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Gas chromatographic–tandem mass spectrometric determination of anabolic steroids and their esters in hair Application in doping control and meat quality control

Yvan Gaillard[a,*], François Vayssette[a], Antoine Balland[b], Gilbert Pépin[a]

[a]*Laboratoire TOXLAB, 18 Rue André Del Sarte, 75018 Paris, France*
[b]*Laboratoire ACE, 20 Rue André Del Sarte, 75018 Paris, France*

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Abstract

We have developed a powerful and simple sensitive method for testing hair for anabolic steroids and their esters. A 100-mg amount of powdered hair was treated with methanol in an ultrasonic bath for extraction of esters, then alkaline digested with 1 *M* NaOH for an optimum recovery of other drugs. The two liquid preparations were subsequently extracted with ethyl acetate, pooled, then finally highly purified using a twin solid-phase extraction on amino and silica cartridges. The residue was derivatized with *N*-methyl-*N*(trimethylsilyl)-trifluoracetamide (MSTFA) prior to injection. Analysis was conducted by gas chromatography coupled to a triple quadrupole mass spectrometer. The generally chosen parent ion was the molecular ion while two daughter ions were selected for each compound with collision energies ranging from −16 to −21 eV. Internal standards were nandrolone d_3 for non-esterified drugs and testosterone phenyl propionate for esters. The limits of detection calculated from an analysis of the blanks (n=30) were 0.08 pg/mg for nandrolone, 6.20 pg/mg for boldenone, 0.07 pg/mg for methyl testosterone, 0.15 pg/mg for ethinyl estradiol, 2.10 pg/mg for metandienone, 0.86 pg/mg for testosterone propionate, 0.95 pg/mg for testosterone cypionate, 1.90 pg/mg for nandrolone decanoate, 3.10 pg/mg for testosterone decanoate and 4.80 pg/mg for testosterone undecanoate. Application to doping control has been demonstrated. In a series of 18 sportsmen, two tested positive for anabolic steroids in hair whereas urinalysis was negative for both of them. The first positive case was nandrolone and the second case concerned the identification of testosterone undecanoate. Measured in 10 white males aged between 22 and 31 years, the testosterone concentration was in the range 1.7–9.2 pg/mg (mean=5.0 pg/mg). The method was also applied in meat quality control. Of the 187 analyses realized based upon hair and urine sampling in slaughter houses, 23 were positive for anabolic steroids in hair: one case for boldenone, one case for metandienone, two cases for testosterone propionate, three cases for nandrolone, five cases for testosterone decanoate and 11 cases for methyl testosterone. In the meantime, urinalysis was always negative for these drugs or their metabolites.

Keywords: Hair; Doping; Anabolic steroids; Steroids; Testosterone esters

*Corresponding author.

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1. Introduction

1.1. Illegal human use of anabolic steroids

Doping is understood to be the utilization of one or more substances or procedures likely to produce an artificial enhancement of the mental or physical conditioning of an athlete [1,2].

Use of anabolic steroids was officially banned in the mid-1970s by sports authorities.

In 1974 testosterone esters attracted a great deal of interest when it became known that they were undetectable as esters in urine where there is physiological testosterone. Thus, athletes became interested in using testosterone esters as a alternative doping several weeks before competition. The situation in former East Germany shown by Franke and Berendonk [3], although well documented [4–8], is not the exception. A California doctor, Robert Kerr, admitted prescribing anabolic steroids to over 20 Olympic medallists of the 1984 Games in Los Angeles [9]. During the 1998 Tour de France bicycle race newspapers and afterwards two books [55,56] showed that doping in some teams was almost always organized by doctors, trainers and the cyclists themselves [10].

It is well known that a latency period is all that is necessary to invalidate these urine tests, which explains the small percentage of positive results from the IOC Laboratory.

Random testing of cyclists in the Flanders region of Belgium from 1987–1994 revealed a positive rate of 7.8% for 4374 analyses [11]. Heading the list of banned substances were amphetamines, ephedrine, nandrolone and testosterone. During anti-doping tests performed in 1988 by the 20 laboratories accredited by the International Olympic Committee (IOC), 47,069 samples were tested and 1153 positive cases were found: nandrolone had the most occurrences (304), followed by 155 for testosterone [12]. The relatively small number of positive cases is directly related to the matrix used. More advanced techniques of detection such as tandem mass spectrometry (MS–MS) or high-resolution mass spectrometry (HRMS) should allow one, in theory, to investigate back further in the past. However there has been no increase in the number of positive cases. Why? Because doping habits have changed. When questioned about their drug treatments, some anonymous cyclists of the Tour de France acknowledged that anabolic steroid use still takes place but that the treatment had taken place during the winter months and had stopped one to two months before the trials [13–16]. Another traditional option is testosterone esters. Sometimes during competition hormonal precursors to testosterone would be consumed, notably dehydroepiandrosterone (DHEA), androstenedione or androstenediol. For other sports like track and field, human growth hormone (hGH) might be taken during competition.

1.2. Illegal use of anabolic steroids in livestock

The use of anabolic steroids and other products to increase body mass was prohibited in Europe in 1989. This was a response to the sharp increase in the administration of hormones, which had never been proven safe for human health. The US, however, has never questioned the use of five hormones: testosterone, estradiol, progesterone, trenbolone and zeranol. These products are freely sold in the form of implants that are placed under the skin of the animals' ears. The evaluation report of these five hormones was adopted in 1995 by the Codex alimentarius (dependent on the FAO and the WHO), setting maximum limits for residue of the two synthesis hormones. No limit was set for natural hormones, thus leaving the possibility for abuse. An epidemic of premature sexual development and ovarian cysts affected about 3000 Puerto Rican children from 1979 to 1981. These toxic effects were traced to hormonal contamination of meat products due to the presence of zeranol and high concentrations of estradiol. Increased rates of uterine and ovarian cancers in adult women were also associated with this contamination [17]. It seems there is a significant carcinogenic risk due to estrogenic feed additives, particularly for hormonally sensitive tissues such as breast tissue. Incidence rates in white women in the US and other industrialized nations increased by 53% from 1950 to 1989 [18]. Moreover, there has been no study of carcinogenesis dealing with the association of several hormones [17,18]. The different associations of products found can be sometimes very astonishing. We reported a case in France in which the analysis of an injection site revealed the simultaneous presence of testosterone, testosterone

cypionate, estradiol, estradiol benzoate, methandriol, stanozolol and norethandrolone [19].

Debruyckere et al. [20] reported three suspicious cases in Belgium in 1989 in which nandrolone metabolites were found in human who had consumed tainted meat. The sole use of a urine matrix is largely insufficient to ensure non-treatment of livestock, as we will show later. Hair analysis, on the other hand, as we have already demonstrated in the case of clenbuterol, covers a much greater period. In that study, urine testing for clenbuterol was negative at the time of slaughter, but hair analysis showed a positive result several months after the treatment had been interrupted [21].

1.3. Present analytical state

Radioimmunoassays have been developed [22,23]. These methods however, lack specificity and cannot be retained in doping control. Indeed, gas chromatography (GC)–MS represents the method of choice for testing for anabolic steroids in urine. Several screening procedures have been reported in the selected-ion-monitoring mode for the detection of anabolic steroids banned by the IOC [24–27]. The use of ion trap GC–MS–MS for the detection of 23 steroids at trace levels was reported [28]. This very sensitive procedure makes it possible to confirm true positive cases which could not have been confirmed using routine GC–MS methods. Decreasing the limits of detection thus increased the period of detection. Of 2000 urine samples analyzed by Munoz-Guerra et al. [28], 45 positive cases could not have been confirmed by GC–MS. Furthermore, long-term detection and identification of metandienone and stanozolol abuse in athletes by GC–HRMS was described. Of the 116 positives cases reported by Schanzer et al. [29], 41 were detected by GC–MS and 75 by GC–HRMS. Sample clean up involves a solid-phase extraction (SPE) on C_{18} cartridges followed by an enzymatic hydrolysis and a liquid–liquid extraction. Alternately, a preparative immunoaffinity chromatography or a high-performance liquid chromatography (HPLC) fractionation were described. HPLC with UV–particle beam MS was reported for the detection of anabolic steroids in oil-based injectables, but the method lacks sensitivity for urinalysis [30]. Electrospray HPLC–MS of testosterone esters has proven to be an effective tool in the detection of the 17-β-fatty acid esters of testosterone in human plasma [31].

Conversely and curiously, identification of steroids in hair has been rarely performed since the science of determining these compounds is very new. Stanozolol was identified in rat hair by negative chemical ionization GC–MS [32]. Rats were given stanozolol 20 mg/kg intraperitoneally once daily for three days. Measured concentrations in the hair collected after two weeks were 362 ± 332 pg/mg in pigmented hair and 90 ± 47 pg/mg in non-pigmented hair. Hair samples were digested using NaOH and solid-phase extracted prior to analysis. In a fatal case of a 32-year-old male bodybuilder who died of a cardiac infarction metandienone, epimetandienone, 6-β-hydroxy-metandienone, metenolone enantate, nandrolone decanoate, and various esters of testosterone (propionate, isocaproate, phenyl propionate, enantate and decanoate) were identified in the hair of the deceased person. Purification of the sample (methanolic sonication) was done by HPLC while identification was realized using GC–HRMS–MS [33]. Quantification of the substances was not realized. Kintz [34] reported the detection by GC–MS of stanozolol (135 and 156 pg/mg), nandrolone (196 and 260 pg/mg) and testosterone (46 and 71 pg/mg) in hair of two bodybuilders. Gleixner and Meyer published detection using HPLC–enzyme immunoassay (EIA) of estradiol and testosterone in the hair of cattle [35]. The same authors also reported the detection of methyl testosterone and ethinyl estradiol in hair of food-producing animals by EIA after a SPE and HPLC purification of the hair extract (overnight with ether, HCl, 1,4-dithiothreitol and sodium dodecyl sulfate) [36]. Veal calves were fed with 3.5 μg of ethinyl estradiol and 35 μg methyl testosterone per kg of body mass, twice daily for 10 days. The mean measured concentration of ethinyl estradiol 1.4 pg/mg ($n=8$) whereas the mean measured concentration of methyl testosterone was 2.7 pg/mg ($n=8$). The French government has rapidly understood the interest of such a method and voted in December 1998 on a low that would authorize the sportsman to ask for additional sampling, if he desired so, (blood, hair or other) in the establishment of the scientific proof [37]. Unfortunately, it is not yet an obligation. Moreover, detection of clenbuterol

in hair represents henceforward an official method of the French Ministry of Agriculture [38].

Considering the need and the interest of testing hair for anabolic steroids both for humans and animals, we have developed a very powerful GC–MS–MS method using a triple quadrupole technology. The other principal interest of hair analysis would be of making possible the detection of esters of testosterone that otherwise are not detectable by urinalysis. Since we need to obtain a specific detection at levels as low as 1 pg/mg, it was very important to obtain an extract that would be as clean as possible resulting in lowering the analytical noise. The sample clean up procedure was thus sophisticated enough yielding a high signal-to-noise ratio. It was however very simple, making the method accessible to every laboratory.

2. Experimental

2.1. Reagents

Methanol, chloroform, dichloromethane, ethyl acetate, 1 *M* and 0.1 *M* sodium hydroxide, of analytical grade were from Carlo Erba (Milan, Italy). *N*-Methyl-*N*(trimethylsilyl)-trifluoracetamide (MSTFA) was from Regis (Morton Grove, IL, USA). 2-Mercaptoethanol and ammonium iodide (NH_4I) were purchased from Prolabo (Paris, France).

Boldenone, estradiol, ethinyl estradiol, metandienone, methyl testosterone, nandrolone, nandrolone decanoate, testosterone, testosterone cypionate, testosterone decanoate, testosterone phenyl propionate and testosterone propionate were obtained from Sigma–Aldrich (Saint-Quentin Fallavier, France). Testosterone undecanoate was a gift from Organon (Brussels, Belgium). Nandrolone d_3 was purchased from Cambridge Isotope Labs. (Andover, MA, USA).

The internal standard (I.S.) working solution was a mixture at 1 μg/ml in methanol of nandrolone d_3 ($I.S._1$ of non-esters) and testosterone phenyl propionate ($I.S._2$ of esters). This solution was stored at −20°C for one year in 1-ml screw cap vials.

Stock solutions of the anabolic steroids were prepared in methanol at 10, 1 and 0.1 μg/ml and stored in screw cap vials at −20°C for one year.

The derivatizing agent was a mixture of MSTFA–2-mercaptoethanol–NH_4I (1000:6:2, v/v/w) and stored at −20°C for two weeks in screw cap vial.

2.2. Instrumentation

The gas chromatograph was a 5890 series II plus from Hewlett-Packard (Les Ulis, France) equipped with an A200S autosampler from Finnigan Mat distributed by Thermo Quest (Les Ulis, France). The GC system was interfaced to a triple quadrupole mass spectrometer TSQ 7000 from Finnigan Mat (Manchester, UK). The analytical column was a CP SIL 8 CB, 30 m×0.25 mm I.D., 0.25 μm film thickness from Chrompack (Les Ulis, France). Helium was used as the carrier gas at a flow-rate of 1.1 ml/min in the constant flow mode (i.e., 56 kPa at 80°C). Pulsed splitless injection was done at 290°C and 180 kPa during 0.75 min. Injection volume was 2 μl. MS temperatures were: interface=300°C, ion source=160°C, quadrupole=70°C. The initial oven temperature was 80°C for 2 min and was increased to 310°C at 15°C/min and held for 14 min. The chromatographic run time was 31 min. The MS instrument was operated in the electronic impact ionization mode. The collision gas was argon at 1.7 mTorr (1 Torr=133.322 Pa). Dwell time per parent ion was set at 200 ms. Three retention windows were retained with, respectively, 4, 5 and 5 parent ions selected (t_R<17.8 min, t_R<21 min and t_R<30 min). Scan times were thus 0.8, 1 and 1 s. Parent ions corresponding to the molecular ion of the 14 compounds (except for ethinyl estradiol and metandienone) were selected with a mass resolution of 1.2 u. Two daughter ions were chosen based upon criterion of specificity and abundance and collected by the third quadrupole with a mass resolution of 1.7 u. These resolutions were judged adequate for optimizing the signal without a noticeable increase of the analytical noise. Table 1 summarizes the retention times (t_R values) relative retention times (RRTs), parent ions, daughter ions and collision energies of the screened molecules.

For SPE, we used NH_2, 500 mg/3 ml (Isolute) cartridges from IST supplied by Touzart et Matignon (Courtaboeuf, France), Silica 500 mg/3 ml (Isolute) cartridges from IST and a Vac Elut sample process-

Table 1
Main chromatographic and MS parameters for the 14 anabolic steroids

Compound	t_R (min)	RRT	Parent ion (m/z)	Collision energy (eV)	Daughter ion 1 (m/z)	Daughter ion 2 (m/z)
Nandrolone d_3 (I.S.$_1$)	17.3	–	421.2	−21	133	287
Nandrolone	17.3	1.002[a]	418.2	−21	182	194
Estradiol	17.5	1.012[a]	416.2	−21	129	285
Testosterone	17.6	1.013[a]	432.2	−21	209	301
Boldenone	18.0	1.038[a]	358.1	−16	122	147
Methyl testosterone	18.1	1.043[a]	446.2	−18	301	356
Ethinyl estradiol	18.2	1.047[a]	425.2	−18	193	231
Metandienone	18.6	1.072[a]	282.1	−16	122	161
Testosterone propionate	18.7	0.659[b]	416.3	−18	209	401
Testosterone cypionate	25.5	0.900[b]	484.3	−21	209	469
Nandrolone decanoate	26.4	0.931[b]	500.3	−21	182	194
Testosterone decanoate	27.1	0.956[b]	514.3	−21	209	499
Testosterone phenyl propionate (I.S.$_2$)	28.3	–	492.3	−21	209	477
Testosterone undecanoate	29.4	1.038[b]	528.4	−21	209	513

[a] Relative to nandrolone d_3.
[b] Relative to testosterone phenyl propionate.

ing station (Analytichem International) from Prolabo (Paris, France).

The ball mill was a Model EI 4000 from Kleco (Visalia, CA, USA).

2.3. Sample collection

Human hair is best collected from the area at the back of the head, called the *vertex posterior*. This area has less variability in hair growth rate than other areas. Moreover the hair is less subject to age and sex-related influences [39]. The sample size collected was at least 200–250 mg. When head hair was not available or too short, pubic or axillae hairs were collected.

Cows hair was collected at the time of slaughtering together with a 50-ml urine sample. The collection of hair was done using an electric clipper, Aesculap from Werke (Tuttlingen, Germany), which had a 7-cm blade. The hair was cut on the head of the animal. No distinction was made regarding hair color if the animal was a two-colored spotted cow.

The problem of the external contamination is very important for drugs of abuse as people can be exposed to a smoked or dusty environment. The approach is of course very different for anabolic steroids as the drugs are supposed to be normally taken orally or by another therapeutic route under a normal pharmaceutical presentation. According to the recommendation of the Society of Hair Testing, our washing procedure included a wash with methanol during 2 min followed by two washes with hot water during 3 min and by two 3-min long washes with dichloromethane [39,40].

The samples were then finely cut with scissors and processed in the ball mill for 6 min for cattle hair and 2 min for human hair.

2.4. Drug incorporation

Drug substances, which are present in the systemic circulation due to deliberate drug consumption and are taken up during histogenesis, represent the endogenous pathway. Molecules absorbed or transferred to the keratinized hair from perspiration (sweat, sebum and transdermal excretion) represent the endogenous–exogenous pathway. Finally, drugs deposited from the external environment (pollution, cosmetic hair treatments) can enter the keratinized hair by absorption and represent the exogenous pathway.

The mechanism generally proposed for the endogenous pathway of molecules into growing hair is passive diffusion from the blood supply at the base of the hair follicle. This favors the accumulation of undissociated basic drugs in the compartment with the lower pH. The isoelectric pH of hair being close to 6, clearly indicates the acidic nature of the hair [41], and thus the good incorporation of basic drugs.

Another important point is represented by the drug interaction with melanin. In contrast to matrix cells, the melanocytes are a highly specialized cell population. The intracellular pH for melanocytes has been estimated in the range 3–5 [42]. Accumulation of basic substances is thus favored in the cytosol. Being entrapped during melanin granule formation, a permanent concentration gradient will occur for drugs with a high melanin affinity and the influx into a melanocyte will be higher than into a matrix cell.

Howells et al. [43] have investigated the binding of several drugs (including clenbuterol, nandrolone, diethylstilbestrol and trenbolone) to melanin from *Sepia officinalis*. Basic and hydrophobic drugs were the most strongly bound. Desorption by ethanol was complete for neutral drugs but partial for the basic drugs. This suggests that the binding of these latter molecules also involves an ionic component. This also suggests that the methanolic sonication of a powdered hair sample should be an effective pretreatment of the matrix when testing for anabolic steroids. The same authors have measured the melanin capacity to be higher for clenbuterol (5.9 nmol/mg) than for the neutral hydrophobic drug nandrolone (0.56 nmol/mg). According to the melanin and acidic protein concentrations contained in white or black hair, it is natural to consider that black hair can concentrate basic drugs more efficiently while white hair could incorporate acidic and neutral drugs slightly more effectively. This was particularly true for clenbuterol as already noticed by several authors. Nevertheless, this difference in term of concentration between black and white hair should be less true for steroids. As an illustration of such an affirmation, Gleixner and Meyer measured testosterone and estradiol in white or black hair of female and male calves [35]. Mean results (*n* between 4 and

9) were 10 (±2), 11 (±7), 2 (±0.5) and 11 (±4) pg/mg for estradiol in females for white, yellow/red, gray and black hair, respectively. In females for testosterone, the results were 2 (±0.1), 5 (±4), 2 (±0.5) and 3 (±2) pg/mg, respectively. In male calves, estradiol concentrations were 4 (±0.4), 7 (±2), 7 (±3) and 4 (±1) pg/mg, respectively (*n* between 4 and 8), while testosterone levels were equal to 10 (±5), 5 (±2), 3 (±0.3) and 12 (±3) pg/mg, respectively, for white, yellow/red, gray and black hair. The sensitivity of testing hair for anabolic steroids must be certainly less color-dependent than it is usually observed for other drugs.

2.5. Sample pretreatment

Non-ester anabolic steroids are stable under strong alkaline conditions, therefore molar sodium hydroxide digestion seems to be an effective pre-treatment of the sample that would give the best recovery of the drugs from the solid hair. Unfortunately, esters of testosterone and of nandrolone are readily hydrolyzed. Extraction of the steroids from hair was thus divided into two steps.

A 100-mg amount of powdered hair were introduced in a screw cap glass tube together with 10 μl of I.S. working solution at 1 μg/ml (containing both nandrolone d_3 and testosterone phenyl propionate) together with 1.5 ml of methanol and sonicated in an ultrasonic bath for 2 h at 50°C. After centrifugation the methanolic fraction was decanted and evaporated to dryness under a stream of nitrogen at 50°C. The remaining hair was then digested by adding 2 ml of 1 *M* NaOH for 15 min at 80°C. This second step was used to compete the extraction of the non-esters anabolic agents as effectively as possible.

2.6. Sample clean up

2.6.1. Liquid–liquid extraction

Reconstitution of the dry residue obtained after the methanolic sonication was done with 2 ml of distilled water and 100 μl of 0.1 *M* NaOH. Addition of 2 ml of ethyl acetate allowed an efficient liquid–liquid extraction of the compounds of interest by gentle inversion of the tube for 10 min. After centrifugation the organic layer was removed in another clean glass tube. This liquid–liquid extraction was also conducted by the same way for the digested hair using the same volume of ethyl acetate. The two organic solvent fractions were combined in the same tube and allowed for further purification. The stability of esters was checked at room temperature for 1 h when diluted in 2 ml distilled water and 100 μl of 0.1 *M* NaOH. The degradation was always less than 2% for all compounds ($n=5$ at 100 pg/ml).

2.6.2. Solid-phase extraction on NH_2 cartridges

Purification of the steroids could be successfully obtained after fixation of ionized impurities on an amino column [44]. The cartridges were activated with 3 ml of ethyl acetate. Samples were thus deposited on the cartridges but the eluate containing the steroids were immediately collected since the compounds are not retained on this stationary phase. A final elution was done with 1 ml of ethyl acetate. Evaporation was done under a stream of nitrogen at 50°C.

2.6.3. Solid-phase extraction on silica cartridges

An ultimate purification could be obtained under normal-phase conditions. The previous residue was reconstituted with 1 ml of chloroform. After activation of the silica cartridges with 3 ml of chloroform, the esterified forms of testosterone and nandrolone could be directly collected after deposit of the sample on the column. A further elution was made with 1 ml of chloroform. Next, the non-ester steroids were eluted using 2×1 ml of chloroform–ethyl acetate (3:1, v/v). Other more polar impurities were still retained on the stationary phase. In the described protocol, the two fractions were combined (they could also be analyzed separately) and evaporated to dryness. The final residue was reconstituted with 20 μl of the derivatizing agent at 80°C for 20 min. Finally, 2 μl was injected into the chromatograph.

2.7. Derivatization

Some anabolic steroids do not exhibit good chromatographic behavior mainly because of the presence of hydroxyl and keto groups in their structure [45]. Among the several reagents described for the derivatization of hydroxyl groups, trimethylsilylation has been particularly used. MSTFA appeared as the reagent of choice. The presence of a catalyst is

however highly recommended to fully derivatize the molecules. Comprehensive methods involving NH_4I plus a reduction agent (dithioerythritol, ethanethiol, 2-mercaptoethanol) seemed to be preferable though utilization of trimethylsilyl iodide was also widespread. When analyzing trimethylsilyl (TMS) derivatives, the quality of the liner port and of the stationary phase of the column can strongly affect the chromatographic behavior of the compounds. The analytical column must be exclusively dedicated to the analysis of TMS derivatives as it could dramatically diminish the behavior of other derivatives.

2.8. Hair calibration

Drug-free hair samples were collected from screened human volunteers. Biological matrices were pooled, extracted and analyzed by the present methods. No peak was obtained which corresponded to the compounds of interest. The same sampling was done on cows of Charolaise breed which are white-colored cattle originating from the center of France.

Standard solutions of anabolic steroids were added producing concentrations of 1, 10, 50, 100 and 1000 pg/mg both for humans and cattle.

2.9. Surveillance of the bovine production in France

In order to check French beef for hormones, an interprofessional committee of ranchers decided to test slaughtered animals in different French regions. A veterinarian targeted animals and realized the sampling. One to two large tufts of animal hair were collected from the head area with an electric shaver. After butchering the animal, 50 ml of urine was collected directly from the bladder using a syringe and needle. All samples were sent to our laboratory within 24 h and analyzed during the week. To date, 173 analyses, as well as 14 others not directly dependent on the conditions described, have been completed for a total of 187 analyses. Urine testing was done according to protocol from directives issued by the Agriculture Minister [46,47].

3. Results of the analytical method

A complete analytical validation was carried out to ensure correct quantitation of the targeted drugs. This was done for two reasons (a) to evaluate in a further study if a cut-off value for the testosterone level could be established and (b) to investigate a possible correlation between drug dosage and concentration in hair.

3.1. Precision and recovery

The relative standard deviations (RSDs) of the within-day precision ($n=8$) were always less than 15.2% for each compound. RSDs of the between-day precision study ($n=10$) for cattle are given in Table 2 together with the overall recovery of the drugs measured at 100 pg/mg ($n=10$). Results with human hair as the sample are not significantly different from those reported for cattle. Figs. 1 and 2 (non-esters) and Fig. 3 (esters) display the chromatogram of an extract of 100 mg of cattle hair standard spiked with 50 pg/mg of each drug.

3.2. Linearity

Analysis of variance (ANOVA) is a powerful and very general method which separates the contributions to the overall variation in a set of experimental data and tests their significance. The sources of variations are each characterized by a sum of square (SS), i.e., the sum of a number of squared terms representing the variation in question, a number of degrees of freedom (df values), and a mean square, which is the former divided by the latter and which can be used to test the significance of the variation contribution by means of the F-test. In analytical calibration experiments, only variation in the y-direction is considered. This variation is expressed as the sum of the squares of the distances of each calibration point from the mean y value [48,49]. This is the total SS of y_{mean}: S_T. There are two contributions to this overall variation. One is the SS due to the regression: S_1, and the second source of variation is the SS about regression, i.e., residual: S_0. The residual variation can be divided itself into two contributions: lack-of-fit and pure analytical error

Table 2
Between-day precision study (%) and overall extraction recovery (%) in cattle hair ($n=10$)

Compound	RSD at 1 pg/mg	RSD at 10 pg/mg	RSD at 100 pg/mg	RSD at 1000 pg/mg	Recovery at 20 pg/mg
Nandrolone	16.4	13.0	9.7	8.7	94
Estradiol	NE[a]	21.2	12.4	9.8	93
Testosterone	NE	16.2	11.2	8.4	91
Boldenone	ND[b]	17.8	15.8	13.7	76
Methyl testosterone	18.8	16.9	12.4	10.4	93
Ethinyl estradiol	25.4	18.3	16.7	9.2	92
Metandienone	26.8	18.7	16.5	14.0	61
Testosterone propionate	15.6	12.3	11.7	9.2	81
Testosterone cypionate	26.0	25.4	12.6	11.5	80
Nandrolone decanoate	ND	22.3	17.9	13.6	80[c]
Testosterone decanoate	ND	20.5	11.5	8.9	79[c]
Testosterone undecanoate	ND	26.3	16.2	8.8	77[c]

[a] NE: Not evaluated because concentrations of testosterone and estradiol are generally superior or close to 1 pg/mg.
[b] ND: Not determined because inferior to LOD.
[c] Recovery was determined at 50 pg/mg.

[48]. For example, F_{cal} (methyl testosterone, human)=2866.1>>F_{theor}; F_{theor} is for (1, $n-2$) df= 5.32, so the source of variation is well described by the regression, F'_{cal} (methyl testosterone, human)= 2.31<F'_{theor}; F'_{theor} is for [$(n-2)-n/2$, $n/2$] df= 5.41, so the model (linear regression) can be considered as correct. The same assertion was verified for all compounds for the calibration in humans and in cattle: F_{cal} values were always superior to 396.1 whereas F'_{cal} was always found to be inferior to 4.12.

3.3. Choice of the model

After affirmation of linearity, the model of the linear regression must be chosen. In a wide dynamic range of calibration, the *y*-errors tend to increase as *x* increases. In other words, the variances are not homogeneous. Hartley's test (*r*) can be employed to easily decide on the necessity of a weighted linear regression in biomedical analysis. The calculated *r* value is the ratio between the highest and the lowest variance in the range of *k* points of calibration [50]. If the variances are non-homogeneous, the regression line must be a weighted one. The equation for this weighted line differs from classical equations because a weighting factor, ω_i, must be associated with each calibration point x_i, y_i. This factor is inversely proportional to the variance of y_i, s_i^{-2}. Calculation of the weighted regression lines is evidently more complex than a classical mean square computation. However, we must encourage such a model since it gives proper estimates of standard deviations and confidence limits when the weights vary significantly with x_i.

3.4. Accuracy

In a first step, a hair standard of known concentration is prepared (standard solutions of anabolic steroids were added producing concentrations of 1, 10, 50, 100 and 1000 pg/mg) and measured *n* times. From the values of the peak-areas, we can calculate the mean (*m*), and the standard deviation (SD) that we consider as being the true value [50].

In a second step, from the equation of the linear regression: $Y\omega=aX\omega+b$, we can calculate the measured peak-area corresponding to this point: y_t. Then we test the accuracy by the mean of a *t*-statistic, by comparing a mean to a point as follows: $t_{observed}=(m-y_t)/(SD/n^{1/2})$. If $t_{observed}<t_{table}$, the null hypothesis is accepted. In other words, the measured value and the true value are not different. t_{table} is given with $n-1$ df.

Using this procedure, only internal errors were

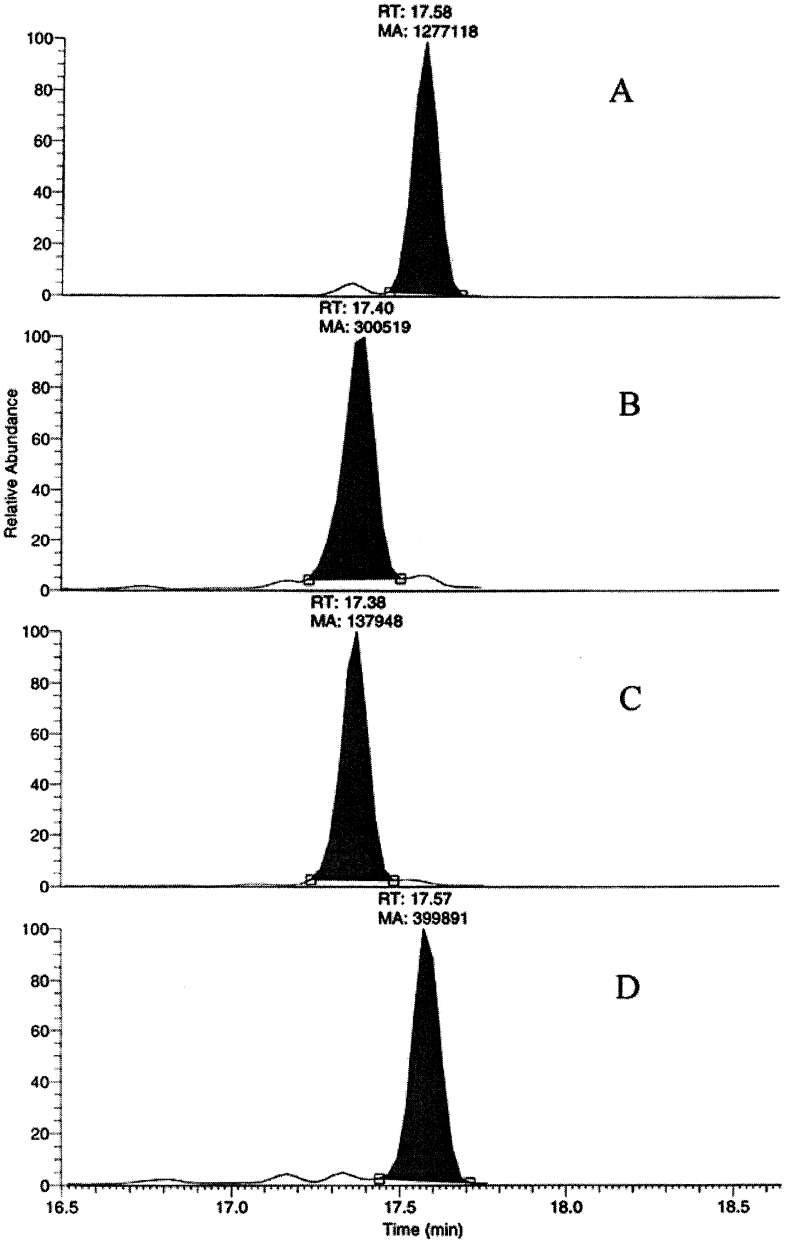

Fig. 1. Chromatogram of an extract of 100 mg of a standard powdered human hair spiked with 50 pg/mg of each drug. Peaks: A=estradiol, sum of ions m/z 129+285 daughters of m/z 416, B=nandrolone, sum of ions m/z 182+194 daughters of m/z 418, C=nandrolone d_3, sum of ions m/z 133+287 daughters of m/z 421, D=testosterone, sum of ions m/z 209+301 daughters of m/z 432.

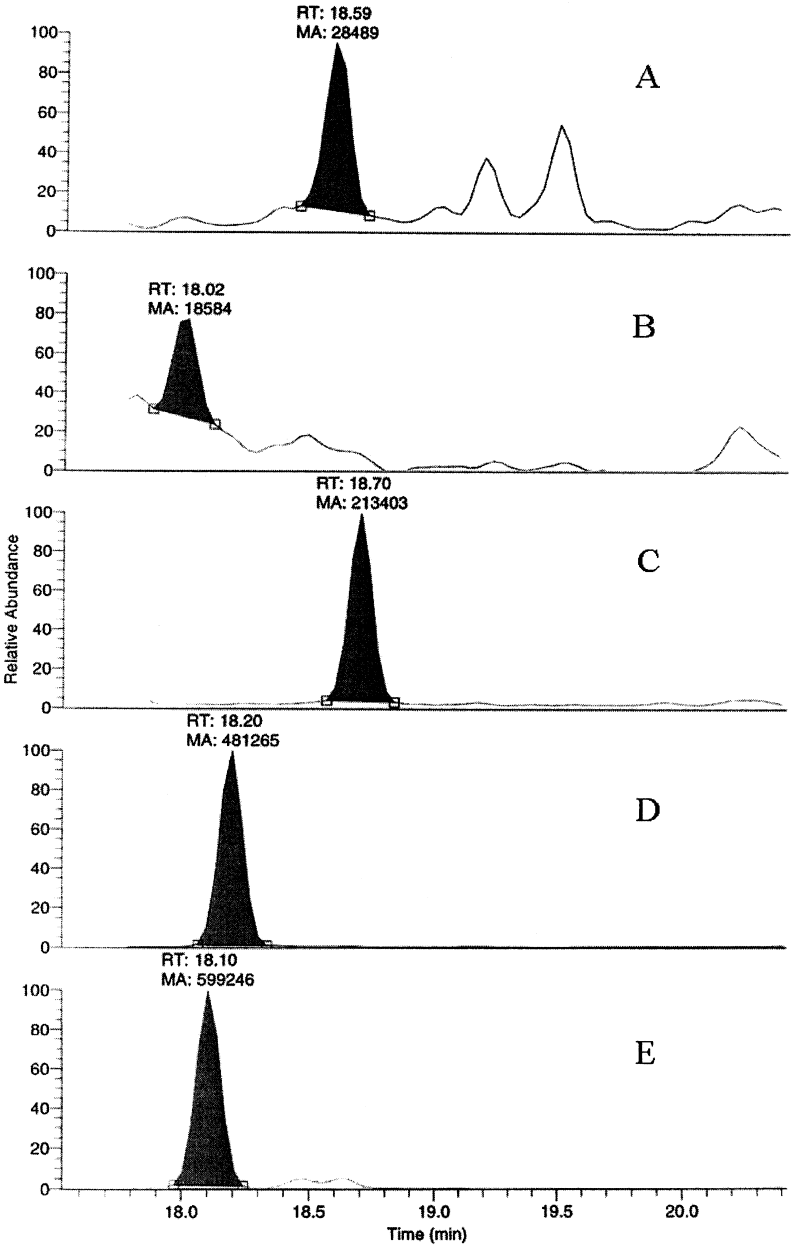

Fig. 2. Chromatogram of an extract of 100 mg of a standard powdered human hair spiked with 50 pg/mg of each drug. Peaks: A=metandienone, sum of ions m/z 122+161 daughters of m/z 282, B=boldenone, sum of ions m/z 122+147 daughters of m/z 358, C=testosterone propionate, sum of ions m/z 209+401 daughters of m/z 416, D=ethinyl estradiol, sum of ions m/z 193+231 daughters of m/z 425, E=methyl testosterone, sum of ions m/z 301+356 daughters of m/z 446.

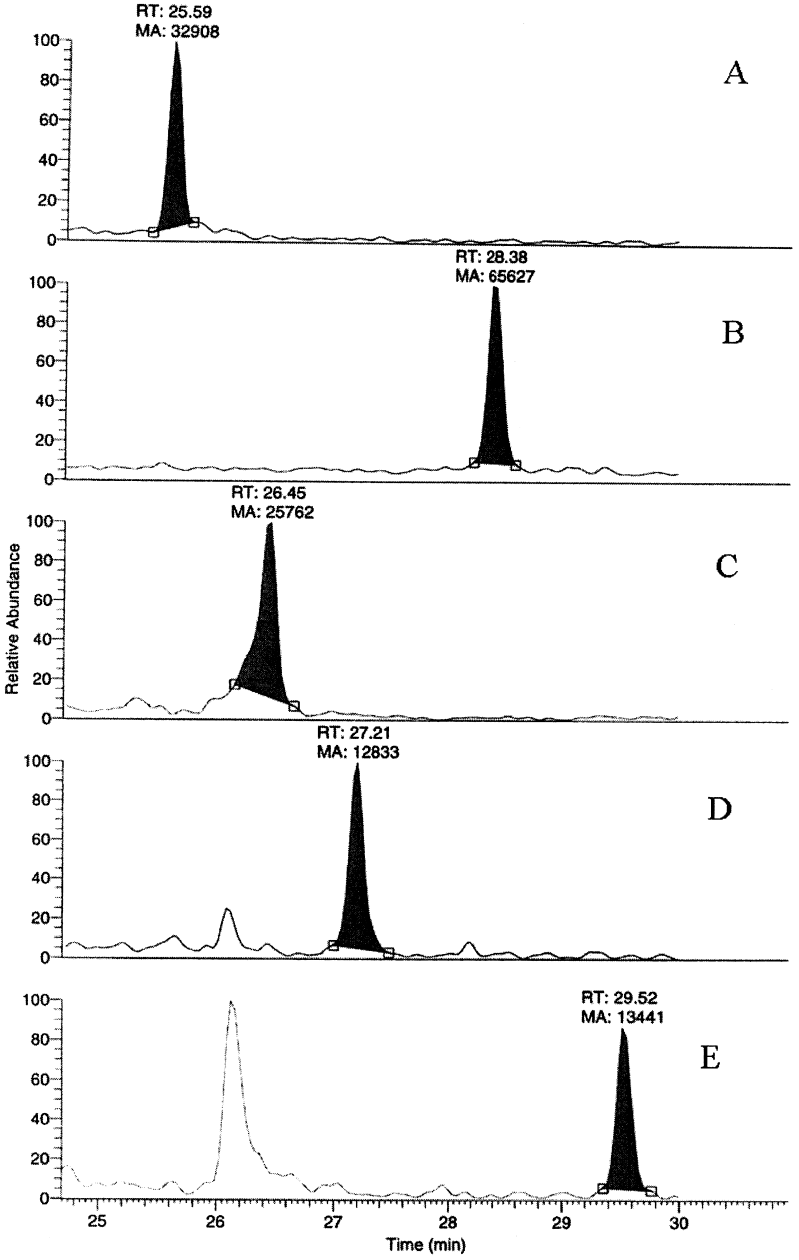

Fig. 3. Chromatogram of an extract of 100 mg of a standard powdered human hair spiked with 50 pg/mg of each drug. Peaks: A=testosterone cypionate, sum of ions m/z 209+469 daughters of m/z 484, B=testosterone phenyl propionate (I.S.$_2$), sum of ions m/z 209+477 daughters of m/z 492, C=nandrolone decanoate, sum of ions m/z 182+194 daughters of m/z 500, D=testosterone decanoate, sum of ions m/z 209+499 daughters of m/z 514, E=testosterone undecanoate, sum of ions m/z 209+513 daughters of m/z 528.

Table 3
Limits of detection (LODs) of anabolic steroids in cattle hair ($n=30$)

Compound	LOD (pg/mg)
Nandrolone	0.08
Estradiol	NR[a]
Testosterone	NR
Boldenone	6.20
Methyl testosterone	0.07
Ethinyl estradiol	0.15
Metandienone	2.10
Testosterone propionate	0.86
Testosterone cypionate	0.95
Nandrolone decanoate	1.90
Testosterone decanoate	3.10
Testosterone undecanoate	4.80

[a] NR: Not realized because blank hair sample for estradiol and testosterone are not available.

tested. Since the way for standard preparation was always the same, we cannot estimate the lack of accuracy due to the external errors (incorrect weight or volumes of standards or samples, or inaccurate dilution of primary standards).

Accuracy was tested at 10 and 100 pg/mg for both humans and cattle. Under these conditions, $t_{observed}$ was always inferior to $t_{table}=2.26$ ($\alpha=5\%$, $n=10$).

3.5. Limits of detection (LODs)

The LOD is given by the mathematical formula: $LOD=m_{blank}+6s_{blank}$, where m_{blank} is the mean of the blanks and s_{blank} corresponds to the SD of the blanks ($n=30$) [50,51]. Under these conditions, LODs are listed in Table 3.

4. Results in biological matrices

4.1. Application in doping control

Application in human doping control has been demonstrated in a judicial context of drug trafficking. In a series of 18 sportsman belonging to two different teams, two individuals were isolated as steroid users. One identified case was nandrolone at 5.1 pg/mg (see Fig. 4). Urinalysis gave negative results (less than 0.05 ng/ml) for norandrosterone (NA) and norethiocholanolone (NE) in a specimen sampled at the same time as the hair. Indeed, it has been very recently proven that an unknown endogenous route in adult males could produce these metabolites. LeBizec et al. [52] have detected a NA concentration in the range 0.05–0.60 ng/ml in more than 50% of the analyzed urine (from 40 persons known not to be treated with steroids). NE was never detected. Dehennin et al. [53] made the same remark on 30 healthy men free of anabolic supply, with NA concentrations ranging between 0.01–0.32 ng/ml. The origin of NA and NE in urine however, has never been proven as coming from nandrolone exclusively and the detection of these metabolites is maybe not related to the presence of nandrolone in human biofluids. Kintz et al. [54] have demonstrated that orally taken capsules of 19-norandrostenediol and 19-norandrostenedione led also to the urinary excretion of NA and NE. A positive result for nandrolone in hair should thus be considered as a highly potent positive case of nandrolone intake.

The second case concerned the detection of testosterone undecanoate at a concentration of 15.2 pg/mg (see Fig. 5). In the urine of the sportsman, the testosterone/epitestosterone ratio was clearly less than 6 since it was established to be 1.07.

When confronted by the results, the two sportsmen spontaneously recognized their doping practices.

We have measured the testosterone concentration in hair of 10 different drug-free male volunteers (aged between 22 and 31 years). It was in the range 1.7–9.2 pg/mg (mean 5.0 pg/mg, $n=10$). This was only a preliminary study that should be conducted later to verify if a possible cut-off could be established for this natural hormone. Such a study should envisage different ethnic populations, and various times of sampling (seasonal) on a significant number of volunteers.

4.2. Application in meat quality control

Of the 187 analyses performed, 11 were positives for methyl testosterone, one for metandienone, one for boldenone, three for nandrolone, two for testosterone propionate and five for testosterone decanoate (a methyl testosterone positive is given in Fig. 6). Mean measured concentrations and extreme figures are noted in Table 4. Prevalence of only one drug

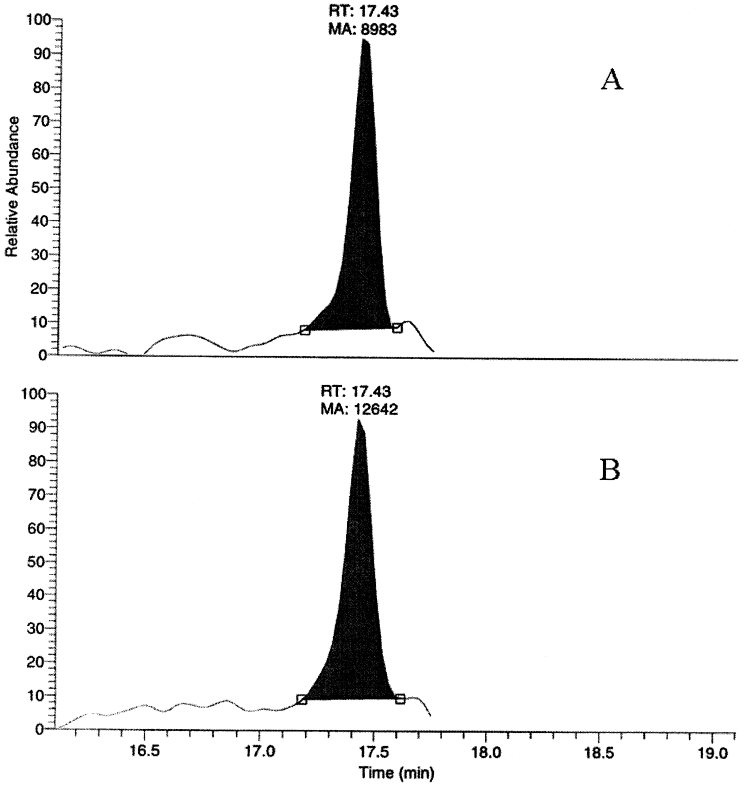

Fig. 4. Chromatogram of an extract of 50 mg of powdered hair taken in a sportsman tested positive for nandrolone at a measured concentration of 5.1 pg/mg. Peaks: A=ion m/z 182 daughter of m/z 418, B=ion m/z 194 daughter of m/z 418.

among all seeking molecules was extremely surprising. Methyl testosterone represents 48% of the positive cases (11 cases out of 23). Detection in urine of all specimens was also conducted by GC–MS and confirmed by GC–MS–MS. Results in this matrix were extremely eloquent since urine was never tested positive for the parent drugs or their metabolites (see Table 4).

The range of standard hair calibration could appear a little too wide since these results seem to indicate that the measured concentrations are contained between 1 and 100 pg/mg. In another case of growth promoter abuse however, we have measured methyl testosterone concentrations ranging from 0.7 to 4312 pg/mg (n=71). These measures were realized in white colored animals (Charolaise breed). In the same breed, measured 30 times on females, estradiol concentration was in the range 0.2–2.9 pg/mg (mean=1.4) while testosterone concentration was in the range 1.2–9.7 pg/mg (mean 4.5). For comparison, measured by HPLC–EIA, Gleixner and Meyer published an estradiol concentration of 1±0.2 pg/mg (n=5 and one isolated case at 121 pg/mg) and a testosterone concentration of 7±2 pg/mg (n=6) in white colored cows. This case at 121 pg/mg of estradiol found by the authors was not discussed. It could correspond to a real non-explained high level of estradiol, to an analytical interference with the enzyme immunoassay technique or to a growth promoting abuse using administration of estradiol or by an ester of the drug (the authors stipulated that the history or anamnesis of most of the animals was not known).

In the presented study, urine was never found positive out of 23 positives in hair. This suggests without ambiguity that urinalysis is an ineffective tool in testing growth promoting use in meat producing animals.

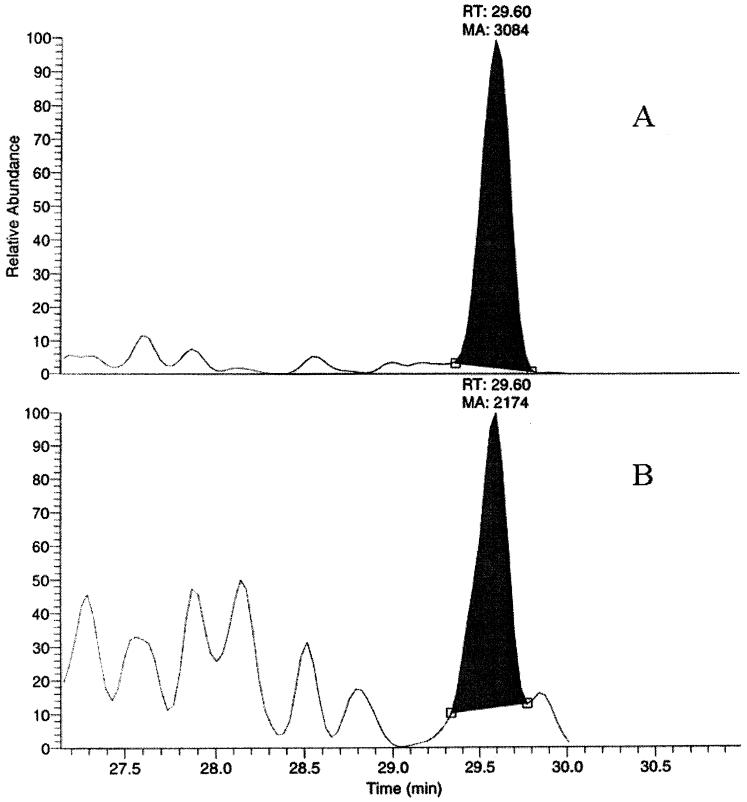

Fig. 5. Chromatogram of an extract of 50 mg of powdered hair taken in a sportsman tested positive for testosterone undecanoate at a measured concentration of 15.2 pg/mg. Peaks: A=ion *m/z* 209 daughter of *m/z* 528, B=ion *m/z* 513 daughter of *m/z* 528.

5. Conclusion

We have developed a simple GC–MS–MS method for the simultaneous measurement of several anabolic steroids in hair. This matrix appeared as the sample of choice for testing testosterone abuse in its ester forms, as it is the sole matrix that could document without ambiguity such a treatment. When testing for these banned substances, urinalysis has never gave a positive result out of two positives in hair for doping control and out of 23 for in meat producing animals. This suggests that urine testing is

Table 4
Results of hair analysis and urinalysis of 187 sampling taken in slaughter houses

Compound	Hair (pg/mg)			Urine (ng/ml)		
	n	Mean value	Extreme values	*n*	Mean value	Extreme values
Boldenone	1	48.6	–	0	–	–
Metandienone	1	15.2	–	0	–	–
Methyl testosterone	11	4.5	1.0–10.4	0	–	–
Nandrolone	3	34.1	15.2–45.2	0[a]	–	–
Testosterone decanoate	5	17.0	4.9–51.3	0	–	–
Testosterone propionate	2	4.5	2.9–6.1	0	–	–

[a] Tested urinary metabolite of nandrolone was epinandrolone derivatized with *N*-methyl-*N*-(*tert*.-butyldimethylsilyl)trifluoracetamide containing 1% *tert*.-butyldimethylchlorosilane mixed with 0.5% NH_4I, 7.5% acetonitrile and 2.5% pyridine.

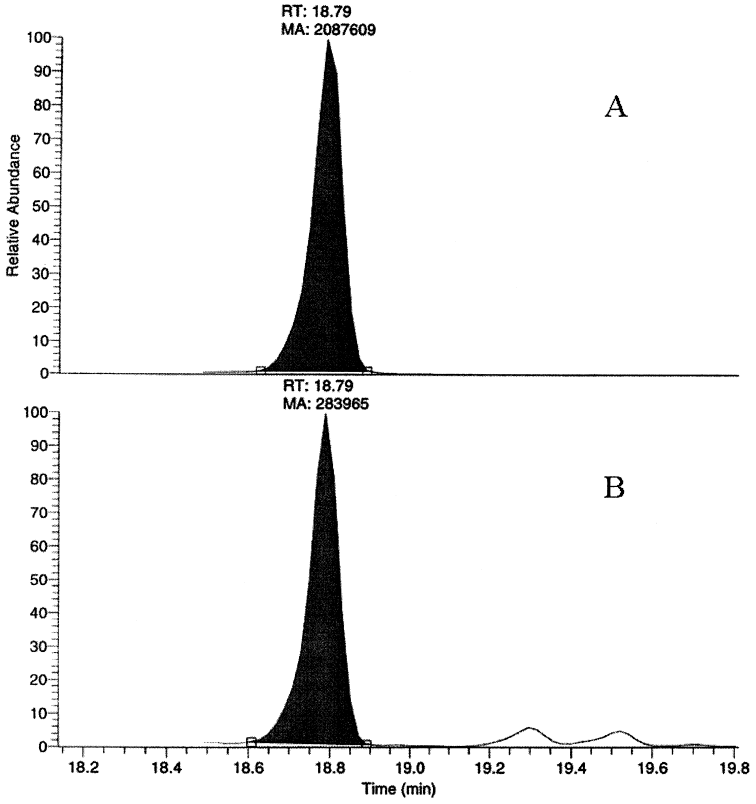

Fig. 6. Chromatogram of an extract of 100 mg of a powdered white colored cow hair tested positive for methyl testosterone measured at a concentration of 3.4 pg/mg. Peaks: A=ion m/z 301 daughter of m/z 446, B=ion m/z 356 daughter of m/z 446.

an ineffective tool when testing for such substances. The French Ministries of Agriculture and Justice have well understood the interest of the technique and have recently used hair testing for sports (drug trafficking) and food additives controls. The IOC still do not recognize the hair matrix.

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