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ORIGINAL RESEARCH

Androgen Bioassay for the Detection of Nonlabeled Androgenic Compounds in Nutritional Supplements

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Both athletes and the general population use nutritional supplements. Athletes often turn to supplements hoping that consuming the supplement will help them be more competitive and healthy, while the general population hopes to improve body image or vitality. While many supplements contain ingredients that may have useful properties, there are supplements that are contaminated with compounds that are banned for use in sport or have been deliberately adulterated to fortify a supplement with an ingredient that will produce the advertised effect. In the present study, we have used yeast cell and mammalian cell androgen bioassays to characterize the androgenic bioactivity of 112 sports supplements available from the Australian market, either over the counter or via the Internet. All 112 products did not declare an androgen on the label as an included ingredient. Our findings show that six out of 112 supplements had strong androgenic bioactivity in the yeast cell bioassay, indicating products spiked or contaminated with androgens. The mammalian cell bioassay confirmed the strong androgenic bioactivity of five out of six positive supplements. Supplement 6 was metabolized to weaker androgenic bioactivity in the mammalian cells. Further to this, Supplement 6 was positive in a yeast cell progestin bioassay. Together, these findings highlight that nutritional supplements, taken without medical supervision, could expose or predispose users to the adverse consequences of androgen abuse. The findings reinforce the need to increase awareness of the dangers of nutritional supplements and highlight the challenges that clinicians face in the fast-growing market of nutritional supplements.

Keywords: sports doping, undeclared androgens, steroids, performance enhancement

Athletes of all ages, from amateurs to professional, use sport supplements. Reasons for supplement use include enhanced performance or recovery, muscle mass gain, or improved appearance (Kiertscher & DiMarco, 2013). The use of supplements by athletes in some sports (e.g., bodybuilding) is reportedly 100%, with an average of 58% for other sports (Knapik et al., 2016). The prevalence varies with the type of sport, gender, and level of competition (Maughan et al., 2011). The prevalence of sport supplement use in adolescents (aged 14–18) was recently reported at 47.7% (Evans et al., 2012). Besides their use in competitive sports, these products are also commonly used by recreational gym users and amateur athletes (Goston & Correia, 2010).

Sport supplements are widely available through websites, supplement shops, and gyms. The global

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sale of supplements was $US96 billion in 2012, with the market estimated to increase ("Nutrition Business Journal Global Supplement and Nutrition Industry Report 2014", 2014). The supplements are marketed on claims of enhanced strength, endurance, speed, weight loss, appearance, or concentration (Dascombe et al., 2010; Lun et al., 2012); however, for the majority of supplements, solid scientific data to support the claims is not available. Moreover, rigorous safety and efficacy testing has not been reported.

As in other countries, sport supplements are only lightly regulated in Australia. They are controlled by the Therapeutics Goods Administration, and to be listed, the supplement need only show proof that it does not contain ingredients that are banned by custom laws. Importantly, there is no requirement for proof of benefit claims. Given the prolific marketing of sport supplements through widespread media outlets (online, magazines, brochures, etc.), there is very little regulation of the marketing of these products.

Companies can take full advantage of the poor quality control. Many studies have now reported non-labeled substances like stimulants and anabolic androgenic steroids, in over-the-counter or internet-purchased supplements (Ayotte et al., 2001; Cadwallader & Murray, 2015; Catlin et al., 2000; Kamber et al., 2000; Pipe & Ayotte, 2002). In some cases, manufacturing practices may have led to the contamination of supplements with trace amounts of an undeclared substance (Judkins et al., 2010). There are, however, reported instances of sport supplements intentionally adulterated with undeclared substances (Geyer et al., 2000, 2004, 2008; Judkins & Prock, 2012; Kamber et al., 2000; Maughan et al., 2011).

Androgens have well-described ergogenic effects. All androgens, including designer androgens, are grouped together with anabolic androgenic steroids, which remain the class of banned substance most likely to be abused in sport (50% of all positive findings in 2015; WADA, 2015). Sport supplements that contain undeclared androgens therefore pose a risk to professional and/or elite athletes because of a potential positive doping test. More importantly, use of such supplements by the unsuspecting consumer can have serious adverse health effects.

It has been recognized since 2004 that undeclared androgens are prevalent in over-the-counter or online available sport supplements at rates as high as 10–25% (Geyer et al., 2004). This has been confirmed many times since as reviewed in Geyer et al. (2008). It is difficult to assess current prevalence (Gilard et al., 2015), as random screenings are seldom performed. Recently in the Australian market, an Australian Sports Anti-Doping Agency (ASADA) study determined that undeclared banned substance prevalence in sport supplements was as high as 19% (LGC, 2016). This included three supplements positive for anabolic androgenic steroids. This study used a chemical analyte approach to detect known banned substances. This study is in keeping with the reported 6.4% of doping violations between 2006 and 2013 in Australia associated with supplement use (Outram & Stewart, 2015).

Androgen bioassays are a useful screening tool for supplements because they are based on androgen biology, rather than analyte structure. Therefore, bioassays can detect androgen presence in supplements, even if unknown designer steroids have been included as an undeclared ingredient. Such practice by manufacturers to incorporate pharmaceutical analogs, or designer steroids, into their products have been reported (FDA, 2015).

In this study, we used tandem yeast cell and mammalian cell androgen bioassays to screen 112 sports supplements from the Australian market. The 112 supplements were randomly chosen to cover the range of sports supplements, including powders, capsules, and tablets. The only inclusion factor was that the labeled ingredients did not include an androgen.

Methods

Details of Sport Supplements Tested in This Study

Sport supplements (n = 112) were purchased from seven different stores. Four of these stores were based in Sydney, NSW; two were based in Brisbane, Queensland; and one online US store that sold sport supplements in Australia. All supplements tested did not declare an androgen on the label. The sport supplements were in the form of powders (74.2%), capsules (19.6%), tablets (5.3%), or liquids (0.9%; see Table 1). The sport supplements represent 77 different companies. Eleven companies were based in Australia, one in Canada, and one in New Zealand, with the remaining companies based in the United States (USA). A supplement which declared the presence of dehydroepiandrosterone (DHEA) was purchased in Sydney and was used as an androgen-positive control supplement in this study.

Steroid Extraction Procedure

The amount of each supplement used for the extraction varied, dependent on the manufacturer's recommended serving size. Steroid extracts were prepared using a standard solid-phase extraction (SPE) method, with minor alterations (Rijk et al., 2009). Briefly, capsules were emptied and solid tablets were pulverized using a mortar and pestle. The powders were suspended in water/methanol 1:1 v/v and dissolved by sonication for 5 min. The suspension was then centrifuged at 3000 g for 10 min and the pellet was discarded. The pH of the supernatant was adjusted to 4.8 using 4N acetic acid and sodium acetate. The supernatant then underwent SPE using a C18 column (0.5 g Bond Elut, Agilent Technologies) that was previously conditioned with 4 mL methanol/sodium acetate (pH 4.8). After sample loading, the columns were washed sequentially with

Table 1 Types of Sports Supplements Screened in this Study

Product Category	No. of products tested	No. of different companies
Protein Powders	25 (22.3%)	19
Stimulants/Nitric Oxide (preworkouts)	24 (21.4%)	21
Amino Acids	12 (10.7%)	12
Testosterone and Growth Hormone Boosters	18 (16.1%)	11
Fat Metabolizers	9 (8%)	9
Carbohydrates	2 (1.8%)	2
Creatine Formulations	6 (5.4%)	6
Vitamins and Herbal Extracts	16 (14.3%)	16

Note. No. of products tested denotes the number of products screened belonging to the respective product category. *No. of different companies* shows how many companies were represented by that respective product category.

2 mL water, 1.5 mL sodium carbonate (10% w/v), 2 mL water, and 2 mL water/methanol (1:1, v/v). The columns were air-dried and the sample was eluted with 4 mL acetonitrile. The eluate was concentrated by evaporation then resuspended in 50 µL 100% ethanol.

Cell Culture

The yeast and human hepatocyte cell line (HuH7) cell-based androgen bioassays have previously been described (Akram et al., 2011). Briefly, yeast cells were cultured overnight at 30°C with shaking (300 rpm) in CSM-leu-ura (MP Biomedicals). After overnight culture, the yeast culture was subcultured in fresh medium and allowed to grow until early mid-log phase ($OD_{600} = 0.5–0.7$).

HuH7 cells were cultured in DMEM-L-glutamine containing high glucose (4500 mg/L) supplemented with 10% FCS and 5.5 µg/mL puromycin dihydrochloride at 37°C under 5% CO_2.

Yeast Cell-Based Androgen Bioassay

Early to mid-log phase cells were sub-cultured in 24-well plates (500 µL/well) and androgen receptor (AR) expression was induced with 100 µM $CuSO_4$. Cells were treated with steroids (5 µL/well) over a concentration range (from 1×10^{-6} M to 1×10^{-10} M) to generate a sigmoidal dose-response curve to calculate the EC_{50}. For agonist assays, yeast cells were treated with sports supplement extracts (5 µL/well) in duplicate. All steroids and extracts were diluted in ethanol with a final concentration of 1%. Yeast cells were incubated overnight at 30°C with shaking (300 rpm) before being lysed and assayed for β-galactosidase activity as previously described (McRobb et al., 2008).

HuH7 Cell-Based Androgen Bioassay

HuH7 cells were seeded in 96-well plates at a concentration of 1×10^5 cells/mL (200 µL/well) and allowed to recover overnight in phenol red-free DMEM supplemented with 10% charcoal-stripped FCS, L-glutamine and 5.5 µg/mL puromycin dihydrochloride. Cells were treated with steroids for 24 hours (2 µL/well) over a concentration range (from 1×10^{-6} M to 1×10^{-11} M) to generate a sigmoidal dose-response curve to calculate the EC_{50}. For sports supplement extracts, HuH7 cells were treated in duplicate with 2 µL per well. Culture supernatant was transferred to white opaque 96-well plates and heat treated for 35 min at 65 °C to deactivate endogenous alkaline phosphatase before SEAP activity was measured using a commercially available assay (Clontech Laboratories, Mountain View, CA, USA).

Results

Validation of the Yeast Androgen Bioassay

Initially, we determined the sensitivity of our yeast and HuH7 androgen bioassays. Using DHT as our reference steroid, the most potent endogenous androgen, we showed that the yeast cell-based androgen bioassay could detect this steroid at concentration $\geq 3.9 \times 10^{-10}$ M. For the HuH7 androgen bioassay, the sensitivity was 8×10^{-11} M. Converting these concentrations into absolute amounts, this corresponds to 0.5 pg and 0.16 pg, respectively. These values are in keeping with our earlier validation publications (Akram et al., 2011; Cooper et al., 2017; Death et al., 2004, 2005)

With sensitivity limits established for the assays, we next determined our detection limit for androgens recovered from a sport supplement matrix. To this end, we spiked 1 g portion of a powdered from of a creatine supplement with T ranging in concentration from 500 ng T to 1 ng T. The spiked supplements then underwent SPE extraction and the extracted steroid subjected to the yeast cell-based androgen bioassay. Figure 1 shows that we could reliably detect a starting T amount of ≥20 ng (13.9×10^{-9} M). This sensitivity is in keeping with what has been previously described for yeast-based bioassays for the detection of androgens in matrices (Zierau et al., 2008).

To ensure that the bioassay did not produce false negative or positive results, we spiked a number of supplements with 20 ng T (Figure 2). This experiment relied on the host supplement being completely androgen

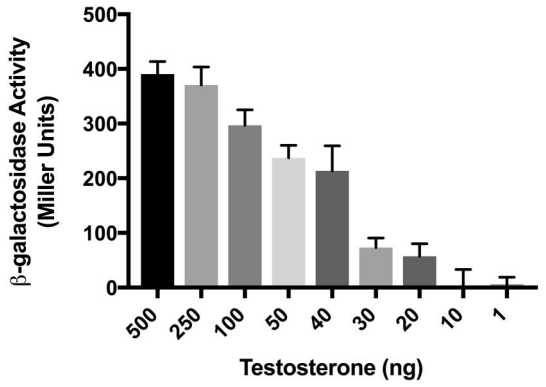

Figure 1 — Detection limit of the yeast androgen bioassay. A creatine powder supplement was spiked with 200 µl of testosterone (T) ethanolic solution. Final T concentrations ranged from 1–500 ng/1 g supplement. Spiked supplement was subjected to SPE extraction. The steroidal extract was then analyzed in the yeast androgen bioassay for β-galactosidase activity.

free so we only spiked supplements that had been certified by the LGC group (www.lgcgroup.com/australiansupplementsurvey) as prohibited substance free. Figure 2a and 2b show that extracts from the different supplements showed negligible AR activation, regardless of whether they were tested with the yeast (Figure 2a) or HuH7 (Figure 2b) assays. However, when spiked with 20 ng T and extracted, there was measurable AR activation in both the yeast and HuH7 assays. We also show that the yeast AR bioactivity was able to detect designer steroids, madol and hemapolin, or selective androgen receptor modulator (SARM), 93746, when 20 ng was spiked into the host supplement. Finally, we showed that the bioassay was able to detect testosterone if the supplement was an oil-based matrix rather than a powder. Together, these data help to assure that the bioassays can detect different structures across varied matrices.

Androgen Bioactivity of 112 Sports Supplements

To determine the presence of undeclared androgens in 112 sport supplements, we analyzed the bioactivity of steroidal extracts prepared from each supplement using the yeast cell-based androgen bioassay. We first assessed 24 from the "preworkout" category and found that 3 (Supplements 2, 5, and 6) had measurable androgen bioactivity (Figure 3). Next, we assessed 18 from the "testosterone and growth hormone-booster category" and found that 2 (Supplement 1 and 4) were positive for androgen bioactivity (Figure 3). Finally, we tested the remaining 70 supplements belonging to creatine (1/6), protein powder (0/25), fat metabolizer (0/9), carbohydrate (0/2) and amino acid (0/12) supplement, and herbal extract (0/16) categories and found that one (Supplement 3) in the creatine group tested positive for androgenic bioactivity (Figure 3).

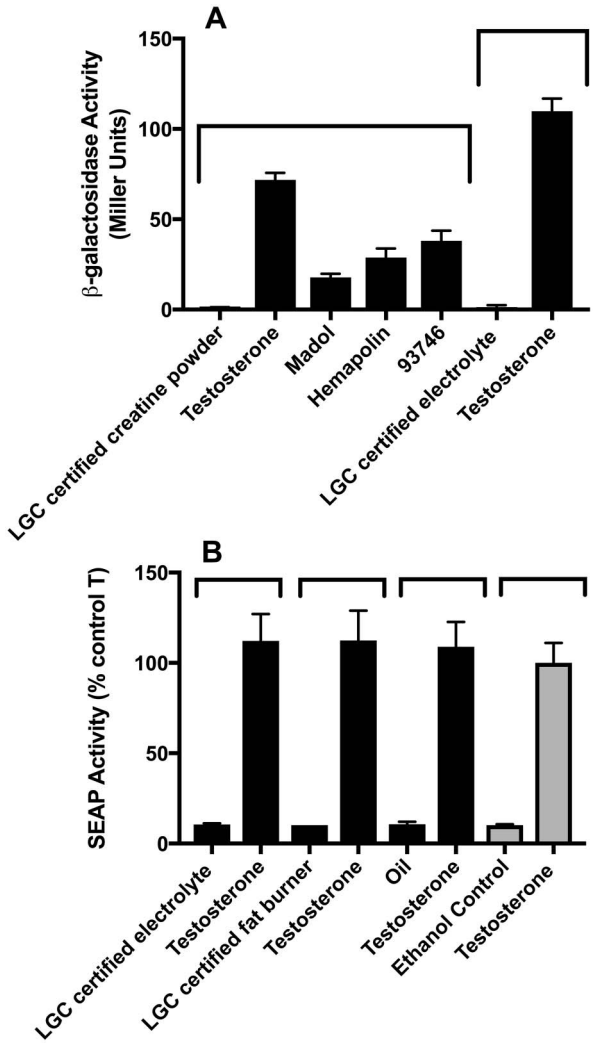

Figure 2 — Sensitivity of the androgen bioassays. (A) An LGC certified creatine or electrolyte powder was tested for androgen activity with (false negative) and without (false positive) spiked androgenic molecules. The powders were spiked with 20 ng of testosterone, madol, hemapolin, or SARM, 93746, extracted, then tested for β-galactosidase activity using the yeast bioassay. (B) An LGC certified electrolyte or fat burner powder or corn oil was tested for androgen activity with (false negative) and without (false positive) spiked testosterone, extracted, then tested for SEAP activity using the HuH7 bioassay. Results are presented as mean ± SEM (n = 3). Purified testosterone and ethanol (vehicle) were included as positive and negative controls, respectively.

The six supplements represent four different companies. Supplements (1–4, 6) were manufactured by USA-based companies, while one supplement (supplement 5) was manufactured in New Zealand. There were zero products manufactured in Australia that tested positive. The ingredients listed on the label of the six positive supplements are detailed in Table 2.

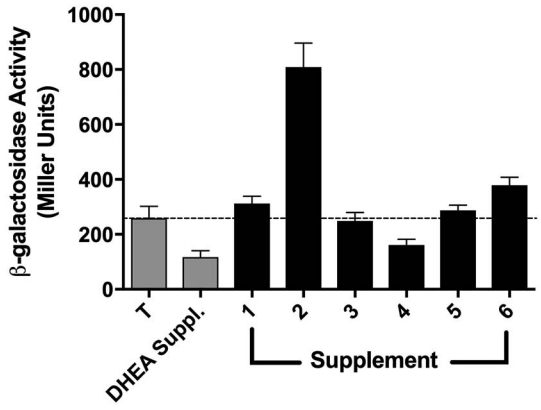

Figure 3 — Six supplements tested positive in the yeast androgen bioassay. Steroid extracts from sport supplements were tested for androgen receptor activation in the yeast androgen bioassay. Results are presented as mean ± SEM (n = 3). Testosterone and a DHEA-containing supplement were included as positive controls.

Three of the 112 supplements could not be assessed using the yeast androgen bioassay because of cytotoxic effects that stopped us being able to culture the yeast (data not shown). These supplements were from the fat metabolizer, testosterone booster, and preworkout supplement categories, respectively. Toxicity of supplements has also been reported previously (Peters et al., 2010).

Progestin Bioactivity

Although separate progesterone, androgen, and estrogen receptors exist, complete selectivity of action is not guaranteed because of the close structural similarities among the hormones. The progesterone receptor (PR) and the AR show significant homology and most progestins have significant cross-reactivity with the AR. For this reason, we next tested whether the six supplements found to be positive for androgenic activity also showed progestogenic activity. This could indicate that a progestin (e.g., a milk product) rather than an androgen was responsible for the positive result in the AR bioassay.

Using the yeast progestin bioassay, an EC_{50} of 3.8 nM for progesterone was measured in keeping with published findings (McRobb et al., 2008). Supplement 6 showed a progestin response suggesting that this supplement contains a steroid molecule that is both progestogenic and androgenic. Supplement 1 showed a weak progestogenic response. All other supplements did not activate the PR (Figure 4).

Prohormone Bioactivity

Sport supplements may contain prohormones that are not active as androgens until they undergo some form of metabolic processing. Such prohormones are usually weakly androgenic, if at all. Therefore, it would be difficult to detect prohormones with the yeast cell androgen bioassay as these cells do not express the steroid metabolizing enzymes required for metabolic activation.

Mammalian cell lines do express steroid metabolizing enzymes so we next tested the 112 supplement extracts using a HuH7 cell androgen bioassay (Akram et al., 2011; Cooper et al., 2013). We determined that five of the 112 steroid extracts showed AR activation (Figure 5). Notably, all five that tested positive in the HuH7 bioassay also tested positive in the yeast cell bioassay. Supplement 6 that was positive in the yeast cell bioassay did not show an androgen response in the HuH7 bioassay.

Discussion

We report here that six of 112 supplements tested positive for androgen bioactivity, despite not declaring an androgen as a listed ingredient. Our findings are in agreement with previous studies that have also reported discrepancies between ingredients listed on the product label and the actual ingredients in the products (Geyer et al., 2000, 2004, 2008; Judkins & Prock, 2012; Judkins et al., 2010; Kamber et al., 2000; Maughan et al., 2011). Six of 112 represents 5.4% of products tested, a percentage that is lower than the 10–25% reported in other studies (Geyer et al., 2004) but on par with what was recently reported for 67 products screened by the LGC group on behalf of the Australian Sports Anti-Doping Authority (ASADA), with three of 67 products tested found to contain anabolic androgenic steroids (LGC, 2016). For all six supplements, the level of androgen bioactivity that was measured by us was above that measured for 20 ng T (>57 β-galactosidase units), the detection limit of the assay for extracted steroids. This concentration of androgen represents a real problem because it exposes an athlete to the risk of a positive doping violation, while for the young or female consumer it is a potential health risk (Geyer et al., 2008).

Sport supplements contaminated or adulterated with AR positive steroidal molecules are a major cause of concern because of the possible health risks to the unsuspecting consumer. The concealment of ingredient information may affect a consumers decision to purchase a product, especially those that would opt for natural products that are supposedly free of androgens. Importantly, the supplements are consumed without expert clinical practitioner advice. Our findings would support the growing recognition of the need of independent laboratory screen testing of all sport supplements for contaminants and/or adulterants on a regular basis so as to reduce the health risks for unaware customers.

The analysis of supplements for undeclared androgens is especially important for elite athletes. Athletes are at risk of a positive doping test by consuming sport supplements (Parr et al., 2011; Striegel et al., 2005). For

Table 2 Ingredients Listed on the Labels of Positive Supplements

Supplement	Serving Size	Ingredients
1	1 capsule	Proprietary matrix: 727 mg Basella Alba Extract, Nettle Root Concentrate, Bitter Melon Extract, Resveratrol, Conjugated Coleonol Extract
2	6650 mg	Proprietary blend: 6650 mg Beta-alanine, Arginine-AKG, creatine monohydrate, Karbolyn™, N-carbamylglutamate, Fibersol-2, Caffeine, theobromine, 1,3 Dimethylamylamine, R-beta methylphenethylamine, evodiamine
3	3 capsules	Creatine di-phosphate 2250 mg
4	1 capsule	Proprietary blend: 575 mg Ptychopetalum Olacoides extract, basella Rubra Extract, (25R)-5-alpha-Spirostan-6-one-3b-ol, (25R)-4-Spirosten-3b,6b-diol
5	1–3 capsules	Enzogenol extract 150 mg (equiv. Pinus radiate dry bark (15,000 mg); d-alpha-tocopheryl acid succinate (Nat. Vit E 181 IU) 150 mg; Calcium ascorbate 128.2 mg (equiv. ascorbic acid (Vitamin C) 100 mg; s-alpha lipoic acid 50 mg; Zinc (as amino acid chelate) 5 mg; Manganese (as amino acid chelate) 500 mcg; Copper (as gluconate) 25 mcg; Selenium (as selenomethionine) 8 mcg
6	2 capsules	Ilex paraguariensis (yerba mate) leaf 336 mg; Paullinia cupana (Guarana) seed 285 mg; coffea; Arabica (Coffee) 194 mg; Turnera diffusa (Damiana) 108 mg; Theobroma cacao (Cocoa) 10 mg; Malpighia (Acerola) 25 mg; Phaseolus vulgaris (Kidney bean) (seed) 10 mg; Cynara scolymus (artichoke) 10 mg; Tyrosine 20 mg; Inositol 15 mg; Schisandra chinensis fruit 10 mg; Eleutherococcus senticosus root 10 mg; Crataegus pinnatifida (Hawthorn) 10 mg; Zingiber officinale (Ginger) root 10 mg; Vitis vinifera 10 mg; Rubus idaeus (Raspberry) leaf 10 mg; Histidine 10 mg; Thiamin hydrochloride 1.1 mg; Riboflavin 1.7 mg; pyridoxine hydrochloride 975 micrograms; folic acid 16.6 micrograms; cyanocobalamin (Vitamin B12) 16.6 micrograms; calcium pantothenate 5.4 mg; calcium carbonate 111 mg; chromium picolinate 131 micrograms

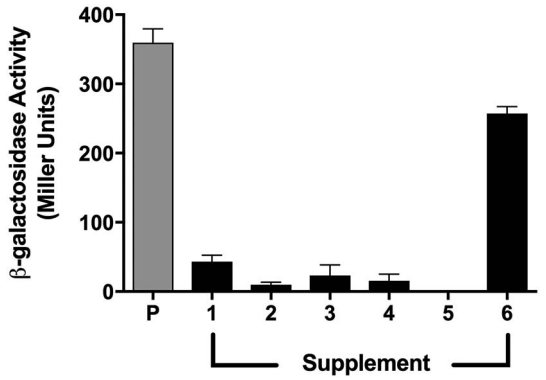

Figure 4 — One supplement tested positive in the yeast cell progestin bioassay. The six steroid extracts that tested positive for androgen bioactivity were tested for progestogenic activity using the yeast cell progesterone receptor bioassay. Results are presented as mean ± SEM (n = 3). Progesterone (3.8 nM, EC_{50} concentration for assay) was used as positive control.

example, Watson et al. reported that ingestion of 5 μg of 19-norandrosterone led to the positive detection in the urine of 15/20 volunteers (Watson et al., 2009). Similar findings were reported in a number of other studies demonstrating trace contamination presents a high risk for testing positive in a sport doping test (Catlin et al., 2000; van der Merwe & Grobbelaar, 2005). This risk would be reduced with rigorous screening for banned substances. However, the risk would not be eliminated because of the pervasive introduction of novel designer steroids.

The source of the steroid molecule activating the AR in the six positive supplements remains unclear. The yeast androgen bioassay is able to detect and characterize a range of anabolic androgenic molecules as our previous investigations show (Akram et al., 2011; Cawley et al., 2016; Cooper et al., 2017; Death et al., 2004, 2005) It may be due to contamination of the supplement as a consequence of a poor manufacturing process. This is a reasonable option as two of the supplements were manufactured by a company that

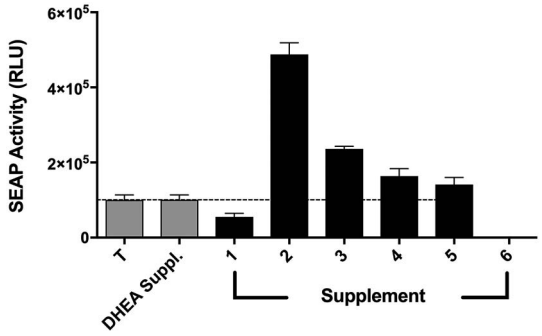

Figure 5 — Five supplements tested positive in the HuH7 cell androgen bioassay. The six steroid extracts that tested positive for androgen bioactivity in the yeast cell bioassay were tested for bioactivity in HuH7 cell bioassay. Results are presented as mean ± SEM (n = 3). Testosterone and a DHEA-containing supplement were included as positive controls.

also produced supplements that have declared androgens on the label. Alternatively, the positive results may be due to deliberate addition of androgenic molecules to products. Both pathways of positive detection have been previously reported for sports supplements (Judkins et al., 2010; Maughan et al., 2011). Our study has a detection limit of 20 ng T from extracted supplements. This suggests that the unknown steroid concentration is within this same range, although the concentration could be higher if they are weak androgens or lower if they are strong androgens. Regardless of actual amount, AR is activated and would mediate subsequent androgenic effects. Geyer et al. report that prohormones at similar concentrations occurred through cross-contamination during the manufacturing process (Geyer et al., 2008). Since 2002, it has also been documented that nutritional supplements have been purposely spiked with 1 mg/g of anabolic androgenic steroids (Geyer et al., 2008). Given our reported β-galactosidase levels, it is more likely our androgenic bioactivity is representative of contamination, rather than adulteration, of the supplements with androgens.

While not yet able to be integrated into routine testing laboratories because of their well-described limitations in their current forms, bioassays remain a useful screening tool for research applications (Cooper et al., 2013). In this study, tandem yeast and HuH7 cell bioassays were used. The yeast cell bioassay successfully identified six positive supplements, with the PR equivalent assay ruling out progestin cross-activation creating false positives, for five of the six supplements. The HuH7 cell assay showed that five of the six supplements were positive, even with exposure to steroid metabolizing enzymes indicating that the steroid molecules may well be potent androgens, as they show resistance to metabolism. For supplement 6, however, the HuH7 result suggests the androgenic/progestagenic molecule is readily metabolized. The relative metabolism of these extracts indicated by the HuH7 bioassay may help guide chemists to the eventual identification of the androgenic molecule.

There are several limitations to the approach used in the current study. First, sports supplements are marketed in a variety of forms including powders, oils, tablets, and capsules. To test their bioactivity, a steroid extraction procedure was necessary to produce a form of the supplement amenable for use in the bioassays. Matrix effects prohibiting efficient extraction may have resulted in false negatives. The method used is well-described and used successfully for steroid extraction however whether it is 100% effective for all matrices is contentious (Parr et al., 2004). Regardless, the use of bioassays has identified six positive findings and so if the SPE method is limiting steroid extraction, our findings will be an underestimate of the prevalence of undeclared androgens in sports supplements sold on the Australian market. The other significant limitation is that the bioassay does not identify the androgen in the supplement. However, any significant androgen bioactivity represents a health risk for the nonsuspecting consumer, or a career risk for the unaware athlete, and the bioassay screening quickly provides this information.

In summary of the 112 products tested, six were found to test positive for activating the androgen receptor in an androgen bioassay. This represented 5.4% (or approximately 1 in 20) of products tested. The positive supplements represented primarily the preworkout category, and hormone booster categories (5/6). Of the formulations tested, capsules gave the highest number of positive findings (5/6), with the other positive being a powder (1/6). The results of this study once again highlight that consuming sports supplements involves risk: (1) for the athlete, there is the potential for a reputation-damaging, positive doping test and (2) for all consumers, there is the risk of adverse health consequences from consuming exogenous androgens.

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References

Akram, O.N., Bursill, C., Desai, R., Heather, A.K., Kazlauskas, R., Handelsman, D.J., & Lambert, G. (2011). Evaluation of androgenic activity of nutraceutical-derived steroids using mammalian and yeast in vitro androgen bioassays. *Analytical Chemistry, 83*(6), 2065–2074. PubMed doi:10.1021/ac102845y

Ayotte, C., Levesque, J.F., Cle roux, M., Lajeunesse, A., Goudreault, D., & Fakirian, A. (2001). Sport nutritional supplements: Quality and doping controls. *Canadian Journal of Applied Physiology, 26*(Suppl.), S120–S129. doi:10.1139/h2001-047

Cadwallader, A.B., & Murray, B. (2015). Performance-enhancing drugs I: Understanding the basics of testing for banned substances. *International Journal of Sport Nutrition and Exercise Metabolism, 25*(4), 396–404. PubMed doi:10.1123/ijsnem.2014-0185

Catlin, D.H., Leder, B.Z., Ahrens, B., Starcevic, B., Hatton, C.K., Green, G.A., & Finkelstein, J.S. (2000). Trace contamination of over-the-counter androstenedione and positive urine test results for a nandrolone metabolite. *JAMA, 284*(20), 2618–2621. PubMed doi:10.1001/jama.284.20.2618

Cawley, A.T., Smart, C., Greer, C., Liu Lau, M., & Keledjian, J. (2016). Detection of the selective androgen receptor modulator andarine (S-4) in a routine equine blood doping control sample. *Drug Testing and Analysis, 8*(2), 257–261. PubMed doi:10.1002/dta.v8.2

Cooper, E.R., McGrath, K.C., & Heather, A.K. (2013). In vitro androgen bioassays as a detection method for designer androgens. *Sensors (Basel), 13*(2), 2148–2163. doi:10.3390/s130202148

Cooper, E.R., McGrath, K.C., Li, X., Akram, O., Kasz, R., Kazlauskas, R., ... Heather, A.K. (2017). The use of tandem yeast and mammalian cell in vitro androgen bioassays to detect androgens in internet-sourced sport supplements. *Drug Testing and Analysis, 9*, 545–552. PubMed doi:10.1002/dta.2000

Dascombe, D.J., Karunaratna, M., Cartoon, J., Fergie, B., & Goodman, C. (2010). Nutritional supplementation habits and perceptions of elite athletes within a state-based sporting institute. *Journal of Sports Science & Medicine, 13*, 274–280. doi:10.1016/j.jsams.2009.03.005

Death, A.K., McGrath, K.C., & Handelsman, D.J. (2005). Valproate is an anti-androgen and anti-progestin. *Steroids, 70*(14), 946–953. PubMed doi:10.1016/j.steroids.2005.07.003

Death, A.K., McGrath, K.C., Kazlauskas, R., & Handelsman, D.J. (2004). Tetrahydrogestrinone is a potent androgen and progestin. *Journal of Clinical Endocrinology and Metabolism, 89*(5), 2498–2500. PubMed doi:10.1210/jc.2004-0033

Evans, M.W., Jr., Ndetan, H., Perko, M., Williams, R., & Walker, C. (2012). Dietary supplement use by children and adolescents in the United States to enhance sport performance: Results of the National Health Interview Survey. *The Journal of Primary Prevention, 33*(1), 3–12. PubMed doi:10.1007/s10935-012-0261-4

FDA. (2015). *Tainted products marketed as dietary supplements_CDER*. Silver Spring, MD: U.S. Food and Drug Administration. Retrieved from www.accessdata.fda.gov

Geyer, H., Mareck-Engelke, U., Reinhart, U., Thevis, M., & Schanzer, W. (2000). Positive doping cases with norandrosterone after application of contaminated nutritional supplements. *Deutsche Zeitschrift Fur Sportmedizin, 51*, 378.

Geyer, H., Parr, M.K., Koehler, K., Mareck, U., Schanzer, W., & Thevis, M. (2008). Nutritional supplements cross-contaminated and faked with doping substances. *Journal of Mass Spectrometry, 43*(7), 892–902. PubMed doi:10.1002/jms.v43:7

Geyer, H., Parr, M.K., Mareck, U., Reinhart, U., Schrader, Y., & Schanzer, W. (2004). Analysis of non-hormonal nutritional supplements for anabolic-androgenic steroids-results of an international study. *International Journal of Sports Medicine, 25*(2), 124–129. PubMed doi:10.1055/s-2004-819955

Gilard, V., Balayssac, S., Tinaugus, A., Martins, N., Martino, R., & Malet-Martino, M. (2015). Detection, identification and quantification by 1H NMR of adulterants in 150 herbal dietary supplements marketed for improving sexual performance. *Journal of Pharmaceutical and Biomedical Analysis, 102*, 476–493. PubMed doi:10.1016/j.jpba.2014.10.011

Goston, J.L., & Correia, M.I. (2010). Intake of nutritional supplements among people exercising in gyms and influencing factors. *Nutrition, 26*(6), 604–611. PubMed doi:10.1016/j.nut.2009.06.021

Judkins, C., & Prock, P. (2012). Supplements and inadvertent doping-how big is the risk to athletes. *Medicine and Sport Science, 59*, 143–152. PubMed doi:10.1159/000341970

Judkins, C.M., Teale, P., & Hall, D.J. (2010). The role of banned substance residue analysis in the control of dietary supplement contamination. *Drug Testing and Analysis, 2*(9), 417–420. PubMed doi:10.1002/dta.v2:9

Kamber, M., Baume, N., Saugy, M., & Rivier, L. (2000). Nutritional supplements as a source for positive doping cases? *International Journal of Sport Nutrition and Exercise Biochemistry, 11*, 258–262. doi:10.1123/ijsnem.11.2.258

Kiertscher, E.D., & DiMarco, N.M. (2013). Use and rationale for taking nutritional supplements among collegiate athletes at risk for nutrient deficiencies. *Perfomance Enhancement & Health, 2*, 24–29. doi:10.1016/j.peh.2013.04.002

Knapik, J.J., Steelman, R.A., Hoedebecke, S.S., Austin, K.G., Farina, E.K., & Lieberman, H.R. (2016). Prevalence of dietary supplement use by athletes: Systematic review and meta-analysis. *Sports Med, 46*, 103–123. PubMed doi:10.1007/s40279-015-0387-7

LGC. (2016). *Australian supplement survey © LGC 2016,* 1–8.

Lun, V., Erdman, K.A., Fung, T.S., & Reimer, R.A. (2012). Dietary supplementation practices in Canadian high-performance athletes. *International Journal of Sport Nutrition and Exercise Metabolism, 22*(1), 31–37. PubMed doi:10.1123/ijsnem.22.1.31

Maughan, R.J., Greenhaff, P.L., & Hespel, P. (2011). Dietary supplements for athletes: Emerging trends and recurring themes. *Journal of Sports Sciences, 29*(Suppl. 1), S57–S66. doi:10.1080/02640414.2011.587446

McRobb, L., Handelsman, D.J., Kazlauskas, R., Wilkinson, S., McLeod, M.D., & Heather, A.K. (2008). Structure-activity relationships of synthetic progestins in a yeast-based in vitro androgen bioassay. *The Journal of Steroid Biochemistry and Molecular Biology, 110*(1–2), 39–47. PubMed doi:10.1016/j.jsbmb.2007.10.008

Nutrition Business Journal Global Supplement and Nutrition Industry Report 2014. (2014). *Nutrition Business Journal*. Retrieved from http://newhope360.com/site-files/newhope360.com/files/uploads/2014/Global_Report%20summary.pdf

Outram, S., & Stewart, B. (2015). Doping through supplement use: A review of the available empirical data. *International Journal of Sport Nutrition and Exercise Metabolism, 25*(1), 54–59. PubMed doi:10.1123/ijsnem.2013-0174

Parr, M.K., Geyer, H., Reinhart, U., & Schanzer, W. (2004). Analytical strategies for the detection of non-labelled anabolic androgenic steroids in nutritional supplements. *Food Additives & Contaminants, 21*(7), 632–640. PubMed doi:10.1080/02652030410001701602

Parr, M.K., Opfermann, G., Geyer, H., Westphal, F., Sonnischsen, F.D., Zapp, J., . . . Schanzer, W. (2011). Seized designer supplement named "1-Androsterone": Identification as 3β-hydroxy-5α-androst-1-en-17-one and its urinary elimination. *Steroids, 76*, 540–547.

Peters, R.J., Rijk, J.C., Bovee, T.F., Nijrolder, A.W., Lommen, A., & Nielen, M.W. (2010). Identification of anabolic steroids and derivatives using bioassay-guided fractionation, UHPLC/TOFMS analysis and accurate mass database searching. *Analytica Chimica Acta, 664*(1), 77–88. PubMed doi:10.1016/j.aca.2010.01.065

Pipe, A., & Ayotte, C. (2002). Nutritional supplements and doping. *Clinical Journal of Sport Medicine, 12*(4), 245–249. PubMed doi:10.1097/00042752-200207000-00008

Rijk, J.C., Bovee, T.F., Wang, S., Van Poucke, C., Van Peteghem, C., & Nielen, M.W. (2009). Detection of anabolic steroids in dietary supplements: The added value of an androgen yeast bioassay in parallel with a liquid chromatography-tandem mass spectrometry screening method. *Analytica Chimica Acta, 637*(1–2), 305–314. doi:10.1016/j.aca.2008.09.014

Striegel, H., Vollkommer, G., Horstmann, T., & Niess, A.M. (2005). Contaminated nutritional supplements—legal protection for elite athletes who tested positive: A case report from Germany. *Journal of Sports Sciences, 23*, 723–726. PubMed

van der Merwe, P.J., & Grobbelaar, E. (2005). Unintentional doping through the use of contaminated nutritional supplements. *South African Medical Journal, 95*(7), 510–511. PubMed

WADA. (2015). *2015 Anti-doping testing figures report*. Retrieved from www.wada-ama.org/sites/default/files/resources/files/2015_wada_anti-doping_testing_Figures_report_0.pdf

Watson, P., Judkins, C., Houghton, E., Russell, C., & Maughan, R.J. (2009). Urinary nandrolone metabolite detection after ingestion of a nandrolone precursor. *Medicine & Science in Sports & Exercise, 41*(4), 766–772. PubMed doi:10.1249/MSS.0b013e31818edaeb

Zierau, O., Lehmann, S., Vollmer, G., Schanzer, W., & Diel, P. (2008). Detection of anabolic steroid abuse using a yeast transactivation system. *Steroids, 73*(11), 1143–1147. PubMed doi:10.1016/j.steroids.2008.04.015