Anal Bioanal Chem (2011) 399:861–875
DOI 10.1007/s00216-010-4319-y

ORIGINAL PAPER

Accelerated sample treatment for screening of banned doping substances by GC–MS: ultrasonication versus microwave energy

M. Galesio · M. Mazzarino · X. de la Torre · F. Botrè · J. L. Capelo

Received: 18 June 2010 / Revised: 15 September 2010 / Accepted: 10 October 2010 / Published online: 29 October 2010

Abstract A comparison between ultrasonication and microwave irradiation as tools to achieve a rapid sample treatment for the analysis of banned doping substances in human urine by means of gas chromatography–mass spectrometry (GC–MS) was performed. The following variables were studied and optimised: (i) time of treatment, (ii) temperature, (iii) microwave power and (iv) ultrasonic amplitude. The results were evaluated and compared with those achieved by the routine method used in the World Anti-Doping Agency (WADA) accredited Antidoping Laboratory of Rome. Only under the effect of the ultrasonic field was it possible to enhance the enzymatic hydrolysis reaction rate of conjugated compounds. Similar reaction yield to the routine method was achieved after 10 min for most compounds. Under microwave irradiation, denaturation of the enzyme occurs for high microwave power. The use of both ultrasonic or microwave energy to improve the reaction rate of the derivatisation of the target compounds with trimethyliodosilane/methyl-*N*-trimethylsilyltrifluoroacetamide (TMSI/MSTFA/NH_4I/2-mercaptoethanol) was also evaluated. To test the use of the two systems in the acceleration of the reaction with TMSI, a pool of 55 banned substances and/or their metabolites were used. After 3 min of ultrasonication, 34 of the 55 compounds had recoveries similar to those obtained with the classic procedure that lasts for 30 min (Student's *t* test, $n=5$), 18 increased to higher silylation yields, and for the compounds 13β,17α-diethyl-3α,17β-dihydroxy-5α-gonane (norboletone metabolite 1), metoprolol and metipranolol the same results were obtained increasing the ultrasonication time to 5 min. Similar results were obtained after 3 min of microwave irradiation at 1,200 W. In this case, 30 of the 55 compounds had recoveries similar to the classic procedure (Student's *t* test, $n=5$) whilst 18 had higher silylation yields. For the compounds 3α-hydroxy-1α-methyl-5α-androstan-17-one (mesterolone metabolite 1), 17α-ethyl-5β-estrane-3α,17β,21-triol (norethandrolone metabolite 1), epioxandrolone, 4-chloro-6β,17β-dihydroxy-17α-methyl-1,4-androstadien-3-one (chlormetandienone metabolite 1), carphedon, esmolol and bambuterol the same results were obtained after 5 min under microwave irradiation.

Keywords Doping substances · GC–MS · β-Glucuronidase hydrolysis · MSTFA derivatisation · Ultrasonic energy · Microwave energy · Sample treatment · Anti-doping

M. Galesio · J. L. Capelo
REQUIMTE, Departamento de Química,
Faculdade de Ciências e Tecnología,
Universidade Nova de Lisboa,
2829-516 Monte de Caparica, Portugal

J. L. Capelo (✉)
BIOSCOPE Group, Physical Chemistry Department,
Science Faculty, University of Vigo,
Ourense Campus,
32004 Vigo, Spain
e-mail: jlcapelom@uvigo.es

M. Mazzarino · X. de la Torre · F. Botrè
Laboratorio Antidoping di Roma,
Federazione Medico Sportiva Italiana,
Largo Giulio Onesti 1,
00197 Rome, Italy

Introduction

The global fight against doping is becoming a problem of increasing complexity. To protect safe and fair competition, antidoping organisations together with the national antidoping laboratories are making a huge effort to put into

practice in and out of competition programs for the control of banned substances. Furthermore, every year, new substances are added to the World Anti-Doping Agency (WADA) list of banned substances and, consequently, laboratory analyses are becoming more complex, which may result in increasing workloads, delaying reports and raising costs of each test.

Doping control of androgenic anabolic steroids (AAS) and other banned substances, such as diuretics, stimulants, β_2-agonists and beta-blockers, is based on the detection of such compounds and their metabolites in urine samples from athletes [1–3]. In urine, these compounds are mostly in their conjugated form [4, 5]. The main pathway for conjugation and inactivation of these substances in the human body is glucuronidation [5, 6]. Therefore, most compounds will be excreted in urine as glucuronate conjugates. Currently, most methods for routine detection of these compounds and their metabolites, comprising both screening and confirmatory analysis, are based on chromatographic-spectrometric techniques, mainly GC–MS techniques [2, 7–11]. It is important to stress that to be analysed by GC–MS techniques, the target analyte must be both volatile and thermally stable. Since most of the banned substances mentioned above, either in the conjugated or free form, do not fulfil this requirement, as a general rule, the analysis of such substances by GC–MS techniques requires a (i) previous enzymatic hydrolysis of the conjugating groups to produce the free parent compound, and (ii) the derivatisation of the free analyte to enhance its volatility, thermal stability and mass spectrometry properties [12, 13]. These two steps of the sample treatment play a critical role in the analysis of doping substances, especially in terms of time and, more importantly, analytical sensitivity. Both steps are enhanced by conventional heating. The cleavage of the glucuronide moiety to produce the free compound is performed at 55 °C for 1 h with the β-glucuronidase enzyme from *E. coli*, which is highly specific for β-glucuronides [5]. After hydrolysis, the incorporation of a trimethylsilyl (TMS) group in the free analyte is performed at 78 °C for 30 min with methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), the most popular derivatisation reagent routinely used by the WADA accredited laboratories [14, 15]. These experimental conditions have been reported by Ali Shareef and co-workers as the optimum conditions to achieve maximum derivatisation yield; yet for a few compounds, generally those with more than one position available for the TMS group, the formation of more than one final product can occur, resulting in sensitivity losses and low reproducibility [16].

Recently and due to some drawbacks mentioned above for the GC–MS analysis of AAS and other banned compounds, several methodologies that use liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) are being rapidly introduced as a better alternative [17–21]. These strategies allow the direct determination of the conjugate analytes and overcome the marginal GC–MS properties of some compounds, even after derivatisation. Nevertheless, the detection of conjugated banned substances is still confined to a small number of compounds because only few conjugated standards are available. Furthermore, concerning the determination of the free AAS, even for LC–MS/MS, derivatisation to enhance their ionization properties is generally carried out due to the limited capability of electrospray to ionize these compounds [20].

Modern sample preparation methods using ultrasonic (US) and microwave energy (MW) are receiving growing interest as an alternative to conventional heating methods, particularly as they offer enhancement of reaction rates and, occasionally, increment in their yields.

When the ultrasonic waves cross a liquid solution different chemical and physical phenomena take place. The reason why ultrasonic energy can affect chemical reactions is linked to the formation of cavitation bubbles [22, 23]. This phenomenon occurs by means of compression and decompression as ultrasonic waves are continuously applied to a liquid medium [22, 23]. These bubbles can grow, oscillate, split and implode. As a result of cavitational bubble implosion, extreme temperatures and pressures are generated, acting like microreactors inside the liquid media [24–27]. Moreover, the formation of highly reactive chemical radicals may also take place inside the liquid medium [23]. Several studies have highlighted the use of ultrasonic energy to enhance numerous physicochemical applications and more recently to improve enzymatic reactions, which is a remarkable advance in the use of ultrasonic energy for sample treatment in analytical chemistry [28–30]. Although the mechanism by which ultrasonication accelerates the enzymatic kinetics is not well understood, some authors have pointed out that the contact area between phases is increased due to cavitation, allowing a reduction of mass transfer limitations in the enzyme–substrate system [23, 24].

The cup horn sonoreactor and the ultrasonic probe are common devices used nowadays to efficiently boost enzymatic reactions kinetics [31, 32]. A minimum knowledge of the properties of such devices is required otherwise unexpected effects can be obtained. As an example, the intensity of sonication, which ultimately dictates the properties of cavitation, is considerably different among the above-mentioned systems. Additionally, it must be taken into account that the ultrasonic probe needs to be inserted into the sample and its applications are done in open vessels, which can be inconvenient for some applications where cross-contamination may occur if the tip is not correctly cleaned and the experimental handling carefully done. All these problems are avoided by using the

cup horn sonoreactor, which, in practical terms, can be considered as a powerful mini-ultrasonic bath.

Concerning microwave energy, several authors have described its use in different fields of chemistry to increase reaction yields and kinetics [33–36]. The microwave heating is produced when microwaves interact with some molecules that have the ability to transform electromagnetic energy into heat [37]. Therefore, in contrast to the conventional heating, the microwave heating process is generated internally through material–microwave interaction, making the heating process very fast [36]. Nevertheless, the so-called microwave effect cannot be explained by the fast heating phenomenon alone, and some theories based on specific radiation effects have been described [36].

In the present work, we study and compare the effects caused by the introduction of microwave and ultrasonic energy in both hydrolysis and derivatisation steps of the routine sample treatment for the determination of banned doping substances in human urine. Although the assessment of ultrasonication for the analysis of AAS has been recently performed in our research group, in the present work, we will evaluate its use in the workflow of two screening methods applied in WADA antidoping laboratories, which includes other banned substances, such as diuretics, stimulants, β-blockers and β_2-agonists. The effect of microwave energy will be for the first time, to the best of our knowledge, evaluated for the overall sample treatment procedure. The effects of time of treatment, heating, ultrasonic amplitude and microwave power were investigated and compared to the conventional heating procedure.

Experimental

Apparatus

An ultrasonic cup horn sonoreactor (SR), model UTR200 (200 W, 24 kHz), from Dr. Hielsher (Teltow, Germany) and a microwave oven MARS 3 from CEM Corporation (North Carolina, USA) were used to accelerate the enzymatic hydrolysis and the TMS derivatisation procedure. A Simplicity 185 from Millipore (Milan, Italy) was used to obtain Milli-Q water. The enzymatic hydrolysis of conjugated compounds and the subsequent derivatisation reaction were performed in 10-mL glass vessels from 3 V Chimica (Rome, Italy).

Standards and reagents

The standards testosterone, testosterone D3, epitestosterone, androsterone, etiocholanolone, etiocholanolone D5, 5α-androstane-3α,17β-diol, 5β-androstane-3α,17β-diol, dehydroisoandrosterone (DHEA), 11-keto-etiocholanolone, 11-hydroxy-etiocholanolone, 11-hydroxy-androsterone, 5β-estran-3α-ol-17-one (nandrolone metabolite 2), 9α-fluoro-17,17-dimethyl-18-norandrosta-4,13-diene-11β-ol-3-one (fluoxymesterone metabolite 1), 9α-fluoro-17α-methyl-4-androsten-3α,6β,11β,17β-tetra-ol (fluoxymesterone metabolite 2), 6β-hydroxyfluoxymesterone (fluoxymesterone metabolite 3), 3α-hydroxy-1α-methyl-5α-androstan-17-one (mesterolone metabolite 1), 17β-hydroxy-7α,17α-dimethylandrost-4-en-3-one (bolasterone), 17β,4-hydroxyandrost-3-one (4-hydroxytestosterone), 17α-methyl-5β-androstene-3α,17β-diol (17α-methyltestosterone metabolite 2), 17β-hydroxy-17α-methyl-2-oxa-5α-androstan-3-one (oxandrolone), 17α-hydroxy-17α-methyl-2-oxa-5α-androstan-3-one (epioxandrolone), 4-chloro-4-androsten-3a-ol-17-one (clostebol metabolite 1), 17α-ethyl-5β-estrane-3α,17β,21-triol (norethandrolone metabolite 1), methyldienolone, 13β,17α-diethyl-3α,17β-dihydroxy-5α-gonane (norboletone metabolite 1), 13β,17α-diethyl-3α,17β-dihydroxy-5β-gonane (norboletone metabolite 2), 4-hydroxy-19-nortestosterone (oxabolone), 4-chloro-6β,17β-dihydroxy-17α-methyl-1,4-androstadien-3-one (chlormetandienone metabolite 1), 17α-ethynyl-17β-hydroxyandrost-4-eno[2,3-d]isoxazole (danazol), 2-hydroxymethyl-17α-methylandrostadiene-11α,17β-diol-3-one (formebolone metabolite 1), formestane, 6β-hydroxymethandienone (methandienone metabolite 2), 17α-methyl-5β-androstane-3α,17β-diol (methandienone metabolite 3), triamterene, probenecid, pemoline, oxilofrine, octopamine, indenolol, pholedrine, etilefrine, etamivan, norfenefrine, 4-phenylpiracetam (carphedon), 6-hydroxy-bromantan, benzoylecgonine, 4-hydroxy-amphetamine, timolol, bisoprolol, betaxolol, salmeterol metabolite, procaterol, penbutolol, nebivolol, metoprolol, metipranolol, esmolol, carteolol, bambuterol and acebutolol were purchased from NARL (Pymble, Australia). The steroid 17α-methyltestosterone was purchased from Riedel-de Haën (Seelze, Germany).

Individual stock standard solutions of each compound (500 mg/L) were prepared by weighing 0.0125 g of analyte in a 25-mL volumetric flask and making it up to volume with methanol. These standard solutions were stored in the dark at −20 °C. Working standard solutions were prepared by dilution of the stock standard solutions in the appropriate volume of methanol.

A solution of β-glucuronidase from *Escherichia coli* K12 with a specific activity approximately of 140 U/mg at 37 °C and pH 7 with nitrophenyl-β-D-glucuronidase as substrate (1 mL contains at least 140 U) was purchased from Roche Diagnostic (Mannheim, Germany). All chemicals (sodium bicarbonate, sodium phosphate, sodium hydrogen phosphate, *tert*-butylmethyl ether) were from Carlo Erba (Milano, Italy). The derivatisation reagent *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) was

from Macherey-Nagel (Düren, Germany) via the Italian distributor (Delchimica Scientific, Napoli, Italy). Ammonium iodide and dithioerythritol (DTE) were supplied by Sigma-Aldrich (Milano, Italy). The derivatisation agent was a mixture of MSTFA/NH_4I/DTE (1,000:2:4, v/w/w) stored at 4 °C for a maximum of 2 weeks.

Sample preparation

Urine hydrolysis procedure

Urine samples (2 mL) were hydrolysed with 50 μL of the commercial solution of β-glucuronidase, after the addition of 0.750 mL of phosphate buffer (0.8 M, pH 7). The hydrolysis was accelerated by means of three different systems as follows: heating at 55 °C for 60 min, or using ultrasonic and microwave energy provided by a sonoreactor and microwave reactor, respectively.

Liquid–liquid extraction of target analytes

After cooling to room temperature, 0.5 mL of carbonate buffer (pH 9) was added to alkalinise the hydrolysed solution. Liquid–liquid extraction was carried out by agitation with 5 mL of *tert*-butyl methyl ether for around 10 min; after centrifugation, the organic phase layer was transferred to a new vessel.

Derivatisation procedure

The procedure for derivatisation with MSTFA was performed as follows: The collected organic phase was dried under a gentle nitrogen stream at 40 °C. After the addition of 50 μL of MSTFA, the vial was closed and the derivatisation reaction was then performed by means of three different systems as follows: heating at 78 °C for 30 min, or using ultrasonic and microwave energy provided by a sonoreactor and a microwave reactor, respectively.

Urine samples

Urine specimens were collected in clean plastic disposable containers and kept at −20 °C until being analysed. Urine samples used in this work were obtained from healthy volunteers from our research team.

GC–MS instrumentation and operating conditions

Gas chromatographic (GC) analyses were carried out on an HP6890 gas chromatograph coupled to a 5973 mass spectrometric detector (Agilent Technologies Italia, Milan, Italy). Chromatographic separations were carried out in a phenyl-methylsilicone column (HP1, 17 m×0.2-mm i.d., 0.11-μm film thickness) from Agilent Technologies Italia. The injection was done in split mode with a split ratio of 1:10 and the injection volume was 1 μL. Helium was used as carrier gas at a constant pressure of 75 kPa. The injection port was set at 280 °C. Two different screening methods were used to determine all banned substances employed in this study: *Screening method 1* – the oven temperature was programmed as follows: initial temperature 188 °C, held for 2.5 min; 3 °C/min ramp to 211 °C, held for 2.0 min; 10 °C/min ramp to 238 °C; and 40 °C/min ramp to 320 °C and held for 3 min. The transfer line temperature was 250 °C, and the ionization source temperature was 230 °C. *Screening method 2* – the oven temperature was programmed as follows: initial temperature 150 °C; 10 °C/min ramp to 200 °C, held for 0.5 min; 20 °C/min ramp to 230 °C, held for 0.5 min; 12 °C/min ramp to 290 °C; and 30 °C/min ramp to 320 °C and held for 1 min. The transfer line temperature was 250 °C, and the ionization source temperature was 230 °C.

For both screening methods the mass detection was performed in selected ion monitoring (SIM) mode using specific fragment ions (see Table 1), with a solvent delay time of 2.6 min. The dwell time in both screening methods was 20 ms. The quantification of all compounds was carried out using the "diagnostic" ions presented in the "Quantification ion" column of Table 1, whereas the "diagnostic" ions in the "Qualification ion" column were used to confirm the identity of the substance. Quantitative measurement of all compounds was performed by response factor calibration (RFC). The values of the urinary concentration of the exogenous compounds were calculated by using the ratio of the areas of the target ions and 17-α-methyltestosterone, which was used as internal standard (ISTD). The concentrations of the endogenous AAS were calculated in relation to the respective deuterated standards.

Experimental design

The routine sample treatment used for the analysis of anabolic steroids and other substances detectable as TMS derivatives by the WADA accredited Anti-Doping Laboratory of Rome is depicted in Fig. 1.

Optimisation of hydrolysis procedure

For the enzymatic hydrolysis optimisation study, the natural endogenous steroids androsterone, etiocholanolone, 11-keto-androsterone, 11-hydroxy-etiocholanolone and 11-hydroxy-androsterone were used. The experimental procedure was followed as described in Sect. "Sample preparation". Deuterated AAS were used as internal standards. It is important to notice that after the hydrolysis study, the derivatisation procedure was carried out by conventional means for all experiments.

Table 1 Diagnostic ions (*m*/*z*) used in SIM mode for detection of banned substances

	Derivative	Diagnostic ions (*m*/*z*)		GC–MS method
		Quantification ion	Qualification ion	
AAS compounds				
Testosterone	bis-*O*-TMS	432	417	1
Epitestosterone	bis-*O*-TMS	432	417	1
Androsterone	bis-*O*-TMS	419	434	1
Etiocholanolone	bis-*O*-TMS	419	434	1
5α-Androstane-3α,17β-diol	bis-*O*-TMS	241	256	1
5β-Androstane-3α,17β-diol	bis-*O*-TMS	241	256	1
DHEA	bis-*O*-TMS	237	327	1
11-Keto-etiocholanolone	bis-*O*-TMS	505	415	1
11-Hydroxy-etiocholanolone	bis-*O*-TMS	522	417	1
11-Hydroxy-androsterone	bis-*O*-TMS	522	417	1
Nandrolone m2	bis-*O*-TMS	405	420	1
Fluoxymesterone m1	bis-*O*-TMS	462	208	2
Fluoxymesterone m2	tetra-*O*-TMS	143	462	2
Fluoxymesterone m3	tetra-*O*-TMS	640	605	2
Mesterolone m1	bis-*O*-TMS	433	448	1
Bolasterone	bis-*O*-TMS	445	315	1
4-Hydroxy-testosterone	tri-*O*-TMS	520	505	1
Methyltestosterone m2	bis-*O*-TMS	143	255	1
Oxandrolone	mono-*O*-TMS	143	308	1
Epioxandrolone	mono-*O*-TMS	143	308	1
Clostebol m1	bis-*O*-TMS	451	453	1
Norethandrolone m1	bis-*O*-TMS	157	331	1
Methyldienolone	bis-*O*-TMS	430	415	1
Norboletone m2	bis-*O*-TMS	157	435	1
Norboletone m1	bis-*O*-TMS	157	435	1
Oxabolone	bis-*O*-TMS	506	491	1
Chlormetandienone m1	bis-*O*-TMS	143	315	1
Danazol	bis-*O*-TMS	481	452	1
Formebolone m1	tri-*O*-TMS	367	222	2
Formestane	tri-*O*-TMS	518	503	2
Metandienome m2	tri-*O*-TMS	517	532	2
Metandienome m3	bis-*O*-TMS	206	444	2
17α-Methyltestosterone	bis-*O*-TMS	301	446	1
Diuretics				
Triamterene	tri-*N*-TMS	432	417	2
Probenecid	mono-*O*-TMS	432	417	2
Stimulants				
Pemoline	tri-*N*,*O*-TMS	178	392	2
Oxilofrine	bis-*O*-TMS	58	267	2
	tri-*O*-TMS	130	382	2
Octopamine	tetra-*N*,*O*-TMS	174	426	2
Pholedrine	mono-*O*-TMS	58	179	2
	bis-*O*-TMS	130	294	2
Etilefrine	bis-*O*-TMS	268	310	2
	tri-*N*,*O*-TMS	130	382	2
Etamivan	mono-*O*-TMS	223	294	2

Table 1 (continued)

	Derivative	Diagnostic ions (*m/z*)		GC–MS method
		Quantification ion	Qualification ion	
Norfenefrine	tetra-*N*,*O*-TMS	174	426	2
4-Phenylpiracetam (carphedon)	bis-*N*-TMS	272	257	2
6-Hydroxy-bromantan	mono-*N*-TMS	393	395	2
Benzoylecgonine	mono-*O*-TMS	82	240	2
4-Hydroxy-amphetamine	bis-*N*,*O*-TMS	116	280	2
	tri-*N*,*O*-TMS	188	352	2
Beta-blockers				
Timolol	mono-*O*-TMS	373	186	2
Bisoprolol	bis-*N*,*O*-TMS	72	405	2
Betaxolol	mono-*O*-TMS	72	263	2
	bis-*N*,*O*-TMS	144	264	2
Salmeterol metabolite	bis-*O*-TMS	260	369	2
Indenolol	mono-*O*-TMS	72	319	2
	bis-*O*-TMS	144	204	2
Penbutolol	mono-*O*-TMS	86	348	2
Nebivolol	tri-N,*O*-TMS	116	368	2
Metoprolol	mono-*O*-TMS	72	223	2
	bis-*N*,*O*-TMS	144	224	2
Metipranolol	mono-*O*-TMS	152	265	2
	bis-*N*,*O*-TMS	144	266	2
Esmolol	mono-*O*-TMS	251	352	2
	bis-*N*,*O*-TMS	144	424	2
Carteolol	bis-*N*,*O*-TMS	235	241	2
Acebutolol	tri-*N*,*O*-TMS	350	365	2
Beta-2 agonists				
Procaterol	tri-*N*,*O*-TMS	407	100	2
Bambuterol	mono-*O*-TMS	86	439	2

m1 metabolite 1, *m2* metabolite 2, *m3* metabolite 3

After optimisation, both ultrasonic and microwave methods were applied to the same control sample and compared with the conventional heating procedure. Ten target steroids were analysed in this study: testosterone, epitestosterone, androsterone, etiocholanolone, DHEA, 5α-androstane-3α,17β-diol, 5β-androstane-3α,17β-diol, 11keto-etiocholanolone, 11-hydroxy-etiocholanolone and 11-hydroxy-androsterone.

Optimisation of derivatisation procedure

The optimisation of the TMS derivatisation study was performed with 25 target banned substances. The experimental procedure described in Sect. "Derivatisation procedure" was applied to 0.5 mL of a methanolic solution spiked with the 25 compounds. The concentration value ranged from 150 to 600 ng/mL.

The effectiveness of the MSTFA derivatisation procedure assisted by microwave energy depends mainly on two critical experimental variables: microwave power and reaction time. To investigate and optimize the effect of the microwave power on the derivatisation yield, as a first approach, the irradiation time was set constant at 3 min and the irradiation power was studied in the range 360 to 1,200 W. Subsequently, the time of irradiation was optimized for the microwave power presenting better results.

After optimisation, both accelerated procedures were applied to a methanolic solution with 55 target compounds comprising 30 anabolic steroids, 2 diuretics, 11 stimulants, 10 beta-blockers and 2 β_2-agonists. The results were compared with the routine procedure. In this set of experiments the optimum conditions to achieve maximum derivatisation yield with the microwave and ultrasonic energy procedure were applied to the same control sample. The steroid 17-α-methyltestosterone was used as ISTD. It is important to stress that for the derivatisation study the

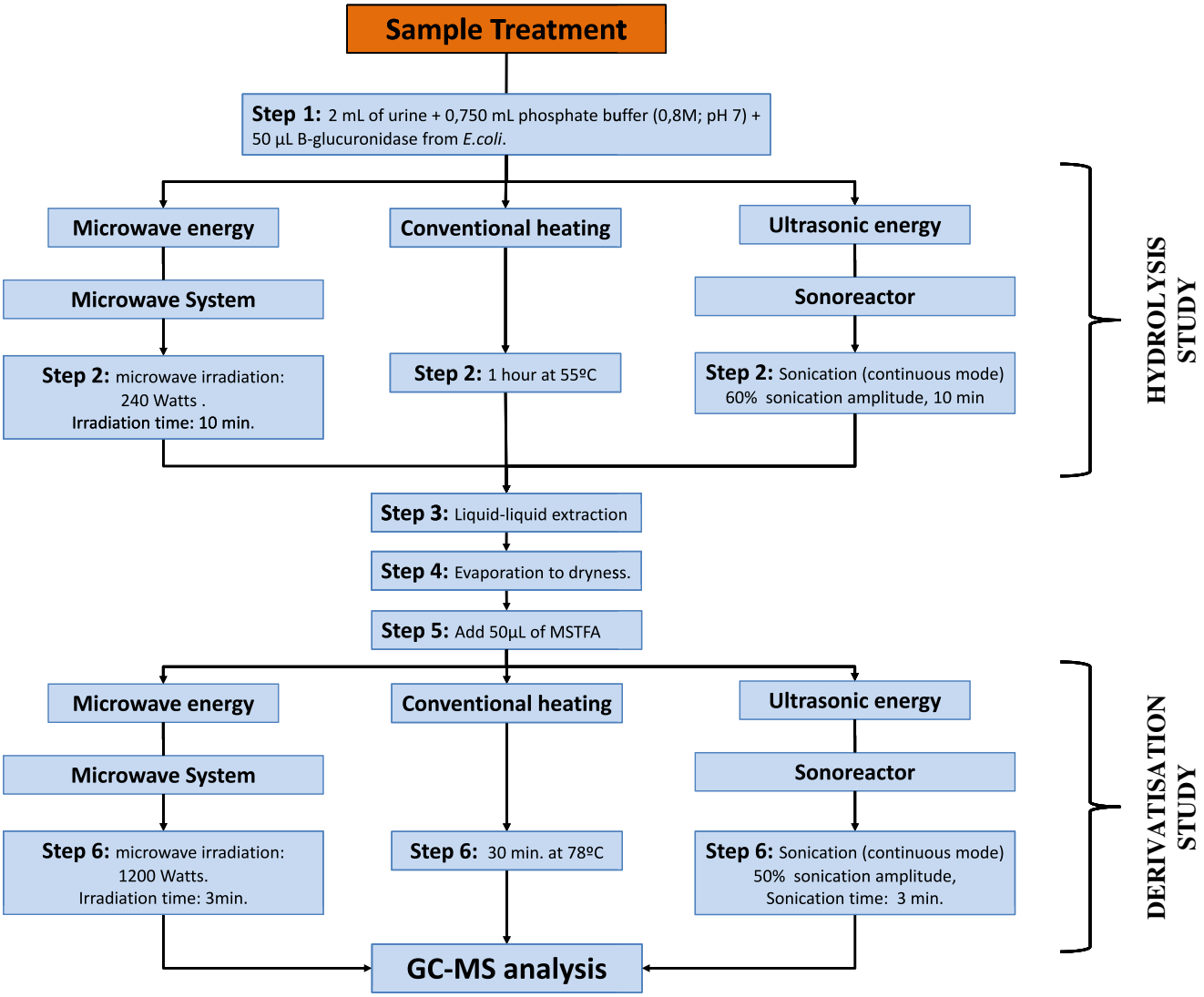

Fig. 1 Comprehensive scheme of the classic, ultrasonic and microwave protocols for the determination of androgenic anabolic steroids

ISTD was derivatised by the conventional procedure and added to all samples at the end of both accelerated and conventional procedures. The final concentration of ISTD in each sample was 200 ng/mL.

Application to real samples

The overall applicability of both accelerated procedures was tested in a real urine sample. The results were compared with the routine procedure. The natural conjugated endogenous AAS from each urine sample were used in this experiment. Each sample was prepared in triplicate.

Statistical analysis

In order to test if both the ultrasonic and the microwave methods are comparable to the conventional method, the *t* test for the comparison of two experimental means was used [38].

Results and discussion

In the comprehensive scheme depicted in Fig. 1, the two critical steps in which the ultrasonic and microwave energy were applied are highlighted. The first step entails the enzymatic hydrolysis process, where the glucuronate conjugates are transformed into the free parent compound. This reaction takes place at 55 °C for 1 h. Once the free steroids have been extracted and pre-concentrated through evaporation to dryness, in the sixth step, they are derivatised by adding MSTFA. The reaction is enhanced by heating at 78 °C for 30 min.

It is important to stress that the results obtained by using the analytical approaches proposed here have been

compared with those obtained with the routine sample treatment. The GC–MS analyses were carried out using two different screening methods, described in Sect. "GC–MS instrumentation and operating conditions", from the WADA accredited Anti-Doping Laboratory of Rome.

Effect of ultrasonic and microwave energy in enhancement of enzymatic hydrolysis reaction

To evaluate the performance of the ultrasonic and microwave energy in the sample treatment, the first set of experiments was devised maintaining all the handling as described in Fig. 1 unaltered except the step where the enzymatic reaction takes place. For the enzymatic hydrolysis study, the ultrasonic and microwave energy were applied to steroid glucuronates. The following five target steroid glucuronates, known to have the longest reaction times for enzymatic hydrolysis, were used in this preliminary study: androsterone, etiocholanolone, 11-keto-androsterone, 11-hydroxy-etiocholanolone and 11-hydroxy-androsterone.

Effect of microwave energy

To perform the microwave-assisted hydrolysis, the time of treatment and the microwave power were optimised. The temperature of the samples during the treatment was followed by using a thermocouple. The optimisation of the microwave-assisted procedure was done keeping the reaction temperature below 60 °C, to avoid thermal denaturation of the enzyme.

To optimise the microwave power the hydrolysis time was set at 10 min, whilst the output power varied between 60 and 480 W. Above this power value, the temperature after 10 min exceeded the 60 °C upper limit. Results shown in Fig. 2 show that as the energy produced by the microwave oven increases from 60 to 240 W so the recovery yields are increased till a maximum value is achieved. Above 240 W the yields decreased constantly. The worst reaction yield was observed for the hydrolysis of the steroid glucuronates 11-hydroxy-etiocholanolone and 11-hydroxy-androsterone, whose recoveries were around 45% of the conventional hydrolysis procedure. These results are consistent with a previous study developed in our laboratory, which stresses the role of the hydroxy group at the C_{11} position in the hydrolysis efficiency, since it is the only structural difference between these and the other steroids studied [39]. For the other compounds, the reaction yields ranged between 80 and 100%. Results shown in Fig. 2 clearly indicate that the protocol using 10 min of irradiation with microwave energy to accelerate the hydrolysis reaction performs worse than the conventional heating protocol, because the hydrolysis yields are lower for the majority of the target steroids studied. The low efficiency of microwave energy in accelerating the enzymatic hydrolysis of steroids is in agreement with literature that highlights partial enzyme inactivation as consequence of conformational changes promoted by microwave irradiation [40].

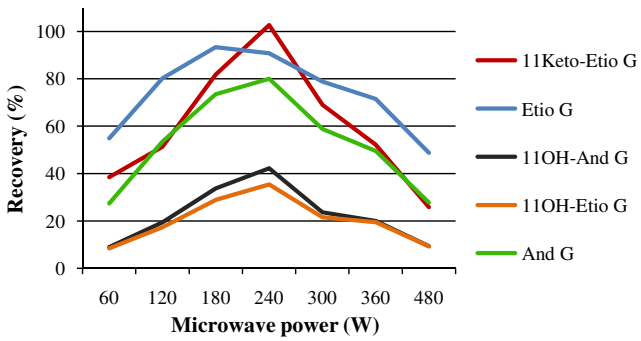

Fig. 2 Effect of microwave power on hydrolysis yield of the natural endogenous steroids androsterone, etiocholanolone, 11-keto-androsterone, 11-hydroxy-etiocholanolone and 11-hydroxy-androsterone (10 min of treatment; n=3). The results are given in percentages (100%=result via the classic method)

To study the effect of time, we developed a set of experiments in which the microwave power was set at 180 W and 240 W, whilst the time of irradiation was increased from 10 to 20 min. Results shown in Fig. 3 suggest that the hydrolysis yields can be increased to almost the same levels as those obtained with the conventional procedure for almost all target steroids. Nevertheless, 11-hydroxy-etiocholanolone and 11-hydroxy-androsterone were hydrolysed in low yields, 50% and 60% respectively. Yadav et al. recently pointed out that microwave energy and conventional heating interact in a similar manner when polar solvents are employed, and therefore the results should be nearly the same when both methods of heating are used [41]. The results obtained in the above described set of experiments suggest that no great improvement arises from the use of microwave energy in the hydrolysis step, since the time required approaches the time of the conventional procedure.

Effect of ultrasonic energy

The use of ultrasonic energy to enhance the enzymatic hydrolysis of steroid glucuronates was evaluated in an earlier study developed in our laboratory [39]. The experimental conditions were optimised there by varying the time of treatment and the amplitude of ultrasonication, and a similar result to the conventional hydrolysis procedure, for the majority of the steroids studied, was achieved in 10 min with the sonoreactor device at 60% of amplitude [39].

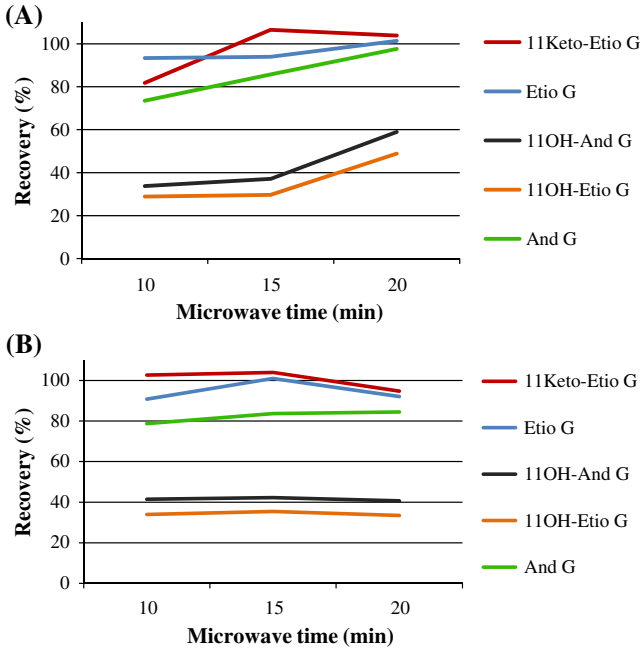

Fig. 3 Effect of microwave time of treatment on hydrolysis yield of the natural endogenous steroids androsterone, etiocholanolone, 11-keto-androsterone, 11-hydroxy-etiocholanolone and 11-hydroxy-androsterone (microwave power **a** 180 W, **b** 240 W; n=3). The results are given in percentages (100%=result via the classic method)

Comparison between microwave and ultrasonic energy

To evaluate and compare the effect of both microwave and ultrasonic energy in the hydrolysis step, the two procedures were applied to the same control sample and compared with the conventional heating procedure. Ten target steroids from human urine were determined in this study: testosterone, epitestosterone, androsterone, etiocholanolone, DHEA, 5α-androstane-3α,17β-diol, 5β-androstane-3α,17β-diol, 11-keto-etiocholanolone, 11-hydroxy-etiocholanolone and 11-hydroxy-androsterone.

The results obtained are shown in Table 2. As expected, except for the endogenous steroids 11β-hydroxy-androsterone and 11β-hydroxy-etiocholanolone, the efficiency of the hydrolysis when the ultrasonic energy was applied was comparable to the conventional heating method. The information taken from all studied compounds is important to the endogenous profile of the athlete. In any case, the 11β-hydroxy group present in these two compounds inhibits the steroid–receptor interaction by steric interference, thereby decreasing both androgenic and anabolic potencies when compared with the other ones under study, which makes them less significant to the endogenous profile of the athlete. Concerning the microwave hydrolysis, the reaction yield is not comparable to the conventional hydrolysis for two other important steroids, androsterone and 5β-androstane-3α,17β-diol.

A parallel set of hydrolysis experiments of the target conjugated compounds was devised to test if 10 min at 55 °C was enough to obtain similar results to those obtained with the sonoreactor. In general, 10 min at 55 °C was insufficient to achieve the same hydrolysis yields. Only for testosterone, epitestosterone and DHEA was 10 min at 55 °C enough to hydrolyse the steroid glucuronides to their

Table 2 Hydrolysis yield of AAS glucuronides from urine. Comparison of the thermal conductivity procedure (1 h at 55 °C) with the ultrasonic procedure (60% of amplitude, 10 min) and the microwave procedure (240 W of power, 10 min)

	Conventional procedure		Microwave method			Ultrasonic method		
	ng/mL; n=6	CV	ng/mL; n=5	Reaction yield (MW/conventional method×100, x ± SD)	$\|t_{cal}\|$	ng/mL; n=5	Reaction yield (US/conventional method×100, x ± SD)	$\|t_{cal}\|$
Testosterone	32±1	1	32±1	100±2	0.25	32±1	100±2	0.51
Epitestosterone	121±3	2	118±4	97±3	1.70	121±1	100±1	0.40
Androsterone	4,213±100	2	3,683±186	87±4	6.06	4,230±98	100±2	0.28
Etiocholanolone	2,215±65	3	2,146±127	97±6	1.17	2,236±67	101±3	0.53
5α-Androstane-3α,17β-diol	122±3	2	124±3	102±3	1.04	125±3	102±2	1.42
5β-Androstane-3α,17β-diol	245±14	6	211±5	86±2	5.22	226±16	92±6	2.11
DHEA	50±2	4	51±1	102±1	1.23	49±3	98±7	0.67
11-Keto-etiocholanolone	2,388±180	8	2,234±196	94±8	1.36	2,608±286	109±12	1.56
11-Hydroxy-androsterone	1,372±72	5	681±64	50±5	16.64	1,072±82	78±6	6.47
11-Hydroxy-etiocholanolone	266±17	6	118±13	44±5	15.81	197±11	74±4	7.78

t_{cal} is a t test that is calculated by comparison of the two experimental means obtained from the two different methods. The difference between both methods is significant when $|t_{cal}|$ is higher than the critical value given by the theoretic t test at a determinate probability distribution. $t_{theoretic}$ (US) = 2.26; $t_{theoretic}$ (MW) = 2.26; p=0.05

n = number of replicates

respective free compound, with 100% of yield when compared to 1 h at 55 °C (data not shown). For the other glucuronides the hydrolysis yield was very low, ranging from 50 to 75% for etiocholanolone, androsterone and 11-keto-etiocholanolone, and below 35% for 11β-hydroxy-androsterone and 11β-hydroxy-etiocholanolone (data not shown).

Effect of ultrasonic and microwave energy in enhancing the derivatisation reaction

The second part of this work focused on the sixth step of the sample treatment, the derivatisation process, as shown in Fig. 1. Among the several derivatisation reactions described in the literature, the production of TMS derivatives is by far the most employed due to its simplicity, high reaction yields and robustness under controlled conditions. However, some of the studied compounds have more than one reaction position available for the TMS group, which may result in the formation of more than one final product leading to losses in sensitivity and reproducibility. Therefore, the formation of the TMS derivative is a critical stage of the analytical procedure.

Effect of microwave energy

To investigate and optimise the effect of the microwave power on the derivatisation yield, 25 banned compounds were used and the irradiation time was set constant at 3 min. The irradiation power was studied in the range 360 to 1,200 W. The sample's temperature after the microwave heating treatment varied from 60 to 85 °C with the increasing of the microwave power. Figure 4 shows that the derivatisation yield after 3 min of irradiation at 1,200 W is comparable to the yield of the routine procedure that lasts for 30 min. Moreover, for diTMS and triTMS derivatives the yield at 1,200 W is higher than those obtained by the routine thermal heating method (78 °C for 30 min) employed in the Rome antidoping laboratory—increments of reaction yield up to 29% higher. This improvement is most likely because of the harsher conditions generated by the microwave energy. The results presented are concordant with the work recently published by Bowden et al. who investigated the effects of microwave energy applied to different derivatisation solvents for GC–MS analysis of steroids. Microwave heating with MSTFA afforded the same yield of derivatisation as the conventional heating for the studied steroids. However, since only monoTMS and diTMS steroid derivatives were studied by Bowden et al., their conclusions were simply focused on the reduction of the reaction time and failed to notice a great improvement in the reaction yield [42].

To optimize the microwave time, the derivatisation reaction was performed at 1,200 W of intensity within five different times, 30 s, 60 s, 90 s, 120 s and 180 s. Figure 5 shows that 30 s was enough to achieve the same hydrolysis yields as with 30 min at 78 °C for most compounds, especially those with just one reaction position for the TMS group. For the compounds with more than one reaction position for the TMS group higher treatment times are needed; therefore for further experiments the time used was 180 s.

Effect of ultrasonic energy

As mentioned above for the hydrolysis study, the effect of the ultrasonic energy in the derivatisation reaction of AAS was previously optimised. The yields of AAS derivatisation after 3 min of ultrasonication with the sonoreactor at 50% of amplitude were comparable, in terms of precision and accuracy, to those obtained by the heating method at 78 °C [39]. Furthermore, for tri- and tetra-TMS derivatives, an improvement of the reaction yield was clearly achieved.

Comparison between microwave and ultrasonic energy

In this set of experiments the optimum conditions to achieve maximum derivatisation yield with the microwave and ultrasonic energy procedure were applied to the same control sample and the results compared with the routine procedure.

As may be seen in Table 3, the yields of derivatisation after 3 min of reaction with both microwave and ultrasonic procedures are excellent. Regarding the ultrasonic energy based procedure, of the 55 banned substances, 34 showed results comparable, in terms of precision and accuracy, to those achieved by the routine procedure. For the microwave energy based procedure, of the 55 compounds, 30 presented similar results to the routine procedure.

Moreover, for several compounds studied, an improvement of the reaction yield was clearly achieved for both procedures; higher reaction yields were achieved for 18 compounds for both the microwave and the ultrasonic procedure. It must be pointed out that for those substances, relative increments of about 10–30% for 8 compounds and 10–48% for 14 compounds were achieved when the microwave and the ultrasonic procedure were employed, respectively. These results are even more impressive if we take into account that not all WADA accredited laboratories employ 78 °C in the TMS derivatisation reaction; some perform this reaction at 60 °C for 0.5 h [43]. For the compounds norboletone metabolite, metoprolol and metipranolol the results achieved were comparable to the conventional procedure after 5 min of ultrasonication. Regarding the microwave procedure, similar results to the conventional procedure were also achieved after 5 min of reaction for the compounds mesterolone metabolite, nor-

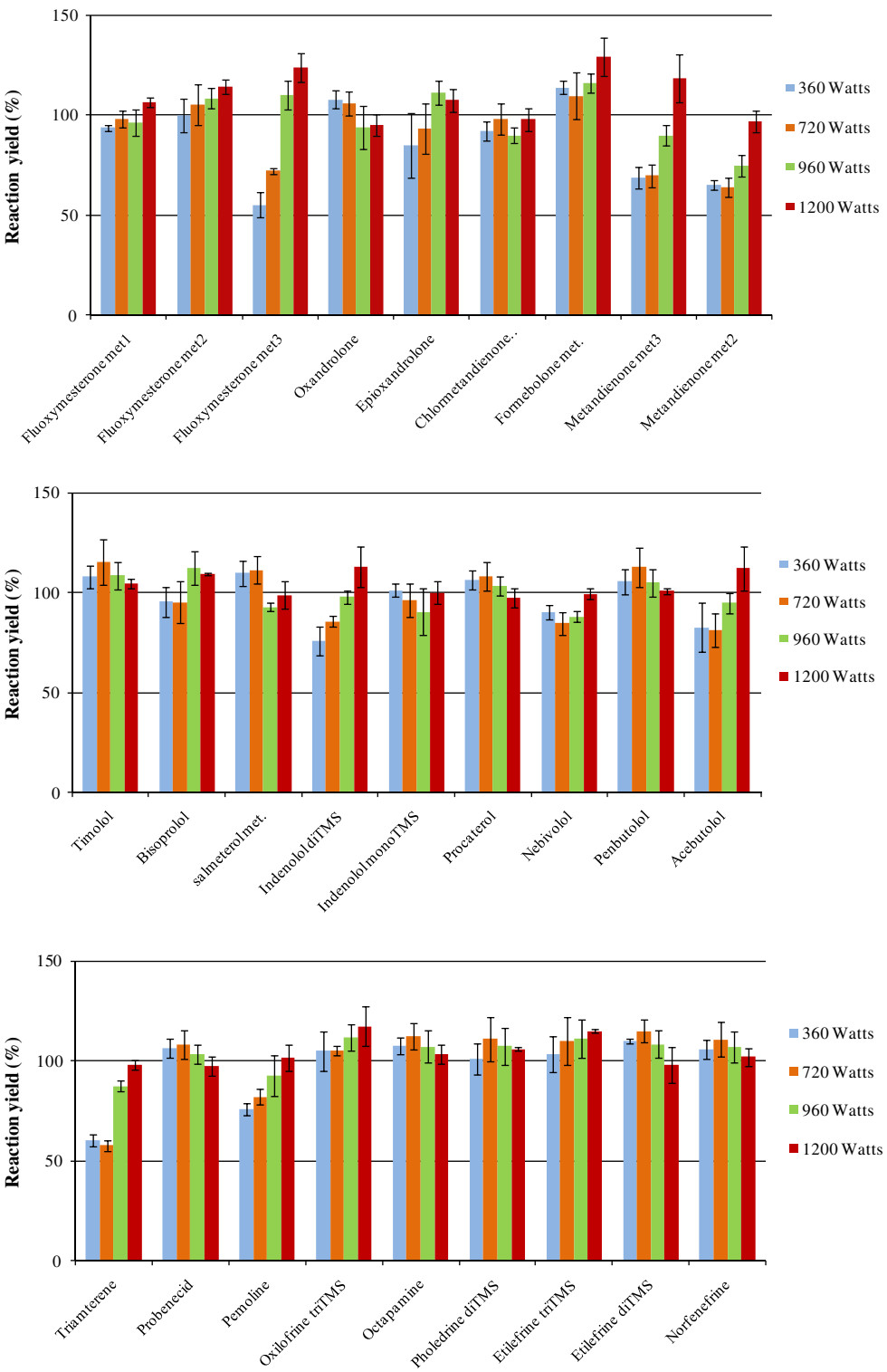

Fig. 4 Effect of microwave power in the derivatisation yield of 25 banned substances (3 min of treatment; n=3). The results are given in percentages (100% = result via the classic method)

ethandrolone metabolite, norboletone metabolite, chlormetandienone metabolite, carphedon, esmolol and bambuterol. For the other compounds the yields were not significantly improved by increasing the ultrasonication and microwave time from 3 to 5 min. In any case, for further experiments the time was set at 3 min for both methods.

Analytical applications

In order to study the reliability of the method, both accelerated procedures were applied to a control urine sample. For this experimental assay only the endogenous AAS from urine were used because no more than a few conjugated standards are commercially available. Both

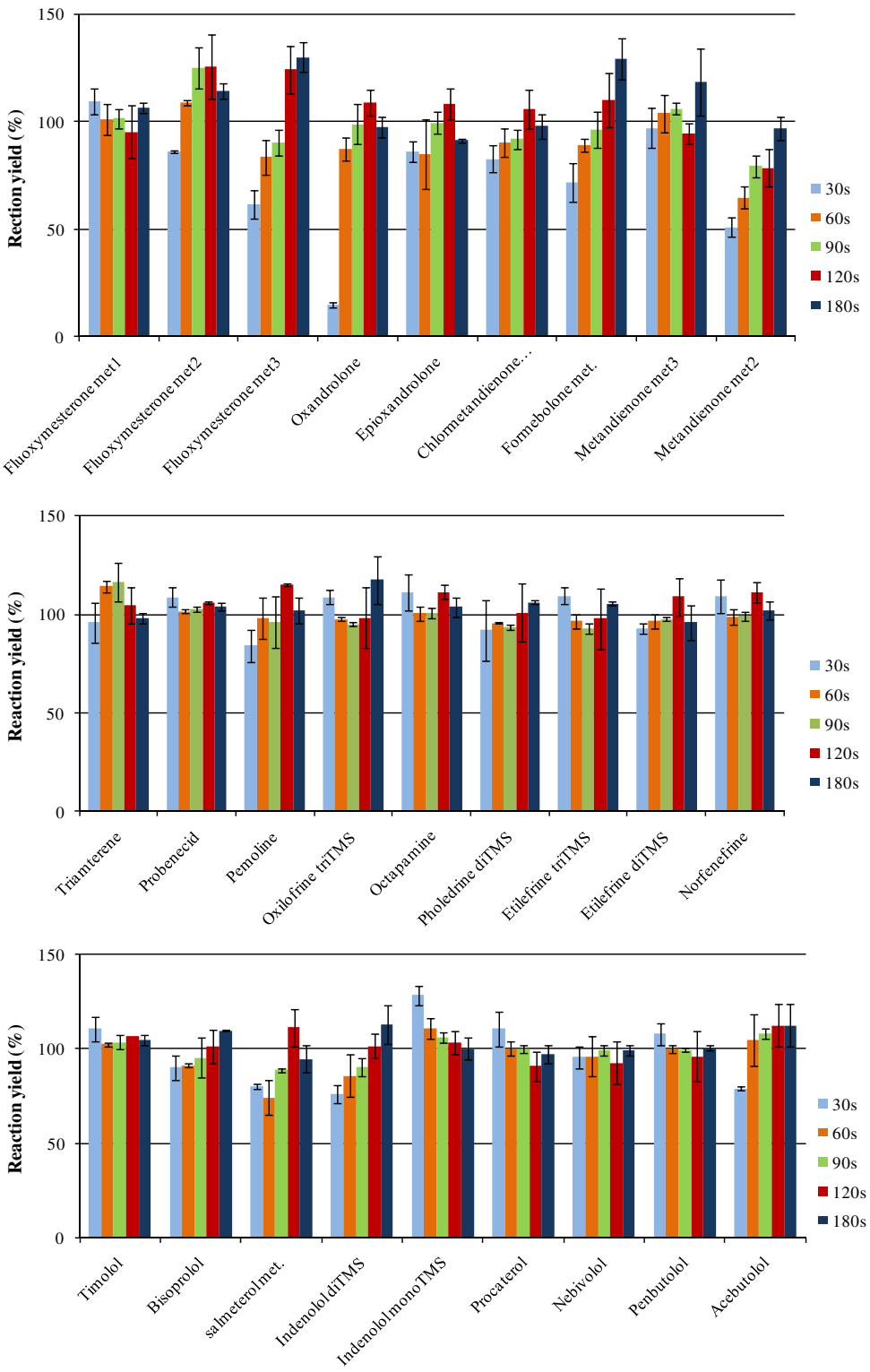

Fig. 5 Effect of microwave time of treatment in the derivatisation yield of 25 banned substances (microwave power 1,200 W; n=3). The results are given in percentages (100% = result via the classic method)

hydrolysis and derivatisation steps were accelerated by using the microwave and ultrasonic methods. Concerning ultrasonication, the hydrolysis step was accelerated with the sonoreactor operating at 60% of amplitude for 10 min and the derivatisation accelerated with the sonoreactor operating at 50% of amplitude for 3 min. Concerning the microwave energy, the hydrolysis reaction was carried out inside the microwave system operating at 240 W for 10 min and the derivatisation was accelerated at 1,200 W for 3 min. The results were compared with the conventional procedure (hydrolysis at 55 °C for 1 h and derivatisation at 78 °C for 30 min) and are shown in Table 4. Only with the ultrasonic

Table 3 Derivatisation yield of TMS derivatives formation with MSTFA. Comparison of the thermal conductivity procedure (30 min at 78 °C) with the ultrasonic procedure (50% of amplitude, 3 min) and the microwave procedure (1,200 W of power, 3 min)

	Conventional procedure		Microwave method			Ultrasonic method		
	(A_{AAS}/A_{ISTD})	CV	(A_{AAS}/A_{ISTD})	Reaction yield (MW/conventional method×100, x±SD)	$\|t_{cal}\|$	(A_{AAS}/A_{ISTD})	Reaction yield (US/conventional method×100, x±SD)	$\|t_{cal}\|$
Testosterone	$(22\pm1)\times10^{-2}$	4	$(23\pm1)\times10^{-2}$	103±4	1.41	$(21\pm0)\times10^{-2}$	97±1	1.44
Epitestosterone	$(64\pm2)\times10^{-2}$	2	$(68\pm3)\times10^{-2}$	105±4	2.39	$(65\pm1)\times10^{-2}$	101±1	0.93
Androsterone	$(110\pm2)\times10^{-1}$	2	$(112\pm4)\times10^{-1}$	102±4	0.94	$(105\pm5)\times10^{-1}$	96±4	1.98
Etiocholanolone	$(97\pm3)\times10^{-1}$	2	$(98\pm4)\times10^{-1}$	100±4	0.44	$(92\pm4)\times10^{-1}$	95±4	2.14
5α-Androstane-3α,17β-diol	$(12\pm1)\times10^{-2}$	6	$(12\pm0)\times10^{-2}$	94±3	1.88	$(12\pm0)\times10^{-2}$	99±1	0.49
5β-Androstane-3α,17β-diol	$(50\pm3)\times10^{-2}$	3	$(51\pm2)\times10^{-2}$	101±4	0.35	$(48\pm1)\times10^{-2}$	95±1	2.03
DHEA	$(12\pm1)\times10^{-3}$	5	$(12\pm1)\times10^{-3}$	101±5	0.22	$(11\pm1)\times10^{-3}$	92±5	2.15
11-Hydroxy-etiocholanolone	$(20\pm1)\times10^{-1}$	3	$(23\pm1)\times10^{-1}$	113±5	5.48	$(22\pm0)\times10^{-1}$	109±1	6.48
11-Hydroxy-androsterone	$(37\pm1)\times10^{-2}$	4	$(40\pm1)\times10^{-2}$	108±4	3.15	$(30\pm0)\times10^{-2}$	105±1	2.71
Nandrolone m2	$(22\pm3)\times10^{-4}$	13	$(20\pm3)\times10^{-4}$	92±11	1.05	$(23\pm4)\times10^{-4}$	104±18	0.43
Fluoxymesterone m1	$(38\pm1)\times10^{-3}$	2	$(41\pm2)\times10^{-3}$	107±2	5.46	$(43\pm2)\times10^{-3}$	113±6	4.42
Fluoxymesterone m2	$(35\pm2)\times10^{-3}$	7	$(40\pm1)\times10^{-3}$	114±4	3.94	$(48\pm4)\times10^{-3}$	135±10	6.28
Fluoxymesterone m3	$(36\pm3)\times10^{-4}$	8	$(46\pm1)\times10^{-4}$	130±7	6.17	$(52\pm5)\times10^{-4}$	148±13	6.84
Mesterolone m1	$(33\pm3)\times10^{-4}$	10	$(28\pm3)\times10^{-4}$	84±10	2.62	$(31\pm6)\times10^{-4}$	95±18	0.55
Bolasterone	$(225\pm4)\times10^{-4}$	2	$(226\pm7)\times10^{-4}$	101±3	0.33	$(236\pm7)\times10^{-4}$	105±3	2.86
4-Hydroxy-testosterone	$(48\pm5)\times10^{-4}$	11	$(42\pm5)\times10^{-4}$	86±11	2.03	$(56\pm6)\times10^{-4}$	115±13	2.01
Methyltestosterone m2	$(17\pm1)\times10^{-3}$	9	$(16\pm2)\times10^{-3}$	96±11	0.67	$(18\pm1)\times10^{-3}$	105±7	1.03
Epioxandrolone	$(107\pm6)\times10^{-4}$	6	$(98\pm1)\times10^{-4}$	91±1	3.34	$(149\pm26)\times10^{-4}$	139±25	3.47
Clostebol metabolite	$(11\pm1)\times10^{-4}$	11	$(10\pm1)\times10^{-4}$	91±9	1.43	$(11\pm1)\times10^{-4}$	103±9	0.42
Norethandrolone m1	$(63\pm1)\times10^{-4}$	2	$(55\pm6)\times10^{-4}$	87±9	3.07	$(63\pm4)\times10^{-4}$	100±7	0.03
Methyldienolone	$(39\pm5)\times10^{-4}$	14	$(43\pm4)\times10^{-4}$	110±10	1.31	$(50\pm5)\times10^{-4}$	127±14	3.06
Norboletone m2	$(28\pm1)\times10^{-3}$	3	$(28\pm1)\times10^{-3}$	100±5	0.10	$(30\pm3)\times10^{-3}$	106±9	1.42
Norboletone m1	$(11\pm2)\times10^{-3}$	15	$(10\pm1)\times10^{-3}$	93±5	0.97	$(9\pm1)\times10^{-3}$	81±9	2.38
Oxabolone	$(29\pm3)\times10^{-4}$	10	$(33\pm4)\times10^{-4}$	113±10	1.55	$(43\pm1)\times10^{-4}$	148±2	10.33
Chlormetandienone m1	$(73\pm3)\times10^{-4}$	4	$(64\pm6)\times10^{-4}$	88±8	3.06	$(68\pm7)\times10^{-4}$	93±9	0.71
Danazol	$(32\pm5)\times10^{-4}$	15	$(31\pm3)\times10^{-4}$	98±9	0.29	$(31\pm2)\times10^{-4}$	97±7	0.45
Formebolone m1	$(39\pm3)\times10^{-4}$	9	$(51\pm4)\times10^{-4}$	130±10	5.07	$(35\pm2)\times10^{-4}$	91±4	2.13
Formestane	$(152\pm14)\times10^{-3}$	9	$(137\pm7)\times10^{-3}$	90±5	2.10	$(150\pm10)\times10^{-3}$	98±6	0.32
Metandienome m2	$(42\pm3)\times10^{-4}$	8	$(43\pm5)\times10^{-4}$	102±12	0.33	$(47\pm6)\times10^{-4}$	112±15	1.63
Metandienome m3	$(113\pm2)\times10^{-3}$	1	$(134\pm18)\times10^{-3}$	119±16	2.64	$(109\pm4)\times10^{-3}$	97±4	2.00
Pemoline	$(38\pm2)\times10^{-2}$	6	$(39\pm3)\times10^{-2}$	102±7	0.45	$(48\pm3)\times10^{-2}$	126±7	6.21
Oxilofrine triTMS	$(163\pm5)\times10^{-1}$	3	$(192\pm27)\times10^{-1}$	118±16	2.36	$(172\pm17)\times10^{-1}$	105±11	1.08
Octopamine	20±1	5	21±1	104±5	1.20	22±1	112±11	2.24
Pholedrine diTMS	$(43\pm2)\times10^{-1}$	4	$(46\pm1)\times10^{-1}$	106±1	3.72	$(43\pm5)\times10^{-1}$	101±11	0.17
Etilefrine triTMS	$(99\pm3)\times10^{-1}$	3	$(104\pm1)\times10^{-1}$	105±1	3.66	$(104\pm12)\times10^{-1}$	105±12	0.93
Etamivan	$(121\pm2)\times10^{-2}$	2	$(118\pm2)\times10^{-2}$	98±2	2.10	$(136\pm16)\times10^{-2}$	112±13	1.95
Norfenefrine	$(104\pm5)\times10^{-1}$	5	$(107\pm5)\times10^{-1}$	102±4	0.73	$(117\pm12)\times10^{-1}$	112±11	2.25
Carphedon	$(40\pm1)\times10^{-2}$	2	$(33\pm2)\times10^{-2}$	81±4	9.00	$(36\pm4)\times10^{-2}$	89±11	2.29
6-Hydroxy-bromantan	$(82\pm3)\times10^{-2}$	4	$(87\pm1)\times10^{-2}$	106±2	3.10	$(92\pm1)\times10^{-2}$	111±2	6.03
Benzoylecgonine	$(192\pm4)\times10^{-2}$	2	$(206\pm5)\times10^{-2}$	107±3	4.57	$(220\pm17)\times10^{-2}$	115±9	3.52
4-OH-amphetamine triTMS	$(12\pm1)\times10^{-2}$	7	$(14\pm1)\times10^{-2}$	119±9	3.88	$(14\pm2)\times10^{-2}$	125±16	3.17
4-OH-amphetamine diTMS	$(35\pm1)\times10^{-1}$	4	$(36\pm1)\times10^{-1}$	104±3	1.94	$(37\pm5)\times10^{-1}$	107±14	1.14
Triamterene	$(347\pm8)\times10^{-3}$	2	$(341\pm8)\times10^{-3}$	98±2	1.29	$(335\pm40)\times10^{-3}$	96±11	0.70
Probenecid	$(344\pm8)\times10^{-2}$	2	$(358\pm6)\times10^{-2}$	104±2	2.97	$(382\pm28)\times10^{-2}$	111±8	2.83
Timolol	$(40\pm1)\times10^{-2}$	3	$(42\pm1)\times10^{-2}$	105±3	2.48	$(45\pm4)\times10^{-2}$	111±9	2.46

Table 3 (continued)

	Conventional procedure		Microwave method			Ultrasonic method		
	(A_{AAS}/A_{ISTD})	CV	(A_{AAS}/A_{ISTD})	Reaction yield (MW/conventional method×100, x±SD)	$\|t_{cal}\|$	(A_{AAS}/A_{ISTD})	Reaction yield (US/conventional method×100, x±SD)	$\|t_{cal}\|$
Betaxolol	$(12±2)×10^{-1}$	13	$(13±1)×10^{-1}$	102±8	0.29	$(18±2)×10^{-1}$	149±19	4.76
Indenolol diTMS	$(153±9)×10^{-2}$	6	$(174±16)×10^{-2}$	113±10	2.49	$(140±10)×10^{-2}$	91±7	2.12
Procaterol	$(91±3)×10^{-2}$	3	$(89±4)×10^{-2}$	97±5	1.03	$(96±9)×10^{-2}$	106±10	1.24
Penbutolol	$(353±11)×10^{-2}$	3	$(356±5)×10^{-2}$	101±1	0.57	$(381±40)×10^{-2}$	108±11	1.50
Nebivolol	$(39±3)×10^{-2}$	7	$(38±1)×10^{-2}$	99±3	0.18	$(38±1)×10^{-2}$	99±2	0.23
Metoprolol diTMS	$(68±4)×10^{-1}$	5	$(65±2)×10^{-1}$	97±3	1.29	$(61±1)×10^{-1}$	91±2	3.70
Metipranolol diTMS	$(106±5)×10^{-1}$	5	$(113±2)×10^{-1}$	106±1	2.79	$(96±2)×10^{-1}$	90±2	4.08
Esmolol diTMS	$(88±3)×10^{-1}$	3	$(79±2)×10^{-1}$	90±2	5.79	$(87±1)×10^{-1}$	99±2	0.75
Carteolol	$(62±2)×10^{-1}$	4	$(60±3)×10^{-1}$	95±5	1.67	$(70±6)×10^{-1}$	113±9	2.85
Bambuterol	$(41±1)×10^{-2}$	3	$(37±1)×10^{-2}$	90±3	4.99	$(44±3)×10^{-2}$	108±7	2.30
Acebutolol	$(10±1)×10^{-2}$	11	$(11±1)×10^{-2}$	104±3	1.78	$(14±2)×10^{-2}$	143±21	4.17

t_{cal} is a t test that is calculated by comparison of the two experimental means obtained from the two different methods. The difference between both methods is significant when $|t_{cal}|$ is higher than the critical value given by the theoretic t test at a determinate probability distribution. $t_{theoretic}$= 2.31; p=0.05

n = 5 replicates for each method

procedure were the results obtained comparable with the conventional method. Furthermore, the precision and accuracy of the ultrasonic procedure indicates that this method shows sufficient robustness for quantitative analysis under routine conditions. Regarding the microwave method, the hydrolysis yields are still far from those of the conventional method for compounds of great importance such as androsterone and 5β-androstane-3α,17β-diol, not to mention the 11-hydroxy-etiocholanolone and 11-hydroxy-androsterone, whose yields are around 50%.

Table 4 Comparison of the microwave and ultrasonic procedures with the classic procedure. The recoveries are calculated as the ratio yield between the microwave and ultrasonic method and the classic method and are expressed as percentages

	Conventional procedure		Microwave method			Ultrasonic method		
	ng/mL; n=5	CV	ng/mL; n=5	Reaction yield (MW/conventional method×100, x ± SD)	$\|t_{cal}\|$	ng/mL; n=5	Reaction yield (US/conventional method×100, x ± SD)	$\|t_{cal}\|$
Testosterone	78±3	4	77±1	99±1	0.65	77±1	99±1	0.65
Epitestosterone	94±2	2	95±1	101±1	0.60	94±2	100±2	0.02
Androsterone	3,923±135	3	3,095±234	79±6	6.85	3,625±256	92±7	2.30
Etiocholanolone	2,837±68	2	2,633±257	93±9	1.72	2,755±46	97±2	2.24
5α-Androstane-3α,17β-diol	103±8	8	99±8	96±8	0.78	93±6	91±6	2.17
5β-Androstane-3α,17β-diol	225±8	3	192±9	85±4	6.05	210±16	93±7	1.83
DHEA	41±2	5	36±1	89±2	4.48	39±1	95±2	2.16
Pregnanediol	468±20	4	459±14	98±3	0.78	455±3	97±1	1.48
11-Hydroxy-etiocholanolone	924±26	3	417±55	45±6	18.55	677±51	73±6	9.62
11-Hydroxy-androsterone	240±9	4	125±16	52±7	14.21	174±9	72±4	11.76

t_{cal} is a t test that is calculated by comparison of the two experimental means obtained from the two different methods. The difference between both methods is significant when $|t_{cal}|$ is higher than the critical value given by the theoretic t test at a determinate probability distribution. $t_{theoretic}$= 2.31; p=0.05

n = number of replicates

Conclusions

This work shows that both ultrasonic and microwave energy can be used to improve and simplify the sample treatment in routine analysis of many banned substances for doping control. Under ultrasonic energy the two key steps of the sample treatment were improved. The new approach is less time consuming and has the same reproducibility in terms of accuracy as the traditional method. Moreover, for 18 of the banned compounds studied the ultrasonic assisted method was able to improve the derivatisation yields. Concerning the microwave energy assisted method this study demonstrates that it can also be successfully used to improve the derivatisation reaction time and, most importantly, the analytical sensitivity of some compounds, since for 18 of the studied substances the derivatisation yield was improved. Regarding the derivatisation reaction, both accelerated methods showed similar results; however, the microwave energy assisted method presents higher sample throughput. Nevertheless, the microwave energy fails in efficiently enhancing the enzymatic hydrolysis of the majority of the steroids tested.

Overall, this study suggests that the use of ultrasonic energy could be very effective as a tool to accelerate doping control tests carried out at major international events, where one of the main tasks for the analytical laboratory is to perform analyses as quickly and efficiently as possible.

Acknowledgements M. Galésio acknowledges the Fundação para a Ciência e a Tecnología (FCT, Portugal) for his doctoral grant SFRH/BD/31652/2007. J.L. Capelo-Martínez acknowledges the Xunta de Galicia (Spain) for their Parga-Pondal Research contract.

References

1. Donike M (1993) Steroid profile in Cologne. In: Donike M, Geyer H, Gotzmann A, Mareck-Engelke U, Rauth S (eds) Proceedings of the 10th Cologne workshop on dope analysis, 7–12th June 1992. Sport und Buch Strauss Edition Sport, Koln, pp 47
2. Botrè F (2008) J Mass Spectrom 43:903–907
3. Schänzer W, Delahaut P, Geyer H, Machnik M, Homing S (1996) J Chromatogr B 687:93–108
4. Dehennin L, Matsumoto AM (1993) J Steroid Biochem Mol Biol 44:179–189
5. Gomes RL, Meredith W, Snape CE, Sephton MA (2009) J Pharm Biomed Anal 49:1133–1140
6. Mulder GJ, Coughtrie MWH, Burchell B (1990) Glucuronidation. In: Mulder GJ (ed) Conjugation reactions in drug metabolism: an integrated approach. Taylor & Francis, London, pp 51–105
7. Jiménez C, Ventura R, Segura J (2002) J Chromatogr B 767:341–351
8. Carvalho VM, Nakamuraa OH, Vieira JGH (2008) J Chromatogr B 872:154–161
9. Chatman K, Hollenbeck T, Hagey L, Vallee M, Purdy R, Weiss F, Siuzdak G (1999) Anal Chem 71:2358–2363
10. Pozo OJ, Van Eenoo P, Deventer K, Delbeke FT (2008) TrAC Trends Anal Chem 27:657–671
11. Marcos J, Pascual JÁ, de la Torre X, Segura J (2002) J Mass Spectrom 37:1059–1073
12. Wang Y, Karu K, Griffiths WJ (2007) Biochimie 89:182–191
13. Van Thuyne W, Van Eenoo P, Delbeke FT (2008) J Chromatogr A 1210:193–202
14. Schanzer W, Donike M (1993) Anal Chim Acta 275:23–48
15. Zuoa Y, Zhang K, Lin Y (2007) J Chromatogr A 1148:211–218
16. Shareef A, Angove MJ, Wells JD (2006) J Chromatogr A 1108:121–128
17. Thevis M, Schänzer W (2007) Anal Bioanal Chem 388:1351–1358
18. Thevis M, Guddat S, Schänzer W (2009) Steroids 74:315
19. Mazzarino M, de la Torre X, Botrè F (2008) Anal Bioanal Chem 392:621
20. Pozo OJ, Van Eenoo P, Deventer K, Delbeke FT (2008) TrAC Trends Anal Chem 27:657
21. Hintikka L, Kuuranne T, Leinonen A, Thevis M, Schanzer W, Halket J, Cowan D, Grosse J, Hemmersbach P, Nielen MWF, Kostiainen R (2008) J Mass Spectrom 43:965
22. Suslick KS (1990) Science 247:1439
23. Mason TJ, Lorimer JP (2002) Applied sonochemistry: the uses of power ultrasound in chemistry and processing. Wiley-VCH, Weinheim
24. Mason TJ (1999) Sonochemistry. Oxford University Press, Oxford
25. Capelo JL (2009) Ultrasound in chemistry: analytical applications. Wiley-VCH, Weinheim
26. Flannigan DJ, Suslick KS (2005) Nature 434:52
27. Didenko YT, Suslick KS (2002) Nature 418:394
28. Capelo JL, Galesio M, Felisberto GM, Vaz C, Pessoa JC (2005) Talanta 66:1272–1280
29. Santos HM, Rial-Otero R, Fernandes L, Vale G, Rivas MG, Moura I, Capelo JL (2007) J Proteome Res 6:3393–3399
30. Carreira RJ, Cordeiro FM, Moro AJ, Rivas MG, Rial-Otero R, Gaspar EM, Moura I, Capelo JL (2007) J Chromatogr A 1153:291–299
31. Santos HM, Capelo JL (2007) Talanta 73:795–802
32. Rial-Otero R, Carreira RJ, Cordeiro FM, Moro AJ, Santos HM, Vale G, Moura I, Capelo JL (2007) J Chromatogr A 1166:101–107
33. de Koning S, Janssen H, Brinkman UAT (2009) Chromatographia 69:33–78
34. Zu YG, Zhang S, Fu YJ, Liu W, Liu ZG, Luo M, Efferth T (2009) Eur Food Res Technol 229:43–49
35. Alterman M, Hallberg AJ (2000) Org Chem 65:7984–7989
36. Goni MA, Montgomery S (2000) Anal Chem 72:3116–3121
37. de la Hoz A, Diaz-Ortiz A, Moreno A (2005) Chem Soc Rev 34:164–178
38. Miller JN, Miller JC (2001) Statistics and chemometrics for analytical chemistry, 4th edn. Prentice Hall, New Jersey
39. Galesio M, Rial-Otero R, Simal-Gándara J, de la Torre X, Botrè F, Capelo JL (2010) Rapid Commun Mass Spectrom 24:2375–2385
40. Rejasse B, Lamare S, Legoy MD, Besson T (2007) J Enzyme Inhib Med Chem 22:518–526
41. Yadav G, Borkar IV (2009) Ind Eng Chem Res 48:7915–7922
42. Bowden JA, Colosi DM, Stutts WL, Mora-Montero DC, Garrett TJ, Yost RA (2009) Anal Chem 81:6725–6734
43. Mareck U, Geyer H, Opfermann G, Thevis M, Schanzer W (2008) J Mass Spectrom 43:877–891