigma Aldrich and β-glucuronidase is purchased from Roche (Indianapolis, IN).

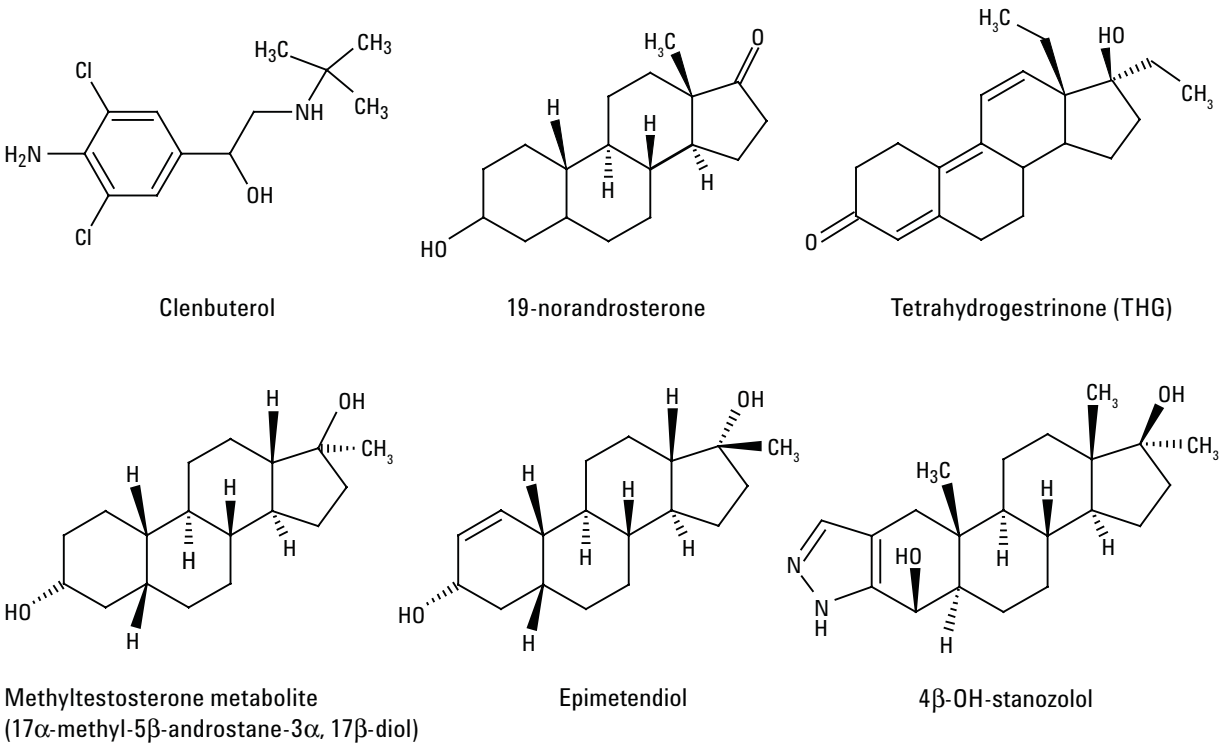

Figure 1. Structures of anabolic substances analyzed in this work.

To 3 mL urine negative control sample, 1 mL 0.8 M potassium phosphate buffer, pH 7.0, is added. A further 25 µL β-glucuronidase is added and then the mixture is incubated at 50 °C for one hour. A 750-µL mixture of 20% (w/v) $K_2CO_3/KHCO_3$ (1:1) mixture is then added. Extract with methyl-t-butyl ether and then remove and dry the organic extract.

The same extraction procedure used for GC/MS screening is employed except that the compounds are not derivatized as usual for GC/MS analysis. Rather, the samples are dried and then reconstituted in 100 µL of LC mobile phase.

As part of the reconstitution step for LC/MS/MS analysis, 20 µL methanol, followed by 8 µL of 1M GRP in 50 mM ammonium acetate buffer, pH 4.2, is added. Incubation at room temperature for one hour is then followed by LC/MS/MS analysis.

Of the compounds analyzed, only the 19-norandrosterone is reactive with the GRP derivative. This compound has been problematic in LC/MS/MS analysis and the GRP improves sensitivity.

The MeTest internal standard has a fixed concentration of 10 ng/mL.

LC / MS Method Details

LC Conditions

Agilent 1200 Series binary pump SL, wellplate sampler, thermostatted column compartment, inline filter 0.5 µm between needle seat and injector valve.

Column:	Agilent ZORBAX XDB-CN 2.1 × 100 mm, 3.5 µm (p/n 961764-905)
Column temp:	50 °C
Mobile phase:	A = 0.1% formic acid in water B = 0.1% formic acid in methanol
Flow rate:	0.4 mL/min; injection vol: 2 µL

Gradient:

Time (min)	%B
0–1	5
3	15
3.01	40
12	50
15	95

Stop time = 15 min; Post-run time = 3 min.

MS Conditions

Mode:	Positive ESI using the Agilent G1948B ionization source
Nebulizer:	40 psig
Drying gas flow:	9 L/min
Drying gas temp:	350 °C
V_{cap}:	4000 V
Q1 resolution:	0.7 amu
Q2 resolution:	0.7 amu

MRM transitions shown in Table 2. Chromatographic retention times (RTs), fragmentor (Frag), collision energy (CE), and dwell times are included. Time segments in which the MRM transitions are implemented are also noted.

Results and Discussion

The chromatographic elution profile of all compounds at their equivalent 10 × MRPL is shown in Figure 2. The responses vary quite significantly among the compounds and the background interference from the matrix is evident.

Concentration levels ranging from 1/2 × to 10 × MRPL are run in triplicate injections. The results for clenbuterol are shown in Figures 3a to 3c. Linearity over this range has a correlation coefficient of $R^2 > 0.999$ using the most conservative

Table 2. Data Acquisition Parameters for MRM Transitions

Compound	RT (min)	MRM	Frag (V)	CE (V)	Dwell (msec)
Segment 1 (0–4.0 min)					
Clenbuterol	2.74	277.0 > 203.1	100	15	200
Segment 2 (4.0–6.3 min)					
19-norandrosterone	5.82	410.3 > 331.3	130	30	75
Segment 3 (6.3– 6.93 min)					
4β-OH-stanozolol	6.64	345.2 > 327.2	140	15	200
Segment 4 (6.93–7.55 min)					
MeTest (IStd)	7.19	303.2 > 97.1	140	25	75
Segment 5 (7.55–8.8 min)					
THG	7.88	313.2 > 295.1	150	15	100
MeTest metabolite	8.08	271.2 > 161.2	110	20	100
Segment 6 (8.8–12.0 min)					
Epimetendiol	9.47	269.2 > 105.1	90	20	200

curve fit settings of linear, ignored origin, and no weighting. A closer look at the reproducibility of the lowest three level replicates is included in Figure 3a. The limit of detection (LOD), which is defined here as being a peak-to-peak signal-to-noise (S/N) ratio of 3:1, the S/N of the lowest level (1/2 × MRPL) is measured first. Then the same factor that is applied to this S/N, in order to obtain a S/N of 3:1, is also applied to the lowest level.

For example, in Figure 3b the S/N is nearly 60:1 for all three injections at the 1/2 × MRPL. A factor of 20 is applied to achieve 3:1 so that the LOD is 1/20th the concentration of this level, or 1/40 × MRPL.

To determine the on-column injection amount it should be noted that the original sample corresponds to 3 mL of urine. Since the MRPL of clenbuterol is 2 ng/mL, according to Table 1, then the 1/2 × MRPL contains 3 ng clenbuterol in the 3 mL urine sample. Following extraction and evaporating to dryness, this 3 ng of clenbuterol is reconstituted in 100 µL of LC mobile phase. Of this volume, 2 µL is injected. Therefore, the on-column injection amount of clenbuterol at the 1/2 × MRPL corresponds to 2/100 × 3 ng = 60 pg. The LOD is therefore 1/20 × 60 pg, or about 3 pg on-column.

The LOD for clenbuterol is given in Figure 3b. Note that the negative quality control (NQC) is also shown as evidence that the calculated S/N is justifiable.

Figure 3c shows the replicate injections at the lowest three levels.

The results for THG, MeTest metabolite, epimetendiol, and 4β-OH-stanozolol are shown in Figures 4, 5, 6, and 7, respectively.

As can be seen from Figure 5a, the 1/2 x MRPL does not appear to be a limit of detection because an S/N of 3:1 does not seem possible. However, in comparison to the matrix blank (NegQC) this level is certainly detectable. For this reason, including the fact that the 1/2 × MRPL replicate injections are at the lowest end of the range investigated and linear with the curve fit, the 1/2 × MRPL of the MeTest metabolite is considered the LOD.

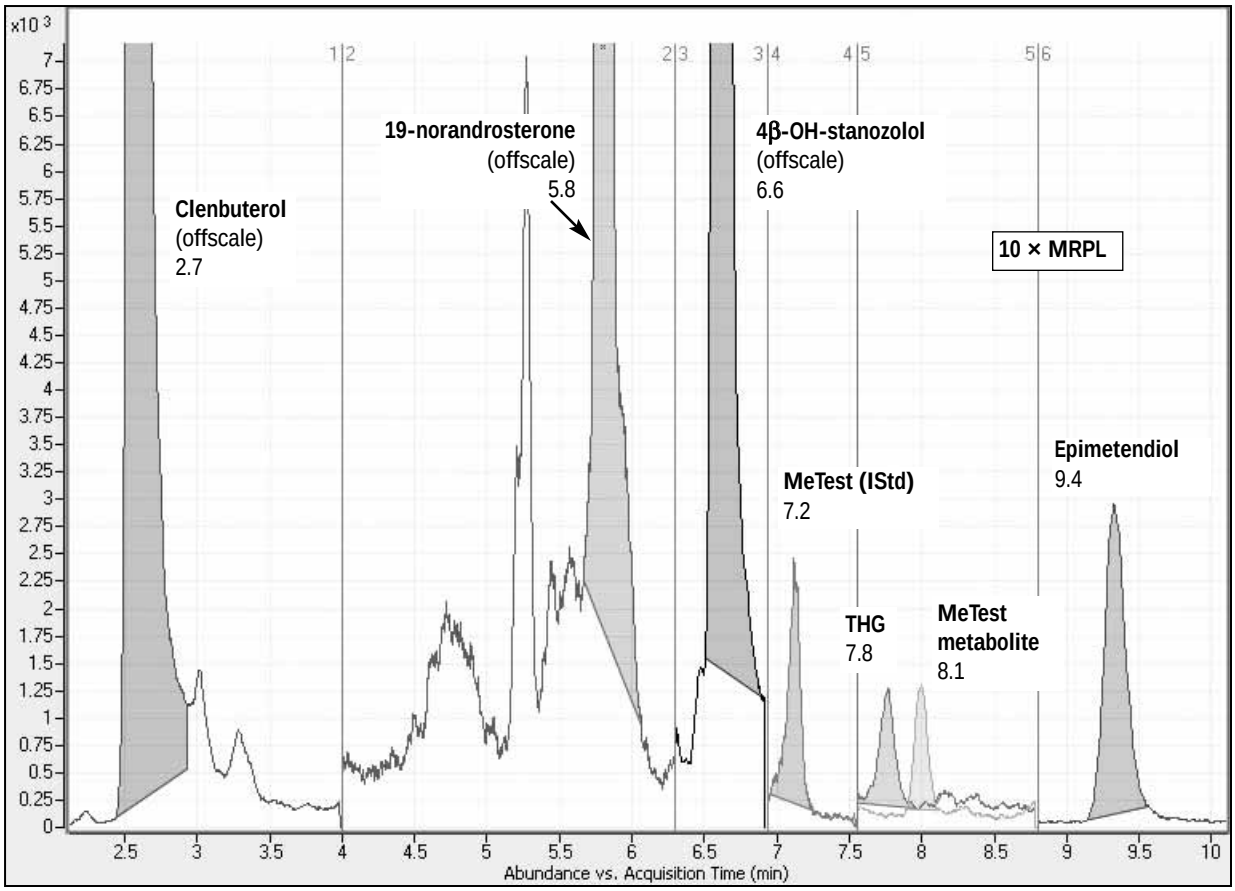

Figure 2. Chromatographic profile of 10 × MRPL extract in urine.

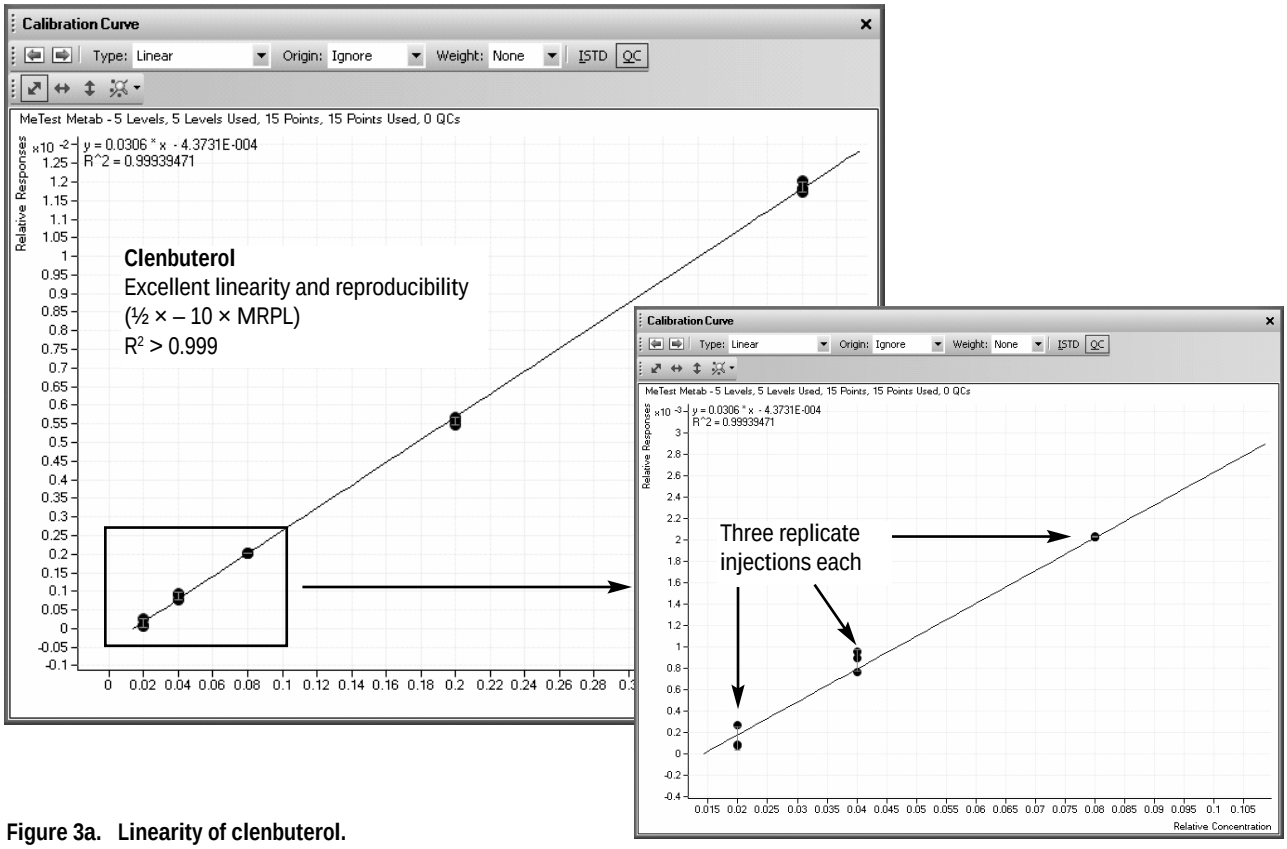

Figure 3a. Linearity of clenbuterol.

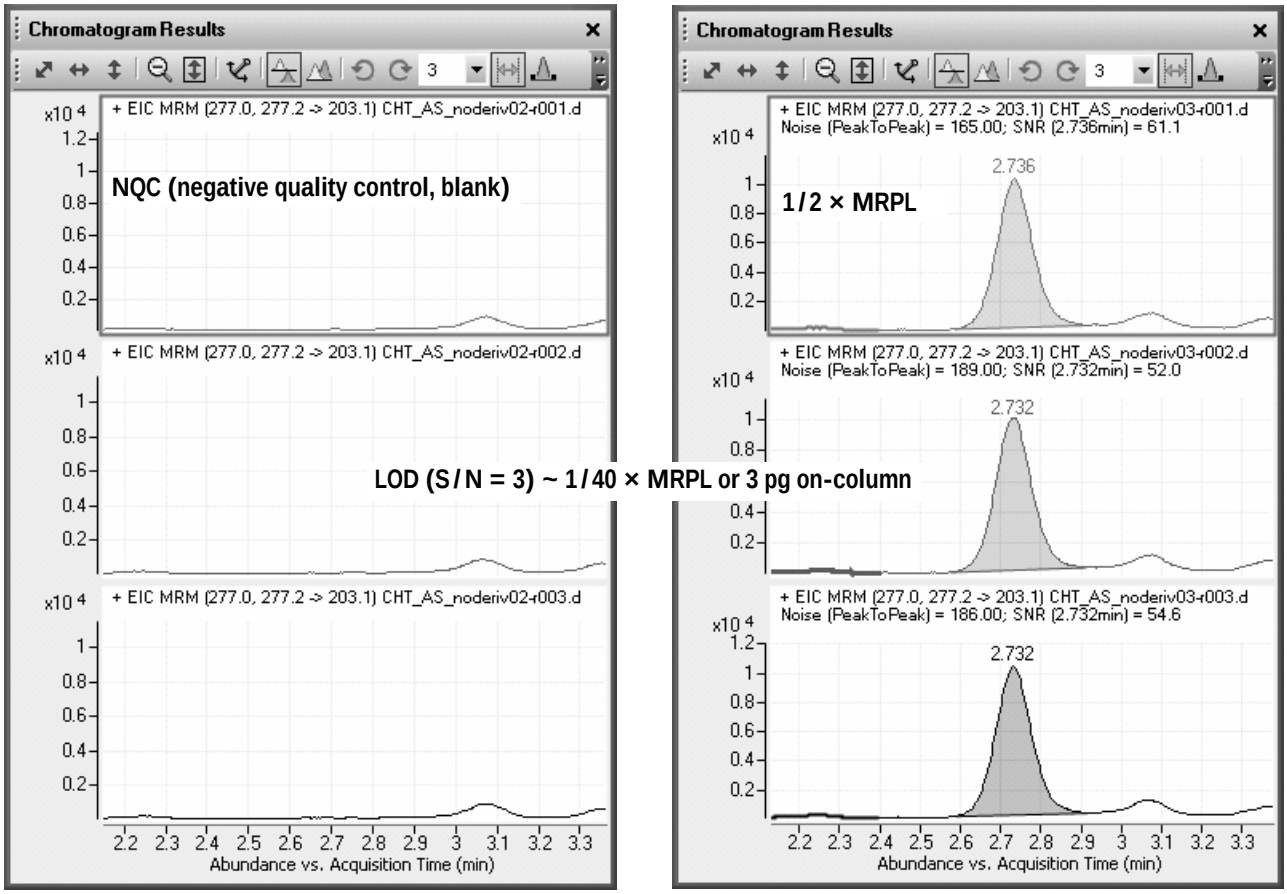

Figure 3b. Estimate of LOD for clenbuterol.

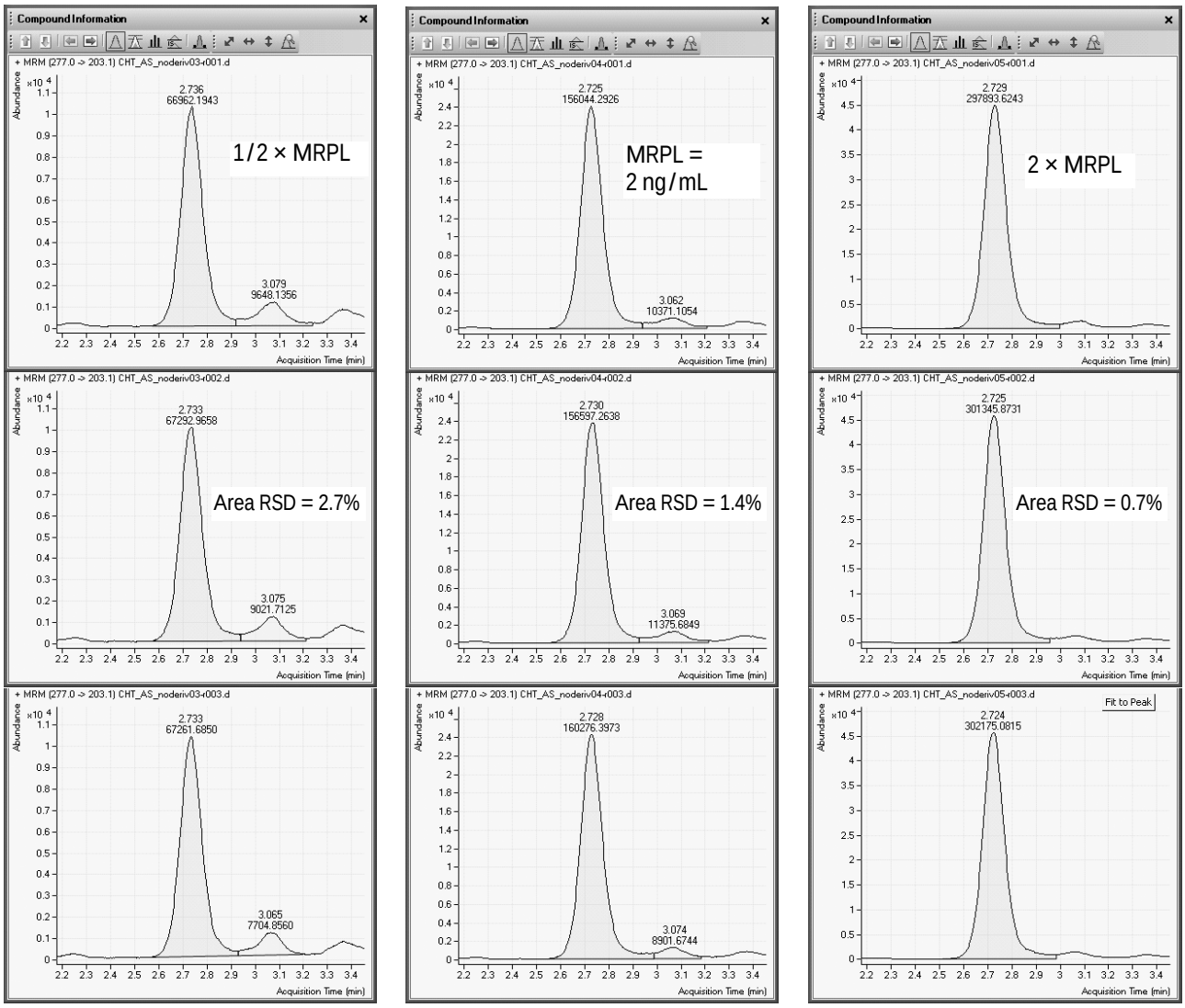

Figure 3c. Triplicate injections of the lowest three levels of clenbuterol.

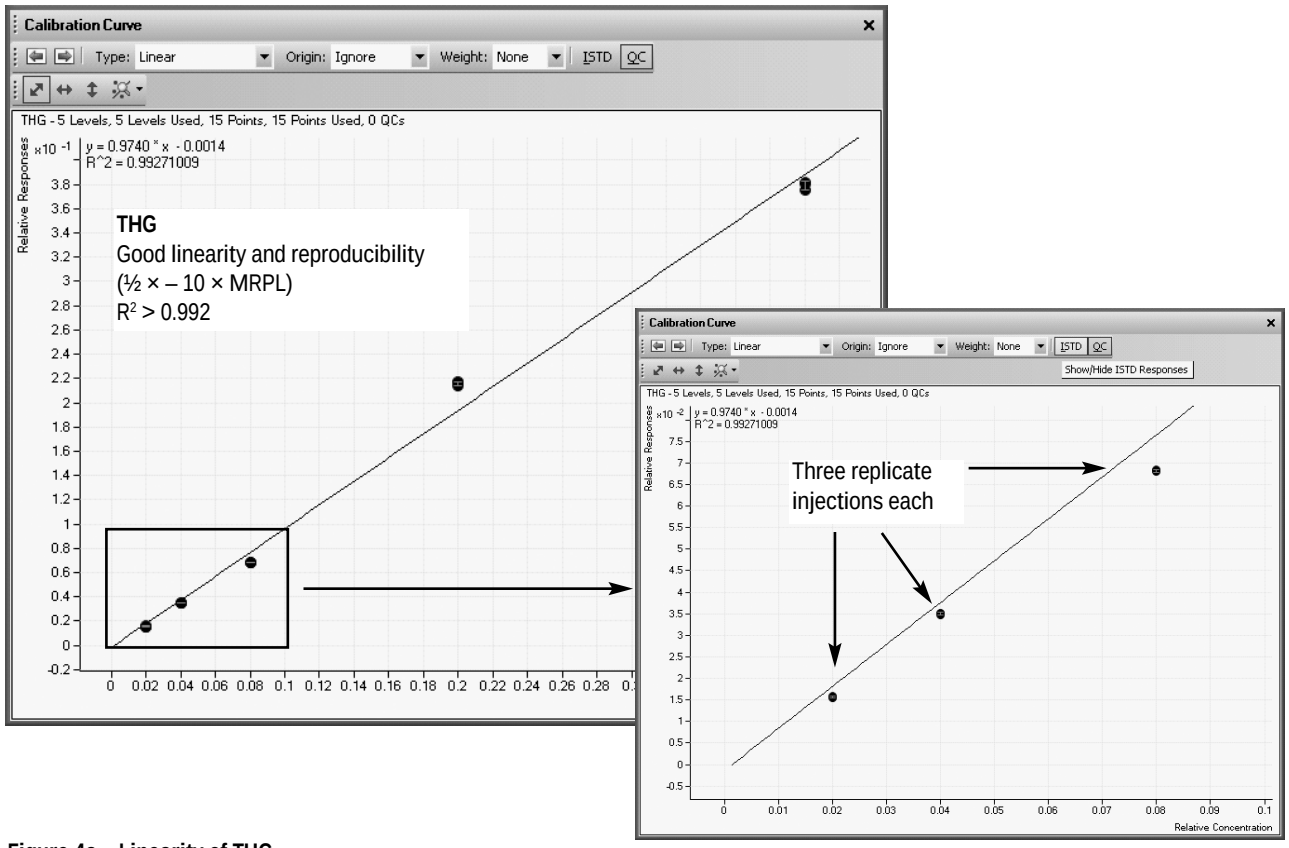

Figure 4a. Linearity of THG.

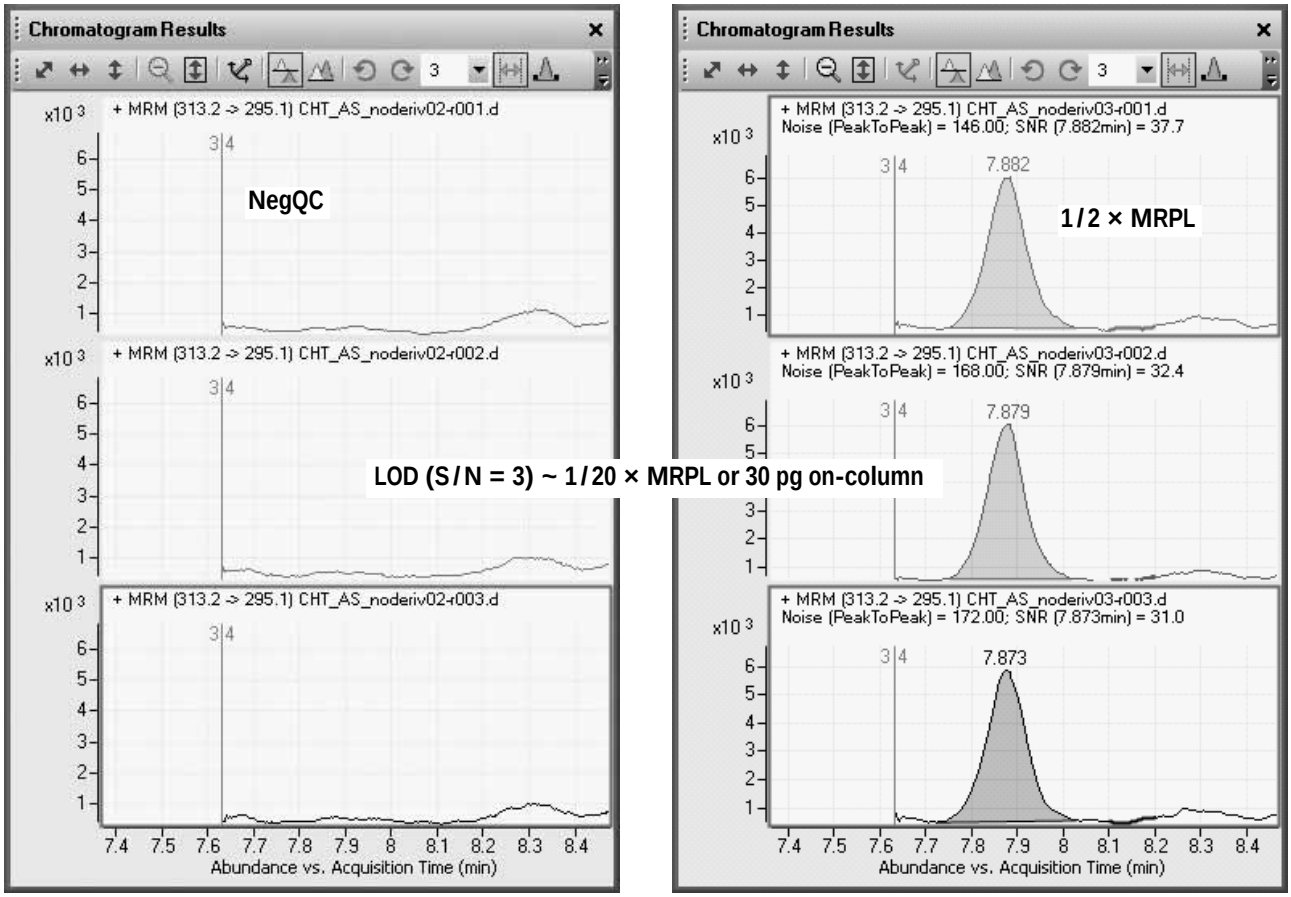

Figure 4b. Estimate of LOD for THG.

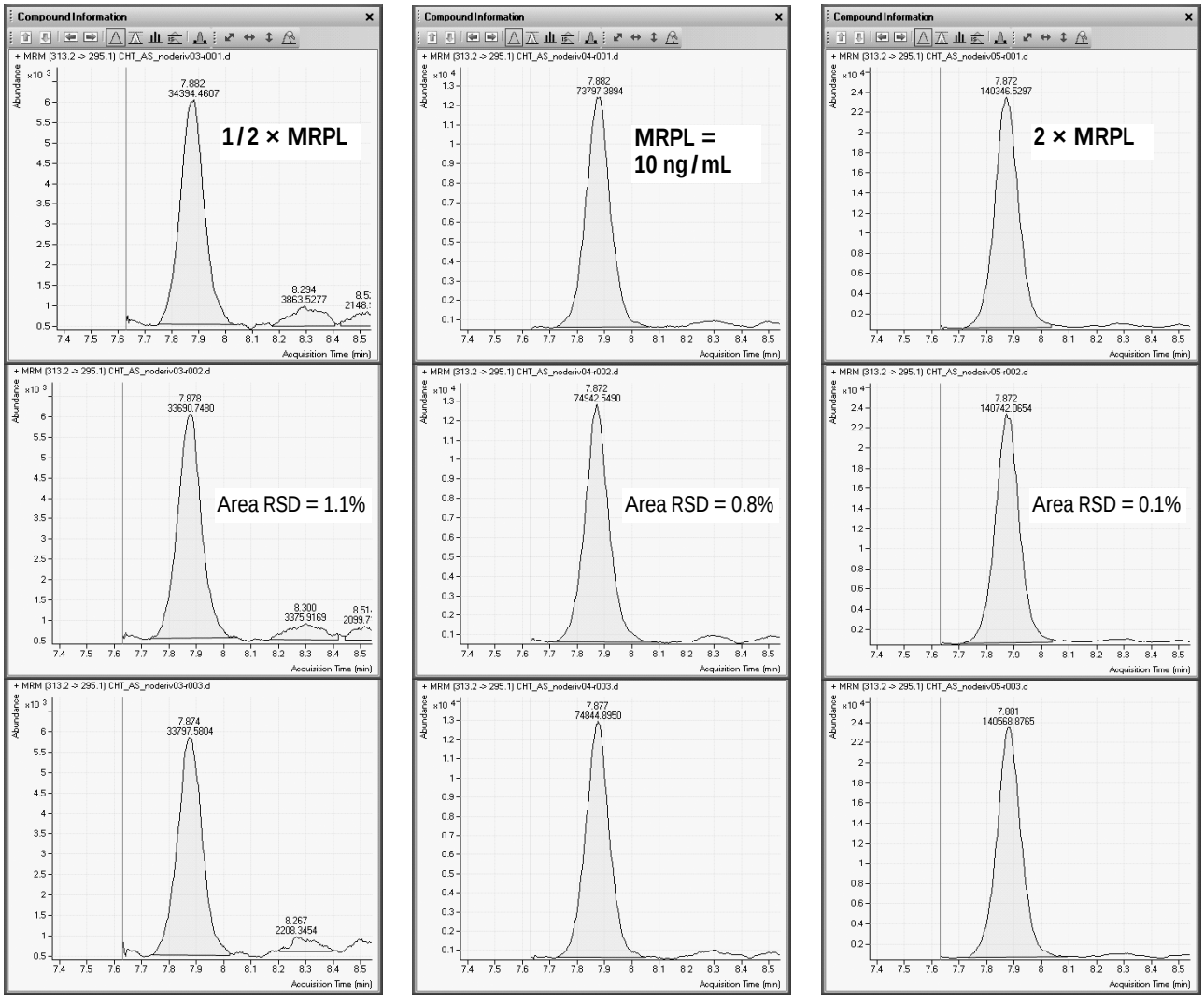

Figure 4c. Triplicate injections of the lowest three levels of THG.

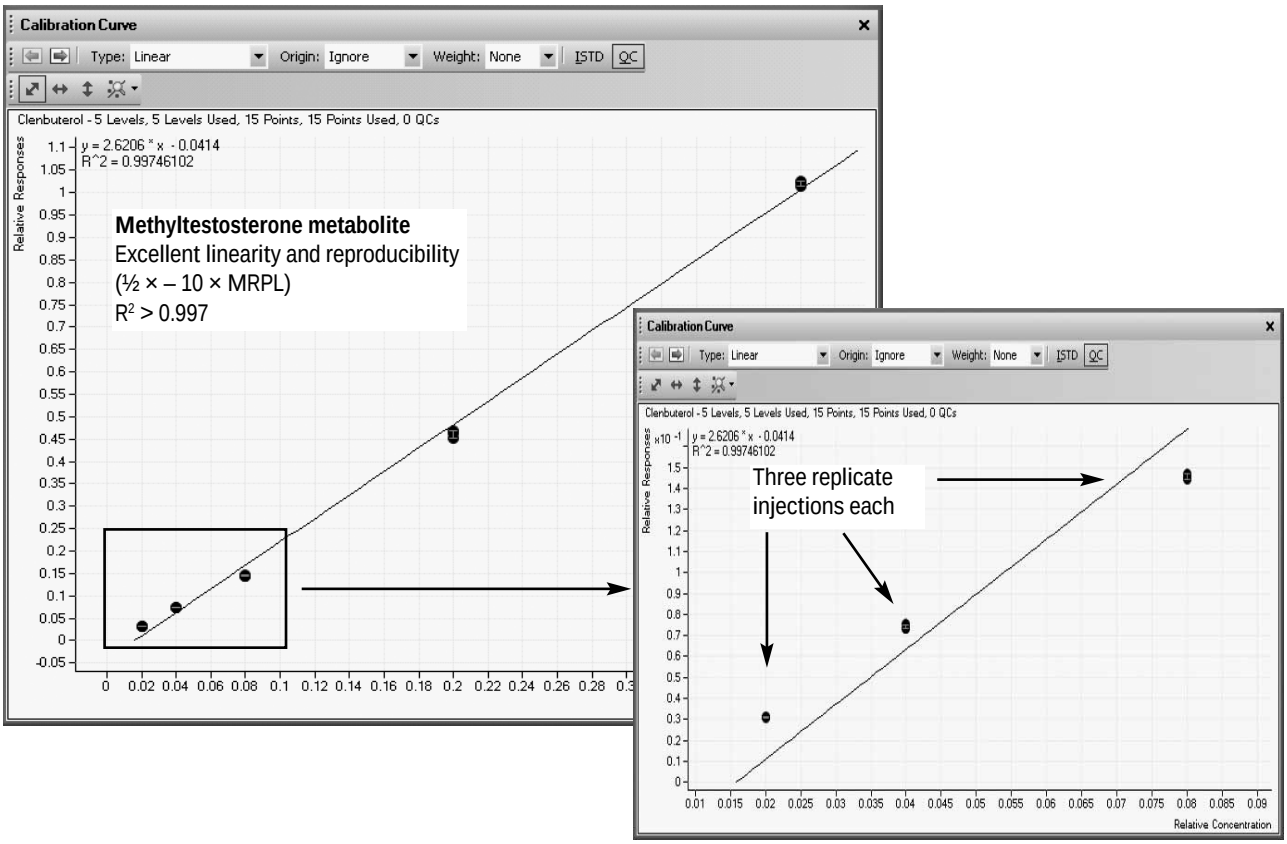

Figure 5a. Linearity of methyltestosterone metabolite.

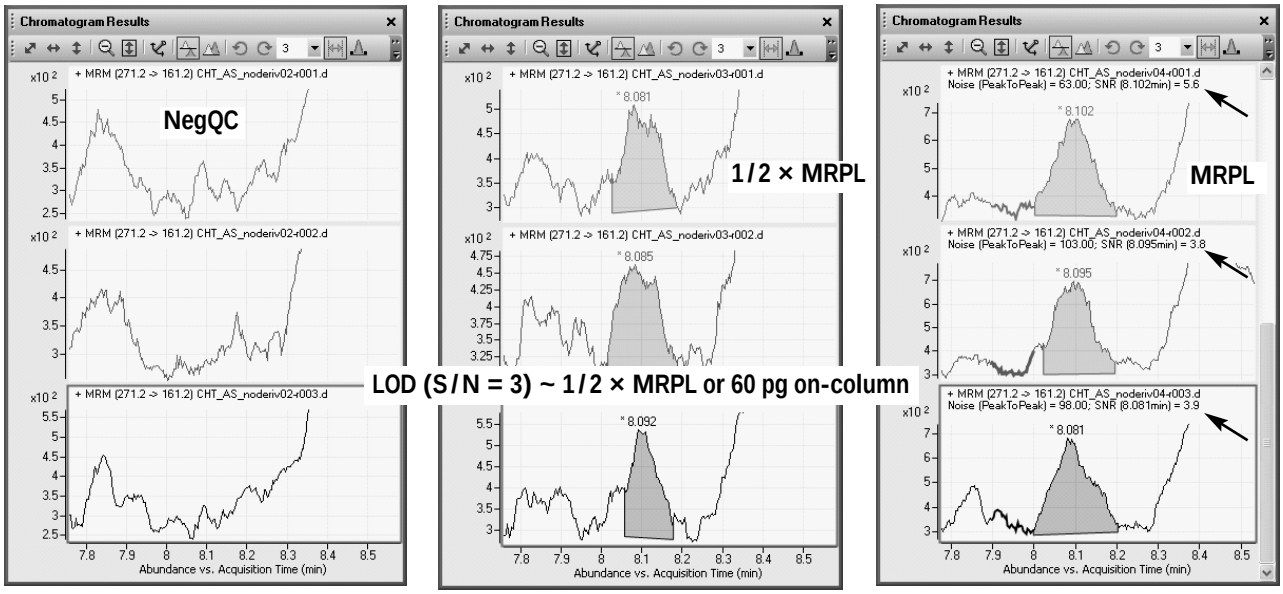

Figure 5b. Estimate of LOD for methyltestosterone metabolite.

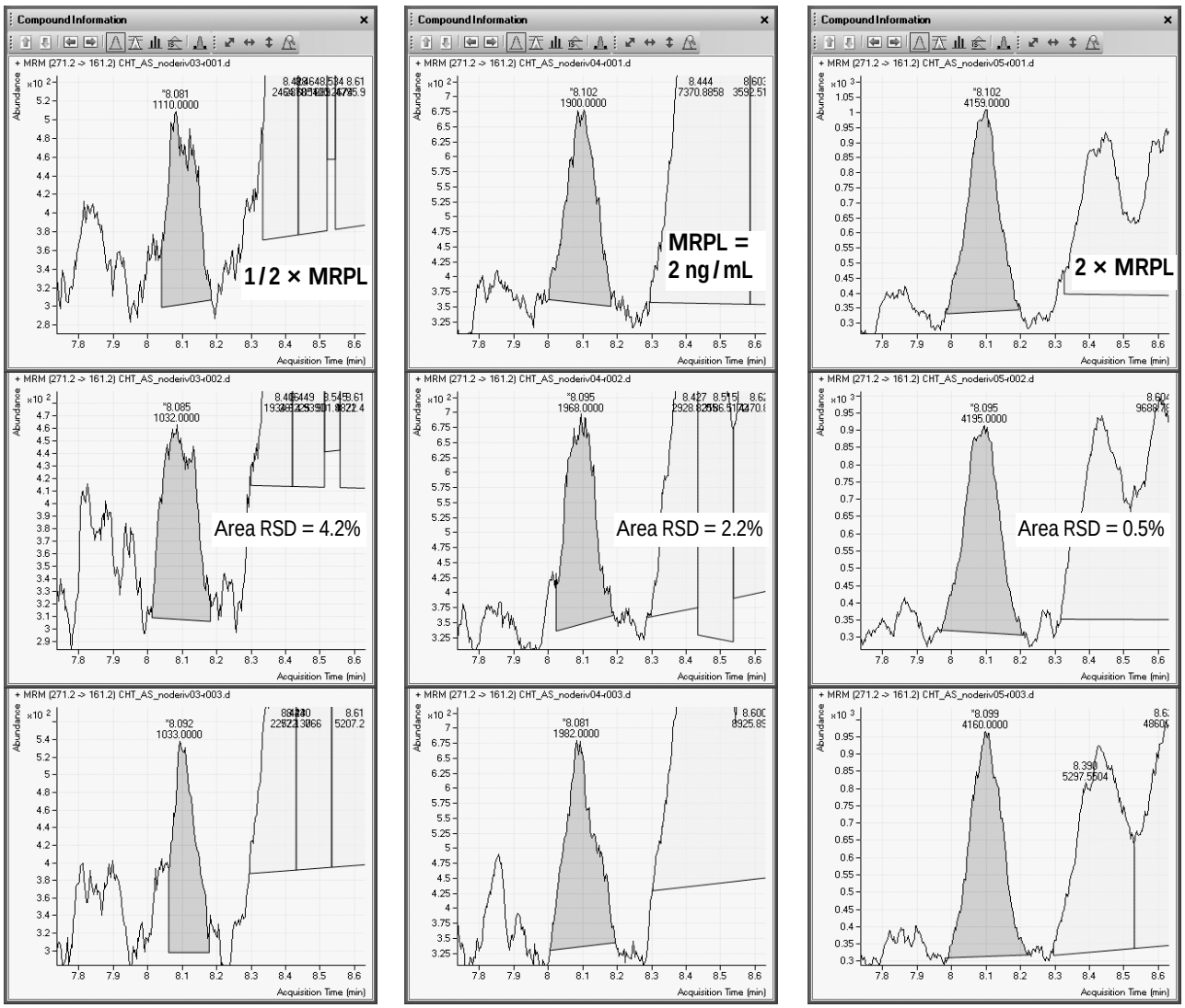

Figure 5c. Triplicate injections of the lowest three levels of methyltestosterone metabolite.

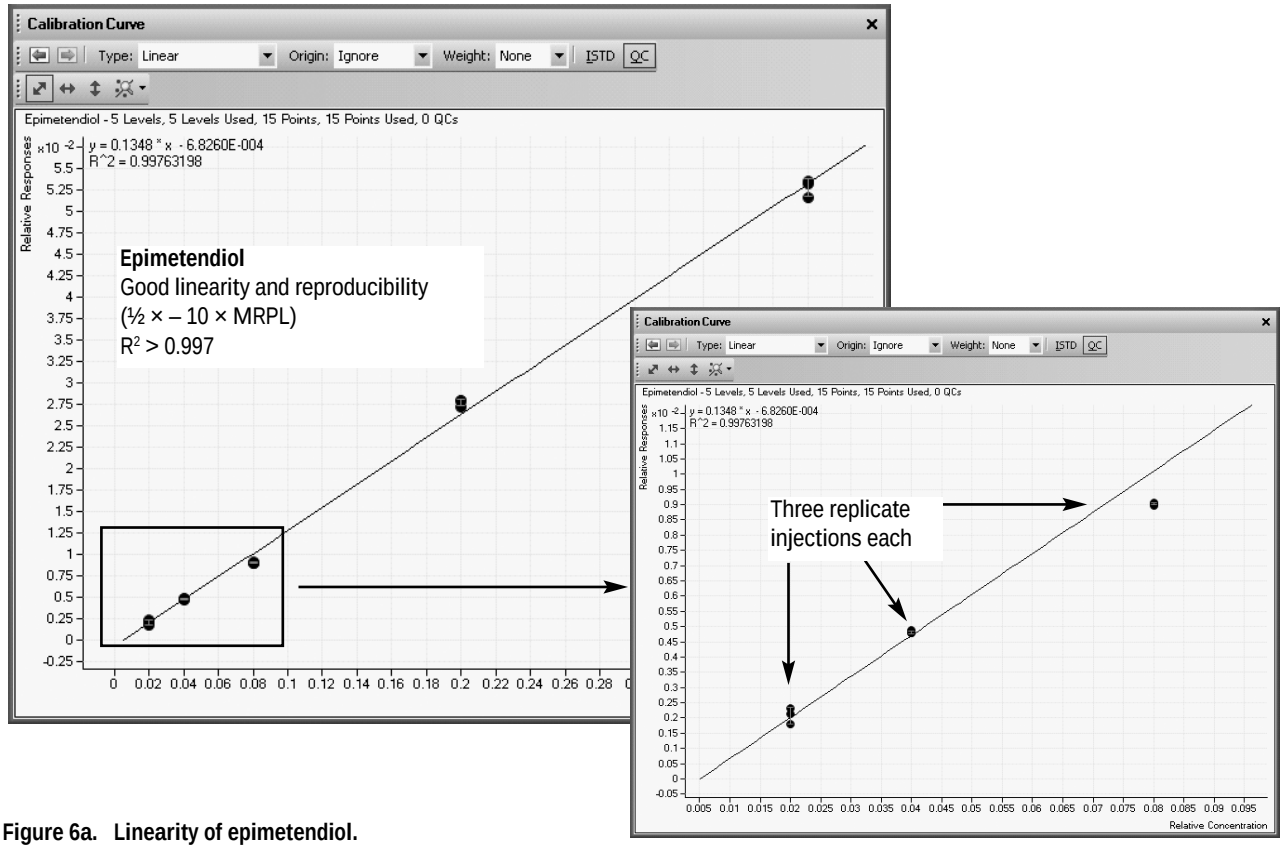

Figure 6a. Linearity of epimetendiol.

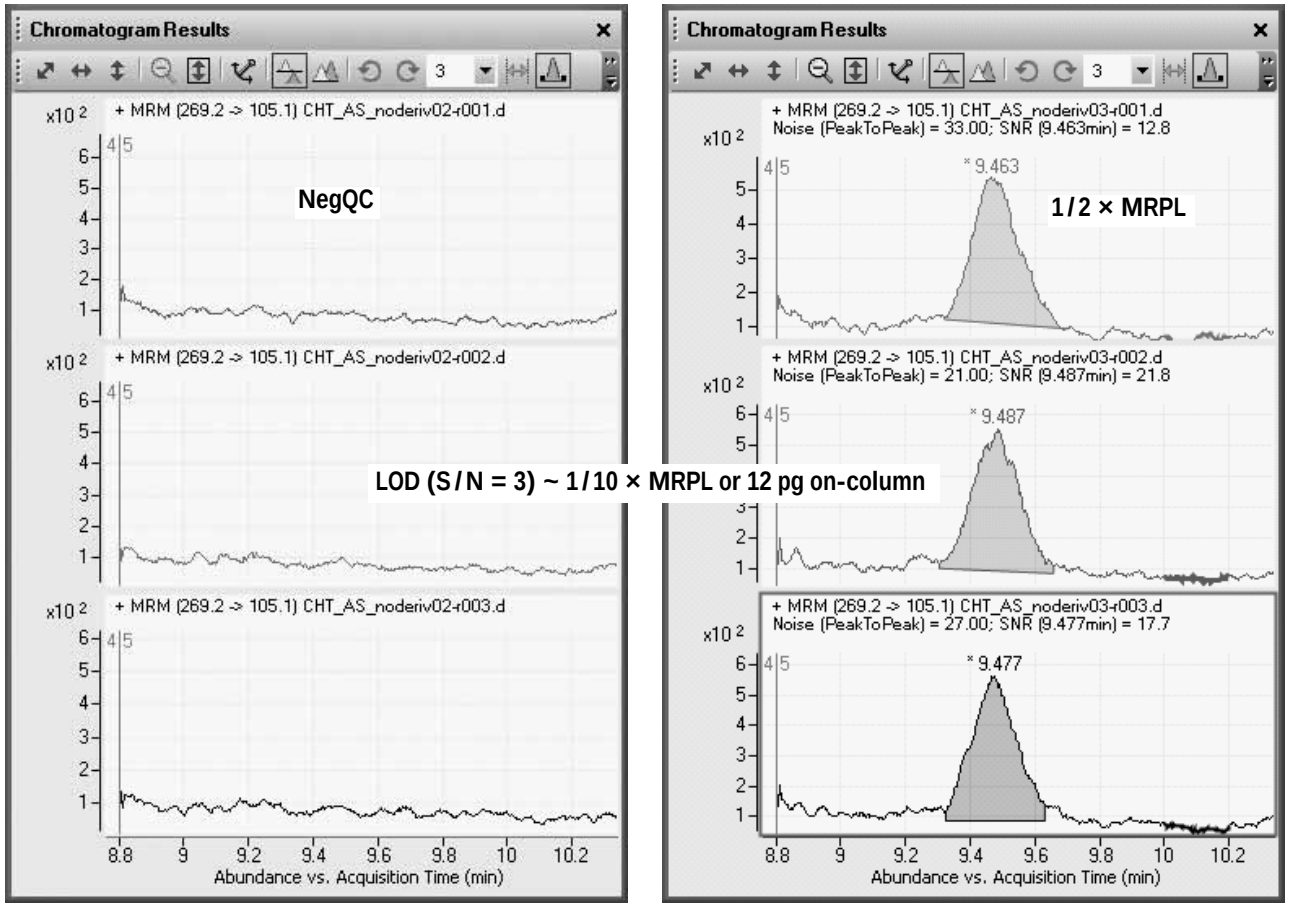

Figure 6b. Estimate of LOD for epimetendiol.

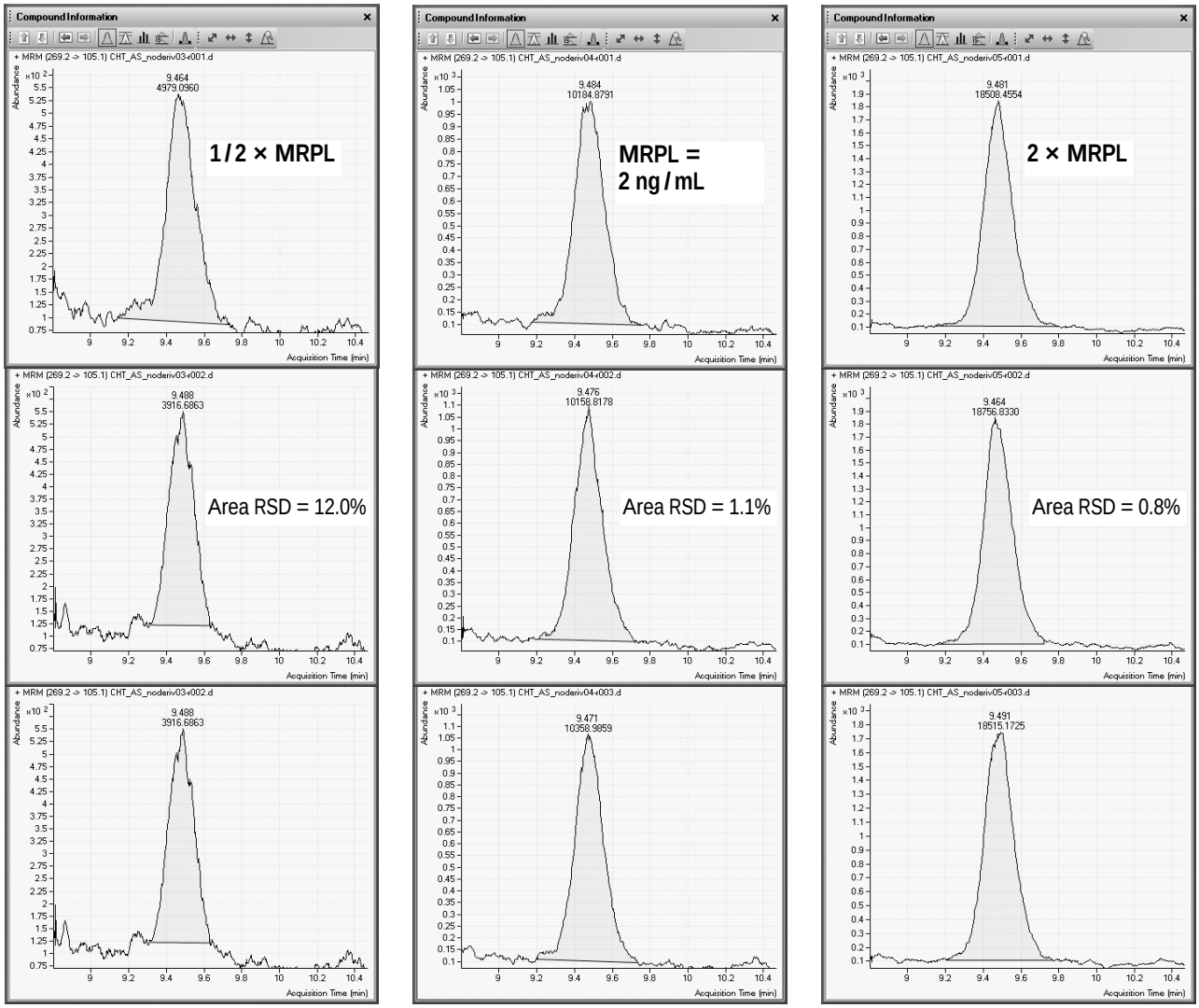

Figure 6c. Triplicate injections of the lowest three levels of epimetendiol.

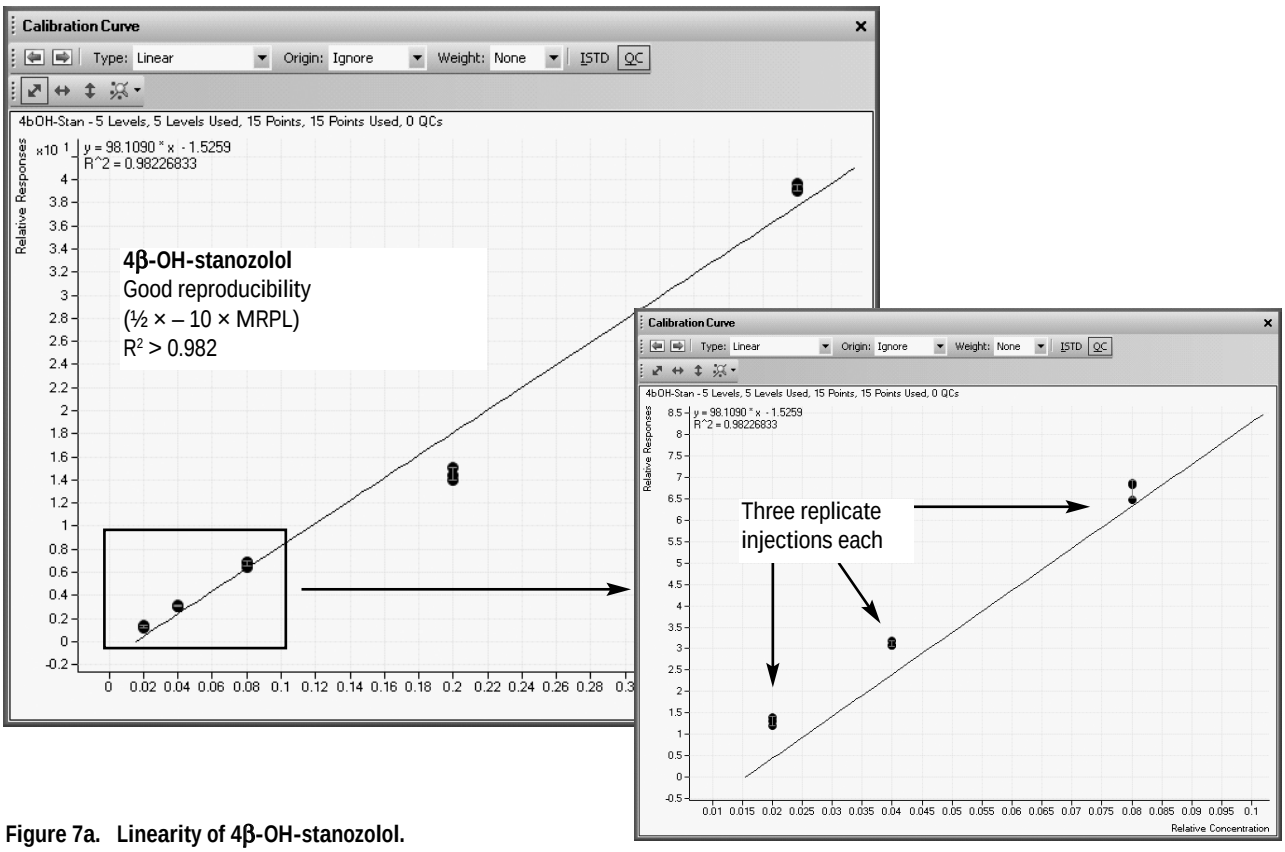

Figure 7a. Linearity of 4β-OH-stanozolol.

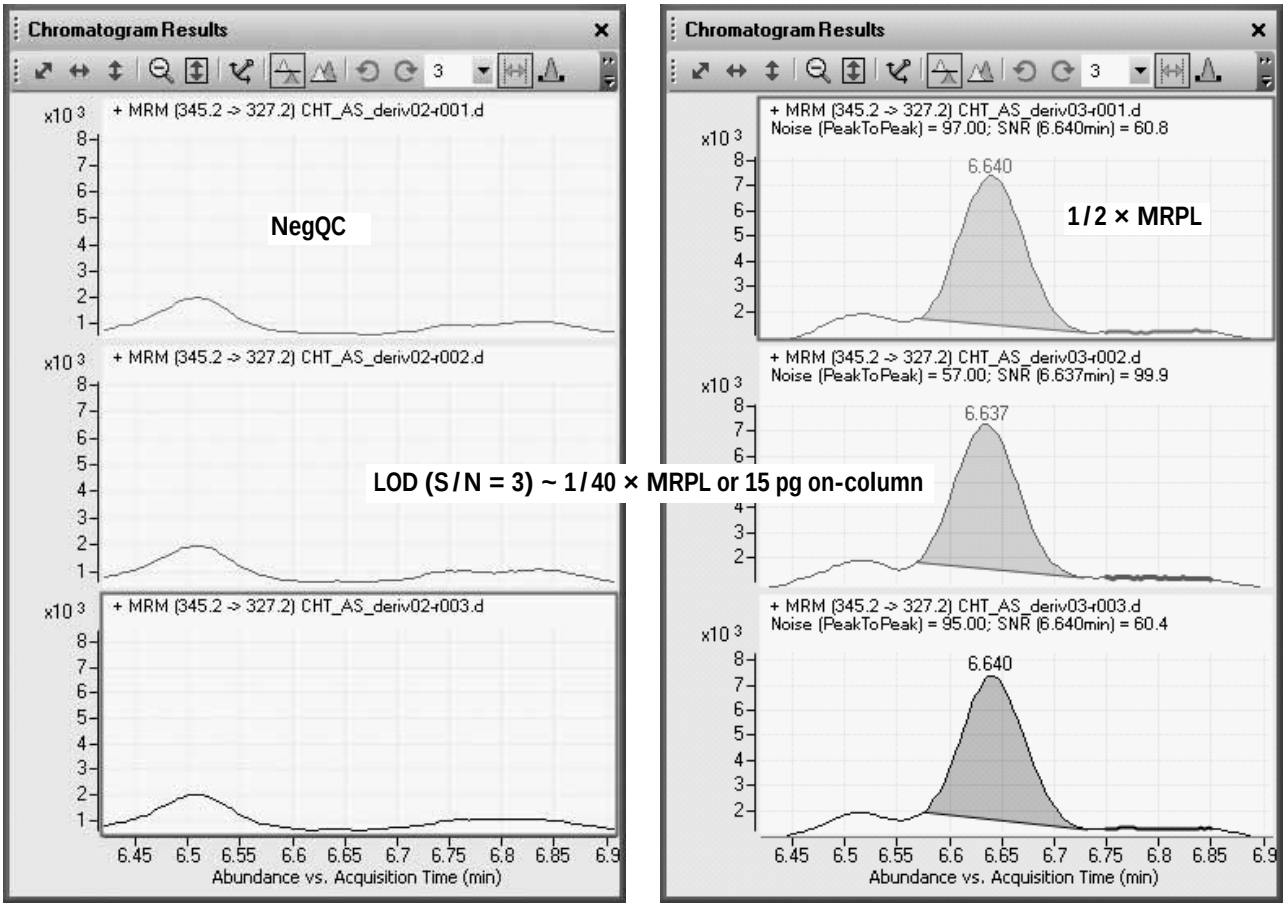

Figure 7b. Estimate of LOD for 4β-OH-stanozolol.

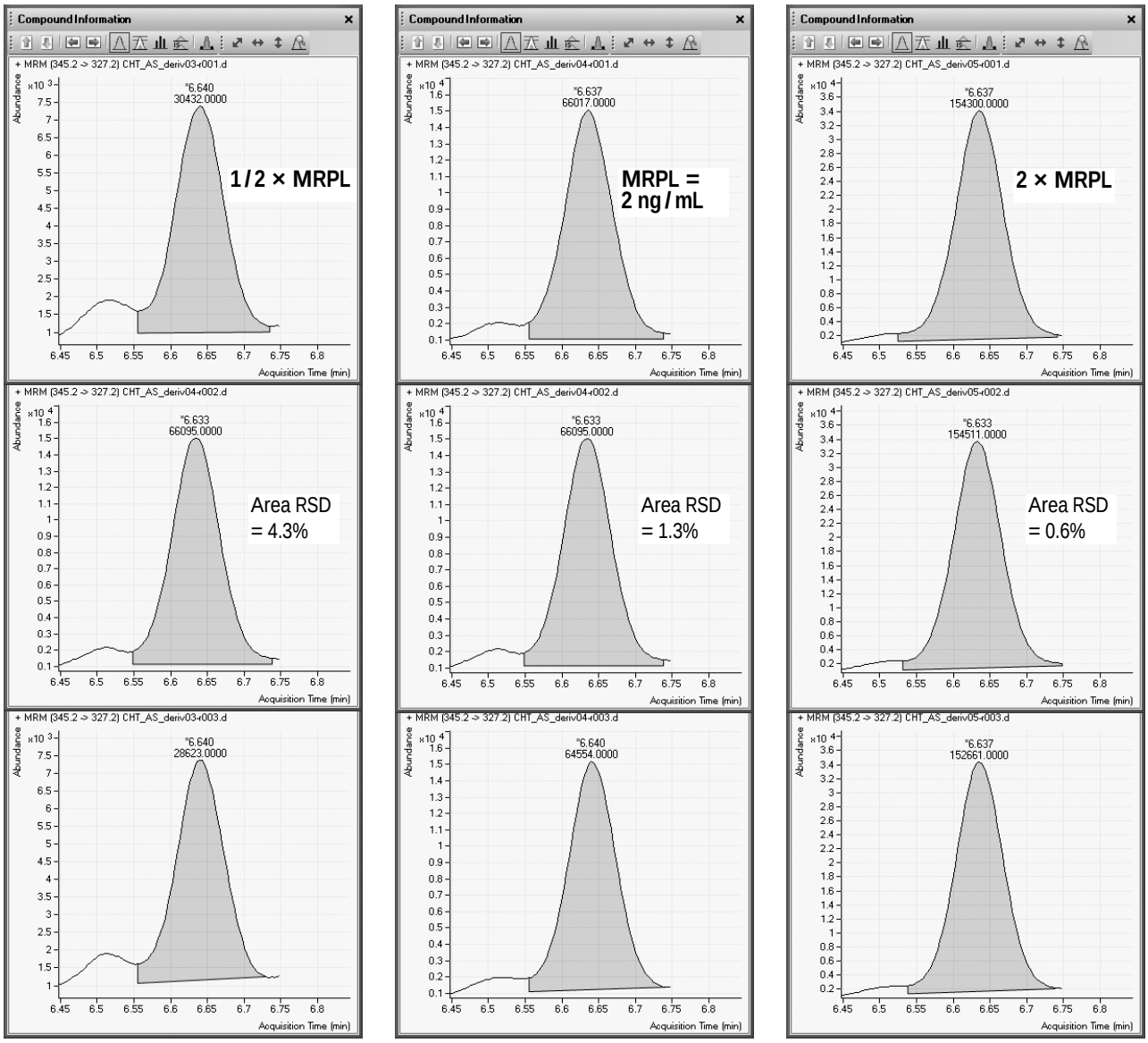

Figure 7c. Triplicate injections of the lowest three levels of 4β-OH-stanozolol.

In Figure 8 the reason for using the GRP derivative is shown by comparing the sensitivity of analyzing the 19-norandrosterone with and without the derivative.

Figures 9a to 9c show the linearity, LOD, and the lowest three level replicate injections for 19-norandrosterone. In Figure 9b we see noticeable signal in the negative quality control. However, this signal definitely comes from the matrix itself as it is not seen in the solvent blank.

The results for all compounds are summarized in Table 3.

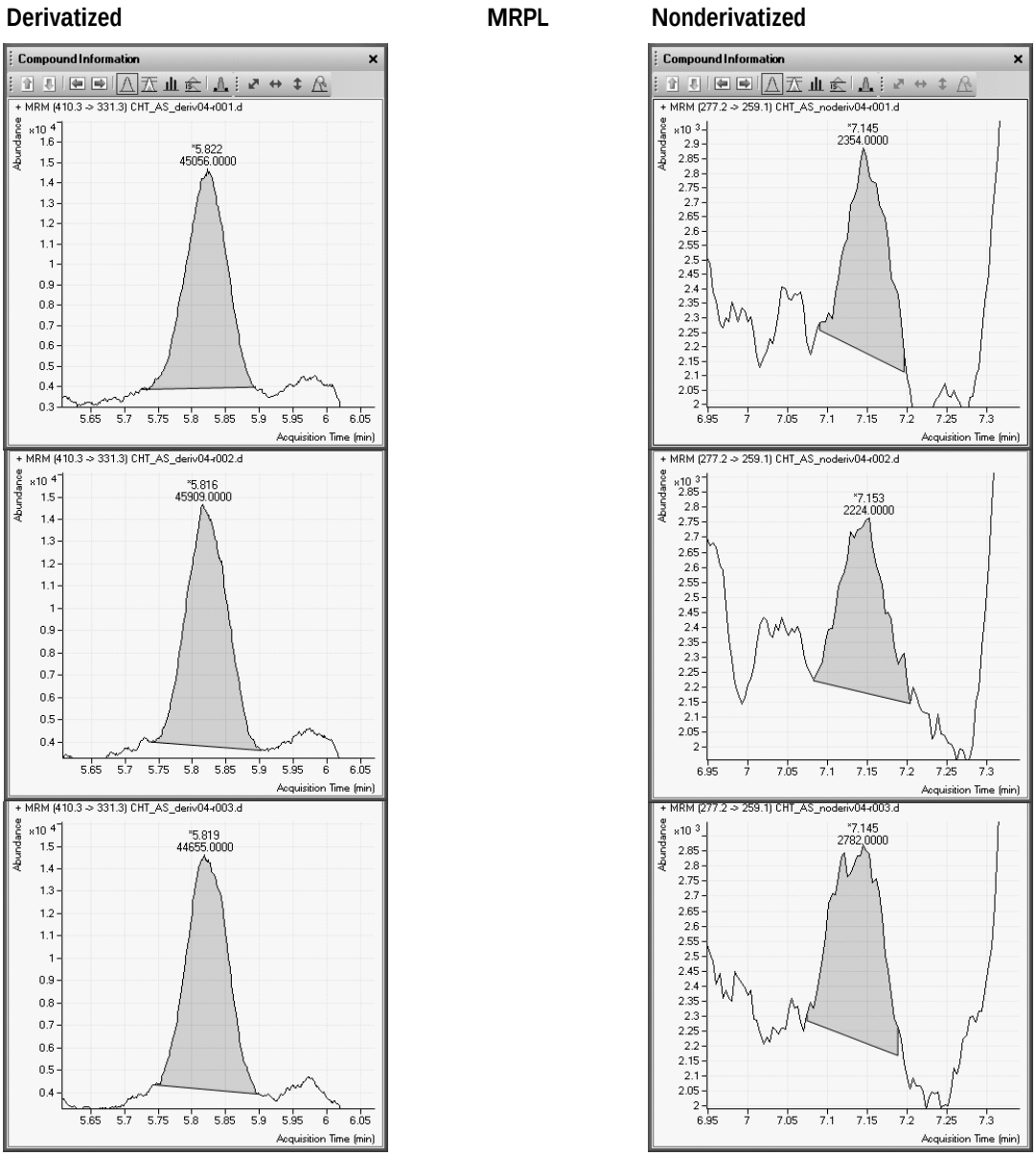

Figure 8. Comparison of signal response for the derivatized (left) versus nonderivatized forms of 19-norandrosterone.

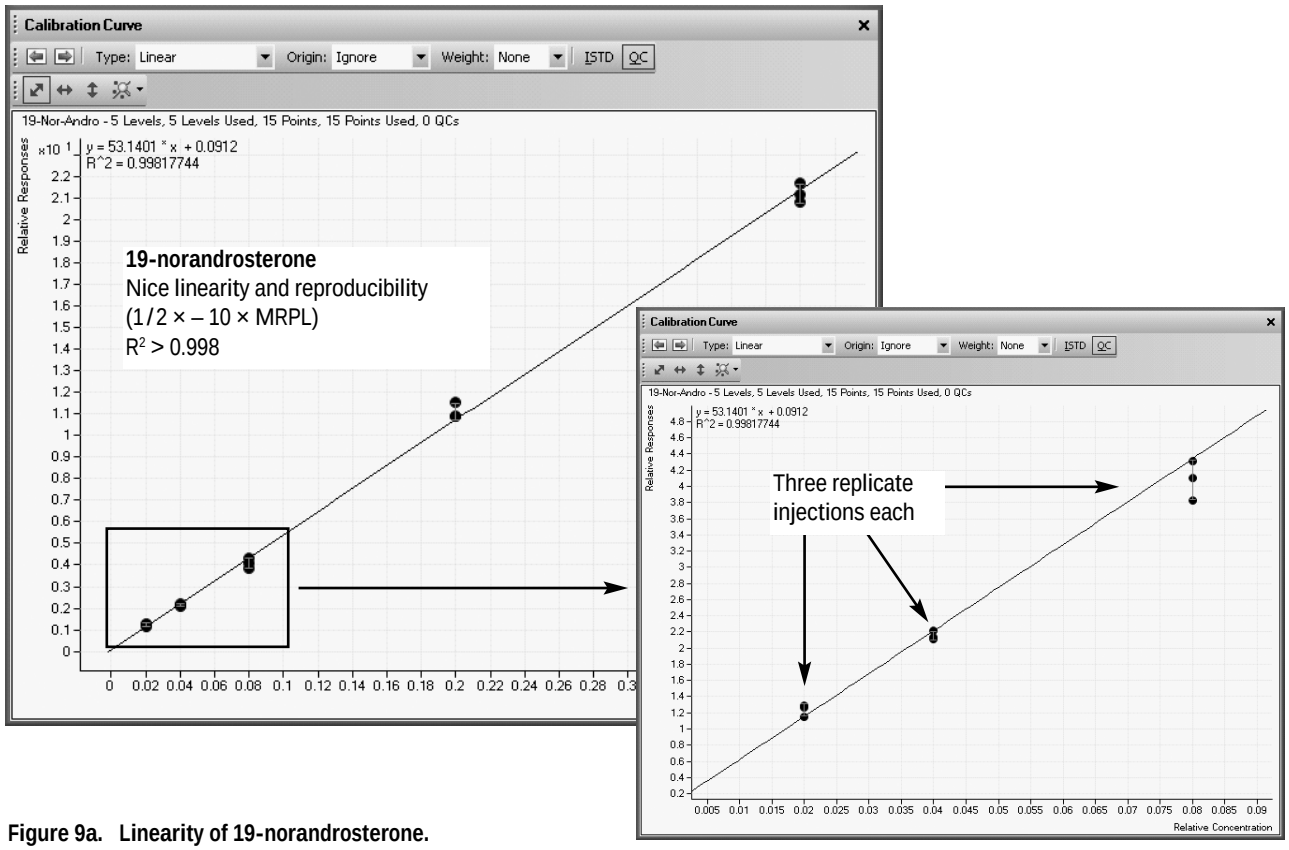

Figure 9a. Linearity of 19-norandrosterone.

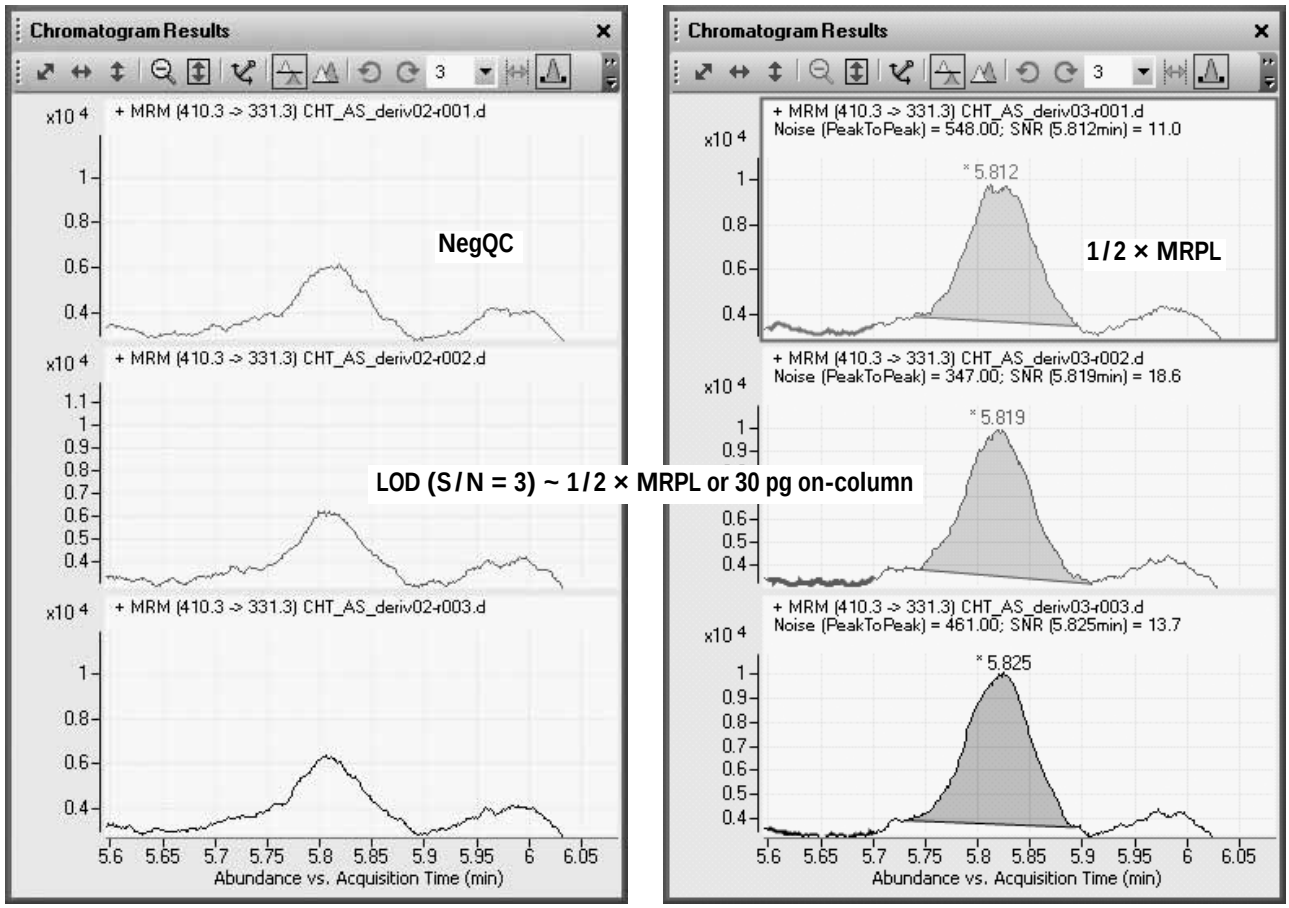

Figure 9b. Estimate of LOD for 19-norandrosterone.

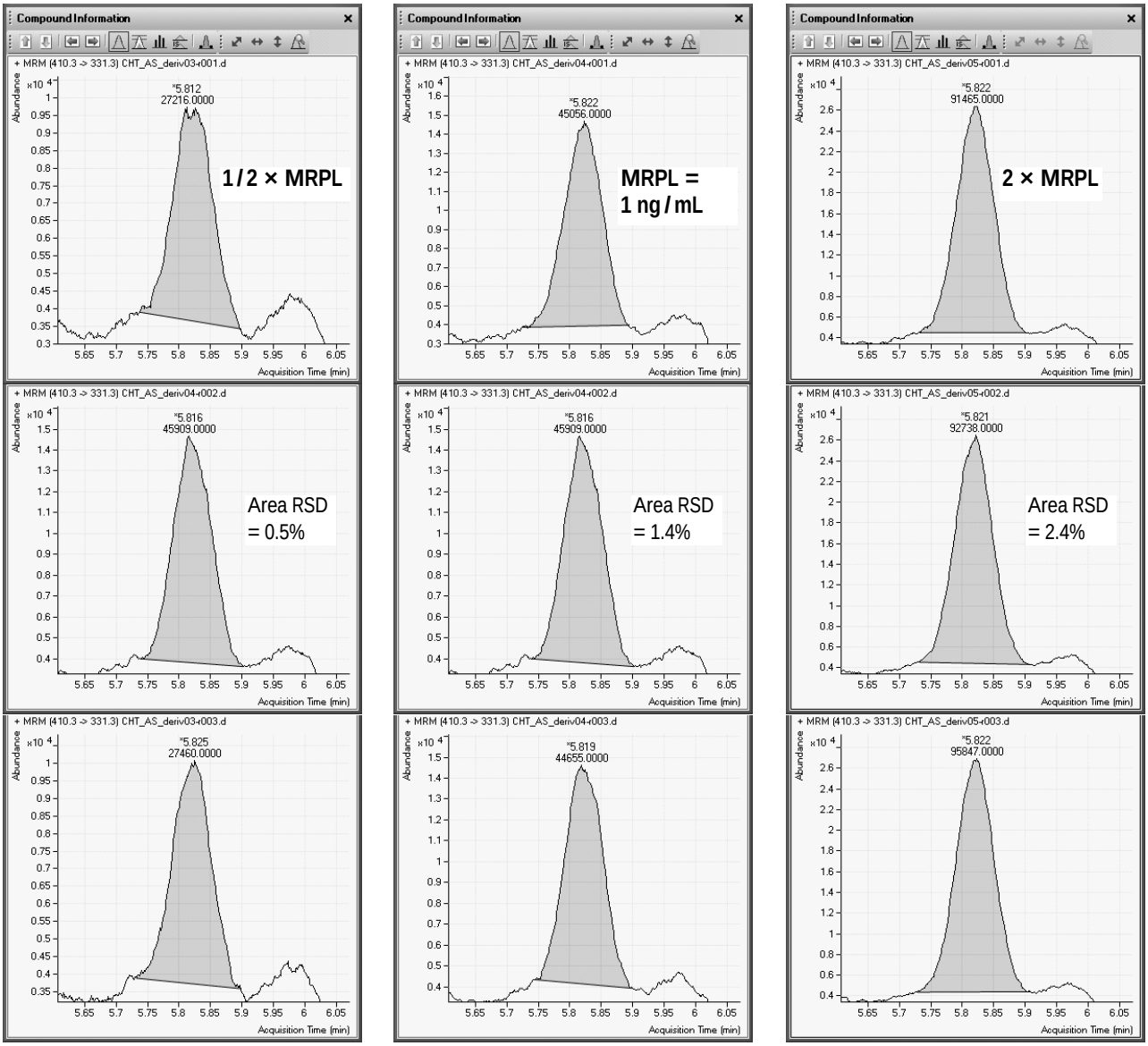

Figure 9c. Triplicate injections of the lowest three levels of 19-norandrosterone.

Table 3. Linearity, Reproducibility, and Calculated Sensitivity for All Compounds Analyzed

Compound	Linearity R^2	% RSD at 1 / 2 × MRPL	LOD on-column (pg)	LOD MRPL (×)
Clenbuterol	> 0.999	1.1	3	1 / 40
THG	> 0.992	1.1	30	1 / 20
MeTest metabolite	> 0.997	4.2	60	1 / 2
Epimetendiol	> 0.997	12.0	12	1 / 10
4β-OH-stanozolol	> 0.982	4.3	15	1 / 40
19-norandrosterone	> 0.998	0.5	30	1 / 2

Conclusions

The analysis of anabolic substances in urine can be difficult and may require the sensitivity of a triple quadrupole mass spectrometer as seen in this work. Linearity over the range of 1/2 × to 10 × MRPL for each compound is demonstrated and shown to be very good, especially for clenbuterol, which has a correlation coefficient of more than 0.999. The liquid chromatography in this work only uses solvents of water and methanol, with the addition of formic acid for a simple gradient. Limits of detection at levels lower than the minimum required performance levels are demonstrated with percent relative standard deviations of peak areas ranging from 12.0% to as low as 0.5%. The addition of Girard's Reagent P solution shows a marked improvement in sensitivity for the 19-norandrosterone compound.

References

1. H. Pereira, M. Marques, I. Talhas, and F. Neto, Analysis of Androgenic Steroids, Beta-2-Agonists and Other Substances by GC-MS-ITD. In W. Schanzer, H. Geyer, A. Gotzmann, and U. Mareck, *Recent Advances in Doping Analysis.* (2003) Sport und Buch Straub, Koln, 11, 259.

2. G. Trout, S. Soo, and R. Kazlauskas, Single Screen for Steroids Using HRMS. In Recent *Advances in Doping Analysis.* (2003) Sport und Buch Straub, Koln, 11, 249.
3. C. Borges, M. Slawson, J. Taccogno, D. Crouch, and J. Hughes, Screening and Confirmation of Anabolic Steroids Using Accurate Mass LC/MS. (2006) Agilent Technologies publication 5989-4738EN.
4. C. Borges, N. Miller, M. Shelby, M. Hansen, C. White, M. Slawson, K. Mont, and D. Crouch, Analysis of a Challenging World Anti-Doping Agency-Banned Steroids and Antiestrogens by LC-MS-MS, *Journal of Analytical Toxicology* (2007), 31, 125.

For More Information

For more information on our products and services, visit our Web site at www.agilent.com/chem.

For more details concerning this note, please contact Michael Zumwalt at Agilent Technologies, Inc.

Printed in the USA
April 19, 2007
5989-6506EN

Agilent Technologies