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Mass spectrometric identification and characterization of urinary metabolites of isopropylnorsynephrine for doping control purposes

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Abstract

Isopropylnorsynephrine (isopropyloctopamine, deterenol, 4-(1-hydroxy-2-(isopropylamino)ethyl)phenol), a beta-selective and direct-acting adrenergic agonist, has been reported in the past as declared as well as non-declared ingredient of dietary supplements. The proven biological activity and the structural similarity of isopropylnorsynephrine to substances classified as prohibited compounds according to the World Anti-Doping Agency's (WADA's) regulations could necessitate the inclusion of this sympathomimetic amine into routine doping control analytical assays. Therefore, information on urinary metabolites is desirable in order to allow for an efficient implementation of target compounds into existing multi-analyte testing procedures, enabling the unequivocal identification of the administration of isopropylnorsynephrine by an athlete. In a pilot study setting, urine samples were collected prior to and after the oral application of ca. 8.7 mg of isopropylnorsynephrine, which were subjected to liquid chromatography-high resolution/high accuracy (tandem) mass spectrometry. The intact drug, hydroxylated and/or glucurono- or sulfo-conjugated isopropylnorsynephrine were detected up to 48 h post-administration, with isopropylnorsynephrine sulfate representing the most abundant urinary target analyte. No relevant amounts of the dealkylation product (octopamine) were observed, indicating that merely moderate adaptations of existing test methods (or data evaluation strategies) are required to include isporpoylnorsynephrine in antidoping analytics, if required.

KEYWORDS
doping, mass spectrometry, sport, stimulants

1 | INTRODUCTION

The selective beta-adrenergic activity of isopropylnorsynephrine (IPNS, isopropyloctopamine, deterenol, 4-(1-hydroxy-2-(isopropylamino)ethyl)phenol, WIN 833, Figure 1) was identified in the late 1940s,[1] and specifically the mechanism of its in vitro-observed lipolytic effect on adipose tissue was continuously investigated since,[2,3] attributed to a substantial β_3-adrenoceptor agonism of the substance.[4] While in vitro and animal study data (*eg*, on its toxicity[5]) exist, clinical data are scarce, and yet IPNS was reported as ingredient

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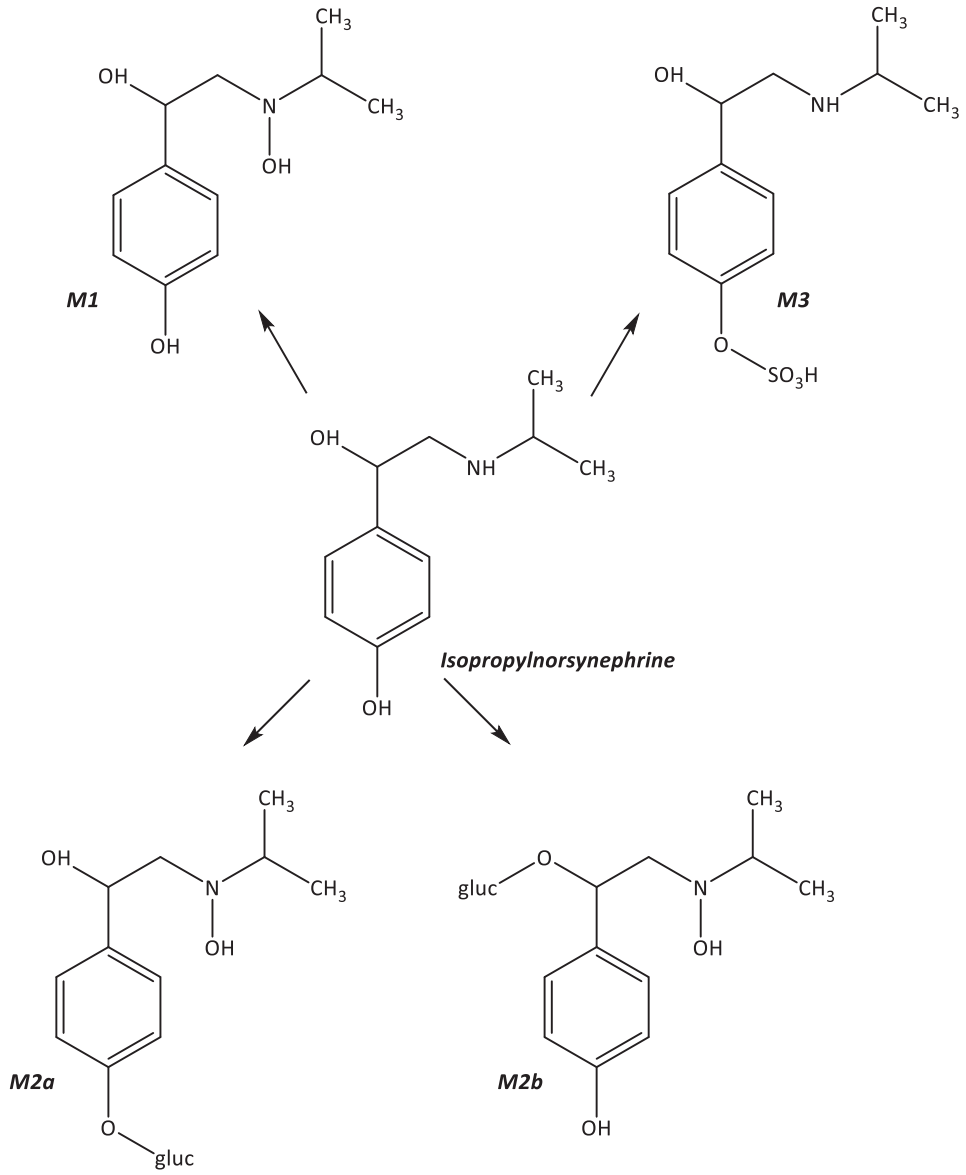

FIGURE 1 Structures of isopropylnorsynephrine (IPNS, center) and tentatively identified metabolites

of dietary supplements on several occasions,[6–10] indicating its facile availability and, thus, potential presence also in doping control samples. IPNS is currently not explicitly listed on the World Anti-Doping Agency's (WADA's) Prohibited List[11]; however, octopamine is classified as stimulant and, consequently, information on urinary metabolites of IPNS are desirable to enable the implementation of the substance into anti-doping testing programs as well as to probe for the potential biotransformation of IPNS into octopamine and/or octopamine sulfate. Here, a reporting limit of 1000 ng/mL applies for the sum of urinary octopamine and its phase-II sulfate metabolite,[12] above which octopamine concentrations constitute an adverse analytical finding.

Hence, pilot studies were conducted with a commercially available dietary supplement containing IPNS. Following a single oral dose of 50% of the recommended amount of the supplement, the formation of urinary metabolites of IPNS and their elimination profiles were investigated by chromatographic – mass spectrometric approaches, and structural information on selected major metabolites derived from high resolution/accurate mass measurements as well as derivatization experiments was used to suggest tentatively assigned structures to the observed metabolic products.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

Iodomethane (purum), potassium carbonate (p.a.), IPNS (Deterenol acetate), 3-chloroperbenzoic acid (<77%), acetone, lithium aluminium hydride (powder, 95%), potassium carbonate (>99%), sulfur trioxide pyridine complex (97%), and octopamine hydrochloride (analytical standard) were obtained from Sigma-Aldrich (Deisendorf, Germany), acetonitrile, formic acid, and ammonium acetate (all analytical grade)

were purchased from Merck (Darmstadt, Germany), and solid-phase extraction cartridges (OASIS HLB, 3 mL, 60 mg sorbent) were obtained from Waters (Eschborn, Germany). The internal standard IPNS-d_7 (deterenol-d_7) was provided by LGC Standards GmbH (Wesel, Germany).

2.2 | Liquid chromatography-mass spectrometry

All analyses were conducted using a Thermo Fisher Scientific (Dreieich, Germany) Vanquish LC system interfaced via electrospray ionization (ESI) to an Exploris 480 quadrupole/orbitrap mass spectrometer. The LC was equipped with a Thermo Accucore C-8 (100 × 2.1 mm, 2.7 µm particle size) analytical column. The LC method employed 5 mM aqueous ammonium acetate (containing 0.1% acetic acid, solvent A) and acetonitrile (solvent B) and gradient elution starting with 99.5% A for 0.5 min, decreasing to 98% A in 4.5 min, decreasing further to 92% A in 3 min and then 50% A in 2 min, before flushing the column at 0% A for 1.5 min and subsequent re-equilibration at starting conditions for 3.5 min. The overall run time was 15 min. The ESI source was operated in positive mode using a spray voltage of 3.0 kV, and full scan (*m/z* 100-800) as well as product ion scan experiments were conducted at a resolution of 30.000 (full width at half maximum at *m/z* 200). Here, the isolation window of the quadrupole for the product ion experiments was set to 1.3 Da. The collision gas was nitrogen (provided by a CMC nitrogen generator, CMC Eschborn, Germany), and the collision energy was set to 25 eV. The system was calibrated using the manufacturer's calibration procedure ensuring mass accuracies better 2 ppm.

2.3 | Dietary supplement testing

A dietary supplement labeled to contain 20 mg of IPNS per serving (11 g) was obtained via Internet order. The material consisted of water-soluble powder and, after homogenization, 55 mg were dissolved in 50 mL of deionized water/acetonitrile (9:1, *v/v*). Eight aliquots of 10 µL each were prepared, seven of which were spiked with either 10, 25, 50, 75, 100, 150, or 200 ng of IPNS reference substance (10, 25, 50, 75, 100, 150, or 200 µL of an aqueous solution containing 1 µg/mL), and all eight samples were finally topped-up to 1 mL with deionized water. The content of IPNS was calculated from the resulting standard addition curve. Analogously, the supplement was tested for the presence of octopamine.

2.4 | Elimination study

Following informed written consent, two healthy male volunteers (46 and 47 years) ingested 5.5 g of the dietary supplement (i.e. 50% of the recommended dose and corresponding to ca. 8.7 mg of IPNS as determined by standard addition analysis), dissolved in 150 mL of tab water. Blank urine samples were collected immediately before the supplement consumption, and post-administration specimens were sampled up to 48 h. The study was conducted with approval of the local ethics committee of the German Sport University Cologne (#167/2020).

2.5 | Sample preparation

Urine samples (0.5 mL) were aliquoted into 2 mL Eppendorf tubes and prepared for analysis by the addition of 100 ng of the internal standard IPNS-d_7, followed by dilution of the sample with deionized water (1:1, *v/v*), thorough vortexing for 10 s, and subsequent centrifugation at 3300 x *g*. A volume of 200 µL of the sample was finally transferred to a glass vial for LC-MS(/MS) analysis.

In order to allow for methylating phenolic hydroxyl functions, carboxyl and amino residues for structure elucidation,[13,14] urine (1 mL) was solid-phase extracted on a HLB cartridge after conditioning of the adsorber resin with 2 mL of methanol and 2 mL of water. After washing with another volume of 2 mL of water, the retained analytes were eluted with 2 mL of methanol, which was evaporated to dryness under reduced pressure at 40°C in a centrifuge. The dry residue was reconstituted in 200 µL of a mixture containing 185 µL of acetonitrile and 15 µL of iodomethane, and 200 mg of potassium carbonate was added prior to heating the mixture to 50°C for 60 min. After cooling, the supernatant was transferred to a new test tube, evaporated to dryness under reduced pressure at 40°C in a centrifuge, and reconstituted in 200 µL of acetonitrile/water (1:1, v/v) for LC-MS(/MS) analysis.

2.6 | Estimation of urinary IPNS concentrations

Urinary IPNS concentrations observed in post-administration samples were estimated by means of a calibration curve prepared by spiking a blank urine sample with IPNS reference material at 0.01, 0.02, 0.1, 0.5, 5, and 1 µg/mL. The samples were prepared and analyzed in accordance to the above-mentioned 'dilute-and-inject' protocol, and peak area ratios of IPNS and the internal standard were utilized.

3 | RESULTS AND DISCUSSION

3.1 | Mass spectrometric characterization of major urinary metabolites

The obtained dietary supplement was labeled to contain 20 mg of IPNS per serving (11 g), and standard-addition analyses confirmed an IPNS concentration of ca. 1.6 mg/g. The resulting dosage per recommended serving matches roughly earlier reports concerning IPNS-containing tablets from 2014,[6] where ca. 20–40 mg per serving (tablet) was found in the context of follow-up investigations into serious adverse events. Urine samples collected after the ingestion of ca. 8.7 mg of IPNS yielded signals attributed to intact and unmodified IPNS, IPNS sulfate, hydroxylated IPNS, and two species of hydroxylated and glucurono-conjugated IPNS (Figure 1) as supported by product ion mass spectra illustrated in Figure 2. The protonated molecule of IPNS

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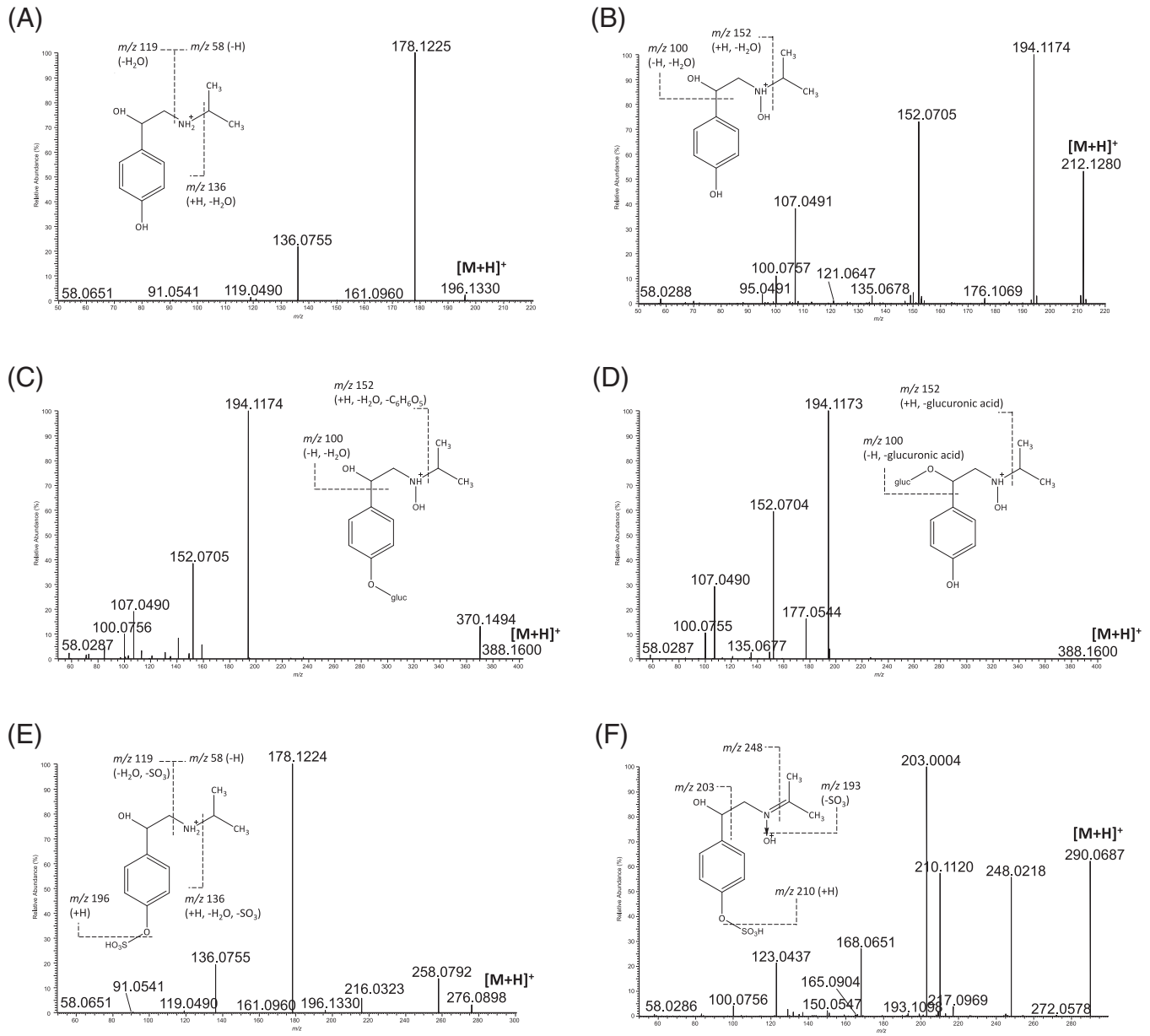

FIGURE 2 ESI product ion mass spectra of (A) isopropylnorsynephrine (IPNS) ($[M+H]^+ = m/z$ 196.13); (B) hydroxylated isopropylnorsynephrine (**M1**) ($[M+H]^+ = m/z$ 212.13); (C) hydroxylated and glucurono-conjugated isopropylnorsynephrine (**M2a**) ($[M+H]^+ = m/z$ 388.16, RT = 1.9 min); (D) hydroxylated and glucurono-conjugated isopropylnorsynephrine (**M2b**) ($[M+H]^+ = m/z$ 388.16, RT = 8.1 min); (E) sulfo-conjugated isopropylnorsynephrine (**M3**) ($[M+H]^+ = m/z$ 276.09), and (F) sulfo-conjugated isopropylnorsynephrine *N*-oxide (**M4**) ($[M+H]^+ = m/z$ 290.07)

at *m/z* 196 was suggested to eliminate water (18 u) and propene (42 u) to form the product ions at *m/z* 178 and 136, respectively, which was proposed to further dissociate to *m/z* 119 and 91 by consecutive losses of ammonia (17 u) and carbon monoxide (28 u) (Figure 2A, Scheme 1) as supported by the corresponding accurate masses of the observed ions as well as by pseudoMS^3 experiments using *m/z* 178, 136, or 119 as in-source CID-generated precursor ions (data not shown). The ion at *m/z* 58 was assigned to propan-2-imimium. Hydroxylated IPNS (**M1**) gave rise to a protonated molecule at *m/z* 212, and its dissociation pathway suggested the location of the newly introduced hydroxyl group at the phenylethanolamine residue by mass shifts of 16 u observed with all product ions proposed to include the norsynephrine structure (Figure 2B). The presence of a catechol moiety was excluded based on pseudoMS^3 experiments with norepinephrine, where $[M+H]^+$ - 18 at *m/z* 152 was used as precursor, which yielded a product ion mass spectrum deviating from that of *m/z* 152 obtained from **M1** under pseudoMS^3 conditions (Figure 3). While the hydroxylated IPNS (**M1**) eliminated a hydroxyl radical from *m/z* 152 to form *m/z* 135, norepinephrine exclusively eliminated ammonia to yield *m/z* 135 (Figure 3). Therefore, in case of **M1**, the formation of *m/z* 152 as an *N*-hydroxylated species must be taken into consideration, either *ab initio* (*cf.* methamphetamine[15–18]) or by rearrangement of

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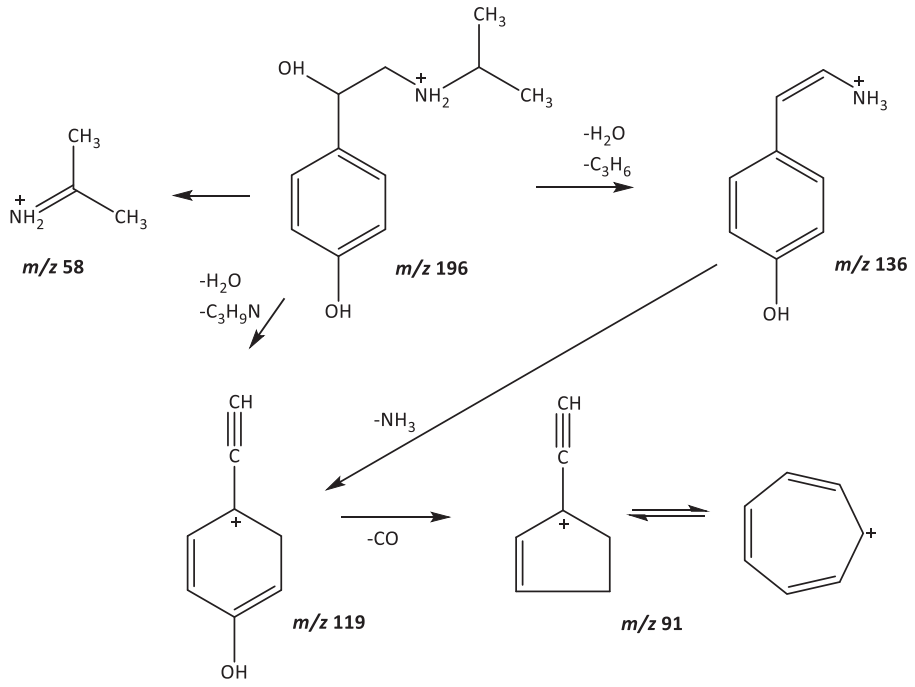

SCHEME 1 Proposed dissociation pathway of protonated isopropylnorsynephrine (IPNS)

a metabolically introduced phenolic hydroxyl group. The existence of a *N*-hydroxylated species is further supported by the presence of *m/z* 100 (elemental composition: $C_5H_{10}NO$) in the product ion mass spectrum of **M1** (Figure 2B) as well as the appearance of *m/z* 58 (elemental composition: C_2H_4NO) in both the MS/MS and the pseudoMS^3 mass spectrum of *m/z* 152 of **M1**, attributable to 4*H*-1,2-oxazet-2-ium, and the formation of the product ion at *m/z* 119 (C_8H_7O, Figure 3), the generation of which necessitates the elimination of hydroxylamine (NH_2OH, 33 u) as observed in earlier studies investigating metabolites of *N*-hydroxylated secondary amine structure.[19] Further corroborating evidence for the metabolic formation of *N*-hydroxyl isopropylnorsynephrine was obtained by chemical microsynthesis of the putative metabolite. Following established synthetic routes,[20] IPNS was first converted into its *N*-oxide by means of 3-chloroperbenzoic acid, and subsequently reduced by lithium aluminium hydride to yield the corresponding *N*-hydroxide. The product ion mass spectrum of the obtained product is depicted in Figure 3C, which plausibly matches the product ion mass spectrum of **M1** shown in Figure 2B.

Two species of glucuronic acid conjugates of **M1** were detected with protonated molecules at *m/z* 388, separated chromatographically by 6.2 min (Figure 2C: **M2a**; Figure 2D: **M2b**, typical chromatogram depicted in Figure 5A). The earlier eluting metabolite **M2a** was of substantially lower abundance compared to the later eluting **M2b**, and featured $[M+H]^+$-18 at *m/z* 370 in contrast to **M2b**, which (in analogy to IPNS) suggested the site of glucuronidation at a phenolic hydroxyl group. Conversely, the glucuronidation of **M1** at the alcoholic hydroxyl group is proposed to result in **M2b** that, upon protonation and collisional activation, eliminates immediately glucuronic acid (194 u) to form the product ion at *m/z* 194.

A signal attributed to the sulfo-conjugate of IPNS (**M3**) was detected, representing the most abundant metabolite of the sympathomimetic amine. The product ion mass spectrum derived from $[M+H]^+$ at *m/z* 276 is illustrated in Figure 2E, exhibiting a product ion at *m/z* 258, which indicates a (predominant) conjugation at the phenolic hydroxyl group. By means of a microsynthesis of sulfo-conjugated IPNS in accordance to established protocols,[21] a reference spectrum was obtained as illustrated in Figure 4A, which plausibly matches the spectrum generated from **M3** (Figure 2E). Further investigations into the conjugation site were conducted by selective methylation of the metabolic products isolated from elimination study urine samples, affecting the secondary amine and phenolic but not alcoholic hydroxyl groups. The presumed sulfo-conjugate of IPNS (**M3**) produced a dimethylated derivative with both newly introduced methyl groups located at the nitrogen atom, forming an aminium residue (and thus a M^+ at *m/z* 304) as evidenced by the presence of the product ion at *m/z* 88, attributed to *N*,*N*-dimethylpropan-2-aminium (Figure 4B). A free phenolic hydroxyl group would be methylated under the chosen conditions, yielding a M^+ precursor ion at *m/z* 318 and, consequently, an asulfate product ion at *m/z* 238 rather than the ion at *m/z* 224 seen in Figure 4B. The combination of a precursor ion at *m/z* 318 and a product ion at *m/z* 238 was found but only at a relative abundance of ca. 2% in comparison to the precursor/product ion pair of *m/z* 304/224, indicating that a minor share of sulfo-conjugation at the alcoholic hydroxyl group might also exist (data not shown).

Also, a signal attributable to the sulfo-conjugate of IPNS *N*-oxide (**M4**) was observed with a product ion mass spectrum shown in Figure 2F, supported by the characteristic product ions observed at *m/z* 248 (- propene), *m/z* 210 (-SO_3), and *m/z* 203 (assigned to sulfo-conjugated hydroxyl(4-hydroxyphenyl)methylium).

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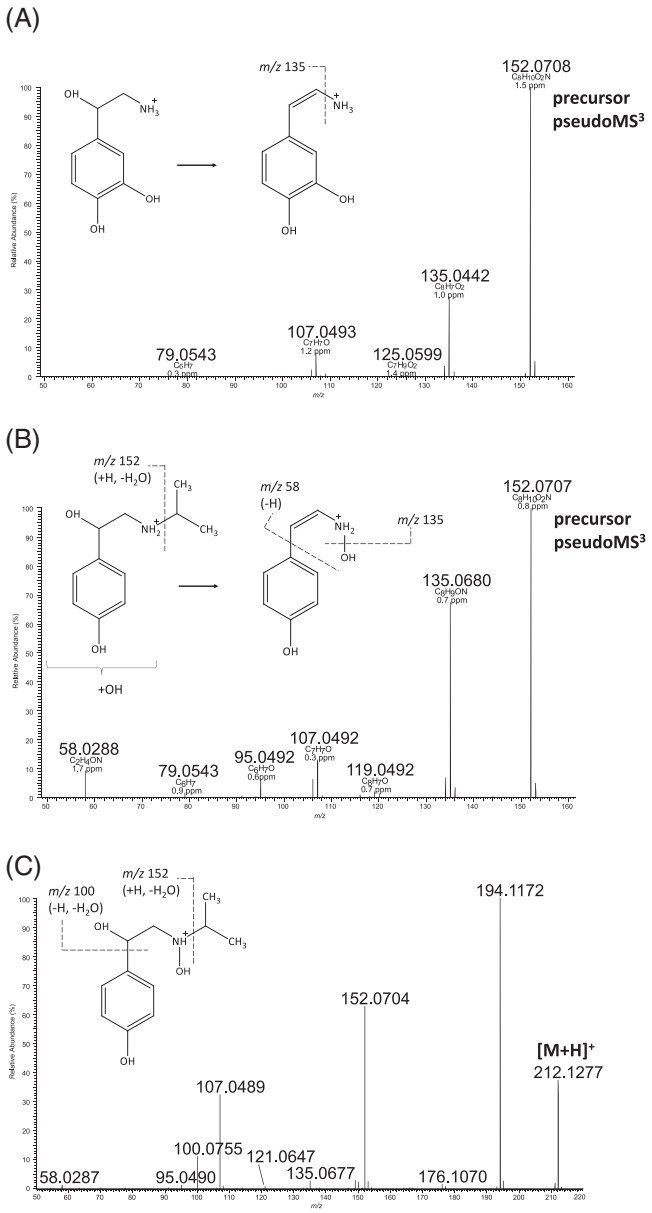

FIGURE 3 pseudoMS^3 mass spectra of (A) norepinephrine ($[M+H]^+ = m/z$ 170.13 → 152.07), (B) hydroxylated isopropylnorsynephrine (**M1**) ($[M+H]^+ = m/z$ 212.13 → 152.07), both recorded at collision energies of 30 eV, and product ion mass spectrum of (C) synthesized *N*-hydroxylated IPNS ($[M+H]^+ = m/z$ 212.12)

3.2 | Urinary excretion profile

The extracted ion chromatograms of major target analytes (ISTD, IPNS, **M2** and **M3**), measured in product ion scan mode and presented by means of accurate masses of product ions ± 0.02 *m/z*, of a urine sample collected 5.5 h post-administration are illustrated in Figure 5A. Below the top pane, which depicts the signal of the internal standard, the peak of IPNS is shown at 2.39 min. The signal at 2.01 min in the same ion trace is attributed to the partial in-source dissociation of IPNS sulfate (**M3**), which is corroborated by the intact **M3** presented at identical retention time in the third pane of Figure 5. The bottom pane exhibits

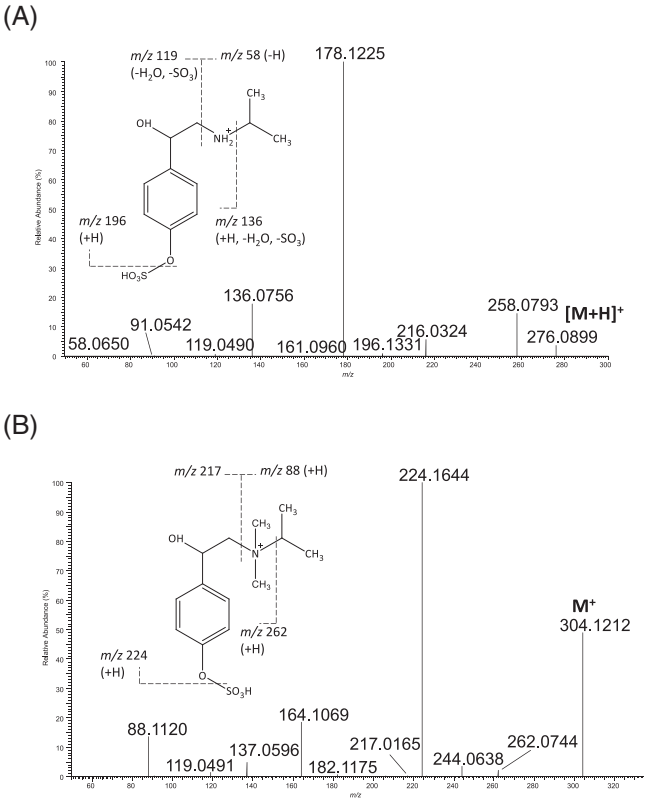

FIGURE 4 Product ion mass spectra of (A) synthesized sulfo-conjugated IPNS (**M3**) with $[M+H]^+ = m/z$ 276.09; and b) dimethylated sulfo-conjugated IPNS (**M3**) with $[M+H]^+ = m/z$ 304.12, presenting a product ion at *m/z* 224.16 that matches the asulfate of **M3** bearing two methyl groups at the nitrogen atom

three signals, all tentatively assigned to hydroxylated and glucurono-conjugated IPNS with **M2a** at 1.87 min and **M2b** at 8.10 min. The latter features a minor additional signal at 7.96 min with identical product ion mass spectrum (data not shown), which is suggested to represent a stereoisomer of **M2b**.

By means of an external calibration curve and internal standard, the urinary concentration of IPNS was estimated in post-administration samples as depicted in Figure 5B. Peak concentrations (specific gravity-adjusted) of ca. 2.3 µg/mL were observed 1 h after oral administration of ca. 8.7 mg of IPNS contained in the dietary supplement, declining below 50 ng/mL within approximately 12 h. In the absence of reference material for the tentatively identified metabolites **M2**-**M3**, peak area ratios of the analytes/internal standard were employed to plot elimination profiles (Figure 5C), outlining the comparably high abundance of IPNS sulfate (**M3**) and OH-IPNS (**M2b**), which contributes to qualifying as target analytes in routine doping controls. Of note, only negligible (if any) amounts of octopamine and octopamine sulfate were detected. Hence, is appears unlikely that the use of IPNS will result in an adverse analytical finding for octopamine in doping control samples; yet, probing for the presence of diagnostic metabolites of IPNS such as *e.g.* the aforementioned **M2b** and **M3** complements the analytical data and supports subsequent result managing processes.

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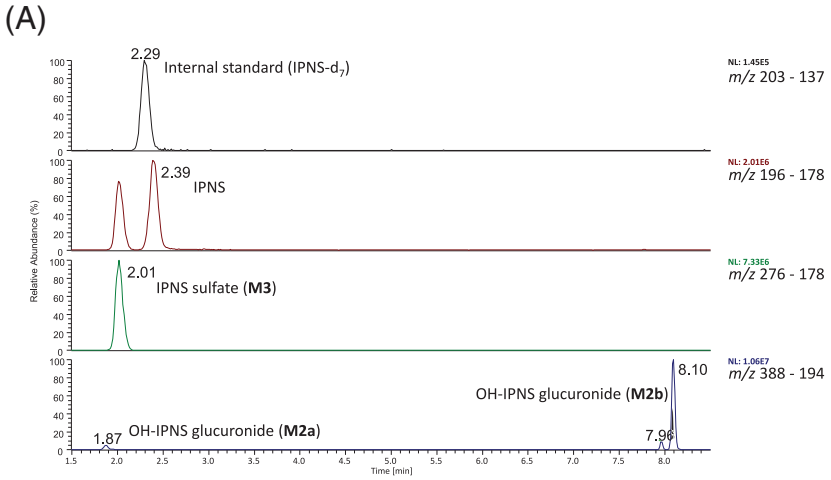

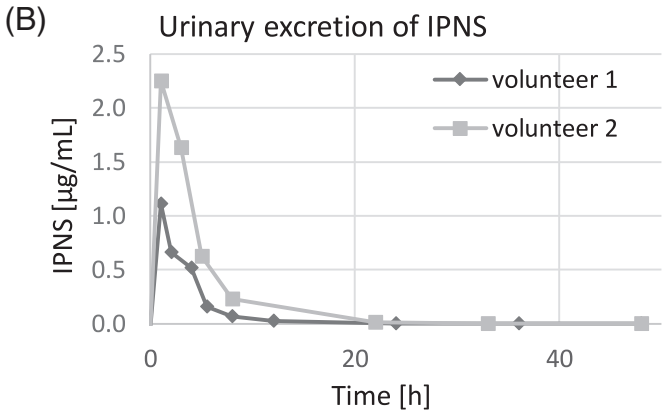

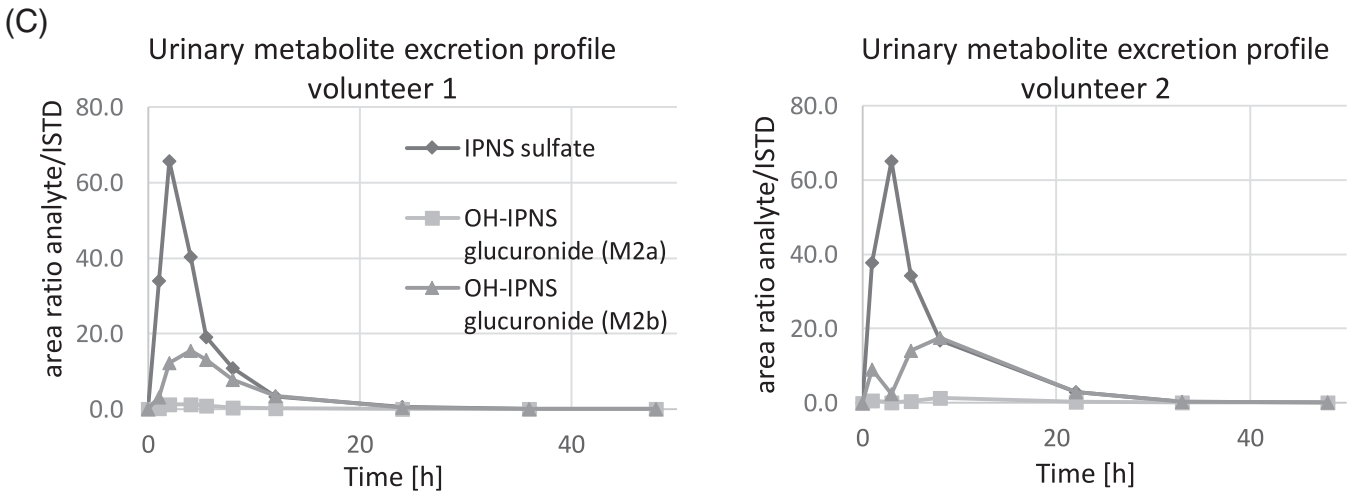

FIGURE 5 (A) extracted ion chromatograms obtained from a post-administration urine sample collected 5.5 h after ingestion of ca. 8.7 mg of IPNS; (B) estimated urinary concentrations of IPNS in post-administration samples collected over a period of 48 h; (C) elimination profiles of IPNS main metabolites, presented as peak area ratios of the target analytes/internal standard

4 | CONCLUSION

Isopropylnorsynephrine has overtly been advertised as active ingredient in commercially available dietary supplements, and adverse health events were associated with the use of products labeled to contain, amongst other pharmacologically active components, IPNS. Albeit chemically closely related to octopamine, the presented data do not suggest a substantial conversion of IPNS into octopamine in both individuals tested within this study; instead, IPNS itself plus diagnostic phase-II metabolites are eliminated into urine that enable the

unequivocal detection of an IPNS administration at dosages commonly suggested by supplement manufacturers.

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DATA SHARING

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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