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Pitfalls in trimethylsilylation of anabolic steroids New derivatisation approach for residue at ultra-trace level

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Abstract

Mixtures such as *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA), ammonium iodide and dithioerythreitol (DTE) or MSTFA, trimethyliodosilane and DTE were used for derivatisation of anabolic steroids extracted from 2 g kidney fat and present at ng kg^{-1} level. They are leading to unexpected products. Their identity and mechanism of formation have been discussed. A new silylation mixture was developed to overcome these pitfalls: *N*,*O*-bis-trimethylsilyl-acetamide was used in combination of 2.5% of MSTFA/I_2 (1000:10 (v/w)). A single product consisting in ether-TMS and/or enol-TMS derivative was observed for all tested steroids with a stability demonstrated for at least 48 h. Quantitative application was proved even at the low ng kg^{-1} level in a complex biological matrices, i.e. kidney fat.

Keywords: Gas chromatography–mass spectrometry; Androgens; Trimethylsilylation; Derivatisation; Enolisation

1. Introduction

Residue analyses in relation with public health, international trade or environment must be based on an integrated analytical chemistry approach. In this field, specific and sensitive methods have been developed and validated to detect and identify these analytes at trace levels in various biological matrices. Combination of gas chromatography and mass spectrometry (GC–MS) is preferred approach for steroid analysis because of its superior separation capabilities [1]. However, the protection of steroids is required before GC–MS analysis, and many reagents are available for this purpose. Trimethylsilyl (TMS) derivatives are probably the most widely employed [2–10]. A variety of trimethylsilylating reagents with different properties are available including TMS-halogen, TMS-amines, TMS-esters and TMS-amides [11,12]. The TMS amides, *N*,*O*-bis-trimethylsilyl-trifluoroacetamide (BSTFA) and *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA), are amongst the most popular. The derivatisation of hindered alcohol function is only possible when catalyst such as trimethylsilylimidazole (TMSIm) is added [13]. For keto-steroids, several approaches are classically used. Oxime derivatives is one possibility with formation of methoxime (MOX) derivative [14–16]. The other possibility is based on the enolisation of the keto group either by acylation with acid anhydride with ad hoc solvent or silylation combining classical silylating reagent and a catalyst such as potassium acetate [17] or trimethyliodosilane (TMIS) [18]. In both case, the molecular weight of the derivative is increased, its stability generally improved and its fragmentation consequently reduced; the sensitivity of the corresponding derivative is commonly enhanced. Alternative approaches to the classical TMIS catalyst have already been proposed; ammonium iodide (NH_4I) is often cited and used [19–25]. Addition of reduction agents such as dithioerythreitol, ethanethiol or 2-mercaptoethanol minimises the formation of iodine, and postpones the degradation of the derivatisation mixture.

The first objective of this work was to evaluate testosterone and estradiol metabolite residues in bovine edible

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tissues. Because of the very low residue concentrations involved (ng kg^{-1}) (ppt) [26], a quantitative method based on gas chromatography coupled to double focusing mass spectrometry ($R = 6000$) was developed for free forms in kidney fat. Quantification was performed using 17β-estradiol-d_3, $^{13}C_3$, 17β-testosterone and $^{13}C_3$, androstenedione as internal standards. Our first approach used derivatisation mixtures such as MSTFA/TMIS/DTE (1000:5:0.25 (v/v/w)) or MSTFA/NH_4I/DTE/CH_3CN (1000:5:0.25:100 (v/w/w/v)). As internal standards of testosterone or 4-androstenedione were not detected and multiple unexpected peaks were observed at the sub-nanogramme leading to confusion in the identification and quantification processes, we try to understand the mechanisms involved in the by-products making, and then, on this basis, to develop an improved derivatisation reagent to get a single and stable derivative product for each target steroid. Four groups of steroids were selected and studied; their structures covered the behaviour of most estrogens and androgens: steroids with alcohol functions (17β-estradiol, 17α-estradiol), steroids with hydroxyl and ketone functions (androsterone), steroids with hydroxyl and α-enone functions (17β-testosterone, 17α-testosterone) and steroids with ketone and α-enone functions (androstenedione).

2. Experimental

2.1. Chemicals and reagents

Reagents and solvents were analytical grade. Methanol, dioxide ethyl ether, acetonitrile, acetone, dichloromethane, cyclohexane, hexane and propanolol-2 were provided by Solvants Documentation Synthesis (SDS, Peypin, France). (2,3,4)$^{13}C_3$,androstenedione was synthesised in LABERCA (Nantes, France). 17β-Estradiol-16,16,17-d_3 (17βE_2-d_3) was supplied by RIVM (Bilthoven, The Netherlands). 17β-Estradiol, 17β-testosterone were purchased from Sigma–Aldrich (L'Isle d'Abeau Chesnes, France). Steraloids (Wilton, NY, USA) provided 17α-testosterone, 17α-estradiol, androstenedione and androsterone. Dr. Kockert from Institut für Organische Chemie (Wien, Austria) provided (2,3,4)$^{13}C_3$,17β-testosterone. MSTFA, BSTFA, TMIS, trimethylbromosilane (TMBrS) and NH_4I were purchased from Fluka (Buchs, Switzerland). Dithioerythreitol was from Aldrich (Milwaukee, WI, USA). *N*,*O*-Bistrimethylsilyl-acetamide (BSA) was from Pierce (Rockford, USA). Iodine (I_2) was obtained from Merck (Darmstadt, Germany).

2.2. Animals

Hereford steers (200 kg mean body weight) were monitored in this study. Kidney fat samples were collected and stored frozen at −18 °C until assay.

2.3. Gas chromatographic and mass spectrometric conditions

A JEOL (Tokyo, Japan) SX-102A double focusing mass spectrometer coupled to a Hewlett-Packard Model 5890 (Palo alto, CA, USA) gas chromatograph equipped with a A200SE autosampler (CTC Analytics, Zwingen, Switzerland) was used. The capillary column was a MDN 5S, 30 m × 0.25 mm i.d., 0.25 μm film thickness from Supelco. Helium was used as GC carrier gas at a flow rate of 1 mL/min. Sample injection of 2 μL was performed using the splitless mode (purge time = 1 min). The injector temperature was set at 270 °C. The oven temperature was programmed as following: 100 °C (2 min), 20 °C/min up to 230 °C (2 min), 5 °C/min up to 270 °C (0 min), 25 °C/min up to 300 °C (8 min). Transfer line were set at 280 °C. The mass spectrometer was operated at middle resolution (6000 at 10% valley) in the selected ion monitoring (SIM) acquisition mode after EI ionisation (70 eV). Source temperature was set at 280 °C. Perfluorokerosene (PFK) was used to provide a "lock" mass. Quantification of analytes was made focusing on *m*/*z* 416.256 (17β-estradiol-3,17-diTMS and 17α-estradiol-3,17-diTMS), *m*/*z* 419.275 (17β-estradiol-16,16,17-d_3-3,17-diTMS), *m*/*z* 430.272 (androstenedione-3,17-diTMS), *m*/*z* 432.288 (17β-testosterone-3,17-diTMS, 17α-testosterone-3,17-diTMS), *m*/*z* 433.282 ($^{13}C_3$,androstenedione-3,17-diTMS), *m*/*z* 435.298 ($^{13}C_3$,17β-testosterone-3,17-diTMS), *m*/*z* 434.303 (androsterone-3,17-diTMS), *m*/*z* 360.248 (17β-testosterone-17-TMS, 17α-testosterone-17-TMS), *m*/*z* 358.232 (androstenedione-3-TMS), *m*/*z* 362.268 (androsterone-3-TMS).

2.4. Sample preparation

Two gram fat of fat sample were introduced in a 50 mL centrifuge tube. After addition of 500 pg 17β-estradiol-$d_3$$^{13}C_3$,17(-testosterone and $^{13}C_3$,androstenedione as internal standards, steroid extraction was done using 5 mL ultra pure water/methanol (20:80 (v/v)). After agitation and centrifugation 30 min at 6000 × *g*, upper phase was collected and reduced to 500 μL under a gentle stream of N_2(4.5). 1.5 mL buffer acetate (0.2 M, pH = 5.2) were added. Liquid/liquid extraction was performed three times with 4 mL diethyl ether. The organic phase was evaporated to dryness and completed with 200 μL acetonitrile and 1.8 mL ultrapure water. Steroid purification was performed on SPE C18 (Ref 505471 (6 mL, 1 g), Supelco, L'Isle d'Abeau Chesnes, France) activated with 10 mL methanol and 10 mL ultrapure water. The column was rinsed with 5 mL ultrapure water. Analytes were eluted with 10 mL water/acetonitrile/acetic acid (35:65:0.5 (v/v/v)) and reduced to 1.5 mL. Alkaline liquid/liquid clean-up was applied: 1 mL sodium carbonate (10%) and 4 mL diethyl ether were added; upper phase was collected. This step was repeated twice and organic phase was evaporated before performing a further purification on a SPE Diol (Ref 57016 (3 mL), Su-

pelco). The cartridge was activated with 30 mL cyclohexane; after sample application, the stationary phase was rinsed with 4 mL cyclohexane and 4 mL cyclohexane/toluene (40:60 (v/v)). Steroids were eluted with 7 mL cyclohexane/dichloromethane/acetone (50:30:20 (v/v/v)). Final purification was performed on an HPLC column based on an Nucleosil® aminopropyle stationary phase (125 mm × 3 mm i.d., 5 μm particle, Macherey Nagel, Hoerdt, France). The mobile phase (hexane:isopropanol (v/v)) was delivered by an HP-1050 system at 2 mL/min. The composition was 95:5 from 0 to 15 min, 40:60 at 22.5 min, 40:60 from 22.5 to 25 min, 95:5 at 30 min and 95:5 from 30 to 35 min. The volume injected was 40 μL. Fractions 2–19 were collected and evaporated to dryness under a gentle stream of N_2.

2.5. Derivatisation

The final extract was transferred into an amber-coloured Chromacol glass vial with crimp top (0.7 mL) (Cluzeau Info Labo CIL, Sainte Foy La Grande, France) and evaporated to dryness under a gentle stream of N_2. Different derivatisation mixtures were tested:

- Protocol A: 20 μL of MSTFA/TMIS/DTE (1000:5:0.25 (v/v/w), 60 °C, 30 min).
- Protocol B: 20 μL of MSTFA/NH_4I/DTE/CH_3CN (1000:5:0.25:100 (v/w/w/v), 60 °C, 30 min).
- Protocol C: 20 μL BSA, then immediately completed with 0.5 μL MSTFA/I_2 (100:1 (v/w)) and finally heated for 30 min at 60 °C.

In order to evaluate a new derivatisation approach, many assays were achieved:

- Four vials containing each 1500 pg androsterone 17β-testosterone and 17α-estradiol were evaporated to dryness and derivatised respectively in 60 μL BSA (Protocol D), MSTFA (Protocol E). BSTFA (Protocol F). BSA/TMBrS/DTE (1000:5:0.25 (v/v/w)) (Protocol G). MSTFA/I_2(100:1 (v/w)) (Protocol H). Each vial was heated for 30 min at 60 °C and injected each 30 min.
- Protocol I: One vial containing 1500 pg of androsterone, 17α-estradiol, 17β-testosterone and androstenedione were derivatised in 60 μL BSA and then immediately completed with 1.5 μL MSTFA/I_2 (100:1 (v/w)). This vial was kept at room temperature, and injected each 30 min. After 18 h, the vial was heated 30 min at 60 °C and injected.
- Protocol J: Three vials containing respectively 50, 100 and 250 pg 17β-testosterone, androsterone, androstenedione and 17β-estradiol as well as 500 pg of each internal standard (17β-estradiol-d_3,$^{13}C_3$-17β-testosterone and $^{13}C_3$-androstenedione) were evaporated to dryness and derivatised in 20 μL BSA, then immediately completed with 0.5 μL MSTFA/I_2 (100:1 (v/w)) and finally heated for 30 min at 60 °C. Each vial was injected at t_0, t_0 + 2 h 30 min, t_0 + 20 h t_0 + 48 h.

For each protocol, septa were sealed by crimping an aluminium top (Ref 8AC3, Cluzeau Info Labo CIL, Sainte Foy La Grande, France).

3. Results and discussion

3.1. Formation of by-products following extracted steroids derivatisation with MSTFA/TMIS/DTE (Protocol A) and MSTFA/NH_4I/DTE/CH_3CN (Protocol B)

GC–MS ion chromatograms of kidney fat samples showed a weak repeatability of abundances of 3,17-diTMS derivatives of $^{13}C_3$,17β-testosterone and $^{13}C_3$,androstenedione, whereas 17β-estradiol-d_3 remained constant (internal standards were added at the same concentration in all samples). In some samples, $^{13}C_3$,androstenedione and $^{13}C_3$,17β-testosterone were not detected. Even when $^{13}C_3$,androstenedione and $^{13}C_3$,17β-testosterone were detected immediately after derivatisation, the injection of the same vial 24 h after showed the absence of the expected signals. The observation of the liner after few injections revealed a spot onto the wall, always at the same location. It was easily explainable by the needle structure which was bevelled so that the droplets were always sprayed with a perfect repeatability at the same position in the liner. Some authors [27] underlined in previous studies that the combination of MSTFA/TMXS (X = halogen) generated the corresponding highly reactive halogenide X^-. These nuclophilic halogenides present in the liner wall spot would be responsible of the weak repeatability of the signal especially observed at low residue levels (Fig. 1). The keto function is in fact highly polarisable and the enol tautomer is electrophilic. For steroids with α,β-unsaturated ketone (i.e. α-enone function), the enolisation generate the delocalisation of electron doublets: many electrophilic sites are available. Nucleophilic reagents in the reaction medium (and/or in the liner) can lead to attacks of there electrophilic sites. The nucleophilic attacks have been pointed out by numerous authors, and have been called either artefacts or silylation by-product [28]. Such phenomenon are also noted in cases of derivatisation of standards with reaction mixtures comparing to pure reagent The phenomenon is more obvious in crude sample comparing to pure standard. Co-extracted components probably play a crucial and unexpected role in the reaction [28–31]. Thus, the attack of a trimethylsilyl radical on the double bond of enol-trimethylsilyl ether for α,β-ketones such as testosterone [32] that subsequently eliminates a trimethylsilyl group was observed (Fig. 2).

We oriented our investigation toward the finding of a derivatisation mixture allowing enolisation and *O*-alkylation by $Si(CH_3)_3^+$ and avoiding these numerous secondary occurring nucleophilic attacks.

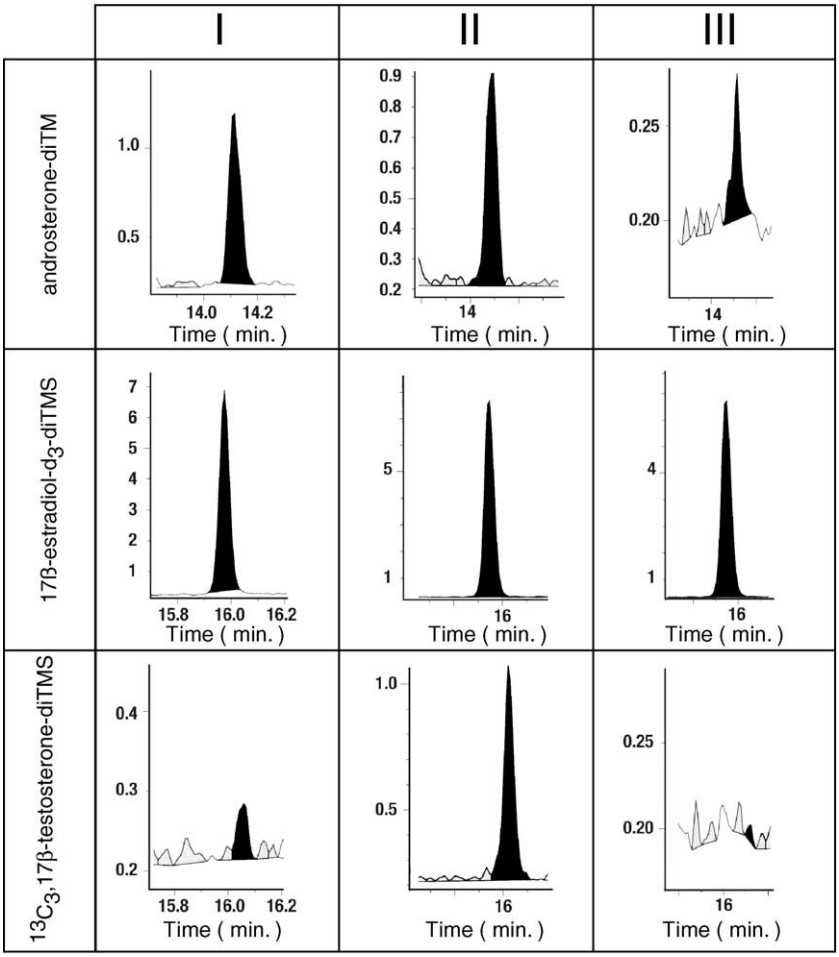

Fig. 1. Weak repeatability of androgen di-TMS derivatives (Protocol A). GC–MS chromatograms of 17β-estradiol-d_3-diTMS, $^{13}C_3$, 17β-testosterone-diTMS and androsterone-diTMS after injection of a extract fat (droplet was sprayed at the spot in the liner) (I). The same vial was immediately injected after rotating needle in the liner (droplet was sprayed at a clean position in the liner) (II). The same vial was then immediately injected after rotating needle in the liner (droplet was sprayed at the spot in the liner) (III).

3.2. Evaluation of a new derivatisation approach on standards

MSTFA, BSTFA and BSA permitted the introduction of a TMS group both in position 3 and 17 for estradiol (Fig. 3). For testosterone (α-enone), the 3,17-diTMS derivative was observed for BSA and MSTFA, as well as for BSTFA but with a lower yield in the last case. BSA was the only one leading to a second mesomer product (Fig. 3C). For androsterone (17-keto), BSA was the only reagent able to produce

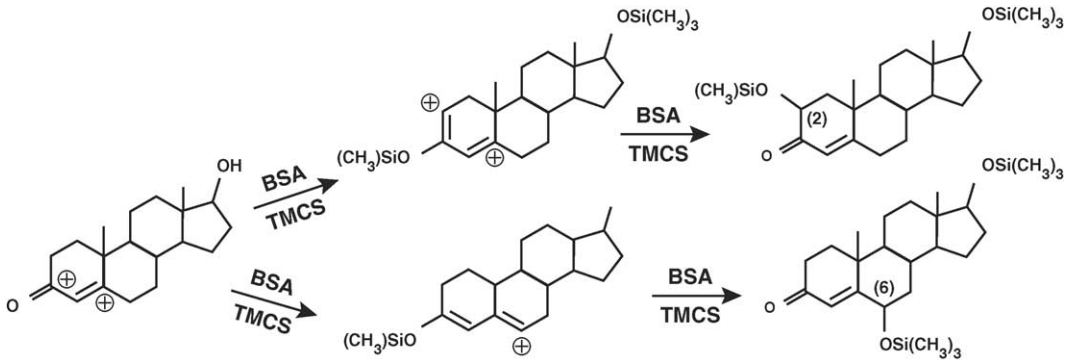

Fig. 2. Attack of a trimethylsilyl radical on the double bond of enol-trimethylsilyl ether for α,β-ketones such as testosterone [32]. The TMS positions (2) and (6) proposed by the authors are well explained by the high electrophilic character (⊕) of the C_2 and C_6 sites, due to the delocalisation of the electron doublets during enolisation and *O*-alkylation by $Si(CH_3)_3^+$.

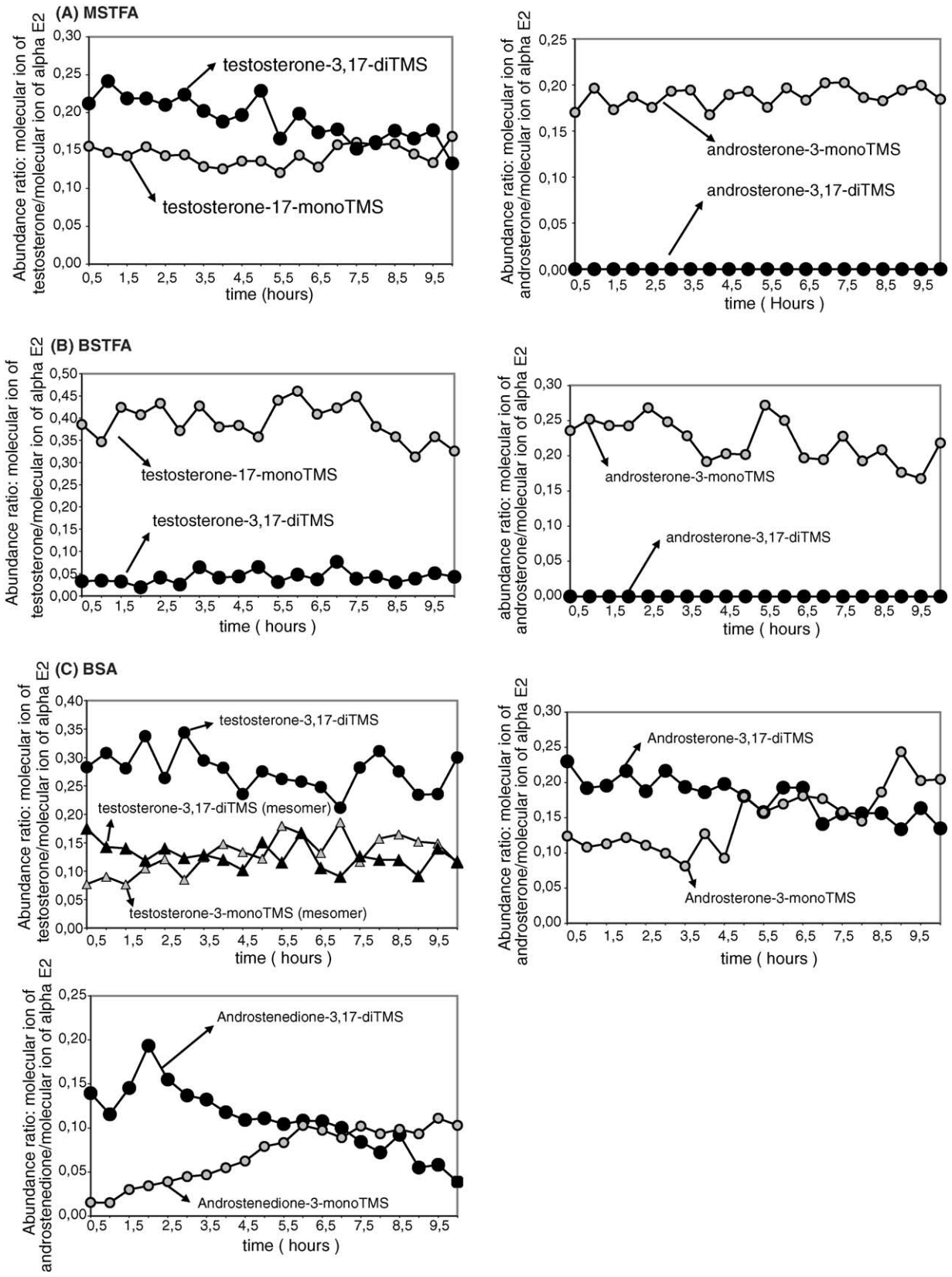

Fig. 3. Derivatisation kinetics of androsterone, androstenedione and 17β-testosterone with MSTFA (Protocol E), BSTFA (Protocol F) and BSA (Protocol D). Evolution of abundance ratio between molecular ion of each androgen (as underivateised, monoTMS and diTMS forms) and molecular ion of 17α-estradiol-diTMS. Androstenedione was not detected with MSTFA and BSTFA.

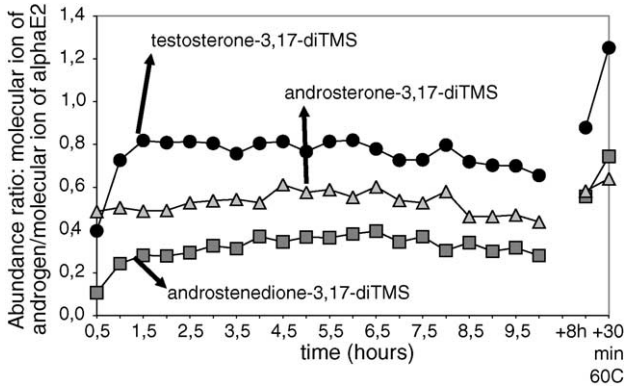

Fig. 4. Derivatisation kinetics of 17β-testosterone, androstenedione and androsterone in BSA and MSTFA/I_2 (Protocol I). Evolution of abundance ratio between molecular ion of 17β-testosterone-diTMS/17α-estradiol-diTMS, androstenedione-diTMS/17α-estradiol-diTMS and androsterone-diTMS/17α-estradiol-diTMS. After 18 h, vial was heated for 30 min at 60 °C and injected.

the 17-TMS-enol derivative whereas MSTFA and BSTFA led to the 3-ether-TMS product (Fig. 3C). These observations are undoubtedly linked to the basicity of BSA which is the strongest one amongst the three reagents (pK_a = 5.82 versus 1.34 (MSTFA) and 1.82 (BSTFA)). The TMS-enolisation of a keto function involved an effect in its first stage the transformation of the ketone into the corresponding enolate: alkaline conditions enhanced this reaction. The 17-keto group (ketone function) is more difficult to enolise than the corresponding 3-enone group (α-enone function). At this level, BSA is promising even if the first one led to two different derivatisation products. Nevertheless, the abundances of derivatised keto-steroids (measured on the total ionic current) was found far below those of diolsteroids such as estradiol. The next step was consequently to improve the production yield of 3,17-diTMS-ketosteroids with different catalysts and avoiding these numerous secondary occurring nucleophilic attacks. With a mixture BSA and TMBrS (a weaker halogenide as TMIS) (Protocol G), a single 3,17-di-TMS derivative was observed for testosterone, as already described (Fig. 3C) and the yield was found good with similar abundance than those detected for estradiol. However, the di-TMS derivative of testosterone was concluded unstable because of by-products production after 10 h. In conclusion, whatever the combination of MSTFA or BSA with TMIS or TMBrS, the ideal derivative was not observed for all tested estrogens and androgens. As the main problem was mainly linked to the instability of the derivative of α,β-unsaturated carbonyl, we decided to evaluate the behaviour of a mixture based on MSTFA and iodine (I_2) already used for a group of steroids particularly sensitive to this phenomenon, i.e. the estra-4,9,11-trien-3-one compounds, in other word trenbolone-like steroids [8,9]. Some preliminary assay with MSTFA/I_2 (100:1 (v/w)) (Protocol H) [9] on 17β-testosterone led to the non-observation of the expected derivative (*m*/*z* 432.2880), and rather oxidised products. The substitution of MSTFA by BSA was made impossible because of the natural unsolubility of I_2 in BSA. The pre-solubilisation of I_2 with solvent such as dichloromethane or DMF did not lead to acceptable results. Our decision was to solubilise I_2 in MSTFA (1:100 (w/v)), and then to add a trace of this so-called catalyst into BSA (0.5:20 (v/v)) (Protocol I).

A unique 3,17-diTMS derivative for testosterone was synthesised and the derivatisation reaction was characterised by an excellent yield but only after the final heating (Figs. 4 and 5). The derivative was found stable at least 20 h. The derivatisation of the α-enone is quasi instantaneous whatever the concentration of 17β-testosterone (Fig. 6). The derivatisation of the alcohol function is total, immediate and stable whatever the concentration of steroid in the medium, as shown for estradiol (Fig. 6). The full derivatisation of ketone function is only achievable after 48 h (Fig. 6). The corresponding 3,17-diTMS derivative was found stable in time. These kinetics showed how difficult it is to determine optimal conditions (time, temperature, ...) for a wide range of steroids. For instance, the increasing of the temperature would be favourable to the formation of the 17-enol-TMS for androsterone, but would be probably responsible for by products formation in α-enone steroids such as testosterone or androstenedione. As a general rule, the quantification of these

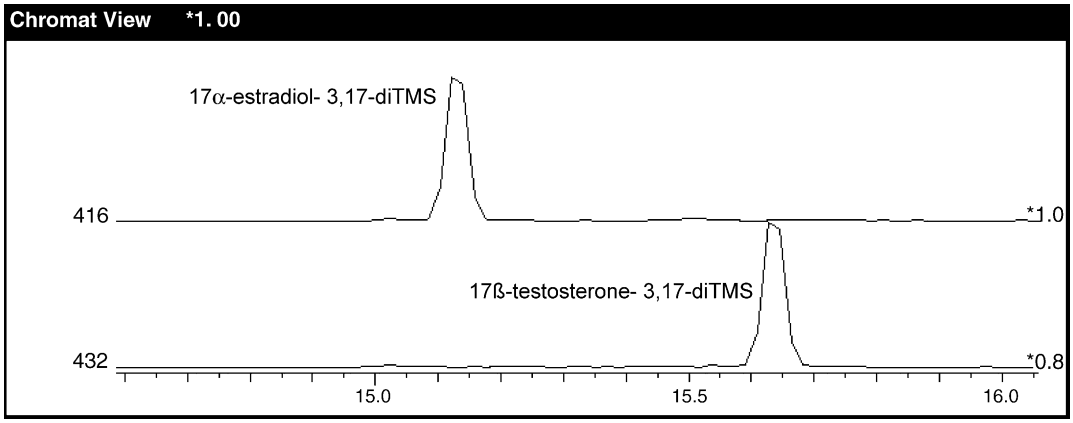

Fig. 5. GC–MS chromatograms of 17β-testosterone-3,17-diTMS and 17α-estradiol-3,17-diTMS derivatised in BSA and MSTFA/I_2 (Protocol C). An unique 3,17diTMS derivative was synthetised for each steroid with an excellent yield: abundance ratio of molecular ion of 17β-testosterone-diTMS/17α-estradiol-diTMS = 1.2.

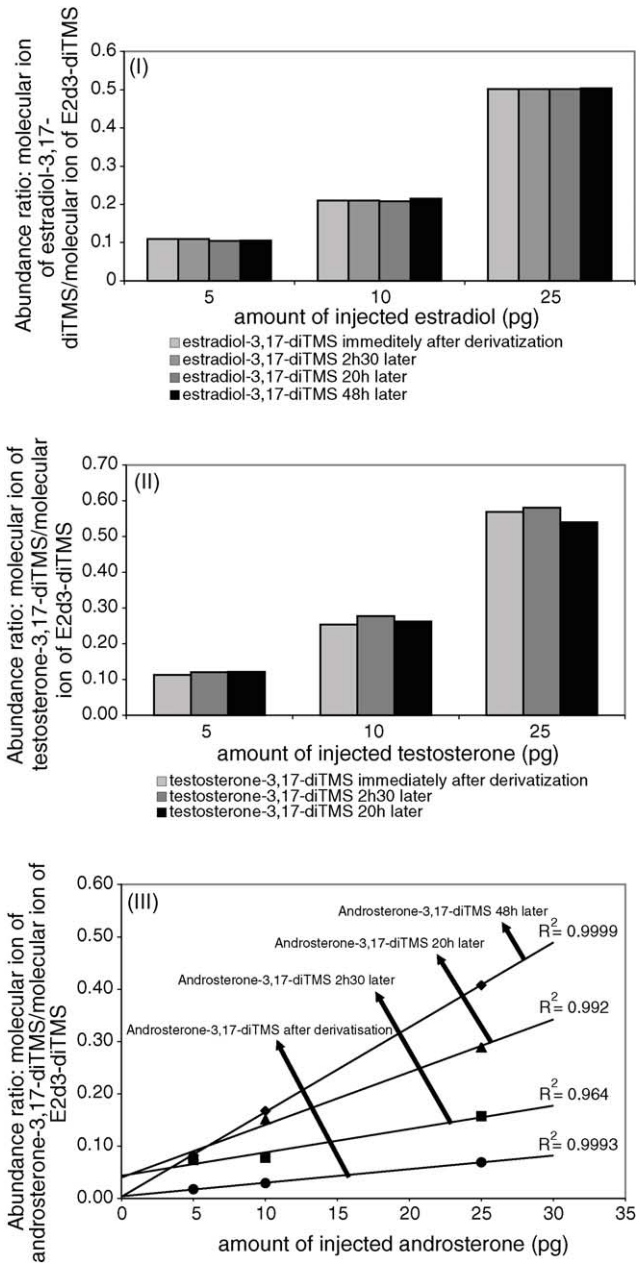

Fig. 6. Response linearity and derivative stability of 17β-estradiol (I), 17β-testosterone (II) and androsterone (III) after derivatisation in BSA and MSTFA/I_2 (Protocol J).

keto-steroids should implicate the systematic use of the corresponding internal standards, and preferably ^{13}C-labelled compounds to avoid any proton-deuterium exchanges as frequently observed with ^{2}H-labelled steroids. The derivatisation would be more practical if extemporaneous mixture of BSA/MSTFA/I_2 would be used but the quantitative formation of the corresponding 3,17-diTMS ketosteroids is worse. It means that the pre-action of BSA is essential. Finally, this mixture conducts to a decreasing in time of 3,17-diTMS derivatives of androstenedione and testosterone, and formation of oxidised TMS derivatised steroids with signals at *m/z* 518, 520, 522 for androstenedione, testosterone and androsterone respectively. Concentrations of this oxidised TMS derivatives were too low to obtain representative full scan mass spectra.

3.3. *Performances of derivatisation with BSA and MSTFA/I_2 (100:1 (v/w)) (Protocol C) on fat extracts*

The chemical behaviour described for steroids in standard preparation was identically observed in real matrix extracts for alcohol functions (estradiol, androstenediols) in terms

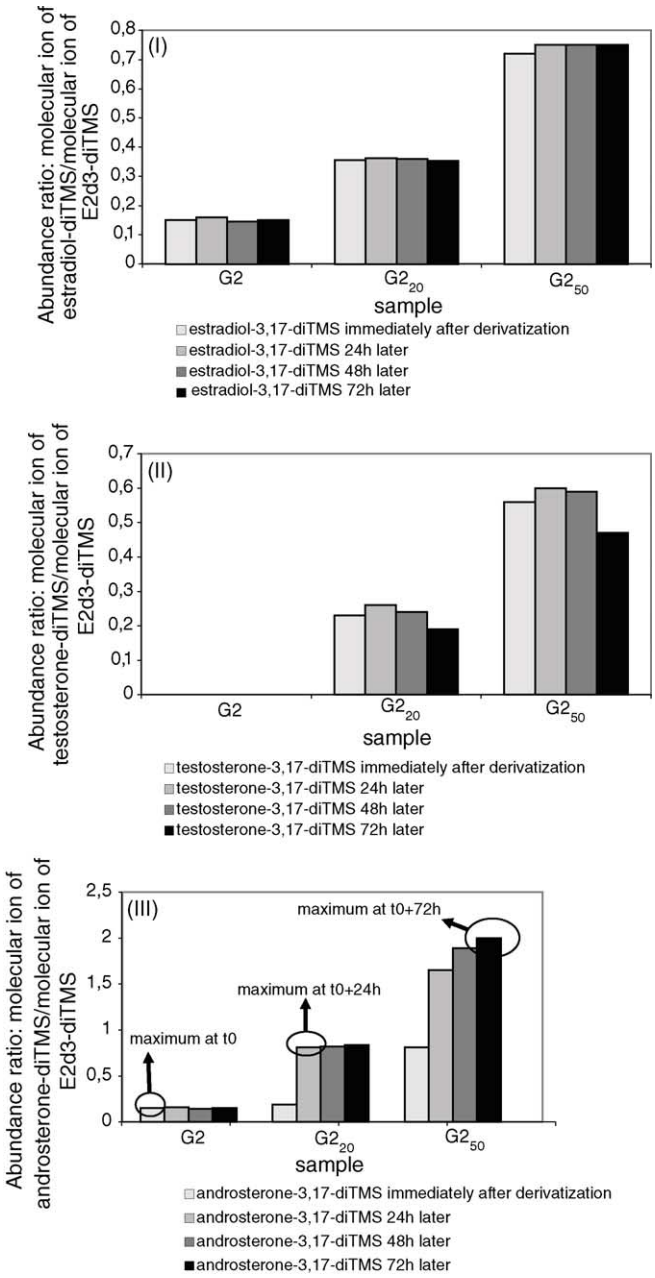

Fig. 7. Response linearity and di-TMS derivative stability of extracted 17β-estradiol (I), 17β-testosterone (II) and androsterone (III) after derivatisation in BSA and MSTFA/I_2 (Protocol C). Steroids were extracted from perirenal fat sample (G2) and spiked G2 at 20 ppt (G2$_{20}$) and 50 ppt (G2$_{50}$). Samples were analysed as described in Section 2.4.

of stability, reaction time (Fig. 7). For 17β-testosterone and androsterone, the necessary derivatisation time for α-enone function and mainly unconjugated ketone is clearly more important on matrix extract (Fig. 7). The presence of multiple co-extracted interferences able to be derivatised would delay the efficient derivatisation of steroid analytes. Repeatability has been daily assessed with the analysis of two spiked fat samples at very low levels (i.e. 20 and 50 ppt) (quality control samples) (Fig. 7) and a systematic calibration curve containing ultra-trace levels of target steroids (four points: 0, 5, 10 and 25 pg injected). The linearity was found excellent ($R^2 = 0.99$ for 17β-estradiol and 17β-testosterone, $R^2 = 0.98$ for androsterone), the limit of detection were in the order of 5 ng kg^{-1} (17β-estradiol), 10 ng kg^{-1} (17β-testosterone) and below 30 ng kg^{-1} (androsterone). This new derivatisation mixture is able to quantify main natural estrogenic and androgenic steroids in complex biological matrices even at the low ng kg^{-1}.

4. Conclusion

Mixtures such as MSTFA/TMIS/DTE, MSTFA/NH_4I/DTE, MSTFA/TMBrS/DTE, BSTFA/TMIS/DTE and BSA/TMBrS/DTE for derivatisation of steroids with ketone and especially α-enone functions at ng kg^{-1} levels are leading to by-products This artefact formation is due to the attack of electrophilic sites of ketosteroids by different nucleophilic reagents in derivatisation mixtures. Although the results described in this paper are inadequate to obtain fully detailed mechanistic overview on such attacks in steroid analysis, they illustrate the still insufficient knowledge of the silylation mechanism. Moreover, these artefacts lead to confusion in the identification and quantification processes [28,31]. A new derivatisation reagent mixture consisting in BSA then completed with (MSTFA/I_2) (100:1 (v/w)) in proportion to (20:0.5 (v/v)) and finally heated for 30 min at 60 °C was developed for the simultaneous trimethylsilylation of a wide range of natural steroids with various chemical functions at ng kg^{-1} levels in kidney fat. The derivative proved to be unique and stable for each steroid; the developed reaction can be considered simple a robust. For quantitative purposes, the linearity was proved even at ng kg^{-1} levels in a complex biological matrice, i.e. kidney fat. As the kinetic is dependent of the substrate concentration, the main difficulty is to decide a priori the necessary time for complete derivatisation of unconjugated keto function especially when the amount of co-extracted molecules is not known. Nevertheless, the use of ad hoc labelled internal standards can easily overcome this difficulty.

References

[1] B. Le Bizec, P. Marchand, D. Maume, F. Monteau, F. André, Chromatographia 59 (2004) S3.
[2] D.R. Knapp, Handbook of Analytical Derivatisation Reactions, Wiley, New York, 1979, p. 741.
[3] E. Houghton, G.A. Oxley, M.S. Moss, S. Evans, Biomed. Mass Spectrom. 5 (2) (1978) 170.
[4] L. Aringer, P. Eneroth, J.A. Gustafsson, Steroids 17 (3) (1971) 377.
[5] M. Donike, J. Chromatogr. 42 (1969) 103.
[6] R. Massé, C. Laliberté, L. Tremblay, J. Chromatogr. 339 (1985) 11.
[7] B. Le Bizec, S. Le Boulaire, M.-P. Montrade, D. Maume, F. Monteau, G. Audusseau, F. André, in: N. Haagsma, A. Ruiter (Eds.), Proceedings of Euroresidue III Conference, Veldhoven, 1996, p. 243.
[8] D. De Boer, M.E. Gainza Bernal, R.D. van Ooyen, R.A.A. Maes, Biol. Mass Spectrom. 20 (1991) 459.
[9] D. Maume, B. Le Bizec, P. Marchand, M.P. Montrade, F. André, Analyst 123 (1998) 2645.
[10] A.E. Pierce, Silylation of Organic Compounds, Pierce Chemical Company, Rockford, IL, 1968, p. 332.
[11] R.P. Evershed, in: K. Blau, J.M. Halket (Eds.), Handbook of Derivatives for Chromatography, second ed., Chichester, Wiley, 1993, p. 51.
[12] G. van Look, Silylating Agents, Fluka Chemie AG, Buchs, Switzerland, 1995;
J. Segura, R. Ventura, C. Jurado, J. Chromatogr. B 713 (1998) 66.
[13] R.P. Evershed, J.G. Mercer, H.H. Rees, J. Chromatogr. 390 (1989) 357.
[14] V.P. Uralets, P.A. Gillette, B. DuVall, R.K. Latven, in: M. Donike, H. Geyer, A. Goltzmann, U. Mareck-Engelke (Eds.), Recent Advances in Doping Analysis, vol. 3, Sport and Buch Strauss, Editio Sport, Köln, 1996, p. 83.
[15] C.H.L. Shackleton, J. Chromatogr. 379 (1986) 91.
[16] M. Ueki, A. Ikekita, M. Fujisaki, T. Hiruma, M. Okano, in: M. Donike, H. Geyer, A. Goltzmann, U. Mareck-Engelke (Eds.), Recent Advances in Doping Analysis, vol. 3, Sport and Buch Strauss, Editio Sport, Köln, 1996, p. 115.
[17] S.G. Webster, J. Chromatogr. 333 (1985) 186.
[18] M. Donike, H. Geyer, A. Goltzmann, M. Kraft, F. Mandel, E. Nolteernsting, in: P. Belloti, G. Benzi, A. Ljungqvist (Eds.), Proceedings International Athletic Foundation World Symposium on Doping in Sport, International Athletic Foundation, 1988, p. 53.
[19] W. Schänzer, S. Horning, G. Opfermann, M. Donike, J. Steroid Biochem. Mol. Biol. 57 (1996) 363.
[20] R. Massé, C. Ayotté, R. Dugal, J. Chromatogr. 489 (1989) 23.
[21] R. Massé, H. Bi, P. Du, Anal. Chim. Acta 247 (1991) 211.
[22] W. Schänzer, M. Donike, Biol. Mass Spectrom. 21 (1992) 3.
[23] W. Schänzer, H. Geyer, M. Donike, J. Steroid Biochem. Mol. Biol. 38 (1991) 441.
[24] J. Segura, J.A. Pascual, R. Ventura, J.I. Ustaran, A. Cuevas, R. Gonzalez, Clin. Chem. 39 (1993) 836.
[25] S. Prevost, T. Nicol, F. Monteau, F. André, B. Le Bizec, Rapid Commun. Mass Spectrom. 15 (2001) 2509.
[26] D. Maume, Y. Deceuninck, K. Pouponneau, A. Paris, B. Le Bizec, F. André, APMIS 109 (2001) 32.
[27] R. Massé, C. Ayotte, R. Dugal, J. Chromatogr. 489 (1989) 23.
[28] J.L. Little, J. Chromatogr. A 844 (1999) 1.
[29] E.C. Horning, G.M. Maume, Tetrahedron Lett. 5 (1969) 343.
[30] G. Opfermann, W. Schänzer, in: W. Schanzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (Eds.), Proceedings of the 14th Cologne Workshop on Dope Analysis, Sport and Buch Strauss, Cologne, 1996, p. 247.
[31] D.H. van de Kerkhof, R.D. van Ooijen, D. de Boer, R.H. Fokkens, N.M.M. Nibbering, J.W. Zwikker, J.H.H. Thijssen, R.A.A. Maes, J. Chromatogr. A 954 (2002) 199.
[32] E.M. Chambaz, G. Maume, B. Maume, E.C. Horning, Anal. Lett. 1 (1968) 749.