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Experiences of testing dietary supplements

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SUMMARY

Determination of the prohibited active ingredients, such as drugs and their designer compounds, of dietary supplements products intended for athletes is an important, but not yet well-known and regulated area of food analysis.

In our laboratory, the analysis of three major product groups is carried out. The first group includes products intended for athletes. They can be dietary supplements or a group of products that had been classified as foodstuffs for particular nutritional uses before 2016, for example, high protein content products promoting great muscular effort, intended primarily for athletes and workers performing heavy labor, which were transferred to the category of dietary supplements or normal foods after 2016. For athletes, when consuming the products, in addition to preserving health and the physiological effects to be achieved, it is important that the products do not contain even trace amounts of substances that are on the prohibited list of WADA (the World Anti-Doping Agency), because they are solely responsible for the presence of these in their bodies.

The second group is that of weight loss products. Regarding the prohibited substances used in dietary supplements, products belonging to this group show a high degree of similarity to the group of products containing stimulants prohibited for athletes.

The third group consists of products used for the treatment of sexual dysfunctions. In these, phosphodiesterase inhibitors, widely known as drugs, may be present as prohibited active ingredients or contaminants. The most common ingredients of these products include plant extracts.

In all three cases, the extent of contamination may vary widely in the groups examined, from trace amounts to pharmaceutical active ingredient levels.

12 years ago, when the analysis of products has been started at our laboratory intended for athletes, our objective was to develop screening analytical methods that are able to determine prohibited stimulants with detection limits of 100 ng/g and anabolic agents with detection limits of 10 to 50 ng/g efficiently, in a robust way, with sufficient measurement (detection) accuracy. The analysis of potency enhancers was launched three years later. Since the initial analysis of 40 components, the number of types and components of active ingredients analyzed has increased significantly and, in addition, in the case of stimulants and narcotics, the detection limit improved to 10 ng/g. Currently, accredited analysis of 126 prohibited ingredients is performed in the laboratory. During this time, more than 10,000 raw materials and finished products

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have been tested. For the analysis of anabolic agents, hormone and metabolic modulators, GC-MS and LC-MS/MS methods have been used, while LC-MS/MS techniques have been used for the determination of stimulants, narcotics, beta-blockers, beta2 agonists and potency enhancers, following appropriate sample preparation.

In our manuscript, of the compound groups examined in the laboratory, the analyses of anabolic agents and aromatase inhibitors, which belong to the group of hormone and metabolic modulators, are presented, in products intended for athletes and their raw materials.

It should be noted that for the names of the compounds in dietary supplements contained in this paper, the Anglo-Saxon version common in international practice is used.

Introduction

According to ESZCSM decree 37/2004 (IV. 26.), the concept of *dietary supplements* is defined as follows: *„it is a food supplementing the traditional diet containing nutrients or other substances with nutritional or physiological effects in a concentrated form, individually or in combination; in a pre-dispensed or dispensable form (for example, capsules, pastilles, pills, powders in packets, dispensable powders, ampoules, drop bottles or other similar powder or liquid forms suitable for dispensing small amounts)"*. The decree also defines the concept of nutrients, which are the minerals and vitamins listed in the annex to the decree. However, other substances with nutritional of physiological effects are not specified. This group includes, for example, a number of products containing plant extracts.

Authority and state tasks related to dietary supplements are divided among a number of organizations in Hungary. Authorities and organizations involved in the control include, among others, the National Institute of Pharmacy and Nutrition (OGYÉI - NIPN), the Ministry of Human Capacities (EMMI - NHC), the National Food Chain Safety Office (NÉBIH - NFCSO) and several other organizations. It is also important to emphasize the importance of self-regulation. Organizations committed to market purity support product inspection within their own frameworks. These include, among others, the Association of Hungarian Dietary Supplement Manufacturers and Distributors (MÉKISZ - AHDSMD), the Hungarian Association of Pharmaceutical Wholesalers (GYNSZ - HAPW), the Hungarian Chamber of Pharmacists (MGYK - HCP), the Hungarian Pharmaceutical Manufacturers' Association (MAGYOSZ - HPMA) and the National Board Against Counterfeiting (HENT - NBAC) **[1]**. For example, based on a Hungarian notification, as a result of official investigations, in 2018, 9 potency enhancers were added to the RASFF (Rapid Alert System for Food and Feed) alarm system and were withdrawn because of prohibited pharmaceutical active ingredient content.

By the end of the 20th century, the size of the market for dietary supplements and products intended for athletes had increased significantly. This increase is still ongoing today. In parallel with the increase in market size, the incidence of product counterfeiting and of contamination with prohibited pharmaceutical active ingredients has also increased. In the USA, a case study was already published in 1975, which reported health damage resulting from the use of counterfeit Chinese herbal products **[2]**. The products in question contained the active ingredients aminopyrine and phenylbutazone not included on the product label. In the early 1990s, 2,609 traditional Chinese herbal products used in 8 hospitals by their patients, were examined by Taiwanese authors. According to their studies, 23.7% of the products contained unlabeled active ingredients. More than half of the contaminated products contained more than one synthetic active ingredients. These included, but were not limited to caffeine, anti-inflammatory and anti-pyretic agents, glucocorticosteroids, non-steroidal anti-inflammatory drugs, diuretics and sedatives **[3]**.

Supplements became popular among athletes in the 1990 as an alternative to doping agents. In competitive sports, both during the preparation phase and during competitions of different levels, the use of adjuvant agents (e.g., vitamins, minerals, other supplements) provides an advantage. The requirement for these products is that they shorten the regeneration time of the body, ensure the energy supply of greater than average muscle work, improve the oxygen supply of muscles and prevent muscle cramps **[4, 5]**. In addition to efficacy, it is essential for athletes that the product used does not contain any components that are on the prohibited list of WADA **[6]**. We would like to dispel the misconception that these products do not contain, because they cannot contain, components that are on the prohibited list, as they are available in normal trade without restriction. The opposite of this statement has been proven by a number of positive doping cases related to the application of supplements used by athletes **[7, 8]**. Products used by athletes may be categorized in different ways based on their use. One of the possible classifications is as follows:

- sports foods (sports drinks, sports gels, energy bars);

- medical supplements (iron, calcium, multivitamins, minerals, probiotics);
- ergogenic supplements (caffeine, beta-alanine, bicarbonate, creatine);
- functional and super foods (herbs, seaweed, plant fibers, seeds, e.g., chia);
- other supplements (a wide range of plant extracts and concentrates with special effects, such as potency enhancement weight loss, energy increase) **[5]**.

Of course, the above list is not a food law classification, but the analytical performance characteristics of the analytical methods developed in our laboratory are suitable for the analysis of the selected product groups. Since dietary supplements and products intended for athletes are foodstuffs from a food law point of view, the rules for the production and marketing of foods are applicable to them.

The analysis of products intended for athletes for prohibited active ingredients began in the mid-1990s. Up until the end of 2004, products containing prohormones could be marketed freely in the USA. Typically, they contained the prohormones of testosterone, 19-nortestosterone and boldenone. These active ingredients were detected in products intended to increase muscle strength and testosterone levels in a number of cases, even when they were not indicated on the product label [**9, 10, 11**].

The first large-scale, comprehensive study was conducted by the International Olympic Committee in 2000-2001. 634 samples, declared to be hormone-free, were purchased from 215 different distributors in 13 countries. 289 of the products tested came from companies also producing prohormones, while 345 products came from companies not producing prohormones. 14.8% (94) of all samples contained prohormones. It is an interesting fact from an analytical point of view that 66 samples (10.4%) could not be analyzed satisfactorily due to matrix effects **[12]**.

Components that are on the prohibited list of WADA and which appear often in products intended for athletes and weight loss products are listed in ***Table 1***. The indicated active ingredients may be contained in the products as either declared active ingredients or as contaminants. This way, dehydroepiandrosterone (DHEA) and 7-keto-DHEA can be found in several products among the ingredients indicated. It also happens that prohibited substances are listed among the ingredients under names which makes them impossible to find on the prohibited list of WADA.

Anabolic agents

The anabolic agents group of WADA includes exogenous and endogenous anabolic androgenic steroids and other anabolic agents.

Anabolic androgenic steroids

Steroids are organic compounds of the lipid family with a sterane nucleus. The backbone of the molecule is formed by seventeen carbon atoms, with three cyclohexane (A, B and C) and a cyclopentane (D) rings forming the gonane basic structure (***Figure 1***). The diversity of steroids is provided by the degree of oxidation of the rings and the functional groups connected to them. Also adds to the diversity of the compounds the positions of the side chains, the number of methyl groups connected and the additional functional groups on the rings.

Anabolic androgenic steroids in the human body include endogenously produced testosterone, dihydrotestosterone (DHT), testosterone prohormones and their metabolites, as well as exogenous anabolic androgenic steroids, which are synthetic derivatives of testosterone. Testosterone, the primary male sex hormone, has both anabolic and androgenic properties. The molecule is synthesizes by the Leydig cells of the testes from cholesterol. Testosterone is also excreted in small amounts by the ovaries and the adrenal glands.

The first androgenic steroids, androsterone and trans-dehydroandrosterone (dehydroepiandrosterone, DHEA) were isolated from urine in 1932 **[13]**. A few years later, in 1935, the male sex hormone testosterone was synthesized and characterized by Adolf Butenandt. In 1939, for his work on sex hormones, he was awarded a shared chemical Nobel prize together with Leopold Ružička.

The metabolic processes of testosterone and its prohormones are shown in ***Figure 2*** [**14**].

Synthetically produced exogenous anabolic androgenic steroids, such as metandienone, stanozolol, methandriol and methyltestosterone, are structurally similar to testosterone (***Figure 2***), and they are used as drugs. Despite their numerous adverse side effects and strict legal regulations, anabolic androgenic steroids are marketed illegally in several countries. In several cases, they also appeared in dietary supplements as unlabeled contaminants [**15, 16, 17, 18**]. In order to circumvent existing laws, their structure is often modified. Designer steroids are mainly marketed in products intended for bodybuilders via the internet. In the absence of clinical studies, little is known about the pharmacological properties and metabolism of the steroids thus produced (***Figure 3***) [**19**], which poses a high risk to consumers [**19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29**]the steroids intentionally added to the

\"nutritional supplements\" are testosterone analogues with some structural modifications. In this study, the analyzed product was bought online and a new anabolic steroid known as methylstenbolone (2,17α-dimethyl-17β-hydroxy-5α-androst-1-en-3-one.

Other anabolic agents

This group includes substances that are not anabolic steroids but have an anabolic side effect. This includes clenbuterol, an effective bronchodilator used in the treatment of asthma. In addition to this property, it increases endurance and stamina, increases muscle mass and decreases the amount of fat. It has also been described in dietary supplements as a contaminant not listed among the ingredients [**30**]. For athletes, it is important that the product consumed does not contain clenbuterol because, according to the WADA code, there is no threshold value below which it would not be prohibited in urine samples [**31**]5-dichlorobenzyl alcohol. This means that the smallest amount of clenbuterol that can be detected in urine is considered to be a positive test result. In this respect, the limit value is the limit of detection (LOD).

Selective Androgen Receptor Modulators (SARMs) are non-steroidal substances, selectively targeting the androgen receptors of muscle tissue and triggering rapid muscle growth. They are typically distributed as black market drugs, but also appear on the market as products that look like dietary supplements.

Hormone and metabolic modulators

Hormone and metabolic modulators are a group of substances that are not limited to hormones themselves. This group of substances often modifies the function of hormones, either by blocking a hormone or by increasing the activity of a hormone. Based on their biological activity, they are divided into several subgroups. One of the subgroups includes aromatase inhibitors. In products intended for bodybuilders, one can often find as ingredients 4-androstene-3,6,17-trione (6-oxo), 2-androstenol (5α-androst-2-ene-17-ol), 2-androstenone (5α-androst-2-ene-17-one), 3-androstenol (5α-androst-3-ene-17-ol); 3-androstenone (5α-androst-3-ene-17-one) [**18**, **22**].

Description of the analytical method

Chemicals and standards

Analytical standards were obtained from LGC Standards GmBH and Sigma in analytical purity. Formic acid and potassium hydroxide was provided by Merck GmBH, ethanethiol by Sigma, ammonium iodide by Riedel-de Haën, anhydrous sodium sulfate by Molar Chemicals and N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) by TCI Europe. n-Pentane was produced by Promochem and LC-MS grade methanol by Honeywell.

The components analyzed are listed in **Tables 2** and **3**.

GC-MS parameters

Gas chromatography measurements were carried out on an Agilent 6890 gas chromatograph coupled with an Agilent 5973 inert MSD. Separation was performed on a Restek Rxi-5Sil MS (30m; 0.25 mm; 0.25 µm) column. The injector temperature was 270 °C, the temperature program started at 180 °C and ended at 300 °C. Injection was performed in split mode.

LC-MS/MS parameters

Liquid chromatographic separation was performed on an Agilent 1290RRLC instrument, consisting of a binary pump, a vacuum degasser, a thermostated autosampler and a column thermostat. For detection, an Agilent 6495 triple quadrupole tandem mass spectrometer was used with an ESI source. Chromatographic separation was performed on a reverse phase Thermo Hypersil Gold C18 (50x3mm; 1.9 µm) column. Eluent A was 0.1% formic acid in high purity water, eluent B was 0.1% formic acid in methanol. The initial eluent composition was 70/30 water/methanol. The amount of injected sample was 3 µl, the temperature of the column thermostat was 40 °C. Separation was carried out using gradient elution.

Sample preparation

For the analysis, 2 g of the homogenized sample was weighed, and then it was processed by a small modification of the method described by W. Thuyne and F.T. Delbeke [**32**]. The internal standard was a 1 µg/ml solution of testosterone-d3. The n-pentane extract obtained during sample preparation was divided into two portions and the solvent was evaporated to dryness under nitrogen. For the LC-MS/MS measurement, the evaporation residue was dissolved in 200 µl of 70/30 water/methanol mixture. For the GC-MS measurement, the evaporated sample was dissolved in 50 µl of MSTFA/NH_4I/ethanethiol (320/1/2) silylating agent and derivatization was carried out at 60 °C for 15 minutes.

In 2019, 25 new components were added to the number of prohibited active substances analyzed as part of our flexible scope of accreditation. The method is for qualitative determination. ***Tables 2*** and ***3*** show the data of the components analyzed and the typical parameters of the measurements. During the validation of the method, the desired limit of detection was 10-250 ng/g, depending on the component and the matrix, while the measurement uncertainty was less than 30%.

In recent years, in addition to the testing of finished products, responsible manufacturers had our laboratory carry out the analysis of several raw materials, mainly different plant extracts. In the course of the analyses, prohibited active ingredients have been found in the samples tested on several occasions.

Table 4 summarizes the raw materials containing prohibited ingredients and the prohibited components detected in the products. The limit of detection (LOD) was 10 ng/g.

Figure 8 shows the MRM transitions of a positive Salix alba extract, one of the raw materials tested. Semiquantitative amounts of the prohibited substances found in the sample are summarized in ***Table 5***.

The chromatographic peaks in the top row of ***Figure 8*** correspond to a standard solution of 10 ng/g concentration. In the bottom row, the MRM chromatogram of the *Salix alba* extract is shown. The order of the components: 1, ADD; 2, AD; 3, B; 4, epi-T; 5, T.

Conclusions

The results of analyses of raw materials for prohibited active ingredients indicate that plant-based raw materials may contain the anabolic agents described in this article as contaminants. In our experience, in addition to anabolic agents, the stimulant ephedrine and its derivatives, as well as strychnine can be present in plant-based raw materials. Products made from raw materials that contain prohibited active ingredients pose a great risk to both professional and amateur athletes. Test results can be used by the manufacturers of dietary supplements to select companies that produce raw materials of adequate quality and for the risk assessment of the manufacture of finished products. Production and distribution of high quality products is in the common interest of manufacturers, distributors and consumers.

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