

1 **Potential of atmospheric pressure chemical ionization source in gas**
2 **chromatography tandem mass spectrometry for the screening of**
3 **urinary exogenous androgenic anabolic steroids**

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23 **Abstract**

24 The atmospheric pressure chemical ionization (APCI) source for gas
25 chromatography-mass spectrometry analysis has been evaluated for the screening
26 of 16 exogenous androgenic anabolic steroids (AAS) in urine. The sample
27 treatment is based on the strategy currently applied in doping control laboratories
28 i.e. enzymatic hydrolysis, liquid-liquid extraction (LLE) and derivatization to form
29 the trimethylsilyl ether-trimethylsilyl *enol ether* (TMS) derivatives. These TMS
30 derivatives are then analyzed by gas chromatography tandem mass spectrometry
31 using a triple quadrupole instrument (GC-QqQ MS/MS) under selected reaction
32 monitoring (SRM) mode. The APCI promotes soft ionization with very little
33 fragmentation resulting, in most cases, in abundant $[M+H]^+$ or $[M+H-2TMSOH]^+$
34 ions, which can be chosen as precursor ions for the SRM transitions, improving in
35 this way the selectivity and sensitivity of the method. Specificity of the transitions
36 is also of great relevance, as the presence of endogenous compounds can affect the
37 measurements when using the most abundant ions. The method has been
38 qualitatively validated by spiking six different urine samples at two concentration
39 levels each. Precision was generally satisfactory with RSD values below 25 and 15
40 % at the low and high concentration level, respectively. Most the limits of detection
41 (LOD) were below 0.5 ng mL^{-1} . Validation results were compared with the
42 commonly used method based on the electron ionization (EI) source. EI analysis
43 was found to be slightly more repeatable whereas lower LODs were found for
44 APCI. In addition, the applicability of the developed method has been tested in
45 samples collected after the administration of 4-chloromethandienone. The highest
46 sensitivity of the APCI method for this compound, allowed to increase the period in
47 which its administration can be detected.

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49 *Keywords:* Anabolic Androgenic Steroids (AAS), Atmospheric Pressure Chemical
50 Ionization (APCI), Gas Chromatography (GC), Tandem Mass Spectrometry
51 (MS/MS), Triple Quadrupole (QqQ), Doping Control Analysis

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55 1. Introduction

56 Since 2004, the World Anti-Doping Agency (WADA) publishes a list of prohibited
57 substances and methods in sport which is yearly updated [1]. Among the groups of
58 substances included in the list, androgenic anabolic steroids (AAS) are the most
59 frequently reported ones [2]. AAS are mainly used due to their anabolic effects
60 such as muscle and strength growth among others [3].

61 AAS are prohibited at all times i.e. in and out of competition. This prohibition
62 makes that any evidence of AAS misuse (e.g. the mere presence of traces of the
63 AAS and/or its metabolites) is sufficient for reporting an adverse analytical finding
64 [4]. The detection of AAS misuse is a constant analytical challenge due to their low
65 concentration in urine, the complexity of the matrix and the similarity between
66 endogenous and exogenous AAS. Thus, sensitivity and selectivity of analytical
67 methods are key factors and requirements for AAS detection have evolved hand in
68 hand with instrumental developments.

69 AAS have been traditionally determined by gas chromatography mass spectrometry
70 (GC-MS) methods working in selected ion monitoring mode (SIM) using electron
71 ionization (EI) sources [5]. After some preparation steps [6] i.e. hydrolysis with β -
72 glucuronidase, liquid-liquid extraction and conversion of both hydroxyl and
73 carbonyl function into the corresponding TMS *ether/enol-TMS* ethers, these
74 methods allowed the detection of most of AAS metabolites at concentrations below
75 10 ng mL^{-1} . For this reason, the minimum required performance level (MRPL) for
76 most AAS was set at 10 ng mL^{-1} . However, these methods failed for the detection
77 of several AAS at the required MRPL, mainly those with difficulties in the
78 derivatization step. Among them, stanozolol and AAS bearing a 4,9,11-triene
79 nucleus like tetrahydrogestrinone (THG) [7].

80 The occurrence of high resolution mass spectrometry opened new possibilities for
81 the detection of stanozolol [8], although the scenario drastically changed after the
82 introduction of liquid chromatography tandem mass spectrometry (LC-MS(/MS)) in
83 doping control laboratories [9,10]. Several methods have been developed for the
84 LC-MS/MS detection of AAS with poor derivatization properties like stanozolol
85 and THG [11, 12, 13]. Thus, both GC-MS(/MS) and LC-MS(/MS) have been
86 employed as complementary techniques in doping control laboratories in order to
87 reach the required MRPLs. Qualitative methods for the detection of exogenous
88 AAS in urine by LC-MS/MS with triple quadrupole (QqQ) analyzers and

89 electrospray ionization source (ESI) [11] have been reported, as well as GC-
90 MS/MS methods with EI [14, 15] or chemical ionization (CI) sources [16].

91 In the last years, the commercialization of triple quadrupole instruments coupled to
92 GC has allowed for increasing the sensitivity of the previous GC-MS methods.
93 Thus, several GC-EI-MS/MS methods in selected reaction monitoring mode (SRM)
94 have been published either for the detection of target analytes [14, 15] or for
95 metabolic studies [17, 18, 19]. Nowadays, this technique has become the gold-
96 standard in AAS analysis for doping control purposes. Due to the sensitivity
97 improvement, the MRPL for AAS has been recently reduced to 2-5 ng mL⁻¹ for
98 most analytes [4]. This fact illustrates the impact of new analytical technologies in
99 the detection of AAS. Therefore, it is valuable to test the performance of emerging
100 analytical tools in this field.

101 As an alternative to EI, different “soft” ionization sources for GC have been tested
102 for the detection of AAS in doping analysis, i.e. CI [16], heated nebulizer
103 microchip atmospheric pressure photoionization (μ APPI) [20, 21] or atmospheric
104 pressure chemical ionization (APCI) [22, 23]. The recently commercialized APCI
105 source for GC represents an attractive alternative in several application fields [24,
106 25, 26]. APCI promotes soft ionization for the generation of $[M+H]^+$ or M^+ ions as
107 the base peak of the spectrum, by means of protonation or charge transfer
108 mechanisms, deeper explained in literature [22, 27]. This soft ionization presents an
109 advantage in the selection of specific precursor ions in MS/MS based methods.

110 In the present work, the potential of APCI source using GC-MS/MS was evaluated
111 for the development of a screening method for the detection of selected exogenous
112 AAS in urine. After validation, the performance of the GC-APCI-MS/MS method
113 has been compared with the conventional GC-EI-MS/MS, by analyzing a group of
114 samples prepared under the same conditions. The applicability of the method was
115 also evaluated in a set of samples collected at different times after the
116 administration of 4-chloromethandienone (4Cl-MTD).

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2. Experimental

2.1. Chemical and reagents

The structures of the selected AAS are shown in Figure 1. Boldenone (BD) was obtained from Sigma (St. Louis, MO, USA). 17 β -hydroxy-5 β -androstan-1-ene-3-one (Boldenone metabolite, BDmet), 17 β -methyl-5 β -androst-1-en-3 α ,17 α -diol (Methandienone metabolite, MTDmet3), 1-testosterone (1-T), 5 α -androstan-17 α -methyl-3 α ,17 β -diol (Methyltestosterone metabolite, MeTmet1), 5 β -androstan-17 α -methyl-3 α ,17 β -diol (Methyltestosterone metabolite, MeTmet2), 5 β -androstan-7 β ,17 α -dimethyl-3 α ,17 β -diol (Calusterone metabolite, CALUSmet), 17 α -methyl-1-testosterone (Me-1-T), 5 β -androstan-7 α ,17 α -dimethyl-3 α ,17 β -diol (Bolasterone metabolite, BOLASmet), 13 β ,17 α -diethyl-5 β -gonane-3 α ,17 β -diol (Norbolethone metabolite, NORBOLmet2) 6 β -hydroxy-4-chloromethandienone (6OH-4Cl-MTD) and 4-hydroxy-testosterone (4OH-T) were purchased from NMI (Pymble, Australia). Fluoxymesterone (FLU) was obtained from Steraloids (Newport, RI, USA). 5 α -Androstan-2 α ,17 α -dimethyl-3 α ,17 β -diol (Methasterone metabolite, METHASmet) was a kind gift from the World Association of Anti-Doping Scientists (WAADS). Oxymesterone (OXY) and madol (MADOL) were provided by the Toronto Research Chemicals (Toronto, Canada).

AAS stock standard solutions at 10 and 100 $\mu\text{g mL}^{-1}$ in methanol were stored at -20 $^{\circ}\text{C}$. Working MIX solutions at appropriate concentration levels for validation were prepared in acetone and also stored at -20 $^{\circ}\text{C}$, whereas individual standard solutions were employed for the transition optimization step and for potential cross-talk evaluation.

β -glucuronidase solution (*Escherichia coli*, type K12) was purchased from Roche Diagnostics (Mannheim, Germany). Analytical grade potassium carbonate, potassium hydroxide pellets, sodium hydrogen phosphate, di-sodium hydrogen phosphate, *tert*-butyl-methyl ether and ammonium iodide were acquired from Merck (Darmstadt, Germany). The derivatization reagent preparation *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) was purchased from Karl Bucher Chemische Fabrik GmbH (Waldstetten, Germany) and 2-mercaptoethanol from Sigma-Aldrich (St Louis, MO, USA). Milli Q water was obtained using a Milli-Q purification system (Millipore Ibérica, Barcelona, Spain). Formic acid and

154 ammonium formate (LC/MS grade), acetonitrile and methanol (LC gradient grade)
155 were purchased from Merck (Darmstadt, Germany).

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158 **2.2. Instrumentation**

159 **2.2.1. GC-APCI-MS/MS**

160 An Agilent 7890A GC system (Palo Alto, CA, USA) equipped with an Agilent
161 7693 autosampler was coupled to a triple quadrupole mass spectrometer, Xevo TQ-
162 S (Waters Corporation, Manchester, UK), using an APGC source, operating in
163 APCI mode. The GC separation was performed using an HP Ultra 1 capillary
164 column, (length 16 m × I.D. 0.20 mm × film thickness 0.11 μm). For the named as
165 *gradient 1*, the oven was programmed as follows: 185 °C (0.5 min); 25 °C min⁻¹ to
166 230 °C; 10 °C min⁻¹ to 290 °C; 70 °C min⁻¹ to 310 °C (2.5 min), being the total run
167 time 11.6 min. *Gradient 2* programme was: 180 °C (1 min); 3 °C min⁻¹ to 230 °C;
168 40 °C min⁻¹ to 310 °C (3 min), total run time 22.7 min. Split injections (ratio 1:10)
169 of 2 μL using a straight deactivated liner with glass wool were carried out at 280
170 °C. Helium 99.999% (Carbueros Metálicos, Spain) was used as carrier gas at 2 mL
171 min⁻¹.

172 The interface temperature was set to 300 °C using N₂ as auxiliary gas at 250 L h⁻¹,
173 make up gas at 300 mL min⁻¹, and cone gas at 170 L h⁻¹. The temperature in the
174 source was set at 150 °C. The APCI corona pin was operated at 1.55 μA and a cone
175 voltage of 20 V was selected. The water used as modifier when working under
176 proton-transfer conditions was placed in an uncapped vial, which was located
177 within a holder placed in the source door. For MS/MS measurement, argon
178 99.995% (Carbueros Metálicos, Spain) was used as collision gas at a pressure of
179 4.15×10^{-3} mbar in the collision cell (Table 1).

180

181 **2.2.2. GC-EI-MS/MS**

182 For all EI experiments, a 7890A gas chromatograph equipped with a 7693
183 autosampler and coupled to a 7000A Series Triple Quadrupole GC/MS (Agilent
184 Technologies) was employed. The same column and chromatographic conditions
185 detailed in the APCI section (*gradient 1*) were used.

186 Nitrogen was used as collision gas at a flow rate of 1.5 mL min⁻¹, and helium
187 (Abello-Linde) as a quenching gas at a flow rate of 2.25 mL min⁻¹. The electron
188 impact source was kept at 230 °C and the quadrupoles at 150 °C.

189

190 **2.3. Sample preparation**

191 Urine samples were treated as previously described in literature [14, 15]. Briefly,
192 25 µL of internal standard solution (methyltestosterone, 10 µg mL⁻¹) was added to
193 2.5 mL of urine. Then, the solution was hydrolyzed by the addition of 1 mL
194 phosphate buffer (pH 7) and 30 µL of β-glucuronidase solution (55 °C, 1h). After
195 cooling at room temperature, 200 mg of NaHCO₃/Na₂CO₃ buffer (1:2; w/w) was
196 added (pH 9.5). A LLE step was carried out by adding 6 mL of methyl tert-butyl
197 ether (MTBE). After centrifugation (4350 rpm, 5 min.), the organic phase was
198 separated and evaporated to dryness (45 °C). Finally, in order to obtain the *enol-*
199 *trimethylsilyl* (TMS) derivatives of the analyte, 50 µL of a mixture of
200 MSTFA/NH₄I/2-mercaptoethanol (1000/2/6; v/w/v) was added to the dry extract
201 and then kept at 60 °C for 20 min.

202

203 **2.4. Validation**

204 Following the WADA criteria [4], the validation of the screening method was
205 designed in order to confirm the suitability of the method to detect half the MRPL
206 of the compound. The method validation was performed using spot urine samples
207 collected from six volunteers (three male and three female which did not take any
208 steroid). Two spiking levels were selected taking into account the current MRPL
209 for the compounds (Table 2). Low concentration levels (LCL) of 1 ng mL⁻¹ and 2
210 ng mL⁻¹, and high concentration levels (HCL) of 10 ng mL⁻¹ and 20 ng mL⁻¹ were
211 selected for AAS with MRPLs of 2 ng mL⁻¹ and 5 ng mL⁻¹, respectively. In this
212 sense, around 0.5xMRPL and 5xMRPL levels were assayed in both cases.

213 For the evaluation of the extraction recoveries of each analyte, six blank samples
214 were spiked at the high concentration level and extracted. The same samples were
215 extracted and spiked after the extraction. The extraction recovery was calculated by
216 comparing peak areas for each analyte in both cases.

217 Relative standard deviation (RSD) of the ratio between the peak areas of each
218 compound and the internal standard were calculated. Repeatability (expressed as
219 RSD) for each analyte was evaluated at the two concentration levels tested.

220 Based on WADA suggestions [4], the limit of detection (LOD) for each analyte was
221 estimated as the concentration that produced a peak signal of three times the
222 background noise in the chromatogram at the lowest fortification level.

223 Selectivity was tested by analyzing 10 different blank urines and monitoring the
224 absence of interferences with signal to noise (S/N) ratios above 3.

225

226 **2.5. Application to real samples**

227 To study the applicability of the validated method, samples from an excretion study
228 of 4-chloromethandienone (4Cl-MTD) were analyzed. A single dose of 20 mg of
229 4Cl-MTD (oral Turinabol) was administrated to a male volunteer (49 years, 85 kg)
230 and different urine samples at intervals of 0-4 h, 4-8 h, 8-12 h, 12-24 h, 24-36 h, 48-
231 56 h and 72-84 h were collected.

232 The study was conducted in accordance with the Declaration of Helsinki. Subject
233 signed an informed consent before participation. Treatment was well tolerated by
234 the subjects and no serious adverse events were observed.

235

236 **3. Results and discussion**

237

238 **3.1. Transition optimization**

239 Two transitions were optimized for each compound to improve the reliability of the
240 method. The first step was the acquisition of a full scan spectrum for each
241 individual TMS-derivative standard. Once the main precursor ions were selected,
242 $[M+H]^+$, $[M+H-TMSOH]^+$ or $[M+H-2TMSOH]^+$, depending on the structure of the
243 steroid [21], product ion spectra were obtained at different collision energies (10,
244 20, 30 and 40 eV) (Figure S1, supplementary information). Based on this
245 information, the largest number of possible optimized SRM transitions was
246 preselected. Then, ten blank urine extracts and ten extracts spiked at the LCL were
247 tested in order to choose the best transitions for each analyte in terms of sensitivity
248 and specificity. Transitions showing the maximum S/N and the minimum influence
249 of the background of the matrix interferences were selected.

250 For most analytes, the most sensitive transition was found to be specific enough
251 since matrix interferences were not observed. Therefore, it was selected for
252 detection of the compound in the screening. However, in some cases such as OXY,
253 (Figure S2, supplementary information), the selected transition was not the most

254 abundant (535.2>269.2), because of the presence of matrix interferences. Thus, a
255 less sensitive but more specific transition was selected (535.2>389.5). In the case of
256 NORBOLmet2 and BD in APCI, it was not possible to select any specific transition
257 because of the presence of endogenous steroids with the same transitions at the
258 same retention times under the selected conditions.

259 A list of the selected SRM transitions used in APCI, facing EI ones, is summarized
260 in Table 1.

261

262 **3.2. Method validation**

263 Table 2 summarizes the results obtained for extraction recovery, repeatability and
264 LOD by using GC-APCI-MS/MS. Suitable extraction recovery values between 67
265 and 89% were obtained in all cases except for Me-1-T (47%) and BDmet (53%).

266 Repeatability was evaluated by the RSD at both LCL and HCL (n=6 for each level).
267 Values between 3% and 30% were obtained confirming the satisfactory precision of
268 the method. As expected, better repeatability was observed at the HCL, being in
269 most cases below 15%, except for 6OH-4Cl-MTD (RSD 22%).

270 In terms of selectivity, no interferences were detected in the ten blank samples for
271 the transitions selected for each compound. Regarding LOD, most of them were
272 lower than 0.5 ng mL⁻¹ and always below the established MRPL (Table 2). As
273 stated in the previous section the main exceptions for this behaviour were
274 NORBOLmet2 and BD, which were interfered by the presence of matrix
275 components irrespective of the selected transition. Chromatographic separation was
276 found to be critical for the proper validation of these compounds. The use of a
277 longer gradient (gradient 2 in the experimental section) allowed for the
278 discrimination between analytes and the matrix interferences (Figure S3,
279 supplementary information). Using this gradient all analytes were adequately
280 validated. In order to isolate as much as possible the effect of the interface, results
281 using gradient 1 will be discussed. Only in the case of NORBOLmet2 and BD
282 results for gradient 2 are discussed.

283 It is well-known that, differently to EI, atmospheric pressure ionization is more
284 affected by matrix constituents that lead to possible matrix-induced
285 suppression/enhancement of the analytes ionization. Since the main goal of the
286 developed method was not quantification of the analytes but the
287 detection/identification of all selected AAS at the LCL,, this effect was not

288 evaluated as that qualitative objective was satisfactorily reached independently on
289 the matrix effects that might affect to ionization. However, matrix effect may be
290 behind the higher RSD observed in APCI and it should be evaluated if the purpose
291 of the analyses was quantification of analytes.

292 Figure 2 shows typical chromatograms obtained for a blank urine sample compared
293 with those of a sample spiked at the LCL.

294

295 **3.3. Comparison APCI vs EI**

296 In order to evaluate the performance of the developed GC-APCI-MS/MS method,
297 the validation results were compared with those obtained by GC-EI-MS/MS. All
298 the factors involving the detection (urine used, extraction, derivatization, column
299 and gradient of temperatures) were controlled in order to isolate as much as
300 possible the effect of the ionization source. Ideally, both sources should be coupled
301 to the same analyzer. Unfortunately, this ideal situation is currently not affordable,
302 i.e. both interfaces are not interchangeable, and, therefore, every source was
303 coupled to a different QqQ analyzer. Thus, although the discussion will be focused
304 on the effect of the interface, a potential influence of the specific analyzer on the
305 results cannot be discarded.

306 Validation results for both methodologies are shown in Table 2. Regarding
307 repeatability, in general, RSD values were lower for EI. At LCL, RSDs ranged
308 from 0.6% to 14% in EI whereas in APCI increased up to 3.2-18%. At HCL, RSDs
309 in the range 0.3-5% and 3-14% were obtained for EI and APCI, respectively. Some
310 values higher than 20 were punctually obtained. Thus, although RSDs from both
311 studies can be considered acceptable, a slightly better repeatability of the EI source
312 was found in this study. The lower repeatability of APCI might be due either to
313 factors affecting the ionization process such as the amount of water in the interface
314 or to the potential matrix effect suffered by APCI.

315 Regarding sensitivity, the results largely depended on the MS behaviour of the
316 steroid. Thus, analytes with an abundant $[M+H]^+$ in APCI (BDmet, 1-T, Me-1-T,
317 4OH-T, FLU, 6OH-4Cl-MTD, BD and OXY) exhibited LODs in the sub-ng/mL
318 range (below 0.4 ng/mL) i.e. more than 10 times lower than the current MRPL for
319 most AAS. For these AAS, LODs estimated for APCI were between 5 and 20 times
320 lower than for EI (Figure 3a), even in those cases in which an abundant M^+ was
321 also present in EI.

322 Worse LODs (typically between 0.3 and 1 ng/mL) were obtained for AAS showing
323 an abundant $[M+H-nTMSOH]^+$ in APCI (MADOL, MTDmet3, MeTmet1,
324 CALUSmet, MeTmet2, METHASmet, NORBOLmet2 and BOLASmet). For these
325 compounds, LODs using APCI were commonly in the same range as those obtained
326 by EI (Table 2, Figure 3b). The low specificity of the product ions can be behind
327 this fact. After the in-source neutral loss of all TMS present in the molecule (lost as
328 TMSOH), the remained hydrocarbon skeleton was selected as precursor ion. Under
329 these conditions the selection of a specific product ion was troublesome. Thus, most
330 of the product ions obtained was not specific and both matrix interferences and high
331 background decreased the sensitivity of the method. Only for BOLASmet, a
332 specific product ion (m/z 175) could be obtained. In this case, the sensitivity was
333 similar to those AAS exhibiting a $[M+H]^+$.
334 The selection of abundant and specific precursor ions was a key factor when aiming
335 at the maximum sensitivity. In the light of obtained results, it is noteworthy to
336 mention that the presence of $[M+H]^+$ in APCI led to the best results in terms of
337 sensitivity. Future work in the search of diverse derivatizing agents that maximize
338 the protonated molecule in APCI would be valuable.

339

340 **3.4. Application to real samples**

341 To check the applicability of the developed methodology, samples collected after
342 4Cl-MTD administration were analyzed by GC-APCI-MS/MS and the results were
343 compared with those obtained by GC-EI-MS/MS.

344 As expected, the main metabolite of 4-chloromethandienone (6OH-4Cl-MTD) was
345 detected by both methods in the urines collected during the first hours after
346 administration (Figure 4a). Owing the better sensitivity provided by APCI, the
347 misuse of 4Cl-MTD could be detected in samples in which the metabolite was
348 undetectable by the commonly used GC-EI-MS/MS methods (Figure 4b).
349 Therefore, the period of time in which the misuse can be detectable increased from
350 56 h to 84 h (the last sample collected) by using APCI. This fact illustrates the
351 potential and future of this source in the detection of AAS misuse.

352

353 **4. Conclusions**

354 The suitability of GC-APCI-MS/MS for sensitive detection of AAS has been
355 demonstrated by the validation of a method for the detection of 16 exogenous AAS
356 in urine.

357 The present work illustrates the potential of the new APCI source as an adequate
358 alternative to the traditional EI source in GC-MS methodologies. Due to the
359 endogenous steroids present in urine, the selection of a specific transition has been
360 found to be a key factor in the method development. Optimization of the
361 chromatography was found to be critical for the correct detection of two of the
362 analytes (BD and NORBOLmet2). Although suitable precision (RSD values below
363 25 and 15% at LCL and HCL, respectively) was obtained with the APCI method, it
364 was slightly higher than the one obtained with EI.

365 Sensitivity was found to be higher with APCI for the majority of compounds tested,
366 with LODs commonly lower than 0.5 ng mL^{-1} . These LODs are similar to the
367 obtained with other soft ionization sources like CI [16]. The higher sensitivity
368 obtained can be related with the abundance of a specific product ion. This, in
369 around 50% of the analytes, the soft ionization of provided by the APCI source
370 allowed for the selection of the $[M+H]^+$ as precursor ion. In the rest of analytes,
371 ions resulting from one or two losses of TMSOH from the derivatizing reagent
372 were selected as precursor. These ions keep still the steroidal skeleton helping in
373 the selection of specific product ions.

374 Anyway, the presence of an abundant $[M+H]^+$ in the mass spectra and its selection
375 as precursor ion was found to be related with a higher sensitivity. Since the selected
376 derivative (TMS) does not favor the protonation, the use of derivatives with higher
377 proton affinity would theoretically improve the sensitivity of the method. Further
378 research in order to investigate the applicability of other derivatizing agents able to
379 generate specific fragments would be desirable.

380 The notable improvement in sensitivity provided by the use of APCI source in GC-
381 MS/MS methods is of great relevance in doping control field, as revealed in the
382 application of the method for the detection of 4-chloromethandienone misuse.
383 Therefore, the use of GC-(APCI) MS/MS based methods could increase the period
384 of time in which the misuse of the AAS can be detected, and opens interesting
385 possibilities in the near future.

386

387

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498 **FIGURE CAPTIONS**

499 **Figure 1.-** Structures of the selected analytes.

500 **Figure 2.-** APCI optimized transitions of selected AAS in (a) blank urine sample
501 and (b) urine sample spiked at LCL.

502 **Figure 3.-** Comparison between APCI and EI for selected compounds: (a) BDmet
503 and (b) MADOL.

504 **Figure 4.-** Comparison between APCI and EI in urine samples collected after
505 administration of 4CI-MTD. Chromatograms of samples collected between (a) 24-
506 36 h after administration and (b) 72-84 h after administration.

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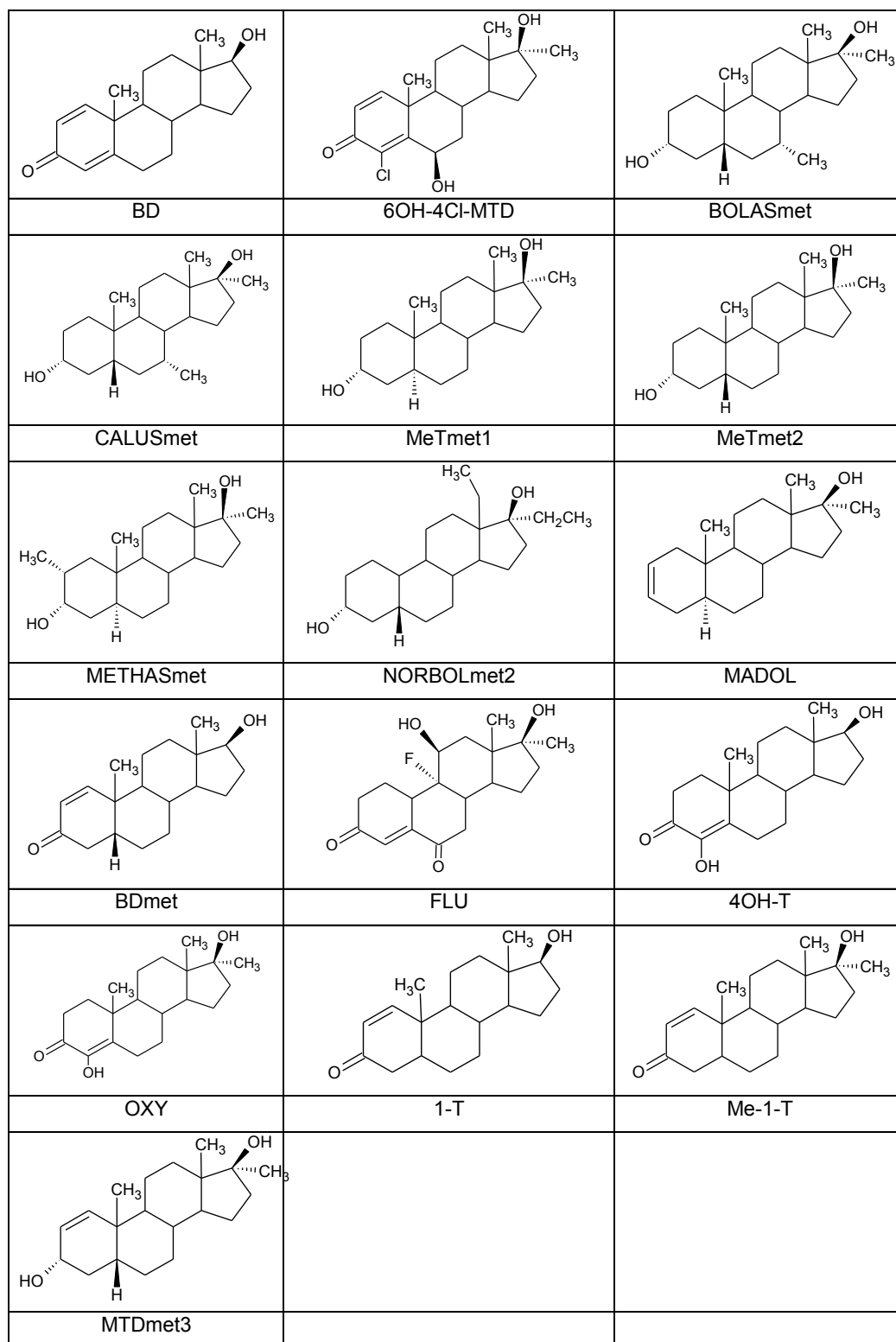
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526 **Figure 1.**

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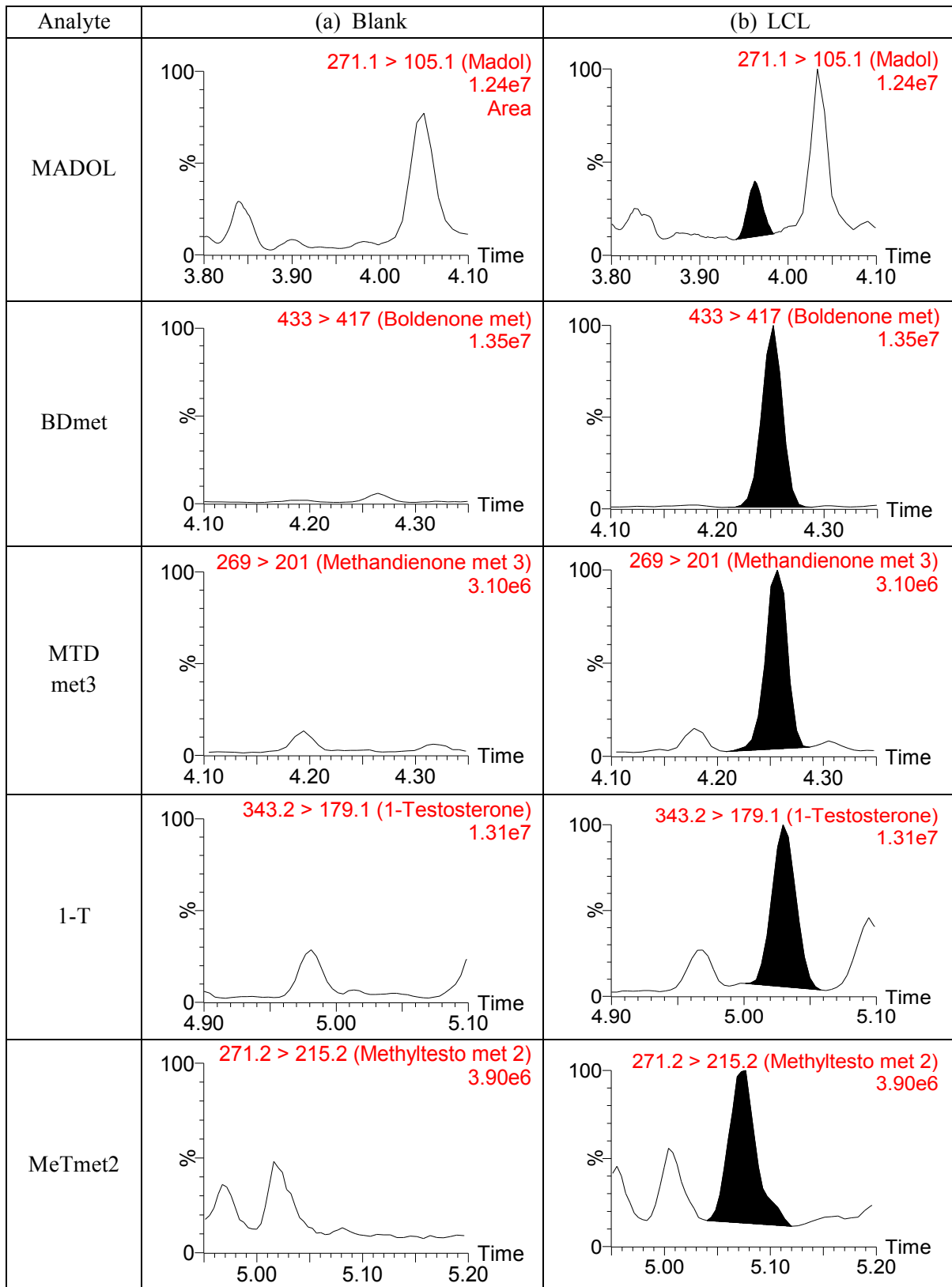


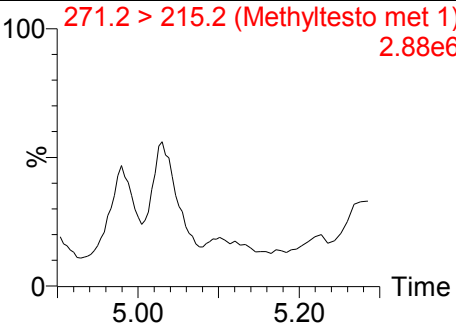
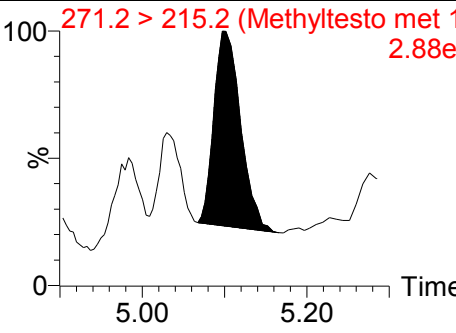
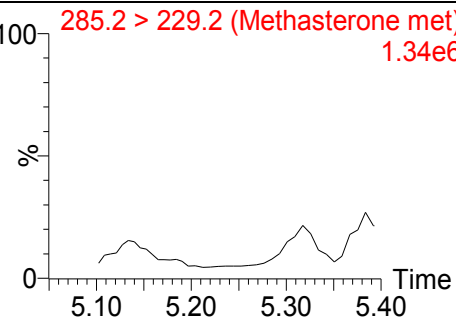
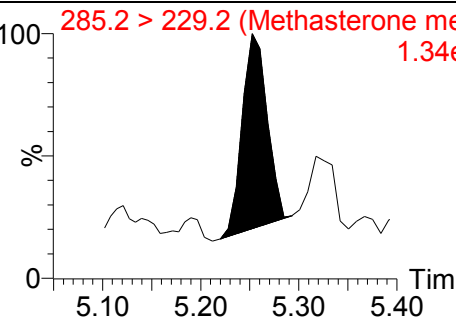
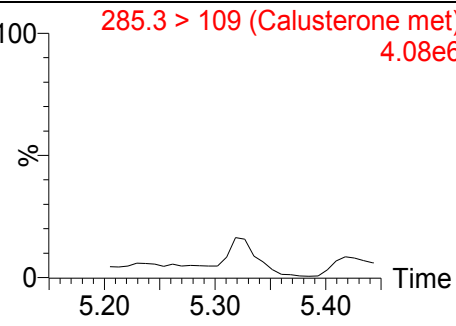
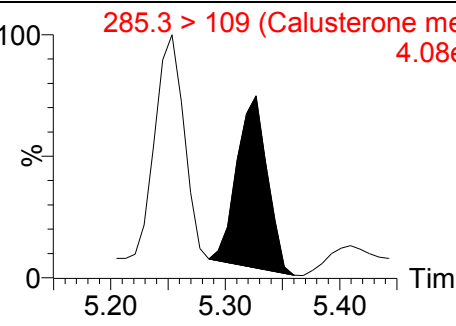
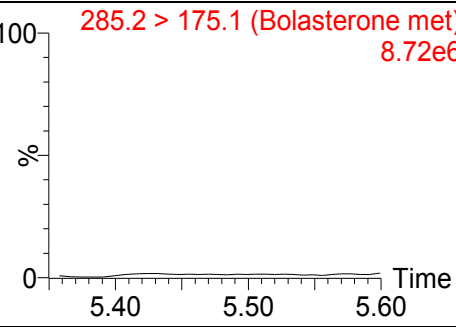
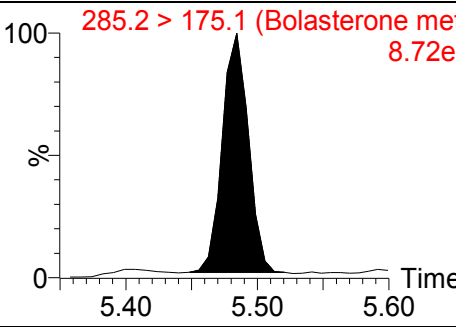
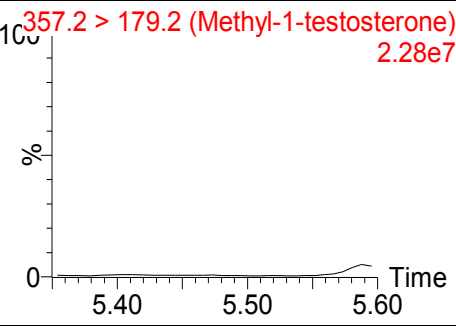
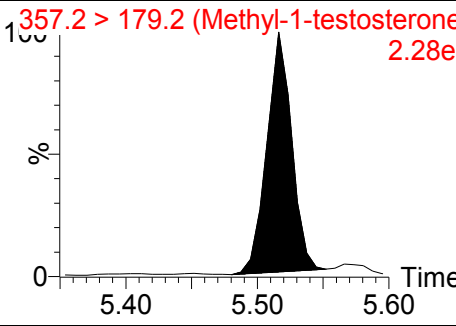
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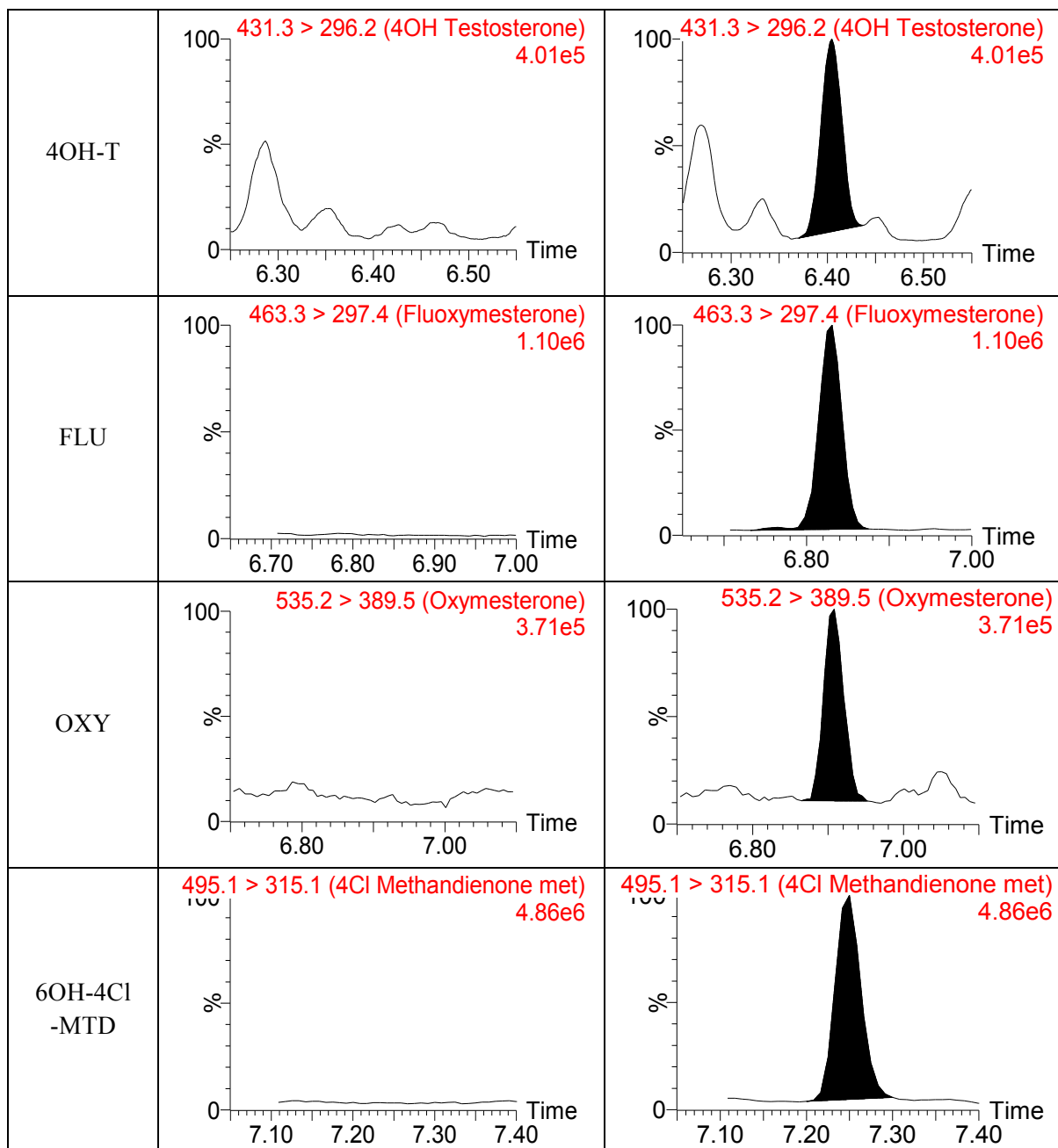
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531 **Figure 2.**



MeTmet1	<p>271.2 > 215.2 (Methyltesto met 1) 2.88e6</p> 	<p>271.2 > 215.2 (Methyltesto met 1) 2.88e6</p> 
METHAS met	<p>285.2 > 229.2 (Methasterone met) 1.34e6</p> 	<p>285.2 > 229.2 (Methasterone met) 1.34e6</p> 
CALUS met	<p>285.3 > 109 (Calusterone met) 4.08e6</p> 	<p>285.3 > 109 (Calusterone met) 4.08e6</p> 
BOLAS met	<p>285.2 > 175.1 (Bolasterone met) 8.72e6</p> 	<p>285.2 > 175.1 (Bolasterone met) 8.72e6</p> 
Me-1-T	<p>357.2 > 179.2 (Methyl-1-testosterone) 2.28e7</p> 	<p>357.2 > 179.2 (Methyl-1-testosterone) 2.28e7</p> 

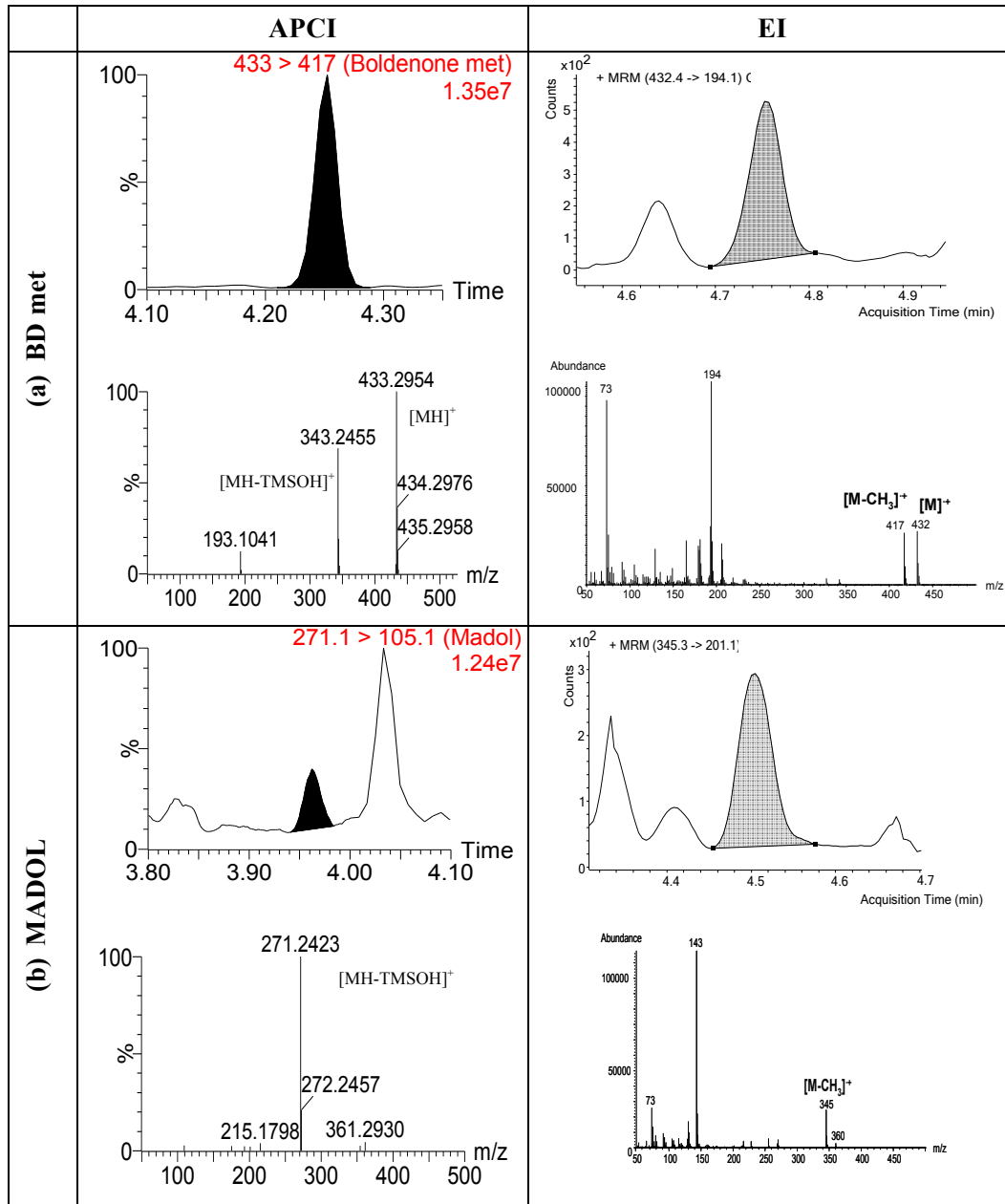


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535 **Figure 3.**



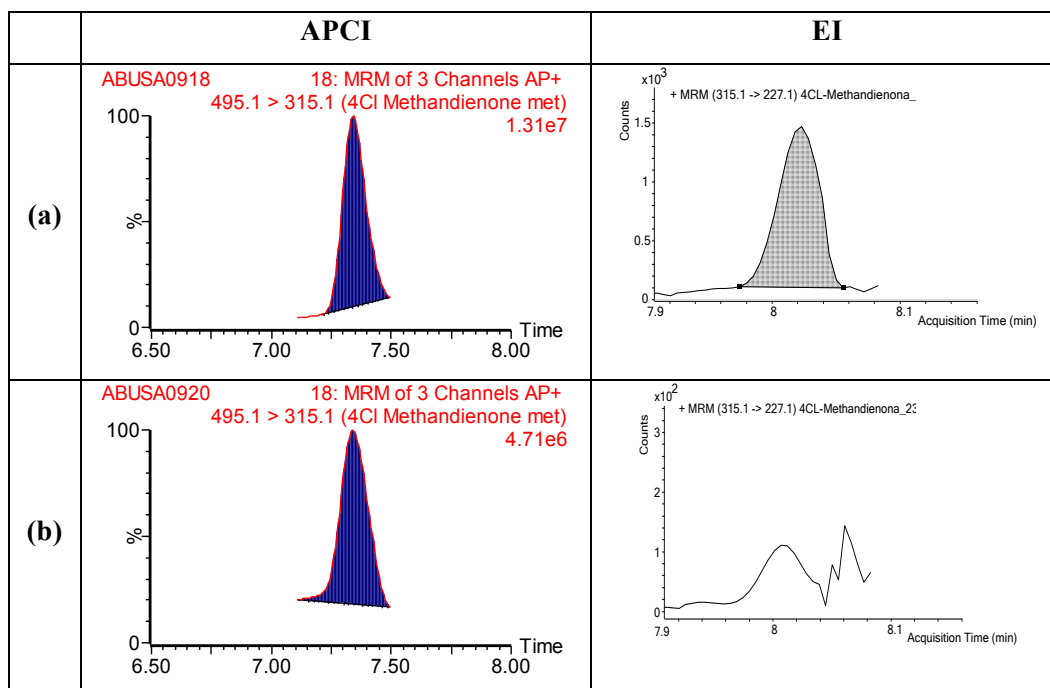
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540 **Figure 4.**



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544 TABLES

545 **Table 1.-** Selected acquisition conditions for the SRM method for both GC-APCI-
546 MS/MS and GC-EI-MS/MS.

Analyte	Derivative	Mw [M+H] ⁺	APCI			EI		
			RT (min)	Transition	CE (eV)	RT (min)	Transition	CE (eV)
MADOL	mono-O-TMS	361	3.96	271.1>105.1*	30	4.49	345.3>201.1	15
				271.1>90.9	30		345.3>255.1	15
BDmet	bis-O-TMS	433	4.27	433>417*	10	4.73	432.4>194.1	14
				433>187	20		432.4>206.1	14
MTDmet3	bis-O-TMS	449	4.27	269>201*	10	4.74	358.3>301.3	12
				269>105	30		358.3>196.1	12
1-T	bis-O-TMS	433	5.03	343.2>179.1*	20	5.61	432.4>194.1	15
				433.3>417	20		432.4>207.2	15
MeTmet2	bis-O-TMS	451	5.07	271.2>215.2*	10	5.64	270.2>213.2	15
				361.2>271.1	10		270.2>199.1	15
MeTmet1	bis-O-TMS	451	5.08	271.2>215.2*	20	5.64	255.2>199.1	25
				361.3>255.2	20		255.2>159.1	25
METHASmet	bis-O-TMS	465	5.25	285.2>229.2*	20	5.84	449.4>269.2	19
				375.3>245.2	20		449.4>213.2	19
BD	bis-O-TMS	431	5.29	341.2>193.1*	20	5.86	430.4>206.2	18
				431.3>193.1	20		430.4>191.2	30
CALUSmet	bis-O-TMS	465	5.32	285.3>109*	20	5.91	284.2>227.2	15
				285.3>175	20		374.3>269.2	13
BOLASmet	bis-O-TMS	465	5.49	285.2>175.1*	20	6.08	284.2>227.2	15
				375.2>245.2	20		284.2>269.2	15
Me-1-T	bis-O-TMS	447	5.52	357.2>179.2*	20	6.14	446.4>194.1	20
				447.3>431.3	20		446.4>143.1	20
NORBOLmet2	bis-O-TMS	465	5.97	375.3>285.2*	10	6.61	435.4>255.2	12
				375.3>231.2	20		435.4>345.3	12
4OH-T	tris-O-TMS	521	6.40	431.3>296.2*	30	7.08	520.4>147.1	33
				521.3>405.3	40		505.4>147.1	10
FLU	tris-O-TMS	553	6.82	463.3>297.4*	30	7.52	552.4>462.4	20
				553.3>353.4	20		552.4>319.3	20
OXY	tris-O-TMS	535	6.91	535.2>389.5*	20	7.62	534.4>429.4	30
				535.2>269.2	30		389.3286.2	30
6OH-4Cl-MTD	bis-O-TMS	495	7.25	495.1>315.1*	10	7.99	315.1>227.1	20
				495.1>155	40		315.1>241.1	15

547 * Most specific transition

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549 **Table 2.-** Validation parameters obtained for extraction recovery (n=6),
 550 repeatability and LOD for APCI and EI analysis.

Analyte	Current MRPL (ng mL ⁻¹)	Extraction recovery (%)	LCL (ng mL ⁻¹)	Repeatability (%)		HCL (ng mL ⁻¹)	Repeatability (%)		LOD (ng mL ⁻¹)	
				APCI	EI		APCI	EI	APCI	EI
MADOL	5	75	2	10	1	20	6	0.3	1	1
BDmet	5	53	2	30	14	20	4.8	5.3	0.1	0.5
MTDmet3	2	70	1	5.3	1.2	10	4.6	0.5	0.3	0.3
1-T	5	79	2	4.2	2.5	20	14	0.5	0.3	2
MeTmet2	2	85	1	4.8	2.3	10	3.9	0.4	0.3	0.5
MeTmet1	2	83	1	12	2.7	10	6.3	1	0.5	0.5
METHASmet	5	78	2	24	8.5	20	9.1	2.3	1	0.3
BD*	5	89	2	3.8	2.5	20	5.9	2	0.1	1
CALUSmet	5	76	2	18	2.3	20	3.5	2	1	2
BOLASmet	5	80	2	23	5.2	20	6.8	1.6	0.1	1
Me-1-T	5	47	2	9.7	5.4	20	4.9	5.1	0.3	1
NORBOLmet2*	5	71	2	13	0.6	20	7.7	1.3	0.4	2
4OH-T	5	68	2	9.4	2.4	20	4.6	1.1	0.4	2
FLU	5	73	2	25	21	20	4	2.3	0.1	1
OXY	5	71	2	13	27	20	3	1.4	0.5	1
6OH-4CI-MTD	2	86	1	3.2	4.2	10	22	0.3	0.2	1

551 * APCI values for BD and NORBOLmet2 were calculated using gradient 2 (see experimental section)

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