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Speeding up the process urine sample pre-treatment: Some perspectives on the use of microwave assisted extraction in the anti-doping field

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ABSTRACT

In this contribution we described a fast and efficient method for the liquid/liquid extraction from human urine of different classes of drugs, included in the list of prohibited substances published every year by the World Anti-doping Agency, using microwave irradiation.

Liquid/liquid extraction was conducted in a temperature controlled single beam microwave oven equipped with an extraction unit and closed vessels. The effects of microwave power and time on the liquid/liquid extraction process were investigated utilizing different organic solvents. The optimum power was found to be 600 W (generating a temperature of 70 °C) with an incubation time of 30–60 s for the most thermolable constituents such as triamcinolone, prednisolone, chlorthiazide, chlorthalidone, epitrembolone and oxandrolone, and 1020 W (generating a temperature of 150 °C) with an incubation time of 30–60 s for the other compounds considered in this study.

The effectiveness of this approach was evaluated by GC–MS (anabolic steroids, beta2-agonists and narcotics) and by LC–MS/MS (diuretics, glucocorticoids and beta-blockers) analyzing more than 20 different urine samples spiked with the compounds considered in this study. The results showed that the effect of microwave irradiation on the liquid/liquid extraction process was very remarkable: the total sample preparation time can be shortened by 9 min compared to the traditional method (30–60 s instead of 10 min); furthermore, a significant increase in the recovery was recorded for specific compounds such as terbutaline and several diuretics. In addition to the above the repeatability of the extraction recoveries, the limits of detection and the matrix interferences were comparable with the reference methods, presently accredited under the ISO17025, followed by the World Anti-doping Agency accredited anti-doping laboratory of Rome.

1. Introduction

The reduction of time and cost of analysis is becoming paramount in the development of modern, effective and practically applicable procedures. In response to this, an increasing interest in techniques and apparatus for performing very fast high-performance separations are encountering a broader and broader diffusion among reference laboratories. Despite the sophisticated arsenal of analytical tools, complete non-invasive measurements are still not possible in most cases. The procedure of extraction can vary in degree of selectivity, speed, and convenience and depends on the approach and conditions used. In this contest the introduction of non-traditional extraction technologies such us microwave assisted extraction (MAE), supercritical fluid extraction (SCFE), pressurized solvent extraction (PSE) has determined a significant improvement in this research field. In particular, the microwave irradiation has been used, since 80s', to extract different compounds especially from solid sample matrices with a remarkable reduction of time and organic solvent [1–7]. The most attractive features of microwave assisted extraction technique is the improvement in the extraction kinetics provided by heating, with less solvent consumption and protection offered to thermolable constituents. Furthermore, the possibility to use closed systems allows: (i) to reach high temperatures because the increased pressure inside the vessel raises to the boiling point of the solvents used and the higher temperatures in turn decrease the time needed for the extraction process, (ii) to completely avoid the losses of volatile substances, and at the end (iii) to use a lower amount of organic solvent [6].

In this study we evaluated the possibility, using microwave irradiation, to improve the ratio sample/time for non-peptide substances, focusing on the possibility to optimize the human

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Table 1
LC–MS/MS: polarity, SRM transitions, MW recovery for beta-blockers, glucocorticoids and diuretics.

Compounds	Polarity	SRM transitions (*m*/*z*)	MW conditions (time, power)	LOD traditional method (ng/mL)	LOD MW method (ng/mL)
Beta-blockers					
Acebutolol	Positive	337/116	30 s, 1020 W	50	50
Alprenolol	Positive	250/91	30 s, 1020 W	50	50
Atenolol	Positive	267/145	30 s, 1020 W	50	50
Betaxolol	Positive	308/121	30 s, 1020 W	50	50
Bisoprolol	Positive	326/116	30 s, 1020 W	50	50
Carteolol	Positive	293/237	30 s, 1020 W	50	50
Carvedilol	Positive	407/224	30 s, 1020 W	60	50
Celiprolol	Positive	380/251	30 s, 1020 W	60	50
Labetalol	Positive	329/162	30 s, 1020 W	60	50
Metoprolol	Positive	268/133	30 s, 1020 W	70	50
Nadolol	Positive	310/254	30 s, 1020 W	70	40
Oxprenolol	Positive	266/225	30 s, 1020 W	70	50
Penbutolol	Positive	292/236	30 s, 1020 W	70	40
Pindolol	Positive	249/116	30 s, 1020 W	100	50
Propranolol	Positive	260/56	30 s, 1020 W	70	50
Sotalol	Positive	273/133	30 s, 1020 W	60	50
Timolol	Positive	317/261	30 s, 1020 W	60	50
Glucocorticoids					
Beclometasone	Positive	409/373; 409/337	30 s, 600 W	10	10
Betamethasone	Positive	393/337; 393/355	30 s, 1020 W	10	3
Budenoside	Positive	431/413; 431/341	30 s, 600 W	10	5
Budenoside metabolita	Positive	377/359; 377/341	30 s, 600 W	15	10
Ciclesonide metabolita	Positive	471/453; 471/323	30 s, 1020 W	15	10
Dexamethasone	Positive	393/337; 393/355	30 s, 1020 W	5	3
Desonide	Positive	417/399; 417/341	60 s, 1020 W	5	3
Fluocortolone	Positive	377/321; 377/303	30 s, 1020 W	5	3
Fludrocortisone	Positive	381/239; 381/343	30 s, 1020 W	15	15
Flumetasone	Positive	411/335; 411/253	30 s, 1020 W	5	5
Flunisolide	Positive	435/397; 435/417	30 s, 1020 W	10	10
Methylprednisolone	Positive	375/357; 375/339	60 s, 600 W	15	15
Prednisolone	Positive	361/343; 361/325	60 s, 600 W	15	15
Prednisone	Positive	359/323; 359/341	60 s, 600 W	15	15
Triamcinolone	Positive	395/357; 395/321	30 s, 600 W	10	5
Triamcinolone acetonide	Positive	435/397; 435/415	30 s, 1020 W	5	5
Diuretics					
Acetazolamide	Negative	221/83;221/58	60 s, 600 W	100	100
Bendroflumethiazide	Positive	422/287; 422/271	30 s, 600 W	100	100
Bumetanide	Positive	365/184; 365/240	30 s, 1020 W	80	50
Canrenone	Positive	341/157; 341/107	30 s, 600 W	50	50
Chlortalidone	Positive	339/135; 339/193	60 s, 600 W	50	50
Chlorthiazide	Negative	294/214; 294/179	60 s, 600 W	80	100
Clopamide	Positive	346/169; 346/55	30 s, 1020 W	100	50
Dichlorphenamide	Negative	303/224; 303/239	60 s, 600 W	80	100
Ethacrynic acid	Negative	301/243	30 s, 600 W	50	50
Furosemide	Negative	329/205; 329/126	30 s, 600 W	80	50
Hydrochlothiazide	Negative	295/205; 295126	60 s, 600 W	80	100
Hydroflumethiazide	Negative	330/303; 330/160	60 s, 600 W	100	100
Indapamide	Positive	366/132; 366/91	60 s, 1020 W	100	50
Piretanide	Positive	363/238; 363/196	60 s, 1020 W	80	50
Torasemide	Positive	349/290; 349/183	30 s, 600 W	100	50
Xipamide	Positive	355/234; 355/274	60 s, 1020 W	80	50

resources needed for every analytical line and, at the same time, to reduce the time and cost of the analytical procedures. In the past years we have presented the possibility of using a microwave oven to speed up some sample preparation steps such us the derivatization [8–10], chemical and enzymatic hydrolysis and the protein digestion; we have now evaluated the effect of microwave irradiation (varying the organic solvent, the extraction time and the applied power) on the efficiency of the liquid/liquid extraction step as carried out in our laboratory for the following classes of compounds: glucocorticoids, beta2-agonists, diuretics, narcotics, anabolic steroids and beta-blockers. The developed method was applied to real urine samples and the results obtained have been then compared with those obtained by the reference methods, i.e. the screening and confirmation methods, accredited according to the ISO17025, and presently followed for the analysis of beta-adrenergic agents, steroids, glucocorticoids, narcotics and diuretics by the WADA-accredited anti-doping laboratory of Rome [10–12].

2. Materials and methods

2.1. Chemicals and reagents

Acebutolol (ACE), acetazolamide (ACET), alprenolol (ALPRE), althiazide (ALTHIA), atenolol (ATE), beclomethasone (BECLO), bendroflumethiazide (BENDRO), betamethasone (BETA), betaxolol (BETAX), bisoprolol (BISO), budesonide (BUDE), bumetanide (BUME), bupranolol (BUPRA), canrenone (CANR), carteolol (CARTE), carvedilol (CARVE), celiprolol (CELI), chlortalidone (CLORTA), chlorthiazide (CLOTIA), clenbuterol (CLENB), clopamide (CLOPA), desonide (DESO), dexamethasone (DEXA), dichlorphenamide (DICLO), ethacrynic acid (ETACR AC), fenoterol (FENO), flumethasone (FLUME), flunisolide (FLUNIS), fludrocortisones (FLUDRO), furosemide (FURO), hydrochlorthiazide (HYDROCLOTIA), hydroflumethiazide (HYDROFLU), indapamide (INDA), labetalol (LABE), levobunolol (LEVO), 16α-methylprednisolone

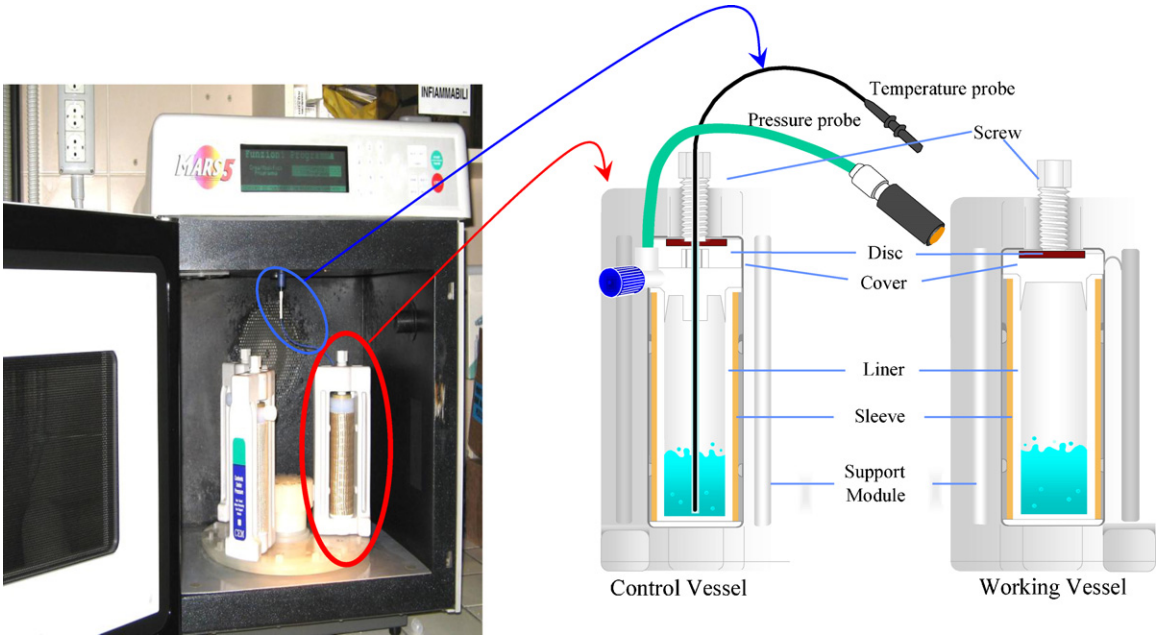

Fig. 1. Microwave oven and microwave extraction unit with closed vessels scheme.

Table 2
GC/MS: ions selected and recovery for beta2-agonists, steroids and narcotics.

Compounds	Ion selected (*m*/*z*)	MW conditions (time, power)	LOD traditional method (ng/mL)	LOD MW method (ng/mL)
Beta2-agonists				
Clenbuterol	86; 335	30 s, 1020 W	1	1
Fenoterol	322; 412	30 s, 1020 W	100	50
Procaterol	100; 407	60 s, 1020 W	60	50
Salbutamol	86; 369	30 s, 1020 W	50	50
Salmeterol	262; 369	30 s, 1020 W	100	60
Salmeterol metabolite	260; 369	30 s, 1020 W	100	60
Terbutaline	86; 356	30 s, 1020 W	100	50
Zilpaterol	308; 405	30 s, 600 W	80	70
Steroids				
Bolasterone	445; 315	30 s, 1020 W	10	5
Beta-boldenone	206; 430	60 s, 600 W	10	10
Boldenone metabolite	194; 417	60 s, 600 W	10	10
Calusterone	445; 315	30 s, 1020 W	10	5
Chlormethandienone metabolite	143; 315	30 s, 1020 W	10	10
Clostebol metabolite	451; 453	30 s, 1020 W	10	5
Danazol	481; 452	30 s, 1020 W	10	10
Danazo metabolite	468; 483	30 s, 1020 W	10	10
Drostanolone metabolite	433; 448	30 s, 1020 W	10	10
Epioxandrolone	143; 308	60 s, 600 W	10	10
Ethylestrenol	241; 270	30 s, 1020 W	10	10
Epi-trembolone	307; 322	30 s, 600 W	10	10
Fluoxymesterone metabolite	462; 208	30 s, 1020 W	10	5
Furazabol metabolite	143; 345; 387	30 s, 1020 W	10	10
Mesterolone metabolite	433; 448	30 s, 1020 W	10	10
Methandienone metabolite (epimethendiol)	143; 358; 253	30 s, 1020 W	10	10
Methandienone metabolita	517; 532	30 s, 1020 W	10	10
Methyltestosterone metabolite 1	143; 255; 360	30 s, 1020 W	10	10
Methyltestosterone metabolite 2	143; 255; 360	30 s, 1020 W	10	10
Mibolerome	301; 446	30 s, 1020 W	10	10
19-Norandrosterone	405; 420	30 s, 1020 W	2	1
19-Noretiocholanolone	405; 420	30 s, 1020 W	2	1
Norethandrolone	157; 331	30 s, 1020 W	10	10
Oxandrolone	143; 308	60 s, 600 W	10	10
Stanazolol metabolite	254; 545; 560	60 s, 600 W	2	2
Narcotics				
Buprenorphine	173; 554	30 s, 1020 W	50	30
Buprenorphine metabolite	173; 468	30 s, 1020 W	50	30
Idromorfone	429; 414	30 s, 600 W	50	30
Morphine	429; 414	60 s, 600 W	50	30
Oxicodone	459; 444	60 s, 1020 W	40	40
Oximorfone	517; 502	60 s, 1020 W	40	40

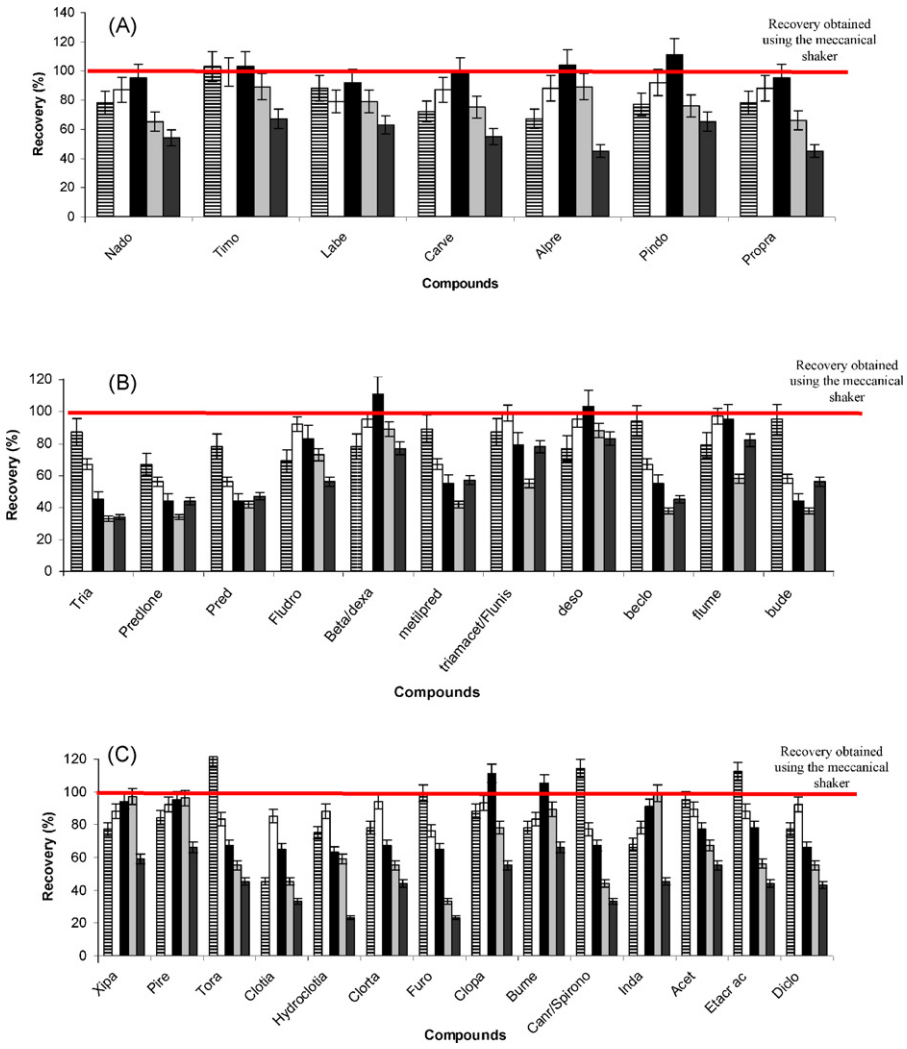

Fig. 2. LC–MS/MS: liquid/liquid extraction recovery in a urine sample spiked with *beta-blockers*: nadolol (NADO), timolol (TIMO), labetalol (LABE), carvedilol (CARVE), alprenolol (ALPRE), pindolol (PINDO), propranolol (PROPRA) (A); *glucocorticoids*: triamcinolone (TRIA), prednisolone (PREDLONE), prednisone (PRED), fludrocortisone (FLUDRO), betamethasone (BETA), dexamethasone (DEXA), 16α-methylprednisolone (METILPRED), triamcinolone acetonide (TRIAMACET), flunisolide (FLUNIS), desonide (DESO), beclomethasone (BECLO), flumetasone (FLUME) and budesonide (BUDE) (B); and *diuretics*: xipamide (XIPA), piretanide (PIRE), torasemide (TORA), chlorthiazide (CLOTIA), hydrochlorthiazide (HYDROCLOTIA), chlorthalidone (CLORTA), furosemide (FURO), clopamide (CLOPA), bumetanide (BUME), canrenone (CANR), spironolactone (SPIRONO), indapamide (INDA), acetazolamide (ACET), etacrynic acid (ETACR AC), dichlorphenamide (DICLO) (C) at the WADA MRPL concentration, using microwave irradiation: (dashed) 30 s 600 W; (open bar) 60 s 600 W; (black) 30 s 1020 W; (light grey) 60 s 1020 W; (grey) 30 s 1200 W. All results are relative to a control sample containing the compounds studied at the WADA MRPL concentration which were extracted for 10 min using a mechanical shaker.

(METILPRED), 17α-methyltestosterone (used as internal standard), metolazone (METO), moprolol (MOPRO), nadolol (NADO), oxprenolol (OXPRE), penbutolol (PENBU), pindolol (PINDO), piretanide (PIRE), procaterol (PROCA), prednisolone (PREDLONE), prednisone (PRED), propranolol (PROPRA), salbutamol (SALBU), spironolactone (SPIRONO), sotalol (SOTA), terbutaline (TERBU), timolol (TIMO), torsemide (TORA), triamcinolone (TRIA), triamcinolone acetonide (TRIAMACET) and xipamide (XIPA) were supplied by Sigma–Aldrich (Milano, Italy). Bolasterone (BOLA), beta-boldenone (b-BOLDE), boldenone metabolita (BOLDEm), calusterone (CALU), chlormetandienone metabolite (CHLORMETm), clostebol metabolite (4-chloro-4-androsten-3α-ol-17-one) (CLOSTm), danazol (DANA), danazol metabolita (DANAm), drostanolone metabolite (2α-methyl-5α-androstan-3α-ol-17-one) (DROSTAm), epioxandrolone (EPIOXA), epi-trembolone (EPITRE), ethylestrenolo (ETHYL), fluoxymesterone metabolite (9α-fluoro-17,17-dimethyl-18-norandrosta-4,13-diene-11β-ol-3-one) (FLUOXYm), furazabol (FURA), furazabol metabolite (16β-hydroxy-furazabol) (FURAm), mesterolone metabolite (MESTEm), methandienone metabolite (epimethendiol) (EPIMET), methenolone (METE), methenolone metabolite (METEm), 17α-methyltestosterone metabolite 1 (17α-methyl-5α-androstene-3α,17β-diolo) (METm1), 17α-methyltestosterone metabolite 2 (17α-methyl-5β-androstene-3α,17β-diolo) (METm2), mibolerone (MIBO), 19-norandrosterone (19-NA), 19-noretiocholanolone (19-NE), norethandrolone metabolita (NOREm), oxandrolone (OXA), stanozolol metabolite (3′-hydroxystanazolol) (STANAm) and zilpaterol (ZILPA) were purchased from NMI (National Measurement Institute, Pymble, Australia). Fluocortolone (FLUOC) and budesonide metabolite (16α-hydroxyprednisolone) (BUDEm) were kindly supplied by the WADA-accredited anti-doping laboratory of Belgium (Ghent DoCoLab). Buprenorphine (BUPRE), buprenorphine metabolite (BUPREm), oxycodone (OXYC), oxymorphone (OXYM), hydromorphone (IDROM) and morphine (MORPH) were obtained from LGC Promochem (Teddington, UK).

All reagents and solvents (sodium bicarbonate, potassium carbonate, sodium phosphate, sodium hydrogen phosphate,

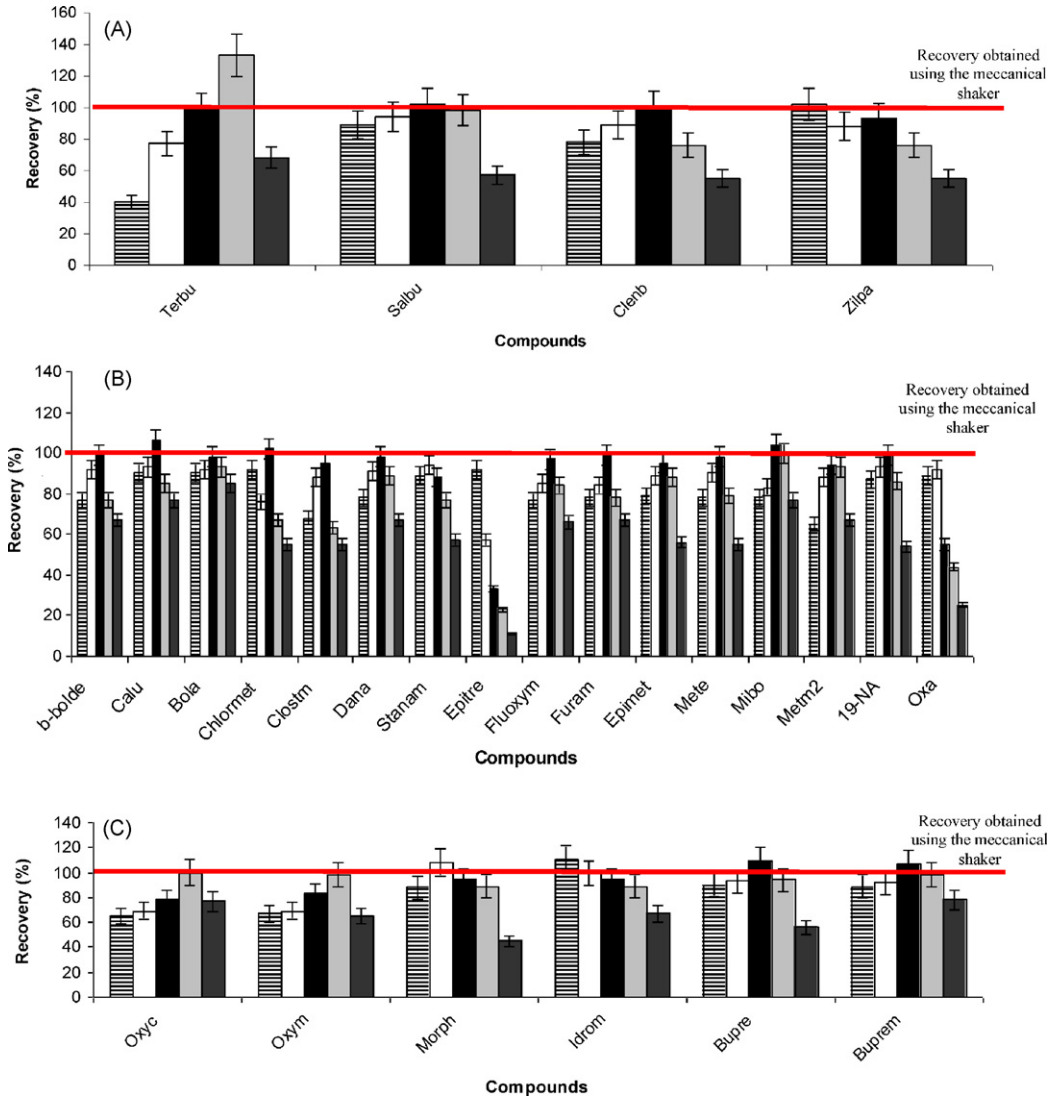

Fig. 3. GC–MS: liquid/liquid extraction recovery in a urine sample spiked with *beta2-agonists*: terbutaline (TERBU), salbutamol (SALBU), clenbuterol (CLENB), zilpaterol (ZILPA) (A); *steroids*: beta-boldenone (b-BOLDE), calusterone (CALU), bolasterone (BOLA), chlormethandienone metabolite (CHLORMET), clostebol metabolite (CLOSTm), danazol (DANA), stanozolol metabolite (STANAm), epi-trembolone (EPITR), metonolone (METE), mibolerone (MIBO), metandienonene metabolite 2 (METm2), 19-norandrosterone (19-NA), oxandrolone (OXA) (B); and *narcotics*: oxycodone (OXY), oxymorphone (OXYM), morphine (MORPH), hydromorphone (IDROM), buprenorphine (BUPRE), buprenorphine metabolite (BUPREm) (C) at the WADA MRPL concentration using microwave irradiation: (dashed) 30 s 600 W; (open bar) 60 s 600 W; (black) 30 s 1020 W; (light grey) 60 s 1020 W; (grey) 30 s 1200 W. All results are relative to a control sample containing the compounds studied at the WADA MRPL concentration which were extracted for 10 min using a mechanical shaker.

formic acid, *tert*-buthlmethyl ether, acetonitrile, diethylether, dichloromethane, ethyl acetate, formic acid) were of analytical or HPLC grade and provided by Carlo Erba (Milano, Italy); the enzyme β-glucronidase from *E. coli*, needed for the enzymatic hydrolysis of glucuronate conjugates, was purchased from Roche (Monza, Italy). The distilled water used was of Milli-Q-grade (Waters, Milano, Italy). Standard solutions were prepared at 1 mg/mL in methanol, the working solutions were prepared monthly and obtained by successive dilutions at concentrations from 100 to 10 μg/mL. All solutions were stored at −20 °C in the dark.

The derivatizing agent was a mixture of MSTFA/NH_4I/dithioerythreitol (1000:2:4, v/w/w) stored in screwed cap vials at 4 °C for maximum of 2 week. N-methyl-N(trimethylsilyl)-trifluoroacetamide (MSTFA) was supplied by Alfathech (Genova, Italy). Ammonium iodide (NH_4I) and dithioerythrytol (DTE) were supplied from Sigma–Aldrich (Milano, Italy).

2.2. Sample preparation procedures

2.2.1. GC–MS

We evaluated the compound recoveries first in water and then in urine samples. The determination of the compounds here considered was carried out by GC–MS after a pre-treatment procedure, accredited according to the ISO17025 and presently followed by the WADA-accredited anti-doping laboratory of Rome and already presented and discussed [10,11]. Briefly, to 3 mL of urine, 1.5 mL of phosphate buffer (pH 7.4), 50 μL of the enzyme β-glucuronidase from *E. coli* and 50 μL of internal standard (17α-methyltestosterone, 12 μg/mL) were added and incubated for 60 min at 50 °C. After hydrolysis, 1.5 mL of carbonate buffer (pH 9) were added to alkalinize the hydrolyzed solution. Extraction was carried out using 10 mL of the organic solvent (the solvents tested were: ethyl acetate, a mixture of chloroform/*tert*-butylmethyl

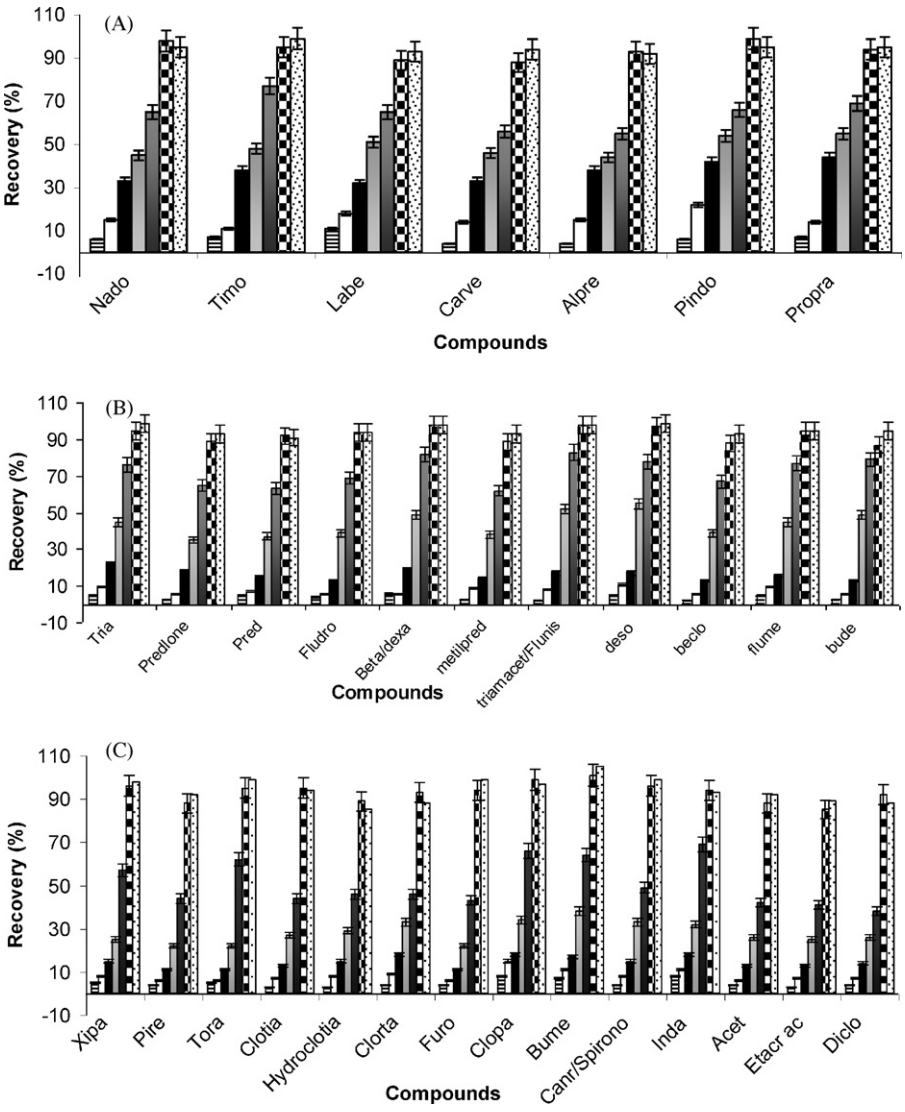

Fig. 4. LC–MS/MS: liquid/liquid extraction recovery in a urine sample spiked with *beta-blockers*: nadolol (NADO), timolol (TIMO), labetalol (LABE), carvedilol (CARVE), alprenolol (ALPRE), pindolol (PINDO), propranolol (PROPRA) (A); *glucocorticoids*: triamcinolone (TRIA), prednisolone (PREDLONE), prednisone (PRED), fludrocortisone (FLUDRO), betamethasone (BETA), dexamethasone (DEXA), 16α-methylprednisolone (METILPRED), triamcinolone acetonide (TRIAMACET), flunisolide (FLUNIS), desonide (DESO), beclomethasone (BECLO), flumetasone (FLUME) and budesonide (BUDE) (B); and *diuretics*: xipamide (XIPA), piretanide (PIRE), torasemide (TORA), chlorthiazide (CLOTIA), hydrochlorthiazide (HYDROCLOTIA), chlorthalidone (CLORTA), furosemide (FURO), clopamide (CLOPA), bumetanide (BUME), canrenone (CANR), spironolactone (SPIRONO), indapamide (INDA), acetazolamide (ACET), etacrynic acid (ETACR AC), dichlorphenamide (DICLO) (C) at the WADA MRPL concentration, using a mechanical shaker: (dashed) 30 s; (open bar) 60 s; (black) 120 s; (light grey) 240 s; (grey) 300 s (chess) 600 s and (dots) 1200 s. All results are relative to the analysis of a standard solution containing the compounds studied at the WADA MRPL concentration.

ether and dichloromethane/*tert*-butylmethyl ether, diethylether and *tert*-butylmethyl ether) for 10 min on a mechanical shaker or for 30–60 s on microwave oven. After centrifugation, the organic layer was transferred and evaporated to dryness under vacuum. The residue was then derivatized at 70 °C for 20 min, using 50 μL of a mixture of MSTFA/NH_4I/dithioerythreitol (1000:2:4, v/w/w) and 1 μL of the derivatized extract was injected into the GC–MS system.

2.2.2. LC–MS/MS

We evaluated the compound recoveries first in water and then in urine samples. The determination of the compounds here considered was carried out by LC–MS/MS after a pre-treatment procedure, accredited according to the ISO17025 and presently followed by the WADA-accredited anti-doping laboratory of Rome and already presented and discussed [12]. Briefly, to 3 mL of urine, 1.5 mL of phosphate buffer (pH 7.4), 50 μL of β-glucuronidase from *E. coli* and 50 μL of the ISTD (17α-methyltestosterone, 12 μg/mL; furosemide d5, 20 μg/mL) were added and incubated for 1 h at 50 °C. After hydrolysis the liquid/liquid extraction was carried out with 10 mL of the organic solvent (the solvents tested were: ethyl acetate, a mixture of chloroform/*tert*-butylmethyl ether and dichloromethane/*tert*-butylmethyl ether, *tert*-butylmethyl ether and diethylether) for 10 min on a mechanical shaker or for 30–60 s on microwave oven. After centrifugation the organic layer was joined to the first organic layer and evaporated to dryness. The residue was reconstituted in 50 μL of mobile phase and an aliquot of 10 μL was injected into the LC–MS/MS system.

2.3. Instrumental conditions

2.3.1. GC–MS

All GC–MS experiments were performed using an Agilent 5890/5973A (Agilent technologies SpA, Cernusco sul Naviglio, MI, Italy), in electron impact ionization (70 eV), using a 17 m fused silica capillary column cross-linked methyl silicone (HP1), ID 0.20 mm, film thickness 0.11 μm. The carrier gas was helium (flow rate:

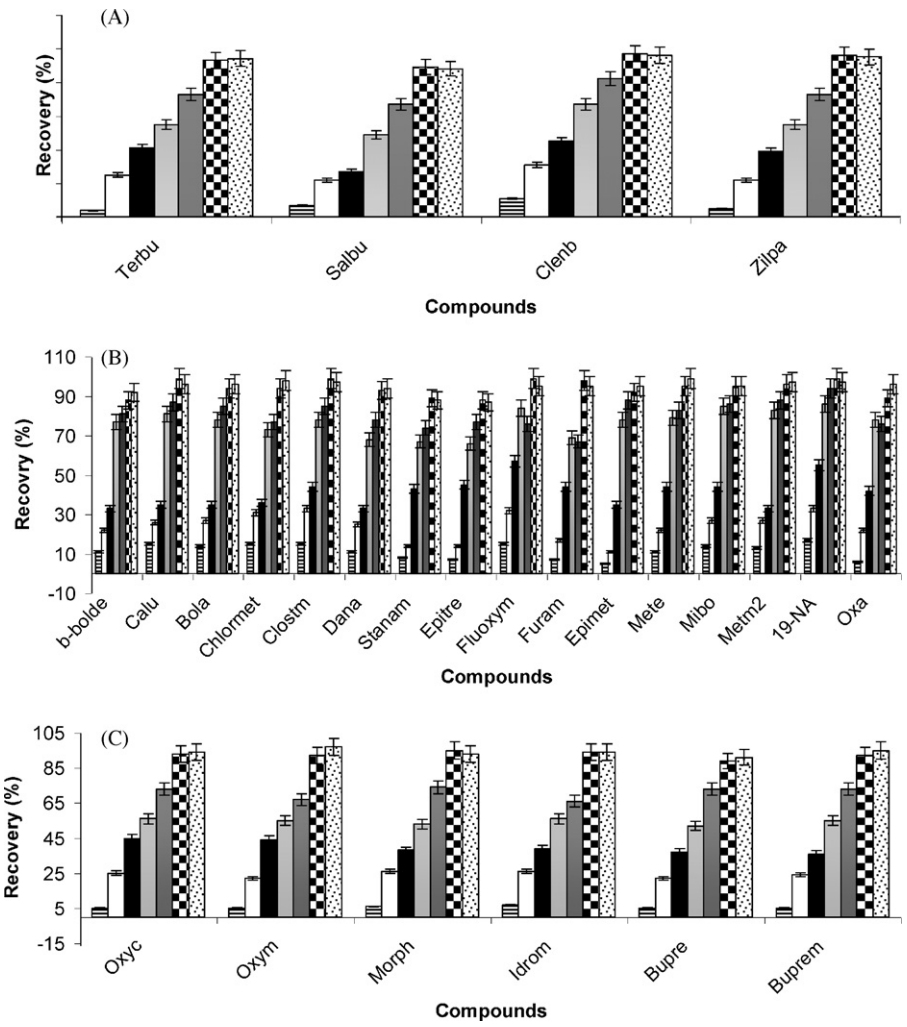

Fig. 5. GC–MS: liquid/liquid extraction recovery in a urine sample spiked with *beta2-agonists*: terbutaline (TERBU), salbutamol (SALBU), clenbuterol (CLENB), zilpaterol (ZILPA) (A); *steroids*: beta-boldenone (b-BOLDE), calusterone (CALU), bolasterone (BOLA), chlormethandienone metabolite (CHLORMET), clostebol metabolite (CLOSTm), danazol (DANA), stanozolol metabolite (STANAm), epi-trembolone (EPITR), metonolone (METE), mibolerone (MIBO), metandienonene metabolite 2 (METm2), 19-norandrosterone (19-NA), oxandrolone (OXA) (B); and *narcotics*: oxycodone (OXY), oxymorphone (OXYM), morphine (MORPH), hydromorphone (IDROM), buprenorphine (BUPRE), buprenorphine metabolite (BUPREm) (C) at the WADA MRPL concentration using a mechanical shaker: (dashed) 30 s; (open bar) 60 s; (black) 120 s; (light grey) 240 s; (grey) 300 s (chess) 600 s and (dots) 1200s. All results are relative to the analysis of a standard solution containing the compounds studied at the WADA MRPL concentration.

1 mL/min, split ratio 1:10), and the temperature program was as follows: 180 °C (hold 4.5 min), 3 °C/min to 230 °C, 20 °C/min to 290 °C, 30 °C/min to 320 °C; transfer line temperature: 280 °C. Acquisition was carried out in selected ion monitoring (SIM); the diagnostic ions monitoring for each compounds are listed in Table 1.

2.3.2. LC–MS/MS

All LC–MS/MS experiments were performed using an Agilent 1100 Series HPLC pump with binary gradient system and automatic injector (Agilent Technologies SpA, Cernusco sul Naviglio, MI, Italy). Reversed-phase liquid chromatography was performed on Supelco Discovery C18 column (2.1 mm × 150 mm, 5 μm). The solvents were: water containing 0.1% (v/v) formic acid (eluent A) and acetonitrile containing 0.1% (v/v) formic acid (eluent B). A gradient program started at 15% B and increasing to 60% B in 7 min and then, after 6 min to 100% B in 1 min. The column was then flushed for 1 min at 100% B and finally re-equilibrated at 15% B for 4 min. The flow rate was set at 250 μL/min.

Data were acquired using an Applied Biosystems (Applera Italia, Monza, Italy) API4000 triple-quadrupole instrument with positive electro-spray ionization. The ion source was operated at 550 °C, the applied capillary voltage was 5500 V and selected reaction monitoring (SRM) experiments were performed employing collision-induced dissociation (CID) using nitrogen as collision gas at 5.8 mPa, obtained from a dedicated nitrogen generator system (Parker-Balston model 75-A74, gas purity 99.5%). The transitions used for SRM method are reported in Table 1. Data acquisition was divided into three segments based on expected retention time to improve the method sensitivity and to increase data point sampling along the chromatographic peaks.

2.4. Microwave conditions

Liquid/liquid extraction was conducted in a temperature controlled single beam microwave oven for organic synthesis MARS5 (Microwave Apparatus CEM Corporation, Matthews, NC, USA). The microwave used has a maximum power output of 1200 W and a nominal frequency of 2.45 GHz. It is equipped with an extraction unit with closed vessels (see Fig. 1 for the microwave system scheme). The vessels are designed with materials that are microwave transparent and relatively inert to solvents. The control vessels have a different cap and cover to enable connection of a fibre-optic temperature probe and a pressure sensing tube to allow monitoring of the internal temperature and pressure of the vessel.

The fibre-optic temperature probe is microwave transparent and allows for temperature control of the extraction process.

The extraction studies were carried out on water and urine samples spiked with all compounds at a WADA minimum required performance level (MRPL) concentrations.

The water and urine samples were treated according to the procedure described before, and the extraction was sampled at intervals of 10, 30, 60, 120, 240, 300, 600 and 1200 s.

3. Results

Figs. 2 and 3 show the liquid/liquid extraction recoveries at different microwave powers and extraction times. We can notice that using the microwave procedures the liquid/liquid extraction was faster (30–60 s instead of 10 min, see Figs. 2–5), and in some instances (e.g. several narcotics, diuretics, beta-blockers and beta2-agonists) also more effective (see Figs. 2 and 3) than the traditional method. More specifically the graphs in Figs. 2 and 3 show that the microwave assisted extraction was 100% complete after 30–60 s; while using a mechanical shaker, the extraction process was <20% complete at 60–120 s and 100% complete in 5–10 min (see Figs. 4 and 5).

The optimum power was 600 W for the most thermolable compounds such as epi-trembolone, oxandrolone, triamcinolone, prednisolone and diuretic thiazides; and 1020 W for the other substances studied. At higher power and times the liquid/liquid extraction recoveries decreased (see Figs. 2 and 3).

We evaluated, also, the effects of microwave irradiation on the compound recoveries using different extraction solvents. The recoveries of narcotics, steroids and glucocorticoids followed the order: ethyl acetate ≥ diethylether ≈ *tert*-butylmethyl ether > *tert*-butylmethyl ether/chloroform ≈ *tert*-butylmethyl ether/dichloromethane (data not shown). While the recovery of diuretics, beta2-agonists and beta-blockers followed the order: ethyl acetate ≥ *tert*-butylmethyl ether/chloroform ≈ *tert*-butylmethyl ether/dichloromethane > diethylether ≈ *tert*-butylmethyl ether (data not shown).

The validation data showed that the repeatability of the extraction recovery (CV% <10 for all target compounds), obtained after microwave assisted extraction of 20 different urine samples spiked with the compounds of interest, was comparable with the results obtained by the traditional method. The limits of detection were in the range between 1 and 10 ng/mL for androgenic anabolic steroids, 3 and 15 ng/mL for glucocorticoids, 2 and 100 ng/mL for diuretics and beta2-agonists and 30 and 50 ng/mL beta-blockers and narcotics (see Tables 1 and 2), thus satisfying, the minimum required performance levels set by the World Anti-doping Agency [13]. Particularly, the requirements for the accredited laboratories include a minimum required performance limit of 10 ng/mL for the androgenic anabolic steroids (with a lower limits for specific target compounds: 2 ng/mL for the stanozolol metabolite, epimetendiol and 17α-methyltestosterone metabolite 2 and 1 ng/mL for 19-norandrosterone), of 30 ng/mL for the whole class of glucocorticoids, 50 ng/mL for the narcotics (with a cut off value set at 1000 ng/mL for morphine), 500 ng/mL for the whole class of beta-blockers of 100 ng/mL for the beta2-agonists (with a cut off value set at 1000 ng/mL for salbutamol).

4. Discussion and conclusions

This preliminary study demonstrates that this is possible using microwave irradiation to speed up the liquid/liquid extraction step of diuretics, narcotics, glucocorticoids, beta-blockers and beta2-agonists, all included in the list of prohibited substances published every year by the World Anti-doping Agency [14]. Microwave power, extraction time and extraction solvent were optimized to achieve an increase in the recovery efficiency and decrease in liquid/liquid extraction time and solvent volume. Microwave assisted extraction is the process of heating solvent in contact with a sample with microwave energy to isolate compounds of analytical interest from the sample matrix into the solvent. The principle of heating using microwave is based upon its direct impact with polar materials/solvents and is governed by two phenomenona: ionic conduction and dipole rotation, which in most cases occurs simultaneously [6]. The above mechanisms clearly indicate that only dielectric material or solvents with permanent dipole get heated up under microwave irradiation. In our experiments the presence of water produced a “superheating” effects with a consequent increase in the diffusivity of the analyte in the matrix. Thus the solvent choice, generally related, also, to the microwave absorbing properties of the solvent; was in our case related, only, to the interaction between solvent and matrix and to the solubility of the target analyte on the organic solvent. Other observation accomplished with the results obtained by the upgrade of the traditional liquid/liquid extraction procedure are reported: (i) the optimum power was found to be 600 W (generating a temperature of 70 °C) for the most thermolable constituents such as for example triamcinolone, prednisolone, chlorthiazide, chlorthalidone, epi-trembolone and oxandrolone, and 1020 W (generating a temperature of 150 °C) for the other compounds considered in this study. Experiments carried out at higher power and times showed a loss of the extraction recovery due to the degradation of the substances at elevated temperature; (ii) for all compounds studied, the best recovery results were obtained using ethyl acetate. Nevertheless using ethyl acetate the matrix effect and ion suppression were higher in confront to the other solvents tested especially if we considered the androgenic anabolic steroids and glucocorticoids for which the use of diethylether or *tert*-butylmethyl ether permits a good recovery and no interferences at the retention times of interest; (iii) the limits of detection were in the range between 1 and 10 ng/mL for androgenic anabolic steroids, 3 and 15 ng/mL for glucocorticoids, 2 and 100 ng/mL for diuretics and beta2-agonists and 30 and 50 ng/mL beta-blockers and narcotics, allowing the detection in urine samples for a period more than adequate for doping purpose; and (iv) for some classes of substances such as diuretics and glucocorticoids the liquid/liquid extraction using microwaves is more convenient for the confirmation analysis. In fact some substances of these classes are thermolable, thus different microwave conditions (power applied) have to be applied.

In conclusion, our results showed that accelerated extraction of low molecular weight compounds under controlled microwave irradiation is practically feasible, without sacrificing sensitivity and specificity. The method proposed is applied to the screening and confirmation analysis in Anti-doping analysis, but can be effective also in other areas such as clinical, toxicological and forensic analysis. The results obtained in this study and in the previous researches carried out in our laboratory indicate the need for continued study in this area of Anti-doping research.

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