

## Nutritional supplements: prevalence of use and contamination with doping agents

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Based upon recent sales numbers, nutritional supplements play a key role in the lifestyle of a substantial proportion of the population. As well as products such as vitamins or minerals, several precursors of anabolic steroids are marketed as nutritional supplements. Another group of commercially available supplements are products for weight loss based upon herbal formulations originating from *Ephedra* species. Apart from supplements indicating the presence of these active compounds, numerous non-hormonal nutritional supplements were found that were contaminated with non-labelled anabolic steroids. Stimulating agents other than naturally occurring analogues of ephedrine were detected. A major group using dietary supplements are sportsmen, ranging from amateur level to elite athletes. Besides the possible health risks associated with the use of dietary supplements, athletes should take care not to violate the rules of the World Anti-Doping Agency because athletes remain responsible for substances detected in their biofluids, irrespective of their origin. Several analytical methods have been developed to determine the presence of doping agents as contaminants. The present review attempts to address the issues concerning the use of nutritional supplements and the detection of doping agents as contaminants in dietary supplements.

### Nutritional supplements: Doping: Prohormones: Stimulants

#### Introduction

The definition of nutritional supplements is important to a discussion of this group of products. Although no universally accepted definition is available, nutritional supplements can be defined as food supplying one or more nutrients in a concentrated form, for example, minerals, vitamins, trace elements and other components that are theoretically present in a normal and balanced diet (Schröder, 2002). The Dietary Supplement Health and Education Act (DSHEA), enacted by the US Congress in 1994, went far beyond a logical definition of nutritional supplements such as the definition from Schröder and included, besides vitamins and minerals, herbs or other botanicals, amino acids, metabolites, constituents, extracts or combinations of any of these ingredients. Although many of these products (particularly herbs) are marketed for their alleged preventive or therapeutic effects, the DSHEA has made it difficult to regulate them as drugs. Since the passage of the DSHEA, even hormones such as dehydroepiandrosterone (DHEA) and melatonin are being sold as dietary supplements. Nutritional supplements are frequently offered in an untypical form of food, for example, tablets, capsules, powders or pills.

The use of nutritional supplements has expanded explosively during the last decade. In 1997 vitamin sales numbers in Germany were estimated at 500 million €, while the total sales of supplements exceeded 1 billion € (Schröder, 2002). US annual sales were estimated at US\$12–15 billion in the late 1990s, of which US\$800 million was spent on sport supplements (Zorpette, 1999; Green *et al.* 2001). In 2000 approximately 3.1 million kg creatine was sold, although a decade earlier nobody had even heard of it (Maughan, 2002). The global market for supplements in 2001 was estimated at US\$46 billion, the US supplement market in 2000 at US\$16.7 billion, as reported in the *Financial Times* newspaper (Maughan *et al.* 2004). An important factor behind this growth was the passage of the DSHEA allowing US customers free access to supplements (Food and Drug Administration, 1994). Other factors influencing the growth of the supplement market are easy access through the Internet, which makes it possible for users all over the world to access all products freely available in the USA, and the ever-improving results and record performances of top-level athletes who promote the use of supplements.

**Abbreviations:** DHEA, dehydroepiandrosterone; DSHEA, Dietary Supplement Health and Education Act; LOD, limit of detection; NZVT, 'Netherlands security system for nutritional supplements for elite sports'; WADA, World Anti-Doping Agency.

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### *Use of nutritional supplements*

Nutritional supplements are used in all layers of the population. General population studies on the use of supplements show a use rate of 40% (Pesko *et al.* 2000). Nevertheless it seems that these products are most frequently used by sportsmen ranging from amateur level to elite athletes (Morrison *et al.* 2004), the category to which they are most aggressively marketed. This high prevalence of use can be seen in a study following the Olympic Games held in Sydney in 2000. In this study the medication use of 2758 athletes selected for doping control was evaluated. Only reported use during the final 3 d before competition was included. Major findings were that 51% of all tested athletes had used vitamins before competition and a large fraction of the tested athletes used other nutritional supplements. Some athletes even reported up to twenty-six different supplements in 1 d (Corrigan & Kazlauskas, 2003). Many surveys dealing with the prevalence of use of nutritional supplements among athletes have been published (Perko *et al.* 2000; Schroder *et al.* 2002; Beshgetoor & Nichols, 2003; Ziegler *et al.* 2003). Sobal & Marquart (1994a) studied a comprehensive analysis of supplement use among athletes. A total of 10 274 athletes, both male and female, were included in this meta-analysis. Their results show that the mean prevalence of supplement use among athletes was 46%. Approximately the same percentage was found for adolescents and high-school athletes (Bell *et al.* 2004; Sobal & Marquart, 1994b). It is not known whether prevalence of use varies widely between the sexes. Sobal & Marquart (1994a) found a significantly higher percentage of use in female athletes compared with male athletes, while a study by Sundgot-Borgen *et al.* (2003) did not find a significant difference between sexes.

Differences can also be observed between sports. Supplement consumption in strength sports such as weight lifting, power lifting and bodybuilding is higher than in other sports (Sobal & Marquart, 1994a; Ronsen *et al.* 1999), confirming the statement that supplement use is most frequent in sports that emphasise muscle size. However, great care should be taken with results obtained from questionnaires, as individuals tend to underrate their use of nutritional supplements (Morrison *et al.* 2004). Also the type of question and the way those questions were put forward to the athletes could significantly influence the outcome. For instance, a higher percentage of runners admitted supplement intake when asked to respond to a frequency questionnaire (69%) than when the supplement use was recorded during a 3 d period (48%) (Nieman *et al.* 1989).

Only a few studies have tried to evaluate the frequency of use of nutritional supplements. In a summary of thirteen studies using time-specified terms (Sobal & Marquart, 1994a), it appears that most athletes take their supplements on a daily or routine basis, rather than occasionally or weekly. Often, these supplements are used more frequently and in higher doses than recommended on the label. The reason for this behaviour is the popular belief that the more supplements are taken, the better a performance will be.

However, reasons for use of dietary supplements differ according to the investigated population. Among high-school students, boys tend to use supplements more for athletic performance improvement and muscle development than

girls do (Sobal & Marquart, 1994b). In general, reasons for supplement use among adolescents are: losing weight and/or keeping weight at a certain level (Sobal & Marquart, 1994a; Bell *et al.* 2004), to gain weight and stimulate muscle development or to compensate for an inadequate diet.

Major reasons for use of supplements by athletes include compensation for an inadequate diet, enhancing performance, meeting the abnormal demands during training and/or competition, keeping up with opponents and following recommendations by parents, coaches and team mates (Sobal & Marquart, 1994a; Maughan, 2002). Athletes are mostly influenced by parents, doctors, coaches and friends, with female athletes being more influenced by their parents and male athletes by their coach (Sobal & Marquart, 1994b).

It is the position of many official authorities, including the American Dietetic Association, Dieticians of Canada and UK Sport that the use of supplements to compensate for needs following strenuous exercise is unnecessary when the athlete has a well-balanced diet. Supplement use without a specific need, disease or deficiency is not recommended (Manore *et al.* 2000). If athletes persist in using supplements for their purported ergogenic effects, it is advised to use counselling in product selection and to use them only after careful evaluation of the product for safety, efficacy, potency and legality. In some cases, especially when the manufacturers' recommendations are not followed, the use can be harmful. Schwenk & Costley (2002) described several non-anabolic nutritional supplements and their potential risks when used in high doses or without the counselling of a physician. Others have also demonstrated that with excessive use most of the supplements harm rather than improve the condition of the user (Haller & Benowitz, 2000; Powers, 2001; Canter & Ernst, 2004).

Again, controversial information can be found on the effectiveness of enhancing sport performances for most marketed supplements. Creatine, for instance, is promoted for its performance-enhancing effect, although studies are contradictory (Mujika *et al.* 1996; Volek *et al.* 1997; Engelhardt *et al.* 1998; Benzi, 2000).

### **Nutritional supplements and doping**

Besides the relatively harmless products (when used properly), such as vitamins, minerals, etc, other types of ingredients can be present in nutritional supplements. Ingredients from plant origin including ephedrine and caffeine can be present in dietary supplements promoting ergogenic effects. Other substances of botanical origin, such as morphine, can be present in the daily diet. In addition, as a consequence of the passage of the DSHEA, prohormones became freely available on the US supplement market. Besides the health risks associated with these products, athletes are also at risk of violating doping rules, as ephedrine, morphine and prohormones figure on the World Anti-Doping Agency (WADA) list of forbidden substances (World Anti-Doping Agency, 2004). A failed doping test resulting from the intake of a supplement can originate from the poor knowledge by the athlete of banned substances indicated on the label, from the fact that the labelled ingredients indeed contain banned substances (for example, Ma Huang herbal products contain ephedrine) or, more

importantly, that the supplement contains a banned substance not indicated on the label.

#### *Prohormones and anabolic androgenic steroids*

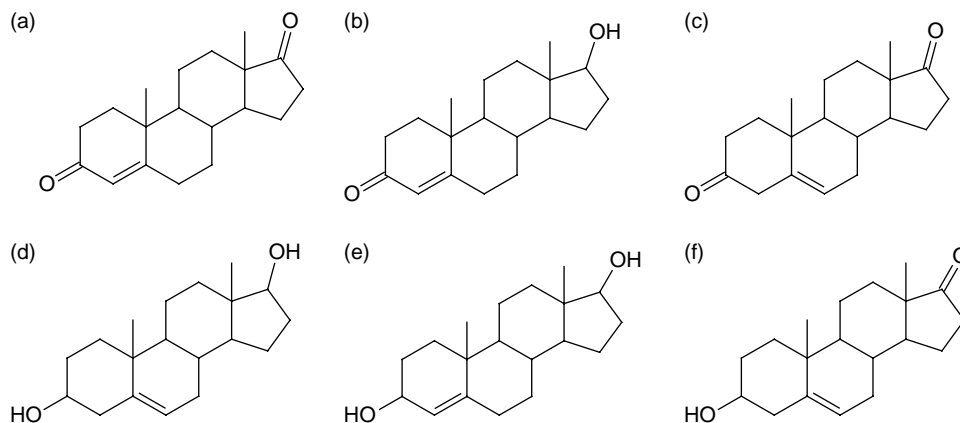
As a result of the passage of the DSHEA, several anabolic androgenic steroids including DHEA, androstenedione, androstenediol and 19-nor-steroids became readily available as over-the-counter products in the USA and through the Internet in the rest of the world. These steroids can be marketed as nutritional supplements as long as the manufacturers make no claims about the use of a dietary supplement to diagnose, prevent, mitigate, treat or cure a specific disease. On the other hand, the DSHEA allowed dietary supplements to bear a 'statement of support' that: (a) claims a benefit related to a classical nutrient deficiency disease; (b) describes how ingredients affect the structure or function of the human body; (c) characterises the documented mechanism by which the ingredients act to maintain structure or function; (d) describes general wellbeing from consumption of the ingredients (Food and Drug Administration, 1994). In this way, the US Food and Drug Administration rules are circumvented and prohormones, precursors of testosterone (Fig. 1) and nandrolone (Fig. 2), became available on the market. These prohormones are converted in the body into the respective anabolic steroids and consequently act in the same way as the latter do.

Supplement manufacturers claim that these products increase serum testosterone concentrations, promote muscle mass and muscle strength, help reducing body fat and enhance mood and sexual performance. However, many studies do not confirm these claims (Kreider, 1999; Broeder *et al.* 2000; Brown *et al.* 2000; Rasmussen *et al.* 2000; Van Eenoo *et al.* 2000). Although in several studies an increase in serum testosterone levels was measured after prohormone use (Leder *et al.* 2000; Broeder, 2003), this does not necessarily result in an increased muscle mass, strength or sexual performance (Broeder *et al.* 2000; Broeder, 2003). In addition, risks associated with the use of prohormones are similar to those observed with anabolic steroids, for example, cardiovascular effects, liver tumours,

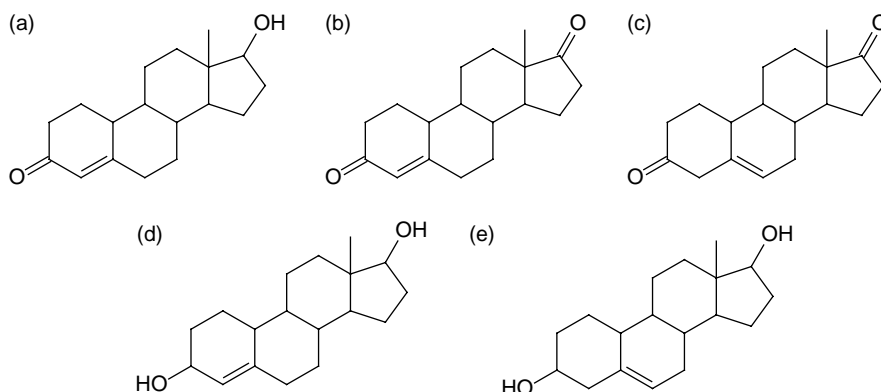
gynaecomastia and aggressive behaviour (Mottram & George, 2000; Müller & Müller-Platz, 2001; Parssinen & Seppala, 2002).

Another issue is the labelled content of supplements. Evidence has been given that prohormones such as androstenedione, sold as nutritional supplements, do not always have the content they claim. Table 1 summarises the labelled *v.* detected content of prohormones, promoted as nutritional supplements. The detected dose rarely approaches the labelled dose and in some cases the alleged prohormone cannot even be detected. More alarming results were obtained in the same studies as it was proven that in several supplements additional steroids, or precursors, other than the labelled prohormones were present. Results from Kamber *et al.* (2001) show that 35% (six out of seventeen) of prohormone supplements contain other substances than indicated on the label. Analysis of a 1-androstene-3 $\beta$ ,17 $\beta$ -diol-containing nutritional supplement revealed the presence of five other anabolic steroids including boldenone and testosterone (Delbeke *et al.* 2002).

In several studies the presence of prohormones in non-hormonal nutritional supplements has been investigated. Results from an international study showed that 14.8% (ninety-four out of 634 samples) of the investigated non-hormonal nutritional supplements contained one or more anabolic androgenic steroid not declared on the label (Geyer *et al.* 2004). The detected substances were DHEA and other precursors of testosterone, including 4-androstene-3,17-dione and 4(5)-androstene-3,17-diol. Twenty-six supplements contained 19-nor-4-androstene-3,17-dione, a precursor of nandrolone. One quarter of the contaminated samples showed a total anabolic androgenic steroid concentration higher than 5  $\mu\text{g/g}$ . The highest percentages of contaminants were found in supplements marketed in the Netherlands (25.8%), Austria (22.7%), UK (18.9%) and USA (18.8%). The vast majority of those supplements originated from the USA. Supplement producers are not obliged to disclose the information where a supplement has been produced. In this way, prohormones or contaminated supplements can enter the European market where they are prohibited. Also interesting to note is that more than 20% of the non-hormonal supplements bought from



**Fig. 1.** Chemical structure of testosterone (b) and some of its major precursors: (a) 4-androstene-3,17-dione; (c) 5-androstene-3,17-dione; (d) 5-androstene-3,17-diol; (e) 4-androstene-3,17-diol; (f) dehydroepiandrosterone.



**Fig. 2.** Chemical structure of nandrolone (a) and some of its major precursors: (b) 19-nor-4-androstene-3,17-dione; (c) 19-nor-5-androstene-3,17-dione; (d) 19-nor-4-androstene-3,17-diol; (e) 19-nor-5-androstene-3,17-diol.

prohormone-selling companies contained anabolic androgenic steroids whereas this percentage is below 10 % for companies not selling prohormones, indicating that improper cleaning of machinery is responsible for a big fraction of the contamination in non-hormonal supplements.

Results obtained in the Ghent Doping Control Laboratory show that 12.72 % ( $n = 36$ ) of all samples ( $n = 283$ ) analysed in the period from December 2002 until August 2005 contained one or multiple prohormones not indicated on the label. Table 2 indicates the rate of incidence of the different detected components. Following these results, multiple prohormone contamination is common. Several contaminated supplements contained six different anabolic androgenic steroids and/or their prohormones.

Some other studies reported on the detection of prohormones as contaminants in nutritional supplements (Geyer *et al.* 2000; De Cock *et al.* 2001; Van Thuyne & Delbeke, 2004, 2005). One of the most commonly detected steroids was 19-nor-4-androstene-3,17-dione. Its presence may lead to a positive doping test for the nandrolone metabolite norandrosterone (Uralets & Gillette, 1999, 2000; Catlin *et al.* 2000; Geyer *et al.* 2000, 2004; De Cock *et al.* 2001). In the study by De Cock *et al.* (2001) approximately 4.8 mg 19-nor-4-androstene-3,17-dione per capsule was found in a supplement of US origin. Its presence was not declared on the label. The recommended dosage was seven capsules per d. Ingestion of only one capsule would have resulted in a positive doping test according to the WADA rules (19-norandrosterone, 2 ng/ml) up to 144 h after the ingestion. Besides these highly contaminated nutritional supplements, low concentrations of nandrolone precursors could also result in positive doping tests (Geyer *et al.* 2004).

Following the results by Van Eenoo *et al.* (1998), the intake of 4-androstene-3,17-dione, present in several nutritional supplements, could result in a positive doping test for 6–9 h after administration.

After the introduction of the prohormones of testosterone such as DHEA on the market in about 1996, the prohormones of nandrolone, and also boldenone and its prohormones, became available on the supplement market (Delbeke *et al.* 2002). Recent

findings report the detection of oxygenated anabolic steroids such as 7-keto DHEA (Delbeke *et al.* 2002) and 4-androstene-3,6,17-trione (6-OXO®) (Van Thuyne *et al.* 2005) in over-the-counter nutritional supplements. Moreover, in two recent studies (Geyer *et al.* 2002; Gmeiner, 2002) the presence of non-labelled metandienone (17 $\alpha$ -methyl-androsta-1,4-dion-17 $\beta$ -ol-3-one) in nutritional supplements was reported. The use of 17 $\alpha$ -alkylated steroids is associated with serious adverse health effects similar to the use of other anabolic steroids but 17 $\alpha$ -alkylation, which makes the steroid orally active, aggravates the situation because of the association with jaundice, hepatic carcinoma and petiociis hepatitis, a fatal degenerative liver condition. Similar remarks can be made about 17 $\alpha$ -methyltestosterone detected in two nutritional supplements analysed in the Ghent Doping Control Laboratory (see Table 2) (Wadler & Hainline, 1989; Habscheid *et al.* 1999; Mottram & George, 2000; Müller & Müller-Platz, 2001; Parssinen & Seppala, 2002). In addition, the intake of these supplements could lead to adverse doping findings.

Numerous dietary supplements are also marketed as containing a natural testosterone booster called *Tribulus terrestris*, a plant containing saponins and diosgenin. Diosgenin has been used as the starting material for industrial hemi-synthesis of androgens; however, there is no evidence that it is metabolised to androgens in man. One study investigated the effect of *T. terrestris* combined with androstenedione supplementation and observed no increase in serum testosterone concentrations (Brown *et al.* 2000). In addition, no effects on the urinary steroid profile after *T. terrestris* were found (Van Eenoo *et al.* 2000).

From 20 January 2005 the US congress enacted the Anabolic Steroid Control Act, publishing a limited list of hormonal substances, including most previously mentioned prohormones that are no longer allowed on the US market. This list is supposed to be exhaustive but unfortunately does not contain some interesting steroids such as DHEA and the recently discovered designer steroid Madol (Sekera *et al.* 2005). In addition, new designer steroids such as 6-oxo steroids or 7-oxo steroids are also not included in this list.

Summarising, athletes should be very careful about dietary supplements. Obviously, athletes should refrain from taking

**Table 1.** Labelled v. detected content of nutritional supplements promoted as prohormones

Reference	Steroid and dose listed on label	Steroids detected	Detected amount (mg)
Catlin <i>et al.</i> (2000)	Androstenedione, 100 mg	Androstenedione	93.1
	Androstenedione, 100 mg	Androstenedione	82.8
	Androstenedione, 100 mg	Androstenedione	103
	Androstenedione, 100 mg	Androstenedione	90
	Androstenedione, 100 mg	Androstenedione	88
	Androstenedione, 100 mg	Androstenedione	85
	4-Androstene-3,17-dione, 50 mg	4-Androstene-3,17-dione	35
	Androstenedione, 50 mg	None	0
Kamber <i>et al.</i> (2001)	4-Androstene-3,17-dione, 250 mg	4-Androstene-3,17-dione	168
		Testosterone	10
	19-Norandrostenedione, 50 mg	19-Norandrostenedione, 33 %	*
	19-Norandrostenediol, 50 mg	19-Norandrostenediol, 52 %	
		Androstenediol, 15 %	
	Androstenediol, 200 mg	Androstenediol	*
		Androstenedione	
		Testosterone	
	5-Androstenediol	5-Androstenediol, 97 %	*
		Testosterone, 2 %	
	5-Androstenediol, 50 mg	5-Androstenediol	*
	Androstenedione, 250 mg	Androstenedione	
	19-Norandrostenedione, 50 mg	19-Norandrostenedione	
	Dehydroepiandrosterone, 150 mg		
Dehydroepiandrosterone, 100 mg	Dehydroepiandrosterone, 85 %	*	
	Androstenedione, 15 %		
Nor-4-androstenedione, 100 mg	Nor-4-androstenedione, 25 %	*	
19-Norandrostenediol, 100 mg	19-Norandrostenediol, 25 %		
	Androstenedione, 42 %		
	Testosterone, 8 %		
Green <i>et al.</i> (2001)	19-Norandrostenediol, 100 mg	Androstenedione	*
	5-Androstenediol, 100 mg	5-Androstenediol	77.3
		Androstenedione	3.3
	4-Androstene-3,17-dione, 250 mg	4-Androstene-3,17-dione	166.3
		Testosterone	10
	4-Androstene-3,17-diol, 100 mg	4-Androstene-3,17-diol	78.5
	Androstenedione, 100 mg	Androstenedione	45.5
	Androstenedione, 100 mg	Androstenedione	51.5
	Androstenedione, 100 mg	Androstenedione	84.5
	Androstenedione, 100 mg	Androstenedione	98.5
	4-Androstene-3,17-dione, 50 mg	4-Androstene-3,17-dione	35.3
	4-Androstene-3,17-dione, 3.33 mg	4-Androstene-3,17-dione	1
	4-Androstene-3,17-diol, 83.33 mg	4-Androstene-3,17-diol	35.8
	5-Androstene-3,17-dione, 3.33 mg	19-Nor-4-androstene-3,17-dione	1
	5-Androstene-3,17-diol, 3.33 mg		
	19-Nor-4-androstene-3,17-dione, 3.33 mg		
	19-Nor-5-androstene-3,17-diol, 3.33 mg		
	5-Androstene-3 $\beta$ ,17 $\beta$ -diol, 50 mg	5-Androstene-3 $\beta$ ,17 $\beta$ -diol	44
	19-Nor-4-androstene-3 $\beta$ ,17 $\beta$ -diol, 50 mg	19-Norandrostenedione	76.5
	19-Norandrostenedione, 100 mg		
Androstenedione, 100 mg	Androstenedione	70	
4-Androstene-3,17-diol, 100 mg	4-Androstene-3,17-diol	87.5	
5-Androstene-3 $\beta$ ,17 $\beta$ -diol, 50 mg	5-Androstene-3 $\beta$ ,17 $\beta$ -diol	88.5	
<i>Tribulus terrestris</i> , 250 mg			
Delbeke <i>et al.</i> (2002)	1-Androstene-3 $\beta$ ,17 $\beta$ -diol, 100 mg	1-Androstene-3 $\beta$ ,17 $\beta$ -diol	*
		Boldenone	*
		Dehydroepiandrosterone	*
		19-Nor-4-androstenediol	*
		Testosterone	*
		5 $\alpha$ -Androstane-3 $\alpha$ ,17 $\beta$ -diol	*

\* Absolute detected amounts not mentioned.

supplements mentioning the presence of one or more prohormones or anabolic androgenic steroids, not only for health reasons, but also to avoid positive doping tests. In addition, athletes should also take care with non-hormonal supplements, as numerous studies have demonstrated that these supplements might be contaminated with substances, resulting in adverse doping tests.

Following WADA rules, athletes remain responsible for the presence of doping substances in their biofluids. To avoid positive doping results following the use of nutritional supplements, athletes should contact supplement manufacturers to get a guarantee on the purity of the products they use. Only laboratories specialised in doping analysis can give this guarantee.

**Table 2.** Incidence of detected compounds in 'non-hormonal' nutritional supplements tested in the Ghent Doping Control Laboratory during the period December 2002 to August 2005

Detected compound	<i>n</i>
Dehydroepiandrosterone	27
Testosterone	18
4-Androstene-3,17-dione	16
19-Nor-4-androstene-3,17-dione	9
Dihydrotestosterone	8
Nandrolone	5
5-Androstene-3 $\beta$ ,17 $\beta$ -diol	5
17 $\alpha$ -Methyltestosterone	2

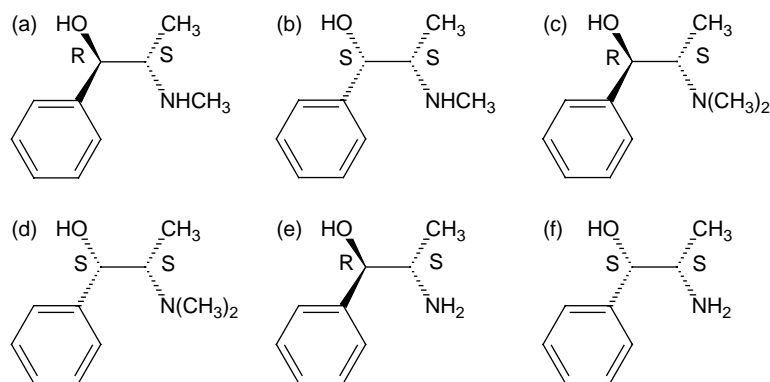
### Stimulants

One of the oldest known medicinal herbs is *Ephedra* or Ma Huang. *Ephedra sinica* is the primary species that has been used in traditional Chinese medicine and is still being used in *Ephedra* preparations and extracts around the world. The six optically active alkaloids present in *Ephedra* species, ranging from 0.02 to 3.4% of the plant material, are summarised in Fig. 3 (Abourashed *et al.* 2003). Decades ago, ephedrine was used for its bronchodilating effect to treat asthma (Chen & Schmidt, 1926). Today, other and more effective products, including  $\beta$ -agonists, have replaced ephedrine in the treatment of asthma. Nevertheless, ephedrine is still promoted as a dietary supplement. The use of ephedrine-containing supplements promoting weight loss or athletic performance enhancement has garnered a lot of success. For instance, more than US\$1 billion was spent on *Ephedra* products in 2000 (Talalay, 2001).

Effects of herbal ephedrine supplements on weight reduction have been well documented. Several studies indicated that the administration of ephedrine to obese individuals resulted in weight loss (Boozer *et al.* 2002; Shekelle *et al.* 2003; Hioki *et al.* 2004). The influence of ephedrine on performance, however, is doubtful. Studies by Bell and colleagues (Bell *et al.* 1998, 2000, 2001; Bell & Jacobs, 1999; Jacobs *et al.* 2003) reported an increase in athletic performance (i.e. increase in exercise time to exhaustion, improved time in Canadian Forces warrior test, increased time to exhaustion in cycle ergometer trial).

However, most of those studies were performed in small populations and caffeine was frequently co-administered with ephedrine which makes it difficult to attribute the obtained effect to ephedrine. In addition, the effect of pseudoephedrine, another optically active alkaloid present in *Ephedra* species and readily available as an over-the-counter nasal decongestant, is not proven on performance (Gillies *et al.* 1996; Chu *et al.* 2002; Chester *et al.* 2003; Hodges *et al.* 2003). In one study only (Gill *et al.* 2000) an improvement in maximum torque performed during an isometric knee extension exercise and in peak power during maximal cycle performance was found, but after doses exceeding the therapeutic dose, making the results questionable.

On the other hand, numerous reports have been published on adverse effects associated with the use of *Ephedra* alkaloids. Haller & Benowitz (2000) reviewed 140 reports of adverse effects related to the use of dietary supplements containing *Ephedra* alkaloids between June 1997 and May 1999. Of these, 31% were considered to be related to the use of *Ephedra* alkaloids and another 31% were deemed to be related. Ten cases resulted in death and thirteen others resulted in permanent health damage. The most frequently reported adverse effects were hypertension, palpitations, tachycardia, stroke and seizures. Furthermore, ephedrine can increase heart rate as well as glucose and insulin concentrations, and in combination with caffeine it could increase systolic blood pressure (Haller *et al.* 2004b). Although misuse is the most probable cause of adverse effects, side effects were also observed in subjects involved in clinical trials with controlled doses (Bell *et al.* 1998, 2000; Hioki *et al.* 2004). As a result of those reports the Food and Drug Administration has issued warnings regarding possible side effects of *Ephedra* alkaloids (Food and Drug Administration, 2004) and several regulatory actions against *Ephedra* and ephedrine alkaloids aiming at the regulation of these compounds have been implemented in the USA (Abourashed *et al.* 2003). After having warned the general public about the risk associated with the use of ephedrine alkaloids, and sending warning letters to manufacturers of these supplements, on 30 December 2003 the Food and Drug Administration issued a regulation prohibiting the sale of dietary supplements containing ephedrine alkaloids that became effective on 11 April 2004.



**Fig. 3.** Chemical structure of the six optically active alkaloids present in *Ephedra* species: (a) (-)-ephedrine; (b) (+)-pseudoephedrine; (c) (-)-N-methylephedrine; (d) (+)-N-methylpseudoephedrine; (e) (-)-norephedrine; (f) (+)-norpseudoephedrine.

However, traditional Asian medicine containing Ephedra alkaloids was excluded from this rule and can still be sold on the US market. In addition, a US federal judge struck down the Food and Drug Administration ban on Ephedra-containing supplements in favour of a supplement-producing company on 13 April 2005. This makes the implementation of this ban doubtful and opens the way for other producers to continue their marketing strategies on ephedrine-containing supplements.

Despite these regulatory efforts, a study by Bents *et al.* (2004) has demonstrated that the use of stimulants amongst college hockey players remains high, with 46 % reporting the use of pseudoephedrine and 38 % using ephedrine.

Similar to other supplements, variation may occur between the labelling of the dietary supplement and the content. Gurley *et al.* (2000) showed that markedly different amounts of Ephedra alkaloids were found in many commercial dietary supplements and even significant batch-to-batch variation occurred. In seven out of the twenty supplements analysed the exact amount of total Ephedra alkaloids was not given and in 80 % of the analysed samples the ephedrine content was not even mentioned on the label. Similar results were noticed by Haller *et al.* (2004a). Not mentioning the active compound on the label of a nutritional supplement could result in serious complications for athletes, for example, positive doping tests, as they are not aware of the fact that Ma Huang contains Ephedra alkaloids. Even more, some supplements declaring Ma Huang on the label seem not to be as 'natural' as they claim, as only ephedrine was present (Gurley *et al.* 1998), indicating an eventual deliberate addition of this substance instead of using herbal products.

Besides alkaloids originating from the *Ephedra* plant, other substances with stimulating properties have been detected in nutritional supplements. In the 1980s a Belgian female marathon runner tested positive for the stimulant phentermine. Analysis of the consumed food supplement promoting weight loss revealed the presence of the anorexic agents phentermine and fenfluramine (Delbeke, 2001). A study conducted by the Dutch Ministry of Health, Welfare and Sports preceding the Olympic Winter Games in Salt Lake City (2002) resulted in the detection of 3,4-methylenedioxymetamphetamine (MDMA or XTC) in a supplement. In conclusion, not only naturally occurring stimulants including ephedrine and analogues, but also synthetic amphetamine analogues and related compounds can be present in nutritional supplements.

Stimulants belong to the classes of prohibited substances according to WADA rules (World Anti-Doping Agency, 2004). A threshold level for ephedrine and methylephedrine of 10 µg/ml and a level of 5 µg/ml for norpseudoephedrine have been introduced to avoid unintentional positive doping results. Nevertheless, because of the wide variety in ephedrine concentrations found in nutritional supplements and the numerous side effects, athletes should be very cautious when using this kind of nutritional supplement and they should be aware of the possibility that supplements can be contaminated with various prohibited substances.

One case of a positive doping test resulting from the administration of a botanical food supplement has been described (Ros *et al.* 1999).

Also, numerous nutritional supplements promoted for performance enhancement and stimulating effects contain caffeine. Some of these products comprise, besides caffeine, extracts of guarana (*Paullinia cupana*), a South American plant which has been shown to contain caffeine (Davies, 1997). Caffeine has a positive influence on endurance sport. This is frequently observed in run and cycle to exhaustion tests but also in swimming and playing tennis (Macintosh & Wright, 1995; Doherty, 1998; Graham *et al.* 1998; Kovacs *et al.* 1998). As a result, extensive research has been done to investigate the mechanisms by which caffeine exerts its performance-enhancing effects. Although not completely resolved, it appears that the caffeine antagonism of adenosine receptors is an important factor in the comprehensive mechanism of action of caffeine (Sinclair & Geiger, 2000). Before 1 January 2004 the use of caffeine-containing nutritional supplements could have resulted in a positive doping test, as caffeine was listed by the International Olympic Committee as a doping agent in class I A 'stimulants' with a threshold level of 12 µg/ml. This is no longer an issue because from 1 January 2004 caffeine was removed from the WADA list of forbidden substances. Nevertheless, athletes should take care when using caffeine because excessive use is associated with withdrawal effects and negative effects on the cardiovascular system (Sinclair & Geiger, 2000).

#### Narcotics

The most popular narcotic analgesic commonly used for the short-term treatment of post-surgery pain and the long-term use for pain relief of cancer patients is morphine. Morphine, just as other alkaloids such as codeine, norcodeine, papaverine and thebaine, is a constituent of the plant *Papaver somniferum*. These active compounds are normally not promoted as dietary supplements but their biological source, *P. somniferum*, can be used in herbal preparations or as an ingredient in the daily diet. The detection of these alkaloids in seeds from the plant *P. somniferum* has led to the suggestion that inclusion of these products in the daily diet may lead to significant concentrations being detected in biological specimens. This 'poppy seed defence' is mainly used by job applicants controlled by the US Department of Defense or by the National Institute on Drug Abuse. A study by Selavka (1991) dealt with the possible contribution of poppy seed-containing food to positive opiate urinalysis results. Similar research demonstrated the plausibility of this poppy seed defence as opiate-positive results (according to the National Institute on Drug Abuse cut-off level of 300 ng/ml) were obtained after consumption of two bread rolls covered with seeds (Meadway *et al.* 1998). Similar results were reported in other studies (Hayes *et al.* 1987; Elsholy *et al.* 1988; Lo & Chua, 1992).

Because of the painkilling effects, athletes can misuse morphine during training or in competition to overcome pain associated with strenuous exercises. As a result, the International Olympic Committee banned morphine. Before 1991, the presence of morphine in human urine, irrespective of its concentration, resulted in a doping offence. This changed in 1991 because the International Cycling Federation introduced a 1 µg/ml threshold level for

morphine as well as for codeine, one of the precursors of morphine. These rules were later adopted by the International Olympic Committee. At present, WADA rules stipulate that the use of codeine and ethylmorphine, another precursor of morphine, is allowed while morphine is forbidden in concentrations higher than 1 µg/ml. Taking into account the previously mentioned results on poppy seeds, athletes should be aware that the use of these products might lead to positive doping tests. Lo & Chua (1992), for example, even found urinary morphine concentrations up to 1.27 µg/ml after the consumption of a poppy seed-containing meal. Other surprising results showed that doping positive results could be obtained after the use of herbal teas containing parts of the plant *P. somniferum* up to 9 h after the administration of two cups of tea (Van Thuyne *et al.* 2003). Urinary morphine concentrations as high as 7.44 µg/ml were detected after the use of a herbal tea. The package of this tea did not mention any sedative or calming effect. Consequently, the actual doping threshold level of 1 µg/ml for morphine is not capable of distinguishing use from abuse. Other markers of poppy seed use need to be found to avoid false-positive results or the threshold level needs to be increased. Awaiting these, athletes should be aware of this problem and should avoid the use of supplements containing *P. somniferum* or parts of this plant.

#### Analysis of nutritional supplements for doping contaminants

Numerous methods have been developed to analyse herbal preparations for the presence of Ephedra alkaloids or to evaluate the labelled content of *E. sinica* (Ma Huang)-containing supplements. These methods include TLC (Dutt & Poh, 1981), immunoassays (Ros *et al.* 1999; Kamber *et al.* 2001), GC (Betz *et al.* 1997; Hansen, 2001; Wiedemer & Thrall, 2004), HPLC (Gurley *et al.* 1998; Hurlbut *et al.* 1998; Okamura *et al.* 1999; Haller *et al.* 2004a; Jacob *et al.* 2004; Li *et al.* 2002; Trujillo & Sorenson, 2003; Roman & Walther, 2004) and capillary electrophoresis (Flurer *et al.* 1995). Identification is best achieved using MS. Separation of the ephedrines using GC-MS is not possible without derivatisation, which could be considered as a technical drawback. On the other hand, several HPLC methods use SDS or triethylamine as a constituent of the mobile phase to increase the theoretical plate number, hampering the HPLC combination with MS due to overloading of the ion source. Only one method has been described for the identification of Ephedra alkaloids in dietary supplements using liquid chromatography-MS (Jacob *et al.* 2004).

Not only naturally occurring stimulants such as ephedrines are detected in supplements, but also synthetic analogues related to amphetamine such as 3,4-methylenedioxymetamphetamine and phentermine.

Several methods for the detection of stimulants in urine using HPLC have been published (Barkan *et al.* 1981; Gal, 1984; Wainer *et al.* 1986; Vandermerwe *et al.* 1994; Imaz *et al.* 2000). GC methods routinely used for the detection of stimulants for doping control purposes have also been described (Coutts *et al.* 1980; Konig & Ernst, 1983; Imaz *et al.* 1993; Vandermerwe & Hendrikz, 1995;

Van Eenoo *et al.* 2001). Based on GC using N-P detection for screening purposes in urine, Parr *et al.* (2003) created a method for the screening of stimulants and other N-containing doping-relevant drugs in nutritional supplements. Confirmation of positive screening results was done using GC-MS after a selective derivatisation using N-methyl-N-trimethylsilyltrifluoroacetamide and N-methyl-bistrifluoroacetamide. Besides ephedrines, amphetamines and related compounds, this method also allows for the identification of methylecgonine, formed during alkaline extraction, as a marker for the presence of cocaine. The only drawback of this method is the relatively high limit of detection for some compounds including methylenedioxy-amphetamine (known as MDA), cocaine and strychnine, as some authorities, such as the 'Netherlands security system for nutritional supplements for elite sports' (NZVT; <http://www.necedo.nl/nzvt/thenetherlandssecuritysystemnutritionalsupplements>) require better analytical sensitivity. The development of a liquid chromatography-MS method for the screening of stimulants in dietary supplements might be a solution.

Analysis of nutritional supplements for the presence of anabolising agents has been described in several papers. Catlin *et al.* (2000) and Green *et al.* (2001) used HPLC to detect contaminants in nutritional supplements marketed as prohormones, but the method was restricted to five prohormones of testosterone and nandrolone. Using this method the detection of low concentrations of contaminants seems questionable as the dietary supplements were diluted numerous times.

A more frequently applied analytical technique for the identification of prohormones in dietary supplements is GC-MS, similar to the screening methods for anabolic steroids in urine for doping control purposes. Full scan MS methods were able to detect contaminants in the mg range (De Cock *et al.* 2001; Kamber *et al.* 2001; Gmeiner, 2002), while the use of selected ion-monitoring methods allowed more sensitive identification of impurities (Geyer *et al.* 2000, 2004; Van Thuyne & Delbeke, 2004, 2005).

Geyer *et al.* (2004) described a method for the screening of twelve compounds including testosterone and prohormones, nandrolone and prohormones, prohormones of boldenone and 5 $\alpha$ -dihydrotestosterone. Limits of detection (LOD) ranged between 5 and 100 ng/g. These LOD allow for the determination of trace contaminations in 'non-hormonal' nutritional supplements.

Two International Organization for Standardization validated methods, ISO 17025, have been published for the screening of twenty-eight different anabolising agents including testosterone and prohormones, nandrolone and prohormones, esters of both compounds, stanozolol and metandienone (Van Thuyne & Delbeke, 2004, 2005). Although until now only one ester of testosterone (testosterone undecanoate) is available for oral administration, these methods are capable of detecting other esters of testosterone and nandrolone when they should become available on the supplement market. A distinction was made between solid (Van Thuyne & Delbeke, 2004) and aqueous (Van Thuyne & Delbeke, 2005) nutritional supplements. LOD ranged from 2 to 40 ng/g for solid supplements



while for aqueous supplements LOD were between 1 and 10 ng/ml. These LOD meet the requirements of official authorities such as the Dutch system (NZVT).

Methods developed for the identification of trace contaminants rely on liquid–liquid extraction. Geyer *et al.* (2004) used a method based on mixing nutritional supplements with methanol. Afterwards an extractive clean-up using pentane and methanol was used. Other methods rely on alkaline extraction using NaOH and pentane–diethyl ether (Van Thuyne & Delbeke, 2004) or K<sub>2</sub>CO<sub>3</sub>–NaHCO<sub>3</sub> and pentane–diethyl ether (Van Thuyne & Delbeke, 2005).

Nutritional supplements form a heterogeneous group of products available in different forms. Consequently, analysis of nutritional supplements for anabolic steroids has proven to be very difficult due to the different matrices. Problems occur most frequently with solid nutritional supplements or oil-based supplements (Van Thuyne & Delbeke, 2005). Parr *et al.* (2004) reported several possible solutions for problems occurring during extraction and analysis of supplements. The proposed modifications include extra derivatisation, additional clean-up steps, alternative sequence of injection and reduction of the matrix. In this way, improvements could be obtained. However, 10.4 % of all investigated samples could still not be analysed. A possible solution for those problems could be a pre-analysis fractionation using HPLC as described by De Cock *et al.* (2001). Promising results in the clean-up step were obtained by Geisendorfer & Gmeiner (2003) using gel permeation chromatography before GC-MS analysis. This method allowed for the determination of twelve different anabolic steroids and prohormones without the occurrence of matrix problems. Although the obtained LOD (20–50 ng/g) were not in compliance with the requirements of some authorities (NZVT), this method could be further developed.

### Conclusion

The use of nutritional supplements seems unnecessary when athletes use a well-balanced diet. Nevertheless, according to supplement sales numbers the use of these supplements has exploded during the last decade. Athletes are influenced by parents, coaches, competitors and high financial stakes to improve their performance. Aggressively marketed dietary supplements promise that the athlete will jump higher, run faster or throw further. As long as products such as vitamins or minerals are used combined with proper medical support this use is rather harmless. The use of prohormones, available on the supplement market as a result of several loopholes in the 1994 DSHEA, is of greater risk. Besides the serious health effects associated with these products, athletes also have to take into account that they have to pass a doping control test. Therefore, the use of supplements by athletes in general is dangerous as numerous nutritional supplements are contaminated with anabolising or stimulating compounds. According to the WADA doping rules athletes are held responsible for the substances detected in their biofluids, irrespective of the origin. The development of methods capable of detecting contamination of nutritional supplements with doping-relevant substances by specialised doping control laboratories is of the utmost

importance. At present athletes or supplement-producing companies may have their supplements tested.

Although several analytical methods are now available, further improvements are needed because presently a fraction of the supplements cannot be analysed, according to the requirements of some testing authorities, due to matrix problems. Clearly, further research is required in this area.

### Acknowledgements

The authors wish to thank the Flemish Ministry of Health for financial support (W. V. T. and P. V. E.).

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