UHPLC−HRMS Method for the Simultaneous Screening of 235 Drugs in Capillary Blood for Doping Control Purpose: Comparative Evaluation of Volumetric and Non-volumetric Dried Blood Spotting Devices

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1. INTRODUCTION

Dried blood spots (DBSs) are a form of bio-samples in which capillary blood by fingertip or arm pricking is applied onto marked circles on untreated/treated cellulose paper or adsorbed on specially manufactured volumetrically controlled polymer-based tips or dots. Since its introduction, the use of DBS, as an alternative matrix, has been progressively extending, now covering many different applications, including therapeutic drug monitoring, forensic analysis, and, more recently, doping analysis. Indeed, DBS provide several advantages compared to conventional venous blood samples: (i) the procedure for the collection of the sample is simplified and minimally invasive, so that it can be successfully performed even by minimally trained personnel, (ii) it offers favorable stability of many analytes, (iii) the collection process reduces the risk of infection, (iv) the risk of bacterial contamination or hemolysis is minimal, (v) the collection devices are generally low cost; and finally (vi) the storage and transport of samples are easier and without additional costs associated with the need of ensuring a rigid temperature control along the chain of custody. Nonetheless, different challenges also need to be faced when DBSs are used and primarily among them are as follows: (i) to ensure a sufficient quality of the spot (mainly in terms of size and homogeneity), (ii) to allow a satisfying recovery of all the target analytes in the case of multi-targeted assays, a critical parameter given the reduced available volume of the sample, (iii) to assess the relevance of hematocrit effects, possibly influencing the performance of the method was confirmed to be fit for purpose, and data obtained in blood can also be used to complement those available in urine, allowing to refine the knowledge concerning the pharmacokinetic profiles.

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analyte concentration and recovery in the case of quantitative determinations, (iv) to take into account the blood-to-plasma ratio (that provides an indication of the binding to erythrocytes of the target analytes), (v) to optimize the timing and procedure for the addition of the internal standard, indispensable in the case of quantitative determinations, (vi) to avoid the loss of volatile or photodegradable analytes, and finally (vii) to specifically consider the effects on the integrity of the sample of environmental conditions, primarily humidity and bacterial growth.\(^{6,7,12-15}\) For the above reasons, different countermeasures were proposed, including (i) the use of plastic bags containing adequate desiccants (i.e., silica gel and bentonite) and humidity indicators, (ii) the use of calibrated capillaries and of volumetric microsampling devices, in the aim to increase the repeatability and reproducibility of the spots, (iii) the use of dried plasma spots (DPSs), in order to overcome multiple hematocrit effects and plasma-to-blood ratio issues, and finally (iv) the measurement of hematocrit directly on the DBS cards to control multiple hematocrit effects.\(^{15-23}\) All the above strategies allowed us to increase the robustness of DBS sampling and the reliability of the quantitative determinations. Nevertheless, the timing of the internal standard addition remains a critical issue for the quantitative assays. The most common approaches consist of the addition of the internal standard into the extraction solvent or directly on the spot, even if in both cases, there is a clear discrepancy between the extraction of the analytes, spotted on the inert support from the capillary and then extracted, and that of the internal standard, added to the spot after the deposition of the sample, with potential effects on the accuracy and precision of the quantitative determination.\(^{21-23}\)

In the doping control field, the blood matrix is primarily used for those substances or parameters that cannot be determined in urine (e.g., markers of blood transfusions, hemoglobin-based oxygen carriers, biomarkers of the hematological module of the athlete biological passport, growth hormone and IGF-1, and anabolic steroid esters). However, the availability of the blood matrix could also be relevant for compounds currently screened for in urine; indeed, the measurement of doping substances and/or their metabolites in the blood, especially if combined with the analysis of a urine sample, would be a source of additional information about (i) the pharmacokinetics of the prohibited compound(s), (ii) the pharmacological effects at the time of the sample collection of substances banned only in-competition and of those substances prohibited only when administered systemically, (iii) the potential manipulation or environmental contamination of urine samples, (iv) the endogenous versus exogenous origin of those compounds that can also be formed ex vivo (e.g., prednisone, prednisolone, and 19-nortestosterone metabolites), (v) the identification of the parent drug, in the case of metabolites that may be formed from different drugs, and (vi) the need of obtaining supplementary evidence in the case of positive results in controversial cases. Moreover, drugs are usually detectable in blood also as unchanged compounds: the availability of blood samples together with the urines might help to better identify the use of novel compounds, whose pharmacological effects may be similar to those of drugs already included in the list of the prohibited substances and methods of the World Anti-Doping Agency (WADA).

To date, being the venous blood collection extremely invasive, only 9% of the doping control tests are performed in blood samples according to the 2019 WADA statistics\(^{24}\) (data of 2020 were significantly affected by the anti-CoViD-19 measures). The use of capillary blood could allow not only to increase the number of doping control tests and consequently the probability to report an Adverse Analytical Finding (AAF) for those compounds detected only in the blood matrix but, as outlined above, also to gather additional information to elucidate controversial cases. For the previously mentioned reasons, the WADA and the International Testing Agency (ITA) have recently encouraged the use of DBS as an alternative matrix to determine both threshold and non-threshold prohibited compounds: a WADA technical document reporting the guidelines for the DBS sample collection equipment (TD2021DBS) was first published in September 2021.\(^{25}\)

Various promising applications of DBS in the doping control field were proposed over the previous 10 years for the analysis of both proteins\(^{52-58}\) and small molecules\(^{49-61}\) included in the WADA’s 2022 Prohibited List.\(^{26}\) However, only a limited number of targets were covered for each class of prohibited small molecules. Thomas et al. published a DBS-based screening focusing on 26 model compounds belonging to different prohibited classes of substances;\(^{49}\) the other studies are mainly focused on the detection of several anabolic steroids and/or their esters and on threshold compounds as well as on few substances prohibited only in competition.\(^{52,53,56,57,60}\) Nonetheless, due to the small volume of biological sample collected when capillary blood is used as an alternative matrix, the development of multi-analyte procedures is of crucial importance to maximize the utility of the test.

Similar to other fields of analytical chemistry in which it is necessary to verify the presence, in a given sample, of a large number of target analytes, also the WADA-approved analytical procedures are based on a two-step process: an “initial testing procedure” (ITP), whose aim is to preliminarily screen for the presence/absence of the panel of target analytes in the sample, and a “confirmation analysis” (CP), which is activated only on those samples that did not result clearly negative at the ITP. This two-step protocol allows for minimizing the risk of both false-positive and false-negative results. Indeed, the sensitivity of the ITP should be good enough to exclude any false-negative result, and the specificity of the CP should be good enough to exclude any false-positive result.

We are here presenting a newly developed multi-targeted ITP for the screening of 235 small molecules (MW < 1000 Da, 225 compounds plus 10 metabolites) in DBS and DPS. The method is based on ultra-high-performance liquid chromatography coupled to high-resolution mass spectrometry (UHPLC–HRMS). The analytical procedure has been fully validated according to both ISO17025 and the requirements of the WADA,\(^{51-53}\) and its actual applicability has been assessed on real samples. The target compounds here selected cover all the classes of prohibited substances (S0, S1, S2, S3, S4, S5, S6, S7, S8, S9, and P1) included in the WADA 2022 Prohibited List\(^{50}\) and include 2 non-approved substances, 18 anabolic agents (plus the metabolite of andarine), 5 confounding factors of the endogenous steroid profile, 5 hypoxia-inducible factor activating agents, 7 beta2-agonists, 13 metabolic modulators (plus the main metabolites of exemestane, GW1516, GW7042, and SR9009), 45 diuretics, 70 stimulants (plus the main metabolite of cocaine), 12 narcotics, 6 cannabimimetics, 26 glucocorticoids (plus the main metabolite of deflazacort), and 16 beta-blockers.

We also addressed the logistic aspects of the expected vast scale application of the method, specifically considering the performance of different microsampling devices presently available on the market (i.e., HemaSpot-HF, Whatman 903 Protein Saver cards, Whatman FTA DMPK-A, B and C cards,
Tasso-M20, and Mitra tips; see Table 1 for details), as well as the recovery and stability of the different classes of prohibited substances in DBS and DPS.

The newly developed workflow was finally applied to analyze post-administration samples containing acetazolamide or delazacort to obtain information concerning the pharmacokinetic profile, the window of detection (compared to that in urine), and the plasma-to-blood ratios, all parameters that, as said above, could provide additional information in the post-analytical activities of result management.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents. The certified standards of the target analytes were purchased from different suppliers (see Tables 2–5 for details). The internal standards morphine d3 (used for the compounds included in section S7), amphetamine d11, and cocaine d3 (used for the compounds included in section S6) were purchased from Cerilliant (Sigma-Aldrich, Milano, Italy). Acetazolamide d3 and bumetanide d5 (used as internal standards for the compounds included in section S5), letrozole d4 (used as an internal standard for the compounds included in section S4), fluconazole d4 (used as an internal standard for the confounding factors), and triamcinolone acetonide d7 (used as an internal standard for the compounds included in section S9) were purchased from Toronto Research Chemicals (TRC, North York, Canada). 17α-Methyltestosterone (used as an internal standard for the anabolic agents and compounds included in sections S0 and S2) was obtained from Steraloids (Newport, RI, USA). Double-labeled formoterol (used as an internal standard for the compounds included in sections S3 and P1) was obtained by AlsaChim (Illkirch, France). JWH018 d5 (used as an internal standard for the compounds included in sections S0 and S2) was obtained from Spot-On Sciences (San Francisco, CA, USA). The HemaSpot-HF Blood Collection Devices were purchased from Spot-On Sciences (San Francisco, CA, USA). The Whatman 903 Protein saver, FTA DMPK A, B, and C cards were purchased from Sigma-Aldrich (Milano, Italy). Tasso-Spot On-Deman push-button devices were purchased from Tasso (Seattle, WA, USA). Mitra VAMS devices (20 μL fixed) were purchased from Neoteryx (Torrance, CA, USA). Table 1 reports the characteristic of each microsampling device considered in this study.

Microlet lancets were obtained from Bayer Health Care (Leverkusen, Germany). Microvette CB300 Lithium Heparin tubes were purchased from Sarstedt S.r.l (Trezzano Sul Naviglio, Milano, Italy).

The reagents used for sample pre-treatment and instrumental analysis (ammonium formate, potassium carbonate, sodium phosphate, sodium hydrogen phosphate, sodium hydrogen carbonate, tert-butylmethylether, ethylacetate, formic acid, acetic acid, ammonia, acetonitrile, methanol, isopropanol, and acetone) were all of analytical grade and supplied by Sigma-Aldrich (Milano, Italy). The Oasis MCX cartridges were purchased from Waters (Milano, Italy).

2.2. Stock Reference Solutions. The standard stock reference solutions of all the compounds under investigation were prepared at a concentration of 1 mg mL−1 in methanol and stored at −20 °C. A working solution containing a mixture of the target analytes was then prepared.

As for the internal standards, an aqueous solution containing morphine d3, amphetamine d11, cocaine d3, acetazolamide d3,...
| Compound       | Elemental Composition | Molecular Ion (m/z)  | RT (min) | LOD (ng mL⁻¹) | RE (%) | ME (%) | Supplier                                      |
|----------------|-----------------------|----------------------|----------|---------------|--------|--------|-----------------------------------------------|
| JTV-519        | C₂₆H₂₆N₂O₅S          | [M + H]⁺ 425.2257    | 7.2      | 0.2           | 65 ± 10| 18     | Sigma-Aldrich (Milano. Italy)                 |
| S107           | C₁₃H₁₃NO₅S           | [M + H]⁺ 210.0947    | 4.4      | 0.2           | 71 ± 10| 21     | Sigma-Aldrich (Milano. Italy)                 |
| ACP 105        | C₂₆H₃₂ClN₃O          | [M + H]⁺ 291.1259    | 10.5     | 1.0           | 61 ± 10| 18     | Toronto Research Chemicals (North York, Canada) |
| andarine       | C₂₆H₂₂F₆N₃O₃        | [M − H]⁻ 440.1075    | 8.6      | 0.1           | 75 ± 10| 15     | Toronto Research Chemicals (North York, Canada) |
| andarine metabolite | C₂₆H₂₂F₆O₃N₂    | [M − H]⁻ 307.0547    | 7.6      | 0.1           | 72 ± 10| 16     | World Association of Anti-Doping Scientists (WAADS) |
| CL-4AS-1       | C₂₆H₂₂ClN₃O₂         | [M + H]⁺ 441.2303    | 12.8     | 1.0           | 62 ± 10| 22     | Santa Cruz Biotechnology (Dallas, USA)         |
| gestrinone      | C₂₆H₂₂O₃             | [M + H]⁺ 3091849     | 9.1      | 0.2           | 82 ± 10| 18     | Toronto Research Chemicals (North York, Canada) |
| GLPG-0492      | C₂₆H₂₂F₆N₃O₃        | [M − H]⁻ 388.0915    | 9.5      | 1.0           | 62 ± 10| 22     | Toronto Research Chemicals (North York, Canada) |
| LGD4033        | C₂₆H₂₂F₆N₃O         | [M + HCOOH-H]⁻ 383.0836| 10.4     | 0.2           | 79 ± 10| 15     | Toronto Research Chemicals (North York, Canada) |
| LY 2452473     | C₂₆H₂₂N₃O₂           | [M + H]⁺ 375.1816    | 9.0      | 0.2           | 65 ± 10| 23     | MedChemExpress (MCE) (D.B.A. Milano, Italy)   |
| methyltrienolone | C₂₆H₂₁O₂              | [M + H]⁺ 285.1849    | 8.3      | 0.2           | 82 ± 10| 28     | Toronto Research Chemicals (North York, Canada) |
| MK 0773        | C₂₆H₂₂F₆N₃O₂         | [M + H]⁺ 480.2769    | 6.3      | 0.5           | 69 ± 10| 24     | MedChemExpress (MCE) (D.B.A. Milano, Italy)   |
| ostarine        | C₂₆H₂₂F₆N₃O₃        | [M − H]⁻ 388.0915    | 9.5      | 0.1           | 77 ± 10| 16     | Toronto Research Chemicals (North York, Canada) |
| RAD 140        | C₂₆H₂₂ClN₃O₂         | [M − H]⁻ 348.0658    | 9.0      | 0.2           | 71 ± 10| 22     | Cayman Chemical (Ann Arbor, MI, USA)          |
| S1             | C₂₆H₂₂ClF₆N₃O₄      | [M − H]⁻ 417.0471    | 12.0     | 0.5           | 68 ± 10| 23     | Toronto Research Chemicals (North York, Canada) |
| S6             | C₂₆H₂₂ClF₆N₃O₃      | [M − H]⁻ 435.0376    | 12.3     | 0.5           | 66 ± 10| 25     | Sigma-Aldrich (Milano. Italy)                 |
| S9             | C₂₆H₂₂F₆N₃O₄         | [M − H]⁻ 401.1077    | 10.7     | 0.5           | 71 ± 10| 24     | Toronto Research Chemicals (North York, Canada) |
| S23            | C₂₆H₂₂ClF₆N₃O₅      | [M − H]⁻ 415.0478    | 11.5     | 0.2           | 65 ± 10| 22     | Toronto Research Chemicals (North York, Canada) |
| stanozolol      | C₂₆H₂₁N₂O₂           | [M + H]⁺ 329.2587    | 8.2      | 0.5           | 63 ± 10| 17     | Toronto Research Chemicals (North York, Canada) |
| tetrahydrogestrinone | C₂₆H₂₄O₂           | [M + H]⁺ 313.2162    | 10.1     | 0.5           | 74 ± 10| 25     | National Measurement Institute (NMI)          |
| TFM-4AS-1      | C₂₆H₂₂F₆N₃O₂         | [M + H]⁺ 475.2567    | 11.9     | 1.0           | 63 ± 10| 22     | Sigma-Aldrich (Milano. Italy)                 |
| daprostatat     | C₂₆H₂₁N₂O₃           | [M − H]⁻ 392.1827    | 16.0     | 1.5           | 62 ± 10| 28     | Biovision (San Francisco, USA)                |
| enarodustat     | C₂₆H₂₁N₂O₄           | [M + H]⁺ 341.12443   | 9.2      | 1.5           | 63 ± 10| 25     | MedChemExpress (MCE) (D.B.A. Milano, Italy)   |
| FG2216         | C₂₆H₂₁N₂O₃           | [M − H]⁻ 279.01781   | 8.9      | 1.0           | 65 ± 10| 22     | MedChemExpress (MCE) (D.B.A. Milano, Italy)   |
| FG4592         | C₂₆H₂₁ClN₃O₄         | [M + H]⁺ 335.11320   | 10.7     | 1.0           | 63 ± 10| 22     | Toronto Research Chemicals (North York, Canada) |
| JOX2           | C₂₆H₂₁N₂O₃           | [M − H]⁻ 351.09864   | 9.7      | 1.0           | 62 ± 10| 25     | Sigma-Aldrich (Milano. Italy)                 |
| bamberterol     | C₂₆H₂₁N₂O₅           | [M + H]⁺ 368.2180    | 5.2      | 2.0           | 65 ± 10| 18     | Sigma-Aldrich (Milano. Italy)                 |
| indacaterol     | C₂₆H₂₁N₂O₅           | [M + H]⁺ 393.2173    | 6.4      | 2.0           | 62 ± 10| 22     | Sigma-Aldrich (Milano. Italy)                 |
| mabuterol       | C₂₆H₂₁ClF₆N₃O₄      | [M + H]⁺ 311.1133    | 5.4      | 2.0           | 63 ± 10| 18     | Sigma-Aldrich (Milano. Italy)                 |
| racotepramine   | C₂₆H₂₁NO₄            | [M + H]⁺ 302.1751    | 6.2      | 1.0           | 65 ± 10| 20     | Sigma-Aldrich (Milano. Italy)                 |
| reprotozol      | C₂₆H₂₁N₂O₅           | [M + H]⁺ 402.1772    | 3.4      | 2.0           | 66 ± 10| 16     | Toronto Research Chemicals (North York, Canada) |
| tuloconazole     | C₂₆H₂₁CINO           | [M + H]⁺ 228.1150    | 4.7      | 0.5           | 67 ± 10| 17     | Sigma-Aldrich (Milano. Italy)                 |
| vilanterol      | C₂₆H₂₁ClNO₃          | [M + H]⁺ 486.1809    | 6.8      | 2.0           | 62 ± 10| 19     | Sigma-Aldrich (Milano. Italy)                 |
| dutasteride     | C₂₆H₂₂F₆N₃O₃        | [M + H]⁺ 545.2333    | 12.2     | 1.0           | 66 ± 10| 22     | Sigma-Aldrich (Milano. Italy)                 |
| finasteride     | C₂₆H₂₁N₂O₄           | [M + H]⁺ 373.2850    | 9.8      | 1.0           | 65 ± 10| 16     | Toronto Research Chemicals (North York, Canada) |
| fluconazole     | C₂₆H₂₁F₂N₃O₂         | [M + H]⁺ 307.1113    | 5.3      | 1.0           | 71 ± 10| 19     | Sigma-Aldrich (Milano. Italy)                 |
Table 2. continued

| compound              | elemental composition | molecular ion (m/z) | RT (min) | LOD<sup>\text{a}</sup> (ng mL<sup>-1</sup>) | RE<sup>\text{a}</sup> (%) | ME<sup>\text{a}</sup> (%) | supplier                  |
|-----------------------|-----------------------|--------------------|---------|------------------------------------------|--------------------------|--------------------------|---------------------------|
| ketoconazole          | C<sub>26</sub>H<sub>34</sub>Cl<sub>2</sub>N<sub>2</sub>O<sub>4</sub> | [M + H]<sup>+</sup> | 531.1560 | 6.6                                      | 1.0                      | 77 ± 10                  | Sigma-Aldrich (Milano, Italy) |
| miconazole            | C<sub>26</sub>H<sub>34</sub>Cl<sub>2</sub>N<sub>2</sub>O | [M + H]<sup>+</sup> | 414.9933 | 8.1                                      | 1.0                      | 75 ± 10                  | Sigma-Aldrich (Milano, Italy) |

<sup>\text{a}</sup>Results obtained by using Mitra and pure methanol as the extraction solvent.

2.3. Samples of Capillary Blood. All validation steps and method development experiments were performed on capillary blood (whole blood and plasma) collected from five male and five female healthy volunteers not taking any medication, with a hematocrit value varying from 32 to 45%. A written consent from the volunteers was obtained. The study was approved by the local ethics committee (Lazio 1).

Capillary blood samples were obtained using a personal lancing device (OneTouch Ultra Soft) with single-use sterile lancets (Microlet). Blood drops from finger pricks were collected with Microvette CB300 Lithium Heparin tubes. Aliquots of 20 μL of whole blood or plasma (the latter obtained after centrifugation) were then applied by means of calibrated pipettes to the volumetric and non-volumetric microsampling devices selected (i.e., HemaSpot-HF, Whatman 903 Protein Saver Card, Whatman FTA DMPK-A, -B, and -C cards, TassoSpot On-Deman push-button device, or Mitra tips). The samples were then dried for 2 h at room temperature (20–25 °C) protected from direct light sources, and then stored with a suitable desiccant (e.g., silica gel and bentonite) in zip-closure foil bags (GE Healthcare, Westborough, MA, USA) until analysis.

For the stability study, three pools of capillary blood samples were spiked with the compounds under investigation at a concentration 5 times the LOD. Aliquots of 20 μL from each pool were then applied to the selected microsampling devices and let dry for 2 h at room temperature. The samples, divided in three batches, were stored at different temperatures: at 50 °C for 1 week and at 25 °C and at 4 °C for 4 weeks.

Post-administration study specimens were collected from subjects under treatment with deflazacort (one male (subject 1) and one female (subject 2) subject, oral administration of one tablet containing 6 mg of deflazacort. Deflan, Laboratori Guidotti, Pisa, Italy) or acetazolamide (two female subjects (subjects 3 and 4), oral administration of half tablet containing 6 mg of deflazacort. Deflan, Laboratori Guidotti, Pisa, Italy) or acetazolamide [two female subjects (subjects 3 and 4), oral administration of half tablet containing 6 mg of deflazacort. Deflan, Laboratori Guidotti, Pisa, Italy) or acetazolamide with those obtained in blood samples, were pre-treated using the validated procedure currently adopted by our laboratory to detect more than 300 prohibited compounds in the occasion of doping control tests. Briefly, an aliquot of 20 μL of urine was fortified with 2 μL of the internal standard solution (morphine d3, amphetamine d11, cocaine d3, acetazolamide d3, bumetanide d5, letrozole d4, JWH018 d5, double-labeled formoterol, 17α-methyltestosterone, fluconazole d4, and triamcinolone acetonide d7 at a final concentration of 50 ng mL<sup>-1</sup>), transferred into a glass tube, and suspended into the extraction reagent. The samples were then centrifuged at 1509g for 5 min.

In the case of extraction with aqueous reagents, the supernatant was transferred into a fresh glass tube, and liquid/liquid (e.g., tert-butylmethylether at pH 7 and ethylacetate at pH 5) or solid-phase extraction (SPE) (e.g., Oasis MCX cartridges, 1 mL, 30 mg) was performed using the protocols already in use in our laboratory. The eluent/extraction solvent was then evaporated to dryness under a gentle stream of nitrogen at 40 °C for 20 min.

In the case of extraction with organic solvents, the supernatant was transferred into a fresh glass tube and evaporated to dryness under a gentle stream of nitrogen at 40 °C for 10 min.

For both protocols, the dry residue was finally reconstituted in 50 μL of mobile phase (initial composition), and an aliquot of 10 μL was injected onto the UHPLC–HRMS system.

2.4.2. Urine Samples. The urine samples, collected to compare the profile and windows of detection of deflazacort and acetazolamide with those obtained in blood samples, were pre-treated using the validated procedure currently adopted by our laboratory to detect more than 300 prohibited compounds in the occasion of doping control tests. Briefly, an aliquot of 20 μL of urine was fortified with 2 μL of the internal standard solution (morphine d3, amphetamine d11, cocaine d3, acetazolamide d3, bumetanide d5, letrozole d4, JWH018 d5, double-labeled formoterol, 17α-methyltestosterone, fluconazole d4, and triamcinolone acetonide d7 at a final concentration of 50 ng mL<sup>-1</sup>), the sample was then hydrolyzed for 1 h at 50 °C using 10 μL of β-glucuronidase and 200 μL of phosphate buffer (0.8 M, pH 7.4). After hydrolysis, urine samples were acidified (pH lower than 5) and purified by using the Oasis MCX cartridges (1 mL, 30 mg), previously conditioned with 1 mL of methanol and 1 mL of ultra-purified water. The cartridges were then washed with 1 mL of water/methanol (80/20). The compounds of interest were finally eluted using 1 mL of methanol/formic acid (95/5) containing 150 mM of ammonium formate. The organic solvent was evaporated to dryness at 40 °C, and the residue was resolved in 50 μL of mobile phase (initial composition). An aliquot of 10 μL was then injected into the UHPLC–HRMS systems.

2.4.3. Real Samples. Capillary blood drops from the fingertips of healthy subjects were collected in Microvette CB 300 Lithium Heparin tubes. Aliquots of 20 μL of whole blood or plasma (the latter obtained after centrifugation) were then applied to both Whatman FTA DMPK-C cards and Mitra tips. The samples were dried for 2 h, and the analytes of interest were extracted by using methanol/acetonitrile in the first case and...
| Compound | Elemental Composition | Molecular Ions (m/z) | RT (min) | LOD (ng mL⁻¹) | RE (%) | ME (%) | Supplier                           |
|----------|-----------------------|---------------------|----------|---------------|--------|--------|------------------------------------|
| aminoglutethimide | C₂₁H₂₈N₂O₃ | [M + H]⁻ 233.1285 | 3.7      | 1.0           | 62 ± 10 | 33     | Sigma-Aldrich (Milano. Italy)     |
| anastrozole | C₁₇H₁₈N₃ | [M + H]⁺ 294.1713 | 7.6      | 0.5           | 75 ± 10 | 22     | Toronto Research Chemicals (North York, Canada) |
| androsta-1,4;6-triene-3;17-dione | C₂₁H₂₈O₂ | [M + H]⁺ 283.1693 | 8.4      | 1.0           | 77 ± 10 | 28     | Toronto Research Chemicals (North York, Canada) |
| bazedoxifene | C₂₆H₂₅N₂O₃ | [M + H]⁺ 471.2642 | 6.6      | 1.5           | 65 ± 10 | 25     | Toronto Research Chemicals (North York, Canada) |
| clomiphene | C₂₆H₂₅ClNO | [M + H]⁺ 406.1932 | 8.3      | 1.0           | 75 ± 10 | 22     | Toronto Research Chemicals (North York, Canada) |
| exemestane | C₂₄H₂₂O₂ | [M + H]⁺ 297.1849 | 9.2      | 1.0           | 79 ± 10 | 26     | Sigma-Aldrich (Milano. Italy)     |
| 17-dihydroxexemestane | C₂₆H₂₅O₂ | [M + H]⁺ 299.2006 | 8.8      | 1.0           | 81 ± 10 | 29     | Toronto Research Chemicals (North York, Canada) |
| GW1516 | C₂₈H₃₈F₅NO₅S₂ | [M + H]⁺ 454.0753 | 15.6     | 2.0           | 61 ± 10 | 16     | Santa Cruz Biotechnology (Dallas) |
| GW1516 sulfone | C₂₈H₃₈F₅NO₅S₂ | [M + H]⁺ 486.0651 | 10.5     | 1.0           | 64 ± 10 | 15     | Toronto Research Chemicals (North York, Canada) |
| GW1516 sulfone | C₂₈H₃₈F₅NO₅S₂ | [M + H]⁺ 470.0702 | 9.4      | 0.5           | 65 ± 10 | 18     | Toronto Research Chemicals (North York, Canada) |
| GW0742 | C₂₈H₃₈F₅NO₅S₂ | [M + H]⁺ 472.0659 | 15.8     | 2.0           | 61 ± 10 | 19     | Sigma-Aldrich (Milano. Italy)     |
| GW0742-sulfone | C₂₈H₃₈F₅NO₅S₂ | [M + H]⁺ 504.0557 | 11.0     | 1.0           | 65 ± 10 | 15     | Toronto Research Chemicals (North York, Canada) |
| GW0742-sulfone | C₂₈H₃₈F₅NO₅S₂ | [M + H]⁺ 488.0608 | 9.8      | 0.5           | 63 ± 10 | 16     | Toronto Research Chemicals (North York, Canada) |
| letrozole | C₂₁H₁₉N₅ | [M − H]⁻ 284.0942 | 7.8      | 0.5           | 81 ± 10 | 18     | Sigma-Aldrich (Milano. Italy)     |
| raloxifene | C₂₆H₂₅NO₅S | [M + H]⁺ 474.1736 | 6.3      | 1.0           | 77 ± 10 | 20     | Sigma-Aldrich (Milano. Italy)     |
| SR9009 | C₂₆H₂₅ClNO₅S | [M + H]⁺ 438.1249 | 15.7     | 1.0           | 82 ± 10 | 18     | Toronto Research Chemicals (North York, Canada) |
| SR9009 M2 | C₂₆H₂₅NO₅S | [M + H]⁺ 314.1169 | 4.7      | 1.0           | 77 ± 10 | 16     | National Measurement Institute (NMI) |
| SR9009 M6 | C₂₆H₂₅ClNO₅S | [M + H]⁺ 283.0353 | 5.6      | 1.0           | 65 ± 10 | 19     | National Measurement Institute (NMI) |
| SR9011 | C₂₆H₂₅ClNO₅S | [M + H]⁺ 479.1878 | 15.5     | 1.0           | 77 ± 10 | 21     | MedChem Express (D.B.A. Milano, Italy) |
| toremifene | C₂₆H₂₅ClNO | [M + H]⁺ 406.1932 | 8.3      | 1.0           | 75 ± 10 | 28     | Toronto Research Chemicals (North York, Canada) |
| 4-amino-6-chloro-1,3-benzenedisulfonamide | C₂₆H₂₅ClNO₅S | [M + H]⁺ 438.9572 | 4.0      | 0.2           | 82 ± 10 | 16     | Sigma-Aldrich (Milano. Italy)     |
| 4-amino-6-trifluoromethyl-benzene-1,3-disulfonamide | C₂₆H₂₅F₃NO₅S | [M + H]⁺ 317.9836 | 5.1      | 2.0           | 72 ± 10 | 16     | Sigma-Aldrich (Milano. Italy)     |
| acetalazolamide | C₂₆H₂₅N₂O₃S | [M + H]⁻ 220.9809 | 3.9      | 0.5           | 85 ± 10 | 18     | Sigma-Aldrich (Milano. Italy)     |
| althiazide | C₂₁H₁₉ClNO₅S | [M − H]⁻ 381.9762 | 7.4      | 2.0           | 65 ± 10 | 15     | Toronto Research Chemicals (North York, Canada) |
| azosemide | C₂₁H₁₉ClNO₅S | [M − H]⁻ 369.0001 | 8.3      | 2.0           | 66 ± 10 | 16     | Toronto Research Chemicals (North York, Canada) |
| bemethazide | C₂₁H₁₉ClNO₅S | [M − H]⁻ 400.0198 | 8.2      | 2.0           | 67 ± 10 | 18     | Toronto Research Chemicals (North York, Canada) |
| bendroflumethiazide | C₂₁H₁₉F₃NO₅S | [M − H]⁻ 420.0305 | 8.3      | 3.0           | 62 ± 10 | 18     | Sigma-Aldrich (Milano. Italy)     |
| benzylnonclogorothiazide | C₂₁H₁₉ClNO₅S | [M − H]⁻ 386.0042 | 7.7      | 2.0           | 66 ± 10 | 17     | Toronto Research Chemicals (North York, Canada) |
| benzthiazide | C₂₁H₁₉ClNO₅S | [M − H]⁻ 429.9762 | 7.9      | 2.0           | 72 ± 10 | 15     | Toronto Research Chemicals (North York, Canada) |
| brinzolamide | C₂₁H₁₉N₂O₃S | [M + H]⁺ 384.0716 | 4.4      | 1.0           | 75 ± 10 | 17     | Sigma-Aldrich (Milano. Italy)     |
| bumetanide | C₂₁H₁₉N₂O₃S | [M + H]⁺ 365.1166 | 8.8      | 1.0           | 77 ± 10 | 16     | Toronto Research Chemicals (North York, Canada) |
| butizide (buthiazide) | C₂₁H₁₉ClNO₅S | [M − H]⁻ 352.0198 | 7.6      | 2.0           | 65 ± 10 | 15     | Toronto Research Chemicals (North York, Canada) |
| canrenone | C₂₆H₂₈O₃ | [M + H]⁺ 341.2111 | 9.5      | 1.0           | 77 ± 10 | 21     | Sigma-Aldrich (Milano. Italy)     |
| **compound**       | elemental composition | molecular ion (m/z) | RT (min) | LOD $^a$ (ng mL$^{-1}$) | DPS/DBS | RE$^+$ (%) DPS/DBS | ME$^+$ (%) DPS/DBS | supplier                        |
|-------------------|----------------------|--------------------|---------|--------------------------|---------|--------------------|--------------------|--------------------------------|
| cyclopenthiazide  | C$_5$H$_6$ClN$_5$O$_3$S$_2$ | [M − H]$^−$ 378.0355 | 8.4     | 2.0                      | 66 ± 10 | 18                 |                   | Toronto Research Chemicals (North York, Canada) |
| cyclothiazide     | C$_5$H$_6$ClN$_5$O$_3$S$_2$ | [M − H]$^−$ 388.0198 | 8.1     | 1.0                      | 72 ± 10 | 19                 |                   | Toronto Research Chemicals (North York, Canada) |
| clofamamide       | C$_5$H$_6$ClN$_5$O$_3$S$_2$ | [M − H]$^−$ 268.9463 | 4.4     | 1.0                      | 77 ± 10 | 18                 |                   | Toronto Research Chemicals (North York, Canada) |
| chlorothiazide    | C$_5$H$_6$ClN$_5$O$_3$S$_2$ | [M − H]$^−$ 293.9416 | 4.2     | 0.2                      | 84 ± 10 | 21                 |                   | Toronto Research Chemicals (North York, Canada) |
| clopamide         | C$_5$H$_6$ClN$_5$O$_3$S$_2$ | [M + H]$^+$ 346.0987 | 6.0     | 1.0                      | 81 ± 10 | 16                 |                   | Toronto Research Chemicals (North York, Canada) |
| conivaptan        | C$_6$H$_6$N$_5$O$_3$S | [M + H]$^+$ 329.9073 | 6.8     | 1.0                      | 77 ± 10 | 22                 |                   | Toronto Research Chemicals (North York, Canada) |
| dorzolamide       | C$_5$H$_6$N$_5$O$_3$S$_3$ | [M + H]$^+$ 325.0345 | 6.0     | 2.0                      | 65 ± 10 | 15                 |                   | Sigma-Aldrich (Milano, Italy) |
| epithiazide       | C$_5$H$_6$ClN$_5$O$_3$S$_3$ | [M − H]$^−$ 423.9480 | 7.7     | 2.0                      | 63 ± 10 | 16                 |                   | Toronto Research Chemicals (North York, Canada) |
| eplerenone        | C$_5$H$_6$O$_3$ | [M + H]$^+$ 415.2115 | 7.6     | 3.0                      | 61 ± 10 | 25                 |                   | Toronto Research Chemicals (North York, Canada) |
| etacrylic acid    | C$_5$H$_6$ClO$_4$ | [M − H]$^−$ 301.0040 | 9.4     | 3.0                      | 62 ± 10 | 16                 |                   | Toronto Research Chemicals (North York, Canada) |
| fenquizone        | C$_5$H$_6$ClN$_5$O$_3$S | [M − H]$^−$ 336.0215 | 6.1     | 1.0                      | 76 ± 10 | 19                 |                   | Toronto Research Chemicals (North York, Canada) |
| furosemide        | C$_5$H$_6$ClN$_5$O$_3$S | [M − H]$^−$ 329.0044 | 7.4     | 0.5                      | 82 ± 10 | 15                 |                   | Toronto Research Chemicals (North York, Canada) |
| hydrochlorothiazide | C$_5$H$_6$ClN$_5$O$_3$S$_2$ | [M − H]$^−$ 295.9572 | 4.4     | 1.0                      | 82 ± 10 | 15                 |                   | Sigma-Aldrich (Milano, Italy) |
| hydroflumethiazide | C$_5$H$_6$F$_3$N$_5$O$_3$S$_2$ | [M − H]$^−$ 329.9836 | 5.5     | 1.0                      | 75 ± 10 | 19                 |                   | Toronto Research Chemicals (North York, Canada) |
| indapamide        | C$_5$H$_6$ClN$_5$O$_3$S$_3$ | [M + H]$^+$ 366.0574 | 7.6     | 2.0                      | 67 ± 10 | 18                 |                   | Sigma-Aldrich (Milano, Italy) |
| lixivaptan        | C$_5$H$_6$ClN$_5$O$_3$S$_3$ | [M + H]$^+$ 474.1379 | 11.3    | 3.0                      | 61 ± 10 | 16                 |                   | Sigma-Aldrich (Milano, Italy) |
| mebutizide        | C$_5$H$_6$ClN$_5$O$_3$S$_3$ | [M − H]$^−$ 380.0511 | 8.7     | 2.0                      | 61 ± 10 | 19                 |                   | Toronto Research Chemicals (North York, Canada) |
| metolazone        | C$_5$H$_6$ClN$_5$O$_3$S$_3$ | [M + H]$^+$ 366.0674 | 7.2     | 2.0                      | 65 ± 10 | 21                 |                   | Toronto Research Chemicals (North York, Canada) |
| methazolamide     | C$_5$H$_6$N$_5$O$_3$S$_2$ | [M − H]$^−$ 234.9965 | 4.7     | 2.0                      | 72 ± 10 | 22                 |                   | Sigma-Aldrich (Milano, Italy) |
| methyclothiazide  | C$_5$H$_6$ClN$_5$O$_3$S$_3$ | [M − H]$^−$ 357.9495 | 7.0     | 2.0                      | 69 ± 10 | 16                 |                   | Toronto Research Chemicals (North York, Canada) |
| moxavaptan        | C$_5$H$_6$N$_5$O$_3$S | [M + H]$^+$ 428.2333 | 6.0     | 3.0                      | 65 ± 10 | 19                 |                   | Sigma-Aldrich (Milano, Italy) |
| piretanide        | C$_5$H$_6$N$_5$O$_3$S$_2$ | [M + H]$^+$ 363.1009 | 8.4     | 1.0                      | 77 ± 10 | 23                 |                   | European Pharmacopoeia Reference Standards |
| polythiazide      | C$_5$H$_6$ClN$_5$O$_3$S$_3$ | [M − H]$^−$ 437.9636 | 8.3     | 0.2                      | 85 ± 10 | 24                 |                   | Toronto Research Chemicals (North York, Canada) |
| quinethazone      | C$_5$H$_6$ClN$_5$O$_3$S$_3$ | [M − H]$^−$ 288.0215 | 4.9     | 2.0                      | 66 ± 10 | 21                 |                   | Sigma-Aldrich (Milano, Italy) |
| relcovaptan       | C$_5$H$_6$ClN$_5$O$_3$S$_3$ | [M + H]$^+$ 620.1020 | 8.6     | 3.0                      | 62 ± 10 | 19                 |                   | Sigma-Aldrich (Milano, Italy) |
| tolvaptan         | C$_5$H$_6$ClN$_5$O$_3$S$_3$ | [M + H]$^+$ 449.1627 | 9.3     | 2.0                      | 62 ± 10 | 19                 |                   | Sigma-Aldrich (Milano, Italy) |
| torasemide        | C$_5$H$_6$N$_5$O$_3$S$_2$ | [M + H]$^+$ 349.1329 | 6.1     | 3.0                      | 61 ± 10 | 15                 |                   | Toronto Research Chemicals (North York, Canada) |
| trichlormethiazide | C$_5$H$_6$ClN$_5$O$_3$S$_2$ | [M − H]$^−$ 377.8949 | 6.7     | 2.0                      | 66 ± 10 | 16                 |                   | Toronto Research Chemicals (North York, Canada) |
| xipamide          | C$_5$H$_6$ClN$_5$O$_3$S$_3$ | [M − H]$^−$ 353.0368 | 8.6     | 0.5                      | 76 ± 10 | 16                 |                   | Sigma-Aldrich (Milano, Italy) |

$^a$Results obtained by using Mitra and pure methanol as the extraction solvent.

**Reference Standards**
- Sigma-Aldrich (Milano, Italy)
- Toronto Research Chemicals (North York, Canada)
- Sigma-Aldrich (North York, Canada)
- Toronto Research Chemicals (North York, Canada)
- Sigma-Aldrich (Milano, Italy)
- Toronto Research Chemicals (North York, Canada)
- European Pharmacopoeia Reference Standards

**LOD** (Limit of Detection) and **RT** (Retention Time) values are provided in the table.
Table 4. Elemental Composition, Molecular Ions, RTs, LODs, REs, ME, and Suppliers of the Model Compounds Included in section S6 of the WADA List

| compound | elemental composition | molecular ion (m/z) | RT (min) | LOD (pg mL$^{-1}$) | RE (%) | ME (%) | supplier                |
|----------|------------------------|---------------------|----------|---------------------|--------|--------|-------------------------|
| 2C-B     | C$_{10}$H$_{12}$BrNO$_2$ | [M + H]$^+$ 260.0281 | 5.1       | 1.0                 | 77 ± 10 | 22     | Cerilliant              |
| 2C-H     | C$_{9}$H$_{13}$NO$_2$  | [M + H]$^+$ 182.1176 | 4.1       | 1.0                 | 75 ± 10 | 33     | Toronto Research Chemicals |
| 2-fluoroamphetamine | C$_{9}$H$_{14}$NF | [M + H]$^+$ 154.1027 | 3.8       | 2.0                 | 65 ± 10 | 19     | Cayman Chemical          |
| 3-fluoromethylthionone | C$_{9}$H$_{14}$FNO | [M + H]$^+$ 182.9076 | 3.6       | 3.0                 | 66 ± 10 | 19     | Cerilliant              |
| 4-fluoromethylthionone | C$_{9}$H$_{14}$FNO | [M + H]$^+$ 182.9076 | 3.6       | 3.0                 | 65 ± 10 | 22     | Toronto Research Chemicals |
| 4-fluoropentadron | C$_{9}$H$_{14}$NF | [M + H]$^+$ 154.1027 | 4.0       | 2.0                 | 65 ± 10 | 23     | Cerilliant              |
| 6-APB    | C$_{9}$H$_{13}$NO$_3$ | [M + H]$^+$ 176.1070 | 4.5       | 0.5                 | 77 ± 10 | 25     | LoGiCal (D.B.A. Milano, Italy) |
| 2S-NBOMe | C$_{9}$H$_{14}$BrNO$_3$ | [M + H]$^+$ 380.0856 | 6.9       | 0.5                 | 85 ± 10 | 18     | Cayman Chemical          |
| 25C-NBOMe | C$_{9}$H$_{15}$CIN$_3$O$_2$ | [M + H]$^+$ 336.1361 | 6.7       | 0.5                 | 85 ± 10 | 19     | Cerilliant              |
| 25H-NBOMe | C$_{9}$H$_{14}$NO$_3$ | [M + H]$^+$ 302.1751 | 6.2       | 0.5                 | 88 ± 10 | 18     | Cayman Chemical          |
| 25I-NBOMe | C$_{9}$H$_{15}$I$_3$NO$_3$ | [M + H]$^+$ 428.0717 | 7.1       | 0.5                 | 86 ± 10 | 20     | Cayman Chemical          |
| adrafinil | C$_{11}$H$_{16}$NO$_3$S$_2$C$_{11}$H$_{14}$ | [M + H]$^+$ 288.0700, [M + H]$^+$ 167.0855 | 6.6       | 1.0                 | 66 ± 10 | 19     | Toronto Research Chemicals |
| α-pyrrolidinovalerenone | C$_{9}$H$_{12}$NO | [M + H]$^+$ 232.1696 | 4.9       | 0.5                 | 82 ± 10 | 18     | Cerilliant              |
| amfepramone | C$_{9}$H$_{12}$NO | [M + H]$^+$ 206.1539 | 4.0       | 2.0                 | 65 ± 10 | 19     | Cerilliant              |
| amphetamine | C$_9$H$_{13}$N$_3$ | [M + H]$^+$ 136.1121 | 3.8       | 3.0                 | 62 ± 10 | 26     | Cerilliant              |
| benzfluorex | C$_{9}$H$_{14}$F$_3$NO$_3$ | [M + H]$^+$ 352.1519 | 7.0       | 3.0                 | 62 ± 10 | 25     | Toronto Research Chemicals |
| benzphetamine | C$_{9}$H$_{14}$N$_3$ | [M + H]$^+$ 240.1747 | 5.7       | 2.0                 | 65 ± 10 | 22     | Alttech (D.B.A. Milano, Italy) |
| benzylpiperazine | C$_{9}$H$_{14}$N$_2$ | [M + H]$^+$ 177.1386 | 2.0       | 3.0                 | 64 ± 10 | 30     | Cerilliant              |
| bupropion | C$_{10}$H$_{12}$CINO | [M + H]$^+$ 240.1150 | 5.5       | 2.0                 | 64 ± 10 | 21     | Cerilliant              |
| butylene | C$_{9}$H$_{12}$NO$_3$ | [M + H]$^+$ 222.1125 | 4.2       | 1.0                 | 72 ± 10 | 29     | Cerilliant              |
| clenbuterol | C$_{9}$H$_{12}$CIN | [M + H]$^+$ 260.1201 | 6.1       | 0.5                 | 85 ± 10 | 18     | LoGiCal (D.B.A. Milano, Italy) |
| cocaine | C$_{9}$H$_{12}$NO$_4$ | [M + H]$^+$ 304.1543 | 5.2       | 0.1                 | 89 ± 10 | 15     | Cerilliant              |
| cocaine metabolite (RZE) | C$_{9}$H$_{12}$NO$_4$ | [M + H]$^+$ 290.1387 | 4.7       | 0.5                 | 88 ± 10 | 18     | Cerilliant              |
| crotropamide | C$_{9}$H$_{12}$N$_2$O$_3$C$_{11}$H$_{14}$NO$_3$ | [M + H]$^+$ 241.1911, [M + H]$^+$ 196.1332 | 7.0       | 0.5                 | 85 ± 10 | 25     | Cerilliant              |
| crotetamide | C$_{9}$H$_{12}$N$_2$O$_3$C$_{10}$H$_{13}$NO$_3$ | [M + H]$^+$ 227.1754, [M + H]$^+$ 182.1176 | 6.0       | 0.5                 | 84 ± 10 | 19     | Toronto Research Chemicals |
| heptaminol | C$_{9}$H$_{10}$NO | [M + H]$^+$ 146.1539 | 2.5       | 2.0                 | 72 ± 10 | 21     | National Measurement Institute (NMI) |
| ethylamphetamine | C$_{9}$H$_{11}$N | [M + H]$^+$ 164.1434 | 4.3       | 3.0                 | 62 ± 10 | 21     | Lipomed (D.B.A. Milano, Italy) |
| ethylamphetamine | C$_{9}$H$_{12}$NO$_2$ | [M + H]$^+$ 182.1176 | 2.4       | 2.0                 | 65 ± 10 | 25     | Sigma-Aldrich (Milano, Italy) |
| N-ethyl hexedrone | C$_{9}$H$_{10}$NO | [M + H]$^+$ 234.1852 | 6.1       | 0.5                 | 88 ± 10 | 19     | Cayman Chemical          |
| famprofazone | C$_{9}$H$_{12}$N$_2$O | [M + H]$^+$ 378.2540 | 6.4       | 0.5                 | 82 ± 10 | 18     | Toronto Research Chemicals |
| fenbutrazoate | C$_{9}$H$_{10}$NO$_3$ | [M + H]$^+$ 368.2200 | 7.1       | 0.5                 | 85 ± 10 | 21     | National Measurement Institute (NMI) |
| fencamfamine | C$_{9}$H$_{12}$N | [M + H]$^+$ 216.1747 | 5.6       | 0.5                 | 85 ± 10 | 20     | Alltech (D.B.A. Milano, Italy) |
| fencamidine | C$_{9}$H$_{12}$N$_2$O$_2$ | [M + H]$^+$ 385.2347 | 4.6       | 0.5                 | 85 ± 10 | 20     | National Measurement Institute (NMI) |
| phenidimazine | C$_{9}$H$_{12}$NO | [M + H]$^+$ 192.1383 | 4.0       | 3.0                 | 62 ± 10 | 19     | Alltech (D.B.A. Milano, Italy) |
| fenethylline | C$_{9}$H$_{12}$N$_2$O$_2$ | [M + H]$^+$ 342.1925 | 4.9       | 0.3                 | 88 ± 10 | 17     | National Measurement Institute (NMI) |
Table 4. continued

| compound | elemental composition | molecular ion (m/z) | RT (min) | LOD* (ng mL⁻¹) | DPS/DBS | RE⁻ (%) DPS/DBS | ME⁻ (%) DPS/DBS | supplier |
|----------|-----------------------|--------------------|----------|-----------------|--------|-----------------|-----------------|----------|
| fenfluramine | C₁₉H₂₅�F₄N | [M + H]^+ 232.1308 | 5.7 | 3.0 | 61 ± 10 | 19 | LoGiCal (D.B.A. Milano, Italy) |
| fenprometamine | C₁₉H₂₈NO | [M + H]^+ 150.1277 | 4.0 | 2.0 | 65 ± 10 | 21 | Toronto Research Chemicals (North York, Canada) |
| fenproporex | C₁₉H₂₈N₂ | [M + H]^+ 189.1386 | 4.2 | 1.0 | 72 ± 10 | 25 | National Measurement Institute (NMI) |
| pheptamine | C₁₉H₂₈NO | [M + H]^+ 150.1277 | 4.3 | 3.0 | 62 ± 10 | 22 | Certiliant (Sigma-Aldrich, Milano, Italy) |
| pholedrine | C₁₉H₂₈NO | [M + H]^+ 166.1226 | 2.6 | 3.0 | 62 ± 10 | 22 | Certiliant (Sigma-Aldrich, Milano, Italy) |
| fenfenorex | C₁₉H₂₈NO | [M + H]^+ 230.1539 | 5.3 | 0.5 | 85 ± 10 | 19 | Toronto Research Chemicals (North York, Canada) |
| isometheptene | C₁₉H₂₈N | [M + H]^+ 142.1590 | 4.7 | 3.0 | 62 ± 10 | 19 | National Measurement Institute (NMI) |
| N-methyl-1,3- benzozyxylbutanetamine (MBDB) | C₁₉H₂₈NO₂ | [M + H]^+ 208.1332 | 4.5 | 2.0 | 65 ± 10 | 28 | Certiliant (Sigma-Aldrich, Milano, Italy) |
| methylenedioxyprovalerone (MDPV) | C₁₉H₂₈N₂O₃ | [M + H]^+ 276.1594 | 5.1 | 0.3 | 88 ± 10 | 16 | Certiliant (Sigma-Aldrich, Milano, Italy) |
| fenmetamethine | C₁₉H₂₈N₂O₂ | [M + H]^+ 180.1019 | 4.0 | 2.0 | 65 ± 10 | 18 | Certiliant (Sigma-Aldrich, Milano, Italy) |
| 3,4-methylenedioxyethylamftetamine (MDMA) | C₁₉H₂₈N₂O₂ | [M + H]^+ 208.1332 | 4.3 | 2.0 | 65 ± 10 | 28 | Certiliant (Sigma-Aldrich, Milano, Italy) |
| methylenedioxyethamphetamine (MDMA) | C₁₉H₂₈N₂O₂ | [M + H]^+ 194.1176 | 4.0 | 3.0 | 62 ± 10 | 25 | Certiliant (Sigma-Aldrich, Milano, Italy) |
| mephedrone | C₁₉H₂₈ClN | [M + H]^+ 212.1201 | 5.2 | 0.2 | 85 ± 10 | 21 | National Measurement Institute (NMI) |
| mephenorex | C₁₉H₂₈NO | [M + H]^+ 178.1226 | 4.2 | 3.0 | 65 ± 10 | 28 | Certiliant (Sigma-Aldrich, Milano, Italy) |
| methamphetamine | C₁₉H₂₈NO | [M + H]^+ 150.1277 | 4.1 | 3.0 | 61 ± 10 | 18 | Certiliant (Sigma-Aldrich, Milano, Italy) |
| methedrone | C₁₉H₂₈NO₂ | [M + H]^+ 194.1176 | 3.9 | 3.0 | 65 ± 10 | 25 | Certiliant (Sigma-Aldrich, Milano, Italy) |
| β-methylphenethylamine | C₁₉H₂₈N | [M + H]^+ 136.1121 | 3.8 | 3.0 | 62 ± 10 | 22 | Toronto Research Chemicals (North York, Canada) |
| methylenedioxyethylamftetamine (MDMA) | C₁₉H₂₈N₂O₂ | [M + H]^+ 208.1332 | 4.3 | 2.0 | 65 ± 10 | 28 | Certiliant (Sigma-Aldrich, Milano, Italy) |
| methylenedioxyethamphetamine (MDMA) | C₁₉H₂₈N₂O₂ | [M + H]^+ 194.1176 | 4.0 | 3.0 | 62 ± 10 | 25 | Certiliant (Sigma-Aldrich, Milano, Italy) |
| methamphetamine | C₁₉H₂₈NO | [M + H]^+ 150.1277 | 4.1 | 3.0 | 61 ± 10 | 18 | Certiliant (Sigma-Aldrich, Milano, Italy) |
| methedrone | C₁₉H₂₈NO₂ | [M + H]^+ 194.1176 | 3.9 | 3.0 | 65 ± 10 | 25 | Certiliant (Sigma-Aldrich, Milano, Italy) |
| β-methylphenethylamine | C₁₉H₂₈N | [M + H]^+ 136.1121 | 3.8 | 3.0 | 62 ± 10 | 22 | Toronto Research Chemicals (North York, Canada) |
| methylenedioxyethylamftetamine (MDMA) | C₁₉H₂₈N₂O₂ | [M + H]^+ 208.1332 | 4.3 | 2.0 | 65 ± 10 | 28 | Certiliant (Sigma-Aldrich, Milano, Italy) |
| methylenedioxyethamphetamine (MDMA) | C₁₉H₂₈N₂O₂ | [M + H]^+ 194.1176 | 4.0 | 3.0 | 62 ± 10 | 25 | Certiliant (Sigma-Aldrich, Milano, Italy) |
| methamphetamine | C₁₉H₂₈NO | [M + H]^+ 150.1277 | 4.1 | 3.0 | 61 ± 10 | 18 | Certiliant (Sigma-Aldrich, Milano, Italy) |
| methedrone | C₁₉H₂₈NO₂ | [M + H]^+ 194.1176 | 3.9 | 3.0 | 65 ± 10 | 25 | Certiliant (Sigma-Aldrich, Milano, Italy) |
| β-methylphenethylamine | C₁₉H₂₈N | [M + H]^+ 136.1121 | 3.8 | 3.0 | 62 ± 10 | 22 | Toronto Research Chemicals (North York, Canada) |
| methylenedioxyethylamftetamine (MDMA) | C₁₉H₂₈N₂O₂ | [M + H]^+ 208.1332 | 4.3 | 2.0 | 65 ± 10 | 28 | Certiliant (Sigma-Aldrich, Milano, Italy) |
| methylenedioxyethamphetamine (MDMA) | C₁₉H₂₈N₂O₂ | [M + H]^+ 194.1176 | 4.0 | 3.0 | 62 ± 10 | 25 | Certiliant (Sigma-Aldrich, Milano, Italy) |
| methamphetamine | C₁₉H₂₈NO | [M + H]^+ 150.1277 | 4.1 | 3.0 | 61 ± 10 | 18 | Certiliant (Sigma-Aldrich, Milano, Italy) |
| methedrene | C₁₉H₂₈NO₂ | [M + H]^+ 194.1176 | 3.9 | 3.0 | 65 ± 10 | 25 | Certiliant (Sigma-Aldrich, Milano, Italy) |
| methamphetamine | C₁₉H₂₈NO | [M + H]^+ 150.1277 | 4.1 | 3.0 | 61 ± 10 | 18 | Certiliant (Sigma-Aldrich, Milano, Italy) |
| methedrene | C₁₉H₂₈NO₂ | [M + H]^+ 194.1176 | 3.9 | 3.0 | 65 ± 10 | 25 | Certiliant (Sigma-Aldrich, Milano, Italy) |

*Results obtained by using Mitra and pure methanol as extraction solvent.*
Table 5. Elemental Composition, Molecular Ions, RTs, LODs, REs, ME, and Suppliers of the Model Compounds Included in sections S7, S8, S9, and P1 of the WADA List

| compound             | elemental composition | molecular ion (m/z) | RT (min) | LOD\(^{\circ}\) (ng mL\(^{-1}\)) | RE\(^{\circ}\) (% DPS/DBS) | ME\(^{\circ}\) (%) DPS/DBS | supplier                        |
|----------------------|-----------------------|--------------------|----------|-----------------------------------|-----------------------------|-----------------------------|---------------------------------|
| allentanil           | C\(_{21}\)H\(_{13}\)N\(_{O}_{3}\) | [M + H]\(^{+}\) 417.2609 | 5.8      | 0.5                              | 72 ± 10                     | 17 Cerilliant (Sigma-Aldrich, Milano, Italy) |
| codeine              | C\(_{18}\)H\(_{17}\)NO\(_{3}\) | [M + H]\(^{+}\) 300.1594 | 3.5      | 1.0                              | 65 ± 10                     | 19 LoGrCal (D.B.A. Milano, Italy)         |
| dextromoramide       | C\(_{25}\)H\(_{32}\)N\(_{2}\)O\(_{2}\) | [M + H]\(^{+}\) 393.2537 | 6.8      | 0.5                              | 85 ± 10                     | 17 Mefarlan Smith Ltd            |
| fentanyl             | C\(_{22}\)H\(_{21}\)NO\(_{2}\) | [M + H]\(^{+}\) 337.2274 | 6.0      | 0.5                              | 72 ± 10                     | 17 Cerilliant (Sigma-Aldrich, Milano, Italy) |
| methadone            | C\(_{21}\)H\(_{15}\)NO | [M + H]\(^{+}\) 310.2116 | 6.9      | 2.0                              | 65 ± 10                     | 21 Cerilliant (Sigma-Aldrich, Milano, Italy) |
| methadone metabolite | C\(_{20}\)H\(_{23}\)N | [M + H]\(^{+}\) 278.1903 | 6.5      | 1.0                              | 77 ± 10                     | 16 Cerilliant (Sigma-Aldrich, Milano, Italy) |
| 3-methylfentanyl     | C\(_{23}\)H\(_{30}\)N\(_{2}\)O | [M + H]\(^{+}\) 351.2431 | 6.3      | 0.1                              | 88 ± 10                     | 16 Cayman Chemical (Ann Arbor, MI, USA) |
| oxycodone            | C\(_{18}\)H\(_{17}\)NO\(_{2}\) | [M + H]\(^{+}\) 316.1543 | 4.0      | 2.0                              | 67 ± 10                     | 18 Cerilliant (Sigma-Aldrich, Milano, Italy) |
| pentazocine          | C\(_{18}\)H\(_{17}\)NO | [M + H]\(^{+}\) 286.2165 | 5.5      | 0.5                              | 85 ± 10                     | 18 Cerilliant (Sigma-Aldrich, Milano, Italy) |
| pethidine            | C\(_{13}\)H\(_{15}\)NO\(_{2}\) | [M + H]\(^{+}\) 248.1645 | 5.4      | 0.5                              | 85 ± 10                     | 21 Cerilliant (Sigma-Aldrich, Milano, Italy) |
| remifentanil         | C\(_{20}\)H\(_{19}\)N\(_{2}\)O\(_{3}\) | [M + H]\(^{+}\) 377.2071 | 5.2      | 1.0                              | 65 ± 10                     | 15 Cayman Chemical (Ann Arbor, MI, USA) |
| sufentanil           | C\(_{20}\)H\(_{29}\)N\(_{2}\)O\(_{2}\)S | [M + H]\(^{+}\) 387.2101 | 6.6      | 0.5                              | 74 ± 10                     | 15 Cerilliant (Sigma-Aldrich, Milano, Italy) |
| tramadol             | C\(_{18}\)H\(_{17}\)NO\(_{2}\) | [M + H]\(^{+}\) 264.1958 | 4.9      | 0.5                              | 77 ± 10                     | 21 Cerilliant (Sigma-Aldrich, Milano, Italy) |

S7: Narcotics

AM2201 | C\(_{24}\)H\(_{17}\)FNO | [M + H]\(^{+}\) 360.1758 | 11.5     | 0.5                              | 75 ± 10                     | 19 Cayman Chemical (Ann Arbor, MI, USA) |
JWH018  | C\(_{24}\)H\(_{17}\)NO | [M + H]\(^{+}\) 342.1852 | 12.0     | 0.5                              | 74 ± 10                     | 21 Cayman Chemical (Ann Arbor, MI, USA) |
JWH073  | C\(_{23}\)H\(_{17}\)NO | [M + H]\(^{+}\) 328.1696 | 12.0     | 0.5                              | 74 ± 10                     | 21 Cayman Chemical (Ann Arbor, MI, USA) |
JWH122  | C\(_{23}\)H\(_{17}\)NO | [M + H]\(^{+}\) 356.2009 | 11.9     | 1.0                              | 72 ± 10                     | 21 Cayman Chemical (Ann Arbor, MI, USA) |
JWH210  | C\(_{28}\)H\(_{27}\)NO | [M + H]\(^{+}\) 354.2216 | 11.5     | 1.0                              | 75 ± 10                     | 22 Cayman Chemical (Ann Arbor, MI, USA) |
JWH250  | C\(_{22}\)H\(_{31}\)NO\(_{2}\) | [M + H]\(^{+}\) 336.1958 | 12.0     | 1.0                              | 71 ± 10                     | 21 Cayman Chemical (Ann Arbor, MI, USA) |

S8: Cannabinoids

beclomethasone       | C\(_{22}\)H\(_{29}\)ClO\(_{3}\) | [M + H]\(^{+}\) 409.1776 | 7.7      | 3.0                              | 65 ± 10                     | 18 Toronto Research Chemicals (North York, Canada) |
betamethasone        | C\(_{22}\)H\(_{29}\)FO\(_{3}\) | [M + H]\(^{+}\) 393.2072 | 7.4      | 1.0                              | 77 ± 10                     | 18 Toronto Research Chemicals (North York, Canada) |
budesonide           | C\(_{23}\)H\(_{34}\)O\(_{6}\) | [M + H]\(^{+}\) 431.2428 | 8.8      | 0.5                              | 85 ± 10                     | 17 Toronto Research Chemicals (North York, Canada) |
ciclesonide          | C\(_{32}\)H\(_{44}\)O\(_{7}\) | [M + H]\(^{+}\) 541.3160 | 16.5     | 3.0                              | 65 ± 10                     | 19 Toronto Research Chemicals (North York, Canada) |
clobetasol           | C\(_{22}\)H\(_{29}\)ClO\(_{4}\) | [M + H]\(^{+}\) 411.1733 | 9.0      | 3.0                              | 65 ± 10                     | 19 Sigma-Aldrich (Milano, Italy) |
cortisol             | C\(_{21}\)H\(_{29}\)O\(_{4}\) | [M + H]\(^{+}\) 363.2166 | 6.7      | 0.8                              | 79 ± 10                     | Steraloids (Newport, RI, USA)            |
cortisone            | C\(_{21}\)H\(_{32}\)O\(_{3}\) | [M + H]\(^{+}\) 361.2010 | 6.9      | 0.5                              | 82 ± 10                     | Steraloids (Newport, RI, USA)            |
deflazacort          | C\(_{25}\)H\(_{31}\)NO\(_{4}\) | [M + H]\(^{+}\) 442.2224 | 7.1      | 2.0                              | 65 ± 10                     | 25 Toronto Research Chemicals (North York, Canada) |
deflazacort metabolite | C\(_{22}\)H\(_{30}\)NO\(_{3}\) | [M + H]\(^{+}\) 400.2119 | 6.7      | 0.5                              | 88 ± 10                     | 18 Toronto Research Chemicals (North York, Canada) |
dexamethasone        | C\(_{22}\)H\(_{29}\)FO\(_{3}\) | [M + H]\(^{+}\) 393.2072 | 7.4      | 1.0                              | 75 ± 10                     | 17 Toronto Research Chemicals (North York, Canada) |
desonide             | C\(_{24}\)H\(_{32}\)O\(_{6}\) | [M + H]\(^{+}\) 417.2272 | 8.0      | 1.0                              | 77 ± 10                     | 17 Toronto Research Chemicals (North York, Canada) |
desoximethasone      | C\(_{22}\)H\(_{39}\)FO\(_{4}\) | [M + H]\(^{+}\) 377.2123 | 8.2      | 2.0                              | 72 ± 10                     | 21 Toronto Research Chemicals (North York, Canada) |
fluocortolone        | C\(_{22}\)H\(_{39}\)FO\(_{4}\) | [M + H]\(^{+}\) 377.2123 | 8.1      | 2.0                              | 72 ± 10                     | 20 Toronto Research Chemicals (North York, Canada) |

S9: Glucocorticoids
The instrumental analysis was carried out by Conditions. The extraction solvent was then evaporated to dryness at 40 °C, reconstituted, and injected in the UHPLC−Mass Spectrometry−DPS/DBS supplier.

### Table 5. continued

| Compound                  | Molecular ion (m/z) | RT (min) | LOD (ng mL⁻¹) | RE (%) DPS/DBS | ME (%) DPS/DBS | Supplier                          |
|---------------------------|---------------------|----------|---------------|---------------|----------------|-------------------------------|
| Fluorometholone           | C₂₂H₂₃F₂O₄          | [M + H]⁺ | 8.2           | 2.0           | 75 ± 10        | 20 Sigma-Aldrich (Milano, Italy) |
| Fludrocortisone           | C₂₁H₂₉F₃O₃          | [M + H]⁺ | 6.9           | 1.0           | 82 ± 10        | 22 Toronto Research Chemicals (North York, Canada) |
| Flumethasone              | C₂₂H₂₉F₃O₃          | [M + H]⁺ | 7.5           | 3.0           | 65 ± 10        | 28 Toronto Research Chemicals (North York, Canada) |
| Fluniolide                | C₂₃H₂₁F₃O₂          | [M + H]⁺ | 7.8           | 0.5           | 85 ± 10        | 22 Toronto Research Chemicals (North York, Canada) |
| 6α-fluprednisolone        | C₂₁H₂₉F₃O₃          | [M + H]⁺ | 6.8           | 3.0           | 66 ± 10        | 28 Toronto Research Chemicals (North York, Canada) |
| Fluticasone furoate       | C₂₇H₃₉F₅O₅S         | [M + H]⁺ | 11.0          | 3.0           | 65 ± 10        | 17 Toronto Research Chemicals (North York, Canada) |
| Fluticasone propionate    | C₂₃H₂₁F₃O₂S         | [M + H]⁺ | 10.9          | 3.0           | 65 ± 10        | 16 Toronto Research Chemicals (North York, Canada) |
| Mepredniosone             | C₂₂H₂₉O₃            | [M + H]⁺ | 7.6           | 2.0           | 66 ± 10        | 19 Toronto Research Chemicals (North York, Canada) |
| 6α-methylprednisolone     | C₂₂H₂₉O₃            | [M + H]⁺ | 7.4           | 2.0           | 72 ± 10        | 30 Sigma-Aldrich (Milano, Italy) |
| Mometasone furoate        | C₂₇H₃₉ClO₅          | [M + H]⁺ | 11.1          | 2.0           | 65 ± 10        | 19 Toronto Research Chemicals (North York, Canada) |
| Prednisolone              | C₂₁H₁₉O₅            | [M + H]⁺ | 6.8           | 0.5           | 85 ± 10        | 29 Sigma-Aldrich (Milano, Italy) |
| Prednione                 | C₂₁H₁₉O₅            | [M + H]⁺ | 6.8           | 0.5           | 88 ± 10        | 25 Sigma-Aldrich (Milano, Italy) |
| Triamcinolone             | C₂₁H₁₉O₅            | [M + H]⁺ | 5.9           | 2.0           | 72 ± 10        | 21 Toronto Research Chemicals (North York, Canada) |
| Triamcinolone acetone     | C₂₃H₁₉O₆            | [M + H]⁺ | 7.8           | 0.5           | 85 ± 10        | 16 Toronto Research Chemicals (North York, Canada) |
| Acebutolol                | C₁₄H₁₉N₂O₄          | [M + H]⁺ | 4.6           | 0.3           | 88 ± 10        | 22 Toronto Research Chemicals (North York, Canada) |
| Atenolol                  | C₁₄H₁₉N₂O₃          | [M + H]⁺ | 3.2           | 0.5           | 85 ± 10        | 16 Toronto Research Chemicals (North York, Canada) |
| Betaxolol                 | C₁₄H₁₉N₃O₃          | [M + H]⁺ | 5.9           | 0.5           | 85 ± 10        | 15 Toronto Research Chemicals (North York, Canada) |
| Bisoprolol                | C₁₄H₁₉N₄O₄          | [M + H]⁺ | 5.5           | 1.0           | 77 ± 10        | 18 Sigma-Aldrich (Milano, Italy) |
| Carbetolol                | C₁₄H₁₉N₂O₃          | [M + H]⁺ | 3.8           | 0.5           | 84 ± 10        | 18 Toronto Research Chemicals (North York, Canada) |
| Carvedilol                | C₁₄H₁₉N₂O₄          | [M + H]⁺ | 6.6           | 0.5           | 86 ± 10        | 17 Toronto Research Chemicals (North York, Canada) |
| Celiprolol                | C₂₀H₁₉N₄O₄          | [M + H]⁺ | 5.2           | 0.3           | 88 ± 10        | 21 Toronto Research Chemicals (North York, Canada) |
| Esmolol                   | C₁₄H₁₉N₄O₄          | [M + H]⁺ | 5.1           | 0.3           | 88 ± 10        | 20 Toronto Research Chemicals (North York, Canada) |
| Indenolol                 | C₁₅H₁₉N₂O₃          | [M + H]⁺ | 5.5           | 0.3           | 88 ± 10        | 15 Skylead Pharmaceutical and Chemicals (Shanghai, China) |
| Mepindolol                | C₁₅H₁₉N₂O₂          | [M + H]⁺ | 4.5           | 0.5           | 85 ± 10        | 19 Toronto Research Chemicals (North York, Canada) |
| Metoprolol                | C₁₅H₁₉N₃O₃          | [M + H]⁺ | 4.7           | 0.3           | 79 ± 10        | 16 Toronto Research Chemicals (North York, Canada) |
| Nebivolol                 | C₂₂H₂₃F₂₄O₄         | [M + H]⁺ | 6.9           | 0.3           | 85 ± 10        | 22 Nebil (Gets pharma, Karachi, Pakistan) |
| Penbutolol                | C₁₄H₂₉N₂O₂          | [M + H]⁺ | 7.0           | 0.5           | 85 ± 10        | 16 Toronto Research Chemicals (North York, Canada) |
| Pindolol                  | C₁₄H₂₉N₂O₂          | [M + H]⁺ | 4.0           | 0.5           | 85 ± 10        | 16 Sigma-Aldrich (Milano, Italy) |
| Sotalol                   | C₁₃H₁₉N₂O₃S         | [M + H]⁺ | 3.1           | 1.5           | 65 ± 10        | 22 Toronto Research Chemicals (North York, Canada) |
| Timolol                   | C₁₃H₁₉N₂O₃S         | [M + H]⁺ | 4.5           | 0.3           | 88 ± 10        | 19 Sigma-Aldrich (Milano, Italy) |

*Results obtained by using Mitra and pure methanol as the extraction solvent.

pure methanol in the second case, and in the experimental conditions optimized in this study (i.e., 500 µL of extraction solvent and 30 min of incubation at 25 °C under ultrasound irradiation). The extraction solvent was then evaporated to
UHPLC–HRMS following the analytical procedure already validated and currently in use in the WADA-accredited anti-doping laboratory of Rome to determine the compounds under investigation in urine samples in the occasion of doping control tests.26

2.5.1. Liquid Chromatography. A Vanquish UHPLC system (Thermo Scientific, Bremen, Germany) was used to carry out the chromatographic separation. Reversed-phase liquid chromatography was performed using a Supelco Ascentis C18 column (150 × 2.1 mm, 2.7 μm) (Sigma-Aldrich, Milano, Italy). Ultrapurified water (eluent A) and acetonitrile (eluent B), both containing 0.1% of formic acid, were selected as the mobile phase. The gradient started at 5% eluent B, was increased to 65% eluent B in 7 min and after 4 min to 95% eluent B in 2 min, held for 4.5 min, decreased to starting conditions of 5% eluent B in 0.31 min, and held for 2 min for re-equilibration. The flow rate was set at 250 μL min⁻¹, the and column temperature was set at 25 °C. The injection volume was 10 μL. After each injection, the needle was washed and purged with H₂O/methanol (2:1, v/v) and H₂O/methanol (4:1, v/v) solutions, respectively. The temperature of the sampler was set to 10 °C.

2.5.2. Mass Spectrometry. A QExactive benchtop Orbitrap-based mass spectrometer (Thermo Scientific, Bremen, Germany) operated in the positive--negative polarity switching mode and equipped with a heated electrospray ionization source was used. The sheath gas and auxiliary gas (both nitrogen) flow rate and the sweep gas flow rate were set at 40, 10, and 1 arbitrary units, respectively. The transfer capillary and source temperature were set at 320 and 350 °C, respectively. The spray voltage was set at +3.8 kV for positive polarity and −3.2 kV for negative polarity. The instrument operated in the full scan mode from m/z 100 to 650 at 35,000 resolving power and a duty cycle of 100 times LODs), and high concentrations (corresponding to 10 times LODs) before spotting on to the card, tips, or dots. The intra-day precision and inter-day precision of the RRT and of the relative response of each analyte was expressed as CV (%).

The recovery of all analytes was estimated by preparing (i) DBS samples (pre-spiked) using drug-free blood samples (whole blood or plasma) fortified with the target analytes at low (corresponding to the LOD), medium (corresponding to 5 times the LOD), and high concentrations (corresponding to 10 times the LOD) before spotting on to the card, tips, or dots and (ii) DBS samples (post-spiked) using drug-free blood samples (whole blood or plasma) spotting on to the card, tips, or dots spiked with the same concentrations as the pre-spike DBS after extraction. The extraction recovery (%) was then calculated by comparing the peak area ratio of the compounds and the peak area of the internal standard of the two sets (pre-spike and post spike) of samples. The internal standard was added after sample pre-treatment in both sets of samples.

The robustness of the method was evaluated by analyzing drug-free blood samples (whole blood or plasma) spiked with the analytes of interest at the LOD concentration. The samples were prepared and analyzed once a week for 7 weeks, randomly changing the instrument employed in routine analyses and the operator involved in the instrumental analysis and in the preparation of the samples.

The stability test was determined on a pool of drug-free whole blood samples fortified with the compounds under investigation at a concentration 5 times the LODs. The fortified samples (whole blood or plasma) were applied to the DBS cards, tips, and dots and allowed to dry for 2 h at 25 °C. The dried DBS and DPS samples were then stored in sealed bags containing desiccant to avoid humidity and contamination at 50 °C for up to 1 week and at 25 and 4 °C for up to 4 weeks. Two replicates were prepared every day for each storage condition in the first week and then once a week.

The stability of the compounds was evaluated by comparing the relative responses obtained after each measurement with the relative responses from freshly prepared samples.

3. RESULTS AND DISCUSSION

3.1. Optimization of the Extraction Protocol. For the selection of the optimal sample pre-treatment protocol, different
incubation times (i.e., 5, 10, 15, 20, 25, 30, 40, and 60 min), temperatures (i.e., 20, 25, 30, 40, and 50 °C), and extraction reagents (composition and volumes) were investigated. Starting from the protocols reported in the literature and considering that the target analytes differ significantly from each other in terms of (physico-)chemical properties (pKₐ, polarity and stability), we have evaluated the extraction efficiency of different aqueous buffers (including phosphate buffer (pH 7), carbonate buffer (pH 9), acetate buffer (pH 5) and ultrapure water), of organic solvents of different polarities (methanol, acetonitrile, tert-butylmethylether, ethylacetate, acetone, and isopropanol), and of mixtures of organic solvents (methanol/acetonitrile, tert-butylmethylether/isopropanol/methanol, methanol/isopropanol, and tert-butylmethylether/methanol/acetone) in different volumes (100, 200, 300, 500, and 1000 μL) and in the presence or absence of 2% of acetic acid or ammonia. The extraction was carried out under ultrasound irradiation.

Ultrapure water, aqueous buffers at pH 7 or at pH 9 was allowed to dissolve completely the spot with high recoveries (REs) for most of the compounds considered; however, the recovery was not satisfying (generally <50%, data not shown) for synthetic cannabinoids and numerous anabolic agents due to their very low solubility in aqueous media. Furthermore, under these conditions, the components of blood including proteins were also co-extracted with the target compounds, causing significant matrix effects (MEs). Further purification steps were, therefore, carried out using either liquid/liquid or SPE following the protocols already in use in our laboratory. As expected, after purification, the extracts were generally cleaner, and the ME was significantly lower, but the entire procedure was significantly longer, more complex, and more costly. The use of the acetate buffer was not sufficient to completely dissolve the dry whole blood or plasma: although cleaner extracts were obtained, liquid/liquid extraction or SPE was still necessary. Moreover, the acidic pH did not allow to effectively recover all the compounds considered.

The organic solvents, on the other hand, were not able to dissolve all the components of the dried whole blood and plasma, and consequently, cleaner extracts were obtained, and further purifications steps were not necessary.

The results obtained using pure organic solvents showed that pure methanol provided an average recovery in the range of 60–90% for all the compounds considered when Mitra or Tasso M20 devices were used (REs lower than 50% were instead registered when the microsampling devices based on untreated/treated cellulose were used), pure isopropanol provided an average of 30–60% recovery for all the compounds considered when both volumetric or non-volumetric devices were used, and finally pure acetonitrile, tert-butylmethyl ether, and ethylacetate provided low extraction yields (lower than 40%) for most of the analytes and microsampling devices under investigation. Pure methanol was therefore selected to extract the compounds under investigation from the devices constituted by porous polymers (see Tables 2–5 for the REs).

Concerning the mixture of solvents, the addition of acetonitrile or isopropanol to methanol significantly increased the REs of most of the compounds considered (with an average improvement of 20%) when the devices based on treated and untreated cellulose were used; moreover, in contrast to pure methanol, cleaner extracts and lower MEs were obtained. Mixtures of methanol and acetonitrile or isopropanol were therefore selected to extract the compounds under investigation from the supports constituted by cellulose paper. The best methanol/acetonitrile or methanol/isopropanol ratio was 1/1; higher percentages of acetonitrile or isopropanol did not significantly increase the REs, whereas in the presence of lower percentages, the REs of several compounds (e.g., stimulants and glucocorticoids) decrease significantly.

Regarding the volume of the extraction solvent, volumes in the 100–1000 μL range were tested. Optimal results were obtained by using 500 μL, higher volumes did not significantly increase the extraction yields, whereas lower volumes were unable to extract effectively all the analytes.

As regards the incubation time and the incubation temperature, interval of time lower than 20 min did not allow to obtain optimal recovery for all the compounds under investigation; our evaluation showed that the optimal extraction time was 30–40 min. Higher extraction time gave slightly higher concentrations of most of the compounds selected apart from the most volatile compounds (e.g., amphetamines) for which the recovery decreases significantly. The optimal incubation temperature was 20–25 °C for all the compounds tested; at higher values, the high volatile compounds were lost. Tables 2–5 reports the REs obtained using the microsampling device Mitra and the experimental conditions optimized in this study (i.e., 500 μL of pure methanol as the extraction solvent and 30 min of incubation at 25 °C).

3.2. Selection of the Microsampling Device. Different microsampling devices (see again Table 1 for details) were comparatively evaluated for the simultaneous analysis of 235 drugs (225 target compounds plus 10 metabolites) in capillary whole blood and plasma for doping control purpose. Both volumetric and non-volumetric devices were evaluated. The non-volumetric devices are all based on cellulose: the Whatman FTA DMPK-A, B cards are chemically treated with reagents that on contact cause lysis of cells, denaturation of proteins, and inactivation of enzymes, also preventing the growth of bacteria: they are therefore suitable for the analysis of small molecular weight analytes, while the Whatman DMPK-C and Whatman 903 Protein Saver are instead untreated and therefore also suitable for the analysis of proteins. The volumetric devices are based either on untreated cellulose (i.e., HemaSpot-HF) or on porous polymers (i.e., Tasso-M20, and Mitra).

Regarding the non-volumetric devices, using the Whatman FTA DMPK-A cards, very low REs were measured for all the compounds under investigation. Optimal results (REs higher than 60% for all the compounds under investigation) were instead obtained by using the Whatman FTA DMPK-B, Whatman FTA DMPK-C, and Whatman 903 Protein Saver cards, with mixtures of methanol/acetonitrile or methanol/isopropanol as the extraction solvent. However, the Whatman FTA DMPK-B gave dirty extracts, maybe due to the presence of the reagents used to treat the cellulose paper, whereas the sensitivity of the analytical procedure was lower with the Whatman 903 Protein Saver cards, due to the lower volume that can be loaded. It has to be stressed out that the use of calibrated pipettes is of crucial importance to obtain homogeneous and repeatable spots.

In regard to the volumetric devices, all the three devices evaluated showed high recovery (60–90%) for all the compounds under investigation by using pure methanol (for the two devices based on porous polymers) or mixtures of methanol/acetonitrile or methanol/isopropanol (for the device based on untreated cellulose) as the extraction solvent. However, the results obtained by using the two devices based
|        | JTV-519 | S-107 | ACP-105 | Andarine |
|--------|---------|-------|---------|----------|
| Andarine metabolite | CL-4AS-1 | Gestrinone | GLPG0492 |
| LGD4033 | LY2452473 | Methyltrienolone | MK0773 |
| Ostarine | RAD140 | S1 | S6 |
| S9 | S23 | Stanozolol | Tetrahydrogestrinone |
| TFM-4AS-1 | Fluconazole | Miconazole | Ketoconazole |
| Daprodustat | FG2216 | FG4592 | Reproterol |
| Tulobuterol | Anastrozole | Androsta-1,4:6-triene-3,17-dione | Bazedoxifene |
| Exemestane | Letrozole | Raloxifene | SR9009 |

Figure 1. continued
Figure 1. (A) Extracted chromatograms at the RTs of 36 model compounds included in sections S0, S1, S2, S3, and S4 in a negative sample. The microsampling device used was Mitra, and the extraction solvent was methanol. (B) Extracted chromatograms at the RTs of 36 model compounds included in sections S0, S1, S2, S3, and S4 in a negative sample spiked with the compounds under investigation at the LOD concentration. The microsampling device used was Mitra, and the extraction solvent was methanol.
|                | Acetazolamide | Azosemide | Bendroflumethiazide | Benzthiazide |
|----------------|---------------|-----------|---------------------|--------------|
| Benzyhydrochlorothiazide |               |           |                     |              |
| Brinzolamide    |               |           |                     |              |
| Canrenone       |               |           |                     |              |
| Chlorthalidone  |               |           |                     |              |
| Chlorothiazide  |               |           |                     |              |
| Clofamamide     |               |           |                     |              |
| Clopamide       |               |           |                     |              |
| Chlorazanil     |               |           |                     |              |
| Conivaptan      |               |           |                     |              |
| Dichlorphenamide|               |           |                     |              |
| Eplerenone      |               |           |                     |              |
| Furosemide      |               |           |                     |              |
| 25B-NBOMe       |               |           |                     |              |
| 2C-H            |               |           |                     |              |
| 2Fluoro-amphetamine |             |           |                     |              |
| 4Fluoro-amphetamine |           |           |                     |              |
| Alpha-pirrolidinvalerofenone |           |           |                     |              |
| Clobenzorex     |               |           |                     |              |
| Cocaine         |               |           |                     |              |
| BEG             |               |           |                     |              |
| Cropropamide    |               |           |                     |              |
| Crotetamide     |               |           |                     |              |
| Fencamfamine    |               |           |                     |              |
| Heptaminol      |               |           |                     |              |
| MDEA/mexedrone/MBDB |           |           |                     |              |
| MDPV            |               |           |                     |              |
| Metedrone/MDMA  |               |           |                     |              |
| Mephedrone      |               |           |                     |              |
| Methylphenidate |               |           |                     |              |
| Ritalinic acid  |               |           |                     |              |
| Modafinil       |               |           |                     |              |
| Morazone        |               |           |                     |              |
| Ortetamine      |               |           |                     |              |
| 4-Methyl-amphetamine |           |           |                     |              |

Figure 2. continued
Figure 2. (A) Extracted chromatograms at the RTs of 36 model compounds included in sections S5 and S6 in a negative sample. The microsampling device used was Mitra, and the extraction solvent was methanol. (B) Extracted chromatograms at the RTs of 36 model compounds included in sections S5 and S6 in a negative sample spiked with the compounds under investigation at the LOD concentration. The microsampling device used was Mitra, and the extraction solvent was methanol.
| Alfentanyl | Codeine | Dextromoramide | Fentanyl |
|-----------|---------|----------------|----------|
| Methadone | Methadone metabolite | Remifentanyl | Oxycodone |
| Pentazocine | Pethidine | Fentanyl | Sufentanil |
| AM2201 | JWH-018 | JWH-073 | JWH-250 |
| Beclomethasone | Betamethasone/Dexamethasone | Budesonide | Flumethasone |
| Fluticasone Furoate | Fluticasone propionate | 6β-methylprednisolone | Mometasone furoate |
| Prednison | Prednisolone | Triamcinolone | Triamcinolone acetonide |
| Cortisone | Flunitolide | |
| Acebutolol | Atenolol | Carteolol | Carvedilol |
| Indenolol | Metoprolol | Nebivolol | Timolol |

Figure 3. continued
Figure 3. (A) Extracted chromatograms at the RTs of 36 model compounds included in sections S7, S8, S9, and P1 in a negative sample. The microsampling device used was Mitra, and the extraction solvent was methanol. (B) Extracted chromatograms at the RTs of 36 model compounds included in sections S7, S8, S9, and P1 in a negative sample spiked with the compounds under investigation at the LOD concentration (B). The microsampling device used was Mitra, and the extraction solvent was methanol.
on porous polymers gave more reproducible results, and for this reason, these devices were preferred for our analytical workflow.

As already described in the previous paragraph, Tables 2−5 report the REs obtained using the microsampling device Mitra and the experimental conditions optimized in this study (i.e., 500 μL of pure methanol as the extraction solvent and 30 min of incubation at 25 °C). Similar results were obtained using Tasso M20 or the Whatman DMPK-C cards. The other devices (Whatman FTA DMPK-A, B, Whatman 903 Protein Saver Card, and HemaSpot-HF) evaluated in this study, as outlined before, demonstrated to be less efficacy and reproducible.

3.3. Method Validation. The initial testing procedure developed in this study was tested and validated in DBS and DPS, in view of the potential application for the determination of the prohibited compounds considered in the framework of forensic investigations. The selected microsampling devices were the non-volumetric devices Whatman FTA DMPK-C cards and the two volumetric devices based on porous polymers (Mitra tips and Tasso M20). The extraction solvents selected were the mixture of methanol/acetonitrile or methanol/isopropanol for the Whatman FTA DMPK-C cards and pure methanol for the two volumetric devices based on porous polymers (Mitra tips and Tasso M20). The parameters considered were specificity, LODs, carry over, ion suppression/enhancement, intra-day and inter-day precision of the RRT and of the relative response, recovery, robustness, and stability.

We stress out that, also according to the ISO17025 definition, the purpose of the ITP, as a screening analysis, is to exclude from additional confirmation analysis all those samples that resulted clearly negative, while a more selective, targeted confirmation analysis must be activated in all other cases. In this way, it is possible to minimize the risk of both “false-negative” results (due to a screening analysis excluding all samples that could not be confirmed at this stage) and “false-positive” results (i.e., samples resulted not clearly negative after the screening analysis but not confirmed by more specific methods). Therefore, in no cases, a positive result (adverse analytical finding) can be issued following the result of the IPT only.

The analyses performed on 10 different negative samples showed that no significant interferences were observed at the retention range of the target analyte considered, thus indicating high method selectivity. All the analytes were clearly distinguishable in the matrices (i.e., whole blood and plasma) considered (see Figures 1A−3A).

The LODs ranged from 0.1 to 3.0 ng mL$^{-1}$ (see again Tables 2−5), low enough to detect the abuse of the compounds under investigation in the occasion of doping control tests according to the data reported in the literature.\textsuperscript{12−15} Data obtained by analyzing negative samples after negative samples spiked with the compounds under investigation at concentrations 20 times the LOD did not show any interfering signals.

The extraction efficiency measured from three replications proved to be reproducible (CV % below ±10) with yields ranging from 60 to 90% depending on the class of substances considered, being lower for the amphetamine-like substances maybe due to their high volatility (see Tables 2−5 for the REs registered using Mitra). Similar results were obtained using the Whatman FTA DMPK-C cards and Tasso M20.

The test for ion suppression/enhancement effects yielded no significant ME (in the range of 15−33%) at the RTs of the analytes considered and of the internal standards for all the three devices considered.

The intermediate and intra-day assay repeatability of the RRTs showed an adequate repeatability (CV % lower than 0.5) for all the analytes under investigation. However, the CV % of
the relative response was in the range of 10−15 for all the analytes tested.

Regarding the investigation of the stability, our examination revealed that most of the analytes considered were stable for no more than 1 day at 50 °C, for up to 2 weeks at 25 °C, and for the full duration of the stability study at 4 °C in all the matrices considered. Highly volatile, light-sensitive, or instable analytes can be lost if the environmental conditions during sample collection, drying, and transportation are not optimal; for this reason, it is of crucial importance after collection to put the samples into plastic bags with an adequate desiccant and humidity indicator.

Figures 1−3 report the results obtained by analyzing a negative sample (Figures 1A−3A) and a negative sample spiked with the compounds under investigation at the LOD concentration (Figures 1B−3B) applied to Mitra and pre-treated using 500 μL of pure methanol for 30 min at 25 °C. As it can be noticed from the 108 extracted chromatograms, all the target analytes were visible and clearly distinguishable from the matrix interferences. Similar results were obtained by using the other two microsampling devices selected in this study: Tasso M20 and methanol as the extraction solvent or Whatman FTA DMPK-C cards and the mixture methanol/acetonitrile or methanol/isopropanol as the extraction solvent.

3.4. Real Samples. The analytical workflow developed and validated in this study to detect prohibited compounds in DBS and DPS was applied to determine the pharmacokinetic profile and windows of detection of acetazolamide and deflazacort after a single oral dose. Indeed, while many data are present in the literature for drugs of abuse, very few are known about the other classes of prohibited compounds.

Capillary blood was collected every 3 h for 3 days after a single oral administration of 6 mg of deflazacort: the analyses revealed that the parent compound was detected in very low levels only in the first 3 h from drug administration, whereas the 21-desacetyl metabolite showed a maximum in the first 3 h from the administration and remained visible for 24 h from the administration. The pharmacokinetic profile in whole blood and plasma was overlapping, indicating that deflazacort did not significantly interact with the red blood cells. In Figure 4, the profile of deflazacort metabolite (i.e., 21-desacetyl-deflazacort) in urine, whole blood, and plasma was reported. In urine, the maximum of excretion was reached after 6 h, whereas in whole blood and plasma, after 3 h from drug administration. The target analyte was detected for more than 24 h in all the matrices tested, enabling the detection of an illicit use of compounds banned only in competition.

Regarding acetazolamide, capillary blood was collected for 4 weeks after a single oral administration of 125 mg of acetazolamide. The samples were collected every 3 h in the first 3 days and then at least once a day. In contrast to deflazacort, acetazolamide was found in much higher levels in whole blood than in plasma, confirming its ability to interact with the proteins of the red cells (see Figure 5 for the results obtained in subject 3). In both subjects, acetazolamide was the only compound detected in blood: it reached maximum levels in the first 3 h from the administration and can be effectively detected for more than 1 month from drug administration (see again Figure 5 for the results for subject 3).

Urine samples were pre-treated by using the analytical procedure currently used in our laboratory to detect glucocorticoids during doping control tests. Urine samples were pre-treated by using the analytical procedure currently used in our laboratory to detect diuretics during doping control tests.

4. CONCLUSIONS

In this study, a multi-analyte initial testing procedure was developed and fully validated to detect 235 compounds (225 target analytes plus 10 among their main metabolites) plus 11...
internal standards in DBS and DPS. The sample pre-treatment was based on ultrasonication at 25 °C for 30 min in the presence of 500 μL of pure methanol, methanol/acetonitrile, or methanol/isopropanol depending on the microsampling device selected (cellulose vs polymer based). The microsampling, pre-treatment, and instrumental analysis workflow were fully validated according to the current WADA and ISO17025 requirements, with satisfying results for all the analytes considered. The total recovery from the selected DBS devices for the different analytes varied between 60 and 90%, whereas the MEs ranged from 15 to 33%. Despite the small volume, the LODs of the analytes varied in the range of 0.1–3.0 ng mL⁻¹ enabling the detection of an illicit use: for indeed, even if, at present, the WADA did not yet fix any minimum required performance levels (MRPLs) for the compounds under investigation in DBS/DPS; nonetheless, most of the analytes could be detected below the WADA MRPL for urine.⁵⁹ When a suspect signal is found, its isotopic pattern (or the fragment ratio for the PRM acquisition) is compared with the isotopic pattern (or the fragment ratio in the case of PRM acquisition) of the target compound in the quality control sample.

As a proof of concept, the entire protocol was successfully applied to the analysis of authentic samples obtained from administration studies with deflazacort and acetazolamide. In both cases, the target compounds were detected for the full duration of the administration study. Nevertheless, the experimental evidence obtained from the analysis of the excretion study samples demonstrated that although the use of plasma could allow to minimize the impact of the hematocrit variability, several compounds are present in the plasma in very low levels due to their interaction with red cells. Consequently, it is still of crucial importance to consider the interaction of the prohibited compounds with red cells and plasmatic proteins before to select the most appropriate blood matrix and analytical protocol.

Finally, in this study, blood was collected with Microvette CB300 and spotted on both volumetric and non-volumetric devices with a calibrated pipette to increase the repeatability and reproducibility of the spots. This point has to be evaluated in depth in the case of actual doping control tests especially when non-volumetric devices are used, and quantitative analyses have to be carried out.

In future, we aim to include in the multi-analyte procedure developed in this study other prohibited compounds and endogenous steroids (androgens and glucocorticoids) (cortisol, cortisone, androstosterone glucuronide, and etiocholanolone glucuronide can be already detected by the analytical procedure developed in this study). We also plan to evaluate the performance of those devices that can directly create DPS (i.e., HemaSpot SE).

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Notes

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