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# Efficient Approach for the Comprehensive Detection of Unknown Anabolic Steroids and Metabolites in Human Urine by Liquid Chromatography−Electrospray-Tandem Mass Spectrometry

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**The detection of new anabolic steroid metabolites and new designer steroids in urine is a challenge in doping analysis. An approach based on precursor ion scanning for the detection of unknown anabolic steroids and metabolites is proposed. The study of the MS/MS spectra of selected anabolic steroids revealed different fragmentation pathways at low and medium collision energy depending on the steroid structure. However, after analysis at high collision energy three common ions at *m/z* 105, *m/z* 91, and *m/z* 77 were found for all studied anabolic steroids. These ions can be explained by the fragmentation of the steroid structure and corresponded to the methyl tropylium, tropylium, and phenyl ions, respectively. Because of the theoretical low specificity of these ions, the simultaneous presence of all of them was used as a starting point to consider a substance as a possible anabolic steroid. Hence, the developed approach is based on the simultaneous acquisition of the precursor ion scan of *m/z* 105, 91, and 77. The specificity of this approach has been checked by the injection of several doping agents including *β*-agonists, corticosteroids, *β*-blockers, and diuretics. In general, only compounds with a steroidal structure showed a signal at all three selected *m/z* values although some exceptions have been found. The applicability of the method was tested for three different scenarios: the detection of steroid metabolites, the detection of unknown steroids, and the analysis of prohormones. In metabolic studies, several recently reported fluoxymesterone metabolites were also found using this method. For detection of unknown steroids, some negative urine samples were spiked with the designer steroid THG and 33 other anabolic steroids and treated as blind samples. Finally, the applicability of the developed approach for the analysis of dietary supplements was checked by the analysis of a prohormone where several impurities and/or degradation products were found.**

Anabolic steroids are one of the most frequently detected compounds in doping analysis. They are normally detected by mass spectrometry coupled either to gas chromatography (GC/MS)[1−3] or to liquid chromatography (LC−MS).[4−8] Most of these methods are based on the target detection of a limited number of known compounds, and therefore they are unable to detect steroids other than the target ones. However, recently the detection of anabolic steroids has become more complex since some nontherapeutically available hormones have been detected either in doping control urine samples (such as norbolethone in 2002[9]) or in formulations (such as THG in 2003[10] and madol in 2005[11]).

Therefore, comprehensive screening methods, which can detect these anabolic steroids without any restriction, are needed to detect new unknown designer steroids. A small number of approaches have been described for this purpose. One of the most attractive is the use of different scan modes available on LC−MS/MS with triple quadrupole-like instruments by operating in precursor ion or neutral loss scanning. The latter has been successfully applied in the detection of corticosteroids[12] in urine.

In the case of anabolic steroids, it is difficult to find a common product ion or neutral loss due to differences in fragmentation of these compounds at medium collision energy which are related

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to their structure.[13] Thevis et al. developed a method based on precursor ion scan by selecting several ions typically appearing for the steroids having a common structure.[14] Although this approach is useful for some steroids, others can remain undetectable if they do not have the preselected structure. Another elegant approach was developed by Nielen et al. using androgen bioassay detection prior to the analysis by quadrupole time-of-flight mass spectrometry (LC−QTOF) identification.[15] This approach allows for the analysis by LC−QTOF of the fraction with androgenic activity. However, the selection of the well with androgen bioactivity in spiked urines is not always easy, and several irrelevant peaks originating from the well plate or endogenous androgens make the detection of the unknown peak difficult.

Another application field where a comprehensive screening method for anabolic steroids can be useful in doping analysis is the study of metabolism. Most of the steroids are metabolized, and therefore the abuse of these substances is normally monitored by the detection of some of their specific metabolites. In doping analysis, GC/MS used to be the selected technique to elucidate steroid metabolism.[2] In this case, the detection and identification of steroid metabolites can be done by extracting common ions such as $m/z$ 143/130 or 231/218.[16]

Research revealed that some steroids and metabolites can be more easily detected by LC−MS/MS.[10] Moreover, some new metabolites can be detected by LC−MS/MS for a longer period than those commonly screened for by GC/MS as stated by Schänzer et al. in the study of metandienone metabolism.[17] Unfortunately, there are still only a small number of papers dealing with metabolic studies by LC−MS/MS in doping analysis. Recently, the use of neutral loss of HF in LC−MS/MS has been used as a target loss in the metabolic study of fluoxymesterone.[18] However this method is only applicable to those steroids containing fluorine atoms, and therefore, a more comprehensive screening method for steroid metabolites is needed in order to increase the applicability of this technique.

In the last years, several new steroids have been commercialized as nutritional supplements. These steroids are commonly referred to as "prohormones" and are available as over-the-counter preparations.[19,20] Several studies indicated that numerous nutritional supplements contain prohormones not declared on the label.[20−22] In order to determine these steroids, target analytical methods based on LC−MS/MS,[23] GC/MS,[24−26] or LC−UV[24] are usually employed. However, other steroids which differ from the targeted can be overseen by these methods, and therefore a general nontarget screening method for steroids can be useful in this field.

The aim of this study was to develop a comprehensive method for the detection of unknown anabolic steroids. The applicability of this approach for metabolic studies was verified by the analysis of urine samples in which the anabolic steroid fluoxymesterone was detected. Additionally, in order to check its applicability for the general detection of steroids, several urine samples were spiked with exogenous steroids at different concentration levels and treated as blind samples. Finally, the applicability in the analysis of dietary supplements was also checked by analyzing a Promagnon-25 prohormone capsule.

## EXPERIMENTAL SECTION

**Chemicals and Reagents.** Structures of the selected analytes are shown in Figure 1. 4$\beta$-hydroxy-stanozolol (4STAN), tetrahydrogestrinone (THG), 4-chloro-17α-methyl-androsta-1,4-diene-6$\beta$,-17$\beta$-diol-3-one (6CLOS), 6$\beta$-hydroxy-metandienone (6DIAN), 5$\beta$-androst-1-ene-17$\beta$-ol-3-one (BOLDm), 19-noretiocholanolone (19NET), 17$\beta$-methyl-5$\beta$-androst-1-ene-3α,17α-diol (EPI), 9-fluoro-17α-methyl-androst-4-ene-3α,6$\beta$,11$\beta$,17$\beta$-tetrol (FLUm1), and 9-fluoro-18-nor-17,17-dimethyl-4,13-diene-11-ol-3-one (FLUm2) were purchased from NMI (Pymble, Australia). 6$\beta$-hydroxy-androst-4-ene-3,17-dione (6-AND), mibolerone (MIB), norclostebol (NCLOS), and 4-hydroxy-testosterone (4T) were purchased from Steraloids (Newport, RI). Gestrinone (GES), boldenone (BOLD), and oxymesterone (OXY) were a kind gift by the Institut für Biochemie (DSHS, Cologne, Germany). 17α-trenbolone (17TREN) and fluoxymesterone (FLU) were purchased from RIVM (The Netherlands) and Pfizer (Puurs, Belgium), respectively. Oxandrolone (OXA) was a kind gift from Searle & Co (Chicago, IL). Testosterone (T), epitestosterone (E-T), and etiocholanolone (ETIO) were purchased from Sigma (St. Louis, MO).

Clenbuterol (CLEN) was obtained from RIVM (The Netherlands) and methylprednisolone (Me-PRED) from Pharmacia (Diegem, Belgium). Atenolol (ATEN) and propanolol (PROP) were purchased from ICI (Kortenberg, Belgium). Bisoprolol (BISO), clopamide (CLOP), and canrenone (CANRE) were purchased from Merck (Overijse, Belgium), Sandoz (Basel, Switzerland), and Sintesa (Brussels, Belgium), respectively. Formoterol (FORM) was a kind gift from Boehringer-Inglheim (Ingelheim, Germany).

The prohormone Promagnon-25 was acquired from Peak Performance Laboratories (New York) via the Internet.

The $\beta$-glucuronidase preparation (type *E. Coli* K12) was purchased from Roche (Mannheim, Germany). Analytical grade potassium carbonate, sodium hydrogen carbonate, and diethyl ether were obtained from Merck (Darmstadt, Germany).

HPLC grade methanol and HPLC grade water were purchased from Acros (Geel, Belgium) and Fischer Scientific (Loughbor-



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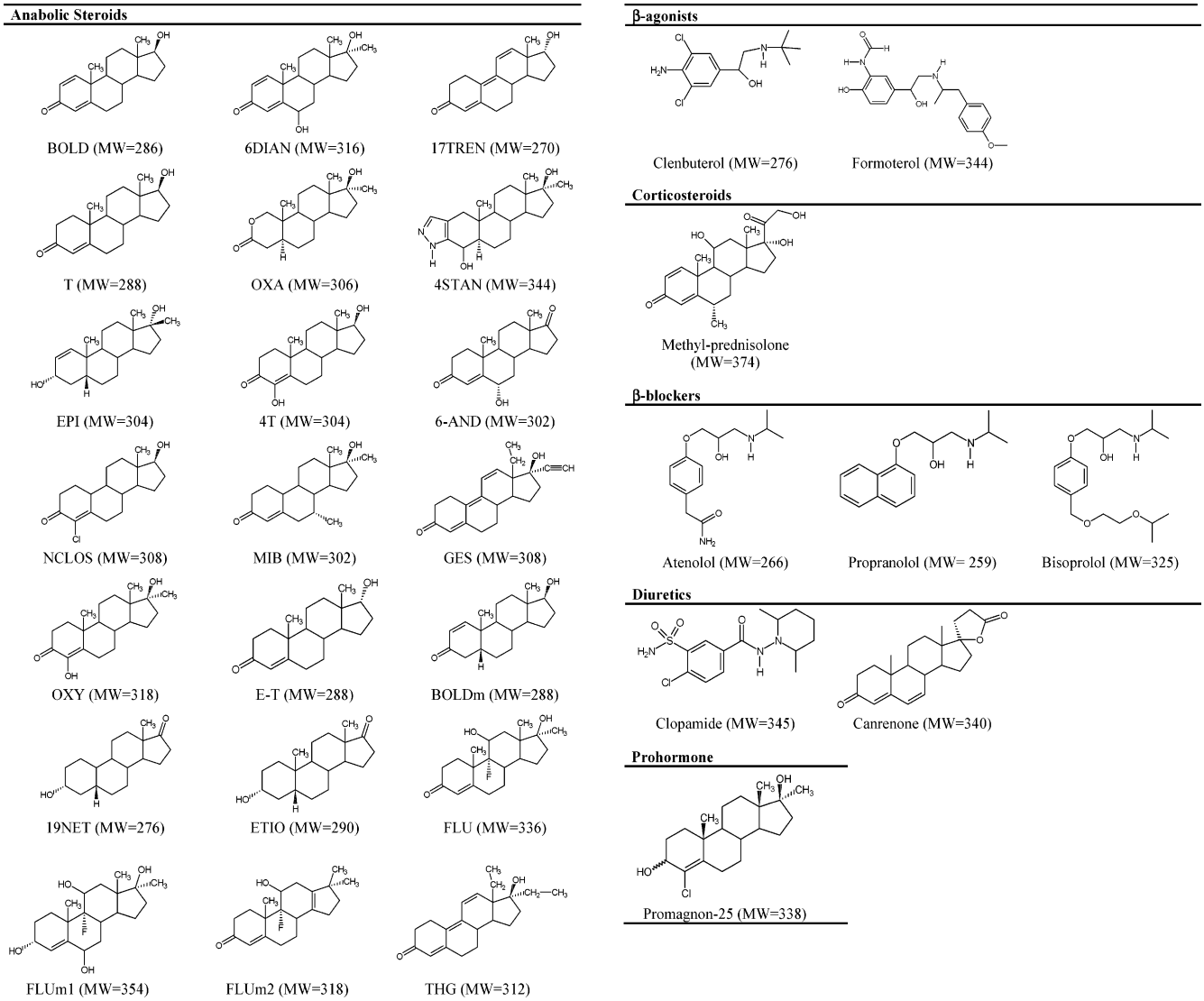


**Figure 1.** Structures and molecular weights of selected compounds.

ough, U.K.), respectively. Ammonium acetate was obtained from Sigma (St. Louis, MO).

Working solutions for each analyte at 1 $\mu$g/mL were obtained by diluting adequate amounts of stock solutions in water−methanol (9:1).

**Instrumentation.** A HPLC Finnigan Surveyor MS pump Plus (Thermo, San Jose, CA) was interfaced to a Finnigan TSQ Quantum Discovery Max triple quadrupole mass spectrometer (Thermo) using the electrospray interface. An amount of 20 $\mu$L of sample was injected into the system using a Finnigan Surveyor autosampler Plus (Thermo). The LC separation was performed using an Omnispher $C_{18}$ column (100 mm × 2 mm i.d., 3 $\mu$m) (Varian, Sint-Katelijne-Waver, Belgium) at a flow rate of 250 $\mu$L/min using a ChromSep guard column (10 mm × 2 mm i. d., 5 $\mu$m) (Varian).

Aqueous ammonium acetate (1 mM) and methanolic ammonium acetate (1 mM) were selected as mobile phase solvents. A gradient program was used; the percentage of organic solvent was linearly changed as follows: 0 min, 30%; 1.5 min, 30%; 8 min, 55%; 15 min, 55%; 29.5 min, 95%; 30.5 min, 95%; 31 min, 30%, 34 min, 30%.

Nitrogen was used as the sheath gas, ion sweep gas, and auxiliary gas at flows of 70, 2, and 5 units, respectively. A spray voltage of 4000 V was used in the positive ionization mode. The capillary temperature was set at 300 °C, and the source CID at 2 units. The collision gas pressure was 1.5 units.

**LC−MS/MS Analysis of Standards.** Standard solutions of five anabolic steroids (TREN, T, OXA, 6DIAN, and BOLD) at 5 $\mu$g/mL were injected into the system. The product ion spectra of the respective $[M + H]^+$ were acquired in the centroid mode between 50 and 400 Da at six different collision energies (15, 20, 25, 30, 40, and 50 eV). The peak width was set at 0.7 mDa and the scan rate at 0.4 s/scan.

**Precursor Ion Scan.** A precursor ion scan method was developed with three acquisition functions selecting the product ions at $m/z$ 105, 91, and 77. The collision energy for these three functions was selected at 45 eV for $m/z$ 105 and 91 and 50 eV for $m/z$ 77. The peak width was set at 0.7 mDa and the scan rate at 0.4 s/scan. Once the data were acquired, the molecular mass of the precursor ion was directly obtained after combining the spectra acquired. In order to know if this precursor ion is the $[M + H]^+$ or another adduct, a chromatogram in full scan mode was

acquired and a background subtracted spectrum of the detected peak was obtained. The presence of other adducts such as $[M + Na]^+$ could help in the assignment of the molecular mass of the analyte.

**Sample Preparation.** Urine samples were treated as previously described.[4] Briefly, for the unconjugated fraction, the pH of an aliquot of 5 mL of urine was adjusted to 9.2 by addition of approximately 0.3 g of sodium hydrogen carbonate−potassium carbonate (2/1;w/w). Liquid−liquid extraction was performed with 5 mL of diethyl ether. After centrifugation, the organic layer was separated and evaporated under nitrogen at 40 °C. For the conjugated fraction, 5 mL of urine was hydrolyzed during 2.5 h at 56 °C after addition of 1 mL of phosphate buffer (pH = 7) and 50 $\mu$L of glucuronidase solution. After cooling to room temperature, extraction was performed as described for the unconjugated fraction. The residue was dissolved into 100 $\mu$L of the initial mobile phase.

For the analysis of prohormones, the content of a capsule was dissolved in 2 mL of methanol. An aliquot of 50 $\mu$L of this solution was diluted into 5 mL of water. After pH adjustment to 9.2, the extraction was performed as described above.

**Application to Metabolic Studies.** Urine samples obtained after the administration of fluoxymesterone were provided by other doping control laboratories, the World Association of Anti-Doping Scientists (WAADS), the International Olympic Committee (IOC), and World Antidoping Agency (WADA) or were samples declared positive in a doping control test at DoCoLab.

Both unconjugated and conjugated fractions of these urines were analyzed together with negative urines using the precursor ion scan method. Results were compared with those previously reported after applying neutral loss experiments.[18]

**Application to the Detection of Unknown Anabolic Steroids.** In order to check the applicability of the method for the detection of unknown steroids, five blank urine samples were spiked with THG at 10 and 50 ng/mL. These samples were extracted and analyzed as described above and both conjugated and unconjugated fractions were treated as blind samples. Additionally, 10 blank urine samples were spiked at 50 ng/mL with 33 anabolic steroids, which exhibited ionization in the positive mode as previously described.[8] These samples were also extracted and treated as blind samples.

**Application to the Analysis of Prohormones.** One capsule of the prohormone Promagnon-25 was extracted and analyzed as described above. The label of the prohormone stated that the only anabolic steroid present in the product is 4-chloro-17α-methyl-androst-4-ene-3ζ-17$\beta$-diol (Figure 1). Those peaks containing the characteristic isotopic pattern for the chlorine atom were considered as structurally related to the analyte, meanwhile compounds without this pattern were considered as impurities caused by other nonchlorinated anabolic steroids.

## RESULTS AND DISCUSSION

**Product Ion Spectrum of Selected Anabolic Steroids.** Five anabolic steroids were selected as model compounds: T, TREN, 6DIAN, OXA, and BOLD (Figure 1). These steroids were selected because they have different structural characteristics, in particular, the number of double bonds and position and number of the functional groups. Therefore, the presence of common ions in these analytes could be extrapolated as a common indicator for a wide variety of anabolic steroids.

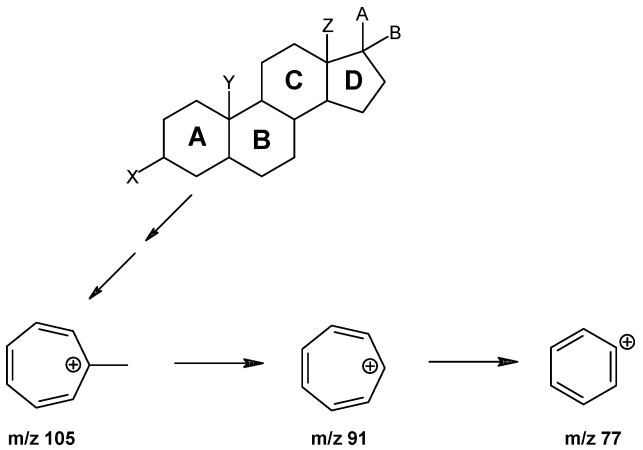


**Figure 2.** Common fragmentation of anabolic steroids at high collision energy.

As previously reported for other anabolic steroids, the product ion spectra of model compounds at low and medium collision energy showed different fragmentation pathways depending on their structure.[13] Hence, at low collision energy (10−15 eV), all compounds exhibited mainly several losses of water. While, TREN and BOLD exhibited one loss of water, OXA, T, and 6AND showed also the ion corresponding to $[M + H - 2H_2O]^+$. In the case of T, these ions were less abundant. At medium collision energy (20−30 eV), T and TREN only showed two ions at $m/z$ 109 and 97 and $m/z$ 253 and 199, respectively, while 6DIAN exhibited four abundant ions at lower $m/z$ (121, 147, 171, and 173). A similar behavior was observed for OXA where four abundant product ions were observed at relatively low $m/z$ (107, 121, 93, and 229). Finally, BOLD only showed an abundant ion at $m/z$ 121 within this range of collision energies.

However, after increasing the collision energy up to 50 eV, a higher number of peaks appeared at low $m/z$ with mass differences of 14 Da. The number of product ions at high collision energy was higher when the ions observed at medium collision energy have high $m/z$, e.g., TREN. After this "molecular explosion", three common ions were detected for all model compounds. These ions at $m/z$ 105, 91, and 77 can be explained by the complete fragmentation of either the B or the C ring, generating the methyl tropylium ion ($m/z$ 105), tropylium ion ($m/z$ 91), and phenyl ion ($m/z$ 77) (Figure 2). In some cases, these ions were combined with other ions such as 115, 121, and 79 but the three selected were usually more abundant under these conditions.

As an example, the MS/MS spectra of three of the selected compounds at three collision energies are presented in Figure 3, which illustrates the fragmentation behavior observed for these anabolic steroids in all three MS/MS conditions.

**Precursor Ion Scan Approach.** On the basis of the data obtained after the MS/MS experiments, a precursor ion scan approach was elaborated in order to detect unknown anabolic steroids. Three precursor ion scan functions were simultaneously used, one for each common product ion found. The presence of the three ions was set as a necessary prerequisite to be considered as a potential anabolic steroid. The molecular mass of the precursor ion scan was restricted between 250 and 385 Da due to the typical structure of anabolic steroids.

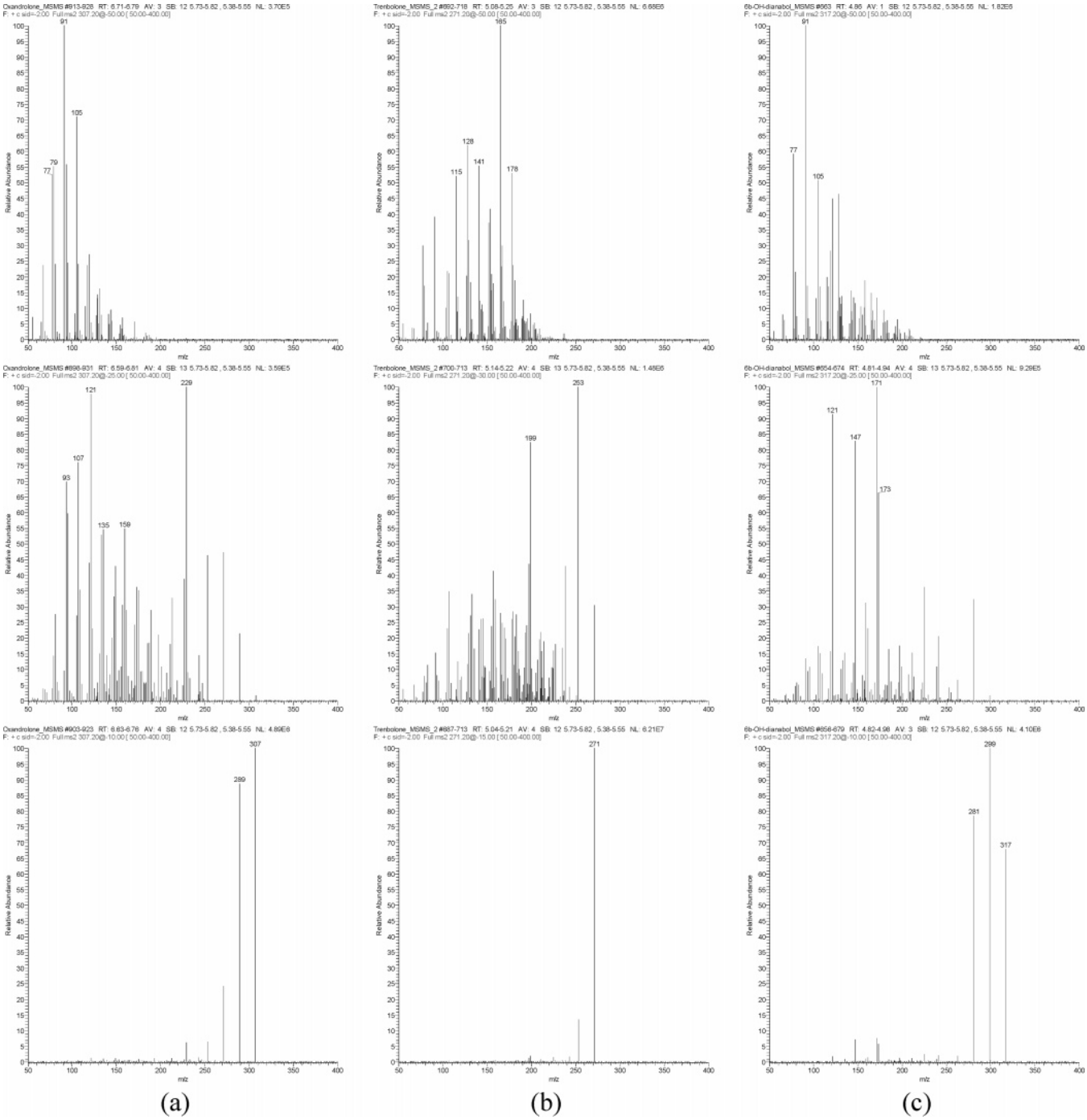


**Figure 3.** Product ion spectra at 10 (bottom), 25 (medium), and 50 eV (top) collision energy for (a) OXA, (b) TREN, and (c) 6DIAN.

This approach was checked by the analysis of standards of the model compounds at different concentration levels. All analytes were detected at concentrations lower than 500 ng/mL, corresponding to 10 ng/mL in urine. Additionally, other anabolic steroids were also checked. As can be seen in Table 1, most of anabolic steroids tested were detected using this approach showing the broad applicability range of the method.

For analytes having a keto group in the 3 position, $[M + H]^+$ was found to be the precursor ion while $[M + H - H_2O]^+$ was observed for those analytes with a hydroxyl group in position 3 and a double bond either in 1 or in 4 (EPI and FLUm1). These results were in accordance with a previous study on the ionization of anabolic steroids.[27] Although some of these analytes also exhibited an abundant sodium adduct ion, ions associated with $[M + Na]^+$ were not detected as a precursor ion of the three ions selected in any of the analytes due to the poor fragmentation of $[M + Na]^+$ adducts. Nevertheless, when a suspected peak appeared, it could be assigned to $[M + H]^+$ or to $[M + H - H_2O]^+$. These options could be easily distinguished after the acquisition of the full scan chromatogram, as steroids normally present other abundant adducts (e.g., $[M + Na]^+$) indicative for the molecular mass of the analyte.

Only those analytes with a keto function in position 17 as a unique protonable moiety such as 19NET and ETIO did not show

(27) Pozo, O. J.; van Eenoo P.; Deventer K.; Delbeke, F. T. *J. Mass Spectrom.* **2007**, *42*, 497−516.

**Table 1. Application of the Developed Approach to Several Anabolic Steroids[a]**

| compound | $m/z$ 77 | $m/z$ 91 | $m/z$ 105 | Anabolic Steroids precursor ion | ratio 77/91 | ratio 105/91 | ratio 105/77 |
|---|---|---|---|---|---|---|---|
| BOLD | YES | YES | YES | $[M + H]^+$ | 1.20 | 0.16 | 0.14 |
| 6DIAN | YES | YES | YES | $[M + H]^+$<br>$[M + H - H_2O]^+$<br>$[M + H - 2H_2O]^+$ | 0.62 | 0.42 | 0.68 |
| 17TREN | YES | YES | YES | $[M + H]^+$ | 0.75 | 0.54 | 0.72 |
| T | YES | YES | YES | $[M + H]^+$ | 1.10 | 0.46 | 0.41 |
| OXA | YES | YES | YES | $[M + H]^+$<br>$[M + NH_4]^+$<br>$[M + H + MeOH]^+$ | 0.56 | 0.67 | 1.19 |
| 4STAN | YES | YES | YES | $[M + H]^+$ | 0.59 | 0.66 | 1.13 |
| EPI | YES | YES | YES | $[M + H - H_2O]^+$ | 0.76 | 0.63 | 0.83 |
| 4T | YES | YES | YES | $[M + H]^+$<br>$[M + H - H_2O]^+$ | 0.63 | 0.40 | 0.64 |
| 6AND | YES | YES | YES | $[M + H]^+$ | 0.52 | 0.75 | 1.42 |
| NCLOS | YES | YES | YES | $[M + H]^+$ | 1.18 | 0.46 | 0.39 |
| MIB | YES | YES | YES | $[M + H]^+$ | 0.55 | 0.60 | 1.09 |
| GES | YES | YES | YES | $[M + H]^+$ | 0.63 | 0.47 | 0.74 |
| OXY | YES | YES | YES | $[M + H]^+$ | 0.40 | 0.83 | 2.09 |
| E−T | YES | YES | YES | $[M + H]^+$ | 0.98 | 0.60 | 0.62 |
| BOLDm | YES | YES | YES | $[M + H]^+$<br>$[M + H + MeOH]^+$ | 0.52 | 0.58 | 1.11 |
| FLUm1 | YES | YES | YES | $[M + H - H_2O]^+$<br>$[M + NH_4 - H_2O]^+$ | 0.65 | 0.96 | 1.47 |
| 19NET | NO | NO | NO | | | | |
| ETIO | NO | NO | NO | | | | |
| | | | | | average<br>0,73<br>RSD (%)<br>34 | average<br>0,57<br>RSD (%)<br>33 | average<br>0,92<br>RSD (%)<br>53 |

[a] YES: A peak for this product ion was found. NO: A peak for this product ion was not found.

any response in the developed method. The ionization of this type of analyte as $[M + H]^+$ is limited by ESI[27] and their fragmentation is normally based on neutral losses of water and acetone,[28] and therefore, they did not exhibit an abundant signal in the precursor ion scan of the three selected ions.

The ion ratios for these three ions were also established for each compound in order to find an additional indicator for the steroid structure. However, high variations were observed, and no relationship between these ratios and the analyte structure could be obtained (Table 1).

**Specificity of the Method.** The major theoretical limitation of this approach was expected to be the low specificity of the selected ions as they are also common ions in the fragmentation of aromatic compounds. In order to evaluate this limitation, the method was applied to other drugs with molecular mass in the selected range and containing aromatic rings or six-membered rings in their structure.

The analysis of these potential interferences showed better specificity than expected. Hence, although generally one of these ions was present in the chromatogram of these compounds, the simultaneous presence of the three selected ions was found a suitable indicator of the steroidal structure (Table 2). As an example, for CLEN no signal was found for the ion at $m/z$ 91 although it had signals for both ions at $m/z$ 105 and $m/z$ 77.

Other compounds such as Me-PRED and CANRE exhibited signals at the three selected ions. Although they are classified as a corticosteroid and a diuretic, respectively, they have a steroidlike structure and therefore the three peaks were observed. As main exceptions to that general rule, ATEN and BISO showed peaks for the three selected ions despite the absence of steroidal structure. However, in these cases, the MS/MS at different collision energies did not show the typical behavior of steroids, i.e., several losses of water at low collision energy, few abundant ions at medium collision energy, and several ions at low $m/z$ after applying high collision energy. Therefore, a steroidal structure for these compounds can be excluded.

**Table 2. Application of the Developed Approach to Other Doping Agents Containing a Six-Membered Ring in Their Structure[a]**

| compound | $m/z$ 77 | $m/z$ 91 | $m/z$ 105 |
|---|---|---|---|
| | $\beta$-Agonist | | |
| CLEN | YES | NO | YES |
| FORM | YES | YES | NO |
| | Corticosteroids | | |
| Me-PRED | YES | YES | YES |
| | $\beta$-Blockers | | |
| ATEN | YES | YES | YES |
| PROP | YES | YES | NO |
| BISO | YES | YES | YES |
| | Diuretics | | |
| CLOP | NO | NO | YES |
| CANRE | YES | YES | YES |

[a] YES: A peak for this product ion was found. NO: A peak for this product ion was not found

(28) Thevis, M.; Schänzer, W. *J. Am. Soc. Mass Spectrom.* **2005**, *16*, 1660−1669.

**Application to Metabolic Studies.** One of the possible applications of the developed method is the detection of new anabolic steroid metabolites. In these studies, the detection of metabolites can be performed by comparison between a blank urine sample and a urine sample collected after drug administration. The complexity of the matrix makes direct comparison of scan chromatograms difficult, and therefore, a more specific method is necessary. This was the case in a fluoxymesterone excretion study where up to six previously unreported metabolites were detected after applying a neutral loss of 20 Da corresponding to HF.[18]

The applicability of the developed precursor ion scan method was evaluated by applying it to excretion urines of fluoxymesterone and comparing the results with those obtained by neutral loss scan for the six main metabolites.[18] The chromatogram of the unconjugated fraction obtained with the precursor ion scanning is shown in Figure 4a. Three abundant endogenous compounds were found between 11.0 and 12.5 min corresponding to precursor ions at $m/z$ 347, 277, and 285 and as these ions were found in several negative urines tested and therefore they were considered as endogenous compounds. Three other peaks were clearly detected at retention times of 13.7, 22.2, and 24.6 min, respectively. These peaks had signals for the three selected ions corresponding to precursor ions at $m/z$ 337 for metabolite 1 and $m/z$ 319 for metabolites 2 and 3 (Figure 4b). In negative urines ($n = 6$), no signals were detected at these retention times and therefore they could be assigned to three fluoxymesterone metabolites.[18]

The evaluation of the early eluting peaks of the chromatogram was more difficult. In this area, several compounds were detected. Some of them did not show a signal for the three selected ions and were therefore not considered as steroids. However, their presence can make the identification of metabolites difficult. Despite these difficulties, three additional metabolites were found in this area after comparing with several negative urines. These compounds showed precursor ions at $m/z$ 337, 351, and 353 (Figure 4b) and were also previously reported as fluoxymesterone metabolites.[18]

Six metabolites of fluoxymesterone were detected in the unconjugated fraction. Only two metabolites reported in the previous study remained undetected using this method, both detected as $[M + Na]^+$ adducts. These adducts are only fragmented in the collision cell to produce a loss of HF[18] and so they cannot be expected to be detected using the described approach.

As stated above, metabolites ionized as $[M + H]^+$ or $[M + H - H_2O]^+$ can be detected by this method. In order to know the molecular mass of the metabolites, a full scan chromatogram was acquired and the spectrum at the corresponding retention time extracted. For example, in the case of metabolite 4, the application of the method revealed an ion at $m/z$ 337 (Figure 4b). The full scan spectrum showed also an abundant ion at $m/z$ 377 and other less abundant at $m/z$ 372. These ions can be related to $[M + Na]^+$ and $[M + NH_4]^+$, respectively, if the ion at $m/z$ 337 corresponds to $[M + H - H_2O]^+$ of metabolite 4. If the ion at $m/z$ 337 is considered as $[M + H]^+$, no reasonable assignments can be performed for the ions at $m/z$ 377 and 372. Therefore, this ion was considered as corresponding to $[M + H - H_2O]^+$ and the molecular mass of the metabolite 4 as 354 Da. The presence of $[M + Na]^+$ in the other peaks confirmed that all of them mainly ionized as $[M + H]^+$.

After analysis of the conjugated fraction, an additional peak at 12.6 min was detected in the chromatogram with a precursor ion assigned at $m/z$ 337 (Figure 4c,d labeled peak 7). This peak could be assigned to fluoxymesterone also in agreement with a previously reported study.[18] The most abundant compounds (1, 2, and 7 in Figure 4c) were clearly detected after hydrolysis. Unfortunately, metabolites 3−6, detected in the nonhydrolyzed fraction, could not be detected in the hydrolyzed fraction due to the increased background probably originating from the high amount of endogenous compounds released after hydrolysis (Figure 4c). The analyst must then distinguish between the endogenous components and the exogenous ones. An in-depth study of the presence of endogenous components by analyzing multiple negative urine samples would help in order to increase the applicability of the method. Nevertheless, the most abundant metabolites could be successfully detected using this approach.

**Application to the Detection of Unknown Anabolic Steroids.** Another promising application of the method is the detection of unknown designer steroids. In order to check for the applicability for this purpose, several urine samples were spiked at 10 and 50 ng/mL with THG and treated as unknown samples. Both unconjugated (Figure 5a,b) and conjugated (Figure 5d,e) fractions were analyzed.

The absence of interferences in the unconjugated fraction favored the detection of THG at the low level (10 ng/mL). At 50 ng/mL, the peak corresponding to THG was one of the most abundant (Figure 5b) allowing its unequivocal detection with a precursor ion at $m/z$ 313 (Figure 5c). As in the case of fluoxymesterone metabolism, the situation was more complex for the conjugated fraction where THG was not detectable at 10ng/mL due to the high number of interferences. Additionally, the high amount of steroids released under these conditions can also increase the ion suppression produced by the matrix reducing also the sensitivity of the method. Despite these interferences, the peak corresponding to THG was clearly detected at the 50 ng/mL level. The spectrum of this ion showed a peak at $m/z$ 313 (Figure 5f). The study of the full scan spectrum showed that this ion can be assigned to the $[M + H]^+$.

In a previous paper, the precursor ion scan of 241 Da was used for the detection of THG structural analogues with a limit of detection of 50 ng/mL.[14] The present approach is, therefore, as sensitive as previously reported but with the main advantage that it can be applied for most of the steroids without almost any structural restriction.

Both unconjugated and conjugated fractions from the 10 blank urines spiked with 33 anabolic steroids were analyzed. These 33 steroids were selected as model compounds for both fractions irrespective of their actual excretion form (conjugated or free) in order to know the detection capability of the method for potentially unknown steroids. Results are summarized in Table 3.

Only three analytes could not be detected using the precursor ion scan method. These three analytes did contain neither a keto function at position 3 nor a conjugated hydroxyl group. The presence of one of these moieties seems to be necessary in order to obtain satisfactory results (Table 1). Additionally, metabolites lacking a keto function, such as those described for methyltest-

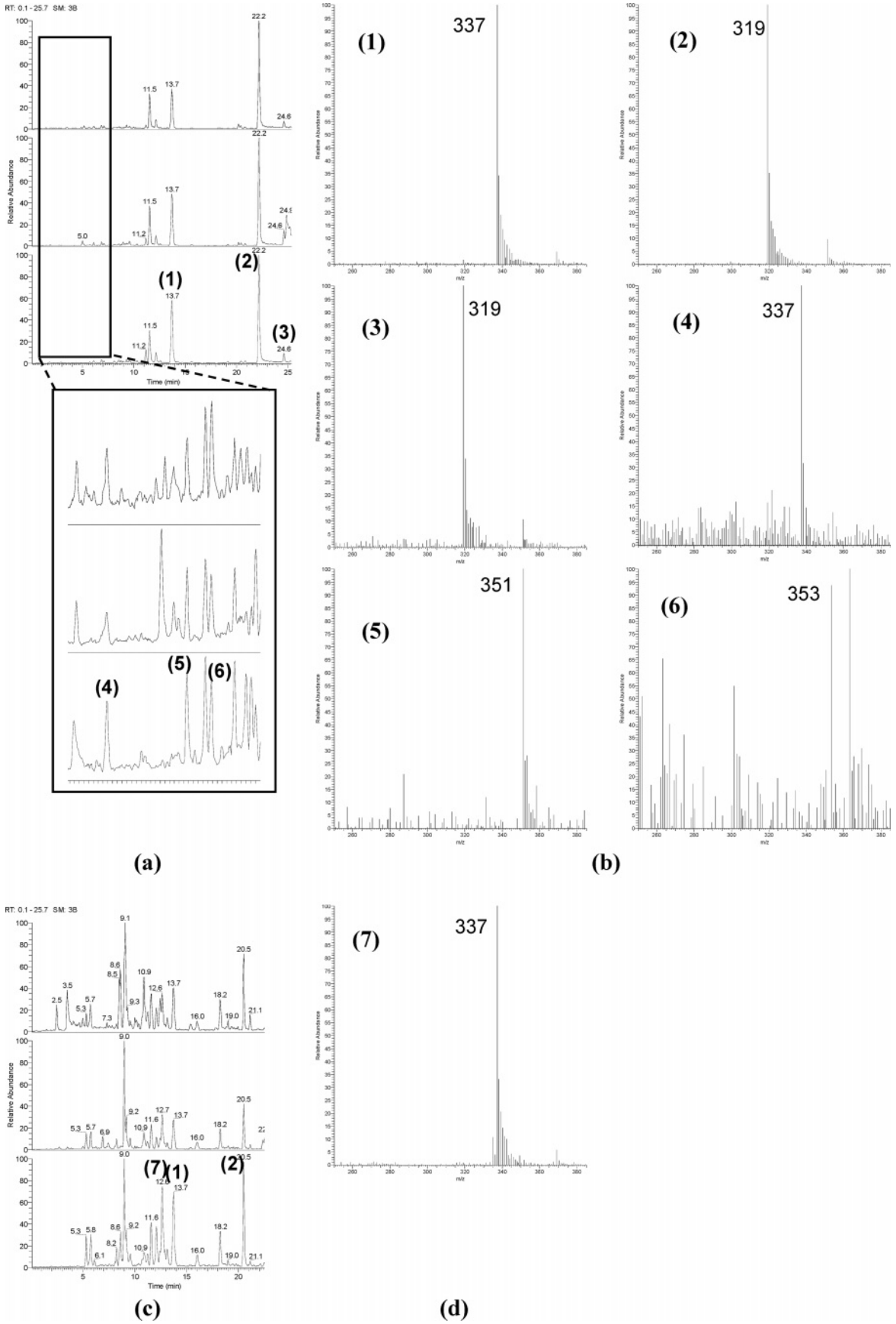


**Figure 4.** Application of the precursor ion approach in the metabolic study of fluoxymesterone. (a) LC−MS/MS precursor ion scan chromatogram (top, *m*/*z* 77; middle, *m*/*z* 91; and bottom, *m*/*z* 105) for the unconjugated fraction, (b) mass spectra of the different precursor ions obtained, (c) LC−MS/MS precursor ion scan chromatogram (top, *m*/*z* 77; middle, *m*/*z* 91; and bottom, *m*/*z* 105) for the conjugated fraction, and (d) mass spectrum of the additional precursor ion obtained.

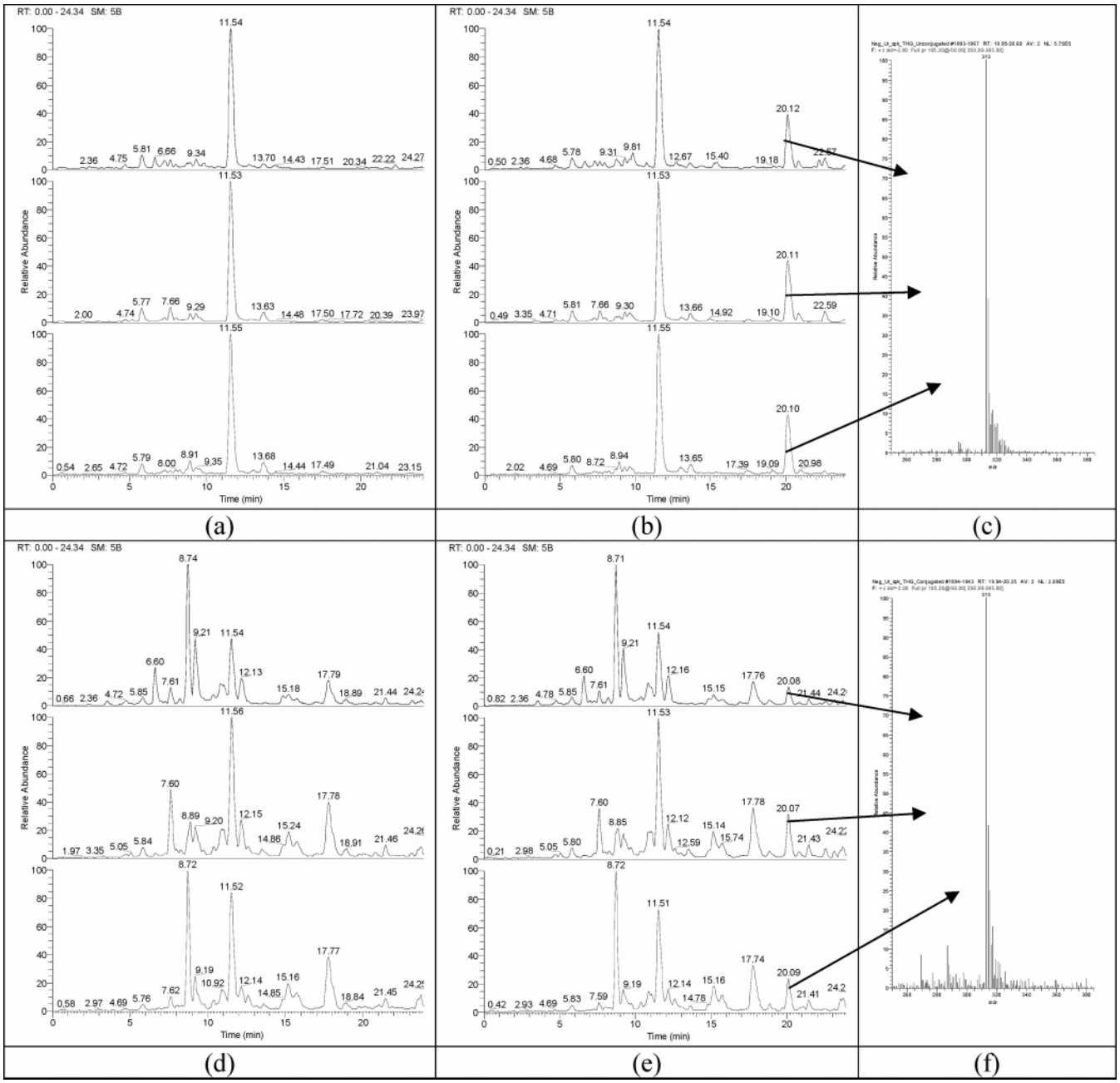


**Figure 5.** Application of the precursor ion approach to the detection of unknown steroids. (a) LC−MS/MS chromatogram of the unconjugated fraction of a negative urine, (b) LC−MS/MS chromatogram of the unconjugated fraction of a urine spiked at 50 ng/mL with THG, (c) mass spectrum obtained for the peak at 20.1 min, (d) LC−MS/MS chromatogram of the conjugated fraction of a negative urine sample, (e) LC−MS/MS chromatogram of the conjugated fraction of a urine spiked at 50 ng/mL with THG, and (f) mass spectrum obtained for the peak at 20.1 min.



osterone,[2] cannot be detected as they are not ionized under these conditions.[27]

In the nonhydrolyzed fraction, the other 30 steroids (91%) were detected in 2 out of 10 samples and 73% of selected steroids were detected in all 10 urine samples. Early eluting compounds (such as FLUm1) showed a lower sensitivity due to both high ion suppression and low extraction recoveries[8] which can make their detection by this method difficult at these low levels. Additionally, 4-hydroxy steroids also exhibited moderate response which decreased the number of positive findings.

As indicated for fluoxymesterone metabolites, the detection of steroids in the hydrolyzed fraction was more difficult due to the increase in endogenous interferences. Most endogenous interferences eluted between 8 and 13 min hampering the detection of some steroids. For example, although FLU, eluting at 12.6 min, showed an excellent ESI behavior due to the conjugation of the keto group (Figure 1), it was not detected in three of the spiked samples due to the endogenous interferences eluting in this area. Oppositely, compounds less protonable such as EPI were detected in all samples because they eluted in a less disturbed area of the chromatogram (26.7 min). Several interferences present in all urine samples caused the undetectability of several steroids at these low concentrations. 6DIAN was not detected due to the presence of one endogenous interference at $m/z$ 299. The $^{13}C$ isotope of the $[M + NH_4]^+$ adduct of this interference ($m/z$ 316) interfered with the $[M + H]^+$ of 6DIAN.

**Table 3. Positive Findings Score after the Analysis of 10 Urine Samples Spiked with 33 Anabolic Steroids**

| analyte | parent | retention time (min) | observed $m/z$ | ion | nonhydrolyzed urine | hydrolyzed urine |
|---|---|---|---|---|---|---|
| EPI | metandienone | 26.7 | 269 | $[M + H - H_2O]^+$ | 10/10 | 10/10 |
| 17TREN | trenbolone | 13.0 | 271 | $[M + H]^+$ | 10/10 | 10/10 |
| 1(5α)-androstene-3,17-dione | | 13.6 | 287 | $[M + H]^+$ | 3/10 | 0/10 |
| | | | 319 | $[M + H + MeOH]^+$ | | |
| BOLD | | 12.7 | 287 | $[M + H]^+$ | 10/10 | 7/10 |
| androst-1,4-diene-3,17-dione | | 11.1 | 285 | $[M + H]^+$ | 10/10 | 10/10 |
| 19-norandrosterone | nortestosterone | n.d | | | | |
| 19NET | nortestosterone | n.d | | | | |
| metandienone | | 14.0 | 301 | $[M + H]^+$ | 10/10 | 10/10 |
| | | | 283 | $[M + H - H_2O]^+$ | | |
| epimetandienone | metandienone | 21.9 | 301 | $[M + H]^+$ | 10/10 | 10/10 |
| | | | 283 | $[M + H - H_2O]^+$ | | |
| 4-hydroxy-androst-4-ene-3,17-dione | | 13.7 | 303 | $[M + H]^+$ | 2/10 | 0/10 |
| | | | 285 | $[M + H - H_2O]^+$ | | |
| 6AND | androstenedione | 8.2 | 303 | $[M + H]^+$ | 10/10 | 10/10 |
| MIB | | 17.8 | 303 | $[M + H]^+$ | 10/10 | 10/10 |
| 4T | | 15.2 | 305 | $[M + H]^+$ | 5/10 | 0/10 |
| | | | 287 | $[M + H - H_2O]^+$ | | |
| OXA | | 13.3 | 307 | $[M + H]^+$ | 10/10 | 3/10 |
| | | | 324 | $[M + NH_4]^+$ | | |
| | | | 339 | $[M + H + MeOH]^+$ | | |
| GES | | 14.8 | 309 | $[M + H]^+$ | 10/10 | 10/10 |
| NCLOS | | 17.8 | 309/311 | $[M + H]^+$ | 10/10 | 10/10 |
| ethisterone | | 15.9 | 313 | $[M + H]^+$ | 10/10 | 7/10 |
| THG | | 20.1 | 313 | $[M + H]^+$ | 10/10 | 10/10 |
| 6DIAN | metandienone | 8.9 | 317 | $[M + H]^+$ | 10/10 | 0/10 |
| | | | 299 | $[M + H - H_2O]^+$ | | |
| | | | 281 | $[M + H - 2H_2O]^+$ | | |
| FLUm2 | fluoxymesterone | 22.2 | 319 | $[M + H]^+$ | 10/10 | 10/10 |
| OXY | | 14.5 | 319 | $[M + H]^+$ | 10/10 | 7/10 |
| 1-methylen-5α-androstan-3α-ol-17-one | metenolone | 320 | 20.6 | $[M + NH_4]^+$ | 2/10 | 0/10 |
| BOLDm | boldenone | 18.3 | 289 | $[M + H]^+$ | 10/10 | 0/10 |
| | | | 321 | $[M + H + MeOH]^+$ | | |
| 1-testosterone | | 18.9 | 289 | $[M + H]^+$ | 10/10 | 0/10 |
| | | | 321 | $[M + H + MeOH]^+$ | | |
| 1α-methyl-5α-androstane-3α-ol-17-one | mesterolone | n.d | | | | |
| FLU | | 12.6 | 337 | $[M + H]^+$ | 10/10 | 6/10 |
| danazol | | 22.4 | 338 | $[M + H]^+$ | 10/10 | 10/10 |
| 16β-hydroxy-stanozolol | stanozolol | 15.1 | 345 | $[M + H]^+$ | 10/10 | 10/10 |
| 3'-hydroxy-stanozolol | stanozolol | 15.1 | 345 | $[M + H]^+$ | 10/10 | 10/10 |
| 4STAN | stanozolol | 15.6 | 345 | $[M + H]^+$ | 10/10 | 10/10 |
| 2-hydroxymethyl-17α-methyl-androsta-1,4-diene-11α,17β-diol-3-one | formebolone | 9.3 | 347 | $[M + H]^+$ | 10/10 | 5/10 |
| 4-chloro-17α-methyl-androsta-1,4-diene-6β,17β-diol-3-one | oral turinabol | 10.5 | 351/353 | $[M + H]^+$ | 3/10 | 0/10 |
| | | | 333/335 | $[M + H - H_2O]^+$ | | |
| | | | 315/317 | $[M + H - 2H_2O]^+$ | | |
| FLUm1 | fluoxymesterone | 3.0 | 337 | $[M + H - H_2O]^+$ | 2/10 | 0/10 |
| | | | 354 | $[M + NH_4 - H_2O]^+$ | | |

Similarly, the presence of endogenous testosterone and epitestosterone hampered the detection of BOLDm and 1T which exhibited the same $[M + H]^+$ at $m/z$ 289 at a similar retention time.

Despite this, 64% of selected steroids could be detected in at least one sample while 45% of the analytes were detectable in all spiked samples. The detectable compounds were mainly highly protonable compounds (with either a conjugated keto or amino group in the structure[27]) eluting in an area of the chromatogram with low interferences.

**Application to the Analysis of Prohormones.** The label of the supplement indicated the presence of one steroid. However, four steroids peaks at 13.7, 17.5, 20.5, and 21.0 min, respectively, were found in the chromatogram from the extract of the prohormone Promagnon-25 (Figure 6). The expected compound does not contain any keto function (Figure 1) and therefore a $[M + H - H_2O]^+$ was postulated. Indeed, for the peak at 20.6 min (peak 1) the main ion observed ($m/z$ 321) corresponded to the $[M + H - H_2O]^+$ of the expected analyte. Additionally, the presence of the $^{37}Cl$ isotope and other ions such as $[M + H - 2H_2O]^+$ ($m/z$ 303) and $[M + NH_4 - H_2O]^+$ ($m/z$ 338) confirmed the assignment of this peak to 4-chloro-17α-methyl-androst-4-ene-3ζ-17β-diol.

One other peak eluting at 21.0 min also presented the characteristic isotopic pattern of a chlorine compound (Figure 6, peak 2). Therefore, it can be considered either as an impurity of the synthesis or as a degradation product. This compound showed $m/z$ 337, which could be associated to the protonated species. Because the $[M + H]^+$ is preferably formed in those steroids possessing a keto group in the position 3,[27] this compound could be assigned to a 3-oxidated Promagnon-25 derivative. However, ultimate confirmation by MS/MS analysis of the standard is necessary.

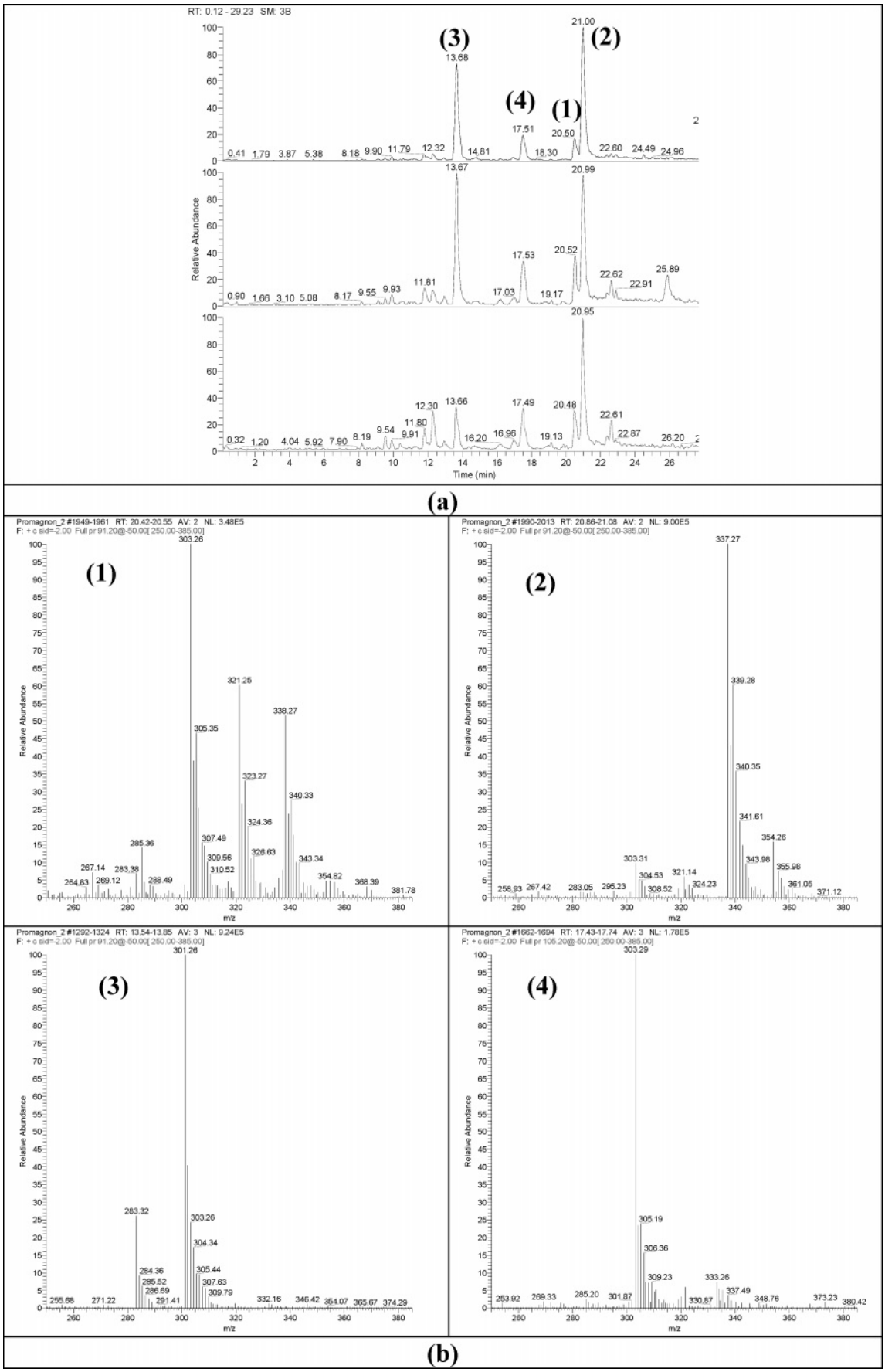


**Figure 6.** Application of the precursor ion approach to the analysis of prohormones. (a) LC−MS/MS chromatogram for Promagnon-25, (b) mass spectra of the different precursor ions obtained.

Two nonchlorinated peaks (retention times 13.7 and 17.5 min) were observed at $m/z$ 301 and 303, respectively, without the presence of the chlorine isotopic pattern (Figure 6, peaks 3 and 4). These peaks were considered as impurities with a steroidal structure. The scan spectra of both compounds revealed that both compounds exhibited an $[M + H]^+$ ion, and therefore the molecular weight of these steroids was considered 300 and 302 Da, respectively. After the injection of steroids with this molecular weight in the MS/MS mode, metandienone was found to be the substance at 13.7 min while methyltestosterone was identified as the steroid eluting at 17.5 min.

## CONCLUSIONS

An LC−MS/MS method based on the precursor ion scan of three ions ($m/z$ 105, $m/z$ 91, and $m/z$ 77) has been developed for the detection of unknown anabolic steroids and metabolites. Although these ions can be common for other groups of substances, the presence of all three has been shown to be an effective way to detect steroidlike compounds. After injection of several steroids, only those not showing an abundant $[M + H]^+$ or $[M + H - H_2O]^+$ could not be detected by the developed method. Most of the anabolic steroids however are ionized as these forms[27] showing the high applicability of this approach. This method can be considered as a complementary tool to existing target screening methods.

The applicability of the method has been tested for three different scenarios: the detection of steroid metabolites, the detection of unknown steroids, and the analysis of prohormones. Although less specific than other approaches such as the neutral loss of HF, the developed method allowed the detection of most of the fluoxymesterone metabolites. Indeed only those compounds which only exhibited $[M + Na]^+$ as the precursor ion remained undetectable. Contrarily to the highly specific method based on the neutral loss, the present approach is not restricted and can be applied to study the metabolism of other anabolic steroids. Moreover, designer steroids, such as THG, can be detected at relatively low concentration levels with the additional advantage that many other steroids can be detected as well.

Because of the high amount of endogenous steroids in urine, the detection was found to be less complex and more sensitive in the unconjugated fraction. Therefore, it is advisable to use this approach first in the unconjugated fraction in order to detect potential steroids excreted free at low concentrations. A subsequent analysis of the conjugated fraction can reveal additional unknown steroids, but the detection of these will be restricted to those analytes either at a high concentration or eluting in a less disturbed area of the chromatogram. Several negative samples needed to be analyzed in order to have a more comprehensive knowledge about the endogenous steroids detected by this approach, which can be useful to distinguish between endogenous and exogenous compounds.

The developed approach has also shown its applicability in the analysis of prohormones. Hence, unknown contaminations and/or degradation products can be detected in prohormones and dietary supplements without predefining any target.

The present methodology allowed the detection of unknown anabolic steroids in different application fields. However, once detected, the steroid structure has to be identified. For this purpose, in depth studies of the MS/MS fragmentation of these analytes could be useful, and the use of complementary techniques such as accurate mass measurements, $MS^n$, or GC/MS will also be helpful.

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