

Influence of synthetic isoflavones on selected urinary steroid biomarkers: Relevance to doping control

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ABSTRACT

In this work we have investigated the influence of the intake of two synthetic isoflavones, methoxyisoflavone and ipriflavone, on the urinary concentration of endogenous steroids, and on their relative ratios, of doping relevance. Specifically, the concentrations of testosterone (T), epitestosterone (E), androsterone (A), etiocholanolone (Etio), 5 α -androstane-3 α ,17 α -diol (5 α Adiol), 5 β -androstane-3 α ,17 α -diol (5 β Adiol), and the ratios T/E, A/T, A/Etio, 5 α Adiol/5 β Adiol, 5 α Adiol/E, were considered, in the framework of the Steroidal Module of the Athlete Biological Passport (ABP). The above set of parameters were complemented by the urinary levels of luteinizing hormone (total LH) and the ratio between T and LH (T/total LH), to assess the possible effects on the biosynthesis of the mentioned steroids.

Five healthy Caucasian male volunteers were selected for the study. Urine samples were collected before and during the administration of (i) methoxyisoflavone (Methoxyisoflavone, MyProtein) and (ii) ipriflavone (Osteofix[®], Chiesi Farmaceutici). For the analysis of the urinary steroid profile, after enzymatic hydrolysis with β -glucuronidase from *Escherichia Coli* (*E. Coli*) and liquid–liquid extraction with *tert*-butylmethyl ether, all samples were analyzed by gas chromatography coupled to tandem mass spectrometry (GC–MS/MS), while for the determination of total LH all urine samples were directly analyzed by a chemiluminescent immunometric assay technique (Siemens Immulite 2000 LH).

Our results show that the administration of either methoxyisoflavone or ipriflavone causes an alteration of the urinary concentrations and concentration ratios of the investigated steroids, in the range 55–80% from the baseline values. Furthermore, an oversecretion of LH after the daily intake of methoxyisoflavone or ipriflavone was also recorded in all volunteers, corresponding to an increase in the biosynthesis and excretion of T and some of its metabolites. These changes trigger a dysregulation in the pattern of urinary excretion of the steroids included in the Steroidal Module of the ABP, which makes more difficult the interpretation of the longitudinal steroid profile based on the definition of individual normality ranges for each athlete. Our data are also consistent with previous evidence regarding the *in vitro* effects of natural and synthetic isoflavones, suggesting that their monitoring in doping control routine analysis would be very beneficial for the result management activities.

1. Introduction

Anabolic androgenic steroids (AAS) are a class of steroid hormones whose chemical structure and bio-pharmacological activity are related to testosterone (T) [1]. In males, the biosynthesis of T (schematically shown in Fig. 1) mainly occurs in Leydig cells, situated in the testicular

interstitium. This process is stimulated by luteinizing hormone (LH), secreted from the anterior pituitary, which is, in turn, stimulated by gonadotropin-releasing hormone (GnRH), secreted from the hypothalamus. The so-called hypothalamus-pituitary-gonadal (HPG) axis is controlled by a negative feedback mechanism, where elevated levels of endogenous steroids, including estrogens and progestogens, send signals

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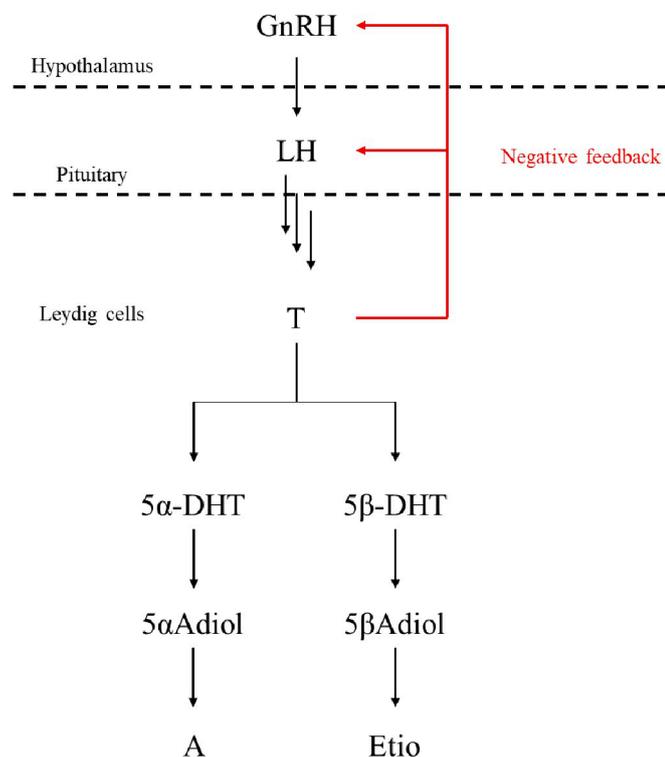


Fig. 1. Simplified schematization of the biosynthesis of anabolic androgenic steroids (AAS).

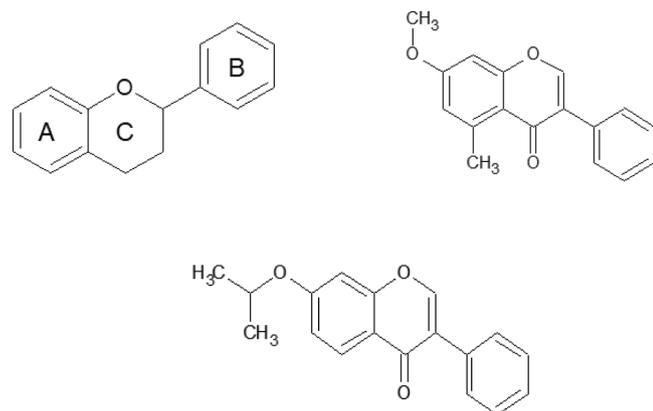


Fig. 2. General structure of flavonoids (a), molecular structure of methoxyisoflavone (b) and of ipriflavone (c).

to the hypothalamus and/or anterior pituitary to decrease the release of GnRH and/or LH [2].

T and its synthetic analogs are available on the market in different forms (gels, patches, suspensions, oral and intramuscular preparations) and dosages. These preparations have been in clinical use for decades. They are administered in two main distinct modes: as male replacement therapy in androgen deficiency, and as pharmacological androgen therapy to obtain specific anabolic or other effects in non-androgen-deficient men with chronic diseases [3]. Because of its anabolic effects, T has been abused to increase sports performance since it was isolated and synthesized [4–5]; it still represents one of the most abused drugs worldwide according to the statistical data of the antidoping laboratories accredited by the World Anti-Doping Agency (WADA) [6]. On the other hand, the main clinical use of LH is typically related to the treatment of infertility, both in men and in women, but the desired effects of the LH misuse in sport are linked to an enhancement of the

testicular production of T [7], especially if potentially suppressed following the previous intake of synthetic AAS. Elevated LH levels can also be connected to the abuse of GnRH and/or of anti-estrogenic drugs [8].

For the above reasons, T, its precursors and synthetic analogs, as well as LH and GnRH, are banned in sports, and are included in the WADA List of Prohibited Substances and Methods, in the sections S1 (Anabolic Agent), and S2 (Peptide Hormones, Growth Factors, Related Substances and Mimetics) respectively. Their use is forbidden both “in-” and “out-” of competition, and in all sport disciplines, but the use of LH is prohibited only in male athletes [9]. The effects of the abuse of T and of structurally related substances are associated with androgenic (e.g., masculinization, virilization) and anabolic (e.g., protein building) effects; while LH can be abused either to enhance muscle strength and/or, as already said, to re-activate the biosynthesis of T, suppressed by the misuse of anabolic steroids [7–8,10–11].

The currently adopted strategy followed by the accredited antidoping laboratories to detect the abuse of the so called “pseudo-endogenous steroids” (endogenous steroids when administered exogenously) involves (i) an “Initial Testing Procedure”, based on the characterization of the urinary steroid profile, which allows the longitudinal monitoring of six target steroids and their relative ratios, and constitutes the backbone of the Steroidal Module of the Athlete Biological Passport (ABP) [12–13]; and (ii) the confirmation of any abnormal urinary steroid profiles by GC-c-IRMS analysis [14]. More in details, the WADA accredited laboratories must measure, in each urine sample, the concentration, normalized for the specific gravity of the sample itself, of testosterone (T), epitestosterone (E), androsterone (A), etiocholanolone (Etio), 5 α -androstane-3 α ,17 α -diol (5 α Adiol), 5 β -androstane-3 α ,17 α -diol (5 β Adiol) as sum of free and glucuronide fractions, together with five ratios (T/E, A/T, A/Etio, 5 α Adiol/5 β Adiol, 5 α Adiol/E) by means of gas-chromatography coupled to mass spectrometry (GC-MS) or tandem mass spectrometry (GC-MS/MS) [12–13]. Individual longitudinal data are integrated in a Bayesian adaptive model with the aim of defining an individualized longitudinal profile whose normality values and upper and lower limits are specific for each athlete: data out of range of normality constitute an atypical result (Atypical Passport Findings, ATPF) [15–18], prompting the activation of the confirmation analysis. In the case of a suspicious sequence of data, a GC-c-IRMS confirmation analysis, giving information about the endogenous or exogenous origin of the target steroid, is mandatory to report an Adverse Analytical Finding (AAF) [14,19].

On the other hand, to detect the abuse of LH in male athletes, the accredited laboratories refer to the WADA Technical Document TD2021CG/LH “Reporting & Management of Human Chorionic Gonadotropin (hCG) and Luteinizing Hormone (LH) Findings in Male Athletes” [20]. According to this document, the accredited laboratories assay for total LH measurement, estimating the levels of the intact heterodimer, of different forms of free β -chain and their degradation products: a “Presumptive AAF” for LH is reported whenever its concentration, normalized for the specific gravity of the sample, is greater than 60 UI/L, using a chemiluminescent Immulite immunoassay, or greater than 40 UI/L using a DELFIA (Dissociation-Enhanced Lanthanide Fluorescent Immunoassay) assay [21]. Furthermore, elevated LH levels can be associated to the illicit use of banned drugs that induce the release of endogenous LH, such as, for example, anti-estrogens and aromatase inhibitors [7–8]. For indeed, anti-estrogens and aromatase inhibitors, together with hCG, synthetic anabolic androgenic steroids, diuretics, masking agents, antifungals (e.g., ketoconazole), 5 α -reductase inhibitors, and alcohol, as well as bacterial contamination, are classified as “exogenous confounding factors”, since they could alter the significance of the urinary concentration values of the target steroids. In view of the above, the confounding factors are monitored in each urine sample analyzed by the WADA-accredited laboratories [12,18,22–24].

Despite this, in the last few years, some research groups investigated the effects of non-banned nor monitored substances on the *in vitro* and *in*

Table 1a
Outline of the methoxyisoflavone excretion study.

Blank						Administration				
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 1	Day 2	Day 3	Day 4	Day 5
7:00	#1	#7	#13	#19	#25	#31	#37	#43	#49	#55
8:00	-	-	-	-	-					
10:00	#2	#8	#14	#20	#26	#32	#38	#44	#50	#56
13:00	#3	#9	#15	#21	#27	#33	#39	#45	#51	#57
16:00	#4	#10	#16	#22	#28	#34	#40	#46	#52	#58
19:00	#5	#11	#17	#23	#29	#35	#41	#47	#53	#59
22:00	#6	#12	#18	#24	#30	#36	#42	#48	#54	#60

in vivo metabolism of the endogenous steroids [25–35]. At the same time, the use of one or more nutritional supplements became more popular among elite athletes, particularly in the last 5–10 days before the competition [36]. Even if their administration cannot be considered a doping practice, some of these nutritional supplements contain substances whose intake could affect the excretion of the endogenous steroids, due to the interaction of the parent compound and/or one or more of its metabolites with the enzymes involved in the endogenous androgenic steroid biosynthesis and metabolism, making more difficult the interpretation of the longitudinal steroid profiles.

Flavonoids are a wide family of chemicals commonly found in fruits and plants and characterized by a basic skeleton composed by 15 carbon atoms articulated in two aromatic rings (ring A and ring B) joined by a propylene “link” which may or may not be closed in a pyran ring (C6–C3–C6) (Fig. 2a). According to the position of the B ring and to the unsaturation and the level of oxidation of the C ring, flavonoids can be classified as flavones, flavonols, catechins, anthocyanins and isoflavones [37]. These compounds are characterized by a huge number of claimed biological activities, such as, for instance, cytostatic, apoptotic, anti-inflammatory, antioxidant, hepatoprotective, and hypolipemic effects, and have attracted attention as possible chemoprotective and/or chemotherapeutic agents [38–39]. Despite this, some natural and synthetic flavonoids are potent modulators of both the expression and the activities of cytochrome P450 genes and proteins [40]. Among flavonoids, natural and synthetic isoflavones may play a role in sport doping [41–42]. They are described as “phytoestrogens” due to their estrogenic/anti-estrogenic activity and could be used in recovering periods after the illicit intake of anabolic steroids (natural and/or synthetic), promoting an increase of the natural production of LH that can reactivate the suppressed biosynthesis of natural androgens [40,43–45]. For this reason, isoflavones-based supplements are often marketed, mostly via non-official retailing channels, as “androgen promoters”, or “testosterone boosters”.

We have already shown in previous works that two synthetic isoflavones, methoxyisoflavone (Fig. 2b) and ipriflavone (Fig. 2c), (i) act as *in vitro* inhibitors of aromatase [46], the enzyme catalyzing the conversion of T to estradiol (E2); and (ii) their urinary metabolites, isolated and characterized by gas-chromatography coupled with high resolution, time of flight mass spectrometry (GC-q-TOF), are easy to detect in samples collected after their oral intake and could be used as markers of exposure. [47].

Despite those experimental evidences, the anabolic and ergogenic effects of natural and synthetic isoflavones have not been specifically assessed yet, mostly due to their reduced oral bioavailability [48]. The

aim of the present work is to study the effects of the administration, for five consecutive days, of a methoxyisoflavone-based supplement (Methoxyisoflavone, MyProtein), commonly used during intensive training periods among bodybuilders as “androgen promoter”, and of an ipriflavone-based drug (Osteofix®, Chiesi Farmaceutici), used to treat the post-menopausal osteoporosis in women, on the urinary levels of selected biomarkers (T, its metabolites, and LH), to assess their potential impact on the strategy followed by the accredited laboratories to detect doping by testosterone and other pseudo-endogenous steroid.

2. Materials and methods

2.1. Standards, chemicals, and reagents

Certified reference standards of methoxyisoflavone and ipriflavone were supplied by LGC Standards (Sesto San Giovanni, Italy); the dietary supplement Methoxyisoflavone was from MyProtein (Cheshire, United Kingdom); the drug Osteofix® containing ipriflavone was purchased from Chiesi Farmaceutici (Parma, Italy). Testosterone (T), epitestosterone (E), androsterone (A) and 17 α -methyltestosterone (used as chromatographic internal standard) were from Sigma-Aldrich (Milano, Italy). Etiocholanolone (Etio), 5 α -androstan-3 α ,17 β -diol (5 α Adiol) and 5 β -androstan-3 α ,17 β -diol (5 β Adiol) were purchased from Steraloids (Newport, RI, USA). 5 α -androstanedione (5 α Adion) and 5 β -androstanedione were from Toronto Research Chemical (North Port, Canada). Deuterated standards (testosterone-*d3* (T-*d3*), epitestosterone-*d3* (E-*d3*), 5 α -androstan-3 α ,17 β -diol-*d3* (5 α Adiol-*d3*), 5 β -androstan-3 α ,17 β -diol-*d5* (5 β Adiol-*d5*), androsterone glucuronide-*d4* (AG-*d4*) and etiocholanolone-*d5* (Etio-*d5*)) were obtained from the National Measurement Institute (NMI Pymble, Australia). The preparation of β -glucuronidase from *Escherichia coli* (*E. coli*), used for the hydrolysis of the glucuronide-conjugated of the endogenous steroids considered in the study, was from Roche Diagnostic (Mannheim, Germany). The derivatizing agent was a mixture of *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA)/mercaptoethanol/ammonium iodide (NH₄I) (1000:6:4 V/V/w) stored in screw-cap vials for a maximum of two weeks. MSTFA was supplied by Chemische Fabrik Karl Bucher GmbH (Waldstetten, Germany); NH₄I and mercaptoethanol were from Sigma-Aldrich (Milano, Italy).

The WHO International Standard Luteinizing Hormone, Human, Pituitary (NIBSC code: 81/535) was purchased from the National Institute for Biological Standards and Controls (NIBSC, Potters Bar, Hertfordshire, United Kingdom).

Solvents (*tert*-butylmethyl ether, ethyl acetate, methanol) and

Table 1b
Outline of the ipriflavone excretion study.

Blank						Administration				
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 1	Day 2	Day 3	Day 4	Day 5
7:00	#1	#7	#13	#19	#25	#31	#37	#43	#49	#55
8:00	-	-	-	-	-	●●	●●	●●	●●	●●
10:00	#2	#8	#14	#20	#26	#32	#38	#44	#50	#56
13:00	#3	#9	#15	#21	#27	#33	#39	#45	#51	#57
16:00	#4	#10	#16	#22	#28	#34	#40	#46	#52	#58
19:00	#5	#11	#17	#23	#29	#35	#41	#47	#53	#59
22:00	#6	#12	#18	#24	#30	#36	#42	#48	#54	#60

Table 2
Selected tandem mass spectrometry transition for the quali-quantitative analysis of the steroids considered in the study.

Analyte	Precursor ion (m/z)	Product ion (m/z)	Collision energy (eV)	Concentration (ng/mL) in calibration samples
T bis-O-TMS	432; 432	209; 196	20; 30	200
E bis-O-TMS	432; 432	209; 196	20; 10	200
A bis-O-TMS	419; 434	329; 419	10; 10	7000
Etio bis-O-TMS	419; 434	329; 419	10; 10	7000
5 α Adiol bis-O-TMS	256; 256	185; 198	10; 10	250
5 β Adiol bis-O-TMS	241; 256	159; 199	20; 10	250
5 α Adion bis-O-TMS	275; 290	155; 185	10; 10	50
5 β Adion bis-O-TMS	275; 275	155; 167	10; 10	50
T- <i>d3</i> bis-O-TMS	435; 435	209; 196	20; 30	100
E- <i>d3</i> bis-O-TMS	435; 435	209; 196	20; 30	25
A- <i>d4</i> bis-O-TMS *	423; 423	231; 333	20; 10	200
Etio- <i>d3</i> bis-O-TMS	424; 424	221; 334	20; 10	200
5 α Adiol- <i>d3</i> bis-O-TMS	259; 259	188; 244	10; 5	50
5 β Adiol- <i>d5</i> bis-O-TMS	246; 261	164; 199	20; 10	50
17 α -methyltestosterone bis-O-TMS	301; 446	169; 301	20; 5	250

reagents (potassium carbonate, sodium phosphate, sodium hydrogen phosphate, sodium hydrogen carbonate) of analytical or high-performance liquid chromatography (HPLC) grade were from Sigma Aldrich (Milano, Italy). Ultra-purified water was obtained from a MilliQ water purification system (Millipore S.p.A., Milano, Italy).

2.2. Sample collection

Urine samples were collected from five male healthy Caucasian volunteers (age 30 ± 5 years, normal body mass index), recruited among those already using synthetic isoflavones as nutritional supplements. All participants undersigned a written informed consent, allowing the use of urine samples for research purposes. The protocol for the controlled administration study was approved by the local Ethical Committee

(Comitato Etico Lazio 1, ref. 297/CE Lazio 1, dated March 8th 2021). A wash-out period of at least six weeks was requested for each subject to be included in the study. After this period, samples collection was performed at multiple time points (six samples/day) for five consecutive days ($n = 30$ samples) before the oral administration of 3 capsules (450 mg/day) of methoxyisoflavone (Methoxyisoflavone, MyProtein) ($n = 30$ samples) and/or 2 tablets (400 mg/day) of ipriflavone (Osteofix[®], Chiesi Farmaceutici) ($n = 30$ samples) (Table 1a-b). Two (vol. 4 and vol. 5) of the five volunteers previously administered with methoxyisoflavone, were then administered with ipriflavone after a washout period of six weeks (when no methoxyisoflavone metabolites were detected). All samples were collected in sterile plastic Falcon[®] tubes and anonymized. An aliquot of 1 mL of each sample was freshly used for the measurement of the specific gravity of the sample and for total LH

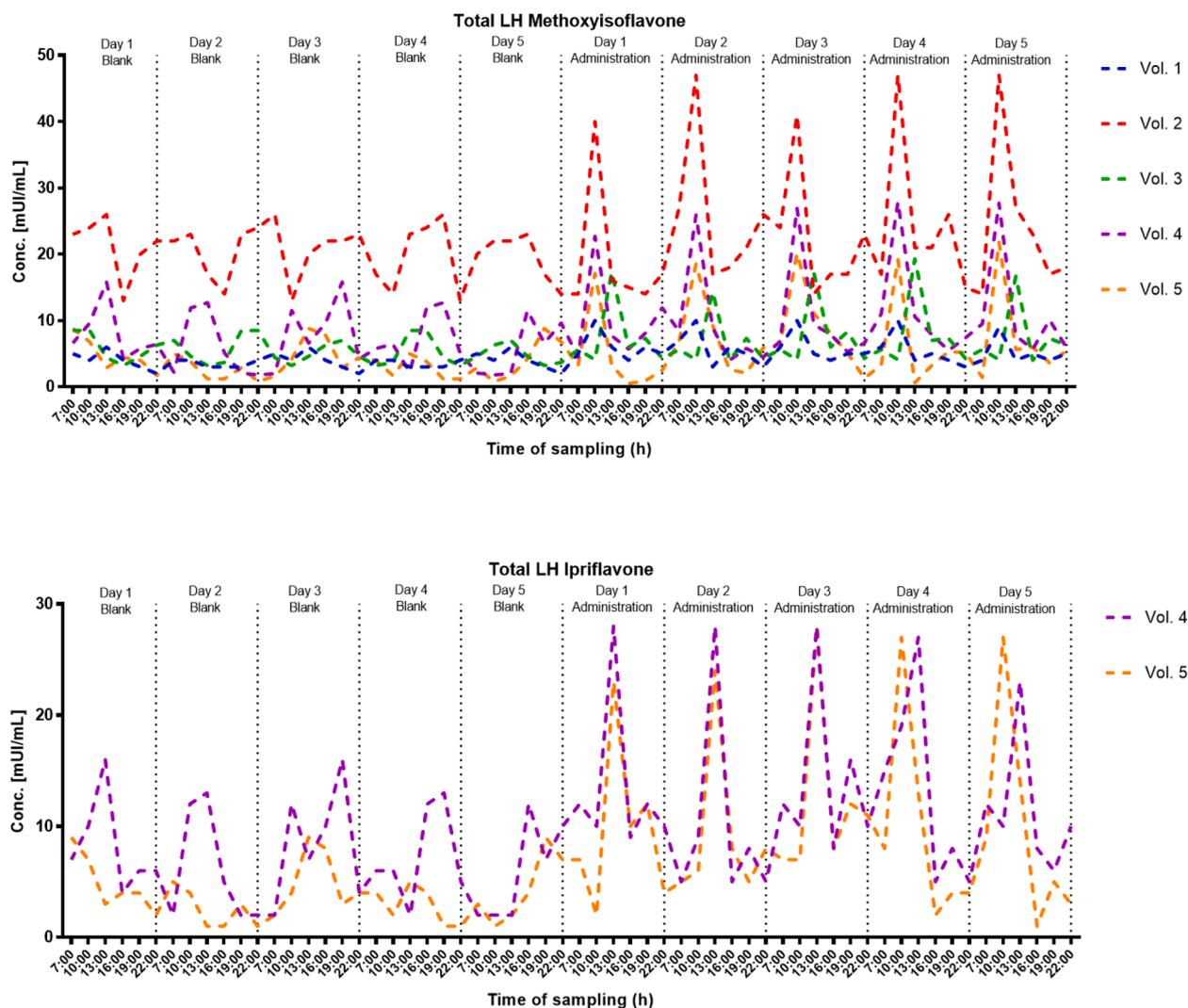


Fig. 3. Urinary excretion of total LH, before and after the administration of methoxyisoflavone (a) and of ipriflavone (b).

analysis. The other part of the samples was stored at -20°C until analysis to prevent changes in the concentrations of endogenous steroids caused by thermal degradation and/or bacterial hydrolysis of the glucuroconjugates. All subjects, after informal consent approval for the observational excretion study, were medically examined to ensure the absence of any diseases and were asked about the use of drugs, alcohol, and dietary habits (the large intake of soy flavonoids, such as quercetin, daidzein, and genistein, could have been a source of interferences with the correct evaluation of the effects of methoxyisoflavone and ipriflavone on the urinary excretion of the selected biomarkers). Pre-administration samples were analyzed to exclude the presence of ipriflavone and methoxyisoflavone. All urine samples were also screened for by the routine methods of the WADA accredited antidoping laboratory of Rome, to confirm the absence of synthetic anabolic steroids, other prohibited substances and any confounding factors described in the WADA Technical Document TD2021EAAS "Endogenous Anabolic Androgenic Steroids. Measurement and Reporting" [12].

2.3. Sample pretreatment

2.3.1. Urinary steroid profile analysis

Sample preparation was performed according to the validated protocol followed in the WADA accredited antidoping laboratory of Rome for the detection of EAAS in human urine [49]. Briefly, 0.75 mL of phosphate buffer (0.8 M, pH 7.4) 50 μL of internal standard (T-d3 100

ng/mL, E-d3 25 ng/mL, AG-d4 200 ng/mL, Etio-d5 200 ng/mL, 5 α Adiol-d3 50 ng/mL, 5 β Adiol-d5 50 ng/mL, 17 α -methyltestosterone 250 ng/mL – final concentrations in 2 mL of urine) and 30 μL of β -glucuronidase from *E. coli* were added to 2 mL of urine, to perform the enzymatic hydrolysis of the glucuro-conjugates of the endogenous steroids. All samples were incubated for 1 h at 55°C . After this period, 0.5 mL of carbonate/bicarbonate buffer (20% w/V, pH 9) were added and a liquid/liquid extraction was performed with 5 mL of *tert*-butylmethyl ether for 5 min on a mechanical shaker. Samples were centrifuged, and the organic layer was transferred to a 10 mL tubes and evaporated to dryness under nitrogen stream at 75°C . The residue was reconstituted in 50 μL of the derivatizing mixture, and the samples were maintained at 75°C for 30 min. Then an aliquot of 2 μL of each sample was injected into the GC-MS/MS system.

2.3.2. Urinary total LH measurement

According to the validated and routinely used method for the determination of total LH, an aliquot of each urine sample was directly analyzed in the Immulite 2000 instrument without any previous pretreatment and following the manufacturer instructions.

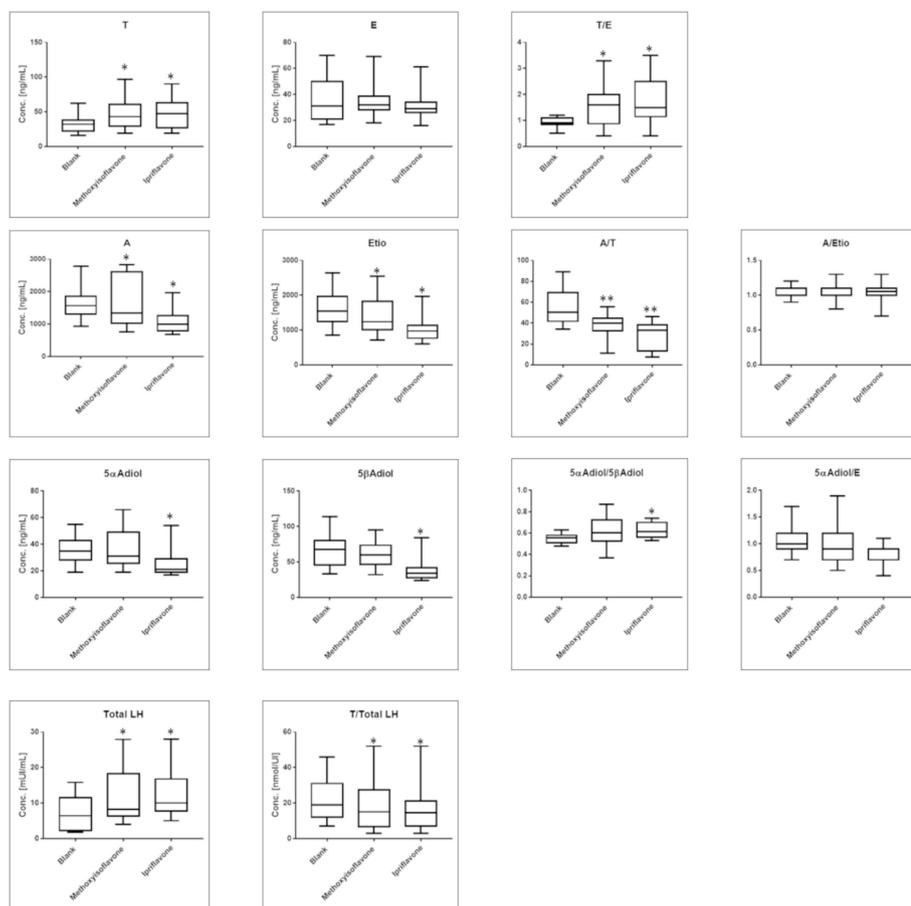


Fig. 4. Boxplots representing data distribution of the concentrations and concentration ratios for all monitored hormone biomarkers measured before the administration (blank samples) and during the administration of 450 mg/day, for five consecutive days, of methoxyisoflavone and 400 mg/day, for five consecutive days, of ipriflavone. Statistical significance variations ($p < 0.05$: *; $p < 0.01$: **) are reported for T (*), A (*), Etio (*), 5 α Adiol (*), 5 β Adiol (*), total LH (*) and for the ratios T/E (*), A/T (**), 5 α Adiol/5 β Adiol (*) and T/total LH (*). Data refer to volunteer 4.

2.4. Instrumental analysis

2.4.1. Gas-chromatography tandem mass spectrometry (GC-MS/MS) conditions

The analysis of urinary steroid profile was carried out according to a previously published method, already applied to assess the influence of other drugs/xenobiotics on the excretion of the endogenous steroids considered in the Steroidal Module of the Athlete Biological Passport [49]. Quantitative analysis was performed on an Agilent 7890A/7000 gas-chromatography tandem mass spectrometer (Agilent Technologies, Cernusco sul Naviglio, Italy), in electron ionization (70 eV) mode, using an Agilent HP-1 17 m fused silica capillary column (cross-linked methyl silicone, i. d. 0.20 mm, film thickness 0.11 μ m, CPS Analitica, Milano, Italy). The GC conditions were as follows: the carrier gas helium at flow rate of 1 mL/min; split ratio 1/20; the temperature program, initial temperature of 188 $^{\circ}$ C for 2.5 min increased at 3 $^{\circ}$ C/minute to 211 $^{\circ}$ C, maintained 2 min, increased at 10 $^{\circ}$ C/minute to 238 $^{\circ}$ C, then at 40 $^{\circ}$ C/minute to 320 $^{\circ}$ C and kept 3.2 min at final temperature; the transfer line and the injection temperature were set at 280 $^{\circ}$ C; the acquisition mode was multiple reaction monitoring (MRM). Identification was based on the specific retention times and on the diagnostic mass-to-charge (m/z) transitions listed in Table 2. Quantitative determination of the urinary concentration of analytes were based on the peak area ratio of the analyte to the corresponding internal standard (T- $d3$ for T, E- $d3$ for E, AG- $d4$ for A, Etio- $d5$ for Etio, 5 α Adiol- $d3$ for 5 α Adiol, 5 β Adiol- $d5$ for 5 β Adiol, 17 α -methyltestosterone for 5 α Adion and 5 β Adion). Calibration samples were prepared in synthetic urine. The concentrations of each analyte in the calibration samples are listed in Table 2.

2.4.2. Immunoanalysis conditions

The analysis of total LH was performed on an Immulite 2000 system,

employing Siemens LH chemiluminescent immunoassay kits (Siemens Medical Solutions Diagnostic, Los Angeles, CA, USA); the method “LH” was selected. The instrument was previously calibrated with the two “adjustors” provided with the diagnostic kit. The detection was made by means of LUMIGEN PPD [4-methoxy-(3-phosphatophenyl)-spiro-(1,2-dioxethane-3,20 - adamantane)] substrate.

2.5. Data analysis

The concentrations of total LH and of those endogenous steroids specifically considered in the Steroidal Module of the Athlete Biological Passport (WADA Technical Document TD2021EAAS “Endogenous Anabolic Androgenic Steroids. Measurement and Reporting” [12]) were measured both in the pre- and post-administration samples, to assess the potential effects of the intake of methoxyisoflavone and ipriflavone on their urinary concentration. The specific gravity of all samples was also measured using a digital refractometer (Mettler Toledo, Novate Milanese, Italy) with the aim to normalize the urinary concentrations to the value of 1.020, according to the TD2021EAAS. Regarding the analysis of the urinary steroid profile, the MassHunter Quantitative Analysis Software 8.0 (Agilent Technologies, Milano, Italy) was used for processing GC-MS/MS data, namely peak integration and analytes quantification. In order to monitor the yield of the hydrolysis and derivatization steps, as well as to evaluate the potential extent of the microbial degradation of the samples, the ratio androsterone-O-mono-TMS/androsterone-O-bis-TMS (A-O-mono-TMS/A-O-bis-TMS), androsterone- $d4$ /etiocholanolone- $d5$ (A- $d4$ /Etio- $d5$) and androsterone/5 α -androstenedione (A/5 α Adion) or etiocholanolone/5 β Adion (Etio/5 β Adion) were calculated. For the quantitative analysis of total urinary LH, the software provided by the manufacturer of the Immulite 2000 was used.

For each compound and ratios considered in this work, data

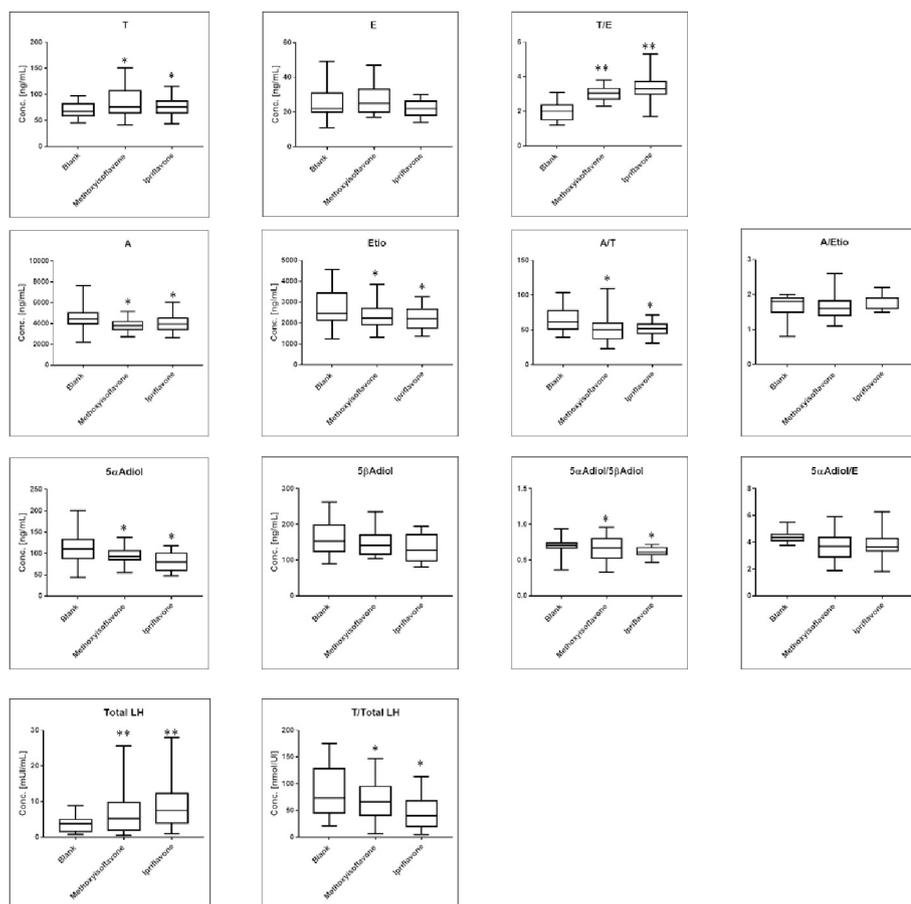


Fig. 5. Boxplots representing data distribution of the concentrations and concentration ratios for all monitored hormone biomarkers measured before the administration (blank samples) and during the administration of 450 mg/day, for five consecutive days, of methoxyisoflavone and 400 mg/day, for five consecutive days, of ipriflavone. Statistical significance variations ($p < 0.05$: *; $p < 0.01$: **) are reported for T (*), A (*), Etio (*), 5 α Adiol (*), total LH (**), and for the ratios T/E (**), A/T (*), 5 α Adiol/5 β Adiol (*) and T/total LH (*). Data refer to volunteer 5.

distribution was assessed, and results were expressed in terms of mean, median, first and third quartiles, minimum and maximum values (box plots). The descriptive statistic's calculations of both total LH and endogenous steroid concentrations and relative ratios and the one-way ANOVA tests were done using GraphPad Prism 9.1.1 (GraphPad Software Inc. La Jolla, CA, USA).

3. Results and discussion

We have investigated the effects on the urinary concentration of total LH, endogenous steroids (T, E, A, Etio, 5 α Adiol, 5 β Adiol) and on their relative ratios (T/E, A/T, A/Etio, 5 α Adiol/5 β Adiol and 5 α Adiol/E) of the intake of two synthetic isoflavones, methoxyisoflavone and ipriflavone, reportedly used by athletes as androgen promoters, but at present not banned nor described as “confounding factors” of the urinary steroid profile by the WADA [12–13]. Furthermore, we also considered the ratio between T and total LH (T/total LH), previously described as an additional parameter to detect the abuse of anabolic agents and of those substances capable of stimulating the biosynthesis of endogenous androgenic anabolic steroids [50–52].

Methoxyisoflavone and ipriflavone were preliminarily characterized for their chromatographic and mass spectrometric profiles. Based on these data, reference solutions obtained from the nutritional supplement Methoxyisoflavone and from the pharmaceutical preparation Osteofix® were analyzed to confirm the actual presence of the two synthetic isoflavones (data not shown). All pre- and post-administration samples were also screened for the potential presence of doping substances/metabolites/biomarkers according to the validated methods currently used in the Laboratorio Antidoping of Rome. Pre-administration samples were also analyzed to exclude also the presence of isoflavone and methoxyisoflavone. The results of the above preliminary analytical

investigations allowed to exclude that the effects – if any – on the biomarkers of the urinary steroid profile were not exclusively due to the intake of the synthetic isoflavones.

As shown in Fig. 3a and 3b, the intake of methoxyisoflavone (450 mg/day) and ipriflavone (400 mg/day) for five consecutive causes an increase in the urinary concentration of total LH in all volunteers, whose extent is in the range 56 and 80% with respect to the baseline values recorded before the administration. However, in none of the samples the concentration of LH exceeded 60 UI/L, that is the decision limit to report a presumptive adverse analytical finding (PAAF) for total LH using an Immulite 2000 assay [20]. The higher values of excretion of total LH are registered, for each volunteer, in the samples collected between 3 and 6 h after the intake of the synthetic isoflavones, likely as a consequence of their effects on the HPG axis. Despite this, due to the poor oral bioavailability of these compounds [48], the concentration of LH rapidly decreases before the intake of the following dose, although still remaining above the pre-administration baseline. Furthermore, we also registered an alteration in the concentrations and concentration ratios of the other urinary biomarkers selected in the study, triggered by the increase of LH secretion that, in turn, causes a disequilibrium in the production of T and its major urinary metabolites.

Figs. 4–5 report the box plots obtained after the analysis of urine samples collected by volunteers 4 and 5 respectively, that were first administered with methoxyisoflavone and, after a wash-out period of six weeks, with ipriflavone. The results were evaluated in terms of mean, median, first and third quartiles, maximum and minimum values both for the samples collected before and during the oral intake of 450 mg/day of methoxyisoflavone or 400 mg/day of ipriflavone, respectively. The significance of the variation between control (“before the administration” samples) and treated (“during the administration” samples) groups for urinary concentrations and concentration ratios was

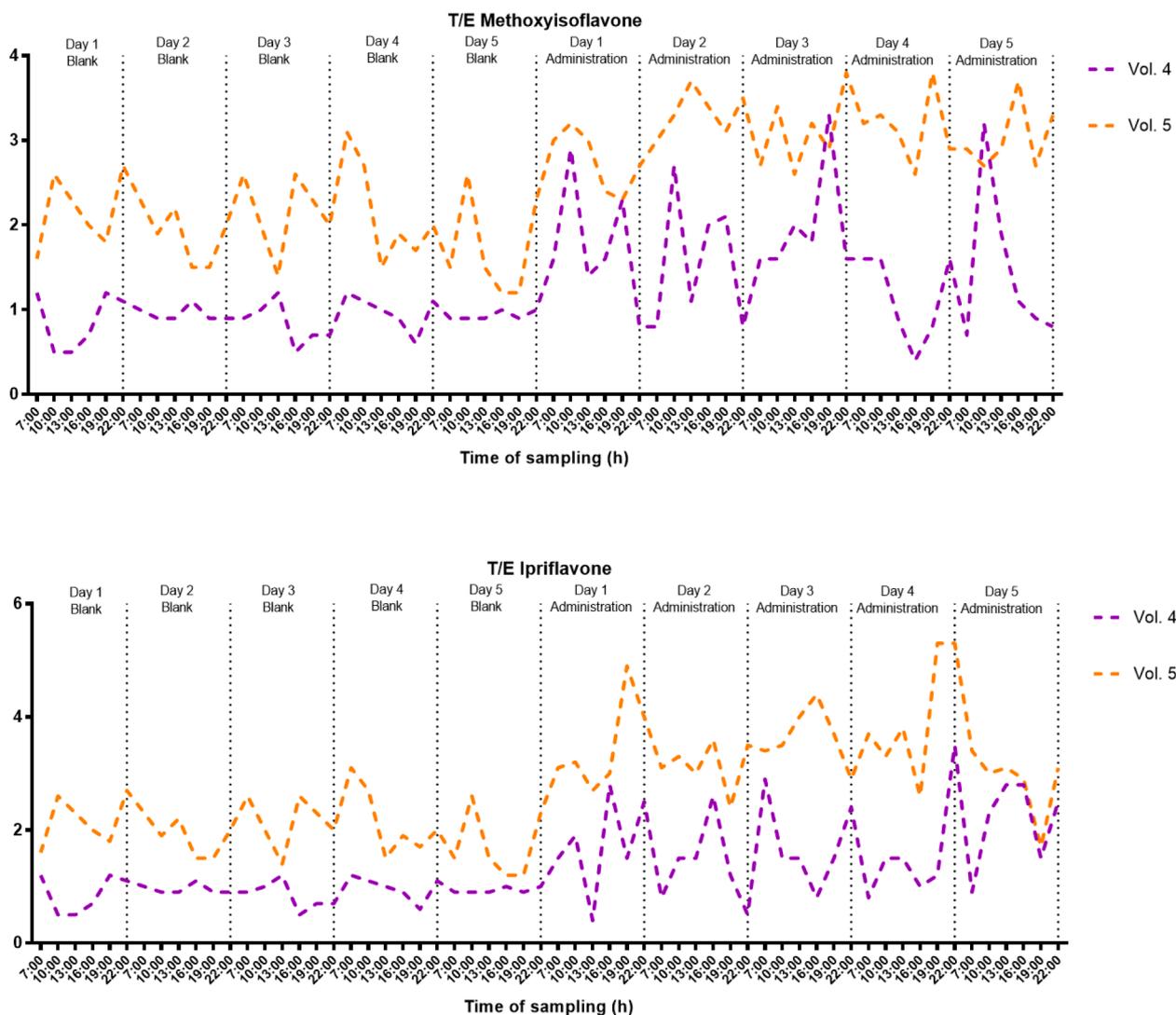


Fig. 6. Longitudinal evaluation of the T/E ratio before and after the administration of 450 mg/day of methoxyisoflavone (a) and of 400 mg/day of ipriflavone, for five consecutive days.

evaluated using a one-way ANOVA test. The p values lower than 0.05 ($p < 0.05$) were considered statistically significant. The oral intake, for five consecutive days, of methoxyisoflavone produces a significant variation, both in concentrations and in data dispersion, of T, A, Etio and total LH. These effects are correlated to statistically significant variations in the ratios T/E, A/T and T/total LH. The order of magnitude of the variations is between 50 and 70% with respect to the pre-administration baseline. The intake of ipriflavone provokes significant variations for all the steroids considered in the Steroidal Module of the Athlete Biological Passport, in the range 65–80% with respect to the pre-administration baseline, except for E, for total LH and for the ratios T/E, A/T, 5α Adiol/ 5β Adiol and T/total LH. This kind of alterations could be traced back to the use of banned drugs and/or substances classified as exogenous confounding factors. In fact, it is well known that, for example, an alteration of the A/T ratio can be correlated to the misuse of pseudo-endogenous steroids, an alteration of the 5α Adiol/ 5β Adiol can be caused by the abuse of a 5α -reductase inhibitor, and that an alteration of the T/total LH ratio can be registered after the intake of an anabolic agent [53].

As already recalled, the Steroidal Module of the Athlete Biological Passport was introduced in 2014 to define longitudinal, subject-based normality ranges for the biomarkers of the urinary steroid profile. In this regard, any exogenous factors capable of altering the concentration

of the described biomarkers must be monitored, to avoid misinterpretation of the experimental data. As we can see in Fig. 6a-b, showing the values of T/E ratio in volunteers 4 and 5 respectively, before and after the intake of methoxyisoflavone and ipriflavone, the non-monitored use of synthetic isoflavones produce a “longitudinal” alteration of the T/E ratio, the most representative parameter considered in the evaluation of the urinary steroid profile, that could lead to an erroneous evaluation of the longitudinal profiles. In this context, the effects here described confirm what already reported following *in vitro* studies [27,29,44–46], where it was proven that also non-banned and non-monitored substances, not yet included among those considered as “confounding factors” in the WADA Technical Document TD2021EAAS [12], could affect the phase I and phase II metabolism of endogenous steroids and the urinary excretion of LH.

Finally, we evaluated the values of the excretion of the total LH and of the T/E ratio for the same volunteer to preliminarily estimate the effects of the two isoflavones on the HPG axis and on the biosynthesis and metabolism of the endogenous steroids considered in the study. As shown in Fig. 7a and 7b, (referring to volunteer 5, but the trend is similar in all subjects), the effect of ipriflavone is more pronounced than the effect of methoxyisoflavone, both on the excretion of total LH and on the T/E ratio. This evidence could be related to the different magnitude of effects of the two synthetic isoflavones on the modulation of LH

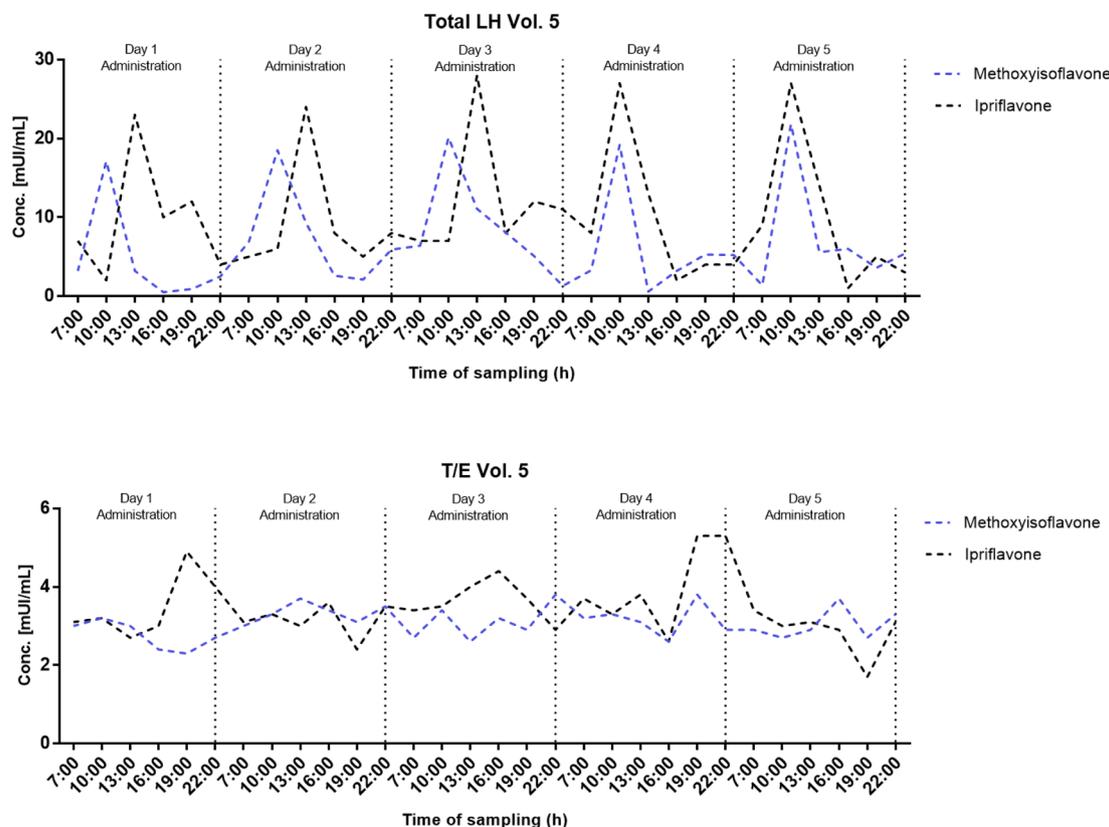


Fig. 7. Longitudinal evaluation of total LH excretion (a) and of the T/E ratio (b) after the administration of methoxyisoflavone (blue) and ipriflavone (black).

biosynthesis and on the enzymatic complex involved in the metabolism of endogenous steroids. In fact, as already reported in the literature, ipriflavone modulates more significantly than methoxyisoflavone the biosynthesis of LH [54], the activity of CYP450 complex [55], and the activity of aromatase [46].

4. Conclusions

We have highlighted the effects of the oral intake of two synthetic isoflavones, methoxyisoflavone and ipriflavone, on the urinary excretion of total LH and on the biomarkers of the Steroidal Module of the Athlete Biological Passport. We have also considered complementary parameters (e.g., the ratio T/total LH) to assess the effects on the hypothalamus-pituitary-gonadal (HPG) axis.

Even though the oral bioavailability of flavonoids is generally very low, we underlined that the oral intake, for five consecutive days, of methoxyisoflavone and ipriflavone induces an alteration of the concentration levels of total LH, of the markers of the urinary steroid profile, and of the corresponding concentration ratios, that could affect the correct interpretation of the longitudinal data collected in the framework of the Steroidal Module of the ABP. Methoxyisoflavone and ipriflavone, marketed as natural supplements and anti-oxidants, could also be misused by the athletes during the recovering period and/or after the administration of anabolic steroids, to restore the suppressed endogenous biosynthesis of testosterone and its precursors.

Although the preliminary data here presented support the view that synthetic isoflavones may cause a confounding effect on the steroidal module of the athlete biological passport, data on a broader population of subjects are necessary to shed further light on the effects of synthetic isoflavones on the HPG axis and on steroidogenesis. We plan to complement the preliminary results obtained in this work with those obtained by extending the administration of synthetic isoflavones to females, and to male and female non-Caucasian volunteers, also in

association with exogenous steroids (e.g., stanozolol or methandienone). However, even if their anabolic and/or androgenic effects are not clear yet, we nonetheless precautionarily suggest the monitoring of the presence of synthetic isoflavones in urine samples collected for doping control analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

- [1] A.T. Kicman, Pharmacology of anabolic steroids, *Br. J. Pharmacol.* 154 (2008) 502–521.
- [2] A.J. Tilbrook, L.J. Clarke, Negative feedback regulation of the secretion and actions of gonadotropin-releasing hormone in males, *Biol. Reprod.* 64 (2001) 735–742.
- [3] D. Handelsman, Testosterone: use, misuse and abuse, *Med. J. Aust.* 185 (2006) 436–439.
- [4] A. Butenandt, G. Hanisch, Über testosterone. Umwandlung des dehydroandrosterons in androstendiol und testosterone; ein weg zur des testosterone aus cholesterin, *Hoppe-Seyler's Zeitschrift für Physiol. Chemie.* 237 (1935) 1–3.
- [5] L. Ruzicka, A. Wettstein, H. Kägi, Sexualhormone VIII. Darstellung von testosterone unter anwendung gemischter ester, *Helv. Chim. Acta* 18 (1) (1935) 1478–1482.
- [6] The World Anti-Doping Agency. WADA 2019 Anti-Doping Testing Figures Reports, https://www.wadaama.org/sites/default/files/resources/files/2019_antidoping_testing_figures_en.pdf (last update: 16/03/2021).
- [7] U.H. Stenman, K. Hotakainen, H. Alfthan, Gonadotropins in doping: pharmacological basis and detection of illicit use, *Br. J. Pharmacol.* 154 (2008) 569–583.

- [8] D. Handelsman, C. Goebel, A. Idan, D. Jimenez, G. Trout, R. Kazlauskas, Effects of recombinant human LH and hCG on serum and urine LH and androgens in men, *Clin. Endocrinol.* 71 (2009) 417–428.
- [9] The World Anti-Doping Agency. WADA International Standards – WADA Prohibited List 2021 https://www.wada-ama.org/sites/default/files/resources/files/2021list_en.pdf (last update: 16/03/2021).
- [10] F. Celotti, P. Negri-Cesi, Anabolic steroids: a review of their effects on muscles, of their possible mechanism of actions and of their use in athletics, *J. Steroid Biochem. Mol. Biol.* 43 (1992) 469–477.
- [11] F. Sjöqvist, M. Garle, A. Rane, Use of doping agents, particularly anabolic steroids, in sports and society, *Lancet* 371 (9627) (2008) 1872–1882.
- [12] The World Anti-Doping Agency. WADA Technical Document TD2021EAAS “Endogenous Anabolic Androgenic Steroids. Measurement and Reporting” https://www.wada-ama.org/sites/default/files/resources/files/td2021eas_final_eng.pdf (last update: 16/03/2021).
- [13] The World Anti-Doping Agency. Athlete Biological Passport (ABP). Operating Guidelines, version 7.1, June 2019 <https://www.wada-ama.org/en/resources/athlete-biological-passport/athlete-biological-passport-abp-operating-guidelines> (last update: 16/03/2021).
- [14] The World Anti-Doping Agency. WADA Technical Document TD2021IRMS “Detection of Synthetic Forms of Endogenous Anabolic Androgenic Steroids by GC/C/IRMS” https://www.wada-ama.org/sites/default/files/resources/files/td2021irms_final_eng.pdf (last update: 16/03/2021).
- [15] P.E. Sottas, N. Robinson, O. Rabin, M. Saugy, The athlete biological passport, *Clin. Chem.* 57 (2011) 969–976.
- [16] P. Van Renterghem, P.-E. Sottas, M. Saugy, P. Van Eenoo, Statistical discrimination of steroid profiles in doping control with support vector machines, *Anal. Chim. Acta* 768 (2013) 41–48.
- [17] M. Saugy, C. Lundby, N. Robinson, Monitoring of biological markers indicative of doping: the Athlete Biological Passport, *Br. J. Sport Med.* 48 (10) (2014) 827–832.
- [18] F. Ponsetto, N. Baume, C. Schweizer, M. Saugy, T. Kuuranne, Steroidal Module of the Athlete Biological Passport, *Curr. Opin. Endocrin. Metab. Res.* 9 (2019) 14–21.
- [19] X. de la Torre, C. Colamonic, D. Curcio, F. Molaioni, F. Botrè, A comprehensive procedure based on gas chromatography-isotope ratio mass spectrometry following HPLC purification for the analysis of underivatized testosterone and its analogues in human urine, *Anal. Chim. Acta* 756 (2012) 23–29.
- [20] The World Anti-Doping Agency. WADA Technical Document TD2021CG/LH “Reporting and Management of Human Chorionic Gonadotrophin (hCG) and Luteinizing Hormone (LH) Findings in Male Athletes” https://www.wada-ama.org/sites/default/files/resources/files/td2021cg-lh_final_eng.pdf (last update: 16/03/2021).
- [21] P. Van Eenoo, P. Van Renterghem, C.H. Dimopoulou, F.T. Delbeke, Estimating measurement uncertainty in quantitative methods non based on chromatography for doping control purposes, *Drug Test. Anal.* 2 (2010) 19–23.
- [22] U. Mareck, H. Geyer, G. Opfermann, M. Thevis, W. Shanzer, Factors influencing the steroid profile in doping control analysis, *J. Mass Spectrom.* 43 (2008) 877–891.
- [23] T. Kuuranne, M. Saugy, N. Baume, Confounding factors and genetic polymorphism in the evaluation of the urinary steroid profile, *Br. J. Sport Med.* 48 (2014) 848–855.
- [24] F. Botrè, X. de la Torre, F. Donati, M. Mazzarino, Narrowing the gap between the number of athletes who dope and the number of athletes who are caught: scientific advances that increase the efficacy of antidoping tests, *Br. J. Sport Med.* 48 (2014) 833–836.
- [25] T. Sten, M. Finel, B. Ask, A. Rane, L. Ekström, Non-steroidal anti-inflammatory drugs interact with testosterone glucuronidation, *Steroids* 74 (12) (2009) 971–977.
- [26] M. Mazzarino, M.C. Braganò, F. Donati, X. de la Torre, F. Botrè, Effects of propyphenazone and other non-steroidal anti-inflammatory agents on the synthetic and endogenous androgenic anabolic steroids urinary excretion and/or instrumental detection, *Anal. Chim. Acta* 657 (1) (2010) 60–68.
- [27] M. Chen, J.E. Drury, T.M. Penning, Substrate specificity and inhibitor analysis of human steroid 5 β -reductase (AKR1D1), *Steroids* 76 (2011) 484–490.
- [28] J.J. Schulze, J.E. Mullen, E.B. Lindgren, M. Ericsson, L. Ekstrom, A.L. Hirschberg, The impact of genetics and hormonal contraceptives on the steroid profile in female athletes, *Front. Endocrinol.* 10 (2014) 8–15.
- [29] A. Palermo, B. Alessi, F. Botrè, X. de la Torre, I. Fiacco, M. Mazzarino, In vitro evaluation of the effects of anti-fungals, benzodiazepines and non-steroidal anti-inflammatory drugs on the glucuronidation of 19-norandrosterone: implications on doping control analysis, *Drug Test. Anal.* 8 (9) (2016) 930–939.
- [30] A. Palermo, F. Botrè, X. de la Torre, I. Fiacco, M. Iannone, M. Mazzarino, Drug-drug interactions and masking effects in sport doping: influence of miconazole administration on the urinary concentrations of endogenous anabolic steroids, *Forensic Toxicol.* 34 (2016) 386–397.
- [31] S. Coll, X. Matabosch, L. Garrosta, N. Monfort, C. Perez-Maña, N. Pizarro, J. A. Mateus, M. Ezzel, R. de la Torre, R. Ventura, The effect of tea consumption on the steroid profile, *Drug Test. Anal.* 10 (9) (2018) 1438–1447.
- [32] E. Isenmann, G. Ambrosio, J.F. Joseph, M. Mazzarino, X. de la Torre, P. Zimmer, R. Kazlauskas, C. Goebel, F. Botrè, P. Diel, M.K. Parr, Ecdysteroids as non-conventional anabolic agent: performance enhancement by ecdysterone supplementation in humans, *Archives Toxicol.* 93 (2019) 1807–1816.
- [33] A. Stoll, M. Iannone, G. De Gregorio, X. de la Torre, F. Molaioni, F. Botrè, M. K. Parr, Influence of pain killers on the urinary anabolic steroid profile, *J. Anal. Toxicol.* 44 (2020) 871–879.
- [34] A. Stoll, M. Iannone, G. De Gregorio, X. de la Torre, F. Molaioni, F. Botrè, M. K. Parr, Influence of indomethacin on steroid metabolism: endocrine disruption and confounding effects in urinary steroid profile of anti-doping analyses, *Metabolites* 10 (2020) 463–482.
- [35] M. Iannone, A. Palermo, X. de la Torre, M. Mazzarino, F. Molaioni, F. Botrè, Influence of Saw palmetto and Pygeum africanum extracts on the urinary concentrations of endogenous anabolic steroids: relevance to doping analysis, *Phytomedicine Plus*, 10.1016/j.phyplu.2020.100005.
- [36] Rapporto di attività controllo antidoping. Ministero della salute. Direzione generale della prevenzione sanitaria, anno 2019 (gennaio-dicembre) http://www.salute.gov.it/imgs/C_17_pubblicazioni_2962_0_alleg.pdf (last update 16/03/2021).
- [37] G.R. Beecher, Overview of dietary flavonoids: nomenclature, occurrence and intake, *J. Nutr.* 133 (2003) 3248S–3254S.
- [38] E. Middleton, C. Kandaswami, T. Theoharides, The effects of plant flavonoids on mammalian cells: implication for inflammation, heart disease and cancer, *Pharmacol. Rev.* 52 (2000) 673–751.
- [39] R.S.R. Zand, D.J.A. Jenkins, E.P. Diamandis, Steroid hormone activity of flavonoids and related compounds, *Breast Cancer Res. Treat.* 62 (1) (2000) 35–49.
- [40] M. Suetsugi, L. Su, K. Karlsberg, Y.C. Yuan, S. Chen, Flavone and isoflavone phytoestrogen are agonist of estrogen-related receptors, *Mol. Cancer Res.* 1 (2013) 981–991.
- [41] T. Incledon, D. Van Gammeren, J. Antonio, The effects of 5-methyl-7-methoxyisoflavone on body composition and performance in college-aged men, *Med. Sci. Sports Exerc.* 33 (5) (2001) S338, <https://doi.org/10.1097/00005768-200105001-01898>.
- [42] C.D. Wilborn, L.W. Taylor, B.I. Campbell, C.K. Kerksick, C.J. Rasmussen, M. Greenwood, R.B. Kreider, Effects of methoxyisoflavone, ecdysterone and sulfoliposaccharide supplementation on training adaptations in resistance-trained men, *J. Int. Soc. Sport Nutr.* 3 (2006) 19–27.
- [43] K.D. Setchell, A. Cassidy, Dietary isoflavones: biological effects and relevance to human health, *J. Nutr.* 129 (1999) 758–767.
- [44] K. KRISTAN, K. KRAJNC, J. KONC, S. GOBEC, J. STOJAN, T. LANISNIKRIŽNER, Phytoestrogens as inhibitors of fungal 17 β -hydroxysteroid dehydrogenase, *Steroids* 70 (9) (2005) 626–635.
- [45] A. Cassetta, J. Stojan, I. Krastanova, K. Kristan, M. Brunskole Šveglj, D. Lamba, T. Lanisnik Rižner, Structural basis for inhibition of 17 β -hydroxysteroid dehydrogenases by phytoestrogens: the case of fungal 17 β -HSDc1, *J. Steroid Biochem. Mol. Biol.* 171 (2017) 80–93.
- [46] M. Iannone, F. Botrè, N. Cardillo, X. de la Torre, Synthetic isoflavones and doping: A novel class of aromatase inhibitors? *Drug Test. Anal.* 11 (2) (2019) 208–214.
- [47] M. Iannone, F. Botrè, S. Parenti, D. Jardines, X. de la Torre, An investigation of the metabolic pathways of synthetic isoflavones by gas chromatography coupled to high accuracy mass spectrometry, *Rapid Commun. Mass. Spectrom.* 33 (2019) 1485–1493.
- [48] J.A. Ross, C.N. Kasum, Dietary flavonoids: bioavailability, metabolic effects and safety, *Annu. Rev. Nutr.* 22 (2002) 19–34.
- [49] M. Iannone, A. Palermo, X. de la Torre, F. Romanelli, A. Sansone, M. Sansone, A. Lenzi, F. Botrè, Effects of transdermal administration of testosterone gel on the urinary steroid profile in hypogonadal men: Implications in antidoping analysis, *Steroids* 152 (2019) 108491, <https://doi.org/10.1016/j.steroids.2019.108491>.
- [50] A.T. Kicman, R.V. Brooks, S.C. Collyer, D.A. Cowan, M.N. Nanjee, G.J. Southan, M. J. Wheeler, Criteria to indicate testosterone administration, *Br. J. Sport Med.* 24 (4) (1990) 253–264.
- [51] D.A. Cowan, A.T. Kicman, C.J. Walker, M.J. Wheeler, Effects of administration of human chorionic gonadotrophin on criteria used to assess testosterone administration in athletes, *J. Endocrinol.* 131 (1991) 147–154.
- [52] D.H. Catlin, D.A. Cowan, Detecting testosterone administration, *Clin. Chem* 38 (1992) 1685–1686.
- [53] T. Piper, H. Geyer, N. Haenelt, F. Huelsemann, W. Schaezner, M. Thevis, Current insights into the Steroidal Module of the Athlete Biological Passport, *Int J Sports Med* (2021), <https://doi.org/10.1055/a-1481-8683>.
- [54] L. Krizova, K. Dadakova, J. Kasparovska, T. Kasparovsky, Isoflavones, *Molecules* 24 (2019) 1–28.
- [55] K. Monostory, L. Vereckzy, F. Levai, I. Szatmari, Ipriflavone as an inhibitor of human cytochrome P450 enzymes, *Br J Pharmacol* 123 (1998) 605–610.